学 位 論 文 の 要 旨

氏名　Abdullah Al Mamun

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)

著 者 名　Abdullah Al Mamun, Michio Hashimoto, Masanori Katakura, Kentaro Matsuzaki, Shahdat Hossain, Hiroyuki Arai, Osamu Shido

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 $A\beta_{1–42}$ 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 $A\beta_{1–42}$-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.

論文審査及び最終試験又は学力の確認の結果の要旨

甲・乙	氏名	Abdullah Al Mamun
学位論文名	Neuroprotective Effect of Madecassoside Evaluated Using Amyloid $\beta_{1\text{-}42}$-Mediated *in Vitro* and *in Vivo* Alzheimer's Disease Models	
学位論文審査委員	主査	長井　篤
	副査	安井　幸彦
	副査	小林　裕太

論文審査の結果の要旨

アルツハイマー病（AD）は記憶認知障害を伴う進行性の神経変性疾患であり、現在、効果的な治療薬はない。AD発症の原因の一つにアミロイドβ（Aβ）タンパク質の脳内への蓄積が挙げられる。本研究では東南アジアなどに自生する食用のセリ科のツボクサ（*Centella Asiatica*）に注目し、その葉に含まれる主要なトリテルペノイド化合物であるmadecassoside（MD）のADに対する効果を３つのレベルで検討した。1）分子レベル；チオフラビンT蛍光法、電子顕微鏡による観察から、MDはAβの凝集と繊維化を抑制すること確認した。2）細胞レベル；ヒト神経芽細胞腫由来SHSY-5Yでは、Aβの細胞毒性によりアポトーシスが誘発され、生存率は低下したが、MDはそのアポトーシスを抑制し、生存率を改善した。3）個体レベル；Aβ脳室内注入ADモデルラットにおいて、MDの前投与はAβによる空間認知機能の低下を抑制した。また、MDは海馬の脳由来栄養因子とpostsynaptic density protein 95タンパク質量を増加させ、Aβ量、酸化ストレスや炎症マーカー量を減少させた。これらの結果から、MDはAβ凝集の抑制作用、抗アポトーシス作用、抗炎症作用や抗酸化作用を介してAβによる認知機能障害を抑制することが示唆された。本研究結果は新たなADの予防法と治療法の提案に繋がる知見ともなるため、本論文は学位に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者はMDの作用に着目し、Aβ凝集の抑制作用、Aβの細胞毒性からの救済、ADモデルラットの症状改善効果を、形態的、行動学的、分子生物学的側面から検討し、機序や治療応用の可能性を考察した。結果の解釈、機序の考察が合理的に行え、関連領域の知識も十分であり、学位授与に値すると判断した。（主査:長井　篤）

申請者は、数多くの実験系を組み、それぞれにおいて明確な結果を示すことによって、Aβによる認知機能障害に対するMDの抑制機構について新たな所見を得、MDがADの予防薬や治療薬として役立つ可能性を示唆した。関連領域の知識も十分であり、学位授与に値すると判断した。（副査：安井幸彦）

申請者はAβの分子レベルでの重合、Aβオリゴマー投与の培養神経細胞に対する毒性、Aβ投与ADモデル動物の発症の3つのレベルで、MDがいずれも抑制を示すことを明らかにした。認知症の治療や予防につながることが期待される研究成果である。公開審査では的確に質疑応答し、関連知識も豊富であることから学位授与に値すると判断した。（副査：小林裕太）

（備考）要旨は、それぞれ４００字程度とする。