The Additive Effects of Low Dose Intake of Ferulic Acid, Phosphatidylserine and Curcumin, Not Alone, Improve Cognitive Function in APPswe/PS1dE9 Transgenic Mice

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Alzheimer’s disease (AD) is the most common form of dementia and its prevention and treatment is a worldwide issue. Many natural components considered to be effective against AD have been identified. However, almost all clinical trials of these components for AD reported inconclusive results. We thought that multiple factors such as amyloid β (Aβ) and tau progressed the pathology of AD and that a therapeutic effect would be obtained by using multiple active ingredients with different effects. Thus, in this study, we treated ferulic acid (FA), phosphatidylserine (PS) and curcumin (Cur) in combination or alone to APPswe/PS1dE9 transgenic mice and evaluated cognitive function by Y-maze test. Consequently, only the three-ingredient group exhibited a significant improvement in cognitive function compared to the control group. In addition, we determined the amounts of Aβ, brain-derived neurotrophic factor (BDNF), interleukin (IL-1)β, acetylcholine and phosphorylated tau in the mouse brains after the treatment. In the two-ingredient (FA and PS) group, a significant decrease in IL-1β and an increasing trend in acetylcholine were observed. In the Cur group, significant decreases in Aβ and phosphorylated tau and an increasing trend in BDNF were observed. In the three-ingredient group, all of them were observed. These results indicate that the intake of multiple active ingredients with different mechanisms of action for the prevention and treatment of AD.

Key words Alzheimer’s disease; ferulic acid; phosphatidylserine; curcumin

INTRODUCTION

Alzheimer’s disease (AD), a neurodegenerative disorder, is the most common form of dementia, characterized by senile plaques composed of amyloid-β (Aβ) peptide and neurofibrillary tangles of tau protein. The prevention and treatment of AD are ongoing concerns worldwide, and many natural components considered effective against AD have been identified. For example, ferulic acid (FA; Fig. 1A) is a phenolic molecule found in various fruits and vegetables and has antioxidant properties. In addition, FA inhibits Aβ production via down-regulation of amyloid precursor protein (APP), β-secretase and γ-secretase; inhibits β-secretase activity and Aβ aggregation; enhances expression of protein phosphatase 2A, which contributes to tau dephosphorylation; promotes clearance of phosphorylated tau via autophagy; activates neuroprotective action; activates acetylcholine receptor; and induces neural proliferation by increasing the levels of cyclic adenosine monophosphate response element binding protein (CREB) phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Phosphatidylserine (PS; Fig. 1B) is a major phospholipid that constitutes approx. 20% of the total phospholipid mass of human cellular membranes. PS is required for healthy neural cell membranes and their proper functioning. As the brain ages, the PS content of neural cell membranes decreases, leading to a decline in the function of receptors, a decrease in neurotransmitter synthesis, and synaptic loss. Studies have reported that PS administration improves these conditions, in addition to cognitive function. PS also promotes acetylcholine release, decreases the acetylcholinesterase (AChE) level, maintains N-methyl-D-aspartate (NMDA) receptor, and contributes to Aβ clearance through exosome secretion. Curcumin (Cur; Fig. 1C) is a natural polyphenol. It is a yellow-colored spice derived from turmeric (Curcuma longa) and has antioxidant and anti-inflammatory properties. It reportedly inhibits Aβ production via APP maturation; α-secretase activation; β-secretase down-regulation; β-secretase inhibition; down-regulation of presenilin 1, which is a main subunit of γ-secretase; inhibition of γ-secretase activity; and modulation of Aβ precursor protein trafficking. Cur also inhibits Aβ aggregation and promotes Aβ clearance via up-regulation of degrading enzymes and induction of autophagy. In addition, Cur also reduces hyperphosphorylated tau via down-regulation and inhibition of glycogen synthase kinase 3β (GSK3β) and inhibition of cyclin-dependent kinase 5, inhibits tau aggregation, exhibits some kinds of neuroprotective effects, and promotes neurogenesis via histone acetylation inhibition and BDNF expression. As a result, the incidence of AD in the United States is higher compared to India, where Cur is consumed on a daily basis. Cur has been demonstrated to improve memory function in nondementia adults. However, almost all clinical trials on its effects on AD have reported negative or inconclusive results so far. Therefore, it seems that using a single active ingredient cannot provide therapeutic effects against AD, because multiple factors such as Aβ and tau are involved in the progress of AD pathology; instead, therapeutic effects might be obtained by using multiple active...
ingredients with different effects.

Since no significant improvement in cognitive function is observed by the use of FA, PS, or Cur alone, in this study, we investigated the adding and synergistic therapeutic effects of a mixture FA, PS, and Cur on cognitive dysfunction in APPswe/PS1dE9 transgenic mice.

MATERIALS AND METHODS

**Test Compounds** FA derived from rice bran was purchased from TSUNO Co., Ltd. (Katsuragi, Japan). PS derived from soy was purchased from NOF Corporation (Tokyo, Japan). Cur was purchased from OMNICA Co., Ltd. (Tokyo, Japan).

**Treatment to APPswe/PS1dE9 Transgenic Mice** APPswe/PS1dE9 transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.), and maintained by crossing the mice with B6C3F1 mice. Genotyping was performed using the PCR method recommended by the Jackson Laboratory, and hemizygote mice were selected for the treatment. Mice were maintained in a regulated environment (temperature 24±3°C; humidity 50±10%; 12-h inverted light-dark cycle) and allowed free access to food and tap water. In the two-ingredient group, FA and PS were mixed into the MF basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) at 0.05% each, and orally administered to mice (n=6). In the three ingredients group, FA, PS and Cur were mixed to the diet at 0.05, 0.05 and 0.01%, respectively, and orally administered to the mice (n=7). In the control group, the MF diet alone was orally administrated (n=7). The administration period was three months (from 6 to 9 months of age). Separately, the MF diet alone or the diet which was mixed with 0.2% Cur was orally administrated to the mice (n=12, each group), and the MF diet alone or the diet which was mixed with 0.2% FA or 0.2% PS was orally administrated to the mice (n=5, each group) in the same way as above. The experimental procedures involving mice and their care were conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee (authorization number; 16-56-1, 16-56-3) and those of the Japanese Pharmacological Society.

**Y-Maze Test** The working memory of the mice was evaluated using the Y-maze test at the start of the treatment and at 4th, 8th and 12th-weeks of the treatment. Each mouse was placed at one arm of a Y-maze, which has three arms 30 cm in length with equal angles between all arms. The mice were allowed to move freely in the maze for 8 min, and the sequence and number of arm entries were recorded. Spontaneous alternation behavior, which is used as a measure of spatial memory, was defined as entry into all three arms on consecutive choices. The percentage of spontaneous alternations was calculated as follows:

$$\text{Number of spontaneous alternations} \times 100 \div \text{Number of total arm entries} - 1$$

**Protein Extraction from Brain Tissue** At the end of treatment, all mice were sacrificed under deep anesthesia with 100 mg/kg sodium pentobarbital (Kyoritsu Seiyaku Corp., Tokyo, Japan) administered intraperitoneally, and the whole brains were removed for biochemical analysis. The brain tissues were homogenized in five or 10 volumes (w/v) of 2× RIPA buffer (Nacalai Tesque Inc., Kyoto, Japan) with 0.5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich Co., St. Louis, MO, U.S.A.), 1× PhosSTOP (Roche, Basel, Switzerland), and 1% protease inhibitor cocktail (Nacalai Tesque, Inc.). The homogenate was centrifuged at 100000×g at 4°C for 20 min. The supernatant was collected as a soluble fraction and used for the measurement of Aβ, BDNF, phosphorylated tau, interleukin (IL)-1β and acetylcholine. The pellet was sonicated in 70% formic acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), neutralized with 20 volumes of 0.9 M Tris buffer (pH 11.0), and collected as an insoluble fraction, which was used for the measurement of insoluble Aβ.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Aβ, BDNF and IL-1β** The levels of Aβ1–42 and Aβ1–40 in the brain were measured using human Aβ ELISA kits (FUJIFILM Wako Pure Chemical Corporation) according to the manufacture’s protocol. Each soluble fraction was diluted 10-fold with the dilution buffer provided with the kit. Each insoluble fraction was diluted 1000-fold with the same buffer. The level of BDNF was measured using BDNF Emax ImmunoAssay Systems (Promega Corporation, Madison, WI, U.S.A.). Each soluble fraction was diluted 3-fold with the dilution buffer provided with the kit. The level of IL-1β was measured using Quantikine ELISA kit for IL-1β (R&D Systems, Minneapolis, MN, U.S.A.). Each soluble fraction was diluted 2-fold with the dilution buffer provided with the kit. A microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used to measure absorbance at 450 nm.

**Western Blotting for Phosphorylated tau Measurement** The level of phosphorylated tau in the brain was measured by Western blotting. Each soluble fraction was mixed with an equal amount of Tris–sodium dodecyl sulfate (SDS) β ME sample buffer (Cosmo Bio Co., Ltd., Tokyo, Japan) and boiled at 100°C for 10 min. Then, 10 μL of each sample was electrophoresed at 200 V for 1 h on a 5–20% polyacrylamide gel (FUJIFILM Wako Pure Chemical Corporation) and transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Merek Millipore, Billerica, MA, U.S.A.), which was run at 15 V for 30 min. After blocking with 2.5% skimmed
milk (Nacalai Tesque, Inc.) in Tris Buffered Saline with 0.05% Tween 20 (TBS-T) (Sigma-Aldrich Corp.) for 1 h, the blots were incubated with anti-phosphorylated (Ser202, Thr205) tau antibody AT8 (1:1000 dilution; Thermo Fisher Scientific, Waltham, MA, U.S.A.), anti-total tau antibody TAU-5 (1:2000 dilution; Thermo Fisher Scientific) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody 14C10 (Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) overnight at 4°C. The blots were washed with TBS-T for 10 min three times. The blots for AT8 and TAU-5 were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse Immunoglobulin G (1:3000 dilution; GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) and the blots for GAPDH were incubated with HRP-conjugated anti-rabbit Immunoglobulin G (1:3000 dilution; GE Healthcare) for 1 h at room temperature and washed with TBS-T for 10 min three times. Phosphorylated tau was detected using Chemi-Lumi One Super (Nacalai Tesque, Inc.). Total tau and GAPDH were detected using Chemi-Lumi One L (Nacalai Tesque, Inc.). The protein bands of the blots were analyzed using an image analyzer (ImageQuant LAS4000; GE Healthcare).

**Measurement of Acetylcholine** The level of acetylcholine in the brain was measured using Amplite™ fluorimetric acetylcholine assay kit (AAT Bioquest Inc., Sunnyvale, CA, U.S.A.) and a multilabel counter ALVO™ MX (Perkinelmer, Inc., Waltham, MA, U.S.A.) according to the manufacture’s protocol. Each sample was diluted two-fold with the dilution buffer provided with the kit.

**Immunohistochemistry (IHC) for Aβ Plaque** The left brain hemispheres were fixed in 4% paraformaldehyde phosphate buffer solution (FUJIFILM Wako Pure Chemical Corporation). The fixed tissues were dehydrated with ethanol (FUJIFILM Wako Pure Chemical Corporation) for 24 h, transferred into xylene (10 min three times), and embedded in paraffin (30 min three times at 60°C). The paraffin-embedded tissue blocks were cut into 5-µm thick sections with a sliding microtome (Nippon Optical Works Co., Ltd., Tokyo, Japan) and extended on Matsunami-Adhesive Silane-coated micro slide glasses (Matsunami Glass Industry, Ltd., Osaka, Japan) at 60°C for 24 h. The sections were deparaffinized with Lemosol (FUJIFILM Wako Pure Chemical Corporation) and ethanol (100, 90, and 70%). After washing twice in water, 70% formic acid solution was dispensed on to the sections, and they were incubated at room temperature for 5 min. The sections were washed with water (5 min three times) and 0.2% Tween 20 in phosphate buffered saline (PBS) (10 min), and then stained with anti-Aβ antibody 6E10 (1:100 dilution; Covance Inc., Princeton, NJ, U.S.A.) using a M. O. M.™ Immunodetection Peroxidase Kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.), according to the manufacture’s protocol. As a chromogen, 3,3'-diaminobenzidine (FUJIFILM Wako Pure Chemical Corporation) at a concentration of 0.5 mg/mL in PBS with 0.005% hydrogen peroxide was used. After staining, the sections were washed with water, dehydrated with ethanol (70,
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90, and 100%) and Lemosol, and then mounted with mounting medium (Daido Sangyo Co., Ltd., Tokyo, Japan). The sections were observed with a microscope BZ-8100 (Keyence Corp., Osaka, Japan). The ratio of the area of Aβ plaques to that of the whole section was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.).

Data Analysis Each data was expressed as mean ± standard error of the mean (S.E.M.). Data derived from the Y-maze test were analyzed using two-way ANOVA and the Bonferroni posttest. Data derived from biochemical tests and IHC were analyzed using one-way ANOVA and the Dunnett’s posttest or the Mann–Whitney U test. The software Graphpad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.) was used for these analyses, and p < 0.05 was considered statistically significant.

RESULTS

Spatial working memories of APPswe/PS1dE9 transgenic mice were evaluated using Y-maze tests during treatment with two ingredients (FA and PS) or three ingredients (FA, PS, and Cur) (Fig. 2A). At the start of treatment (7-month-old mice), the spontaneous alternation frequency of each group was about approx. 60–63: 60.9 ± 4.6% for the control group, 62.2 ± 3.3% for the two-ingredient group, and 60.6 ± 3.9% for the three-ingredient group. Over 3 months of treatment, the spontaneous alternation frequency of the control group significantly decreased to 42.4 ± 8.8%, while that of the two- and three-ingredient groups did not decrease much: 54.7 ± 7.1% and 58.3 ± 5.5%, respectively. The difference between the three-ingredient group and the control group was significant (p = 0.031 by two-way ANOVA and p < 0.01 at week 12 by the Bonferroni posttest). For comparison, we evaluated the efficacy of Cur alone (Fig. 2B). At the start of treatment (7-month-old mice), the spontaneous alternation frequency of each group was about approx. 60–63: 60.9 ± 4.6% for the control group, 62.2 ± 3.3% for the two-ingredient group, and 60.6 ± 3.9% for the three-ingredient group. Over 3 months of treatment, the spontaneous alternation frequency of the control group decreased to 49.4 ± 2.7% and
Furthermore, we also confirmed the efficacy of FA or PS alone (Fig. 2C). At the start of treatment, spontaneous alternation frequency was 60.1 ± 3.4% for the control group, 61.0 ± 4.4% for the FA group and 60.7 ± 7.1% for the PS group. After 3 months of treatment, the spontaneous alternation frequency of these groups was decreased to 45.9 ± 9.6%, 46.5 ± 7.9%, and 46.5 ± 6.3%, respectively. These results indicated that the combined use of FA, PS, and Cur has additive and synergistic therapeutic or preventive effects on cognitive dysfunction.

We observed a tendency of a decrease in the number of total arm entries in all the groups at week 12 of treatment (Fig. 2D), indicating that an age-related decline in motor function might occur in the mice in this study. Therefore, we did not conduct other memory tests.

After 3 months of treatment, we determined the amounts of Aβ, BDNF, IL-1β, acetylcholine, and phosphorylated tau in the mouse brains (Figs. 3, 4). Insoluble Aβ1-42 (Fig. 3A) and insoluble Aβ1-40 (Fig. 3B) significantly decreased in the three-ingredient group compared to the control group (Aβ1-42: \( p = 0.019 \) by one-way ANOVA and \( p < 0.05 \) by Dunnett test; Aβ1-40: \( p = 0.014 \) by one-way ANOVA and \( p < 0.01 \) by the Dunnett test). There was also a slight decrease in the soluble Aβ in the two- and three-ingredient groups, but no significant difference was observed (Figs. 3C, D). In addition, in the Cur group, Aβ decreased, and there was a significant difference in soluble Aβ1-40 compared with the control group (\( p = 0.038 \) by the Mann–Whitney U test: Fig. 4D). BDNF was slightly higher in the three-ingredient group compared to the control group, but the difference was not statistically significant (\( p = 0.77 \) by one-way ANOVA; Fig. 3E). In addition, BDNF was slightly higher in the Cur group compared to the control group, but again, the difference was not significant (\( p = 0.22 \) by the Mann–Whitney U test; Fig. 4E). IL-1β, which is an inflammatory cytokine, decreased in the two- and three-ingredient groups (\( p = 0.10 \) by one-way ANOVA; Fig. 3F) and was significantly lower in both groups compared to the control group (\( p = 0.036 \) and \( p = 0.023 \) by the Mann–Whitney U test). In
Mann–Whitney p significant difference between the groups (with the results of ELISA for insoluble Aβ area of Aβ were immunostained with anti-Aβ antibody 6E10. As a result, the AT8/TAU-5 ratio was significantly lower in the Cur group compared to the control group (p = 0.049, Mann–Whitney test; Fig. 3I). In contrast, phosphorylated tau decreased in the three-ingredient group compared to the control group (p = 0.12 by one-way ANOVA; Fig. 3K). In addition, there was no significant difference in the acetylcholine levels in the Cur and control groups (p = 0.49, Mann–Whitney U test; Fig. 4K).

Brain sections from the control group and the three-ingredient group showed a significant difference in insoluble Aβ plaques were immunostained with anti-Aβ antibody 6E10 (Fig. 5). The area of Aβ plaques decreased by 29% in the three-ingredient group compared to the control group (p = 0.018 by the Mann–Whitney U test; Fig. 4F).

Phosphorylated tau detected by AT8 normalized by GAPDH increased in the two- and three-ingredient groups compared to the control group (p = 0.045 by one-way ANOVA and p < 0.05 by the Dunnett test; Fig. 3G). Interestingly, we found no change in phosphorylated tau between the two-ingredient group and the control group, and the total tau detected by TAU-5 significantly increased in the three-ingredient group (Fig. 3H). As a result, the AT8/TAU-5 ratio was significantly lower in the three-ingredient group compared to the control group (p = 0.045 by one-way ANOVA and p < 0.05 by the Dunnett test; Fig. 3I). In contrast, phosphorylated tau decreased in the Cur group compared to the control group (p = 0.12 by one-way ANOVA), and there was a significant difference in the AT8/TAU-5 ratio for the Cur and control groups (p = 0.049 by the Mann–Whitney U test; Fig. 4I).

Acetylcholine slightly increased in the two- and three-ingredient groups compared to the control group but not significantly (p = 0.12 by one-way ANOVA; Fig. 3K). In addition, there was no significant difference in the acetylcholine levels in the Cur and control groups (p = 0.09 by the Mann–Whitney U test).

### DISCUSSION

In AD, Aβ abnormalities precede the onset of cognitive dysfunction by approximately 20 years, so it is too late to administer drugs and/or other therapies after cognitive dysfunction onset. In addition, the currently approved AD drugs, such as AChE inhibitors and NMDA receptor antagonist, are only symptomatic treatments. Realistic measures for AD treatment would include long-term preventive approaches. One such effective long-term approach is the consumption of active ingredients every day. However, spoiling the taste and smell of food by using large amounts of biological ingredients is not desirable. Even when they are taken as dietary supplements, it might be difficult to maintain large daily doses. Therefore, we reasoned that additive and synergistic effects of combining multiple active ingredients, even at low doses, might overcome these difficulties.

In this study, we chose FA, PS and Cur as active ingredients because each has a different mechanism of action (MOA) and their combination might directly improve multiplex AD-specific pathologies. To begin this study, we compared a few ingredients that are considered good for cognitive function in terms of their MOAs (Table 1). As mentioned before, FA inhibits Aβ production via down-regulation of APP and β-secretase, inhibits Aβ aggregation, and protects nerve cells from Aβ-induced neurotoxicity, but few studies have reported improvement of tau pathology, except promotion of tau dephosphorylation and phosphorylated tau clearance. Therefore, FA is expected to act relatively upstream of the pathophysiologic mechanism of AD. On the other hand, many studies have reported that Cur not only inhibits Aβ production but also inhibits tau phosphorylation and its aggregation. Therefore, Cur is also expected to act downstream of the pathophysiologic mechanism of AD.

In addition, FA reportedly activates acetylcholine receptor, but Cur does not. On the other hand, Cur reportedly inhibits AChE activity, but FA does not. On the basis of these findings, we believe that FA and Cur might complement each other.

We also compared a few polyphenols besides FA and Cur. Catechin and its analogs are flavonoid polyphenols that are abundant in green tea. Among them, epigallocatechin gallate (EGCG) reportedly inhibits Aβ production via α-secretase activation and β-secretase inhibition, inhibits Aβ aggregation, promotes Aβ clearance via autophagy, inhibits tau aggregation, protects neural cells from oxidative stress and Aβ-induced neurotoxicity, inhibits AChE activity, and promotes neurogenesis. In addition, more than 10 clinical trials have been conducted on EGCG or green tea and some have shown efficacy in improving cognitive function. However, EGCG also promotes tau phosphorylation via GSK3β up-regulation and AChE activation, which might worsen AD. Therefore, we did not select EGCG and other catechins in this study.

Other flavonoid polyphenols, such as quer cetin, myricetin, and taxifolin, also reportedly are effective against AD. Quercetin inhibits Aβ production via α-secretase up-regulation and β-secretase, γ-secretase inhibition, inhibits Aβ aggregation, promotes Aβ clearance, protects nerve cells from Aβ-induced cytotoxicity, and activates the CREB/BDNF pathway. Myricetin inhibits Aβ production via
| Mechanism of action | The ingredients in the present study | Polyphenols | Polyunsaturated fatty acids | Phospholipids |
|---------------------|--------------------------------------|-------------|-----------------------------|---------------|
|                     | FA  | PS  | Cur | Catechin | Quercetin | Myricetin | Taxifolin | Resveratrol | DHA, EPA | α-Linolenic acid | Phosphatidylcholone | Plasmalogen |
| Aβ generation inhibition | APP downregulation | 6 |
|                       | APP maturemation inhibition | 28 |
|                       | α-Secretase upregulation | 29 |
|                       | α-Secretase activation | 6 |
|                       | β-Secretase downregulation | 53 |
|                       | β-Secretase inhibition | 87 |
|                       | β-Secretase moduration | 7 |
|                       | γ-Secretase downregulation | 6 |
|                       | γ-Secretase inhibition | 53 |
|                       | APP trafficking moduration | 7 |
| Aβ aggregation inhibition | Aβ aggregation inhibition | 8 |
|                       | IDE upregulation | 37 |
|                       | Neprilysin upregulation | 37 |
|                       | Autophagy induction | 38 |
|                       | Proteasome activation | 70 |
|                       | Blood flow improvement | 91 |
| Aβ clearance promotion | Aβ aggregation inhibition | 8 |
|                       | Neural exosomes secretion | 27 |
|                       | IDE upregulation | 37 |
|                       | Neprilysin upregulation | 37 |
|                       | Autophagy induction | 38 |
|                       | Proteasome activation | 70 |
|                       | Blood flow improvement | 91 |
| Tau phosphorylation inhibition | GSK3β downregulation | 39 |
|                       | GSK3β inhibition | 32 |
|                       | CDK5 inhibition | 40 |
|                       | PP2A upregulation | 9 |
| Tau aggregation inhibition | Tau aggregation inhibition | 41 |
|                       | Autophagy induction | 56 |
| Tau clearance promotion | Tau aggregation inhibition | 41 |
|                       | Autophagy induction | 56 |
| Neuroprotection | Oxidative stress | 11 |
|                       | Aβ cytotoxicity | 12 |
|                       | Glutamate cytotoxicity | 45 |
|                       | Cell membrane recovery | 16–19 |
| Neurotransmission | Acetylcholine release | 22–24 |
|                       | AChE downregulation | 25 |
|                       | AChE inhibition | 31 |
|                       | Acetylcholine receptor activation | 13 |
|                       | NMDA receptor regulation | 26 |
| Neurogenesis | CREB/BDNF pathway activation | 14 |
|                       | Histone acetylase inhibition | 47 |
|                       | Sonic hedgehog pathway activation | 62 |

MOAs, mechanisms of action; AD, Alzheimer’s disease.
α-secretase activation and β-secretase inhibition, inhibits Aβ aggregation, and promotes an increase in BDNF. Taxifolin inhibits Aβ production via β-secretase up-regulation, inhibits Aβ aggregation, and inhibits AChE activity. Although a clinical trial on a quercetin-rich onion powder was conducted, it was difficult to judge the effectiveness of the powder because the scale of the trial was very small (N = 4 to 5 people). In addition, the reported MOA almost overlapped with the MOAs of FA and Cur. Therefore, these ingredients were excluded from this study.

Resveratrol, a stilbenoid polyphenol, is known as a sirtuin activator. Sirtuin is involved in α-secretase activation and tau aggregation inhibition. In fact, studies have reported that resveratrol reduces Aβ via α-secretase activation. Sirtuin also has neuroprotective effects, and resveratrol reportedly protects neural cells from Aβ-induced neurotoxicity via activation of the sirtuin pathway. Resveratrol also inhibits Aβ production via β-secretase inhibition, inhibits Aβ aggregation, promotes Aβ clearance, reduces phosphorylated tau via GSK3 inhibition, protects nerves cells from Aβ-induced cytotoxicity, inhibits AChE activity, regulates proper expression of the NMDA receptor, and promotes an increase in BDNF. However, a clinical trial on resveratrol for AD treatment showed no positive effect; in fact, resveratrol treatment seemed to worsen the brain condition compared with the placebo, so we did not include it in this study.

Polysaturated fatty acids, such as docosahexaenoic acid (DHA) and omega-3 eicosapentaenoic acid (EPA) from fish oil, can also be effective against AD. Polysaturated fatty acids are important components of the cell membrane and contribute to proper nerve cell function. In fact, in an AD patient’s brain, DHA decreases. Therefore, the dietary intake of these polysaturated fatty acids could be effective in preventing and treating AD. DHA reportedly inhibits Aβ production via β-secretase inhibition, inhibits Aβ aggregation, promotes Aβ clearance, protects neural cells from oxidative stress, promotes acetylcholine release, regulates the NMDA receptor, and promotes neurogenesis. Several clinical trials on DHA/EPA (as fish oil) for AD patients have been conducted, with some of them showing positive results.

In addition, α-linolenic acid, which is a precursor of DHA/EPA and rich in linseed oil, also reportedly has anti-AD actions such as α-secretase up-regulation, β-secretase inhibition, γ-secretase inhibition, neuroprotection, acetylcholine receptor regulation, and promotion of BDNF expression. However, α-linolenic acid, DHA, and EPA are easily susceptible to oxidation. Not only does oxidized fish oil show no positive effects, but it may also have negative effects on health. We believed it was difficult to prevent oxidation of polysaturated fatty acids when mixed with food, so these were excluded from this study.

Some kinds of phospholipids, such as phosphatidylcholine (PC; also called lecithin) or plasmalogens other than PS, also seem effective in preventing and treating AD. PC, like phosphatidylserine (PS; also called lecithin) or plasmalogens other than PS, oxidizes polyunsaturated fatty acids when mixed with food, so these were excluded from this study.

In this study, only the three-ingredient group exhibited a significant improvement in cognitive function despite a significant decrease in Aβ level and phosphorylated tau in other groups. These results indicated that the cognitive function of the APPswe/PS1dE9 transgenic mice improved by using multiple substances with different effective MOAs. In fact, studies have reported that the combined use of FA and other polyphenols ameliorates cognitive dysfunction and AD-like...
pathology in mice.\(^{13,14,15}\) Therefore, such combined use might be an effective AD prevention or treatment strategy, not only in mice models but also for humans.

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**Conflict of Interest** H. Sugimoto is President of Green Tech Co., Ltd. and M. Okuda and Y. Fujita are employees of it.

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