論文題目

The effect of kamikihito on cognitive function
（加味帰脾湯の認知機能に対する作用）

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Contents

Introduction

Nearly 36 million people worldwide are living with dementia, and as the population ages, this number is expected to more than triple by 2050. The countries with the largest number of people with dementia are China (5.4 million), Japan (4.3 million), the United States (3.9 million), India (3.7 million), Germany (1.5 million), Russia (1.2 million), France (1.1 million), Italy (1.1 million) and Brazil (1.0 million). Dementia has become a prominent social issue [1, 2]. Alzheimer's disease (AD), the most common cause of dementia, is characterized by extracellular deposits of amyloid beta (Aβ) protein and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau. Acetylcholinesterase inhibitors (donepezil, galantamine and rivastigmine) and an N-methyl-d-aspartate (NMDA) receptor antagonist (memantine) are currently the only approved drugs for the treatment of AD [3]. However, because these drugs do not target Aβ and hyperphosphorylated tau, it is difficult to prevent, halt, or reverse the disease [4].

In spite of efforts to discover drugs targeting Aβ, no truly effective therapy has been developed to date [5]. Solanezumab and bapineuzumab are one of the most notable immunotherapeutic agents targeting Aβ. In phase 3 clinical trials, the immunotherapies had no effect on behavioral tests of mild- to moderate-AD patients [6]. The results of these clinical trials indicate that reversing the degeneration of neuronal networks after the progression of AD is difficult. We propose that it is important for new anti-AD drugs to reverse the degeneration of neuronal networks after the

appearance of symptoms.

From this point of view, we are investigating drugs used in Japanese traditional medicine (called Kampo), focusing on kamikihito (KKT). KKT is an herbal drug known for its effectiveness in treating insomnia, loss of appetite, amnesia, and depression. We previously reported the effect of KKT in the 5XFAD mouse model of AD [7]. 5XFAD mice exhibit increased Aβ production, Aβ plaques, and gliosis at 2 months of age and synapse loss and memory impairment at 4 months of age [8]. Therefore, 5XFAD mice are considered to be a model of AD after the appearance of symptoms. Our previous study showed that the administration of KKT to 5XFAD mice (4-7 months old) for 15 days improves memory impairment and reverses the degeneration of cortical axons and presynaptic terminals. These effects could be beneficial for AD patients, but the mechanism underlying the effects of KKT remains unknown. The aim of the present study is to investigate the mechanism by which KKT reverses progressive axon degeneration.

Chapter 1

Kamikihito reverses amyloid-β-induced progression of tau phosphorylation and axonal atrophy.

1.1 Introduction

Amyloid beta (Aβ) is widely believed to play a crucial role in AD pathology, and there have been numerous attempts to discover drugs targeting Aβ. However, recent clinical trials have wholly faced failures. In the decade from 2002 to 2012, 244 compounds were attempted in 413 clinical trials for AD, and the only one compound (memantine) was approved for marketing [9, 10]. Anti-Aβ therapies, for example anti-Aβ antibody and γ-secretase inhibitor, were attempted the most in AD clinical trials (70 compounds in 146 trials). Most recently in two phase 3 trials, bapineuzumab, a humanized monoclonal antibody that recognizes the N-terminus of Aβ and binds both soluble and aggregated Aβ, failed to improve cognitive dysfunction in patients with mild- to moderate AD [11]. In two additional phase 3 trials, solanezumab, a humanized monoclonal antibody to Aβ (16–24) that preferentially binds soluble Aβ, also failed to improve cognitive dysfunction [12]. Under the unfortunate situation, it is no wonder to think that targeting Aβ is not sufficient for radical cure of AD [13, 14].

Neurofibrillary tangles, which are composed of hyperphosphorylated tau, also play an important

role in pathologies of AD. Therapeutic approaches targeting hyperphosphorylated tau, such as tau aggregation inhibitors drugs, are currently under investigation, but such efforts are on a very small scale. It was reported that tau aggregation precedes Aβ deposits and is already progressed when AD is diagnosed. Therefore it is important to reverse the tau phosphorylation, not to inhibit, for treatment AD targeting tau.

We investigated the effect of KKT to tau phosphorylation and demonstrated that KKT post-treatment reversed the Aβ-induced increase in tau phosphorylation via protein phosphatase 2A (PP2A) activation [15].

1.2 Materials and Methods

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama.

1.2.1. Preparation of KKT extract

KKT is composed of 14 crude drugs: Ginseng Radix (*Panax ginseng* C.A. Meyer), Polygalae Radix (*Polygalae tenuifolia* Willd.), Astragali Radix (*Astragalus membranaceus* Bunge), Zizyphi Fructus (*Zizyphus jujube* Mill. var. *inermis* Rehd.), Zizyphi Spinosi Semen (*Z. jujube* Mill. var. *spinosa*), Angelicae Radix (*Angelica acutiloba* Kitagawa), Glycyrrhizae Radix (*Glycyrrhiza*

uralensis Fisch), Atractylodis Rhizoma (*Atractylodes japonica* Koidzumi ex Kitamura), Zingiberis Rhizoma (*Zingiber officinale* Roscoe), Poria (*Poria cocos* Wolf), Saussureae Radix (*Saussurea lappa* Clarke), Longanae Arillus (*Euphoria longana* Lam.), Bupleuri Radix (*Bupleurum falcatum* Linne), and Gardeniae Fructus (*Gardenia jasminoides* Ellis) at a ratio of 3 : 1.5 : 2: 1.5 : 3: 2 : 1 : 3 : 1 : 3 : 1 : 3 : 3 : 3, respectively. All crude drugs were purchased from Tochimoto Tenkaido (Osaka, Japan). The mixture of crude drugs (100 g) was extracted with water (500 mL) at 100°C for 50 min, evaporated under reduced pressure, and freeze-dried to obtain a powder extract (12.3 g).

1.2.2. Primary culture

Embryos were removed from a pregnant ddY mouse (Japan SLC, Shizuoka, Japan) at 14 days of gestation. The cerebral cortices were dissected, and the dura mater was removed. The tissues were minced, dissociated and cultured with Neurobasal medium (Gibco BRL, Rockville, MD, USA) containing 12% horse serum, 0.6% D-glucose and 2 mM L-glutamine. Cells were grown in 8-well chamber slides (Falcon, Franklin Lakes, NJ, USA) or 10-cm dishes coated with 5 μg/ml poly-D-lysine at 37°C in a humidified incubator with 10% CO_2. On the next day of culture, the medium was replaced with fresh Neurobasal medium containing 2% B-27 supplement rather than horse serum.

1.2.3. Aβ-induced axonal atrophy

Mouse cortical neurons (E14) were cultured in 8-well chamber slides (Falcon, Franklin Lakes, NJ, USA) at a density of 1.45×10^4 cells/cm^2 for 3 days. To induce axon atrophy, the cells were treated with Aβ (25–35) (10 μM) (Sigma-Aldrich, St Louis, MO, USA). The Aβ (25-35), an active fragment of Aβ, was previously incubated for 4 days at 37°C to induce aggregation. In experiments involving pre-treatment, KKT (10 μg/ml), kenpaullon (KPL, 1 μM; an inhibitor of glycogen synthase kinase-3β: GSK-3β) or vehicle was administered to the cells, and Aβ (25-35) was added to the cells 1 h later. At 24 h after incubation with Aβ, the medium was replaced with Aβ (25-35)-free medium, and the drug treatments were continued for an additional 72 h. In experiments involving post-treatment, cells were incubated with Aβ (25-35) (10 μM) for 24 h. The medium was then replaced to remove Aβ (25-35). The replacement medium contained KKT (10 μg/ml), KPL (1 μM) or vehicle. After drug treatment for 72 h, the cells were fixed with 4% paraformaldehyde and immunostained axons with a monoclonal antibody against phosphorylated NF-H (pNF-H, dilution 1 : 500, Sternberger Monoclonals, Luthervulle, MD, USA) and neuronal cell bodies with a polyclonal antibody against MAP 2a and 2b (MAP2, dilution 1 : 500, Chemicon, Temecula, CA, USA). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1 : 300) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (dilution 1 : 300) (Molecular Probes, Eugene, OR, USA). Fluorescent images were detected by a fluorescence microscope system

(BX-61/DP70, Olympus, Tokyo, Japan). The lengths of axons were measured using the Neurocyte image analyzer (Kurabo, Osaka, Japan), which automatically traced and measured neurite lengths without measuring cell bodies. The total length of the axons was divided by the number of cells in an identical area to calculate the average length of axon per neuron.

1.2.4. Aβ-induced tau phosphorylation

Mouse cortical neurons (E14) were cultured in 8-well chamber slides at a density of 1.45×10^4 cells/cm^2 for 3 days. For pre-treatment experiments, KKT (10 μg/ml), KPL (1 μM) or vehicle was administered to the cells, and Aβ (25-35) (10 μM) was added 1 h later. At 4 h or 72 h after incubation with Aβ, the cells were fixed. For post-treatment experiments, cells were treated with Aβ (25-35) (10 μM) for 24 h. Then, the medium was replaced with medium lacking Aβ (25-35). KKT (10 μg/ml), KPL (1 μM) or vehicle was included in the replacement medium. At 72 h after drug treatment, the cells were fixed and immunostained with a monoclonal antibody against paired helical filament tau (PHF-tau, AT-8 clone, dilution 1: 200, Thermo Scientific, Rockford, IL, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1: 300) was used as the secondary antibody. Images were captured using a fluorescence microscope system (BX-61/DP70, Olympus). The level of tau phosphorylation was measured as the fluorescence intensity in the cell body of each neuron using a CS analyzer (ATTO, Tokyo, Japan).

1.2.5. PP2A activity assay

Mouse cortical neurons (E14) were cultured in a 10-cm-dish at a density of 8.67×10^4 cells/cm^2 for 3 days. KKT (10 μg/ml) or vehicle was administered to the cells, and Aβ (25–35)(10 μM) was added 1 h later. At 72 hours after incubation with Aβ, the cells were harvested and suspended in phosphate storage buffer [2 mM EGTA, 5 mM EDTA, 0.5 mM PMSF, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100 and protease inhibitor cocktail (1:200)]. The cell lysates were sonicated and centrifuged at 16,000 ×g for 10 min, and the supernatants were used for the PP2A activity assay. PP2A activity was measured using a serine/threonine phosphatase assay system (V2460, Promega, Madison, WI, USA) according to the manufacturer's protocol. The cell lysates were passed twice through Sephadex® G-25 Spin Columns to remove endogenous free phosphate. The protein concentration was determined using the Pierce® 660 nm Protein Assay. PP2A reaction buffer (50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol and 0.1 mg/ml bovine serum albumin) and 1 mM phosphopeptide were prepared in a 96-well plate. The enzyme reaction was started by adding 2.5 μg of the protein lysate to each well, and the mixture was incubated for 30 min at 33°C. The reaction was stopped by the addition of 50 μl of molybdate dye-additive mixture, and the plate was incubated at room temperature for 15 min. Phosphate release from the substrate was detected by measuring the absorbance of the molybdate-malachite green-phosphate complex at 630 nm.

1.2.6. Animals

Transgenic mice (5XFAD) [8] were obtained from Jackson laboratory (Bar Harbor, ME, USA). To investigate the effect of KKT on 5XFAD mice, the study used hemizygous 5XFAD mice (female, 9–11 months) and non-transgenic wild-type mice (female, 9–10 months). All mice were housed with free access to food and water and kept in a controlled environment (22 ± 2°C, 50 ± 5% humidity, 12 h light cycle starting at 7:00 am).

KKT was dissolved in physiological saline. KKT (500 mg/kg/day) or vehicle solution was intragastrically administered once a day for 15 days. On the 16th day, the mice were sacrificed, and the cerebral cortex was isolated and homogenized in phosphate storage buffer for the PP2A activity assay.

1.2.7. GSK-3β activity assay

Mouse cortical neurons (E14) were cultured in 8-well chamber slides at a density of 1.45×10^4 cells/cm^2 for 3 days. KKT (10 μg/ml) or vehicle was administered to the cells, and Aβ (25–35) (10 μM) was added 1 h later. At 4 h after incubation with Aβ, the cells were fixed with 4% paraformaldehyde and immunostained with a monoclonal antibody against phosphorylated GSK-3β at Tyr-216 (pY216, dilution 1: 200, BD Biosciences, Franklin Lakes, NJ, USA) as a marker of activated GSK-3β and a polyclonal antibody against GSK-3β (dilution 1: 500, Santa Cruz

Biotechnology, Santa Cruz, CA, USA) as a marker of total GSK-3β. Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1 : 300) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (dilution 1 : 300) were used as secondary antibodies. Images were captured using a fluorescence microscope system (BX-61/DP70, Olympus). Expression of phosphorylated GSK-3β and total GSK-3β was measured by determining the fluorescence intensity in the cell body of each neuron using a CS analyzer (ATTO). The ratio of GSK-3β (pY216) to GSK-3β (total) was calculated.

1.2.8. Statistical analysis

Statistical comparisons were performed through a One-way analysis of variance (ANOVA) with *post hoc* Dunnett's test or Bonferroni's Multiple Comparison Test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). $p < 0.05$ was considered significant. The data are presented as the means ± SE.

1.3. Results

1.3.1. KKT decreases Aβ (25-35)-induced phosphorylation of tau.

We first focused on the effectiveness of KKT against Aβ (25-35)-induced phosphorylation of tau. Full-length Aβ (1-42) and its active fragment Aβ (25-35) have been reported to induce

phosphorylation of tau [16, 17]. Phosphorylation of tau leads to axon transport deficits, synaptic loss and mitochondrial dysfunction, resulting in axonal dysfunction [18-20]. Among the various kinases involved in phosphorylation of tau, GSK-3β plays a critical role in AD pathology [21]. We therefore investigated the effect of KKT (10 μg/ml) and KPL (inhibitor of GSK-3β; Ki = 0.23 μM) (1 μM) on Aβ (25-35)-induced phosphorylation of tau. Drugs were administered pre- or post-treatment with Aβ (25-35).

As shown in Figure 1A, treatment of cortical neurons with Aβ (25-35) for 4 h resulted in a 3.7-fold increase in phosphorylation of tau compared with the control. However, the phosphorylation of tau was attenuated to 2.6-fold by 1 h of pre-treatment with KKT (10 μg/ml). With KPL pre-treatment, phosphorylation of tau was remarkably prevented with 1.4-fold (Figure 1A). Tau remained phosphorylated at 72 h after Aβ (25-35) treatment, although the degree of phosphorylation was lower than at 4 h after treatment (Figure 1B). Phosphorylation of tau in the late phase was remarkably prevented by pre-treatment with KKT, but pre-treatment with KPL resulted in only a slight attenuation of tau phosphorylation.

We next investigated the effects of post-treatment with KKT (10 μg/ml) and KPL (1 μM) on Aβ (25-35)-induced phosphorylation of tau (Figure 2). We added Aβ (25-35) to the cells for 24 h, and the cells were incubated with each drug for the next 72 h without Aβ (25-35). KPL no longer affected the Aβ-induced phosphorylation of tau under these conditions, whereas KKT post-treatment

significantly inhibited the phosphorylation of tau.

These results suggest that KKT reduces the phosphorylation of tau at both early and late phases and that increased tau phosphorylation can be reversed by KKT. In contrast, KPL may inhibit the phosphorylation of tau only in the early phase.

1.3.2. KKT reverses Aβ (25-35)-induced axonal atrophy

Figure 1 shows that KKT remarkably reduced the phosphorylation of tau in the late phases (Figure 1B), and KPL prevented the phosphorylation of tau more completely in the early phases (Figure 1A). Inhibition of tau phosphorylation by KPL no longer occurred when phosphorylation had already progressed (Figure 2). Because it is known that Aβ induces axonal atrophy through phosphorylation of tau, we investigated the effects of KKT and KPL on Aβ (25-35)-induced axonal atrophy.

Treatment of cortical neurons with Aβ (25-35) (10 μM) for 24 h significantly decreased the axonal density (Figure 3A), which did not recover even 72 h after the removal of Aβ (25-35) (Figure 3B). In the pre-treatment protocol, administration of KKT (10 μg/ml) and KPL (1 μM) significantly inhibited Aβ (25-35)-induced axonal atrophy (Figure 3A). The protection of axonal atrophy was equivalent in neurons that were pre-treated with KKT or with KPL. In contrast, in the post-treatment protocol, only administration of KKT reversed Aβ (25-35)-induced axonal atrophy (Figure 3B). These results suggest that KKT, but not KPL, increases axonal density even after axonal atrophy has

already progressed.

1.3.3. KKT reverses the Aβ-induced decrease in PP2A activity

KKT remarkably reduced the phosphorylation of tau in the late phase (Figure 1B). To explain the long-lasting inhibitory effect of KKT on the phosphorylation of tau, we proposed that KKT may play a role in the dephosphorylation of tau. It was previously reported that Aβ increases the phosphorylation of tau by decreasing PP2A activity [22]; we therefore investigated the effect of KKT on PP2A activity under Aβ (25-35) treatment. KKT (10 μg/ml) was added to the cells, and Aβ (25-35) (10 μM) was added 1 h later. PP2A activity was measured 72 h after Aβ addition. As shown in Figure 4A, Aβ treatment decreased PP2A activity to 79.6 % compared with the control group. However, pre-treatment with KKT completely prevented the decrease in PP2A activity (Figure 4A).

PP2A activity was also measured in vivo. KKT (500 mg/kg/day) was administered orally to the 5XFAD mice, and cerebral cortex lysates were prepared for measurement of PP2A activity. Our previous data showed that KKT administration for 15 days completely restored memory impairment in 5XFAD mice [7]. In the present experiment, we further confirmed that memory was improved by KKT treatment (data not shown). Vehicle-treated 5XFAD mice showed a significant decrease in PP2A activity compared with wild-type mice. However, KKT treatment of 5XFAD mice significantly increased PP2A activity compared with vehicle treatment (Figure 4B). These results

demonstrate that KKT enhances the activity of PP2A under Aβ (25-35)-induced down-regulation of PP2A activity.

1.3.4. KKT has no effect on GSK-3β activity

Figures 1 and 2 show that KKT inhibits the Aβ (25-35)-induced phosphorylation of tau in both the early and late phases. However, the effect of KKT in the early phase was lower than that of KPL. Because the effects of KKT and KPL were different, we next investigated whether KKT was also an inhibitor of GSK-3β or not. GSK-3β activity was shown to be regulated by phosphorylation at specific residues; for example, phosphorylation of Ser9 and Ser389 inhibited its kinase activity, whereas phosphorylation at Tyr216 was required for its activation [23]. In this experiment, we measured the ratio of GSK-3β (pY216) to GSK-3β (total) to evaluate the level of GSK-3β activation. As shown in Figure 5, Aβ (25-35) (10 μM) treatment for 4 h significantly increased the amount of phosphorylated GSK-3β (Tyr216). However, pre-treatment with KKT (10 μg/ml) had no effect on this increase (Figure 5). These results suggest that KKT may not be involved in the regulation of GSK-3β activity.

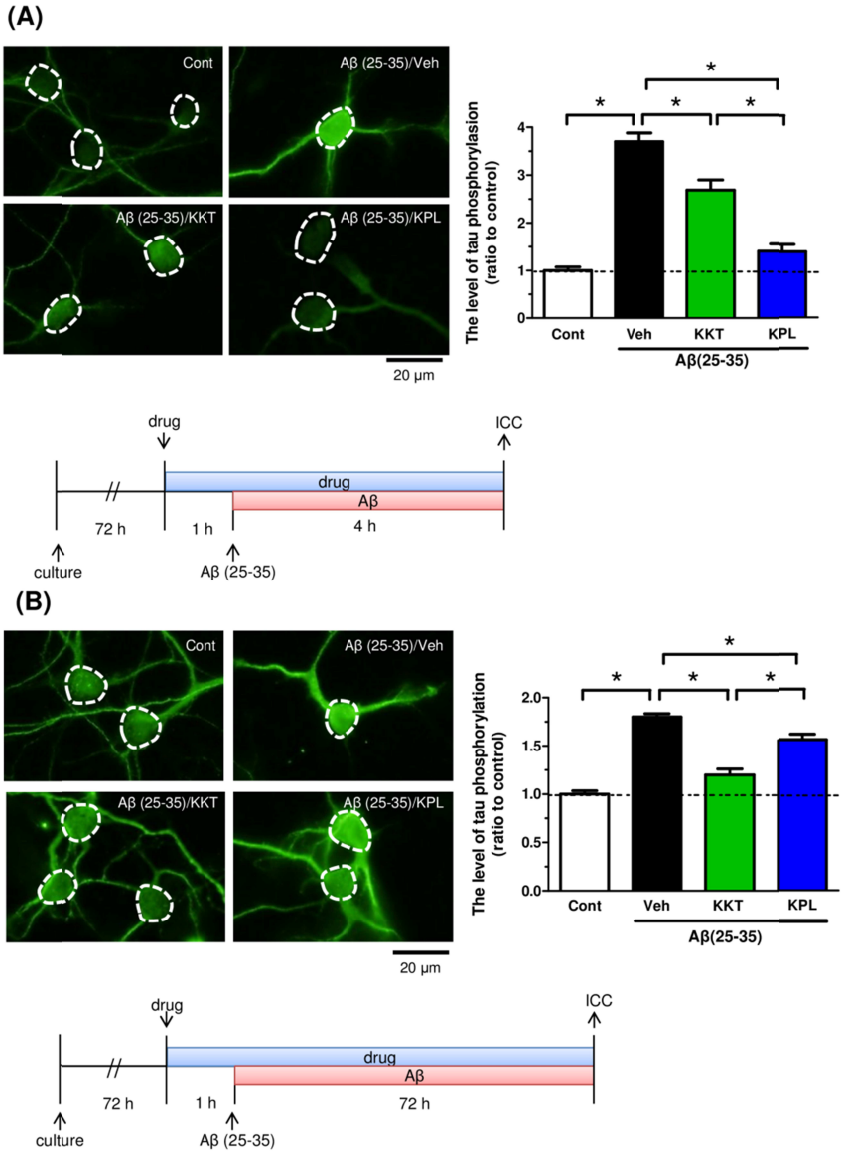

Figure 1. Effect of KKT and KPL pre-treatment on Aβ-induced phosphorylation of tau. Cortical neurons were cultured for 3 days and then treated with vehicle solution, KKT (10 μg/ml) or KPL (1 μM). At 1 h after each treatment, the cells were treated with Aβ (25-35) (10 μM) for 4 h (A) or 72 h (B). After Aβ treatment, the cells were fixed and immunostained with an antibody against paired helical filament tau (PHF-tau). The PHF-tau fluorescence intensity was quantified for each treatment. * $p < 0.05$, (n = 15 – 20, One-way ANOVA, *post hoc* Bonferroni's Multiple Comparison Test).

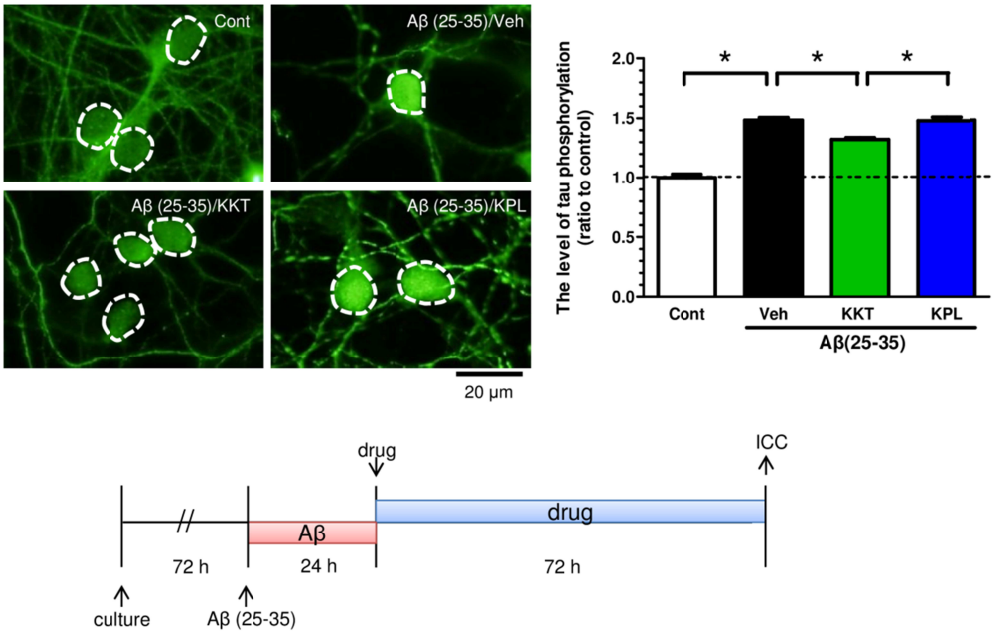

Figure 2. Effect of KKT and KPL post-treatment on Aβ-induced phosphorylation of tau. Cortical neurons were cultured for 3 days and then treated with Aβ (25-35) (10 μM). At 24 h after Aβ treatment, Aβ was removed from the medium, and the cells were treated with vehicle solution, KKT (10 μg/ml) or KPL (1 μM). At 72 h after each treatment, the cells were fixed and immunostained with an antibody against paired helical filament tau (PHF-tau). The PHF-tau fluorescence intensity was quantified for each treatment. * $p < 0.05$, (n = 15 - 17, One-way ANOVA, *post hoc* Bonferroni's Multiple Comparison Test).

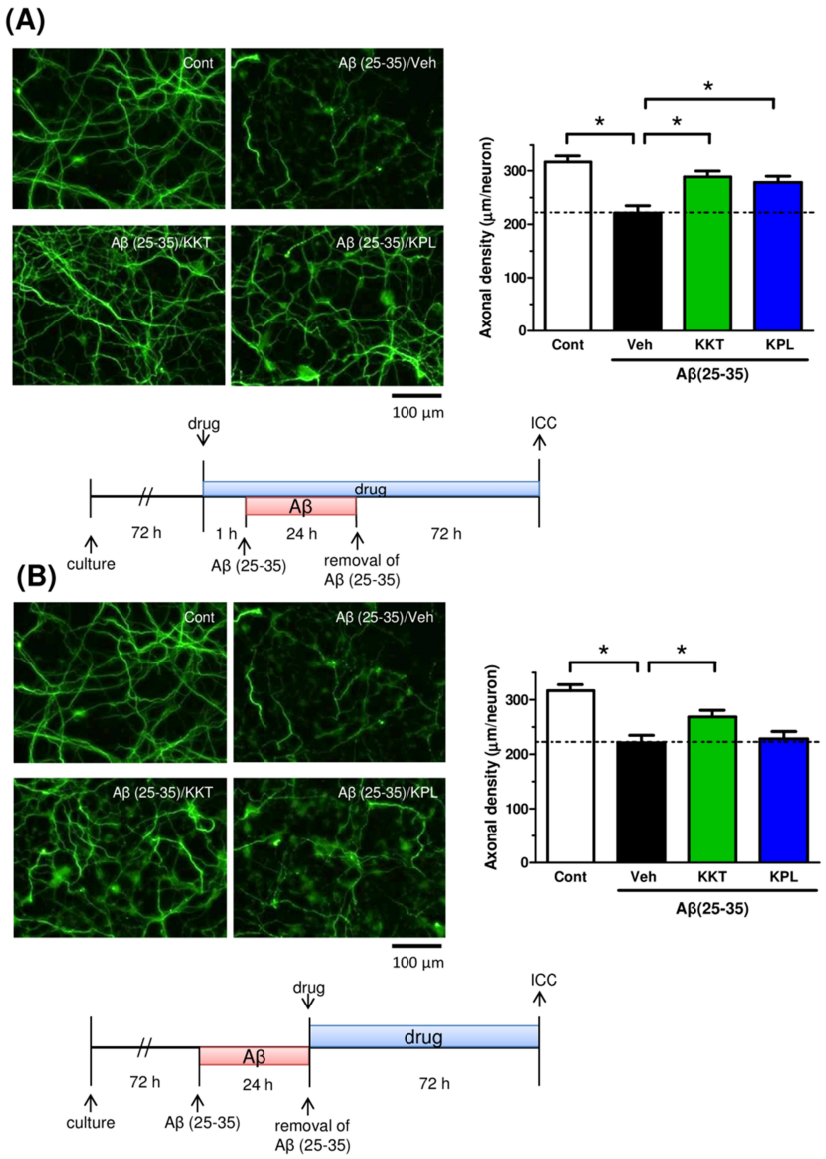

Figure 3. Effect of KKT and KPL pre- and post-treatment on Aβ-induced axonal atrophy.

(A) For pre-treatment, cortical neurons were cultured for 3 days and then treated with vehicle solution, KKT (10 μg/ml) or KPL (1 μM). After 1 h, the cells were treated with Aβ (25-35) (10 μM) for 24 h, and Aβ was removed from the medium. Each drug treatment was continued for 72 h. (B) For post-treatment, cortical neurons were cultured for 3 d and then treated with Aβ (25-35) (10 μM). At 24 h after Aβ treatment, Aβ was removed from the medium and the cells were treated with vehicle solution, KKT (10 μg/ml) or KPL (1 μM) for 72 h. After each drug treatment, the cells were fixed and immunostained with an antibody against phosphorylated NF-H and MAP2. The lengths of phosphorylated NF-H-positive neurites were quantified for each treatment. * $p < 0.05$, (n = 23 - 31, One-way ANOVA, *post hoc* Bonferroni's Multiple Comparison Test).

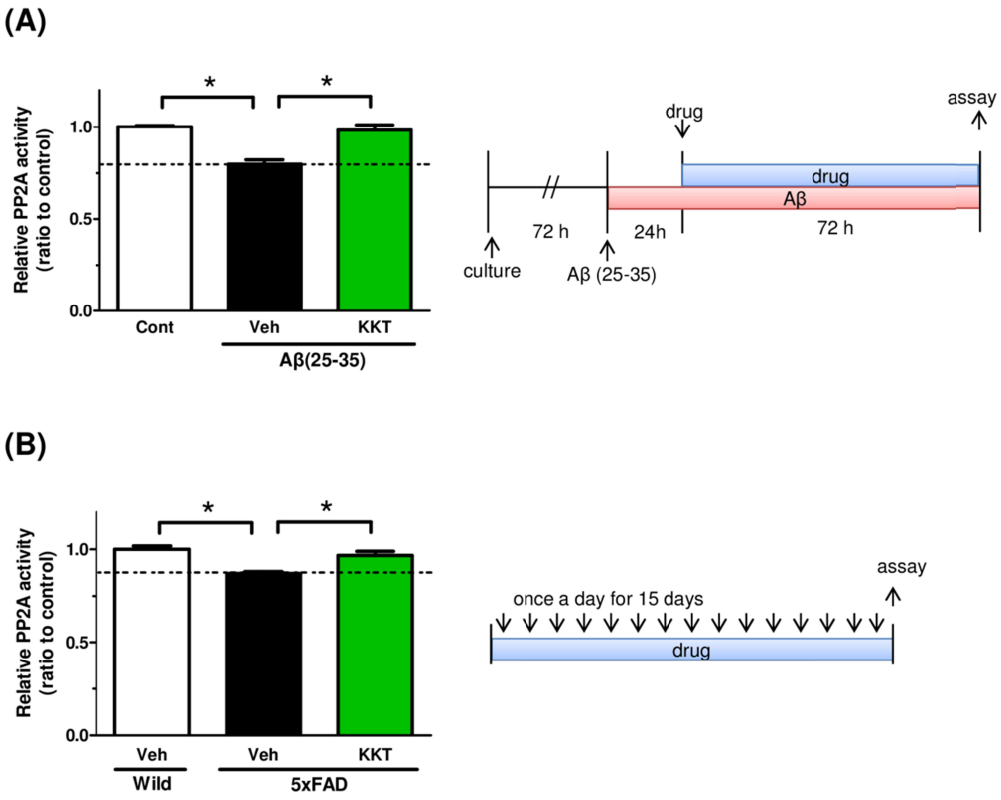

Figure 4. KKT enhances PP2A activity *in vitro* and *in vivo*.

(A) Cortical neurons were cultured for 3 d and then treated with vehicle solution or KKT (10 μg/ml). At 1 h after each treatment, the cells were treated with Aβ (25-35) (10 μM) for 72 h. The cell lysates were prepared for the PP2A activity assay. PP2A activity was measured using a serine/threonine phosphatase assay system. (B) Vehicle solution or KKT (10 μg/ml) was administered once a day for 15 da to wild-type or 5XFAD mice. Cerebral cortex homogenates were used for the PP2A activity assay. * $p < 0.05$ vs Veh, (n = 4 - 8, One-way ANOVA, *post hoc* Dunnett's test).

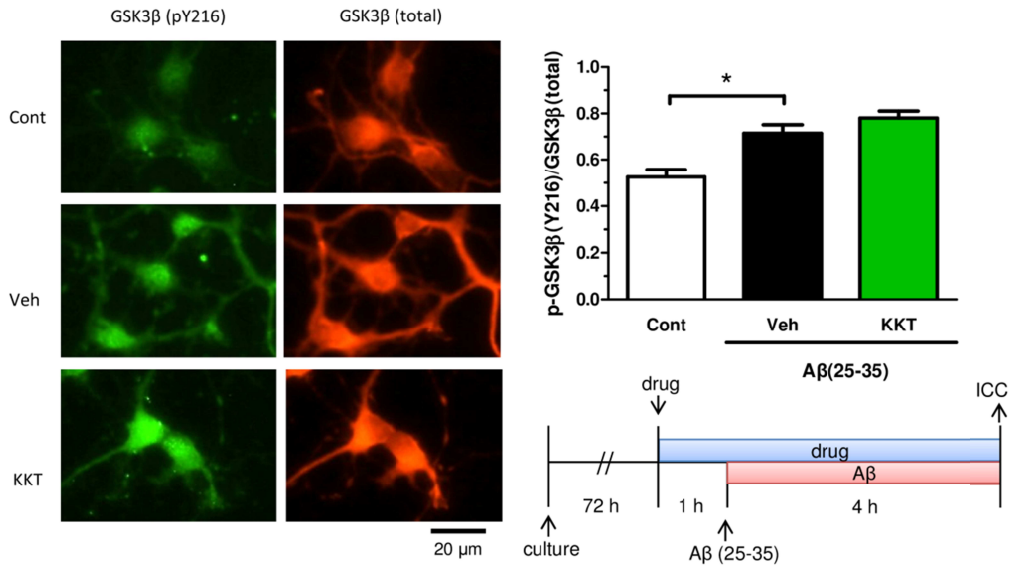

Figure 5. Effect of KKT on Aβ-induced activation of GSK-3β.

Cortical neurons were cultured for 3 d and then treated with vehicle solution or KKT (10 μg/ml). At 1 h after each treatment, the cells were treated with Aβ (25-35) (10 μM) for 4 h. After Aβ treatment, the cells were fixed and immunostained with antibodies against phosphorylated GSK-3β at Tyr-216 (pY216), a marker of activated GSK-3β, and against total GSK-3β. The fluorescence intensity was quantified for each treatment. The ratio of GSK-3β (pY216) to GSK-3β (total) is shown. * $p < 0.05$ vs Veh, (n = 15 - 16, One-way ANOVA, *post hoc* Dunnett's test).

1.4. Discussion

Senile plaques and neurofibrillary tangles are two hallmark pathologies of AD. Senile plaques contain extracellular deposits of fibrillar Aβ, and neurofibrillary tangles are composed of hyperphosphorylated tau. It has been reported that Aβ induces phosphorylation of tau [16, 17] and that the phosphorylation of tau leads to axon transport deficits, synaptic loss, and mitochondrial dysfunction [19–20]. These disturbances eventually lead to axonal degeneration and neuronal death. Consequently, therapeutic approaches targeting tau have been growing steadily in recent years. For example, 2-methyl-5-(3-4-[(S)-methylsulfinyl]phenyl-1-benzofuran-5-yl)-1,3,4-oxadiazole (MMBO) and NP12 have been reported to decrease tau phosphorylation and reverse cognitive deficits in AD mouse models, which are novel GSK-3 inhibitors [24, 25]. In patients with mild to moderate AD, methylene blue (a tau aggregation inhibitor) is effective in treating cognitive deficits [26]. Phase II clinical trials of NP12 (tideglusib) and phase III clinical trials of LMTX, a derivative of methylene blue, are ongoing.

In this study, we investigate the mechanism underlying the effect of KKT. KKT reversed memory impairment in 5XFAD mice in our previous study [7], and here we demonstrate three properties of the effects of KKT. First, posttreatment with KKT reverses the progression of tau phosphorylation; however, posttreatment with KPL has no effect on tau phosphorylation (Figure 2). Second, posttreatment with KKT restores atrophied axons, whereas KPL does not (Figure 3(b)). Third, KKT

enhances the activity of PP2A (Figure 4) but is not involved in the regulation of GSK-3β activity (Figure 5).

Recent reports of therapeutic strategies targeting Aβ, such as solanezumab and semagacestat, demonstrate that decreasing Aβ deposits or plasma Aβ levels after the appearance of symptoms does not improve cognitive dysfunction [6, 27]. In contrast, several reports have shown that tau is essential for Aβ-induced neuronal dysfunction in vitro and in vivo [28, 29]. Therefore, a decrease in tau phosphorylation may improve the symptoms of AD. From this point of view, it is very interesting that KKT reverses the progression of tau phosphorylation and axonal atrophy.

Our data suggest that KKT decreases the phosphorylation of tau through the activation of PP2A; however, the mechanism by which KKT increases PP2A activity remains unknown. The PP2A subtype accounts for more than 70% of all phosphatases in the human brain. In addition, the activity of PP2A decreases more than that of other phosphatases in the brains of AD patients [30]. Thus, PP2A likely plays a critical role in the dephosphorylation of tau. There are reports showing that PP2A activators decrease the phosphorylation of tau and reverse memory deficits in an AD model. For example, memantine enhances PP2A activity indirectly, and 1 year of treatment with memantine significantly decreases the levels of phosphorylated tau in the CSF of AD patients [31, 32]. Other reports have shown that sodium selenite, which induces dephosphorylation of tau via PP2A activation, improves memory deficits in tau transgenic mice [33]. Therefore, activation of PP2A

likely plays an important role in the effects of KKT for AD treatment. However it has also been reported that KKT ameliorates scopolamine-induced spatial memory deficits and methyl-β-carboline-3-carboxylate(β-CCM)-induced-anxiogenic behavior [34, 35]. These reports suggest that KKT may stimulate the cholinergic system and antagonize the benzodiazepine receptor system. Multiple constituents of KKT may therefore contribute to its pharmacological effects.

Our present data indicating that KKT is effective even after Aβ-induced degeneration has occurred are important. Future studies should clarify which compounds in KKT are related to the restorative effect. KKT extract contains many constituents. Among them, at least ginsenoside Rg1, ginsenoside Rb1, saikosaponin a, saikosaponin b1, saikosaponin b2, geniposide, liquiritin, apioliquiritin, glycyrrhizic acid, 6-gingerol, and ligustilide were identified in our previous report [7]. Ginsenoside Rb1, a constituent in Ginseng Radix, attenuated spatial memory deficits in Aβ (25–35)-injected mice [36] and extended axons under Aβ (25–35) treatment (our unpublished data). Therefore ginsenoside Rb1 may be one of the active constituents in KKT.

Also we previously showed that posttreatment with Polygalae Radix extract reversed Aβ (25–35)-induced axonal atrophy [37]; therefore, Polygalae Radix may be at least partially responsible for the effect of KKT.

Chapter 2

A cross-country comparison of kamikihito toward the development of a global study

2.1 Introduction

Traditional medicine has been used in East Asia (China, Korea and Japan) to treat various symptoms since antiquity. These traditional medicines originated in China 3,000 years ago and were introduced to Korea and Japan in the 6th century [38, 39]. Until recently, traditional medicine has evolved independently in each country; therefore, slight differences exist in the diagnostic methods and symptoms targeted even when traditional formulations with the same origin are used. Recently, traditional medicine has been made available to treat intractable diseases in Western medicine, such as dementia [40]. However, little evidence regarding the efficacy of this treatment exists because of the small number of cases, non-randomized controlled research trial designs and insufficient mechanistic explanations [41]. To increase the evidence in favor of traditional medicines, expanded cross-country clinical studies are necessary. Clinical study approaches are also useful for evaluating the differences between ethnic groups and traditional medical systems. Ideally, researchers across countries should be able to use unified formulations of traditional medicine; however, this practice is difficult.

If the same-origin formulations used in each country have equivalent effects, cross-country clinical studies can be easily conducted, even when a unified formulation is not available. Moreover,

the results can be understood across different countries. However, no comparative studies have examined the same-origin but non-identical formulations used in different countries.

We previously reported the effects of kamikihito (Japanese name, KKT) in a 5XFAD mouse model of Alzheimer's disease [7]. The KKT formulations that have the same origin and components are also used in traditional Chinese and Korean medicine (called jia-wei-gui-pi-tang, JGT and kami-guibi-tang, KGT, respectively) to treat insomnia, loss of appetite, amnesia, and depression. Using Chinese characters, KKT, JGT, and KGT are written similarly in Japanese, Chinese, and Korean. To confirm and expand the global usefulness of the formulations, it is important to investigate whether these formulations yield equivalent effects. Thus, this study compared the effects of KKT and KGT preparations on memory function in normal mice. We prepared three types of formulations and investigated the effects of KKT on normal memory [42]. One form of KKT is commercially available from a Japanese pharmaceutical company (Kracie), a second form of KKT is prescribed as a decoction at Toyama University Hospital, and the final form of KGT is used at the Kyung Hee Korean Medical Hospital of Kyung Hee University in Korea.

2.2 Materials and Methods

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama.

2.2.1 Preparation of KKT and KGT extracts

KKT and KGT are composed of 14 crude drugs. The original plant sources of these drugs and the daily human doses administered are shown in Table 1. All crude drugs used for KKT at Toyama University Hospital (KKT-TU) were purchased from Tochimoto Tenkaido (Osaka, Japan). The mixture of crude drugs (3.3 - fold the daily dose; 100 g) was extracted with 500 ml of water at 100°C for 50 min, evaporated under reduced pressure, and freeze-dried to obtain a powder extract (12.3 g, yield 12.3%).

For the KGT at Kyung Hee University (KGT-KHU), all crude drugs were obtained from the Kyung Hee Herb Pharmacy (Wonju, Kangwon, Korea) and E-dam Oriental Medicine Materials (Namyangju, Gyeonggi, Korea). The mixture of crude drugs (160 - fold the daily dose, 4,720 g) was extracted with 80 L of water at 100°C for 3 h. The decoction was filtered, lyophilized, and stored at 4°C. Ultimately, 760 g of water-extracted powder was obtained using wet granulation (yield 16.1%), and 40 g of polyvinylpyrrolidone was added to the extract as an excipient.

The Kracie KKT (KKT-Kra) was obtained from Kracie Pharma, Ltd. (Tokyo, Japan). All crude drugs used for the extract were self-prepared under quality control. The mixture of crude drugs (29.5 g daily dose) was extracted with 300 ml of water at 100°C for 60 min, evaporated under reduced pressure, and spray-dried to obtain a powder extract (5.6 g, yield 19.0%).

2.2.2 Preparation of anchusan (AS)

AS is composed of 7 crude drugs. The original plant sources of these drugs and the daily human doses administered are shown in Table 2. For the AS used at the Toyama University Hospital, all crude drugs were purchased from Tochimoto Tenkaido. The mixture of crude drugs (5.0 - fold the daily dose, 100 g) was extracted with 500 ml of water at 100°C for 50 min, evaporated under reduced pressure, and freeze-dried to obtain a powder extract (7.41 g, yield 7.41%).

2.2.3 Animals

Six-week-old male ddY mice (Japan SLC, Shizuoka, Japan) were housed in groups with free access to food and water and maintained in a controlled environment (22 ± 2°C, 50 ± 5% humidity, and a 12-h light/dark cycle starting at 7:00 a.m.). Each KKT, KGT, or AS dose was dissolved in distilled physiological saline. KKT-TU (500 mg extract/kg/day), KKT-Kra (500 mg extract/kg/day), KGT-KHU (500 mg extract/kg/day), AS (500 mg/kg/day), or a vehicle solution (physiological saline) was administered orally once daily for 13 days. Open field tests for locomotion were performed on Day 7. A novel object recognition test was conducted on Day 8, and an object location test was performed on Day 11 (see Fig. 1A). All tests were performed in the daytime and under the same illuminated conditions (90 lux).

2.2.4 Open field test

The mice were released for 10 min in a square field (30 × 41 cm; height, 36.5 cm), and a digital camera tracked their paths. The distance over which the mice traveled for 10 min (i.e., locomotion activity) was analyzed using EthoVision 3.0 (Noldus, Wageningen, Netherlands). This testing was conducted in a dimly illuminated room.

2.2.5 Object recognition test

Two identical objects (ceramic black cat ornaments) were placed at a fixed distance within the square field (30 × 41 cm; height, 36.5 cm). A mouse was then placed in the center of the field, and the number of times it contacted the two objects was recorded over a 10-min period (training session). The mice were then returned to the same field 48 h after the training session, and one of the familiar objects used during the training session was replaced with a novel object (a ceramic yellow duck ornament). The mice were allowed to explore freely for 10 min, and the number of times that they made contact with each object was recorded (test session). A preference index (i.e., the ratio of the number of times that a mouse contacted any objects during the training session or the novel object during the test session to the number of times that the mice contacted both objects) was used as a measure of objective cognitive function. The interval time 48 h was determined by our experiments as duration when normal mice cannot keep the memory.

2.2.6 Object location test

The object location test began the day after the object recognition test. In the training session, two identical novel objects (ceramic green frog ornaments) were placed at a fixed distance within the open field box. Inside the box, different wall covering patterns were used; one side of the black wall had vertical white strips, and the opposite side of the wall had white circles. The two objects were placed on the floor of the box. A mouse was placed at the opposite end of the field, and the number of explorations of each object was recorded over a 10-min period (training session). After a 48-h interval, the test session was conducted during which one of the objects was moved to a different position in the field. The mice were allowed to explore freely for 10 min, and the number of explorations of each object was recorded (test session). A preference index (i.e., the ratio of the number of times that a mouse contacted any object during the training session or the object at the novel location during the test session to the number of times that the mouse contacted both objects) was used as a measure of spatial memory.

2.2.7 Three-dimensional high-performance liquid chromatography (3D-HPLC) analysis of extracts

Each extract solution (10 mg/ml) was filtered through membrane filter (0.22 μm) and then submitted for HPLC analysis (20 μl). The HPLC apparatus (Hitachi Ltd., Japan) consisted of a pump

(L-2130) with analysis system software (Elite LaChrom); it was equipped with a photodiode array detector (UV 230-400 nm, L-2455), a system controller, an auto injector (L-2200), and a column oven (L-2300). The HPLC conditions were as follows: column, LaChrom Ultra C18 (5 μm, Lot No. 21D5-011; Hitachi Ltd., Japan) with 150 × 4.6 mm I.D.; elutant, (A) H_2O containing 0.1% formic acid and (B) CH_3CN containing 0.1% formic acid (a linear gradient was used from “95% A and 5% B” to “30% A and 70% B” for 90 min); temperature, 20°C; and flow rate, 0.2 ml/min.

2.2.8 Statistical Analysis

Statistical comparisons were performed using a one-way ANOVA followed by a Dunnett's post hoc test or a paired *t*-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ were considered significant. The mean data values are presented together with their associated standard errors (SE).

2.3 Results

2.3.1 KKT and KGT enhance object recognition and location memory in ddY mice

To investigate the effects of each drug on object recognition and location memory, KGT-KHU (500 mg/kg/day), KKT-TU (500 mg/kg/day), KKT-Kra (500 mg/kg/day), AS (500 mg/kg/day), or a

vehicle solution was orally administered once daily for 13 days. On the 7th day of administration, an open field test was performed. The locomotor activity was similar across all groups ($p > 0.05$, one-way ANOVA followed by Dunnett's *post hoc* test, Figure 6B). The novel object recognition test was performed on the 8th day, and the object location test began on the 11th day. In this experiment, AS was used as a reference drug because its components do not overlap with KKT-TU, KGT-KHU, or KKT-Kra (except for Glycyrrhizae Radix). AS is used clinically to treat stomachaches and loss of appetite.

In the novel object recognition test, the vehicle- and AS-treated mice showed equivalent exploratory behaviors that were close to chance (50%) in the test session ($p > 0.05$, paired *t*-test). This finding indicates that the vehicle- and AS-treated mice were unable to maintain memories of the objects presented in the training session. In contrast, the KGT-KHU- and KKT-Kra-treated mice showed significant exploratory behaviors in the presence of a novel object in the test session ($p = 0.015$ and 0.014, paired *t*-test). The KKT-TU-treated mice also showed above-chance exploratory behaviors; however, this result was not significant ($p = 0.079$, paired *t*-test, Fig. 7A).

In the object location test, the vehicle- and AS-treated mice showed similar exploratory behaviors that approximated chance (50%) in the test session ($p > 0.05$, paired *t*-test), which indicated that these mice were unable to maintain the spatial memories acquired during the training session. In contrast, KGT-KHU- ($p = 0.047$), KKT-TU- ($p = 0.003$), and KKT-Kra-treated ($p = 0.001$) mice

showed significant exploratory behaviors towards novel objects (paired t-test, Fig. 7B). These results suggest that KKT and KGT have similar effects on normal memory enhancement.

2.3.2 3D HPLC analysis

The 3D-HPLC profiles of each preparation are shown in Figure 8A. The compound distributions with UV absorbance were plotted by the retention time (x-axis), wavelength (y-axis) and content (color bar). The general profiles of KKT-TU, KGT-KHU, and KKT-Kra were similar, but there were several differences in the peak height. In contrast, the peak profiles of AS were quite different from KKT and KGT.

In the chromatograms at 260 nm (Figure 8B) and 310 nm (Figure 8C), some main compounds were annotated. Geniposide derived from Gardeniae Fructus was present at slightly higher levels in KKT-TU compared with KGT-KHU and KKT-Kra.

6'-O-[6-O-(4-Hydroxy-Z-cinnamoyl-β-D-glucopyranosyl]geniposidic acid methyl ester derived from Gardeniae Fructus was present at comparatively low levels in KGT-KHU. The glycyrrhizin and liquiritin derived from Glycyrrhihizae Radix were present at high levels in KKT-Kra.

Table 1　Compositions of kamikihito and kami-guibi-tang

Crude drug name	Original plant source	Kyung Hee University	University of Toyama	Kracie
		(g/day)	(g/day)	(g/day)
Ginseng Radix	*Panax ginseng*	3	3	3
Atratylodis Rhizoma	*Atratylodes japonica*	3	3	3
Poria	*Poria cocos*	3	3	3
Bupleuri Radix	*Bupleurum falcatum*	3	3	3
Zizyphi Spinosi Semen	*Ziziphus jujuba* var. *spinosa*	3	3	3
Longan Arillus	*Euphoria longana*	3	3	3
Astragali Radix	*Astragalus membranaceus*	2	2	2
Angelicae Radix	*Angelica acutiloba*	2	2	2
Gardeniae Fructus	*Gardenia jasminoides*	2	3	2
Polygalae Radix	*Polygala tenuifolia*	1.5	1.5	1.5
Zizyphi Fructus	*Ziziphus jujuba* var. *inermis*	1.5	1.5	1.5
Glycyrrhizae Radix	*Glycyrrhiza uralensis*	1	1	1
Saussureae Radix	*Saussurea lappa*	1	1	1
Zingiberis Rhizoma	*Zingiber officinale*	0.5	1	0.5

Table 2　Composition of anchusan

Crude drug name	Original plant source	University of Toyama (g/day)
Cinnamomi Cortex	*Cinnamomum cassia*	4
Corydalis Tuber	*Corydalis turtschaninovii*	4
Ostreae Testa	*Ostrea gigas*	4
Foeniculi Fructus	*Foeniculum vulgare*	4
Glycyrrhizae Radix	*Glycyrrhiza uralensis*	1.5
Amomi Semen	*Amomum xanthioides*	1.5
Alpiniae officinari Rhizoma	*Alpinia officinarum*	0.7

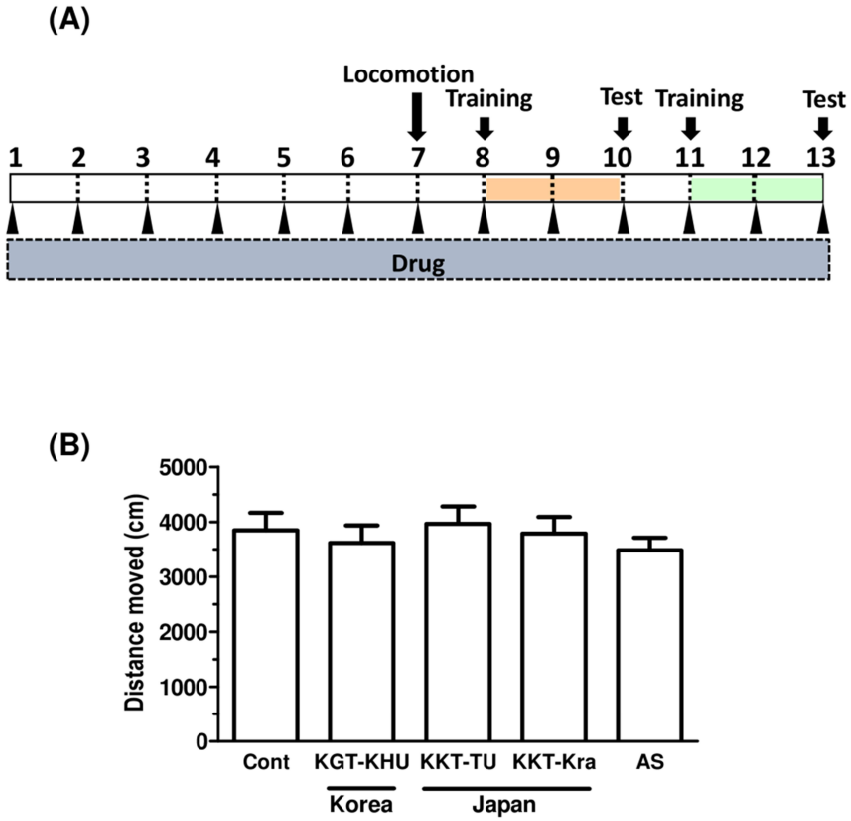

Figure 6. Experimental schedule and locomotion.

(A) The experimental schedule for locomotion, object recognition and object location tests. (B) KGT-KHU (500 mg/kg/day), KKT-TU (500 mg/kg/day), KKT-Kra (500 mg/kg/day), AS (500 mg/kg/day), or vehicle solutions were administered daily to ddY mice for 13 days. On the 7th day of administration, open field tests were performed. The total distances traveled for 10 min in an open field are shown (n = 5, one-way ANOVA with Dunnett's *post hoc* test).

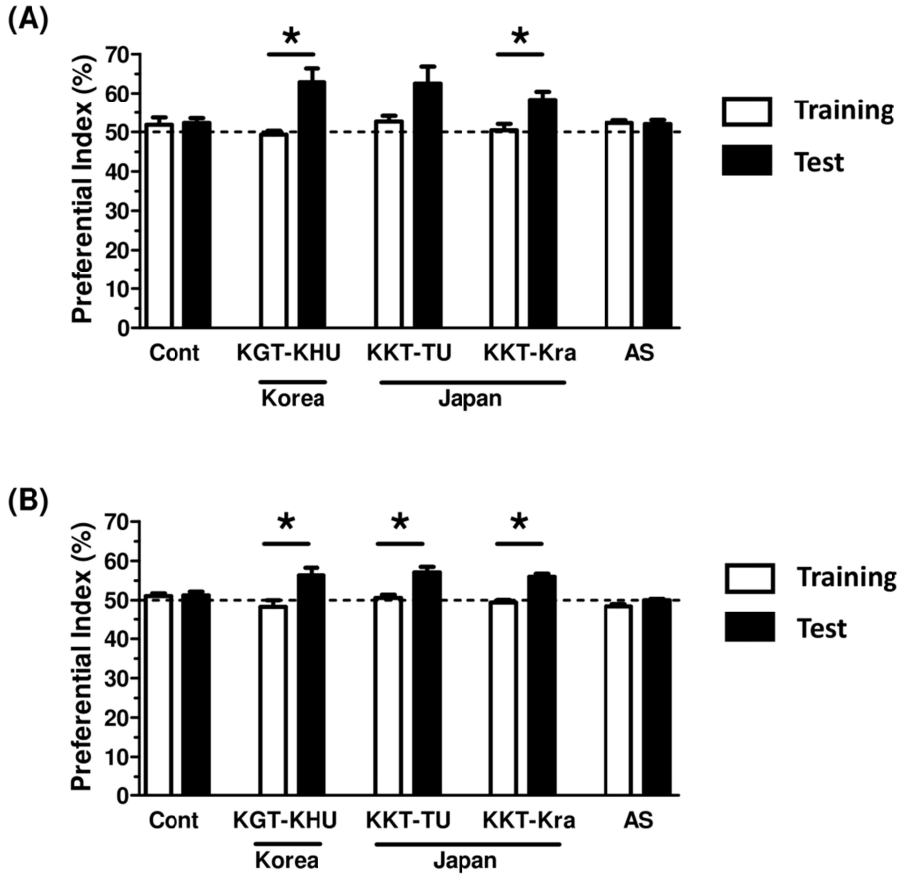

Figure 7. The effects of KKT, KGT, and AS on object recognition and location memories. KGT-KHU (500 mg/kg/day), KKT-TU (500 mg/kg/day), KKT-Kra (500 mg/kg/day), AS (500 mg/kg/day), or vehicle solutions were administered daily to ddY mice for 13 days. A) The novel object recognition test was performed on the 8th day. Preferential indices are shown. The white columns show the results of the training session, and the black columns show the results of the test session. B) The object location test was performed on the 11th day. Preferential indices are shown. The white columns show the results of the training session, and the black columns show the results of the test session. * $p < 0.05$ (n = 5, paired *t*-test)

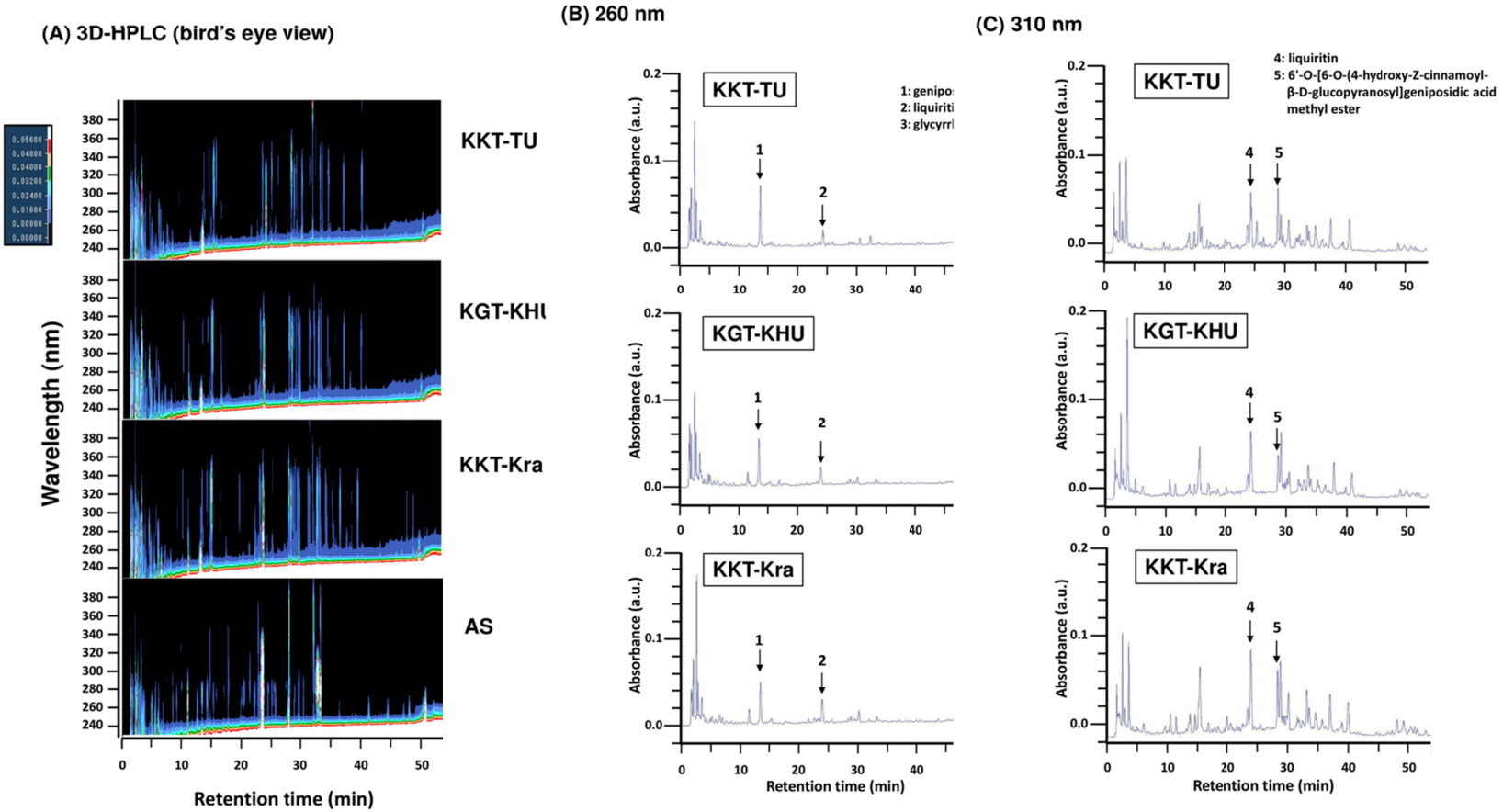

Figure 8. 3D-HPLC analysis.

(A) The 3D-HPLC profile of each preparation is shown at a bird's eye view. The distributions of the compounds were plotted by retention time (*x*-axis), wavelength (*y*-axis), and content (color gradations in the colored bar). (B) Chromatograms at 260 nm. Geniposide, liquiritin, and glycyrrhizin are annotated in each chromatogram. (C) Chromatograms at 260 nm. *L*iquiritin and 6'-*O*-[6-*O*-(4-Hydroxy-Z-cinnamoyl-β-D-glucopyranosyl]geniposidic acid methyl ester are annotated in each chromatogram.

2.4 Discussion

Although the prescribed original plant sources were the same across three formulations, the crude drugs used for each type of formulations were obtained from different places. In addition, the preparation methods for each extracted drug were slightly different. The 3D-HPLC profiles of KGT-KHU, KKT-TU, and KKT-Kra showed a similar distribution of peaks but slight differences in the amount of compounds present. However, all three formulations yielded enhancements in object and spatial memory in normal mice; this finding suggests that KKT formulations prepared in different countries have equivalent effects even if the amounts of constituents are not strictly identical.

This is the first report to show that KKT and KGT are memory stimulants. KGT may also be useful to treat dementia because we previously reported that KKT improved memory dysfunction in the 5XFAD mouse model of Alzheimer's disease [7].

KKT and KGT formulations produced memory enhancement effects attributed to drug-specific components because AS had no effect memory enhancement in normal mice. The components of AS do not overlap with KKT and KGT (except for Glycyrrhizae Radix), and the AS compounds peaks were quite different from the other drugs. In our previous study, Ginseng Radix, Polygalae Radix, and Astragali Radix produced axonal extension effects in cultured neurons and the mouse brain [35, 43, 44]. We also indicated that axonal growth in the brain of normal mice is involved in memory

enhancement [45]. Some of constituents in Gardeniae Fructus including geniposide have neurite outgrowth activity [46]. In addition, Huang et al. reported that tenuigenin, an active compound of Polygalae Radix, enhanced learning and memory in normal mice [47]. These data suggest that the active crude drug in KKT for memory enhancement might not be specified so easily. However, it is important to determine which compounds in these drugs contribute to the memory enhancement effects. As shown in Figure 8A, a thousand compounds are contained in KKT and KGT. Analyzing the pharmacological activities and underlying mechanisms of each compound one by one is very inefficient, and the sum of each event detected in a mono-crude drug treatment does not necessary indicate a net effect of a particular formulation. Therefore, we are identifying the main signaling pathway of KKT, which is crucially related to memory enhancement, as an important first step. If we can identify the main signaling pathway for the memory enhancements of KKT, the active constituents that contribute to the pathway could be focused on more easily.

Yokukansan (called yokukansan in Japan, yi-gan san in China, and ukgansan in Korea) is a traditional herbal medicine that has been used to treat restlessness, insomnia and agitation. Iwasaki et al. reported that yokukansan improved the behavioral and psychological symptoms of dementia (BPSD) in a clinical study performed in Japan [48]. Additional clinical and basic studies have been conducted, and the evidence for yokukansan with regard to BPSD has grown [49]. However, the expansion of these studies has been limited to Japan; specifically, 22 clinical and 39 basic studies

that involved yokukansan have been conducted in Japan. In contrast, only one ukgansan study was reported in Korea that showed neuroprotective effects on MPP^+/MPTP-induced cytotoxicity [50]. A global clinical study of yokukansan has not been performed to date, and the cross-country, reliable effectiveness of yokukansan is not understood. To address this problem, global clinical studies must be conducted.

In this study, same-origin traditional formulations, which were prepared differently in Japan and Korea, showed equivalent effects in in vivo experiments. The 3D-HPLC data showed that most major components in KKT were also present in KGT, but with slight differences. These results suggest that the use of identical formulations is not required for investigating the pharmacological effects of traditional formulations in cross-country clinical studies. These types of studies may expand the usefulness of traditional medicine for the clinical study of dementia in different countries. Moreover, this study is the first to examine KKT and KGT and may support the progression to global clinical studies.

Conclusions

Results in chapter 1 demonstrated that KKT dephosphorylated tau via PP2A activation and improved axonal atrophy, even after those Aβ-induced deteriorative phenomena already occurred [15]. In chapter 2, the effects of KKT used in Japan and Korea were compared and both of them showed normal memory enhancement effects equivalently [42].

Previous our basic research and other clinical study demonstrated that KKT improved the cognitive disorder in AD [7, 51], suggesting that KKT might be effective after onset of AD. We suppose such a therapeutic effect of KKT on AD may be brought at least by PP2A activation. However, the mechanism of KKT leading to regulation of PP2A remains unknown. To investigate the signal pathway of KKT, we have tried to identify the most upstream molecule in KKT-triggered signaling [52].

KKT enhanced the cognitive function in the normal situation, suggesting that effects of KKT are not necessary limited to repairing Aβ-induced damages. Up regulation of basic neuronal activity is doable by KKT treatment. Therefore, KKT might be also applicable to other dementia states such as aging-associated cognitive decline.

Future studies to clarify the whole underlying mechanisms of KKT against cognitive disorder and to confirm effects of KKT on AD or other type of dementia clinically may pave the way for overcoming dementia.

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