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A modified preparation (LMK03) of the oriental medicine Jangwonhwan reduces $A\beta_{1-42}$ level in the brain of Tg-APPswe/PS1dE9 mouse model of Alzheimer disease

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ABSTRACT

Ethnopharmacological relevance: The oriental medicine Jangwonhwan, which is a boiled extract of 12 medicinal herbs/mushroom, has been prescribed for patients with cognitive dysfunction. Recently, a modified recipe of Jangwonhwan (LMK02-Jangwonhwan) consisting of seven medicinal plants/mushroom, was shown to have a therapeutic potential to ameliorate AD-like pathology.
Aim of the study: It was investigated whether a further reduction of Jangwonhwan (LMK03-Jangwonhwan) retains the potency to suppress the AD-like pathology.
Materials and Methods: The transgenic mice of Alzheimer disease, Tg-APPswe/PS1dE9, were fed LMK03-Jangwonhwan consisting of two of the herbs, white *Poria cocos* (Schw.) Wolf and *Angelica gigas* Nakai, which could protect the AD-like pathology at 300 mg/kg/day of dose for 3 months. In vitro cell biological study, immunohistological and ELISA (enzyme-linked immunosorbent assay) analyses were used to assess its neuroprotective effects against Aβ-induced cell death, and the Aβ accumulation and plaque deposition in the brain.
Results: In vitro study with SH-SY5Y neuroblastoma cells showed that LMK03-Jangwonhwan could protect from cytotoxicity induced by hydrogen peroxide or oligomeric $A\beta_{1-42}$. Tg-APPswe/PS1dE9 mice were administered LMK03-Jangwonhwan at 300 mg/kg/day for 3 months from 4.5 months of age. Immunohistological and ELISA analyses showed that LMK03-Jangwonhwan partially reduced $A\beta_{1-42}$ and $A\beta_{1-40}$ levels and β-amyloid plaque deposition in the brain of Tg-APPswe/PS1dE9 mice. However, LMK03-Jangwonhwan poorly suppressed accumulation of reactive oxidative stress in the hippocampus of Tg-APPswe/PS1dE9 mice and inefficiently improved the expression of phospho-CREB and calbindin, the cellular factors that were down-regulated in AD-like brains.
Conclusions: These results suggest that LMK03-Jangwonhwan has a potency to inhibit AD-like pathology at a detectable level, but LMK03 is not likely to retain the major ability of LMK02-Jangwonhwan to modify AD pathology in several AD-related molecular parameters.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive declines and histopathological changes including plaque deposition and neuronal loss. Genetic mutations in β-amyloid precursor protein (APP), presenilin-1 (PS1), and PS2 (Kim and Tanzi, 1997; Selkoe, 1998) produce familial causes of AD (Kowalska et al., 2004) and the ApoE4 allele enhances the risk (Bu, 2009; Zhong and Weisgraber, 2009). However, most AD cases are caused by non-genetic environmental factors (Ferri et al., 2005). Previous studies have reported that abnormal metabolism of APP is critical for AD pathogenesis (Hardy and Higgins, 1992; Lee et al., 2007), Appropriately, much investigative effort has been directed at developing anti-AD drugs by targeting cellular factors regulating Aβ production (Cacabelos, 2008; Francis et al., 2010). However, development of mechanism-driven drugs has been slow, and currently available AD drugs either enhance the synaptic activity of cholinergic neurons or act on the *N*-methyl-D-aspartic acid receptor (Areosa et al., 2006; Chohan and Iqbal, 2006), and thus they are anti-symptomatic. Concerning the slow progress and the

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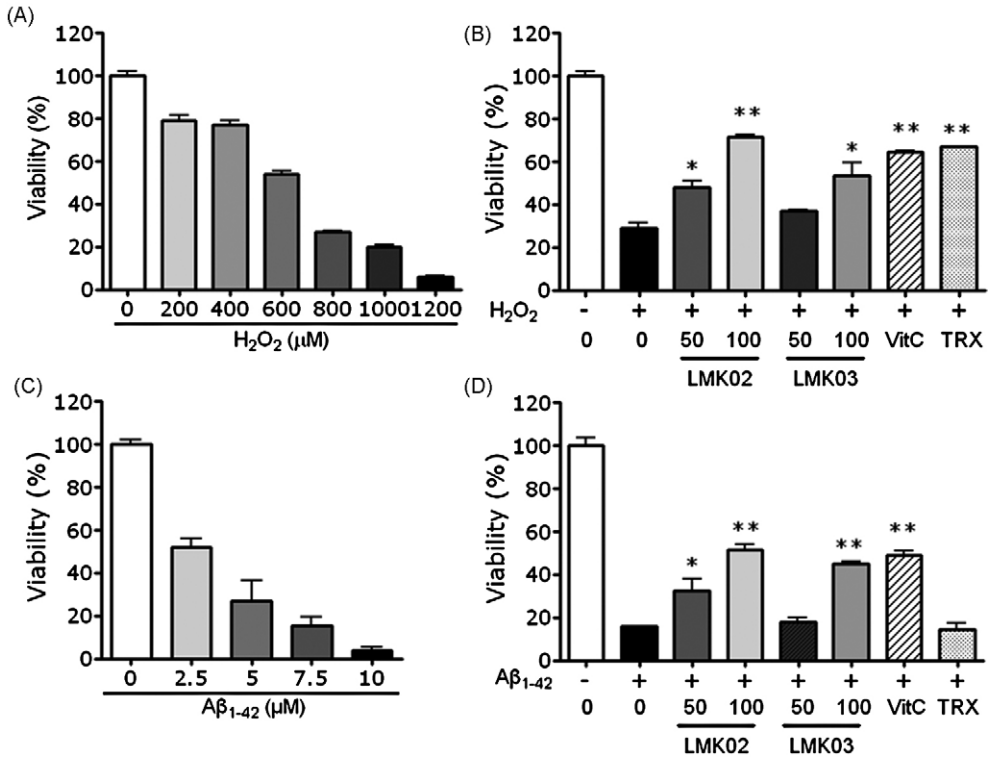

Fig. 1. LMK03 suppressed cytotoxicity of SH-SY5Y neuroblastoma cells induced by H_2O_2 or $A\beta_{1-42}$. (A and B) H_2O_2-induced cell death profile in SH-SY5Y cells. (A) LMK03 inhibited the H_2O_2-induced death of SH-SY5Y cells in a dose dependent manner. (B) Both LMK02 and LMK03-Jangwonhwan inhibited H_2O_2 (800 μM)-induced cell death. (C and D) $A\beta_{1-42}$-induced cell death curve in SH-SY5Y cells. (C) LMK03 suppressed the cell death of SH-SY5Y induced by $A\beta_{1-42}$ in a dose dependent manner. (D) Both LMK02 and LMK03-Jangwonhwan inhibited $A\beta_{1-42}$(7.5 μM)-induced cell death. $A\beta_{1-42}$ was pre-incubated at 4°C for 7 days before use. LMK02 and LMK03 indicated by 50 or 100 were treated at 50 mg/ml or 100 mg/ml of dose. Cell viability was measured using a WST-1 assay after 24 h of drug treatment. VitC, vitamin C (200 μM); TRX, trolox (200 μM). Data points represent means ± SEM ($n = 6$). * and ** denote differences between the indicated group at $p < 0.05$ and $p < 0.01$, respectively (one-way ANOVA, Newman–Keuls multiple comparisons test).

complexity of the known pathophysiology of AD, several new approaches to AD drugs including supplementary and alternative methods have been proposed (Anekonda and Reddy, 2005; Middleton and Yaffe, 2009; Noorbakhsh et al., 2009).

AD is a multifactorial disease in terms of etiology and pathophysiology. AD brains feature distinct pathological changes including enhanced Aβ accumulation, senile plaque deposition, neurofibrillary tangle formation, and neuronal loss. AD brains also display various biochemical and histological alterations, which include down-regulated mitochondrial function (Onyango and Khan, 2006; Wang et al., 2009), and up-regulation of oxidative stress (Frank and Gupta, 2005; Zhu et al., 2007; Lee et al., 2009), the calcium regulatory protein calbindin (Cao et al., 2007), phosphorylation of cAMP response element-binding (CREB) protein (Lee et al., 2006; Ma et al., 2007), and factors for inflammatory responses (Finch and Morgan, 2007). Many of these changes accumulated in the brain in AD and manifest as progressively deteriorating. A number of recent studies with AD mouse models have implicated therapeutic potential for AD (Ghosh et al., 2002; Hills and Vacca, 2007; Imbimbo, 2008). The possible routes include genetic or pharmacological suppression of Aβ production (Schenk et al., 1999; Luo et al., 2001; Panza et al., 2009), activation of Aβ clearance (Fox et al., 2005; Gilman et al., 2005; Solomon, 2007), or supplementation of anti-oxidant capacity (Markesbery and Carney, 1999; Seshadri et al., 2002). These results suggest that various therapeutic methods targeting the levels from pathogenic mechanisms to AD symptoms need to be considered.

Jangwonhwan is a traditional medicine prescribed for over 400 years for the treatment of amnesia and to help enhance cognition (Huh, 1610). However, the neurobiological mechanism of its action has not been explored until recent years. Traditional Jangwonhwan is prepared by a decoction of 12 medicinal plants and mushroom, including red *Panax ginseng* C.A Meyer. In a recent study, we demonstrated the therapeutic potential in AD-like brain of a modified Jangwonhwan, LMK02, which is prepared with a boiled extract of seven medicinal plants/mushroom inclding red *Panax ginseng* C.A. Meyer, *Acorus gramineus* Soland, white *Poria cocos* (Schw.) Wolf, *Angelica gigas* Nakai, *Ophiopogon japonicas* Ker-Gawl, *Scrophularia buergeriana* Miquel, and *Thuja orientalis* L. In the present study, we investigated whether a shortened version of Jangwonhwan, LMK03-Jangwonhwan, prepared with a boiled extract of two medicinal plants, white *Poria cocos* (Schw.) Wolf and *Angelica gigas* Nakai retains the potency to suppress AD-like pathology.

2. Materials and methods

2.1. AD murine model

Tg-APPswe/PS1dE9 transgenic mice overexpressing human mutated APP and PS1 (APPswe/PS1dE9) were initially purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred to maintain in C57BL6 x C3H F1 hybrid as described previously (Jankowsky et al., 2001; Seo et al., 2010). The mice were housed in pairs in normal plastic cages with free access to food and water in a temperature- and humidity-controlled environment under a 12 h light/dark cycle (lights on at 7 a.m.), and were allowed a diet of lab chow and water *ad libitum*. Tg-APPswe/PS1dE9 mice at 4.5 months of age were randomized into control ($n = 7$) and LMK03-Jangwonhwan ($n = 8$) groups. Lab chow containing

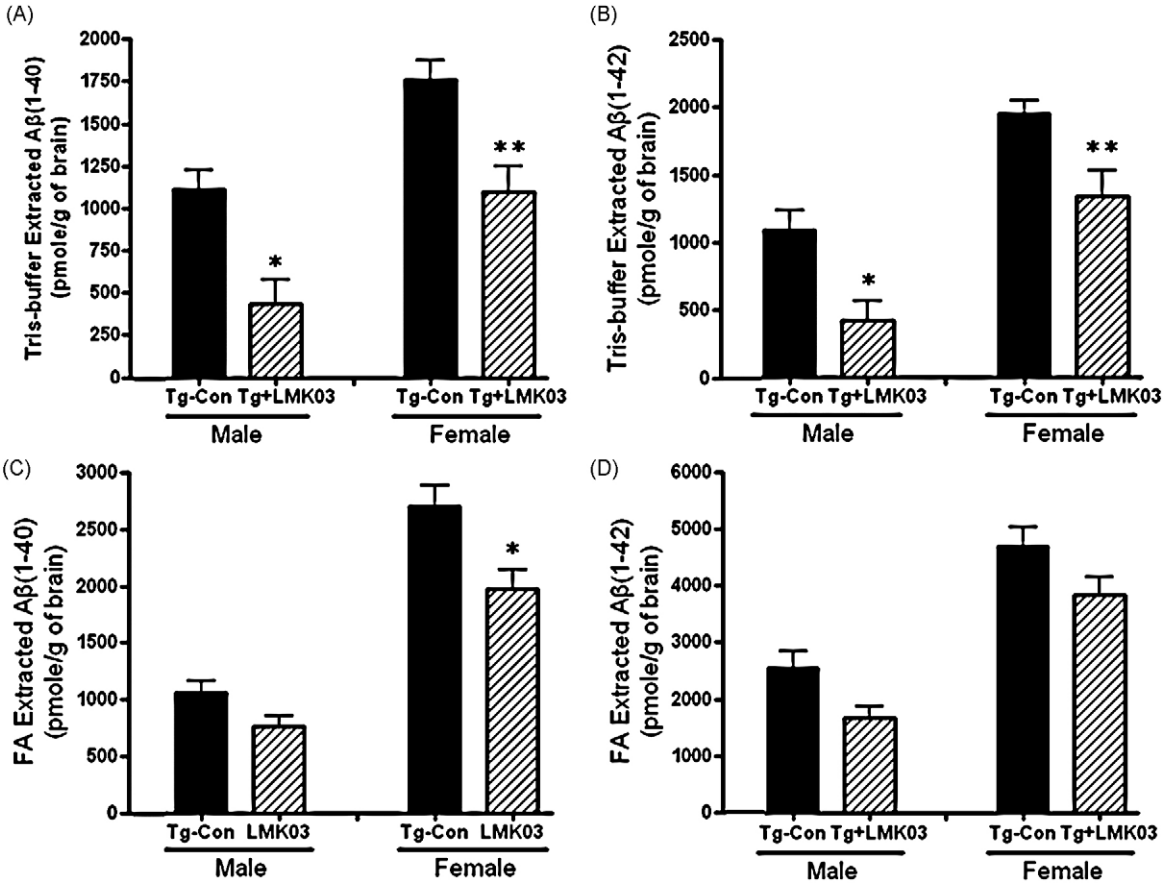

Fig. 2. Administration of LMK03 reduced $A\beta_{1-42}$ and $A\beta_{1-40}$ levels in the brains of Tg-APPswe/PS1dE9 mice. (A–D) ELISA analyses showing that the amounts of Tris buffer-extracted forms (A and B) and formic acid-extracted forms (C and D) of $A\beta_{1-40}$ (A and C) and $A\beta_{1-42}$ (B, D) in the prefrontal cortex of Tg-APPswe/PS1dE9 mice at 7.5 months of age. ELISA analysis was performed for individual animals in duplicate. Data points represent the means ± SEM ($n = 6$). * and ** denote differences between two groups at the $p < 0.05$ and $p < 0.01$ levels, respectively (one-way ANOVA, Newman–Keuls multiple range test). Tg-Con, Tg-APPswe/PS1dE9 mouse control; Tg + LMK03, Tg-APPswe/PS1dE9 mice treated with LMK03.

LMK03 was prepared for a mouse to have 300 mg/kg/day and was gamma-irradiated. The mice were fed lab chow containing LMK03-Jangwonhwan for 3 months beginning at 4.5 months of age. Non-transgenic littermate control mice were also prepared and fed control food. All animals were handled in accordance with the animal care guidelines of Ewha Womans University School of Medicine.

2.2. Preparation of LMK03-Jangwonhwan

Preparation of LMK02-Jangwonhwan was described previously (Seo et al., 2010). LMK03-Jangwonhwan was prepared similarly. Briefly, a decoction of white *Poria cocos* (Schw.) Wolf (Polyporaceae family; sclerotium, 48 g) and *Angelica gigas* Nakai (Apiaceae family; root, 48 g) was prepared by following the standard procedure detailed in The Korean Pharmacopoeia Eighth Edition (Korea Food and Drug Administration, 2008). Each of the dried herbs (96 g in sum) was mixed and cut into small pieces. After adding 840 ml of filtrated drinking water, the ground material was boiled for 3 h at 100 °C in a reflux condenser (MyungSung Stainless Steel, Seoul, Korea). The boiled extract was filtered through Whatman No. 4 filter paper having 20–25 μm pore-size) and concentrated by vacuum evaporation using a Rotavapor R-200, (Bűch, Germany) at 60 °C. The final yield from the whole procedure was approximately 15.5 g of the dried mixture. The dried material was stored at −80 °C until use.

2.3. Cell culture works

The SH-SY5Y neuroblastoma cell line was cultured as described previously (Yu et al., 2003; Seo et al., 2010). Briefly, SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), penicillin (20 units/ml), and streptomycin (20 mg/ml) at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2. $A\beta_{1-42}$ was purchased from U.S. Peptide (Rancho Cucamonga, CA, USA). $A\beta_{1-42}$ was dissolved in dimethyl sulfoxide, diluted to 100 μM in phosphate-buffered saline (PBS), and then incubated at 4 °C for 7 days before use. For assessment of cell viability, cells were incubated with hydrogen peroxide (H_2O_2) or $A\beta_{1-42}$ in serum-free DMEM. Cell viability was determined using a WST-1 assay as described previously (Cho et al., 2003; Seo et al., 2010).

2.4. Enzyme-linked immunosorbant assay (ELISA) of Aβ

ELISA to quantify $A\beta_{1-42}$ and $A\beta_{1-40}$ were performed as described previously (Lee et al., 2009; Seo et al., 2009). Briefly, the left prefrontal cortex was homogenized in Tris-buffered saline (20 mM Tris and 137 mM NaCl, pH 7.6) in the presence of protease inhibitors (Complete Mini; Roche, Indianapolis, IN, USA). Homogenates were centrifuged at 100,000 × *g* for 1 h at 4 °C, and the supernatant was used to quantify the soluble forms of $A\beta_{1-40}$

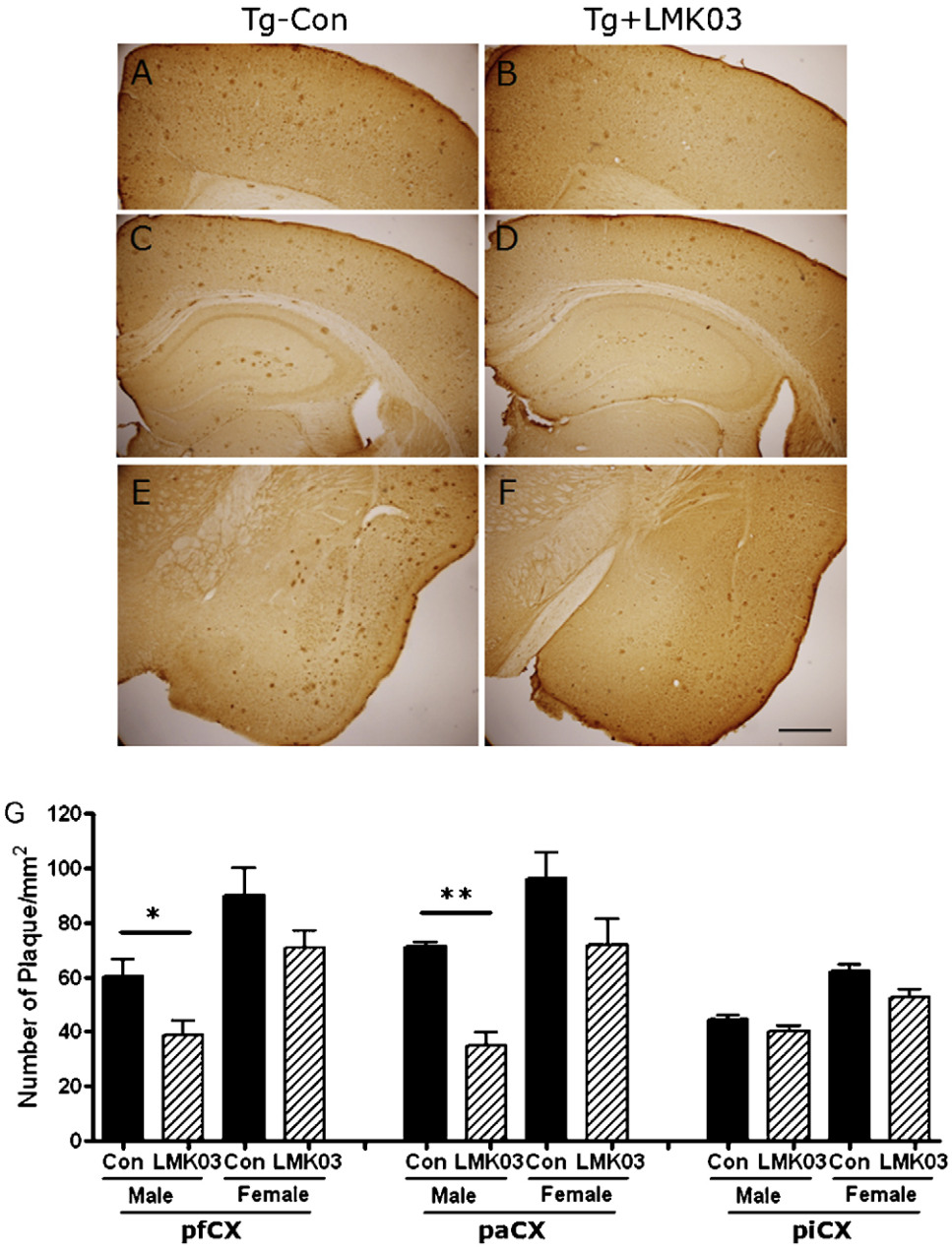

Fig. 3. Administration of LMK03 weakly reduced plaque deposition in the brain of Tg-APPswe/PS1dE9 mice. (A–F) Photomicrographs showing anti-Aβ antibody (Bam-10)-stained prefrontal cortex (A and B), parietal cortex and hippocampus (C and D), and piriform cortex (E and F) of Tg-APPswe/PS1dE9 control mice (Tg-Con; A, C, and E), and Tg-APPswe/PS1dE9 mice fed LMK03 (400 mg/kg/day) (Tg + LMK03; B, D, and F). All images were from female. Scale bar, 500 μm. (G) Quantification of plaque numbers in the superior prefrontal cortex (pfCX), parietal cortex (paCX), and piriform cortex (piCX) of Tg-APPswe/PS1dE9 mice with LMK03 (LMK03) and their Tg-APPswe/PS1dE9 control (Con). Microscopic images of anti-Aβ antibody-stained sections were converted into digital images and plaque numbers were counted using the TOMORO ScopeEye 3.6 program. Data are presented as the means ± SEM. (n = 6–8 animals). * and ** denote differences between two groups at the $p < 0.05$ and $p < 0.01$ levels, respectively (one-way ANOVA, Newman–Keuls multiple range test).

and $A\beta_{1-42}$. The pellet was sonicated in 70% formic acid, centrifuged as above, and the supernatant was collected. The formic acid extract was neutralized with 1 M Tris–HCl buffer (pH 11) by dilution (1:20). The final assays were performed using human β-amyloid $A\beta_{1-40}$ or $A\beta_{1-42}$ Colorimetric Sandwich ELISA kits (BioSource, Camarillo, CA, USA) following the manufacturer's instructions.

2.5. Measurements of lipid peroxidation

Lipid peroxidation levels were assessed by measuring malondialdehyde (MDA) levels using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR, USA), as described previously (Im et al., 2006; Seo et al., 2010). In brief, hippocampal homogenates were prepared in 4 volumes of ice-cold 20 mM PBS (pH 7.4) containing 5 mM butylated hydroxytoluene. After centrifugation at 3000 g for 10 min at 4 °C, the supernatant was recovered. Two hundred microliters of supernatant were mixed with 10 μl of probucol and 640 μl of diluted R1 reagent (diluted 1:3 of methanol:*N*-methyl-2-phenylindole) provided in the Bioxytech MDA-586 kit and 150 μl of 12 N HCl. Each reaction was then incubated at 45 °C for 60 min and centrifuged at 10,000 × *g* for 10 min. The supernatant was used to measure the absorbance at 586 nm. MDA levels were normalized with respect to protein concentration and expressed as a percentage of the sham control value. The protein concentration

was determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.6. Histological examination

Histological examination was conducted as described previously (Seo et al., 2009, 2010). Briefly, transgenic Tg-APPswe/PS1dE9 mice were sacrificed at 7.5 months of age and perfused with 0.9% saline. The right hemisphere was post-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight, and coronally cut into 40 μm thick sections with a Leica VT 1000S vibratome (Leica Instruments, Nussloch, Germany). Free-floating sections were blocked by 5% normal goat serum, 2% bovine serum albumin, and 2% FBS. A biotinylated horseradish peroxidase system was used for color development. Anti-Aβ antibody Bam-10 (A5213) and anti-calbindin (C9848) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-phospho-CREB (06-519) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). For the quantification of plaque levels, microscopic images of anti-Aβ antibody (Bam-10)-stained superior prefrontal cortex, parietal cortex, and piriform cortex were captured using an Oympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus, Tokyo, Japan). The numbers of plaques were determined using the TOMORO ScopeEye version 3.6 program (Techsan Community, Seoul, Korea).

2.7. Statistical analysis

Two-sample comparisons were carried out using the Student's *t*-test, while multiple comparisons were made using one-way ANOVA followed by the Newman–Keuls multiple range test. All data represent the mean ± SEM, and statistical differences were accepted at the 5% level unless otherwise indicated.

3. Results

The previous finding that LMK02-Jangwonhwan consisting of seven medicinal herbs has a therapeutic potential in AD-like brain prompted the present study to investigate whether a further modified Jangwonhwan, LMK03-Jangwonhwan (LMK03 for short), which was prepared by a boiled extract of white *Poria cocos* (Schw.) Wolf and *Angelica gigas* Nakai, also had similar therapeutic potential. Because LMK02-Jangwonhwan exhibited considerable ability to protect against death of SH-SY5Y neuroblastoma induced by H_2O_2 or $A\beta_{1-42}$ (Seo et al., 2010), we examined whether LMK03-Jangwonhan produces neuroprotective effects in a similar context.

The viability of SH-SY5Y cells treated with H_2O_2 declined in a H_2O_2 dose dependent manner (Fig. 1A), in which 800 μM of H_2O_2 produced approximately 30% of cell viability. The viability of SH-SY5Y cells treated with 800 μM H_2O_2 was improved to 50% and 77% in the presence of 50 mg/ml and 100 mg/ml of LMK03, respectively. The neuroprotective effect by LMK03 was significant, but its effect was slightly weaker than that resulting from the corresponding dose of LMK02-Jangwonhwan (Fig. 1B). The next experiment examined the cell viability profile of SH-SY5Y cells in response to $A\beta_{1-42}$, which was primed for 7 days at 4 °C. SH-SY5Y cells treated with oligomeric $A\beta_{1-42}$ showed a reduction of viability in an $A\beta_{1-42}$ dose dependent manner (Fig. 1C). SH-SY5Y cells treated with 7.5 μM of oligomeric $A\beta_{1-42}$ showed approximately 18% viability, whereas viability was improved to 25% and 55% in the presence of, respectively, 50 mg/ml and 100 mg/ml of LMK03. The results were consistent with the view that the protective ability of LMK03 for SH-SY5Y cells from the cytotoxicity induced by H_2O_2 or $A\beta_{1-42}$ is similar to that of LMK02-Jangwonhwan (Fig. 1D).

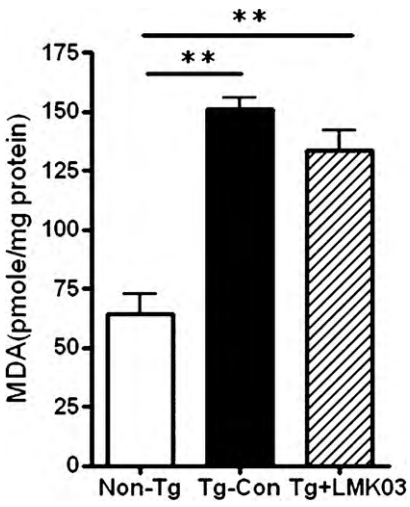

Fig. 4. Administration of LMK03 poorly suppressed lipid peroxidation levels in the brain of Tg-APPswe/PS1dE9 mice. Malondialdehyde (MDA) levels in the brain of Tg-APPswe/PS1dE9 control mice (Tg-Con), Tg-APPswe/PS1dE9 mice fed LMK03 (Tg + LMK03; 300 mg/kg), and their non-transgenic control mice (Non-Tg). Data represent means ± SEM. ($n = 6–8$ animals). ** denotes differences between the indicated group at $p < 0.01$ (one-way ANOVA, Newman–Keuls multiple comparisons test).

To understand the *in vivo* effects of LMK03-Jangwonhwan, Tg-APPswe/PS1dE9 mice were fed lab chow containing LMK03-Jangwonhwan at a dose of 300 mg/kg/day for 3 months from 4.5 to 7.5 months of age. The proportions of white *Poria cocos* (Schw.) Wolf and *Angelica gigas* Nakai among the total weight of dried LMK03-Jangwonhwan were 50% and 50%. Body weight of the LMK03-Jangwonhwan group during the treatment period was similar to the control (data not shown). ELISA analysis with the prefrontal cortex homogenates revealed that the Tris buffer-soluble forms of $A\beta_{1-42}$ and $A\beta_{1-40}$ were partially reduced in Tg-APPswe/PS1dE9 mice treated with LMK03 (Fig. 2A and B). Formic acid-extracted forms of $A\beta_{1-42}$ and $A\beta_{1-40}$ levels tended to be reduced in the LMK03-treated group, but the values were not statistically significant (Fig. 2C and D).

Immunohistochemical analysis with anti-$A\beta_{1-42}$ antibody (Bam-10) revealed that the plaque levels in the prefrontal and parietal cortices of Tg-APPswe/PS1dE9 mice treated with LMK03 were weakly reduced compared with control Tg-APPswe/PS1dE9 mice, but the levels in the piriform cortex or in females were not significantly different from the Tg-APPswe/PS1dE9 control (Fig. 3A–G).

LMK02-Jangwonhwan suppressed the levels of MDA, a by-product of lipid peroxidation, in the brain of Tg-APPswe/PS1dE9 mice at 7.5 months of age (Seo et al., 2010). Next, we examined whether LMK03 produced a similar suppression effect on lipid peroxidation. The levels of MDA in the brain of control Tg-APPswe/PS1dE9 mice were markedly increased, but the enhanced lipid peroxidation level in Tg-APPswe/PS1dE9 mice was not significantly reduced by LMK03 (Fig. 4).

Recent studies by us and others have demonstrated that phospho-CREB levels and calbindin are reduced in the hippocampus of murine models of AD (Leuba et al., 1998; Palop et al., 2003; Lee et al., 2004, 2006; Seo et al., 2009, 2010), and also in the brains of AD patients (Iacopino and Christakos, 1990; Yamamoto-Sasaki et al., 1999). Therefore, we examined expression levels of the biomarkers in the brains of Tg-APPswe/PS1dE9 mice. Immunohistochemical staining with anti-phospho-CREB or anti-calbindin antibody showed that phospho-CREB levels and calbindin levels in the hippocampus of Tg-APPswe/PS1dE9 mice were reduced, but the reduced levels were not significantly attenuated by LMK03 (Fig. 5A–L).

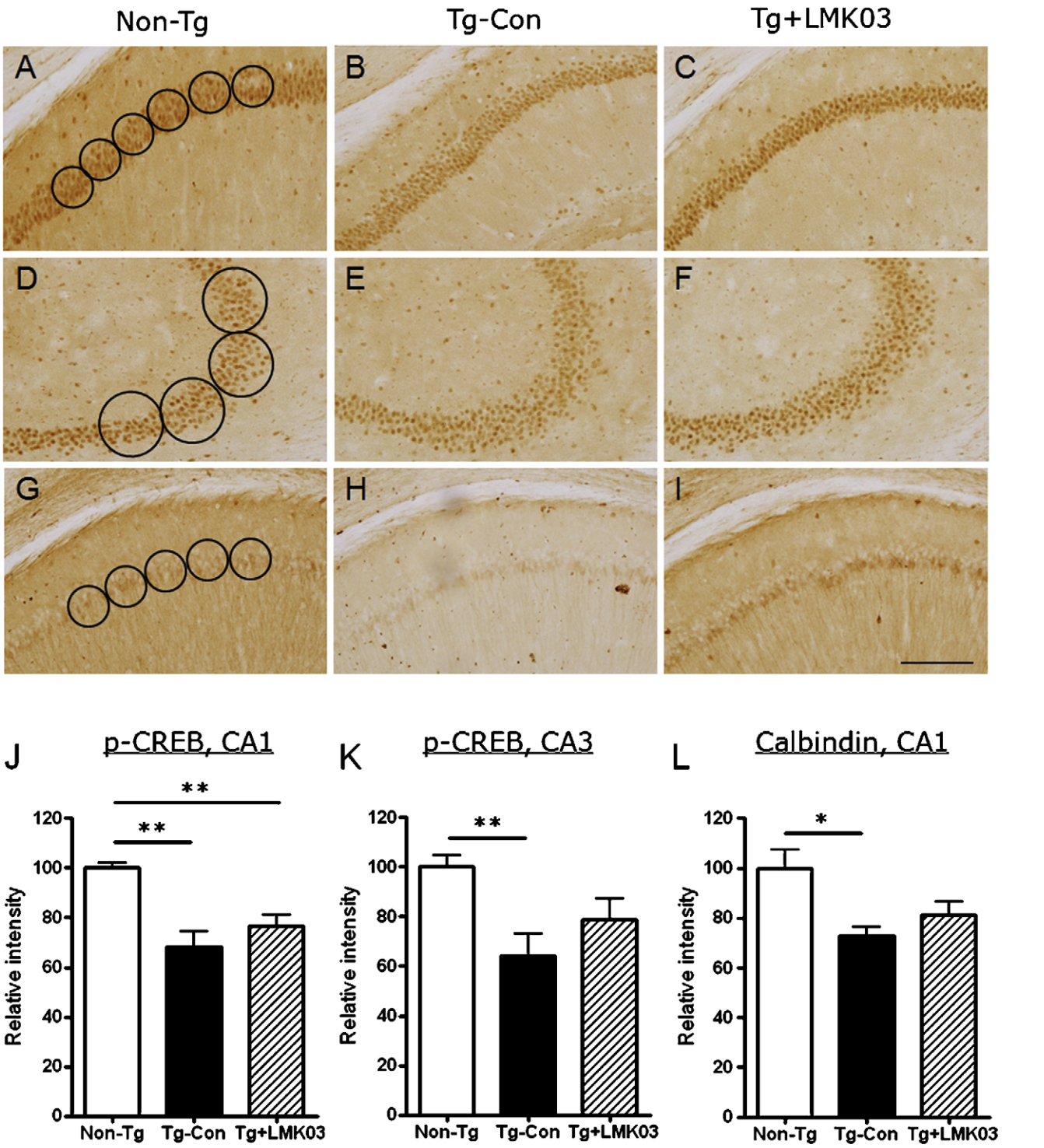

Fig. 5. Administration of LMK03 does not reverse the down-regulated expression of phospho-CREB and calbindin in the hippocampi of Tg-APPswe/PS1dE9 mice. (A–I) Photomicrographs showing anti-phospho-CREB antibody (A–F) or anti-calbindin antibody (G–I)-stained CA1 (A–C and G–I) and CA3 (D–F) of the hippocampus of non-transgenic control mice (Non-Tg; A, D, and G), control Tg-APPswe/PS1dE9 mice (Tg-Con; B, E, and H), and Tg-APPswe/PS1dE9 mice fed LMK03 (Tg + LMK03; C, F, and I). Scale bar, 200 μm. (J–L) Quantification of phospho-CREB (J and K) and calbindin (L) levels in the CA1 (J and L) and CA3 (K). Relative intensities within the 4–5 consecutive circles indicated (A, D, and G) were measured for each brain section of non-transgenic control mice (Non-Tg), control Tg-APPswe/PS1dE9 mice (Tg-Con), and Tg-APPswe/PS1dE9 mice fed LMK03 (Tg + LMK03). Data represent means ± SEM ($n = 6–8$ animals). * and ** denote differences between two groups at the $p < 0.05$ and $p < 0.01$ levels, respectively (one-way ANOVA, Newman–Keuls multiple comparison test).

4. Discussion

Traditional Jangwonhwan, which is composed of a decoction of 12 medicinal herbs/mushrooms, has been prescribed for centuries for patients with amnesia and to help enhance cognition (Huh, 1610). While traditional Jangwonhwan may have compelling medical benefits, it needs to pass the scrutiny of modern-day validation if the medicinal efficacy of Jangwonhwan is to be widely adopted in patient care. For any particular preparation, the mechanism of action and the most effective component or component combination for the proposed therapeutic effects should be known. In a recent attempt to more definitively categorize the functional

nature of Jangwonhwan, we reported that LMK02-Jangwonhwan, which consists of 7 of the 12 medicinal herbs/mushroom of traditional Jangwonhwan, suppresses the accumulation of Aβ and plaque deposition in the brains of Tg-APPswe/PS1dE9 mice (Seo et al., 2010). The present study was undertaken to test whether a further reduction of Jangwonhwan, designated LMK03-Jangwonhwan and consisting of only two medicinal herbs, white *Poria cocos* (Schw.) Wolf and *Angelica gigas* Nakai, retained the ability to inhibit AD-like pathology. The data in the present study demonstrate that LMK03 is effective, but in a diminished capacity. Although LMK03 protected Aβ-induced neuronal toxicity as effectively as LMK02 did in SH-SY5Y neuronal cells in vitro, LMK03 is not likely to retain the major ability of LMK02-Jangwonhwan to modify AD pathology.

These results raise several important issues concerning the therapeutic components of Jangwonhwan. Tg-APPswe/PS1dE9 mice were treated with LMK02-Jangwonhwan at a dose of 400 mg/kg/day for 3 months in the previous study (Seo et al., 2010) or with LMK03-Jangwonhwan at a dose of 300 mg/kg/day for 3 months in the present study. Although the precise amount contributed by each medicinal herb in the crude extract of LMK02- and LMK03-Jangwonhwan needs to be estimated experimentally, we may roughly estimate the contribution in the following way. The proportion of each herb/mushroom among the total weight of dried LMK02-Jangwonhwan constitutes 12–19%, which might correspond to 46–76 mg/kg/day of dosage. Whereas the proportion of each herb among the total weight of dried LMK03-Jangwonhwan constitutes 50% each, which might correspond to 150 mg/kg/day of dosage. This simple calculation further leads us to the realization that, if LMK03 do indeed carry "the most effective component(s)", the effective component was administered to Tg-APPswe/PS1dE9 mice more than that with LMK02, but observed AD modifying effects were weaker compared to those by LMK02-Jangwonhwan (Seo et al., 2010). An interpretation of the above is that the therapeutic effects of LMK02-Jangwonhwan may involve one or more of the five herbs of LMK02-Jangwonhwan that are not present in LMK03.

Our *in vitro* analysis with SH-SY5Y neuronal cells showed that LMK03 has a strong anti-oxidant property and protects from cytotoxicity induced by H_2O_2 or $A\beta_{1-42}$. Moreover, the anti-oxidant property and the protective effects of cell death by LMK03 were as significant as with LMK02-Jangwonhwan (Fig. 1). Nonetheless, in contrast to LMK02-Jangwonhwan, *in vivo* administration of LMK03 was not effective in inhibiting plaque pathology or accumulation of oxidative stress. Thus, the anti-oxidant capability of LMK03 evident *in vitro* is no guarantee of similar efficacy *in vivo*. This reasoning is consistent with our previous speculation that the therapeutic effect of LMK02-Jangwonhwan on the AD-like brain might be produced, at least in part, through their anti-oxidant properties (Seo et al., 2010), and probably involve other mechanisms that have not been explored in our studies. Further systematic reductions in the formulation of LMK02 and testing of their effects might help to address the question and lead to the identification of the most effective component(s) for or combination treatment of AD. These analyses are under way.

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