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Effects of Soy Phytoestrogens on Reference Memory and Neuronal Cholinergic Enzymes in Ovariectomized Rats

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ABSTRACT The effects of soy phytoestrogens on Morris water maze (MWM) performance and neuronal cholinergic enzyme activities and immunoreactivity were studied in ovariectomized (OVX) rats. The rats were assigned to four groups fed control diet (CD), 3.9 mg/kg 17β-estradiol diet (E2), 263.4 mg/kg soy phytoestrogens diet (SP1), and 526.9 mg/kg soy phytoestrogens diet (SP2). In the MWM task, escape latency and path length were significantly less in the E2 and SP2 groups than in the CD group on the second day. Choline acetyltransferase (ChAT) activity in the cerebral cortex and ChAT immunoreactivity in the diagonal band of Broca were significantly greater in the E2, SP1, and SP2 groups than in the CD group. Acetylcholinesterase activity in the hippocampus in the E2, SP1, and SP2 groups was significantly lower than in the CD group. This study suggests that soy phytoestrogens affect the reference memory and neuronal cholinergic system in OVX rats.

KEY WORDS: *• acetylcholine esterase • choline acetyltransferase • Morris water maze • ovariectomy • soy phytoestrogens*

INTRODUCTION

RECENT EVIDENCE HAS IMPLICATED estrogen in memory function. Estrogen deficiency following menopause or after surgical excision of ovaries has been associated with cognitive decline,[1,2] and estrogen replacement therapy has been reported to delay the onset of Alzheimer's disease among women.[3] Several studies have found that estrogen regulates the neuronal cholinergic system and influences memory function.[4,5] Estrogen treatment increases the activity of choline acetyltransferase (ChAT) in the horizontal diagonal band nucleus, frontal cortex, and hippocampus of ovariectomized (OVX) rats.[6,7] In the Morris water maze (MWM) task, estrogen replacement in 14-month-old OVX rats resulted in a better acquisition performance.[5]

Soy phytoestrogens are similar to estrogen in structure and exhibit both estrogenic and antiestrogenic activities. They have been studied for their potential beneficial effects against hormone-dependent cancers and age-related diseases.[8–10] Although little is known about the influence of soy phytoestrogens on memory function and the neuronal cholinergic system, some studies have shown that soy phytoestrogens increased ChAT mRNA in the frontal cortex of retired breeder rats[7] and improved radial arm maze performance in OVX and retired breeder rats.[11]

In our previous study, we observed that soy isoflavones reduced age-related declines in cholinergic neurons and cognitive decline in male rats.[12] However, studies of their effects on ChAT activity are few, and no studies have investigated whether soy phytoestrogens affect acetylcholinesterase (AChE) activity in OVX rat brain. In addition, more studies are needed to find the effective amount of soy phytoestrogens for enhancing memory function. This study, therefore, investigated cholinergic enzyme activities of soy phytoestrogens in the brain areas relating to memory and evaluated reference memory using the MWM task.

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MATERIALS AND METHODS

Preparation of soy phytoestrogens

Phytoestrogens were extracted from soy hypocotyls with 10 volumes of 80% aqueous ethanol with stirring for 2 hours at 70°C. The ethanol extract was condensed with a rotary evaporator at 50°C. Finally, the extract was obtained by freeze-drying the concentrated ethanol extract. Once isolated, the soy phytoestrogen extract was stored at −80°C until analysis and formulation of the experimental diets. High-performance liquid chromatography (HPLC) analysis of three soy isoflavones and their nine derivatives was performed using a diode-array detector (HP1100 system, Agilent, Santa Clara, CA) and an Eclipse XDB C-18 column (Agilent). Ultraviolet detection was at 260 nm, and the injection volume was 5 μL. The mobile phases were 0.1% (vol/vol) acetic acid in H_2O (solvent A) and 0.1% (vol/vol) acetic acid in acetonitrile (solvent B). A flow rate of 1.2 mL/minute under an initial condition of 93:7 (A:B) was held for 25 minutes, brought to 15% B over 25 minutes, to 20% B over 5 minutes, and to 25% B over 15 minutes, all with a linear gradient. Genistein, daidzein, and glycitein were purchased from Sigma (St. Louis, MO), and malonyl, acetyl, and glycoside forms were purchased from Fujico (Kobe, Japan) for use as standards in the HPLC analyses.

Animals and diets

Female Sprague-Dawley rats (12–16 weeks old) were purchased from Bio-Genomics (Seoul, Republic of Korea). They were acclimated in an environmentally controlled animal laboratory and given free access to an AIN-93 diet (Harlan Teklad, Madison, WI) and distilled water. They were kept at a constant temperature (22 ± 2°C) and relative humidity (55 ± 5%) with a 12-hour light/dark cycle. Procedures involving animals and their care conformed to the institutional guidelines, which are in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures.

When the rats were 45 weeks of age and their overall mean body weight was 395.1 g, they were subjected to bilateral ovariectomy. After ovariectomy, they were divided into four groups ($n = 14$ per group): control basal diet-treated group (CD), 17β-estradiol (3.9 mg/kg diet)-treated group (E2), low content of soy phytoestrogen extract (263.4 mg/kg diet)-treated group (SP1), and high content of soy phytoestrogen extract (526.9 mg/kg diet)-treated group (SP2). The dosage of soy isoflavones was based on a previous study that found that soy isoflavones improved cognitive functions in male rats.[12]

All the rats were fed an isonitrogenous and isocaloric diet, based on the AIN-93 diet, for 16 weeks. Distilled water was always available to the rats. During the experimental period, the body weight was measured once per week, and the food intake was measured every day. Behavioral tests were performed at 15 weeks, and the rats were killed for the collection of brains at 16 weeks.

MWM test

The learning and reference memory test of rats was performed using the MWM task.[13] A circular pool (135 cm in diameter and 55 cm deep) was filled with water to a depth of 40 cm, and powdered milk was mixed with the water to produce an opaque medium. The pool was then divided into four quadrants, and a platform was placed in one quadrant 2.5 cm below the water surface. The other three quadrants were used as starting positions, and the location of the submerged platform was not changed throughout the experiment. The order of the start locations was quasirandomized, and the rat never started from the same location on any two consecutive trials. On each trial, the swimming time was restricted to 120 seconds. When the rat reached the hidden platform, it was allowed to rest there for 20 seconds and then removed from the maze and dried off. If it did not find the platform within 120 seconds, it was guided to the platform by the experimenter. The test lasted for 4 days, and each day consisted of four trials. The inter-trial interval was set as 10 minutes. Rat positions in the pool were automatically tracked, and swimming time and distance were automatically calculated by a video tracking system (View Point, Lyon, France) above the center of the pool.

Measurement of activities of cholinergic enzymes

After behavioral testing, the rats ($n = 7$ per group) were decapitated, and cerebral cortex and hippocampus were collected. ChAT activity was determined spectrophotometrically using the method of Chao and Wolfgram[14] with some modification. The tissue was homogenized (1%, wt/vol) in ice-cold 0.05 mol/L sodium phosphate buffer (pH 7.0). In each test tube, 20 μL of 0.05 mol/L phosphate buffer, 1 mol/L choline chloride, 3 mmol/L sodium chloride, 1.1 mmol/L EDTA, 6.5 mmol/L dithioerythritol, 0.76 mmol/L neostigmine bromide, and distilled water were added along with 40 μL of 6.2 mmol/L acetyl-coenzyme A and 0.1 mol/L creatine. The mixture was preincubated at 37°C for 5 minutes, and 200 μL of tissue homogenate was added. For the blank, 200 μL of boiled homogenate was added. The mixture was incubated at 37°C for 20 minutes and boiled at 100°C for 2 minutes; 800 μL of 2.5 mmol/L sodium arsenite was then added. The test tubes were centrifuged for 5 minutes, and 1 mL of suspension was collected. 4-Dithiopyrimidine (10 μL, 1 mmol/L) was added, and the tubes were allowed to stand for 15 minutes before being read spectrophotometrically at 342 nm. One unit of ChAT activity was defined as 1 nmol of coenzyme A˘SH formed/mg of protein/hour.

AChE activity ($n = 7$ per group) was determined by the method of Ellman *et al*.[15] with some modification. In a test tube, 2.6 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0), 0.4 mL of homogenate, and 100 μL of dithiobisnitrobenzoic acid were added. The test tube was mixed, and

Table 1. Soy Phytoestrogen Content of Extracts from Soy Hypocotyls

	Content (mg/100 g of extracts)		
Residue	*Genistein*	*Daidzein*	*Glycitein*
Aglycone	9.83 ± 0.06	32.14 ± 0.29	23.11 ± 0.15
Glucoside	276.54 ± 3.07	741.52 ± 0.11	706.50 ± 3.68
Malonyl	459.09 ± 1.65	1,516.2 ± 14.6	1,176.8 ± 8.27
Acetyl	14.64 ± 0.53	40.21 ± 0.10	21.64 ± 0.08

Data are mean ± SD values.

20 μL of the substrate, acetylcholine iodide, was added. The initial absorbance and the absorbance after 4 minutes were read at 412 nm. One unit of AChE activity was defined as 1 μmol of substrate hydrolyzed/mg of protein/minute. Protein concentration was determined by the method of Bradford.[16]

Immunohistochemistry for ChAT

The animals ($n = 7$ per group) were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 *M* phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The brains were removed and postfixed in the same fixative for 6 hours. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing phosphate-buffered saline.

The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in phosphate-buffered saline for 30 minutes and 10% normal goat serum in 0.05 *M* phosphate-buffered saline for 30 minutes. They were next incubated with diluted rabbit anti-ChAT antibody (1:1,000) (Chemicon, Temecula, CA) overnight at 4°C and subsequently exposed to biotinylated goat anti-rabbit immunoglobulin G and streptavidin–peroxidase complex (diluted 1:200) (Vector, Burlingame, CA). Then, the sections were visualized with 3,3′-diaminobenzidine in 0.1 *M* Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. In order to establish the specificity of primary antibody, the procedure included the omission of the primary antibody (goat anti-rabbit) and substitution of normal goat serum for the primary antibody. A negative control test was carried out using preimmune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

Quantitative analysis

Images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 μm (×40 primary magnification). The resolution of each pixel was of 256 gray levels. The intensity of all ChAT-immunoreactive structures was evaluated by means of a relative optical density (ROD) value, which was obtained after transformation of mean gray values using the formula ROD = log (256/mean gray value). The ROD of the complete field was measured, and the values of background staining were subtracted from the ROD values of all immunoreactive structures before statistically processing. ROD values are presented as ROD units. Also, the results of the western blot study were scanned, and an ROD value was obtained using Scion Image software (Scion Corp., Frederick, MD).

Statistical analysis

The effect of the dietary treatment was assessed by analysis of variance using SPSS version 10.0 (SPSS, Chicago, IL). Significant differences between groups were determined

Table 2. Food Intake and Relative Uterine Weight in Rats Fed Experimental Diets

Experimental group	*Food intake (g/day)*	*Uterine weight (g/100 g of body weight)*
CD	16.97 ± 1.88	0.034 ± 0.011[a]
E2	17.43 ± 3.13	0.125 ± 00.45[b]
SP1	18.07 ± 2.11	0.041 ± 0.003[a]
SP2	18.0 ± 1.57	0.048 ± 0.013[a]

Data are mean ± SEM values ($n = 14$ per group) for the CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens) groups.

Values in the same column not sharing a common superscript letter are significantly different ($P < .05$).

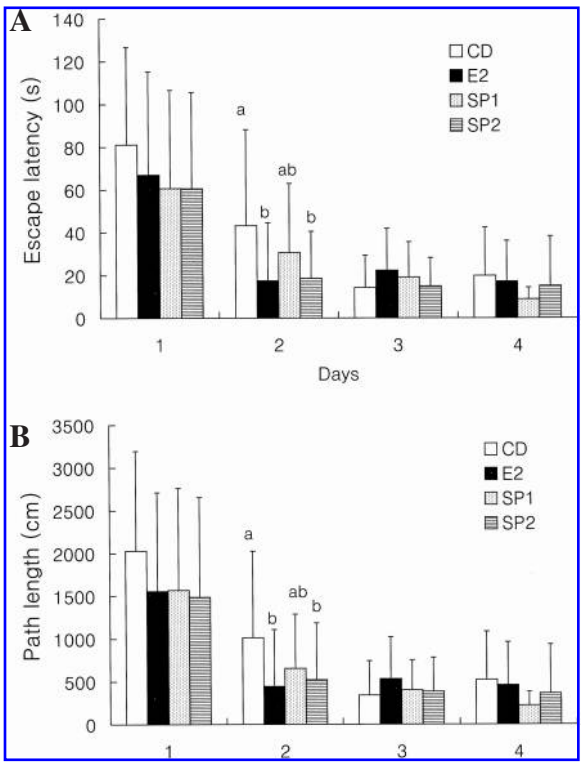

FIG. 1. Effects of the experimental diets on the performance of OVX rats in the MWM: CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens). (**A**) Escape latency was the swimming time to reach the platform. (**B**) Path length was the swimming distance to arrive at platform. Data are mean ± SD values ($n = 14$ per group). Bars for a day without a common superscript letter are significantly different ($P < .05$).

by Duncan's multiple range test at a significance level of $P < .05$.

RESULTS

Soy phytoestrogens and daily intake

The soy phytoestrogen contents of extracts from soy hypocotyls were analyzed by HPLC (Table 1). The glucoside and malonyl forms were dominant in the extract. The experimental diets of the SP1 and SP2 groups contained phytoestrogens at about 263.4 and 526.9 mg/kg of diet, respectively. The daily isoflavone intake per rat in the SP1 group was 4.76 mg of soy phytoestrogens, and that in the SP2 group was 9.48 mg.

Body weight, relative uterine weight, and food intake

All the groups started and ended the study with similar mean body weights (overall mean body weight, 409.6 g), and food intakes were not different (Table 2). However, relative uterine weight was significantly higher in the E2 group (Table 2). This preventive effect of estradiol on uterine atrophy induced by ovariectomy was not shown in rats fed soy phytoestrogens.

Spatial reference memory

Spatial reference memory was measured by escape latency and path length to platform. In each day of trials, escape latency was significantly less in the E2 and SP2 groups than in the CD group at the second day (Fig. 1A). However, there was no significant difference among groups at the third and fourth day. Meanwhile, the E2 and SP2 groups showed significantly shorter path length than the CD group at the second day (Fig. 1B). On the other days of the trials, there was no difference among groups in path length. In addition, the SP1 group was not significantly different from the CD group in escape latency and path length during the test.

Activities of cholinergic enzymes

In the cerebral cortex, ChAT activity was significantly greater in the E2, SP1, and SP2 groups than in the CD group (Fig. 2). The E2 group also had significantly higher ChAT activity in the hippocampus than the CD group, but the rats fed soy phytoestrogens did not. AChE activity in the cerebral cortex was not significantly different among the groups (Fig. 3). However, AChE activity in the hippocampus of the E2, SP1, and SP2 groups was significantly lower than in the CD group.

ChAT immunoreactivity in the diagonal band of Broca

In the CD group, ChAT immunoreactivity was detected in the diagonal band of Broca (Fig. 4). In the E2 group, ChAT immunoreactivity as shown by ROD was significantly higher in the diagonal band of Broca (Fig. 5). In the SP1 and SP2 groups, ChAT immunoreactivity was also

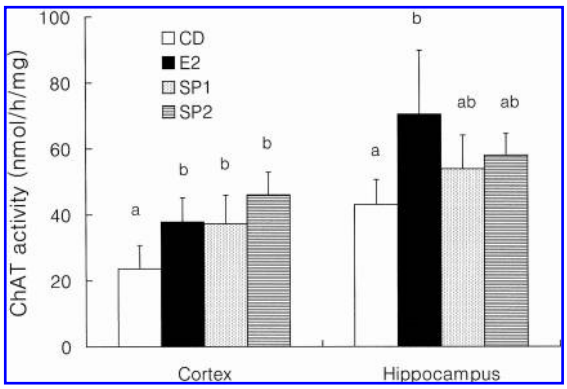

FIG. 2. ChAT enzyme activity in the cerebral cortex and hippocampus of OVX rats: CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens). Data are mean ± SD values ($n = 7$ per group). Bars for a brain section without a common superscript letter are significantly different ($P < .05$).

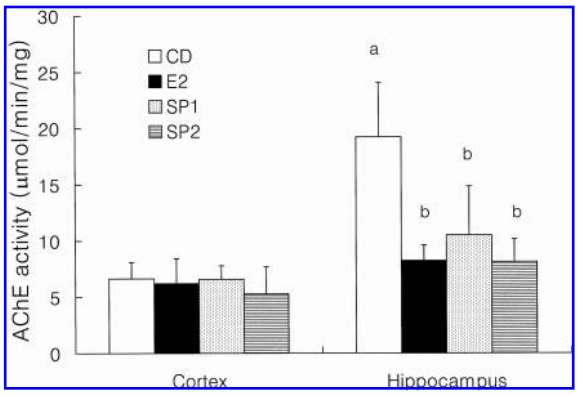

FIG. 3. AChE enzyme activity in the cerebral cortex and hippocampus of OVX rats: CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens). Data are mean ± SD values ($n = 7$ per group). Bars for a brain section without a common superscript letter are significantly different ($P < .05$).

higher in the diagonal band of Broca. In particular, in the SP2 group, ChAT immunoreactivity was about threefold higher than that in the CD group.

DISCUSSION

The present study showed that chronic administration of soy phytoestrogens affected the reference memory, as well as cholinergic enzyme activities in the cerebral cortex and hippocampus in OVX rats. Soy phytoestrogens have an estrogenic effect. It was reported that soy phytoestrogens might improve learning and memory by mimicking the effects of estrogen in the brain.[17] Several studies have shown that soy phytoestrogens improve radial arm maze performance in female rats. Lund *et al*.[18] reported that female rats consuming a lifelong high-phytoestrogen diet showed acquisition of the radial arm maze test faster than female rats consuming a phytoestrogen-free diet. In addition, OVX rats consuming soy phytoestrogen-containing diets for 10 months showed a dose-dependent improvement in their performance in radial arm maze tests.[11] There are some human studies in postmenopausal women suggesting that soy isoflavone supplements improve cognitive functions.[19–23] However, File *et al*.[21] reported that the cognitive improvement was limited to functions in the area of the frontal lobe.

The present study used MWM to evaluate the effect of soy phytoestrogens on spatial memory. All the groups showed normal learning and acquisition behaviors. The escape latency and path length of all the rats were gradually decreased during the test period, and the values among the groups were not significantly different except on the second day. On the second day, the E2 and SP2 groups had significantly lower values than the CD group in escape latency and path length. Although there have been few studies on the effects of soy phytoestrogens on the performance of OVX rats in the MWM task, Markham *et al*.[5] found that

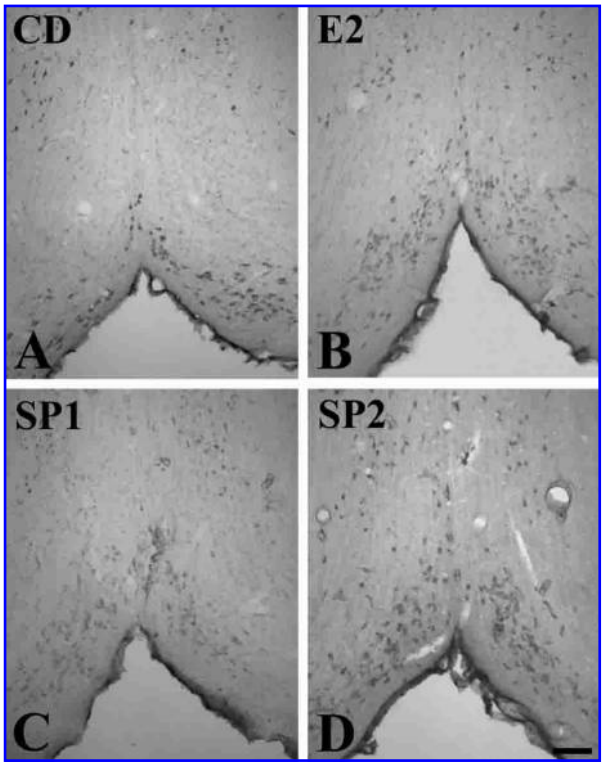

FIG. 4. Immunohistochemical staining for ChAT enzyme in the diagonal band of Broca of OVX rats: CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens). Scale bar = 100 μm.

estrogen-treated OVX rats exhibited improved acquisition of the MWM task. In that study, no treatment groups showed poor acquisition behavior in trial block 3 of the second day, and there were no significant differences in estrogen- and/or progesterone-treated groups. The present study showed similar results. The E2 and SP2 groups had

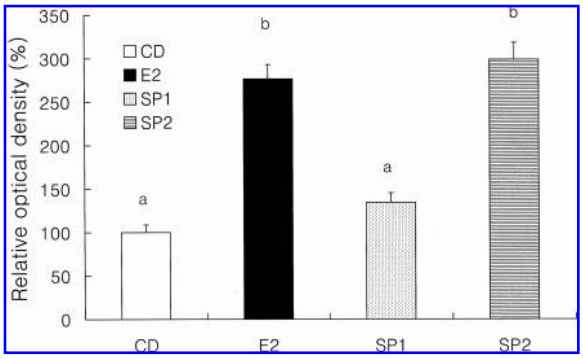

FIG. 5. ROD as percentage values of ChAT immunoreactivity in the diagonal band of Broca of OVX rats: CD, E2 (3.9 mg/kg 17β-estradiol), SP1 (263.4 mg/kg soy phytoestrogens), and SP2 (526.9 mg/kg soy phytoestrogens). Data are mean ± SD values ($n = 7$ per group). Bars having unlike superscript letters are significantly different ($P < .05$).

significantly different values than the CD group in escape latency and path length. However, the SP1 group showed no difference from the CD group. The daily phytoestrogen intake per rat in the SP1 group was 4.76 mg, and that in the SP2 group was 9.48 mg. The different MWM outcomes between the two groups may be due to the different intakes of soy phytoestrogens in OVX rats. That is, the phytoestrogen content of the SP1 diet was too low to benefit the acquisition of the MWM task.

The neuronal cholinergic system is involved in various aspects of cognition and memory,[24,25] and estrogen may play an important role in cholinergic neurons and may improve cognitive function.[26] The present study evaluated neuronal cholinergic enzyme activities in OVX rats fed soy phytoestrogens. The E2 group had significantly higher ChAT activity than the CD group in the cerebral cortex and hippocampus. The soy phytoestrogen-fed rats showed significantly higher ChAT activity than the CD group only in the cerebral cortex. Pan *et al*.[17] reported that ChAT mRNA levels were significantly higher only in the cerebral cortex of OVX rats fed phytoestrogens compared to rats with no treatment. However, the result of AChE activity in the brain was reversed. The AChE activity was not different among groups in the cerebral cortex, but AChE activity in the hippocampus of the E2, SP1, and SP2 groups was significantly lower than that in the CD group. These results show that soy phytoestrogens can affect cognitive function by modulating ChAT activity in the cerebral cortex and AChE activity in the hippocampus.

In brief, the present study suggests that soy phytoestrogens prevent the degeneration of the neuronal cholinergic system and can ameliorate deficits in memory tasks resulting from estrogen deprivation in OVX rats.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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