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Korean Mistletoe (*Viscum album var. coloratum*) Inhibits Amyloid β Protein (25-35)-induced Cultured Neuronal Cell Damage and Memory Impairment

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Abstract – The present study aims to investigate the effect of methanol extract of Korean mistletoe (KM; *Viscum album var. coloratum*), on amyloid β protein (Aβ) (25-35), a synthetic 25-35 amyloid peptide, -induced neurotoxicity in cultured rat cerebral cortical neurons and memory impairment in mice. Exposure of cultured neurons to 10 μM Aβ (25-35) for 24 h induced a neuronal cell death, which was measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining. KM (10, 30 and 50 μg/ml) significantly inhibited the Aβ (25-35)-induced apoptotic neuronal death. KM (50 μg/ml) inhibited 10 μM Aβ (25-35)-induced elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), which was measured by a fluorescent dye, Fluo-4 AM. Glutamate release into medium and generation of reactive oxygen species (ROS) induced by 10 μM Aβ (25-35) were also inhibited by KM (10, 30 and 50 μg/ml). These results suggest that KM may mitigate the Aβ (25-35)-induced neurotoxicity by interfering with the increase of $[Ca^{2+}]_i$ and then inhibiting glutamate release and generation of ROS in cultured neurons. In addition, orally administered KM (25 and 50 mg/kg, 7 days) significantly prevented memory impairment induced by intracerebroventricular injection of Aβ (25-35) (8 nmol). Taken together, it is suggested that anti-dementia effect of KM is due to its neuroprotective effect against Aβ (25-35)-induced neurotoxicity and that KM may have therapeutic role in prevention of the progression of Alzheimer's disease.

Keywords – Korean mistletoe, *Viscum album var. coloratum*, Amyloid β protein, Neuroprotection, Cultured neuron, Memory impairment

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive impairment and progressive memory loss. It is associated with neuronal loss and extracellular senile plaque, whose major constituent is β-amyloid protein (Aβ), a 39-43 amino acid peptide derived from amyloid precursor protein, accumulation.[1] The mechanisms underlying Aβ-neurotoxicity are complex but may involve *N*-methyl-$_D$-aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, sustained elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and oxidative stresses.[2-4] The core toxic fragment of full length Aβ (1-40) is Aβ (25-35). Aβ (25-35) shows faster and stronger, in the similitude with Aβ (1-40), neurotoxic effects such as oxidative damage, inflammatory responses and memory impairment.[5,6]

Semi-parasitic plants, mistletoes, belong to the families Loranthaceae and have long been recognized as therapeutic herbs all over the world.[7] Korean mistletoe (KM; *Viscum album var. coloratum*) has also been traditionally used for analgesic, sedative and anti-arteriosclerosis effect in Korea.[7,8] *In vivo* and *in vitro* studies have shown its predominant antitumor and immunization effects.[9-11] KM dilates the coronary vessels, reduces myocardial ischemic symptoms, decreases the oxygen demand of cardiac tissue, lowers peripheral blood pressure, and thus has been used clinically as an agent to combat cerebral and cardiovascular diseases.[12-14] Various chemical components such as lectin, steroid, triterpene, sesquiterpene lactone, and flavonoids, which have antitumor, hypotensive and antioxidant activities, have been identified from the extracts of KM.[15,16] Although the pharmacological actions of KM in the CNS are not well characterized, we hypothesized that KM might protect neurons against neurodegenerative disease such as AD and ischemia on

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the basis of its traditional use on the CNS and antioxidant activity. We demonstrated that KM exerted a significant inhibitory action on hydrogen peroxide-induced cultured neuronal damage.[17] Therefore, the aim of the present study was to investigate the protective effect of KM against Aβ (25-35)-induced neurotoxicity in cultured rat cortical neurons and impaired memory by the intracerebroventricular (i.c.v.) injection of Aβ (25-35) in mice.

Experimental

Preparation of KM methanol extract and reagents – KM was purchased from an oriental drug store in Daegu, Korea, and authenticated by Professor K.-S. Song, Kyungpook National University and voucher specimen was deposited in College of Veterinary Medicine, Chungbuk National University, Korea. The dried KM (2 kg) was extracted with methanol (2 L × 24 × h × 3) at room temperature. The extract was filtered, and the filtrate was concentrated under reduced pressure using a rotary evaporator. The yield was about 10% (W/W). The methanolic extract from KM showed one major peaks in HPLC analysis using RP-18 column (4.6 × 250 mm, 5 μm, Eclipse XDB-C18, Agilent, CA, USA). It was identified as homoflavoyadorinin B, by comparing its retention time and UV pattern with those of authentic sample (Fig. 1). Authentic homoflavoyadorinin B was obtained from GHAM Bio-Pharm, Daegu, Korea. Typically, dried KM used in this experiment contained 0.08~0.09% of homoflavoyadorinin B. Aβ (25-35) was purchased from Bachem (Bubendorf, Switzerland). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified MEM and poly-$_L$-lysine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 dye, Fluo-4 AM and 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from Gibco (Logan, UT, USA).

Experimental animals – Pregnant Sprague-Dawley (SD) rats and male ICR mice were purchased from Daehan Biolink Co. Ltd. (Chungbuk, Korea), and housed singly and in groups of 10, respectively, in environmentally controlled rooms at 22 ± 2 °C, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the regulations for the Care and Use of Laboratory Animals of the Animal Ethnical Committee of

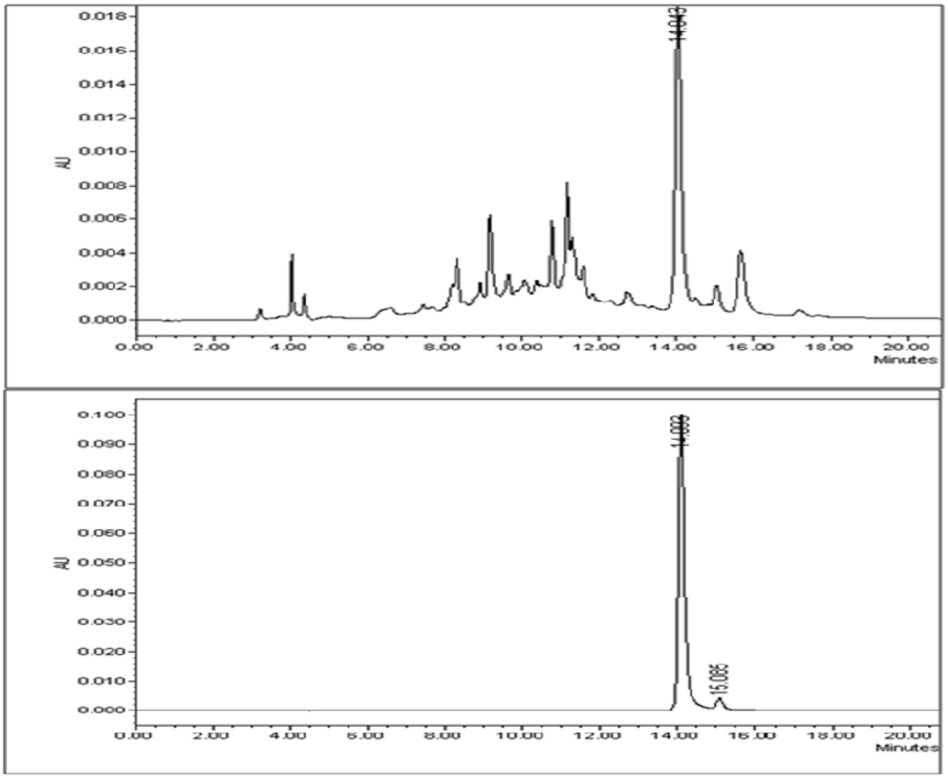

Fig. 1. Representative HPLC chromatogram of the methanol extract of KM (upper) and standard homoflavoyadorinin B (lower). Detection; UV336 nm.

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Induction of neurotoxicity in primary cultures of rat cerebral cortical neurons – Primary cortical neuronal cultures were prepared using embryonic day 15 to 16 SD rat fetuses, as previously described.[18] Neurotoxicity experiments were performed on neurons after 4-5 days in culture. The cultured neurons were treated with 10 μM Aβ (25-35) in serum-free DMEM at 37 ºC for 24 h (unless otherwise indicated) to produce neurotoxicity. Aβ (25-35) stock solution of 2 mM was prepared in sterile distilled water, stored at −20 ºC, and incubated for more than 2 days at 37 ºC to aggregate before use. KM was dissolved in methanol with the concentration of 50 mg/ml and further diluted with experimental buffers. The final concentration of methanol was 0.1% or less, which did not affect cell viability. For each experiment, KM or its vehicle was added 15 min prior to treatment with Aβ (25-35); and it was also present in the buffer during the Aβ (25-35) incubation.

Measurements of Aβ (25-35)-induced neuronal death and intracellular biochemical changes – An MTT assay and Hoechst 33342 staining were performed to measure neuronal cell death and apoptosis, respectively, 24 h after exposure of cultured neurons to 10 μM Aβ (25-35), as previously described.[18] Changes in $[Ca^{2+}]_i$ were measured with Fluo-4 AM, a Ca^{2+}-sensitive fluorescent dye, using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) with 488-nm excitation argon laser and 515-nm longpass emission filters. The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of H_2DCF-DA, and a laser scanning confocal microscope (MRC1024ES, Biorad, Maylands, UK) with 488-nm excitation and 510-nm emission filters were used to monitor the generation of ROS in neurons treated with 10 μM Aβ (25-35) for 24 h. Glutamate secreted into the incubation medium for 6 h from 10 μM Aβ (25-35) treated cells was quantified by a high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (MF series, BAS, IN, USA).[18]

Measurement of memory impairment – I.c.v. injection of the aggregated Aβ (25-35) (8 nmol) was performed to induce memory impairment in mice, as previously described.[19] KM (25 and 50 mg/kg) suspended in distilled water was orally administered to 5-week-old ICR mice 30 min before the injection of Aβ (25-35) and further administered daily for 7 days. Passive avoidance apparatus (Gemini Avoidance System, SanDiego, CA, USA) was used to measure memory acquisition according to the method previously described.[19] Mice were trained on step-through passive avoidance task 30 min after administration of KM on day 7 of i.c.v. injection of Aβ (25-35). Retention trial was given 24 h after the acquisition trial. To discard the possible effects of Aβ (25-35) on motor function, the animals were subjected to an activity monitor, a photobeam monitoring system (AM1051, Benwick Electronics, Benwick, UK), and rota-rod apparatus (Daejong Inc., Seoul, Korea) to examine locomotor activity and motor coordination, respectively. Each mouse was placed in the center of the activity cage, and the total number of mobile count was registered for 5 min and then put on the rota-rod for 2 min.

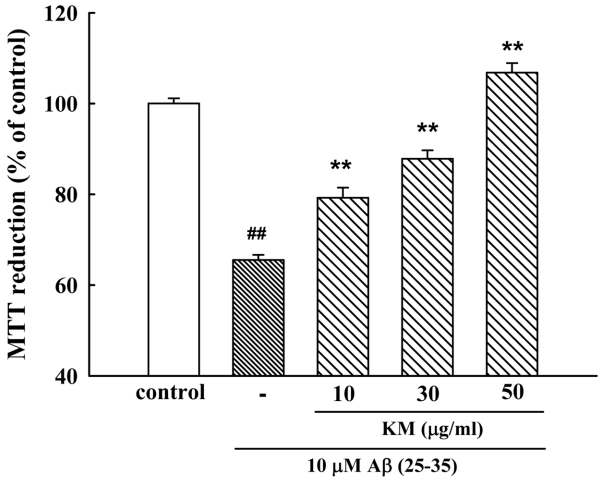

Fig. 2. Inhibitory effect of KM on Aβ (25-35)-induced cell death in cultured cortical neurons. Neuronal death was measured using the MTT assay. The MTT absorbance from non-treated cells was normalized to 100%. Results are expressed as mean ± S.E.M. of data obtained from at least four independent experiments performed in two to four wells. $^{\#\#}P < 0.01$ *vs.* control; $^{**}P < 0.01$ *vs.* 10 μM Aβ (25-35).

Statistical analysis – Data were expressed as the mean ± S.E.M., and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. $P < 0.05$ was considered to be significant.

Results

KM protected Aβ (25-35)-induced neuronal apoptotic death – When cortical neurons were exposed to 10 μM Aβ (25-35), MTT absorbance was 65.5 ± 1.1% of that of the untreated controls, indicating that Aβ (25-35) caused neuronal death. In cultures treated with KM (10, 30 and 50 μg/ml), Aβ (25-35)-induced neuronal death was significantly reduced showing 106.8 ± 2.1% MTT absorbance with 50 μg/ml KM (Fig. 2). This result suggests that KM could also prevent neuronal cell death caused by serum-free stress condition, since neurons incubated in serum-free DMEM for 24 h showed MTT absorbance of 88.8 ±

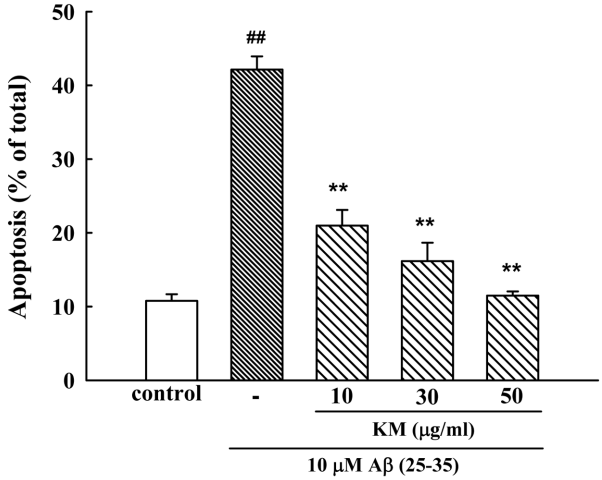

Fig. 3. Inhibitory effect of KM on Aβ (25-35)-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted from 5 to 6 fields per well. Results shown are apoptotic cells as a percentage of the total number of cells and expressed as the mean ± S.E.M. of data obtained from three independent experiments performed in two wells. $^{\#\#}P < 0.01$ *vs.* control; $^{**}P < 0.01$ *vs.* 10 μM Aβ (25-35).

4.7% compared to that of neurons incubated in the normal growth medium containing serum (data not shown).

Hoechst 33342 staining was used to detect condensed or fragmented DNA, which was indicative of Aβ (25-35)-induced neuronal apoptotic death. Treatment of neurons with 10 μM Aβ (25-35) produced apoptosis in 42.2 ± 1.8%, compared with 10.8 ± 0.9% apoptotic neurons in control cultures. The addition of KM (10, 30 and 50 μg/ml) significantly decreased the Aβ (25-35)-induced apoptotic cell death to 11.5 ± 0.6% apoptotic neurons in 50 μg/ml KM (Fig. 3).

KM inhibited Aβ (25-35)-induced elevation of $[Ca^{2+}]_i$, ROS generation, and glutamate release – Accumulation of $[Ca^{2+}]_i$ may play a critical role in the early stages of Aβ-induced neuronal cell damage. In cultured cortical neurons, $[Ca^{2+}]_i$ rapidly increased in response to treatment with 10 μM Aβ (25-35) and maintained a plateau for over 6 min, showing a maximal fluorescence intensity of approximately 190 compared to a base of 100. In contrast, pretreatment with KM (50 μg/ml) significantly inhibited the increase of $[Ca^{2+}]_i$ induced by Aβ (25-35) (Fig. 4). KM did not affect the basal $[Ca^{2+}]_i$ (Data not shown).

In H_2DCF-DA-loaded cerebral cortical neurons, treatment with 10 μM Aβ (25-35) for 24 h increased the fluorescence intensity approximately 5-fold to 207.6 ± 12.6 compared to control neurons (44.1 ± 3.6), indicating the generation of ROS. KM (10, 30 and 50 μg/ml) significantly blocked the Aβ (25-35)-induced increase of

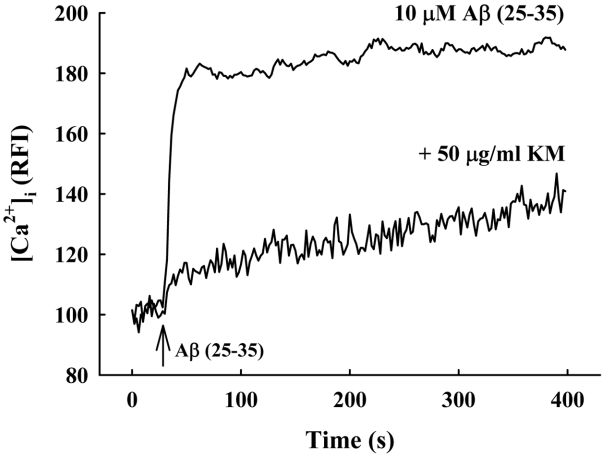

Fig. 4. Inhibitory effect of KM on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using a laser scanning confocal microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least three independent experiments.

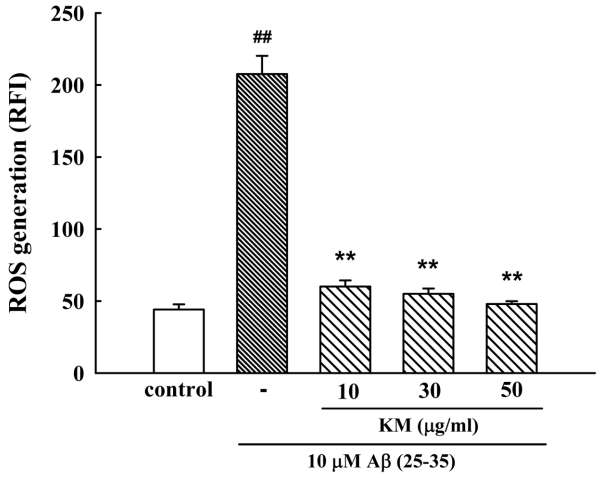

Fig. 5. Inhibitory effect of KM on Aβ (25-35)-induced ROS generation in cultured cortical neurons. (A) Representative photomicrographs of fluorescence density in H_2DCF-DA loaded cultured neurons, observed by a confocal microscope. (B) Results are expressed as the mean ± S.E.M. of RFI obtained from three independent experiments performed in duplicate. $^{\#\#}P < 0.01$ *vs.* control; $^{**}P < 0.01$ *vs.* 10 μM Aβ (25-35).

fluorescence intensity showing 47.9 ± 2.0 in 50 μg/ml (Fig. 5). KM in all doses used in this experiment almost completely inhibited the Aβ (25-35)-induced ROS generation without dose-dependency. This result may imply that the lowest dose of 10 μg/ml was enough to deplete ROS, but not enough to completely block apoptotic death for 24 h.

As shown in Fig. 6, 10 μM Aβ (25-35) markedly elevated the basal glutamate level in culture medium from 0.43 ± 0.09 μM of control neurons to 1.51 ± 0.43 μM.

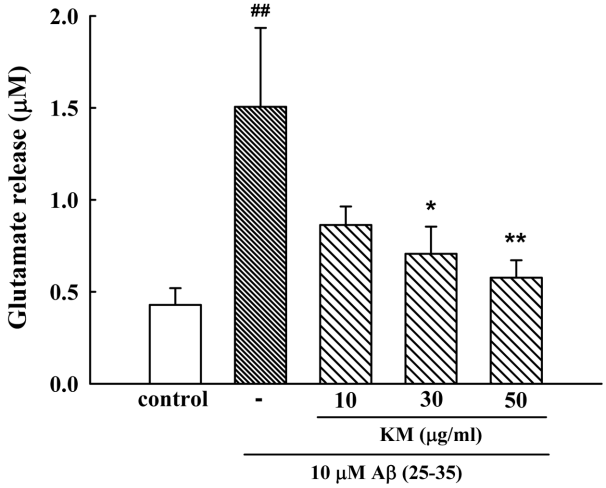

Fig. 6. Inhibitory effect of KM on Aβ (25-35)-induced glutamate release in cultured cortical neurons. The amount of released glutamate for 6 h was measured by HPLC with ECD. Results are expressed as the mean ± S.E.M. of data obtained from three independent experiments performed in three or four wells. $^{\#\#}P < 0.01$ *vs.* control; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* 10 μM Aβ (25-35).

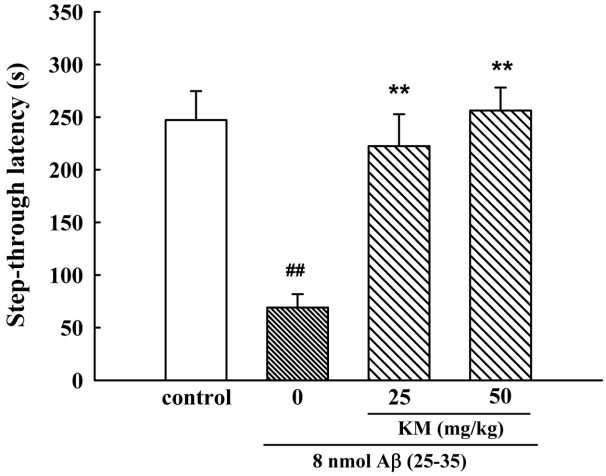

Fig. 7. Protective effect of KM on Aβ (25-35)-induced memory impairments in mice. The learning and memory performance was assessed by the passive avoidance test. Values represent the mean ± S.E.M. of step through latency (n = 10-13). $^{\#\#}P < 0.01$ *vs.* vehicle-treated control group. $^{**}P < 0.01$ *vs.* 8 nmol Aβ (25-35)-treated group.

The addition of KM (10, 30 and 50 μg/ml) significantly inhibited the Aβ (25-35)-induced elevation of glutamate release to 0.58 ± 0.09 μM in 50 μg/ml KM.

KM inhibited Aβ (25-35)-induced memory impairment in mice – In a preliminary experiment, 50 mg/kg KM was enough to completely inhibit Aβ (25-35)-induced memory impairment. Therefore, the doses of 25, an half of maximal dose, and 50 mg/kg were selected for this study. In acquisition trial, the step-through latency did not differ among four groups (control, 8 nmol Aβ (25-35), Aβ (25-35) + 25 mg/kg KM, and Aβ (25-35) + 50 mg/kg KM; data not shown). The step-through latency of Aβ (25-35)-treated group in the retention trial significantly decreased to 69.2 ± 12.7 s, compared to 247.2 ± 27.5 s of control group, indicating that Aβ (25-35) induced memory impairment in mice. Chronically administered KM completely protected the memory impairment produced by Aβ (25-35). The step through latency in KM-administered groups was 222.4 ± 30.3 s and 256.2 ± 21.9 s at the doses of 25 and 50 mg/kg, respectively (Fig. 7).

To elucidate whether KM affects general motor functions, spontaneous locomotor activity and motor coordination were measured in mice. The results showed that both KM and Aβ (25-35) did not significantly affect locomotor and rota-rod activity (Table 1). These results indicate that KM as well as Aβ (25-35) has no effect on general motor function and the improvement of memory was not associated with immobility, which might be caused by KM administration.

Table 1. Effect of KM on locomotor activity and motor coordination in mice

Group	Dose	Locomotor activity[a] (% of prevalue)	Number of mice which fell down
Control	–	76.7 ± 7.1	0
Aβ (25-35)	8 nmol	65.5 ± 8.0	0
+ KM	25 mg/kg	74.4 ± 7.6	0
+ KM	50 mg/kg	72.7 ± 11.8	0

Locomotor activity and motor coordination using an activity monitor and a rota-rod apparatus, respectively, were measured before and after passive avoidance test. [a]Results are expressed as mean ± SEM (n = 10-13).

Discussion

Aβ is the major component of senile plaques and is a major contributor to the pathogenesis of AD. Aβ-induced neurotoxicity has been attributed to Ca^{2+} influx, generation of ROS, and activation of caspase-3.[2-4] We have also demonstrated that Aβ (25-35) caused elevation of $[Ca^{2+}]_i$, glutamate release, ROS generation and then apoptotic neuronal cell death, which was blocked by the treatment with (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine (MK-801), an NMDA receptor antagonist, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME), a nitric oxide synthase (NOS) inhibitor.[18,20] The primary event following Aβ (25-35) treatment of cultured neurons has been suggested to be Ca^{2+} influx, apparently via L-type voltage-dependent Ca^{2+} channel (L-VDCC), since blockage of this channel and/or Ca^{2+} chelation prevents all other

consequences.[21,22] Furthermore, Aβ (25-35)-induced elevation of $[Ca^{2+}]_i$ and neurotoxicity were inhibited by MK-801, suggesting Ca^{2+} influx through NMDA receptor-coupled L-VDCC plays a critical role in the neurotoxicity.[18,20,23,24] In the present study, Aβ (25-35) elicited a rapid and significant $[Ca^{2+}]_i$ increase, which was blocked by KM. KM also significantly inhibited the Aβ (25-35)-induced glutamate elevation. These results indicate that the sustained inhibition on $[Ca^{2+}]_i$ elevation by KM resulted in the decrease of the Aβ (25-35)-induced glutamate release.

KM decreased the Aβ (25-35)-induced increase of ROS generation in the present study. It has been reported that vitamin-E, an antioxidant, blocked the Aβ-induced generation of ROS, but not Ca^{2+} influx, and reduction of extracellular Ca^{2+} inhibited the Aβ-induced increase in intracellular Ca^{2+} as well as generation of ROS, indicating that ROS generation is a consequence of Ca^{2+} accumulation.[25] Our previous data demonstrated that the significant increase of ROS generation took more than 1 h, while the elevation of $[Ca^{2+}]_i$ occurred within seconds after the treatment with 10 μM Aβ (25-35).[20] These results together indicate that KM inhibited the Aβ (25-35)-induced ROS generation via the blockade of $[Ca^{2+}]_i$ increase in the present study. Furthermore, cultured cortical neurons exposed to 10 μM Aβ (25-35) for more than 24 h showed increased chromatin condensation, a typical feature of apoptotic cell death, and KM protected the neuronal cell against Aβ (25-35)-induced apoptotic death. It is thus concluded that KM may prevent the Aβ (25-35)-induced apoptosis of neuronal cells by interfering with the increase of $[Ca^{2+}]_i$, and then by inhibiting glutamate release and ROS generation in cortical neurons.

Aβ (25-35) preferably induces impairments of spatial and non-spatial short-term memory, and these effects remain evident up to 6 months even after a single i.c.v. injection of the peptide.[26] Memory impairment in passive avoidance test was also confirmed in mice 7 days after the i.c.v. injection of Aβ (25-35) in the present work. Chronic treatment with KM (25 and 50 mg/kg) effectively protected the Aβ (25-35)-induced memory deficit. This result is consistent with its protective effect on Aβ (25-35)-induced neurotoxicity in *in vitro*. Studies have indicated that oxidative stress in the pathology of AD is responsible for the onset of the cognitive dysfunction as well as the progression of the disease.[2,27] High level of Aβ is responsible for the increased appearance of ROS such as superoxide (O_2^-) and NO in AD, which produce $ONOO^-$ by a rapid interaction.[28,29] A scavenger of $ONOO^-$ was proved to protect against Aβ (25-35)-induced memory impairment.[30] Aβ (25-35) (10 μM) significantly increased the ROS level in cultured neurons and this was inhibited by KM in the present study. A variety of anti-oxidants such as lectin, alkaloids, triterpens, and flavonoids have been isolated from KM.[15,16] Furthermore, the antioxidant effect of a main component of KM, homoflavoyadorinin B, has been also proposed.[31] Therefore, it is possible that the favorable effect of KM on Aβ (25-35)-induced cognitive deficits is attributable to the inhibition on ROS generation by these components. Meanwhile, the inhibition by KM on Aβ (25-35)-induced increase of $[Ca^{2+}]_i$ could be associated with the protection of memory impairment, because memantine was demonstrated to have therapeutic benefits on AD due to its affinity for the NMDA receptor Ca^{2+} channel.[32] In addition, the possible contribution to the neuroprotective efficacy in AD through the blocking effect on the VDCC by the cholinesterase inhibitor, donepezil, was also reported.[33] The protection by KM against Aβ (25-35)-induced memory deficit was not due to possibly caused immobility as KM did not affect general motor function in mice. Further studies should be performed to clarify the mechanism.

Although Aβ is believed as a major cause in the pathophysiology of AD,[34] it is still controversial whether increase of Aβ formation is sufficient to cause nerve cell degeneration in AD. However, neurotoxicity of Aβ has been demonstrated in both *in vitro* and *in vivo*,[35,36] as probed in the present study. The present study demonstrated that methanol extract of KM significantly inhibited Aβ (25-35)-induced cultured neuronal cell death and markedly improved memory impairment induced by Aβ (25-35) in mice. As far as we know, this is the first report to demonstrate the neuroprotective effect of KM against Aβ-induced toxicity. Mistletoe has been reported to bind to microglia and AD plaque glycoproteins in human brains.[37] These results suggest evidences of the possibility of KM having neuroprotective effect in AD brains with the prevention of the disease progression. In conclusion, the present study provides a mechanistic explanation that KM has protective effect against Aβ (25-35)-induced neuronal cell death and memory impairment. The protection against Aβ (25-35)-induced neurotoxicity by KM may provide the pharmacological basis of its therapeutic promise in prevention of neurodegeneration in AD.

Acknowledgements

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