Journal of Ethnopharmacology 128 (2010) 206–212

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Oriental medicine Jangwonhwan reduces Aβ(1–42) level and β-amyloid deposition in the brain of Tg-APPswe/PS1dE9 mouse model of Alzheimer disease

Ji-Seon Seo[a,b], Jong-Hyun Yun[c], In-Sun Baek[a], Yeo-Hyun Leem[a], Hyung-Won Kang[c], Hyoung Kwon Cho[d], Yeoung-Su Lyu[c], Hyung-Jin Son[b], Pyung-Lim Han[a,b,*]

[a] Department of Chemistry & Nano Sciences, Ewha Womans University, Seoul, Republic of Korea
[b] Department of Brain & Cognitive Sciences, Ewha Womans University, Seoul, Republic of Korea
[c] Department of Neuropsychiatry, College of Oriental Medicine, Wonkwang University, Iksan, Republic of Korea
[d] Hanpoong Pharm. Research Center, Jeonju, Republic of Korea

ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 5 January 2010
Accepted 6 January 2010
Available online 14 January 2010

Keywords:
Oriental medicine
Alzheimer disease
Aβ
β-amyloid plaque
LMK02-Jangwonhwan
Panax ginseng C.A. Meyer
Angelica gigas Nakai

ABSTRACT

Ethnopharmacological relevance: Jangwonhwan, a boiled extract of 12 medicinal plants/mushroom including Korean red ginseng (*Panax ginseng* C.A. Meyer), has been prescribed for patients with cognitive dysfunction and are believed to induce brain activity enhancement, provide light sedation, and facilitate sound sleep.
Aim of the study: The present study was carried out to investigate whether Jangwonhwan has a beneficial effect on the brain of Alzheimer disease.
Materials and methods: The transgenic mice of Alzheimer disease, Tg-APPswe/PS1dE9, were fed a modified recipe of Jangwonhwan consisting of a boiled extract of 7 herbs/mushroom (called LMK02-Jangwonhwan) at 400 mg/kg/day of dose for 3 months from 4.5 months of age. Immunohistological and ELISA analyses were used to assess the Aβ accumulation and plaque deposition in the brain. Other *in vitro* and *in vivo* works were performed to understand the underlying mechanism.
Results: LMK02-Jangwonhwan notably reduced Aβ(1–42) and Aβ(1–40) levels, concomitantly with a reduction of plaque deposition, in the brain of Tg-APPswe/PS1dE9 mice. LMK02-Jangwonhwan partially suppressed oxidative stress accumulation, and prevented the down-regulation of phospho-CREB and calbindin typically seen in the hippocampus of AD-like brains. *In vitro* study with SH-SY5Y neuroblastoma cells showed that LMK02-Jangwonhwan inhibited oxidative stress and Aβ-induced neurotoxicity.
Conclusion: The present study suggests that LMK02-Jangwonhwan confers a therapeutic potential to ameliorate AD-like pathology in the brain of Tg-APPswe/PS1dE9 mice.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by senile plaque deposition, neurofibrillary tangle formation, and neuronal loss. Studies with AD brains and AD mouse models show that abnormal metabolism of amyloid precursor protein (APP) is a key mechanism of AD pathogenesis (Selkoe and Schenk, 2003; Bertram and Tanzi, 2008). AD brains also show a variety of alterations in cellular and molecular functions, including down-regulation of anti-oxidant systems, Aβ clearance capability, mitochondrial function, up-regulation of oxidative stress, inflammatory response and synaptic dysfunction (Bazan et al., 2002; Frank and Gupta, 2005; Finch and Morgan, 2007; Zhu et al., 2007; Lee et al., 2009). It has been demonstrated that intervention strategies against any of these pathways, such as suppression of Aβ production, activation of Aβ clearance, or supplementation of anti-oxidant capacity, confer a therapeutic potential for AD (Ghosh et al., 2002; Hills and Vacca, 2007; Imbimbo, 2008). Given that most cases of AD are sporadic and caused by various non-genetic and/or environmental factors (Selkoe and Schenk, 2003; Goedert and Spillantini, 2006), diverse therapeutic methods for AD warrant investigation.

Jangwonhwan is an oriental medicine that has historically been administered to patients with amnesia. Traditional Jangwonhwan (Huh, 1610) consists of a decoction of 12 medicinal plants and mushroom, including red *Panax ginseng* C.A. Meyer (family: Acoraceae) and white *Poria cocos* (Schw.) Wolf (family: Polyporaceae), and a trace amount of an inorganic material. Jangwonhwan is

* Corresponding author at: Division of Nano Sciences, Ewha Womans University, 11-1 Daehyun-Dong, Seodaemoon-Gu, Seoul 120-750, Republic of Korea. Tel.: +82 2 3277 4130; fax: +82 2 3277 3419.
E-mail address: plhan@ewha.ac.kr (P.-L. Han).

0378-8741/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2010.01.014

prescribed currently for patients with amnesia, but in a modified recipe by adding to, or substracting from, the composition of the original recipe. Therapeutic mechanisms and the efficacies of modified Jangwonhwans are likely varied, but their effectiveness has not been clearly elaborated. In the present study, we investigated whether a modified Jangwonhwan, LMK02, confers a beneficial effect on the brain of Tg-APPswe/PS1dE9 transgenic mice, a murine model of Alzheimer disease, and demonstrated that LMK02-Jangwonhwan has a therapeutic potential to help the AD-like brain.

2. Materials and methods

2.1. AD murine model

Tg-APPswe/PS1dE9 transgenic mice over- expressing human mutated APP and PS1 (APPswe/PS1dE9) were maintained in C57BL6 × C3H F1 hybrids, as described previously (Jankowsky et al., 2001). The mice were housed in pairs with a non-transgenic female 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 the control ($n = 6$) and LMK02-Jangwonhwan ($n = 8$) groups. We prepared lab chow containing of LMK02 for a mouse to have 400 mg/kg/day, which is equivalent to 32.5 mg/kg/day (or 1.95 g/60 kg/day) for human according to the translation formula of Reagan-Shaw et al. (2008). The mice were fed lab chow containing LMK02 for 3 months from 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 the Ewha Womans University School of Medicine.

2.2. Preparation for LMK02-Jangwonhwan and control of standard biomarkers

All medicinal herbs used were purchased from Yoo-Il Pharm. Co. (Seoul, Korea). A decoction of the seven herbs: red *Panax ginseng* C.A. Meyer (root, 20 g) of Araliaceae, *Acorus gramineus* Soland (rhizome, 16 g) of Acoraceae, white *Poria cocos* (Schw.) Wolf (sclerotium, 16 g) of Polyporaceae, *Angelica gigas* Nakai (root, 12 g) of Apiaceae, *Ophiopogon japonicas* Ker-Gawl (rhizome, 12 g) of Ruscaceae, *Scrophularia buergeriana* Miquel (root, 16 g) of Scrophulariaceae, and *Thuja orientalis* L. (seed, 12 g) of Cupressaceae was prepared by the HanPoong Oriental Medicine Pharm. Co. (Jeonju, Korea) by following the standard procedure (The Korean Pharmacopoeia Eighth Edition IX from the Korea FDA, 2008. Seoul. Sinnilbooks Press Inc. p6, p1611, p1466, p1457, p1450, p1526, and p1519). In brief, each of the dried herbs (104 g in sum) was mixed together and ground to small pieces (to a standard size according to K.P. IX). After adding 840 ml of filter-purified drinking water (K.P. IX), the mixture was boiled for 3 h at 100 °C in a reflux condenser (MyungSung Stainless Steel, Seoul, Korea). The boiled extract was then filtered through Whatman No. 4 filter paper (20–25-μm of pore-size) and then concentrated by a vacuum evaporator (Rotavapor R-200, Bűch, Germany) at 60 °C. Boiled extraction of the herbs was repeated with another 840 ml of filter-purified water. The dried extract was ground using a pulverizer and combined with the primary decoction. The final yield from the whole procedure was ~31.7 g of the dried mixture. Overall, 95 g of the dried substance was obtained from 3 preparation trials, then combined and stored at −80 °C until use.

We chosen the ginsenoside Rg3 and decursin as standard molecular markers for red *Panax ginseng* C.A. Meyer and white *Poria cocos* (Schw.) Wolf, respectively, by following the guideline posted by the Korean FDA (2007). The amount of Rg3 in LMK02-Jangwonhwan was determined using a high performance liquid chromatography (HPLC) (Detector, Waters #2487; Pump: Waters #2695, Waters, USA) equipped with the μ-Bondapak NH_2 column (300 mm × 3.9 mm, 10 μm, Waters, USA) by eluting with 2% acetonitrile and 98% water. The column temperature was set at 40 °C, the detection wavelength was set at 203 nm, and the flow rate was at 1.5 ml/min and sample injection volume was 10 μl. The detection wavelength was set at 203 nm. The amount of decursin in LMK02-Jangwonhwan was determined similarly, but using the Capcell Pak C_{18} (250 mm × 4.6 mm, 5 μm, Shiseido Co., Ltd., Japan) column by eluting with 48% acetonitrile and 52% water. The detection wavelength was set at 280 nm, and the flow rate was at 0.7 ml/min. Total 47.0 ± 0.42 μg of Rg3 per gram of red *Panax ginseng* C.A. Meyer ($n = 9$) and 2.71 ± 0.14 mg of decursin per gram of white *Poria cocos* (Schw.) Wolf ($n = 9$) were detected from the preparation of LMK02-Jangwonhwan.

2.3. Assessment of Aβ levels

Transgenic Tg-APPswe/PS1dE9 mice were sacrificed at 7.5 months of age and perfused with 0.9% saline. The right and left hemispheres of the brain were used for histological and biochemical analyses, respectively. ELISA assays for Aβ(1–42) and Aβ(1–40) levels were described in previously studies (Lee et al., 2009; Seo et al., 2009). Briefly, the prefrontal cortex was individually homogenized in Tris-buffered saline (20 mM Tris and 137 mM NaCl, [pH 7.6]) in the presence of protease inhibitor mixtures (Complete Mini; Roche, IN, USA). Homogenates were centrifuged at 100,000 × *g* for 1 h at 4 °C, and the supernatant was used to measure soluble forms of Aβ(1–40) and Aβ(1–42) levels. The pellet was sonicated in 70% formic acid and centrifuged as above, and the supernatant was collected, and regarded as the insoluble Aβ peptide fraction or the formic acid extract. The formic acid extract was collected, and neutralized with 1 M Tris–Cl buffer (pH 11) by 1:20 dilution, and the diluted formic acid extract was used for each assay, as previously described (Lee et al., 2006). The final assays were performed using Human β-Amyloid Aβ(1–40) or Aβ(1–42) Colorimetric Sandwich ELISA kits (BioSource, Camarillo, CA, USA) by following the manufacturer's instructions.

2.4. Immunohistological and microscopic works

For immunohistochemistry, 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 vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Free-floating sections were blocked by 5% normal goat serum, 2% BSA, and 2% FBS. A biotinylated HRP system was used for color development. Anti-Aβ antibody Bam-10 (A5213) and anti-calbindin (C9848) were obtained from Sigma (St. Louis, MO, USA). Anti-phospho-CREB (06-519) was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Microscopic studies were carried out using an Oympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus Co., Tokyo, Japan). 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. The numbers of plaques in each region were measured using TOMORO ScopeEye 3.6 program (Techsan Community, Seoul, Korea).

2.5. In vitro cell culture works

Cell culture of SH-SY5Y neuroblastoma cell line was described in a previous study (Yu et al., 2003). 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. Cells at 70–80% confluence were used for all experiments. Aβ(1–42) was purchased from U.S. Peptide, Inc. (Rancho Cucamonga, CA, USA) and dissolved to the concentration of 5 mM in dimethyl sulfoxide (DMSO). After diluting to 100 μM in phosphate-buffered saline, Aβ(1–42) was incubated at 4 °C for 24 h, which was used in cell viability tests. For cell viability assay, cells were incubated with H_2O_2 or Aβ(1–42) in serum-free DMEM.

Cell viability was determined using WST-1 assay as described previously (Cho et al., 2003). Briefly, after one-tenth volume of WST-1 solution was added to the culture medium, cells were incubated for 2 h at 37 °C in a humidified incubator supplied with 5% CO_2. The tetrazolium salt WST-1 is reduced into a soluble dark red formazan by the mitochondrial enzyme succinate-dehydrogenase, the amount of which is proportional to the number of living cells. The cultured medium was removed, and absorbance was measured at 640 nm for a background absorbance and at 440 nm for viability.

2.6. Measurements of lipid peroxidation

Lipid peroxidation levels were assessed as described previously (Im et al., 2006; Lee et al., 2009). To address this, malondialdehyde (MDA) levels were measured using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR, USA). In brief, hippocampal homogenates were prepared in 4 vol. of ice-cold 20 mM PBS (pH 7.4) containing 5 mM butylated hydroxytoluene. After centrifugation at 3000 × *g*, the supernatant was taken. Two hundred microlitres of supernatant were mixed with 10 μl of probucol and 640 μl of diluted R1 reagent (1:3 of methanol:N-methyl-2-phenylindole) provided in the Bioxytech MDA-586 kit, and 150 μl of 12N 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 an 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 Laboratories, USA).

2.7. Statistical analysis

For statistics, data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Multiple comparisons were made using one-way ANOVA followed by the Newman–Keuls multiple range test. All data were presented as means ± SEM and statistical difference was accepted at the 5% level unless otherwise indicated.

3. Results

Our preliminary analysis of the neuroprotective properties of each of 12 herbal/fungal members of the traditional Jangwonhwan in Aβ-treated cultured neurons and a literature review of the known neurologic properties of each herbal/fungal member of Jangwonhwan led us to a modified version of Jangwonhwan. The preparation, called LMK02-Jangwonhwan (or LMK02 for short), a

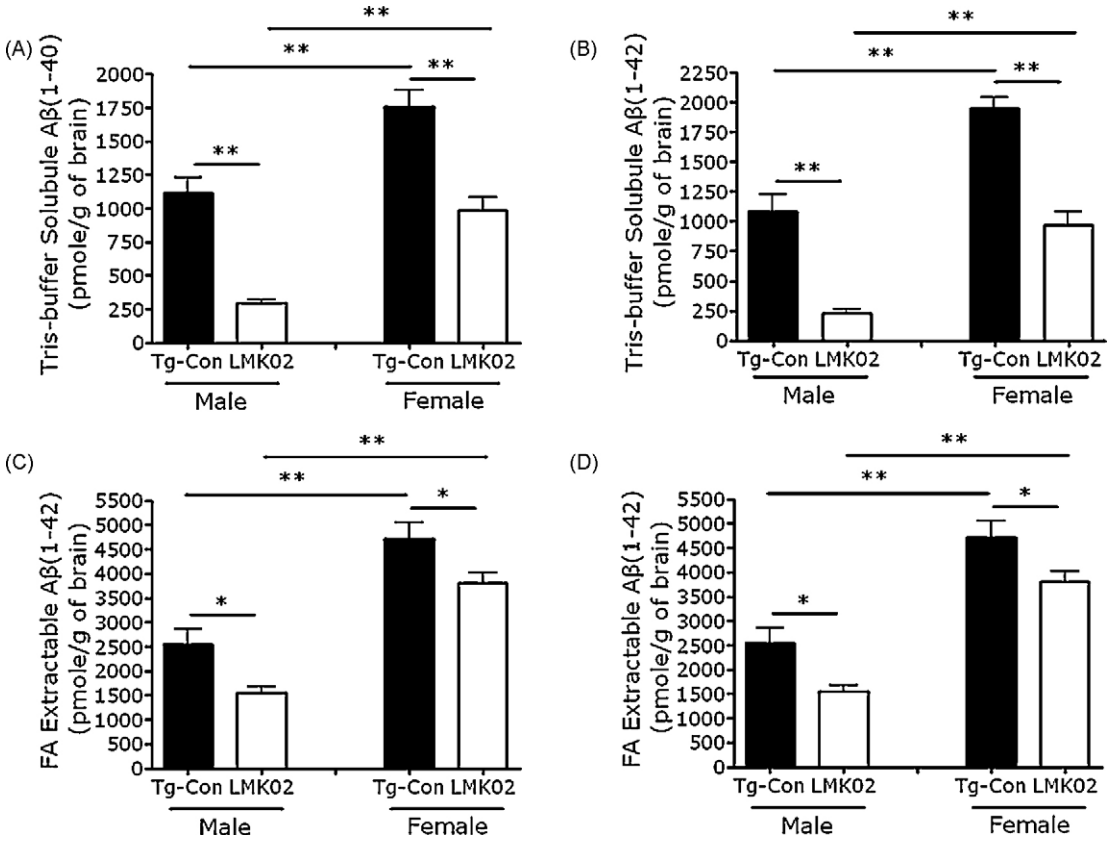

Fig. 1. Administration of LMK02 reduced Aβ(1–42) and Aβ(1–40) levels in the brains of Tg-APPswe/PS1dE9 mice. (A–D) ELISA analysis showing the amounts of Tris-buffer extracted soluble (A and B) and formic acid-extracted insoluble (C and D) forms of Aβ(1–42) and Aβ(1–40) in the prefrontal cortex of Tg-APPswe/PS1dE9 mice at 7.5 months of age. LMK02 was administered at 400 mg/kg/day of dose for 3 months. Each group contained 6–8 animals, and ELISA analysis was performed for individual animals with duplicate. * 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). WT, non-transgenic control; Tg-CON, Tg-APPswe/PS1dE9 mouse control; Tg + LMK02, Tg-APPswe/PS1dE9 mice treated with LMK02.

boiled extract of the following 7 medicinal herbs; 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.

Tg-APPswe/PS1dE9 mice were fed lab chow containing LMK02 at 400 mg/kg/day of dose for 3 months from 4.5 months of age, and the mice were sacrificed at 7.5 months of age. Body weight was not significantly different between the control and LMK02 groups during the treatment period (data not shown).

ELISA analysis with prefrontal cortex homogenates revealed that the soluble forms of Aβ(1–42) and Aβ(1–40), which were prepared in Tris buffer (20 mM Tris and 137 mM NaCl, [pH 7.6]), were markedly reduced in Tg-APPswe/PS1dE9 mice with LMK02 (Fig. 1A and B). Similarly, formic acid-extracted insoluble forms of Aβ(1–42) and Aβ(1–40) levels were reduced in LMK02-treated Tg-APPswe/PS1dE9 mice (Fig. 1C and D).

Consistent with the ELISA data, anti-Aβ(1–42) antibody (Bam-10)-stained brain sections showed that LMK02 significantly

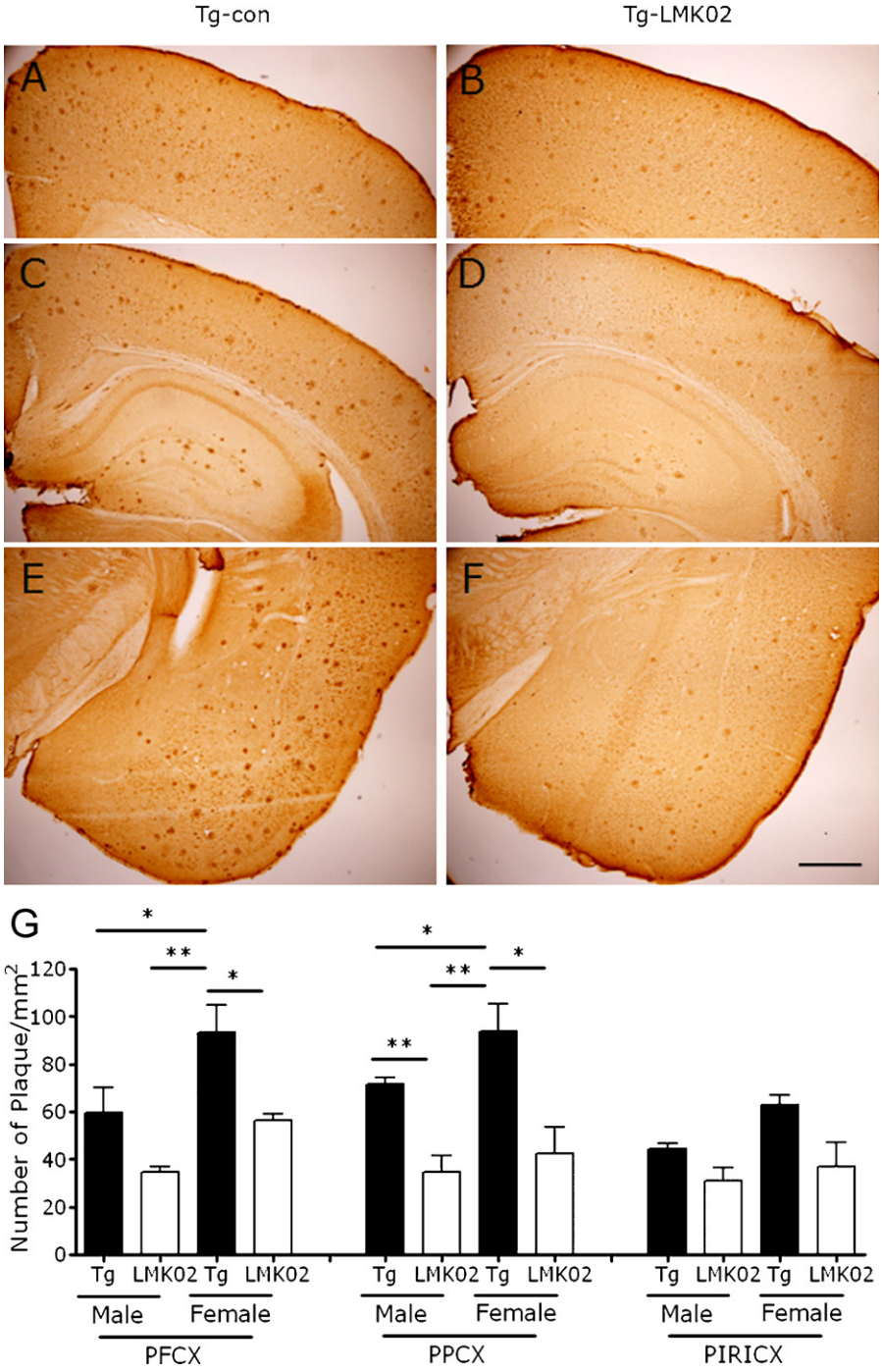

Fig. 2. Administration of LMK02 reduced plaque deposition in the brain of Tg-APPswe/PS1dE9 mice. (A–F) Photomicrographs showing anti-Aβ antibody (Bam-10)-stained prefrontal cortex (A and B), parietal cortex and hippocampus (C and D), and piriform cortex (E and F) of Tg-APPswe/PS1dE9 control mice (A, C and E), and Tg-APPswe/PS1dE9 mice fed LMK02 (400 mg/kg/day) (Tg-LMK02; B, D and F). Scale bar, 500 μm. (G) Quantification of plaque numbers in the superior prefrontal cortex, parietal cortex, and piriform cortex of Tg-APPswe/PS1dE9 mice with or without LMK02. Plaque numbers were counted using a computer-aid image analysis system as described in Section 2. PFCX, prefrontal cortex; PPCX, parietal cortex; PIRICX, piriform cortex. 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).

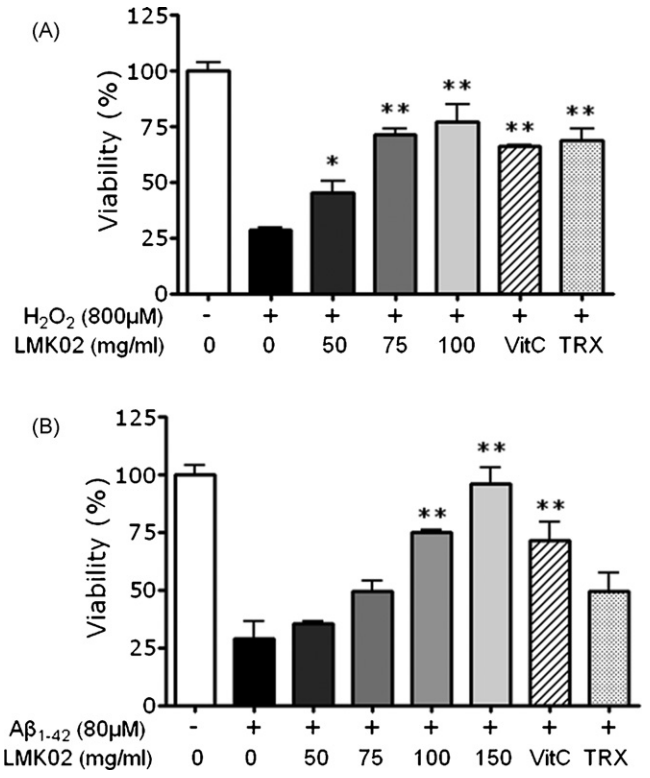

Fig. 3. LMK02 suppressed the cytotoxicity of SH-SY5Y neuroblastoma cells induced by H_2O_2 or Aβ(1–42). (A and B) LMK02 inhibited the cell death of SH-SY5Y induced by H_2O_2 (800 μM; A) or by Aβ(1–42) (80 μM; B) in a dose dependent manner. Cell death profiles of SH-SY5Y in response to increasing doses of H_2O_2 or Aβ(1–42) were obtained prior to experiments. Aβ(1–42) was pre-incubated at 4 °C for 24 h before treatment. VitC, vitamin C (500 μM); TRX, trolox (500 μM). Cell viability was measured using WST-1 assay after 24 h of treatments. Data points represent the 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).

reduced plaque deposition in several areas of the brain, including the prefrontal cortex, parietal cortex, and piriform cortex, of both male and female Tg-APPswe/PS1dE9 mice (Fig. 2A–G).

Next, we examined whether LMK02 reduced oxidative stress in the brain. MDA assay showed that the levels of MDA, which is an oxidized by-product of lipid peroxidation, in the brain of non-transgenic mice and control Tg-APPswe/PS1dE9 mice at 7.5 months of age were, respectively, 64.4 ± 7.3 pmol/g of protein and 151.2 ± 3.4 pmol/g of protein, indicating that Tg-APPswe/PS1dE9 mice show enhanced lipid peroxidation levels in the brain ($p < 0.01$). Whereas the level of MDA in Tg-APPswe/PS1dE9 mice treated with LMK02 was 121.9 ± 13.3 pmol/g of protein, suggesting that the enhanced lipid peroxidation level was significantly reduced by LMK02 ($p < 0.05$).

It was attempted to understand the mechanism underlying LMK02-induced suppression of Aβ pathology and lipid peroxidation levels in the brain of Tg-APPswe/PS1dE9 mice. Treatment of LMK02 in SH-SY5Y neuroblastoma cells suppressed the cell death induced by H_2O_2, suggesting LMK02 confers a potent anti-oxidant property. Moreover, LMK02 suppressed the cell death of SH-SY5Y induced by Aβ(1–42) (Fig. 3A and B).

Recent studies by us and others have reported that phospho-CREB levels and calbindin were reduced in the hippocampus of murine models of AD, including Tg-CTF99/B6 and Tg2576 mice (Leuba et al., 1998; Palop et al., 2003; Lee et al., 2004, 2006; Seo et al., 2009), and also in the brains of AD patients (Iacopino and Christakos, 1990; Yamamoto-Sasaki et al., 1999). These results prompted us to examine whether LMK02 affects the expression of the molecular signatures in AD-like brains. Immunohistochemical staining with anti-phospho-CREB antibody revealed that phospho-CREB levels in the hippocampus of Tg-APPswe/PS1dE9 mice were reduced, and the reduction was reversed by LMK02 (Fig. 4A–F, J and K). Calbindin expression was also reduced in the pyramidal neurons of the hippocampus of Tg-APPswe/PS1dE9 mice, and the reduction was partially attenuated in the hippocampus of Tg-APPswe/PS1dE9 mice treated with LMK02 (Fig. 4G–I and L).

4. Discussion

In the present study, we demonstrated that administration of LMK02-Jangwonhwan, a modified recipe of traditional Jangwonhwan, notably suppressed Aβ levels and plaque deposition in the brains of Tg-APPswe/PS1dE9 mice. Moreover, LMK02 partially reversed the reduced expressions of phospho-CREB and calbindin typically seen in the hippocampus of AD-like brains. These results suggest that LMK02 has beneficial effects on the brain with AD-like pathology.

As described in Section 1, traditional Jangwonhwan was a decoction of 12 medicinal herbs and mushroom, and included a trace amount of an inorganic material, sulphormercury cinnabar. Because cinnabar is toxic to neurons, exclusion of cinnabar, as well as other members of Jangwonhwan, has been attempted in the past years. However, the efficacy of various modified Jangwonhwans has not been systematically analyzed and the evaluation of its efficacy basically relied upon empirical results until recent years. Moreover, the mechanism underlying the therapeutic effects of modified Jangwonhwans, including the traditional mixture, on the brain of Alzheimer disease has not been sought at all. Towards the minimalization of Jangwonhwan to ultimately focus on inclusion of its most effective component(s), in the present study, we investigated and found a therapeutic potential for LMK02-Jangwonhwan, which consisted of 7 medicinal herbs/mushroom selected from the traditional Jangwonhwan. The selected 7 medicinal herbs/mushroom are those that have been widely used in the oriental medicine. For examples, red *Panax ginseng* C.A. Meyer is used for replenishing the digestive system, treating febrile disease and allaying emotional distress and restlessness. *Acorus gramineus* Soland is used as a stimulant. White *Poria cocos* (Schw.) Wolf and *Thuja orientalis* L. are used to promote relaxation. *Angelica gigas* Nakai, *Ophiopogon japonicus* Ker-Gawl, and *Scrophularia buergeriana* Miquel are used to replenish the cardiovascular system and the *Eum(Yin)* state of the body. Moreover, a recent study reported that a decoction of red *Panax ginseng* C.A. Meyer and *Acorus gramineus* Soland confers a neuroprotective effect on the rat brain insulted with ischemia (Lee et al., in press). Though it remains unknown which herb(s)/mushroom in Jangwonhwan confers a beneficial effect on the AD-like brain, the present data support that the LMK02 decoction provides a starting point in the further investigation.

Various natural compounds are effective in suppressing AD-like pathology through their properties as anti-oxidants (Frank and Gupta, 2005; dos Santos-Neto et al., 2006). Curcumin and *Ginkgo biloba* L. extract have beneficial effects on AD-like brains (Lim et al., 2001; Balasubramanyam et al., 2003; Smith and Luo, 2003; Stackman et al., 2003; Bastianetto and Quirion, 2004; Chan et al., 2005). Recently we demonstrated that a naturally occurring oleanolic-glycoside saponin-enriched fraction from the root of *Pulsatilla koreana* Nakai (family: Ranunculaceae) has an anti-oxidant property and suppresses anti-β-amyloid pathology in the Tg2576 mouse model of AD (Seo et al., 2009). These results tempt us to speculate that the therapeutic effect of LMK02 on the AD-like brain might be produced in part through their anti-oxidant properties. A partial suppression by LMK02 of the oxidative stress in the brain of Tg-APPswe/PS1dE9 mice might support this interpretation. In fact, LMK02 suppressed the cytotoxicity of SH-SY5Y neuroblastoma cells

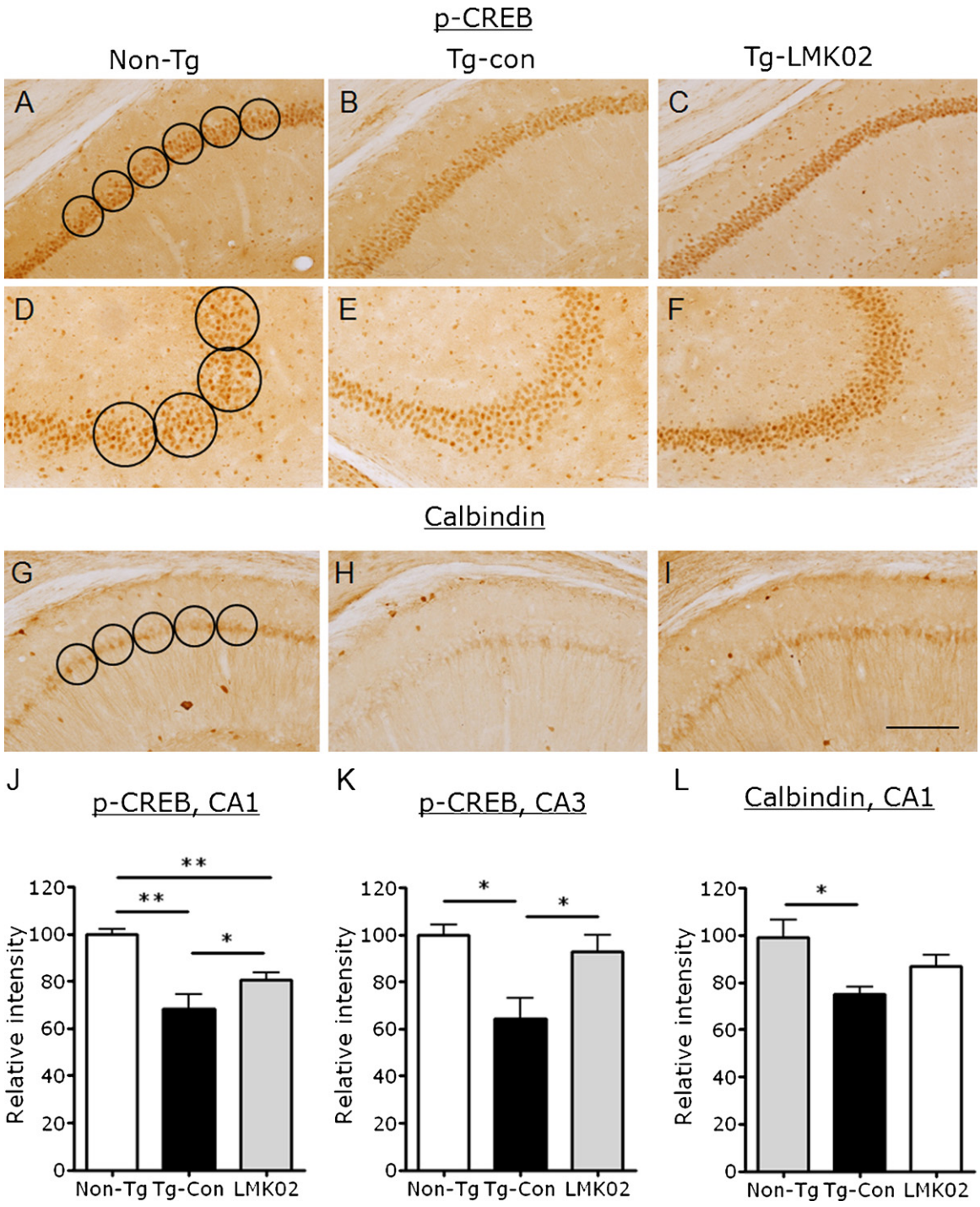

Fig. 4. Administration of LMK02 reversed the down-regulated expression of phospho-CREB and calbindin in the hippocampi of Tg-APPswe/PS1dE9 mice. (A–I) Photomicrographs showing anti-phospho-CREB antibody (A–F) or anti-calbindin antibody (G–I)-stained CA1 (A–C and G–I) and CA3 (D–F) of hippocampus of non-transgenic control mice (A, D, and G), control Tg-APPswe/PS1dE9 mice (B, E, and H), and Tg-APPswe/PS1dE9 mice fed LMK02 (Tg-LMK02; 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). Captured images of phospho-CREB or calbindin-stained hippocampus in the 4–5 consecutive circles as indicated were converted into digital images and used for counting the stained intensities using the TOMORO ScopeEye 3.6 program (Techsan Community, Seoul, Korea). 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 LMK02 (Tg-LMK02). 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 comparison test).

induced by H_2O_2 or Aβ(1–42), indicating that LMK02 has a potent anti-oxidant property (Fig. 3). Further reductions of LMK02, to at least a single herb level, will greatly accelerate the effort to identify the most effective component(s) or combination for treatment of AD.

Acknowledgements

This work was supported by a grant (B070030) from the Korean Health R&D project from the Ministry of Health and Welfare, Republic of Korea.

References

Balasubramanyam, M., Koteswari, A.A., Kumar, R.S., Monickaraj, S.F., Maheswari, J.U., Mohan, V., 2003. Curcumin-induced inhibition of cellular reactive oxygen species generation: novel therapeutic implications. Journal of Biosciences 29, 715–721.

Bastianetto, S., Quirion, R., 2004. Natural antioxidants and neurodegenerative diseases. Frontiers in Bioscience 9, 3447–3452.

Bazan, N.G., Palacios-Pelaez, R., Lukiw, W.J., 2002. Hypoxia signaling to genes: significance in Alzheimer's disease. Molecular Neurobiology 26, 283–298.

Bertram, L., Tanzi, R.E., 2008. Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. Nature Reviews Neuroscience 9, 768–778.

Chan, W.H., Wu, H.J., Hsuuw, Y.D., 2005. Curcumin inhibits ROS formation and apoptosis in methylglyoxal-treated human hepatoma G2 cells. Annals of the New York Academy of Sciences 1042, 372–378.

Cho, I.H., Im, J.Y., Kim, D., Kim, K.S., Lee, J.K., Han, P.L., 2003. Protective effects of extracellular glutathione against Zn^{2+}-induced cell death *in vitro* and *in vivo*. Journal of Neuroscience Research 74, 736–743.

dos Santos-Neto, L.L., de Vilhena Toledo, M.A., Medeiros-Souza, P., de Souza, G.A., 2006. The use of herbal medicine in Alzheimer's disease. Evidence-based Complementary and Alternative Medicine 3, 441–445.

Finch, C.E., Morgan, T.E., 2007. Systemic inflammation, infection, ApoE alleles, and Alzheimer disease: as position paper. Current Alzheimer Research 4, 185–189.

Frank, B., Gupta, S., 2005. A review of antioxidants and Alzheimer's disease. Annals of Clinical Psychiatry 17, 269–286.

Ghosh, A.K., Hong, L., Tang, J., 2002. Beta-secretase as a therapeutic target for inhibitor drugs. Current Medicinal Chemistry 9, 1135–1144.

Goedert, M., Spillantini, M.G., 2006. A century of Alzheimer's disease. Science 314, 777–781.

Hills, I.D., Vacca, J.P., 2007. Progress toward a practical BACE-1 inhibitor. Current Opinion in Drug Discovery & Development 10, 383–391.

Huh, J., 1610. Donguibogam. Reprinted by Namsandang Press in 1994, Seoul.

Iacopino, A.M., Christakos, S., 1990. Specific reduction of calcium binding protein (28-kilodalton calbindin-D) gene expression in aging and neurodegenerative diseases. Proceedings of the National Academy of Sciences of the United States of America 87, 4078–4082.

Im, J.Y., Kim, D., Paik, S.G., Han, P.L., 2006. Cyclooxygenase-2-dependent neuronal death proceeds via superoxide anion generation. Free Radical Biology & Medicine 41, 960–972.

Imbimbo, B.P., 2008. Therapeutic potential of gamma-secretase inhibitors and modulators. Current Topics in Medicinal Chemistry 8, 54–61.

Jankowsky, J.L., Slunt, H.H., Ratovitski, T., Jenkins, N.A., Copeland, N.G., Borchelt, D.R., 2001. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. Biomolecular Engineering 17, 157–165.

Korea Food and Drug Administration, 2008. The Korean Pharmacopoeia IX, ninth ed. Sinnilbooks Press Inc., Seoul.

Korea Food and Drug Administration, 2007. Korean Pharmaceutical Codex, third ed. Sinnilbooks Press Inc., Seoul, p 1533.

Lee, K.W., Lee, S.H., Kim, H., Song, J.-S., Yang, S.-D., Paik, S.G., Han, P.L., 2004. Progressive cognitive impairment and anxiety induction in the absence of plaque deposition in C57BL/6 inbred mice expressing transgenic amyloid precursor protein. Journal of Neuroscience Research 76, 572–580.

Lee, K.W., Im, J.Y., Song, J.S., Lee, S.H., Lee, H.J., Ha, H.Y., Koh, J.Y., Gwag, B.J., Yang, S.D., Paik, S.G., Han, P.L., 2006. Progressive neuronal loss and behavioral impairments of transgenic C57BL/6 inbred mice expressing the carboxy terminus of amyloid precursor protein. Neurobiology of Disease 22, 10–24.

Lee, K.W., Kim, J.B., Seo, J.S., Kim, T.K., Im, J.Y., Baek, I.S., Kim, K.S., Lee, J.K., Han, P.L., 2009. Behavioral stress accelerates plaque pathogenesis in the brain of Tg2576 mice via generation of metabolic oxidative stress. Journal of Neurochemistry 108, 165–175.

Lee, B., Choi, E.J., Lee, E.J., Han, S.M., Hahm, D.H., Lee, H.J., Shim, I., in press. The neuroprotective effect of methanol extract of *Gagamjungjihwan* and *Fructus Euodiae* on ischemia-induced neuronal and cognitive impairment in the rat. Evidence-based Complementary and Alternative Medicine.

Leuba, G., Kraftsik, R., Saini, K., 1998. Quantitative distribution of parvalbumin, calretinin, and calbindin D-28k immunoreactive neurons in the visual cortex of normal and Alzheimer cases. Experimental Neurology 152, 278–291.

Lim, G.P., Chu, T., Yang, F., Beech, W., Frautschy, S.A., Cole, G.M., 2001. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. Journal of Neuroscience 21, 8370–8377.

Palop, J.J., Jones, B., Kekonius, L., Chin, J., Yu, G.Q., 2003. Neuronal depletion of calcium-dependent proteins in the dentate gyrus is tightly linked to Alzheimer's disease-related cognitive deficits. Proceedings of the National Academy of Sciences of the United States of America 100, 9572–9577.

Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. The FASEB Journal 22, 659–661.

Selkoe, D.J., Schenk, D., 2003. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. Annual Review of Pharmacology and Toxicology 43, 545–584.

Seo, J.S., Kim I, T.K., Leem, Y.H., Lee, K.W., Park, S.K., Baek, I.S., Kim, S.K., Im, G.J., Lee, S.M., Park, Y.H., Han, P.L., 2009. SK-PC-B70 M confers anti-oxidant activity and reduces Aβ levels in the brain of Tg2576 mice. Brain Research 1261, 100–108.

Smith, J.V., Luo, Y., 2003. Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by *Ginkgo biloba* extract EGb 761. Journal of Alzheimer's Disease 5, 287–300.

Stackman, R.W., Eckenstein, F., Frei, B., Kulhanek, D., Nowlin, J., Quinn, J.F., 2003. Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. Experimental Neurology 184, 510–520.

Yamamoto-Sasaki, M., Ozawa, H., Saito, T., Rosler, M., Riederer, P., 1999. Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. Brain Research 824, 300–303.

Yu, Y.M., Han, P.L., Lee, J.K., 2003. JNK pathway is required for retinoic acid-induced neurite outgrowth of human neuroblastoma, SH-SY5Y. Neuroreport 14, 941–945.

Zhu, X., Su, B., Wang, X., Smith, M.A., Perry, G., 2007. Causes of oxidative stress in Alzheimer disease. Cellular and Molecular Life Sciences 64, 2202–2210.