学 位 論 文 の 要 旨

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学 位 論 文 名　Neuroprotective Effect of Madecassoside Evaluated Using Amyloid β_{1-42}-mediated *in vitro* and *in vivo* Alzheimer's Disease Models

発 表 雑 誌 名
（巻: 初頁～終頁等，年）　International Journal of Indigenous Medicinal Plants (in press)

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論 文 内 容 の 要 旨

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, characterized by the deposition of amyloid β (Aβ) peptides in the neuritic plaques and neurofibrillary tangles in the brain. $A\beta_{1-42}$, the major amyloid component of AD plaques. The deposition of Aβ leads to oxidative stress, synaptic impairments, neuronal loss and memory deficits. Aβ-mediated memory loss is associated with decrease in plasticity-related neuronal protein molecules including brain-derived neurotrophic factor (BDNF) and postsynaptic density protein-95 (PSD-95).

Madecassoside (MD) is a pharmacologically active triterpenoid glycoside of *Centella asiatica* leaf extract which constitutes 53% of total triterpenoid content. *Centella asiatica* commonly has been used for medicinal purpose for centuries in Asia, Middle East and Africa and its neuroprotective effect has been comprehensively studied. Though MD, possesses several pharmacological activities in biological system, the mechanism by which MD provides neuroprotection in AD remains unknown. Therefore, we investigated its effects on spontaneous $A\beta_{1-42}$ fibril formation and on $A\beta_{1-42}$-induced toxicity in human neuroblastoma SH-SY5Y cells *in vitro*. This study also examined how MD interferes with $A\beta_{1-42}$-mediated pathogenic factors in AD model rats.

MATERIALS AND METHODS

Thioflavin T fluorescence assay and electron microscopy: The $A\beta_{1-42}$ peptide (50 μM) was suspended in the desired volume of assembly buffer (100 μL of 50 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl and 0.01% sodium azide) with or without MD. After incubation

at 37 °C, 40-μL aliquots from each tube were mixed with 210 μL of 5 μM thioflavin T (ThT) in 50 mM glycine–NaOH buffer, pH 8.5, and fluorescence was measured using fluorescence spectrophotometer. A 4-μL sample was placed on a copper grid and stained with 1% uranyl acetate; excess uranyl acetate was removed from the grid with distilled water, air dried, and examined under a Hitachi H-7000 transmission electron microscope (TEM).

Cell culture, morphology study and detection of apoptosis: SH-SY5Y cells were cultured in 96-well plates at a density of 1×10^4 cells per well for 24 h with MD prior to exposure to oligomeric $A\beta_{1–42}$ for 48 h. After 48h of co-treatment with MD, cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell proliferation assay. Morphological examination was performed by Tuj-1 and 4',6-diamidino-2-phenylindole (DAPI) staining. The apoptotic nuclei containing free 3'-OH termini were detected by using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit.

Animals, experimental design and preparation of AD model: Male Wistar rats (5 weeks) were randomly divided into two groups: the control group (n = 22) and the MD group (n = 22). The MD group was orally feed MD (95% pure) dissolved in distilled water at 300 mg/kg BW for 11 weeks and the control group was given adjusted volume of distilled water. After 4 weeks, all rats were subjected to surgery to prepare AD model. $A\beta_{1-42}$ peptide solution (5 µl, 2.5 nmol) was injected through a stainless steel 30-G cannula inserted 3.8 mm into the right ventricle using a Hamilton microsyringe with an infusion rate of 1 µL/min. The control rats other than those from the $A\beta_{1-42}$ infusion group were injected with vehicle alone. The control group was subdivided into rats infused with only solvent used for dissolving Aβ (Vehicle group, n = 10) and an Aβ infused group (Aβ group, n = 12). Similarly, the MD group was subdivided into rats infused with the solvent used for dissolving Aβ (MD + Vehicle group, n = 10) and Aβ infused group (MD +Aβ group, n = 12).

Radial maze learning ability**:** The rats were behaviorally tested for their learning-related cognitive abilities by determining their ability to complete a task in an eight-arm radial maze. Two parameters of memory function were examined: reference memory error (RME), which was determined by the number of entries into unbaited arms, and working memory error (WME), which was estimated by the number of repeated entries into arms that had already been visited within a trial. Lower number of RMEs and WMEs suggested better spatial learning ability.

ELISA and measurement of oxidative status: Aβ, BDNF, PSD-95, TNFα and cathepsin D levels were detected by conventional ELISA. Lipid peroxide (LPO) concentration were measured by the thiobarbituric acid reactive substance assay. The levels of reactive oxygen species (ROS) were quantified from a dichlorofluorescin standard curve. Protein concentration

was estimated using the Lowry method.

RESULTS AND DISCUSSION

The ThT fluorescence assay revealed that MD significantly inhibited fibril formation. The electronic microscopic view further revealed the typical filamentous and branching morphology of $A\beta_{1\text{-}42,}$ while MD-treated samples displayed spaced and beaded structure's, leading to necklace-like diffused proto-fibrillar filaments. The fibril diameter was shorter in presence of MD than those of the $A\beta_{1\text{–}42}$ alone. Based on these data, which showed that MD-mediated anti-$A\beta_{1\text{-}42}$ fibril formation effects *in vitro*, we therefore assessed whether or not MD could protect from $A\beta_{1\text{-}42}$ oligomer-induced toxicity in SH-SY5Y cells. Aβ-induced neurotoxicity in the SH-SY5Y cells was evaluated with MTS assay, morphological study and the extent of apoptosis by TUNEL labeling. MTS assay reveled that co-treatment with MD inhibited the $A\beta_{1\text{-}42}$-induced loss of cellular viability. MD also decreased the extent of apoptosis, as indicated by the reduction of TUNEL-positive nuclei in the $A\beta_{1\text{-}42}$ + MD treated cells.

The *in vitro* anti-$A\beta_{1\text{-}42}$ fibril formation effects, combined with the anti-apoptotic effects of MD observed in SH-SY5Y cells, motivated us to conduct *in vivo* experiments to examine the effect of MD pre-administration on spatial learning in AD model rats. Randomized two factor ANOVA (block and group) revealed that infusion of $A\beta_{1\text{-}42}$ into the ventricle of rat brains caused significant impairment spatial memory, indicating a successful modeling of AD in rats. Subset analyses of the number of RMEs further demonstrated that MD pre-administered rats had lower number of RMEs scores than the AD rats. Furthermore, pre-preadministration of MD reduced amyloid burden in detergent insoluble membrane fractions, level of LPO, ROS, TNF-α and cathepsin D levels with concomitant increases in BDNF and PSD-95 levels predominantly only in the hippocampus.

CONCLUSION

The present experiments indicate that MD can inhibit spontaneous fibril formation of $A\beta_{1\text{-}42}$ and protect from $A\beta_{1\text{-}42}$-induced cytoxicity in SH-SY5Y cells through anti-apoptotic mechanism. The ability of MD to reduce cognitive deficits and prevent neurodegeneration in $A\beta_{1\text{–}42}$-infused rats might involve by the action of i) anti-oxidative and anti-inflammatory effects of MD; ii) reduction of brain Aβ burden; iii) increased levels of plasticity-related proteins, BDNF and PSD 95; and iv) decreased levels of cathepsin D in the hippocampus. Accordingly, this study suggests that MD has the potential to be used as a prophylactic, complementary agent in AD.