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Early Intervention with an Estrogen Receptor β -Selective Phytoestrogenic Formulation Prolongs Survival, Improves Spatial Recognition Memory, and Slows Progression of Amyloid Pathology in a Female Mouse Model of Alzheimer's Disease

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Abstract

Our recent developments have yielded a novel phytoestrogenic formulation, referred to as the phyto- β -SERM formulation, which exhibits an 83-fold binding selectivity for the estrogen receptor subtype β (ER β) over ER α . Earlier studies indicate that the phyto- β -SERM formulation is neuroprotective and promotes estrogenic mechanisms in the brain while devoid of feminizing activity in the periphery. Further investigation in a mouse model of human menopause indicates that chronic exposure to the phyto- β -SERM formulation at a clinically relevant dosage prevents/alleviates menopause-related climacteric symptoms. This study assessed the efficacy, in an early intervention paradigm, of the phyto- β -SERM formulation in the regulation of early stages of physical and neurological changes associated with Alzheimer's disease (AD) in a female triple transgenic mouse model of AD. Results demonstrated that, when initiated prior to the appearance of AD pathology, a 9-month dietary supplementation with the phyto- β -SERM formulation promoted physical health, prolonged survival, improved spatial recognition memory, and attenuated amyloid- β deposition and plaque formation in the brains of treated AD mice. In comparison, dietary supplementation of a commercial soy extract preparation showed no effect on cognitive measures, although it appeared to have a positive impact on amyloid pathology. In overall agreement with the behavioral and histological outcomes, results from a gene expression profiling analysis offered insights on the underlying molecular mechanisms associated with the two dietary treatments. In particular, the data suggests that there may be a crosstalk between ER β

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SUPPLEMENTARY MATERIAL

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and glycogen synthase kinase 3 signaling pathways that could play a role in conferring ER β -mediated neuroprotection against AD. Taken together, these results support the therapeutic potential of the phyto- β -SERM formulation for prevention and/or early intervention of AD, and warrants further investigations in human studies.

Keywords

Alzheimer's disease; early intervention; estrogen receptor β ; glycogen synthase kinase 3; phyto- β -SERM formulation

INTRODUCTION

Phytoestrogens are a diverse group of plant-derived non-steroidal structural analogs of mammalian estrogens that can bind at weak to moderate affinities to estrogen receptors (ERs) and exert estrogenic or antiestrogenic activities [1, 2]. Over the last decade, an increased level of interest in the research of phytoestrogens has been attributed in part to indications of the health-promoting effects of soy foods that are rich in phytoestrogens. A number of epidemiological studies suggest that the high dietary intake of phytoestrogen-enriched soy foods (20–80 mg/d) may contribute to the low incidence of multiple sex hormone-related disorders such as menopausal hot flashes [3] and breast cancers [4] in Asian women, and prostate cancers in Asian men [5], as compared to the incidence in Westerners who consume a much smaller amount of phytoestrogens (<1 mg/d) [6, 7]. The high habitual intake of phytoestrogen-enriched soy foods has also been linked to the lower prevalence of Alzheimer's disease (AD) in Asia compared to the Western world. A summary of 22 epidemiological studies conducted in populations across the continents reveals a 2.5-fold higher prevalence rate for AD in North America and Europe, in comparison to Japan and China, although the prevalence rates for vascular dementia were similar [8].

These positive observational findings, however, lack confirmation from results derived from randomized, controlled trials, which have yielded inconsistent and inconclusive results. For instance, results from seven of eight human studies published in 2000–2007 that sought to determine the impact of soy extracts on cognitive function in postmenopausal women are highly variable [8]. The discrepant outcomes could be caused in part by potentially significant differences in the constitutive composition between the natural forms of soy foods that were focused in observational studies and pharmacological preparations of soy extracts that were used in most of the interventional studies [8]. A great deal of soy extract products sold over the counter are advertised as dietary supplements for use by women to lessen menopausal symptoms such as hot flashes. They are regulated under the Dietary Supplement Health and Education Act of 1994, a much looser regulation than the Food and Drug Administration's authority over prescription drugs, which leaves the public with a serious lack of information about their safety and efficacy [9]. In addition, there is no standardization as to how these soy extract preparations are processed, therefore, results derived from one study can refer only to the specific soy preparation tested, and cannot be extrapolated to other preparations [9].

Our recent developments have yielded a novel formulation composed of a rationally combined mixture of three clinically relevant phytoestrogens, genistein, daidzein, and equol [10]. This formulation exhibits an 83-fold binding selectivity for the estrogen receptor subtype β (ER β) over ER α , thus we refer to it as a phytoestrogenic ER β -selective modulator (SERM) formulation or phyto- β -SERM formulation [10]. Earlier studies in both *in vitro* cell cultures and *in vivo* animal models indicate that the phyto- β -SERM formulation is neuroprotective and promotes estrogenic mechanisms in the brain while devoid of feminizing activity as seen with ER non-selective estrogenic compounds such as 17 β -estradiol in the reproductive system [10]. Further investigation in a mouse model of human menopause reveals that chronic dietary exposure to the phyto- β -SERM formulation at a clinically relevant dosage prevents/alleviates menopause-related physical and neurological changes including the rise in skin temperature, hair thinning/loss, and deficit in spatial learning and memory function [11]. Moreover, at the molecular level, the phyto- β -SERM formulation promotes the expression of hippocampal proteins involved in neural plasticity and amyloid degradation/clearance [11]. These results support the therapeutic potential of the phyto- β -SERM formulation for the management of climacteric symptoms and mitigation of decline in brain responses induced by ovarian hormone loss in menopause [11].

The present study was undertaken to determine the efficacy, in an early intervention paradigm, of a 9-month dietary supplementation with the phyto- β -SERM formulation at the same clinically relevant dosage used in the menopause study [11], in the regulation of early stages of AD-related changes in a female triple transgenic mouse model that contains three mutant genes (A β PP_{swe}, PS1_{M146V}, and tau_{p301L}) relevant to human AD [12].

MATERIALS AND METHODS

Custom diets

Three rodent diets were custom manufactured by Harlan Laboratories (Madison, WI). The composition of each diet is described in our previous report [11]. In brief, the *base/control* diet was prepared from Teklad Global 16% Protein Rodent Diet (Harlan Laboratories), which was ground and repelleted. This diet has a fixed formula and is nutritionally balanced, containing 16% protein and 3.6% fat that support the growth and maintenance of rodents and not containing alfalfa or soybean meal, thus minimizing the levels of natural phytoestrogens. The *phyto- β -SERM diet* was prepared by adding equal parts of genistein, daidzein, and equol (LC Laboratories, Woburn, MA) to the base diet. The *soy extract diet* was prepared by adding a commercial soy extract product, Healthy Women Soy Extract Supplement (Amazon, Seattle, WA), to the base diet. The phyto- β -SERM and soy extract diets were designed to deliver to a mouse a daily intake of 0.25 mg of added total phytoestrogens, or 10 mg/kg (body weight [BW]) mouse per day, assuming a 25-g mouse eating 2.5-g diet per day. This mouse dose is biologically equivalent to a daily intake of 50mg in humans. The conversion of human dose to mouse equivalent dose was based on the conversion factor of equivalent surface area dose from human to mouse [13]: 50 mg/60 kg (BW, human) $\times$ 12 (human to mouse conversion factor) = 10 mg/kg (BW, mouse). This human dose is the estimated average amount of phytoestrogens that Asians regularly ingest from the dietary consumption of soy foods [6] and is the recommended daily serving dose

for many commercial soy extract supplements sold to the women in the United States, including the one tested in this study [14].

Animals and treatment

The use of animals and treatment were approved by the Institutional Animal Care and Use Committee at the University of Southern California (Protocol No: 10780). Three-month-old female triple transgenic AD (3xTg-AD) mice were ovariectomized (OVX) or underwent a sham-OVX operation and immediately fed one of the three custom diets prepared above, for 9 months. Mouse food intake and BW were recorded one to two times every week throughout the study. Two weeks before the treatment was ended, a cognition-behavioral test of spatial working memory function, Y-maze, was administered. At the time of sacrificing the mice, brain tissues were collected and dissected into two hemispheres. One hemisphere was fixed in 4% paraformaldehyde for Campbell-Switzer silver staining analysis of amyloid plaques. The other hemisphere was further dissected into the hippocampus, cortex, and cerebellum. The hippocampal tissues were processed into total protein samples and analyzed by ELISA for changes in the content of amyloid- β ($A\beta$)₁₋₄₀ and $A\beta$ ₁₋₄₂. The cortical tissues were processed into total RNA samples and analyzed by Taqman low-density qRT-PCR arrays for changes in the expression levels of AD-related genes.

Y-Maze cognition-behavioral test

Y-Maze with three identical arms that are evenly spaced with an arm length of 35 cm, arm height of 10 cm, and lane width of 5 cm (Model No. 60180, Stoelting, Wood Dale, IL) was used. The test was conducted in a temperature-controlled test room with accurate configuration of spatial visual cues. The experimenter was blind to treatment conditions. In the one-trial test of spontaneous alternation behavior (SAB), the mice were allowed to move freely within the maze for 5 minutes. The total number and the order of arm entries were recorded. Alternation is defined as successive entries into the three arms in overlapping triplet sets as described previously [15, 16]. The percent alternation is calculated as the ratio of actual to possible alternations: $(\text{the total number of arm entries} - 2) \times 100$. The two-trial recognition memory test consisted of two trials separated by an intertrial interval. In the first acquisition trial, one arm of the maze was closed, and mice were allowed to explore the two other arms for 10 minutes. During the 5-hour interval, the mice were housed in their home cages located in a room other than the test room. In the second retention trial, the mice had free access to all three arms, and were allowed to explore the maze for 5 minutes. The first arm entered, the number of entries into each arm, and the amount of time spent in the novel arm were recorded.

ELISA analyses of $A\beta$ ₁₋₄₀ and $A\beta$ ₁₋₄₂

$A\beta$ ₁₋₄₀ and $A\beta$ ₁₋₄₂ were quantitated using a solid phase sandwich ELISA system (Biosource, Camarillo, CA). Briefly, mouse brain tissues were weighed and homogenized in 4 $\times$ wet mass of cold PBS supplemented with 1 $\times$ Protease Inhibitor Cocktail (Calbiochem). The homogenates were mixed with 10 M guanidine HCl/100 mM Tris HCl (pH 8.0) to yield a solution with 5M final guanidine concentration, and incubated at room temperature (RT) for 4 hours. Mixed samples were diluted at 1:10 with cold reaction buffer BSAT-DPBS (Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween-20, pH 7.4) and

centrifuged at $16,000 \times g$ at 4°C for 10 minutes. Supernatants were collected and diluted at 1:5 with sample diluent buffer. 50 μL /well of human $\text{A}\beta_{1-40}$ (or $\text{A}\beta_{1-42}$) standards and diluted samples were added to a pre-coated 96-well plate with an antibody specific for the NH_2 -terminus of human $\text{A}\beta$ and incubated with 50 μL /well of detection antibody solution (specific for the COOH -terminus of human $\text{A}\beta_{1-40}$ or $\text{A}\beta_{1-42}$) at RT for 3 hours. Plate was washed with wash buffer and incubated with 100 μL /well of anti-rabbit Ig's-HRP solution at RT for 30 minutes. After washing, 100 μL /well of stabilized chromogen were added to the plate and incubated at RT in the dark for 10–30 minutes. 100 μL /well of stop solution were added to the plate and mixed gently. Optical density was measured at 450 nm. Results were calculated from the standard curve.

Campbell-Switzer silver staining of $\text{A}\beta$ plaques

Mouse hemispheres were sectioned using Multi-Brain processing technology at NeuroScience Associates (Knoxville, TN). Briefly, hemispheres were treated with 20% glycerol and 2% DMSO to prevent freeze-artifacts and subsequently embedded together in a gelatin matrix. After curing, the block of embedded hemispheres was rapidly frozen by immersion in isopentane chilled to -70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The block was sectioned at 35 μm in the coronal plane through the hippocampus region of the mouse hemispheres (Bregma -0.5 to -4.5 mm). Cut sections were collected sequentially into 12 containers that were filled with antigen preserve solution (50% PBS, 50% ethylene glycol, and 1% polyvinyl pyrrolidone, pH 7.0). At the completion of sectioning, each container held a serial set of one-of-every-12th section. Each of the large sections cut from the block is a composite section holding individual sections from each of the hemispheres embedded in each block. With such composite sections, uniformity of staining was achieved across treatment groups.

Campbell-Switzer silver staining that labels $\text{A}\beta$ plaques [17] was performed on a serial set of one-of-every-4th (140 μm interval) sections of mouse hemispheres (3 containers; 33 sections for each animal). Briefly, the sections were placed in freshly prepared 2% ammonium hydroxide for 5 minutes. The sections were next placed in a silver-pyridine-carbonate solution for 40 minutes, 1% citric acid for 3 minutes, and 0.5% acetic acid until ready for development. The sections were developed in Physical Developer ABC solution (after Gallyas; containing Na carbonate citric acid, tungstosilicic acid, and formaldehyde) with the development time being visually assessed. The development was stopped by briefly placing the sections in 0.5% acetic acid. The stained sections were then mounted on gelatinized glass slides and viewed under a Zeiss Axiovert 200 M Marianas digital microscopy workstation equipped with 3I Slidebook imaging software. Plaque load was quantitated following conventional stereological procedures. As an indicator of the depth of plaque load, the number of sections affected by plaques was counted for each animal. The percentage of plaques-affected sections out of the total number of sections was calculated for each treatment group. As an indicator of the width/density of plaque load, three sections (sections 26–28 for all animals) that represented the fraction of sections loaded with most plaques across animals were chosen, and the areas occupied by plaques on those sections were measured using the image processing and analysis software ImageJ (<http://rsbweb.nih.gov/>

ij/; developed by Wayne Rasband at NIH, Bethesda, MD). The total size of plaques-occupied areas within the area of interest was calculated for each treatment group.

TLDA qRT-PCR gene expression profiling

Taqman low-density arrays (TLDA) pre-loaded with mouse Taqman assays for qRT-PCR detection of the expression of 91 AD-related target genes and 5 candidate control genes (Supplementary Table 1) were manufactured by Applied Biosystems (Part Number: 4378714; Foster City, CA). Total RNA derived from mouse cortical tissues were isolated using the Pure-Link RNA Mini Kit (Invitrogen, Carlsbad, CA). RNA quantity and quality were analyzed using the Experion RNA StdSens Analysis Kit on an Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). RNA to cDNA synthesis was prepared using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) on a MyCycler Thermal Cycler (Bio-Rad). Taqman qRT-PCR reactions were performed on 50 ng cDNA samples mixed with the TaqMan Universal PCR Master Mix 2× (Applied Biosystems), under the thermal cycling conditions: stage 1: AmpErase UNG activation at 50°C/2 min/100% ramp; stage 2: AmpliTaq gold DNA polymerase activation at 94.5°C/10 min/100% ramp; stage 3: melt at 97°C/30 s/50% ramp, followed by anneal/extend at 59.7°C/1 min/100% ramp, for 40 cycles. Fluorescence was detected on an ABI 7900HT Fast Real-Time PCR System equipped with the Sequence Detection System Software Version 2.3 (Applied Biosystems).

Data were analyzed using the RQ Manager Version 1.2 and DataAssist Version 2.0 (Applied Biosystems). Relative gene expression levels or fold changes relative to the comparison group were calculated by the comparative Ct (C_t) method, with C_t denoting threshold cycle [18, 19]. Selection of the endogenous control gene for normalization was based on the control stability measure (M), which indicates the expression stability of control genes on the basis of non-normalized expression levels. M was calculated using the geNorm algorithm; genes with the lowest M values have the most stable expression. Four-six samples per group were included in the analysis. For each sample, ΔC_t was calculated as the difference in average C_t of the target gene and the endogenous control gene. For each treatment group, mean $2^{-\Delta C_t}$ was calculated as the geometric mean of $2^{-\Delta C_t}$ of the four-six samples in the group. Fold change was then calculated as mean $2^{-\Delta C_t}$ (treatment group) / mean $2^{-\Delta C_t}$ (comparison group). Fold change values greater than one indicate a positive expression or up-regulation relative to the comparison group. Fold change values less than one indicate a negative expression or down-regulation relative to the comparison group. The $2^{-\Delta C_t}$ values for each target gene were statistically compared between the treatment and comparison group using Student's t -test. The statistical significance was indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Hierarchical clustering diagram graphically displayed clusters of treatment groups as well as target genes. Distance correlations based on the ΔC_t values.

Statistical analyses

For all other non-gene-expression data, statistically significant differences between groups were determined by one-way analyses of variance followed by Student-Newman-Keuls pairwise multiple comparison *post hoc* tests. The statistical significance was indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Phyto- β -SERM diet promoted physical health and survival of female 3xTg-AD mice

The present study sought to investigate the impact of three diets, previously studied in a menopausal model [11], in female 3xTg-AD mice. Four treatment groups, seven mice per group, a total of 48 mice were included in the study, including: 1) sham-OVX mice treated with the control diet (sham-OVX+ control group), 2) OVX mice treated with the control diet (OVX+ control group), 3) OVX mice treated with the phyto- β -SERM diet (OVX+ phyto- β -SERM group), and 4) OVX mice treated with the soy extract diet (OVX+ soy extract group) (Table 1). Treatment started when the mice were three months of age and had no signs of any AD-related changes, which was intended to achieve an early intervention paradigm. Treatment ended when the mice were 12 months of age and just beginning to form A β plaques in their brains, which was intended to target the early stage of changes associated with AD to potentially maximize the detection sensitivity for possibly small magnitude of therapeutic efficacy (Fig. 1). Throughout the 9-month study, no differences in food intake among groups were detected. However, it was observed that the OVX mice treated with the control diet underwent a weight loss when they reached 9 months of age, which, nevertheless, did not occur in the mice treated with the phyto- β -SERM diet (Fig. 2A). Moreover, the phyto- β -SERM diet-treated group had evidently reduced mortality. As shown in Fig. 2B, there were a total of 7 mice that died at different points of time throughout the study. For the sham-OVX control group, a total of one death occurred during the first 3-month treatment, resulting in a final mortality rate of 14.3% (Fig. 2B). For the OVX control group, a death of two mice occurred within the first 3-month, followed by one more during the second 3-month treatment, resulting in a final mortality rate of 42.9% (Fig. 2B). OVX mice treated with the phyto- β -SERM diet had a much better survival rate than OVX mice treated with the control diet. Only one death occurred at the very end of the study, resulting in the same final mortality rate of 14.3% as the sham-OVX control group (Fig. 2B). OVX mice treated with the soy extract diet did not differ from those on the control diet; one and two deaths occurred within the first and second 3-month, respectively, resulting in the same final mortality rate of 42.9% as the OVX control group at the end of the 9-month study (Fig. 2B).

Phyto- β -SERM diet promoted spatial recognition memory of female 3xTg-AD mice

Two weeks before the end of the 9-month study, mice underwent a cognition-behavioral test, Y-maze, which is based upon the natural tendency of mice to explore a novel arm rather than a familiar one when both are presented simultaneously to assess the hippocampus-dependent spatial working memory function. The one-trial SAB test was designed to assess the normal navigational behavior of mice when they were free of stress and allowed to explore all three arms of the maze. Success in this test was reflected by a high rate of alternation indicating that mice remembered which arm was last entered (Fig. 3A). Compared with the SAB test, the two-trial recognition test was designed to assess a higher level of cognitive complexity that involves a time delay between the learning and recognition process. In brief, mice were first allowed to explore the maze that had one arm closed. After a 5-hour interval, mice were then brought back to the maze and allowed to freely explore all three arms. The choice to explore the novel arm (the arm closed in the

learning process), as reflected by the first entry, the number of entries, and the amount of time spent in the novel arm, indicates the greater learning and recognition capacity (Fig. 3B). Data revealed that in the one-trial test, there were no significant differences in SAB among groups, which could possibly be due to the simplicity of the task that was insufficient to differentiate the cognitive impact of different treatments (Fig. 3A). Compared to the one-trial test, the two-trial spatial recognition test presented a much greater sensitivity in detecting differences among groups. When compared to the OVX control mice, a significant improvement was achieved in OVX mice treated with the phyto- β -SERM diet, as indicated by both the first entry choice (Fig. 3B; 60% versus 25% to the novel arm) and the amount of time spent in the novel arm (Fig. 3B; 47% increase; $*p < 0.05$). The mice exposed to the soy extract showed no differences from the control diet-treated mice in both measures (Fig. 3B).

Phyto- β -SERM diet attenuated A β deposition and plaque formation in the brains of female 3xTg-AD mice

At the end of the 9-month study, mouse brain tissues were collected, and hippocampi dissected from one hemisphere were subjected to an ELISA analysis of the A β_{1-40} and A β_{1-42} deposition. The other hemisphere was fixed, and a serial set of hippocampal sections (33 sections per brain; 140 μ M interval) were stained with the Campbell-Switzer silver stain that labels A β plaques. Due to the unexpected deaths, the final numbers of mice included in the analyses were: $n = 6$ for the Sham-OVX control group, $n = 4$ for the OVX control group, $n = 6$ for the phyto- β -SERM group, and $n = 4$ for the soy extract group. Data shown in Fig. 4 revealed that no significant differences existed between the sham-OVX control group and the OVX control group. When compared to the Sham-OVX control group, the phyto- β -SERM group showed a significant reduction in the deposition of both A β_{1-40} (Fig. 4A; 66% reduction; $**p < 0.01$) and A β_{1-42} (Fig. 4B; 58% reduction; $**p < 0.01$). Consistent with the ELISA data, the phyto- β -SERM diet-treated mice showed significantly attenuated plaque formation throughout the hippocampal region. This was indicated by both the number of brain sections affected by plaques (Fig. 4C; 40% reduction; $*p < 0.05$) and the size of the areas occupied by plaques on the three most affected sections (sections 26–28 for all animals) (Fig. 4D, E; 84% reduction; $*p < 0.05$). When compared to the OVX control group that had a small $n = 4$ and an apparent outlier, the effect induced by the phyto- β -SERM diet failed to reach a statistical significance in the first three measures (Fig. 4A–C). However, the phyto- β -SERM diet-treated mice showed a significant reduction in the fourth outcome measure of the areas occupied by plaques on those most affected sections (Fig. 4D, E; 83% reduction; $*p < 0.05$). It appeared that the soy extract diet also had a positive impact on the same measures of amyloid pathology but at a smaller magnitude, and only one significant effect was detected when comparing to the Sham-OVX control group on the measure of the areas occupied by plaques on those most affected sections (Fig. 4D, E; 69% reduction; $*p < 0.05$).

Phyto- β -SERM and soy extract diets induced both similar and distinct expression profiles of AD-related genes in the brains of female 3xTg-AD mice

To further understand the impact, at the molecular level, of the dietary treatments, mouse brain cortical tissues were profiled by Taqman low-density qRT-PCR arrays on the expression of a focused set of genes implicated in AD (Supplementary Table 1). Among five

candidate control genes, *Actb* and *Gapdh* exhibited the most stable expression across all samples, thus their arithmetic mean was used as the normalizing control in the following data analyses. Of the 91 target genes assayed, 65 genes with Ct values <35 were analyzed, and among them, 19 genes exhibited a statistically significant change with $p < 0.05$ (Fig. 5 and Table 2). When compared to the OVX mice treated with the control diet, major changes detected in the control diet-treated Sham-OVX mice brain samples included up-expression of *Adam9* (disintegrin and metalloproteinase domain-containing protein 9), *Ide* (insulin-degrading enzyme), and *Ppp2ca* (serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform), and down-expression of *Psenen* (presenilin enhancer 2) (Table 2). When compared to either of the two control diet-treated groups, the phyto- β -SERM and soy extract treatment groups exhibited both similarities and differences in the overall expression profiles. Specifically, when compared to the OVX control group, three genes, *Gap43* (neuronal growth-associated protein 43), *Ide*, and *Lrpap1* (low density lipoprotein receptor-related protein associated protein 1), were similarly increased by both the phyto- β -SERM and soy extract diets. Genes that were significantly changed by the phyto- β -SERM diet but not by the soy extract diet included up-expression of *Apoe* (apolipoprotein E), and down-expression of *App* (amyloid precursor protein) and *Gsk3b* (glycogen synthase kinase 3 beta). Genes that were significantly changed by the soy extract diet but not by the phyto- β -SERM diet included down-expression of *Grin1* (glutamate NMDA receptor subunit zeta 1), *Ncstn* (nicastrin), and *Prkcb1* (protein kinase C beta type) (Table 2 and Fig. 6). Consistent with the amyloid pathology data, more changes were detected when compared to the Sham-OVX control group. Four genes, *Ide*, *Lrpap1*, *Mapt* (microtubule-associated protein tau), and *Prkacb* (cAMP-dependent protein kinase catalytic subunit beta), were similarly influenced by both the phyto- β -SERM and soy extract diets. Genes that exhibited a significant change in response to the phyto- β -SERM diet but not to the soy extract diet included up-expression of *Chat* (choline acetyltransferase) and *Mme* (membrane metallo-endopeptidase, also known as neprilysin), and down-expression of *Apha2* (amyloid precursor protein-binding family A member 2), *App*, and *Gsk3b*. Genes that exhibited a significant change in response to the soy extract diet but not to the phyto- β -SERM diet included up-expression of *Psenen*, and down-expression of *Chrm3* (cholinergic receptor muscarinic 3), *Ncstn*, and *Uchl1* (ubiquitin carboxy-terminal hydrolase L1) (Table 2 and Fig. 6).

DISCUSSION

AD, as the most common form of dementia, currently affects more than 35 million people worldwide, and treatment and care for them costs more than \$604 billion per year. These figures are predicted to triple by the mid of the century [20]. Of those affected with AD, 62% are women [21]. The female vulnerability to AD is partly due to their longer life expectancy and hence the greater age-associated risk for developing AD. However, age is not the only risk factor for women. Numerous studies have indicated a clear association between ovarian hormone loss following menopause and changes in cognitive function and increased risk for AD [22, 23]. Estrogen therapy, when timely initiated at the onset of menopause, can potentially counteract the cognitive changes and reduce the risk of AD [24, 25]. However, the therapeutic application of estrogen therapy, in particular for disease prevention or risk reduction, has been hampered by safety concerns [26, 27].

In search of an alternative therapy that can be continuously used for the purpose of prevention or delay of the onset of AD in postmenopausal women, our recent development efforts have focused on naturally occurring estrogen receptor subtype β -selective ER modulators (β -SERMs) as a potentially safe and effective strategy to activate neural responses against neurodegeneration [8, 28–30]. Starting from target validation followed by *in silico* virtual screening and a series of *in vitro* and *in vivo* testing, we have developed a novel formulation composed of three clinically relevant phytoestrogens (genistein, daidzein, and equol), referred to as the phyto- β -SERM formulation [10, 31–33]. The phyto- β -SERM formulation exhibits an 83-fold binding preference for ER β over ER α , and was found to be efficacious in promoting neuronal survival and brain defense mechanisms including enhancement of brain mitochondrial function and amyloid homeostasis, while devoid of feminizing activity as seen with the endogenous estrogen, 17 β -estradiol, in the reproductive system [10]. Further investigation in a menopause mouse model indicated that chronic exposure to the phyto- β -SERM formulation at a clinically relevant dosage holds a therapeutic potential to prevent or mitigate menopause-related climacteric symptoms including hot flashes, alopecia, and cognitive decline [11].

The present study investigated the efficacy, in an early intervention paradigm, of the phyto- β -SERM formulation in the regulation of early stages of physical and neurological changes associated with AD in a female 3xTg-AD mouse model [12]. Results demonstrated that when initiated prior to the appearance of any signs of AD, a 9-month dietary supplementation with the phyto- β -SERM formulation at the same clinically relevant dosage used in the menopause study [11] was effective to: i) promote the physical health of the treated AD mice, in particular, prevent the weight loss associated with the progression of the disease, an observation consistent with the clinical finding that incident dementia in women was preceded by weight loss by at least a decade [34]; ii) prolong the survival of the treated AD mice; iii) improve the spatial working memory function of the treated AD mice; and iv) attenuate the A β deposition and plaque formation in the brains of the treated AD mice. In comparison, dietary supplementation of a commercial soy extract preparation did not induce a change in the survival and cognitive performance of the AD mice, although it appeared to have a favorable effect on amyloid pathology in the brains of the treated AD mice.

In overall agreement with the behavioral and histological outcomes, results from a gene expression profiling analysis offered insights on underlying molecular mechanisms associated with the two dietary treatments. Consistent with the amyloid pathology data, both the phyto- β -SERM diet and the soy extract diet induced a positive effect on the expression of genes involved in reducing production and increasing clearance of A β . In addition to upregulation of Ide, which serves as one of the major mechanisms in the degradation of A β in the brain [35], other factors that could have contributed to the attenuation of amyloid pathology by the phyto- β -SERM formulation include downregulation of App and increased clearance of A β by Apoe. In comparison, downregulation of Ncstn, a stabilizing cofactor required for the assembly of the γ -secretase complex, the final enzymatic step that leads to the production of A β [36, 37], appeared to be associated with the reduction of amyloid pathology by the soy extract treatment.

Despite the positive impact on amyloid pathology conferred by both dietary treatments, both the behavioral and gene expression data indicated that only the phyto- β -SERM diet, and not the soy extract diet, was beneficial for the cognitive function of the treated mice. In addition to Gap43, a neuronal growth-associated protein playing a crucial role in synaptic function that was similarly upregulated by both treatments, the soy extract diet, but not the phytoSERM diet, downregulated Grin1 that encodes the NR1 subunit of the NMDA receptor [38]. Substantial loss of the NR1 subunit at both the mRNA and protein levels has been found in AD brains [39, 40]. Thus, it can be speculated that the mixed profile as demonstrated by a positive effect on Gap43 while negative on Grin, could have contributed to the flat response in cognitive performance seen in mice treated with the soy extract diet. This data is in line with recent literature indicating that no direct link exists between amyloid burden and cognitive functioning [41, 42]. Such a disconnection could also explain the lack of a difference in amyloid pathology between the sham-OVX and OVX groups, suggesting that, under an AD condition, long-term ovarian hormone loss could pose a greater impact on cognitive function than amyloid pathology.

In addition to those involved in amyloid pathology and cognitive function, another important gene emerging from the gene array study is Gsk3b, which was downregulated by the phyto- β -SERM diet but not by the soy extract diet. Gsk3b encodes the β subtype of glycogen synthase kinase 3, a class of constitutively active, proline-directed serine/threonine kinase involved in a number of cellular processes ranging from energy metabolism to neural development and apoptosis [43, 44]. Gsk3 deregulation has been implicated in a variety of human pathologies including cancer, type 2 diabetes mellitus, and AD [45, 46]. In particular, increased expression and activity of Gsk3 has been associated with the pathological hallmarks of the disease in both sporadic and familial AD cases [47, 48]. As a consequence, much of recent effort has been directed toward the development and characterization of Gsk3 inhibitors as a possible therapeutic strategy for treatment of AD [45, 49, 50]. The existing literature work highlights the significance of the finding of this study, indicating that ER β could play a role in the regulation of Gsk3 signaling in the brain. Such an activity could serve as a mechanism underlying the therapeutic potential of the phyto- β -SERM formulation against AD. Investigations of upstream and downstream regulators involved in this process could further the understanding of the roles of ER β in the regulation of neurological health and diseases.

As discussed in our previous report [11], we postulate that the difference in responses to the phyto- β -SERM formulation and the soy extract preparation could largely be attributed to the composition of the two preparations. Compared to a random mixture often contained within a soy extract preparation, a formulation with a clearly defined composition and synergy could maximize the therapeutic efficacy as exemplified by the phyto- β -SERM formulation. Moreover, the standardization of equol along with genistein and daidzein in the phyto- β -SERM formulation could provide an additional advantage. Equol is metabolized from daidzein by intestinal microbial flora following the intake of daidzein-containing soy products [51]. Approximately 20–35% of Western adults are equol-producers as compared to 55–60% in Asian populations [52–55]. Therefore, clinically, inclusion of equol in the phyto- β -SERM formulation could potentially benefit both equol producers and non-producers. Moreover, the phyto- β -SERM formulation illustrates a mechanistic advantage

associated with its high selectivity for ER β over ER α . ER β has been demonstrated to play a crucial role in brain development, neural plasticity, and hippocampus-dependent learning and memory [29, 56–58]. It has also been shown to have a broad involvement in mediating estrogenic activities in the brain including regulation of neuronal survival, mitochondrial function, and A β degradation [32, 33, 35, 59]. Selective activation of ER β would also minimize proliferative induction and associated cancer risks [60].

In conclusion, the data presented herein provides compelling evidence supporting the therapeutic potential of the phyto- β -SERM formulation for prevention and/or early intervention of AD. These findings, along with those found in a preclinical model of human menopause [11], have recently led to a clinical trial of the phyto- β -SERM formulation designed to evaluate the dosage/safety, pharmacokinetics, and proof-of-concept efficacy to mitigate hot flash frequency and memory deficits in menopausal women ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01723917) identifier: NCT01723917). This pilot development trial will serve as a foundation for further investigations of the effectiveness of the phyto- β -SERM formulation in patients at high risk for developing AD or with early-stage AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Study design: Age-related progression of pathological changes in female 3xTg-AD mice and therapeutic target window. No signs of AD-related changes were detected at the age of 3 months. A significant increase in intraneuronal A β protein deposition started to appear at the age of 9 months, which eventually led to the onset/early formation of extraneuronal A β plaques in select brain regions, with subiculum as the most vulnerable region, at the age of 12 months. At the age of 18 months, substantial plaques were observed throughout hippocampus and some cortical regions, notably the amygdalar complex. A β plaques were labeled by Campbell-Switzer silver stain.

Fig. 2. Phyto- β -SERM diet prevented weight change occurred at the age of 9 months (A) and promoted survival (B) of female 3xTg-AD mice. Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto- β -SERM formulation-containing diet, or a commercial soy extract-containing diet, for 9 months. Mouse food intake, body weight and deaths were recorded throughout the 9-month study.

A. One-Trial Spontaneous Alteration Behavioral (SAB) Test

B. Two-Trial Recognition Memory Test

Fig. 3.

Phyto- β -SERM diet promoted spatial recognition memory of female 3xTg-AD mice. Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto- β -SERM formulation-containing diet, or a commercial soy extract-containing diet, for 9 months. Two weeks before the end of the treatment, a cognition-behavioral test of spatial working memory function, Y-Maze, was administered. A) In the one-trial test of spontaneous alternation behavior (SAB), the total number and the order of arm entries were recorded. B) In the two-trial recognition memory

test that consisted of an acquisition trial followed by a retention trial, the first entry, the number of entries into each arm, and the amount of time spent in the novel arm were recorded. Data are presented as group mean $\pm$ SEM; n/group = 4–6. * $p < 0.05$.

Fig. 4. Phyto- β -SERM diet attenuated A β deposition and plaque formation in the brains of female 3xTg-AD mice. Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto- β -SERM formulation-containing diet, or a commercial soy extract-containing diet, for 9 months. A, B) $A\beta_{1-40}$ and $A\beta_{1-42}$ content in hippocampal tissues derived from one hemisphere were measured by ELISA; (C–E) The other hemisphere was sectioned along the coronal plane, and for each animal, a total of 33 sections collected at 140 μ M intervals throughout hippocampus were

stained with the Campbell-Switzer silver stain that labels A β plaques in black. C) Group comparisons on the percentage of sections that were affected by plaques out of the total sections analyzed; (D, E) Group comparisons on the total size of the areas that were occupied by plaques within the area of interest (defined by the red box) on 3 sections that were loaded with most plaques (S26–S28). Data are presented as group mean $\pm$ SEM; n/group = 4–6. * p < 0.05 and ** p < 0.01.

Fig. 5. Expression profiles of AD-related genes in the brains of female 3xTg-AD mice. Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto- β -SERM formulation-containing diet or a commercial soy extract-containing diet, for 9 months. Cortical tissues derived from one hemisphere were processed and profiled by Taqman low-density qRT-PCR arrays for changes in the expression levels of a focused set of genes related to AD. Here shows the hierarchical cluster diagram on 65 genes with Ct < 35; red indicates high expression, green

indicates low expression. Control group =OVX+ Control; Group 1 = Sham-OVX+ Control; Group 2 =OVX+ Phyto- β -SERM; Group 3 =OVX + Soy Extract.

A. Differentially expressed genes when compared to OVX + Control group

B. Differentially expressed genes when compared to Sham-OVX + Control group

Fig. 6.

Phyto-β-SERM and soy extract diets induced both similar and distinct expression profiles of AD-related genes in the brains of female 3xTg-AD mice. Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto-β-SERM formulation-containing diet or a commercial soy extract-containing diet, for 9 months. Cortical tissues derived from one hemisphere were processed and profiled by Taqman low-density qRT-PCR arrays for changes in the expression levels of a focused set of genes related to AD. Here lists the genes significantly changed by either the phyto-β-SERM diet or the soy extract diet when compared to either of the two control diet-treated groups; red indicates upregulation, green indicates downregulation.

Table 1

Experimental overview

Study animals	Study diets	Study groups (<i>n</i> = 7/group)	Treatment		Measurements
			Start age	End age	
Female 3xTg-AD mice; OVX or Sham-OVX	- Control diet - Phyto-β-SERM diet - Soy extract diet	- Sham-OVX+ Control diet - OVX+ Control diet - OVX+ Phyto-β-SERM diet	3-month-old	12-month-old	- Food intake and body weight –recorded 1–2 times every week - General health and appearance – photographed 1–2 times every two weeks - Y-Maze – conducted 2 weeks before sacrificing the mice - ELISA analyses of Aβ_{1–40}/Aβ_{1–42} deposition in hippocampus –tissues collected from one hemispheres at the time of sacrificing the mice - Taqman low-density array (TLDA) qRT-PCR analyses of gene expression profiles in cortex –tissues collected from one hemispheres at the time of sacrificing the mice - Campbell-Switzer silver staining of Aβ plaque formation –the other hemispheres fixed at the time of sacrificing the mice

Table 2

AD-related gene expression profile comparisons between treatment groups

Gene symbol	Sham-OVX+ control versus OVX+ control		OVX+ Phyto- β -SERM versus OVX+ control		OVX+ Soy extract versus OVX+ control		OVX+ Phyto- β -SERM versus Sham-OVX+ control		OVX+ Soy extract versus Sham-OVX+ control	
	fold change	p-Value	fold change	p-Value	fold change	p-Value	fold change	p-Value	fold change	p-Value
<i>AβPP & Aβ Metabolism</i>										
Adam9	1.42	0.003 **	1.27	0.065	1.30	0.051	0.89	0.182	0.92	0.264
Apha2	1.20	0.338	0.92	0.525	1.13	0.595	0.76	0.030 *	0.94	0.553
App	1.08	0.477	0.76	0.017 *	0.98	0.905	0.70	0.005 **	0.91	0.423
Ide	1.88	0.007 **	1.37	0.025 *	1.40	0.023 *	0.73	0.021 *	0.75	0.046 *
Mme	0.57	0.062	0.83	0.381	0.64	0.145	1.45	0.038 *	1.11	0.406
Nestn	0.99	0.950	0.73	0.266	0.45	0.031 *	0.74	0.221	0.45	0.015 *
Psenen	0.52	0.013 *	0.79	0.374	1.05	0.734	1.53	0.141	2.04	0.028 *
<i>Synaptic & Cognitive Function</i>										
Chat	0.51	0.117	1.07	0.915	1.09	0.791	2.08	0.030 *	2.13	0.095
Chrm3	1.02	0.936	0.85	0.527	0.51	0.130	0.83	0.452	0.50	0.043 *
Gap43	1.27	0.110	1.30	0.024 *	1.44	0.036 *	1.02	0.906	1.13	0.417
Grin1	0.51	0.380	0.88	0.130	0.81	0.036 *	1.72	0.557	1.58	0.816
Uchl1	1.28	0.065	1.14	0.110	0.98	0.833	0.89	0.145	0.77	0.039 *
<i>Cholesterol Trafficking/Protein Kinases/Tau Protein</i>										
Apoe	1.28	0.208	1.20	0.049 *	1.30	0.064	0.93	0.545	1.02	0.980
Lrpap1	0.97	0.759	1.36	0.027 *	1.47	0.003 **	1.40	0.014 *	1.51	0.003 **
Gsk3b	1.08	0.256	0.83	0.030 *	0.88	0.402	0.79	0.008 **	0.83	0.125
Ppp2ca	1.31	0.017 *	1.35	0.106	1.38	0.072	1.03	0.753	1.05	0.581
Prkacb	1.26	0.111	0.83	0.300	0.93	0.667	0.66	0.006 **	0.74	0.027 *
Prkcb1	0.58	0.415	0.73	0.051	0.71	0.016 *	1.24	0.918	1.22	0.869
Mapt	1.07	0.762	0.81	0.230	0.81	0.274	0.75	0.029 *	0.76	0.026 *

Three-month-old female 3xTg-AD mice were OVX or sham-OVX and fed one of the three test diets: a phytoestrogen-free control diet, a phyto- β -SERM formulation-containing diet or a commercial soy extract-containing diet, for 9 months. Cortical tissues derived from one hemisphere were profiled by Taqman low-density qRT-PCR arrays for changes in the expression levels of a focused set of genes related to AD. This table lists all genes that exhibited a significant change between any two of the four treatment groups. Fold changes with $p < 0.05$ are highlighted in bold.

* $p < 0.05$,
 ** $p < 0.01$.