약학석사 학위논문

Neuroprotective Effect of Demethylsuberosin against MPP^+-induced Cell Death in Human Neuroblastoma SH-SY5Y Cells

신경아세포종 SH-SY5Y 세포에서 MPP^+ 로 유도된 세포 사멸에 Demethylsuberosin 의 뇌세포 보호 효과

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Abstract

Neuroprotective Effect of Demethylsuberosin against MPP^+-induced Cell Death in Human Neuroblastoma SH-SY5Y Cells

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1-Methyl-4-phenylpyridinium ion (MPP^+), which is Parkinsonism-inducing neurotoxin, causes specific cell death in human neuroblastoma SH-SY5Y cells. The rapid accumulation of MPP^+ in the mitochondrial matrix inhibits mitochondrial respiratory chain complex I, resulting in the depletion of adenosine triphosphate (ATP) synthesis, the trigger of apoptotic cell death pathway and the decrease of transient receptor potential channel 1 (TRPC1) protein level. In this study, we investigated the protective effect of demethylsuberosin (DMS) against MPP^+-induced cell death in SH-SY5Y cells. *Cudrania tricuspidata (Carr.) Bureau ex Lavallee* is widely distributed over Korea, Japan and China, and its neuroprotective, anti-inflammatory and antioxidant effects have been reported. Recently it was reported that xanthones from the root bark and isoflavones from fruits of *C. tricuspidata* reveleaed neuroprotective effects against 6-hydroxydopamine (6-OHDA)-induced cell death in SH-SY5Y cells. However, the effect of DMS isolated from the root of *C. tricuspidata* against MPP^+-induced cell

death remains unknown. In this study, the effect of DMS isolated from the root of *C. tricuspidata* on MPP^+-induced cell death were investigated. DMS protected neuronal cells against MPP^+-induced cell death, and its protective effect was much better than the positive control, betulinic acid. It has been reported that MPP^+ triggers apoptotic cell death pathway such as increase of Bax and decrease of Bcl-2. In this study, we identified that DMS inhibited the MPP^+-induced apoptotic cell death pathway. Furthermore, DMS attenuated the MPP^+-induced dysfunction of the chymotrypsin-like and caspase-like activities of proteasome in SH-SY5Y cells, and its restorable effect was much better than betulinic acid. DMS attenuated the MPP^+-induced decrease of TRPC1 protein, associated with store-operated Ca^{2+} channels. The MPP^+-induced decrease of TRPC1 protein lead to reduce thapsigargin-stimulated Ca^{2+} influx in cells, and this can cause cell death. DMS also attenuated the reduction of thapsigargin-stimulated Ca^{2+} influx in cells. These results suggest that DMS could have helpful effects on MPP^+-induced cell toxicity by up-regulating TRPC1 protein level.

keywords : *Cudrania tricuspidata (Carr.) Bureau ex Lavallee*, demethylsuberosin, neuroprotection, 1-methyl-4-phenylpyridinium (MPP^+), transient receptor potential channel 1 (TRPC1), Proteasome activity

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Abbreviations

MPP^+	1-methyl-4-phenylpyridinium ion
ATP	adenosine triphosphate
TRPC1	transient receptor potential channel 1
DMS	Demethylsuberosin
BA	Betulinic acid
6-OHDA	6-hydroxydopamine
ROS	reactive oxygen species
UPR	unfolded protein response
UPS	ubiquitin proteasome system
PA700	proteasome activator 700
PA28	proteasome activator 28

1. Introduction

MPP^+ is a Parkinsonism-inducing neurotoxin and it causes specific cell death in dopaminergic neurons. The rapid accumulation of MPP^+ in the mitochondrial matrix inhibits mitochondrial respiratory chain complex I (NADH: ubiquinone oxidoreductase), resulting in the depletion of adenosine triphosphate (ATP) synthesis (Mizuno, Suzuki, Sone, & Saitoh, 1987). The depletion of ATP synthesis generates reactive oxygen species (ROS) and inhibits the function of ATP-dependent UPS, leading to the disruption of the unfolded protein response (UPR), and finally it causes neuronal cell death (Lim, 2007). It has been also reported that MPP^+ inhibits thapsigargin-stimulated Calcium influx in cells by decreasing TRPC1 protein level (Selvaraj et al., 2012).

Cudrania tricuspidata (Carr.) *Bureau ex Lavallee* belonging to the family Moraceae is a small thorny tree widely distributed over Korea, Japan and China, and its neuroprotective (Jeong et al., 2010), anti-inflammatory (Seo et al., 2000) and antioxidant (B. W. Lee et al., 2005) effects have been reported. Recently it was reported that xanthones from the root bark (Kwon et al., 2014) and isoflavones from fruits (Hiep et al., 2015) of *C. tricuspidata* reveleaed neuroprotective effects against 6-hydroxydopamine (6-OHDA)-induced cell death in SH-SY5Y cells. Demethylsuberosin (DMS), a prenylated coumarin, was isolated from the root of *C. tricuspidata*, and it was reported that DMS showed significant feeding deterrence effect against instars of *Spodoptera. Exigua* (Trumble & Millar, 1996) and anti-inflammatory activity (Ma, 2009). Neurodegenerative diseases such as Parkinson's disease are characteristic of the failure of ubiquitin proteasome system (UPS)

(McNaught, Olanow, Halliwell, Isacson, & Jenner, 2001). It was reported that proteasome activator 700 (PA700) and proteasome activator 28 (PA28), the cellular proteasome activators, showed decreased activity in the pars compacta of the substantia nigra in sporadic Parkinson's diseases (McNaught, Belizaire, Isacson, Jenner, & Olanow, 2003). We are reporting DMS potently activates proteasome and it has neuroprotective effect.

2. Materials and Methods

2.1. Isolation and identification of demethylsuberosin

The root bark of *C. tricuspidata* was collected by the Korea Forest Research Institute, Southern Forest Research Center, Jinju, Korea in September 2008 and authenticated by Dr. Hak Ju Lee (Korea Forest Research Institute, Seoul, Korea). A voucher specimen (accession number KH1-4-090814) was deposited at the Department of Biosystems and Biotechnology, Korea University, Seoul, Korea.

The dried root bark of *C. tricuspidata* (13.0 kg) was ground and extracted with MeOH (48 L, 20 L, and 18 L) at room temperature, and the extracts were concentrated *in vacuo* at 35 ℃. The dark brown residue (702.1 g) was suspended in H_2O (4.0 L) and partitioned with *n*-hexane (4.0 × 5 L) and EtOAc (4.0 × 6 L), sequentially. The EtOAc-soluble fraction (213.0 g) was applied to a silica gel column (15 × 60 cm, mesh 230–400) using $CHCl_3$/MeOH (1:0 to 1:1, 6 L for each eluent) to afford seven fractions (F1; 6 L, F2; 6 L, F3; 6 L, F4; 12 L, F5;18 L, F6; 24 L, F7; 18 L). F4 (36.0 g) was fractionated on a silica gel column (10 × 60 cm, mesh 230–400) with *n*-hexane/EtOAc (30:1 to 0:1, 4 L for each eluent) to give

eight fractions (F4.1; 7 L, F4.2; 3 L, F4.3; 4 L, F4.4; 4 L, F4.5; 3 L, F4.6; 7 L, F4.8; 9 L). F4.5 (7.2 g) was chromatographed on a silica gel column (10 × 60 cm, mesh 230–400) using $CHCl_3$/MeOH (1:0 to 5:1, 2 L for each eluent) to afford seven fractions (F4.5.1 to F4.5.7; each 2 L), and F4.5.2 (4.6 g) was then subjected to a C_{18} reversed-phase silica gel column (6 × 70 cm, 75 μm) with MeOH/H_2O (4:1 to 1:0, 5 L for each eluent) to give 15 fractions (F4.5.2.1 to F4.5.2.15; each 1 L). F4.5.2.2 (37.1 mg) was purified by preparative HPLC (YMC Pack ODS-A, 250 × 20 mm i.d., 5 μm, 50 - 85% MeOH in H_2O, flow rate 8.0 mL/min) to afford demethylsuberosin (2.9 mg, > 95%).

2.2. Cell culture

The human neuroblastoma cell line SH-SY5Y (ATCC No. CRL-2266) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin at 37 ℃ in a humidified 5 % CO_2 atmosphere.

2.3. Cell viability test

SH-SY5Y cells in Dulbeco's modified eagle's medium (DMEM) were cultured in 96-well plate (5×10^4 cells/200 μL/well) for 24 h, and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. Cell viability was performed using MTT assay by measuring at 540 nm using microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) as described by Carmichael (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987).

2.4. Proteasome activity

Cell-based proteasome activity was determined using MPP^+-treated SH-SY5Y cells, as described by Henrik Lovborg and coworkers (Lovborg et al., 2006). Briefly, cells (1.0×10^5 cells/300 μL/well) were cultured in 48 well plates for 24 h in DMEM media supplemented with 10% FBS. Samples and MPP^+ (2 mM) were simultaneously treated for 48 h in DMEM media supplemented with 5% FBS. The proteolytic activity of the proteasome was evaluated in cell lysates by using a proteasome activity kit (APT 280; Millipore, USA). In brief, 40 μg of cell lysate was incubated for 2 h at 37 ℃ in the provided buffer with fluorophore-linked peptide substrates. Fluorogenic peptides used as substrates were succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC at 40 μM), *t*-butyloxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin (Boc-LRR-AMC at 40 μM) and benzyloxycarbonyl-Leu-Leu-Glu-4-methyl-coumaryl-7-amide (Z-LLE-MCA at 80 μM) for chymotrypsin-like, trypsin-like and caspase-like proteases activities, respectively. Suc-LLVY-AMC included in the kit was used and Boc-LRR-AMC and Z-LLE-MCA were purchased from Enzo Life Sciences (USA). Reaction mixtures without cell lysates were used as blanks and aminomethylcoumarin (AMC) or methylcoumarylamide (MCA) fluorescence were measured at excitation/emission wavelengths of 380/460 and 380/440 nm, respectively using a microplate reader (SpectraMax Plus 384, Molecular Devices).

2.5. Measurement of intracellular Ca^{2+}

SH-SY5Y cells (1.0×10^5 cells/100 μL /well) were cultured in black 96-well

plates and incubated with MPP^+ (500 μM) and sample for 24 hr at 37 ℃. Cells were washed with PBS buffer and incubated with Fura 2/AM (20 μM) in 100 μL of Tyrode buffer (5 mM KCl, 130 mM NaCl, 10 mM Hepes, 0.6 mM MgCl, 1% BSA, 1% glucose) for 1 hr. The cells were washed three times with PBS buffer and filled with 100 μL of Tyrode buffer. The fluorescence intensity was recorded for 2 hr using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA) at an emission of 512 nm and excitation of 340 and 380 nm. The fluorescence ratio (F_{340}/F_{380}) was used as an indication of a rise the intracellular concentration of Ca^{2+} (Thastrup, Cullen, Drobak, Hanley, & Dawson, 1990).

2.6. Measurement of protein expression

SH-SY5Y cells ($2.0x10^5$ cells/2 mL/well) were cultured in 6-well plates for 24 hr. Cells were then treated with different concentrations of DMS and MPP^+ (2 mM) for 48 hr. Cells were lysed by PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Korea) for 20 min at -20℃. After incubation, lysates were centrifuged at 14,000 rpm for 14 min at 4℃ for clarification. Supernatant was subjected to 10% SDS-PAGE and transferred to PVDF membrane (Immobilon®-P, Millipore, USA). The membrane was incubated with primary antibodies at 4℃ overnight. After washing, the membranes were probed with secondary antibodies for 1 hr and developed with ECL western blotting detection reagent, WEST-ZOL® plus (iNtRON Biotechnology). Bands were visualized using an image analyzer, ImageQuant LAS4000 (Little Chalfont, United Kingdom).

2.7. Statistical analysis

Data obtained were expressed as the mean ± standard deviation (SD). Statistical significance was determined using GraphPad Prism (GraphPad Software, USA). The differences among groups were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison method. P value less than 0.05 was considered to be statistically significant. All the data were obtained from at least 3 independent experiments.

3. Results

3.1. Protective effect of demethylsuberosin on MPP^+-induced cell death

Demethylsuberosin (DMS) (Fig. 1) (> 95% purity) was isolated from the root of *C. tricuspidata*, and its structure was characterized by previously reported spectroscopic data (Jiang & Hamada, 2009). As shown in Table 1, DMS significantly protected neuronal cells against MPP^+-induced cell death among other compounds isolated from the roots. As shown in Fig 2, DMS protected neuronal cells against MPP^+-induced cell death with an EC_{50} value of 0.17 μM, while EC_{50} value of BA was 4.29 μM.

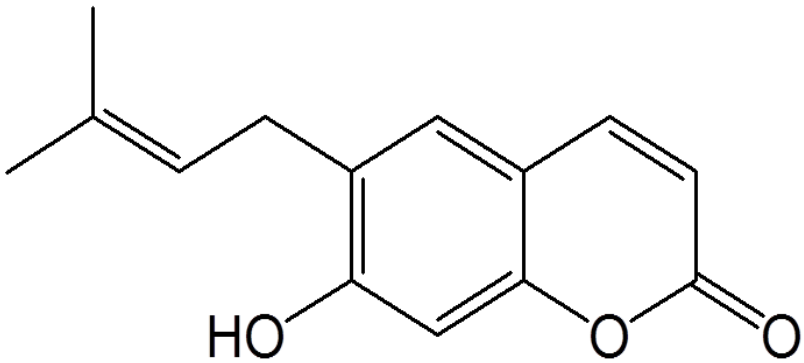

Figure 1. Chemical structure of demethylsuberosin (DMS)

Table 1. Effects of isolated compounds from methanol extract of the roots of *Cudrania tricuspidata* on MPP^+-induced cell death

Compound Name	MPP^+- EC_{50} (μM)
New compound	>50
2,4-Bis(4-hydroxybenzyl)phenol	>50
New compound	>50
Demethylsuberosin	0.17
cis-3',4'-Diisovalerylkhellactone	>50

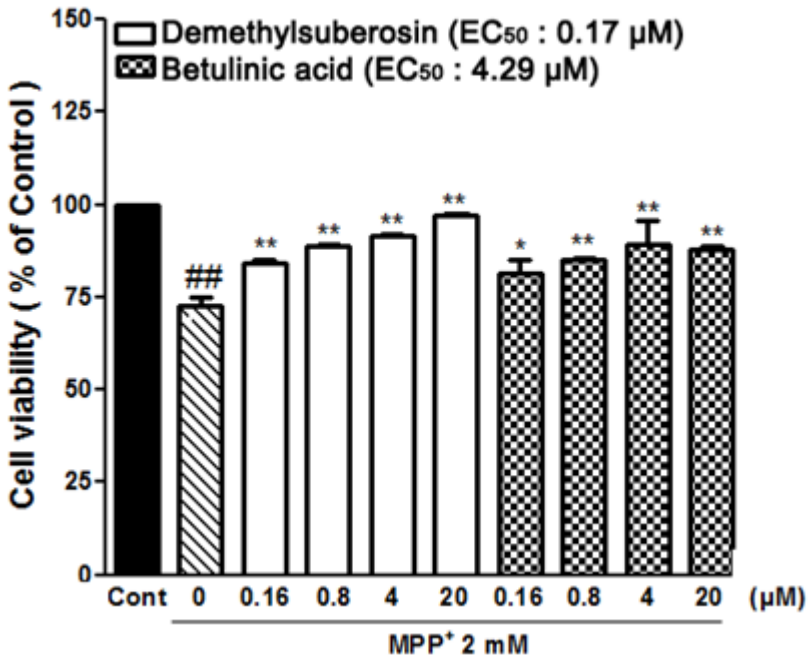

Figure 2. Neuroprotective effects of demethylsuberosin against MPP^+-induced cell death in SH-SY5Y cells. Cells were cultured in 96-well plate for 24 h and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. BA was used as a control compound. The cell viability is given as a percentage of that of the control and data represent the mean ± SD of three independent experiments. $P < 0.005$, compared with control group; $*P < 0.01$ and $**P < 0.005$, compared with MPP^+-induced group, respectively.

3.2. Effect of demethylsuberosin on MPP^+-induced dysfunction of proteasome activities in SH-SY5Y cells

As shown in Fig. 3, DMS attenuated the MPP^+-induced dysfunction of the chymotrypsin-like and caspase-like activities of proteasome in SH-SY5Y cells with an EC_{50} value of 0.76 μM and 0.82 μM, respectively, but not the trypsin-like activity. BA revealed a less potent proteasome activity with an EC_{50} value of 3.56 μM (chymotrypsin-like activity), and 3.66 μM (caspase-like activity).

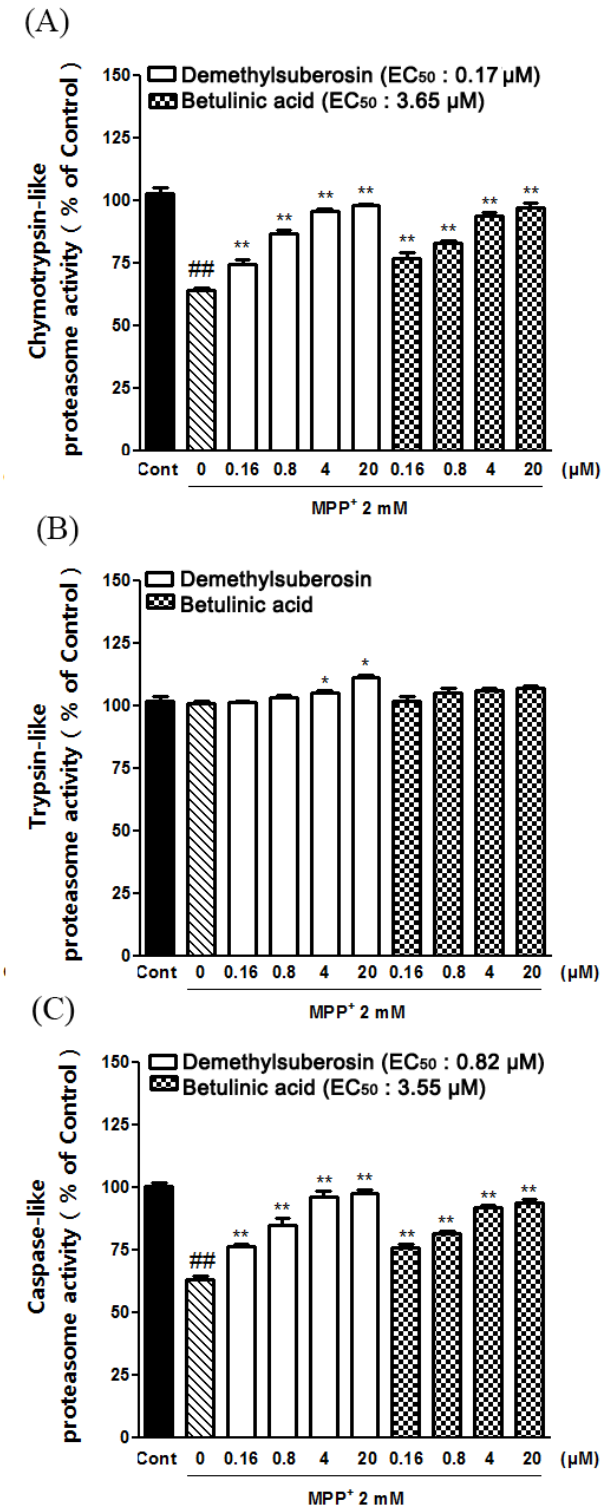

Figure 3. Effects of demethylsuberosin against MPP^+-induced dysfunction of proteasome activities in SH-SY5Y cells. Cells were cultured in 48-well plate for

24 h and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. BA was used as a control compound. The activity is given as a percentage of that of the control and data represent the mean ± SD of three independent experiments. $^{\#\#}P < 0.005$, as compared with control group; $*P < 0.01$ and $**P < 0.005$, as compared with MPP^+-induced group, respectively.

3.3. Effect of demethylsuberosin on MPP^+-induced reduction of thapsigargin-stimulated Ca^{2+} influx in SH-SY5Y cells

MPP^+ is known to inhibit thapsigargin-stimulated calcium influx into the cytosol by decreasing level of TRPC1 protein (Selvaraj et al., 2012). As shown in Fig 4, after the addition of thapsigargin in SH-SY5Y cells, the rapid elevation of intracellular calcium influx was observed in the untreated group. But, the MPP^+-treated group showed that the increase in intracellular Ca^{2+} was significantly decreased. The MPP^+ and DMS-treated group showed that the rapid elevation of intracellular calcium influx was as much observed as the untreated group did.

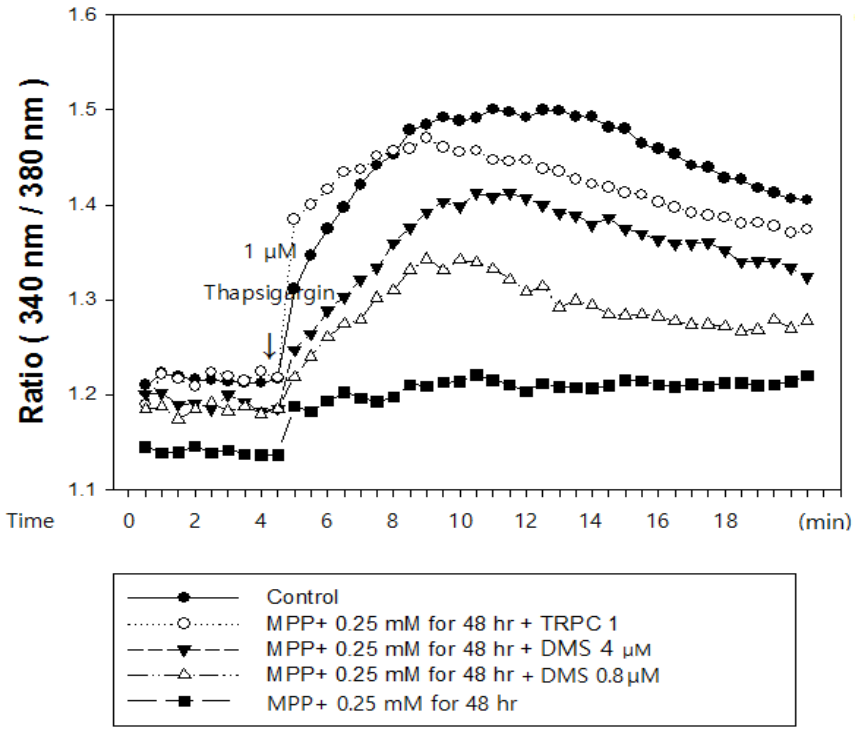

Figure 4. Effects of demethylsuberosin against MPP^+-induced reduction of thapsigargin-stimulated Ca^{2+} influx in SH-SY5Y cells. SH-SY5Y cells were treated with MPP^+ and DMS for indicated time periods and concentrations. Also, cells were treated with TRPC1 overexpression vector 1.25 μg/ml. Fluorescence intensity was measured for 20 min. (excitation; 340 and 380 nm, emission; 512 nm). Values were expressed as a ratio of the fluorescence at the two excitation wavelengths (F_{340}/F_{380}).

3.4. Effect of demethylsuberosin on MPP^+-induced inhibition of TRPC1 protein expression

MPP^+ is known to decrease level of TRPC1 protein, resulting in ER stress (Selvaraj et al., 2012). We investigated the effect of DMS on MPP^+-induced inhibition of TRPC1 protein expression. As shown in Fig 5, MPP^+ showed significant inhibitory effect on TRPC1 protein expression. The MPP^+ and DMS-treated group showed dose dependent increase on TRPC1 protein expression.

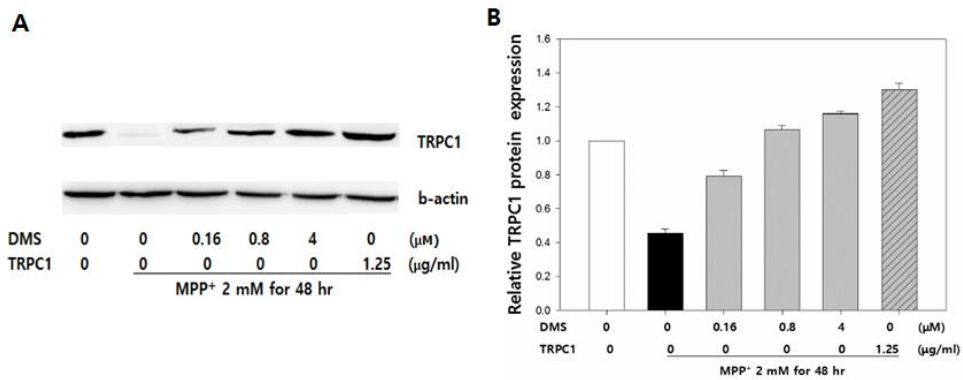

Figure 5. Effects of demethylsuberosin against MPP^+-induced inhibition of TRPC1 protein expression in SH-SY5Y cells. SH-SY5Y cells were treated with MPP^+, DMS, and TRPC1 overexpression vector for the indicated time periods and concentrations. The TRPC1 protein (A) expressions and levels of TRPC1 protein were analyzed using western-blot.

4. Discussion

In dopaminergic neurons, the rapid accumulation of MPP^+ occurs in the mitochondrial matrix and it inhibits NADH dehydrogenase, resulting in the depletion of ATP synthesis and decreasing the function of ATP-dependent UPS (Ramsay, Salach, & Singer, 1986). It was reported that MPP^+ upregulates and aggregates α-synuclein by dysfunction of the UPS and finally causes neuronal cell death (Chiba, Trevor, & Castagnoli, 1985). It also was reported that an antioxidant, a keto-cartenoid astaxanthin, (D. H. Lee, Kim, & Lee, 2011) and verbascoside (Sheng, Zhang, Pu, Ma, & Li, 2002) protected neuronal cells against MPP^+-induced cell death in SH-SY5Y cells.

In this study, we investigated the protective effect of DMS on MPP^+-induced cell death. In comparison with BA, the protective effect of DMS was much better than BA, with an EC_{50} value of 0.17 μM and 4.29 μM. To investigate the effect of DMS on proteasome activity in cells, proteasome activities were examined in cells. DMS attenuated the MPP^+-induced dysfunction of the chymotrypsin-like and caspase-like activities of proteasome in SH-SY5Y cells with an EC_{50} value of 0.76 μM and 0.82 μM, respectively, but not the trypsin-like activity. Based on our data, the protective effect of DMS is partly due to the activation of proteasome in cells.

We also investigated the effect of DMS on the expression of TRPC1 protein and thapsigargin-stimulated calcium influx in cells. As shown in Fig 4, DMS increased calcium influx, compared with MPP^+-treated group. To identify that its increased calcium influx is caused by TRPC1 protein expression, we overexpressed TRPC1 protein by using TRPC1 vector. TRPC1 overexpressed group showed thapsigargin-stimulated calcium influx as untreated group. As shown in Fig 5, DMS increased

the level of TRPC1 protein and showed dose-dependency, compared with MPP^+-treated group. It has been reported that TRPC1 protein and calcium influx are crucial in cells (Selvaraj et al., 2012). Based on our data, the protective effect of DMS on MPP^+ in cells is partly due to the elevation of MPP^+-inhibited proteasome activities, the elevation of MPP^+-decreased TRPC1 protein level. Even though further research is needed in relation to the neuroprotective effect of DMS and the UPS, we can investigate that DMS has a protective effect on MPP^+-induced cell death by up-regulating proteasome activities and TRPC1 protein level.

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국문초록

신경아세포종 SH-SY5Y 세포에서 MPP^+ 로 유도된 세포 사멸에 Demethylsuberosin 의 뇌세포 보호 효과

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파킨슨 증상을 유도하는 신경독성인 1-mehyl-4-phenylpyridinium(MPP^+)는 신경아세포종 SH-SY5Y 세포에서 세포 사멸을 일으킨다. 인간 신경 아세포종 SH-SY5Y 세포에서 Bcl-2 단백질 발현양의 감소, Bax 단백질 발현양의 증가, 유비퀴틴-프로테아좀 시스템에서의 프로테아제 활성 감소, 칼슘 채널 TRPC1 단백질 발현양의 감소를 유발한 것으로 알려져왔다.

이에 본 연구에서는 천연물에서 MPP^+에 대해 세포 보호 효과를 가지는 물질을 연구해 새로운 치료제의 가능성을 살펴보았다. 선행 연구에 따르면 꾸지뽕 나무의 뿌리 추출물은 항염증, 항산화 효과를 가지고 있는 것으로 알려져 있다. 본 연구에서 꾸지뽕 뿌리의 추출물에서 분리한 demethylsuberosin(DMS)이 MPP^+에 의해 유도된 세포 독성에 양성 대

조군인 betulinic acid보다 더 세포 보호 효과가 좋은 것을 확인하였고, 또한 MPP^+에 의해 감소된 Bcl-2 단백질 발현양을 복구하였고 증가된 Bax 단백질 발현양을 감소시켰다. DMS는 MPP^+에 의해 유도된 proteasome activity의 기능장애를 복구시켰다. 또한 DMS는 MPP^+에 의해 유도된 TRPC1 단백질 발현양의 감소를 정상 수준으로 회복시켰다. MPP^+에 의해 TRPC1 단백질이 감소하면 세포 내 thapsigargin 유도 Ca^{2+} 유입량이 감소한다. DMS는 TRPC1 단백질 발현양을 증가시켜 감소한 thapsigargin 유도 Ca^{2+} 유입량을 복구시켰다.

꾸지뽕 뿌리에서 분리한 DMS는 TRPC1 단백질의 발현을 증가시키고, proteasome activity를 증가시켜 MPP^+에 의해 유도된 세포 사멸에 세포보호효과를 보여주었다. 따라서 DMS는 MPP^+에 유도된 파킨슨 증상에 완화에 후보물질이 될 수 있다는 가능성을 보여주었다.

주요어 : *Cudrania tricuspidata (Carr.) Bureau ex Lavallee*, demethylsuberosin, neuroprotection, 1-methyl-4-phenylpyridinium (MPP^+), transient receptor potential channel 1 (TRPC1), Proteasome activity

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감사의 글

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