Silymarin Attenuated the Amyloid β Plaque Burden and Improved Behavioral Abnormalities in an Alzheimer’s Disease Mouse Model

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Alzheimer’s disease (AD) is characterized by progressive cognitive impairment and the formation of senile plaques. Silymarin, an extract of milk thistle, has long been used as a medicinal herb for liver diseases. Here we report marked suppression of amyloid β-protein (Aβ) fibril formation and neurotoxicity in PC12 cells after silymarin treatment in vitro. In vivo studies had indicated a significant reduction in brain Aβ deposition and improvement in behavioral abnormalities in amyloid precursor protein (APP) transgenic mice that had been preventively treated with a powdered diet containing 0.1% silymarin for 6 months. The silymarin-treated APP mice also showed less anxiety than the vehicle-treated APP mice. These behavioral changes were associated with a decline in Aβ oligomer production induced by silymarin intake. These results suggest that silymarin is a promising agent for the prevention of AD.

Key words: silymarin; Alzheimer’s disease; amyloid β; mouse model

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Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid β; APP, amyloid precursor protein; BACE, β-secretase; PBS, phosphate buffered saline; Th-T, thioflavin-T; TBS, tris buffered saline

their high concentrations of functional food factors such as polyphenolic compounds, carotenoids, and antioxidants. Many studies have investigated the inhibitory effects of various flavonoids on Aβ aggregation and neurotoxicity. In particular, curcumin was reported to show potent activity against AD-like pathology in a transgenic AD mouse, and was recently examined in a clinical trial in patients with AD.

Silymarin, the active ingredient of milk thistle extract, is a mixture of flavonolignane diastereomers: silibinin (silybin A + silybin B) [(2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxy-methyl)-2,3-dihydropbenzo[b][1,4]dioxin-6-y1]chroman-4-one], isosilybin (isosilybin A + isosilybin B), silydianin, and silychristin. It has long been used as an anti-hepatotoxic agent without notable adverse effects, especially against the damage caused by alcohol and disturbances in the function of the gastrointestinal tract, but there have been no studies of the utility of silymarin in AD prevention. We suggest that if it has a preventive effect against the Aβ-dependent phenotypes of AD, silymarin is a promising therapeutic agent for AD, since it has already been proven safe for human consumption.

To evaluate the potential role of silymarin in the prevention of AD, we examined the effects of silymarin on Aβ fibril formation and neurotoxicity in PC12 cells and in 6-month-old APP transgenic mice (line J20), a well-established AD mouse model, prophylactically treated with silymarin for 6 months. This is the first report to indicate that silymarin has protective effects against Aβ peptide fibril formation and Aβ-induced neurotoxicity in vitro, and that silymarin treatment of APP transgenic mice significantly alleviates the cerebral plaque burden and microglial activation in the brain and improves the behavioral abnormalities induced by AD pathology.
Materials and Methods

Thioflavin-T fluorescence assay. Aβ40 and Aβ42 peptides were synthesized chemically by standard protocol,20) or purchased from the Peptide Institute (Osaka, Japan). The aggregative abilities of Aβ40 and Aβ42 were evaluated by the thioflavin-T (Th-T) method developed by Naiki et al.21) Measurement was done on a SPECTRA max GEMINI XS at room temperature, as described elsewhere.21) Fluorescence intensity was measured at 424 nm excitation and 485 nm emission. Percentage inhibition was calculated by comparing the fluorescence values of silymarin (lot no. 2397805, LKT Laboratories, St. Paul, Minnesota) samples with those of vehicle solutions without silymarin. HPLC analysis showed that silymarin in this study consisted mainly of (+)-silybin A and (+)-silybin B together with (+)-silydianin and (+)-taxifolin (Supplemental Fig. 1; see Biosci. Biotechnol. Biochem. Web site).

Transmission electron microscopy. The fibril formation of Aβ40 and of Aβ42 was detected using an electron microscope. The experimental procedure was described elsewhere.22)

Estimation of cell survival. PC12 cells (RCBR0009, Riken BioResource Center, Tsukuba, Japan) were used as a neural cell model in order to evaluate the cytotoxicity of Aβ peptides.23) The experimental procedure was a slightly modified version of a previously described method.21) Briefly, after pre-incubation of PC12 culture (2 × 10⁴ cells per well) for 24 h at 37°C, the cells were pre-treated for 1 h with and without silymarin (lot no. 2397805, LKT Laboratories) before treatment with 10μM Aβ42 for another 48 h. Twenty nm silymarin was dissolved in dimethyl sulfoxide (DMSO; Dojindo Laboratories, Kumamoto, Japan) and stored until use. A final concentration of 0.5% v/v DMSO was used to prevent DMSO cytotoxicity affecting the results. The rate at which formazan was produced was evaluated by measuring the optical density at 600 nm (Versamax; Molecular Devices, Sunnyvale, CA). Data are given as percentages of control values (without silymarin or Aβ42) after subtraction of a slight formation of formazan by silymarin itself.

Mice. Heterozygous transgenic mice, AβPP mice (line J20; Jackson Laboratory, Bar Harbor, Maine), which possess the human APP (APP) gene containing the Swedish (670/671KM→K40) and Dutch (717V→E40) mutations driven by the platelet-derived growth factor promoter on a C57BL/6J background were used.24) Genotyping of the transgenic mice was done by PCR using genomic DNA isolated from the tail tip. The tip of each mouse’s tail was cut off and placed in 500 μl of tail buffer (1% SDS, 250 mM NaCl, 25 mM EDTA pH 8.0, and 50 mM Tris–HCl pH 8.0), to which 15 μl of Proteinase K (recombinant PCR grade, Roche, Basel, Switzerland) was added. After incubation at 60°C overnight, the mixture was centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant was diluted 10-fold in distilled water to produce the DNA template. Primers (5’-GGT GAG TTT GTA AGT GAT GCC-3’ and 5’-TCT TCT TCT TCC ACC TCA GC-3’) used to detect the presence of the transgene were used under the following conditions: 1 cycle of 95°C for 15 min; 35 cycles of 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. Non-transgenic mice were used as littermate controls. All the mice were maintained and studied following the guidelines approved by the TMIG Institutional Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology, no. 10014.

Silymarin treatment. Female transgenic mice and their wild-type littermates were assigned to one of four groups: treated transgenic mice (n = 10), untreated transgenic mice (n = 9), treated wild-type mice (n = 9), and untreated wild-type mice (n = 4). The mice in the untreated groups were fed a powdered control diet (CRF-1; Oriental Yeast, Tokyo), and the treated groups were fed the same diet containing 0.1% silymarin (lot no. 2397805, LKT Laboratories) in CRF-1. The animals had ad libitum access to water and standard chow. The powdered food was changed every 3 d. Treatment was initiated when the mice were 6 months of age and was continued for 6 months. After 5 months of treatment, the mice were subjected to behavioral tests, followed by an analysis of AD-related neuropathology. One brain hemisphere was fixed in 4% paraformaldehyde for 3–5 d and used in histological studies, and the opposite hemisphere was frozen rapidly in liquid nitrogen and stored at −80°C until used in biochemical studies.

Immunohistochemistry. Brain sections (5 μm) were deparaffinized, rehydrated, and washed in phosphate buffered saline (PBS), and then treated for 30 s with 99% formic acid. After 30 min of incubation in 3% hydrogen peroxide in methanol to prevent endogenous peroxidation, the sections were blocked with 10% normal goat serum (Gibco, Gaithersburg, MD) in PBS, and then incubated with anti-Aβ (6E10, 1/1000; Covance Laboratories, Princeton, NJ) and anti-Iba1 (1/200; Wako, Osaka, Japan) overnight at 4°C. After the sections were washed 3 times with PBS for 5 min, the primary antibodies were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. The sections were then washed 3 times with PBS for 5 min and treated with the ABC Elite kit (ABC Elite, Vector Laboratories, Burlingame, CA) following the manufacturer’s protocol. 3,3’-Diaminobenzidine (Sigma) was used as chromogen, and the sections were counterstained with hematoxylin.

Imaging studies were acquired with a Photomicroscope Unit VS-100 (Olympus, Tokyo), and the immunoreactive area in the brain occupied, by the hippocampus and cortex, was quantified using Leica Qwin V3 software (Leica Microsystems, Wetzlar, Germany). An average was taken for two sections from each animal.

Tissue preparation. The frozen brains were homogenized in 3 volumes w/v of 50 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl (TBS) and a mixture of protease and phosphatase inhibitors (Complete™, Roche Diagnostics, Indianapolis, IN) supplemented with 0.7 mg/ml pepstatin A (Peptide Institute) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The homogenates were then centrifuged at 186,000 g for 30 min at 4°C using an Optima TL ultracentrifuge and a TL-A55 rotor (Beckman, Palo Alto, CA) to give a TBS-soluble fraction. The TBS-insoluble pellet was then sonicated in an equal volume of 6 M guanidine-HCl (Sigma) containing a mixture of protease inhibitors, and then centrifuged at 186,000 g for 30 min at 4°C. The supernatant was diluted with a 12-fold volume of TBS, and then centrifuged at 15,000 rpm for 10 min at 4°C to generate a guanidine-soluble fraction (the TBS-insoluble fraction). The total protein concentrations of the brain homogenates were determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA) following the protocol of the manufacturer, with bovine serum albumin as standard. Aβ ELISA. Brain Aβ40, Aβ42, total Aβ, and oligomer Aβ levels were measured by sandwich ELISA with a Human β Amyloid ELISA Kit (Aβ40: Cat# 277114, Human amyloid β (1–40)(N); Aβ42: Cat# 277112, Human amyloid β (1–42)(N); total Aβ: Cat# 27729, Human amyloid β (1–x); oligomer Aβ; Cat# 27725, Human amyloid β oligomers (82E1-specific); Immuno Biochemical Laboratories, Gunma, Japan) following the manufacturer’s instructions.

Y-maze. Exploratory behavior was tested using the Y-maze task according to the protocols developed by Mucke et al.25,26) The Y-maze apparatus (Muramochi Kikai, Tokyo) consists of three identical arms (40 × 4 × 12 cm high) made of gray opaque polycarbonate with equal angles between each arm. The mice were placed alone into one arm and allowed to explore the maze for 5 min, and the apparatus was thoroughly cleaned with 70% ethanol between trials. The mice’s performance was monitored with a DV-Track Video Tracking System (Muramochi Kikai).

Elevated plus maze. Anxiety was assessed using the elevated plus maze task.24,26) The elevated plus maze apparatus (Muramochi Kikai) consists of two open arms (30 x 6 cm) and two closed arms of the same size with 14-cm-high gray opaque walls made of polycrylonitrile. The arms and central square are made of gray opaque polycrylonitrile chloride and are elevated to a height of 40 cm above the floor. The arms were placed alone in the central square (6 x 6 cm) of the maze facing each other, and were allowed to explore freely for 10 min. The apparatus was thoroughly cleaned with 70% ethanol between trials. The total time spent in each arm was recorded automatically with a DV-Track Video Tracking System (Muramochi Kikai).
Data analysis. All data are shown as mean ± SEM, and differences were analyzed by Student’s t test (for in vitro experiments) or repeated-measures analysis of variance (ANOVA) by Tukey-Kramer test (for the in vivo experiments). p values < 0.05 were considered statistically significant.

Results

Anti-Ab aggregative ability of silymarin in vitro
To examine the effects of silymarin on Ab aggregation, we performed Th-T fluorescence assays for Ab40 and Ab42 (Fig. 1). In the absence of silymarin, all of the Ab40 (20 μM) had formed Th-T-binding aggregates after 96 h of incubation (Fig. 1A, black triangles), whereas the Ab40 fluorescence level decreased in a silymarin dose-dependent manner. One hundred μM of silymarin completely abrogated Ab40 fluorescence throughout the incubation period (Fig. 1A, white squares). On the other hand, Ab42 began forming aggregates after a lag phase of 4 h, and the maximum level of Th-T fluorescence was reached after 12 h (Fig. 1B, black triangles). When 20 μM silymarin was added, the fluorescence signals of Ab42 fell to 84.4 ± 2.0% at 24 h (Fig. 1B, white circles). Similarly to the findings for Ab40, 100 μM silymarin completely inhibited the fibril formation of Ab42 (Fig. 1B, white squares). Since there is a possibility that Th-T fluorescence is affected by the presence of exogenous compounds such as curcumin, quercetin, and resveratrol,27 we examined the inhibitory effect of silymarin on amyloid fibril formation using transmission electron microscopy (Fig. 1C). At the end of the incubation of Ab40 and of Ab42 (20 μM) in the Th-T test, typical fibrils formed by each peptide were observed, and their formation was significantly reduced by silymarin treatment in a dose-dependent manner (Fig. 1C). These results suggest that silymarin has anti-aggregative effects on Ab40 and Ab42 in vitro.

Neuroprotective effects of silymarin against Ab42-induced toxicity
Ab42 plays a pivotal role in the pathogenesis of AD due to its potent aggregative ability and neurotoxicity.21,28–32 To investigate the neuroprotective effects of silymarin against Ab42-induced neurotoxicity, we carried out an MTT assay using PC12 cells. Ab42 induced cytotoxicity (20.7 ± 1.1% viability) in the cells when it was added at a concentration of 1 μM for 48 h (Fig. 2A). When PC12 cells were pre-incubated with silymarin for 1 h and then treated with Ab42 for 48 h, Ab42 toxicity was significantly alleviated in a silymarin dose-dependent manner. In particular, 100 μM silymarin restored cell...
Fig. 2. Silymarin Prevented Aβ42-Induced Neurotoxicity in PC12 Cells.

A, PC12 cells were pre-treated with and without silymarin for 1 h, and then subjected to 48 h of treatment with 10 µM Aβ42. B, PC12 cells were treated with silymarin alone for 48 h. Cell viability was assessed by the MTT method, and expressed as percentage of the viability of the control, which was measured in the absence of silymarin or Aβ42. Values represent mean ± SEM, n = 3. *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle (without silymarin).

viability to 48.4 ± 2.4% (Fig. 2A). On the other hand, when cells were treated with silymarin alone for 48 h, beneficial effects on cell viability were seen at concentrations of 50 and 100 µM (Fig. 2B). These results indicate that silymarin has anti-Aβ42 cytotoxic and neuroprotective properties.

Silymarin attenuated Aβ pathology in APP transgenic mice

Next we performed in vivo experiments in order to evaluate the preventive role of silymarin, using APP transgenic mice (J20) as a well-established AD mouse model. The transgenic mice and their wild-type littermates were assigned to one of four groups (untreated transgenic mice, treated transgenic mice, untreated wild-type mice, and treated wild-type mice). The mice in the untreated groups were fed a vehicle diet, while those in the treated groups were fed the same diet, containing 0.1% silymarin, whose concentration was based on US Food and Drug Administration criteria (http://www.fda.gov/cber/gdlns/dose.htm). Prophylactic silymarin treatment was started at the age of 6 months, and was continued for 6 months. No plaque formation was observed in the APP transgenic mice at 5 months of age (Fig. 3A). The 11–12 month-old mice were subsequently subjected to behavioral tests. No mice died in the study, and the survival rate was not significantly different between the vehicle and the silymarin group (data not shown).

The Aβ plaque burden was visualized in brain sections by immunohistochemical analysis using anti-Aβ antibody. As shown in Fig. 3B and C, silymarin administration significantly decreased the area of Aβ deposits in the brains of the transgenic mice (3.3 ± 0.1 versus 3.9 ± 0.2%, p = 0.047), and no plaques were observed in the brains of the wild-type mice (data not shown). To confirm the effects of silymarin on the levels of brain amyloid, the Aβ contents of the TBS-insoluble and TBS-soluble fractions of the brain extracts of the mice were measured by ELISA. The TBS-insoluble fractions of the silymarin-treated group showed a significant 29.7% decrease in total Aβ levels as compared with those of the vehicle group (107.2 ± 14.2 versus 156.2 ± 14.5 ng/mg of protein, p = 0.039; Fig. 3D). In the TBS-soluble fraction, there were no significant differences among the Aβ40, Aβ42, or total Aβ levels (Fig. 3E). Although silymarin treatment showed a tendency to decrease the Aβ42 to total Aβ ratio in the TBS-soluble fraction of the transgenic mice as compared to that of vehicle treatment, the difference did not reach significance. Recently, Selkoe et al. developed a specific ELISA to Aβ oligomer (a dimer), in which the same N-terminal Aβ antibody (82E1, IBL) was used for antigen capture and detection, and found a clear correlation between the oligomer increase in plasma and brain homogenates with memory decline.33) Hence, the oligomer level in the silymarin-treated transgenic mice was measured by oligomer-specific ELISA. As shown in Fig. 3F, oligomer formation was significantly reduced, by 32.3%, by silymarin intake (60.2 ± 4.8 versus 40.8 ± 6.7 fmol/mg of protein, p = 0.033). Taken together, these results indicate that silymarin significantly altered Aβ levels in both the insoluble and oligomer proteins of transgenic mouse brains.

Silymarin inhibited neuroinflammation in APP transgenic mice

In the brains of AD patients and model AD mice, an increased inflammatory response is observed in the areas surrounding senile plaques.34–39) We estimated neuroinflammation in the brain using an Iba1 antibody as a microglia marker (Fig. 4). Silymarin markedly decreased the Iba1 immunoreactive area, by 36.3%, in the hippocampi of the transgenic mice, indicating potent activation of microglia as compared with that observed in the vehicle-treated wild-type (Fig. 4B). In contrast, no effects of silymarin treatment were seen in the wild-type group (Fig. 4). This implies that silymarin treatment effectively blocks the neuroinflammation associated with plaque decline.

Silymarin improved behavioral abnormalities in APP transgenic mice

APP transgenic model mice subjected to the Y-maze test exhibit hyperactivity related to behavioral abnormality and entorhinal cortex dysfunction during AD progression.24,40) As shown in Fig. 5A, the silymarin-administered transgenic mice showed a significantly decreased number of arm entries as compared with the vehicle-treated APP mice. However, the difference in spontaneous alternation between the silymarin- and vehicle-treated APP mice did not reach significance (data not shown).

AD model mice also show an altered anxiety and fear phenotype in the elevated plus maze, which is used to assess anxiety and fear.31) The transgenic mice spent significantly less time in the closed arms in the elevated plus maze test, while silymarin treatment significantly extended the time spent in the closed arms of the maze (Fig. 5B). In the wild-type mice, silymarin treatment had no effect on behavioral performance (Fig. 5B), similarly to the results for the Y-maze (Fig. 5A). This indicates that silymarin treatment abrogates the abnormal behaviors of AD model mice.
Discussion

The drugs currently available for AD and dementia such as acetylcholinesterase inhibitors, temporarily improve cognitive dysfunction without affecting plaque formation. On the other hand, there have been many studies on the anti-amyloid aggregation properties of flavonoids and polyphenols and their utility as treatments for several neurodegenerative disorders, including AD and Parkinson’s disease, but further studies are required to determine appropriate doses and treatment durations for the use of these substances in human studies. On the other hand, silymarin has already been established and is used as a safe anti-hepatotoxic agent without adverse effects. Its mechanism involves the enhancement of hepatocyte protein synthesis, the reduction of blood cholesterol, and decreasing the activity of tumor promoters. Moreover, silymarin treatment prevents skin and other types of cancer.

In the present study, we demonstrated the anti-amyloid properties of silymarin in vitro (Figs. 1, 2), and found that chronic administration of it (half a year) significantly reduced the Aβ plaque burden associated with microglial activation (Fig. 3 and 4), Aβ oligomer formation (Fig. 3F), and hyperactivity and disturbed behavior (Fig. 5) in APP transgenic mice. Aβ peptides are processed from APP by β- and γ-secretases. γ-Secretase (BACE) regulates total Aβ levels, whereas γ-secretase enhances Aβ42 production and increases the ratio of Aβ42 to total Aβ. We tested the alternation of BACE1 activity in soluble brain fractions of the transgenic mice. As shown in Supplemental Fig. 2 (see Biosci. Biotechnol. Biochem. Web site), no significant change in BACE1 activity was observed after silymarin treatment. Since silymarin treatment decreased the amount of total Aβ in the TBS-insoluble fraction of the mouse brain homogenate (Fig. 3D) and failed to reduce the Aβ42 to total Aβ ratio in the TBS-soluble

**Fig. 3.** Silymarin Attenuated Aβ Plaque Burden and Oligomer Content in APP Transgenic Mice.

A, Representative images of amyloid deposits immunostained with 6E10 (anti-Aβ) antibody in the brains of 5-month-old wild-type (Wt) and transgenic (Tg) mice. The scale bar represents 300 μm. B, Representative images of amyloid deposits immunostained with 6E10 antibody in the brains of 12-month-old transgenic (Tg) mice treated with vehicle or with silymarin. The scale bar represents 300 μm. C, Quantification of the 6E10-positive Aβ plaque area in the brain (n = 6–7 per group). Values represent mean ± SEM. *p < 0.05. D and E, Aβ40, Aβ42, and total Aβ levels in the TBS-insoluble fraction (D) and the TBS-soluble fraction (E). F, Aβ oligomer levels in the TBS-soluble fraction. Values represent mean ± SEM, n = 9–10 per group. *p < 0.05.
fraction (Fig. 3E), the protective effect of silymarin on Aβ accumulation is attributable to the blockade of its aggregation, not to β-secretase inhibition. Collectively, these results indicate that silymarin strongly suppresses Aβ-dependent neuropathology via its anti-amyloidogenic ability. Recently, Pasinetti et al. reported that a naturally derived grape seed polyphenolic extract prevents both senile plaques and Aβ oligomerization and attenuates cognitive deterioration in vivo. Silymarin is also thought to block the dual pathways of Aβ fibril and oligomer formation. Furthermore, we found that there were no changes in full-length APP band between the silymarin-treated and the untreated Tg mice (Supplemental Fig. 3; see Biosci. Biotechnol. Biochem. Web site), suggesting that these forms of suppression are independent of Aβ production. Silymarin treatment decreased not only the Aβ burden but also oligomer levels, which were strongly correlated with attenuation of behavioral abnormalities. Yamada et al. recently reported prevention of oligomer formation by phenolic compounds using Tg2576, another established AD mouse model, and suggested that rosmarinic acid is the most attractive target in AD prevention, but provided almost no specific information on the essential structure of anti-amyloidogenic flavonoids. Starvaggi Cucuzza et al. reported that silymarin treatment enhanced cell proliferation via activation of protein kinase B and inhibition of caspase-3 activity in bovine and murine mammary cells in a dose-dependent manner. In addition, silymarin can also modulate the activities of key molecules for cell cycle checkpoint, such as cyclin and cyclin-dependent kinases. In the present study, we found that silymarin treatment increased cell viability by 125% in PC12 cells (Fig. 2B). These beneficial effects of silymarin might act to enhance neuronal cell viability as well as to attenuate Aβ toxicity in the brains of AD model mice.

Since it has been reported that silymarin treatment reduced activation of the inflammatory system, the anti-inflammatory effect of silymarin provides evidence of its preventive potential as a treatment for AD. Alternatively, Espana et al. proposed that Aβ accumulation in the amygdala induces fear and anxiety phenotypes in AD model mice, in agreement with our results regarding the prevention of AD-related behavioral abnormalities such as hyperactivity and fear by silymarin treatment through reduction of Aβ levels (Fig. 5). Unexpectedly, no significant repair of memory loss in APP transgenic mice was observed after silymarin treatment in the Morris water maze using the same dose and duration as used in this study (data not shown). Nabeshima et al. recently reported that silibinin, a component of silymarin, prevents acute and subchronic memory loss induced by the intraventricular injection of Aβ25–35 into mice, due to its strong free radical-
scavenging activity\(^{57}\) and its inhibition of nitric-oxide synthase and tumor necrosis factor-\(\alpha\).\(^{58}\) However, no data on AD pathology due to silibinin were given in these reports. The differences in the protective effects of silibinin and silymarin on memory task might be explained by distinct protocols of material (a component of silymarin or crude extract), duration (11 d or 6 months), and in vivo model (acute model by exogenous A\(\beta\) injection or chronic model by APP transgene), respectively.

The pathogenesis of AD is closely related to lipid accumulation.\(^{59,60}\) Recently, Helzner et al. reported that higher levels of total cholesterol and low-density lipoprotein cholesterol induced faster cognitive decline in AD progression.\(^{60}\) Notably, the plasma lipid status of the APP transgenic mice was repaired by silymarin treatment (Supplemental Fig. 4; see Biosci. Biotechnol. Biochem. Web site). There were no changes in liver weight in the APP mice as compared with the littermate wild type (data not shown), indicating that no adverse effects were induced by silymarin treatment within the concentration and duration ranges examined. Hence, we cannot rule out the possibility that the alleviation of AD-like pathology by silymarin is involved in its hepatoprotective role.

In conclusion, we identified the involvement of chronic silymarin intake in AD prevention \(in vitro\) and \(in vivo\). Six months of silymarin treatment had a satisfactory safety profile without any signs of organ toxicity in the mice. The mice were fed 200 mg/kg/d silymarin in their powdered diet, and it was assumed that each mouse ate 5 g of food per d, and that the body weight of each mouse was 25 g. This amount is approximately equivalent to a human dose of 16 mg/kg/d according to the US Food and Drug Administration criteria for converting drug equivalent dosages across species (http://www.fda.gov/cber/gdlns/dose.htm). Since silymarin was used as a hepatic medicine at a dose of 8 mg/kg/d (200 mg, 3 times per day) for 4 months with some adverse effects in a clinical trial,\(^{61}\) the dose used in this study may be applicable in future preclinical trials.

In sum, silymarin is a safe natural product that can be used for AD prevention. Identification of the active components in silymarin and the molecular mechanism of its action remain for the future.

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References

31) Murakami K, Hara H, Masuda Y, Ohigashi H, and Irie K, 
(2005).
33) Xia W, Yang T, Shankar G, Smith IM, Shen Y, Walsh DM, and 
34) Ijagaki S, McGeer PL, Akiyama H, Zhu S, and Selkoe D, 
35) Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao 
36) Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, and 
37) Bornemann KD, Wiederhold KH, Pauli C, Ermini F, Stalder M, 
Schnell L, Sommer B, Jucker M, and Staufenbiel M, 
38) Matsuoka Y, Picciano M, Malester B, LaFrancois J, Zehr C, 
Daeschner JM, Olschowka JA, O'Banion MK, Tenner AJ, Lemere CA, and Duff K, 
39) Yao Y, Chinnici C, Tang H, Trojanowski JQ, Lee VM, and 
24, 54–65 (2009).
43) Millard CB and Broomfield CA, J. Neurochem., 64, 1909–1918 
45) Flora K, Hahn M, Rosen H, and Benner K, Am. J. Gastro-
enterol., 93, 139–143 (1998).
359 (2007).