Oral administration of squid lecithin-transphosphatidylated phosphatidylserine improves memory impairment in aged rats

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A B S T R A C T

Recently, lecithin-derived phosphatidylserine (PS), which originates from marine life, has received much attention as a viable alternative to bovine cerebral cortex PS. In this study, the use of squid phosphatidylcholine-transphosphatidylated PS (SQ-PS) was evaluated through examination of its ameliorating effects on age-associated learning and memory deficits in rats. Aged rats were orally administered SQ-PS (10, 20, or 50 mg/kg per day) once a day for seven days and then subjected to the Morris water maze test. SQ-PS administration produced significant dose-dependent improvements in escape latency for finding the platform in the Morris water maze in the aged rats even though Soy-PS administration also exhibited comparable improvements with SQ-PS. Biochemical alterations in the hippocampal cholinergic system, including changes in choline acetyltransferase and acetylcholinesterase immunoreactivity, were consistent with the behavioral results. In addition, SQ-PS treatment significantly restored age-associated decreases of choline transporter and muscarinic acetylcholine receptor type 1 mRNA expression in the hippocampus. These results demonstrate that orally administered SQ-PS dose-dependently aids in the improvement of memory deficits that occur during normal aging in rats. This suggests that SQ-PS may be a useful therapeutic agent in the treatment of diminished memory function in elderly people.

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1. Introduction

Lecithin-derived phosphatidylserine (PS) is a constituent of membrane phospholipids that are especially abundant in the brain. It elicits a variety of neurochemical activities including the release and turnover of neurotransmitters such as acetylcholine, norepinephrine, and dopamine (Casamenti et al., 1979). PS also results in increased levels of glucose and cAMP in the CNS (Bruni and Toffano, 1982), particularly in amnesic patients (Pepeu et al., 1986).

A number of clinical studies report that the oral administration of bovine cerebral cortex PS (BC-PS) improves the cognitive function of elderly people suffering from age-associated memory impairments, Alzheimer’s disease, and senile dementia with few side effects (Engel et al., 1992). However, the use of BC-PS as medicine or a dietary supplement is now discouraged due to the risk of contamination by the prion that is likely to cause bovine spongiform encephalopathies (BSE) such as mad cow disease and Creutzfeld-Jakob disease (Prusiner, 1991). In addition, only about 3 g of PS can be obtained from one bovine cortex which is too small an amount to adequately maintain a PS supply for the market at the best price (Kato-Kataoka et al., 2010).

To solve these problems, soybean-derived PS (Soy-PS), which is a BSE-risk-free PS, was enzymatically produced from soybean phosphatidylcholine (PC) by phospholipase D-catalyzed transphosphatidylation of soybean PC (Jorissen et al., 2002). Some studies have demonstrated that the oral administration of Soy-PS to aged rats results in a significant improvement of memory and other cognitive functions (Jorissen et al., 2010; Suzuki et al., 2001). However, compared with BC-PS which contains approximately 10% DHA (Chen et al., 1989), Soy-PS, which does...
not contain DHA species, provides little benefit with regard to age-associated memory impairment (Schreiber et al., 2000). Because of some clinical reports indicating a little insufficient activity of soybean PS in improving memory and other cognitive functions in elderly human, much researcher is still investigating to find a new alternative of BS-PS (Schreiber et al., 2000). Therefore, in recent years, a mixture of squid skin PC-derived PS has received attention as a potential alternative to BC-PS since it has substantial amounts of docosahexaenoic (DHA) and eicosapentaenoic acid (EPA; Hosokawa et al., 2000; Lee et al., 2010), active components in the beneficial effects of PS. Furthermore, many reports show that DHA plays an important role in brain function and neurodevelopment (Kim et al., 2011; Mills et al., 2011). Therefore, it may be postulated that phospholipids sourced from animals improve learning and memory ability to a greater extent than those sourced from soybeans, which is probably due to DHA action at the sn-2 position (Sommer Hartvigsen et al., 2004). DHA is released as a result of pancreatic phospholipase action in the body and seems to exert a synergistic effect with the residual PS moiety on the improvement of brain function (Yakhapova et al., 2010).

However, the direct action of orally administered marine-derived PS in the CNS is still in question and its mechanisms remain poorly understood (Drago et al., 1981; Lee et al., 2010). The current study investigates the effect of squid derived PS (SQ-PS) on age-related spatial memory in aged rats in the Morris water maze (MWM) by means of assessing improvement of SQ-PS as a nutritional supplement for age-associated memory impairment. To evaluate the relationship between orally administered SQ-PS and cholinergic function, we examined the neuroprotective effects of SQ-PS on the central acetylcholine system by assessing ChAT and AChE immunohistochemistry of hippocampal neurons as same methods described in our previous study (Lee et al., 2010). The effect of a Soy-PS treatment was also analyzed on the same parameters in young and aged rats in order to compare their effects with those obtained with the SQ-PS treatment.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley (SD) rats weighing 260–280 g (six weeks old) and 500–530 g (15 months old) were obtained from Charles River Laboratories (Yokohama, Japan). The rats were housed in a limited-access rodent facility with up to five rats per polycarbonate cage. The room controls were set to maintain temperature at 22 ± 2 °C with relative humidity at 55 ± 15%. Cages were lit using artificial light for 12 h each day. Sterilized drinking water and standard chow diet were daily supplied. In the case of the AG-SQ-PS50 (50 mg PS/kg body weight) group, orally administered amount of squid PS is 10 mg per day per rat weighing 200 g. Because 40.7 g DHA and 13.5 g EPA are included in 100 g squid PS (Table 2), 4.07 mg DHA and 1.35 mg EPA are daily administered to each rat weighing 200 g. In summary, less than 0.024 g DHA and 0.21 g EPA might be ingested in the standard diet group as a control, respectively, and 4.07 mg DHA and 1.35 mg EPA were orally administered to the rats in the SQ-PS diet groups. The PS was homogenously suspended in saline and orally administered to the subjects once a day for seven consecutive days. MWM testing was performed 30 min subsequent to every PS treatment on each of the seven days.

2.2. Experimental groups

The rats were randomly divided into six groups as follows: 1) saline-treated normal adult group (young group; six week old subjects, n = 8); 2) saline-treated aged group (Aged group; sham control; 15 month old subjects, n = 10); 3) 10 mg/kg SQ-PS-treated aged group (AG-SQ-PS10, n = 7); 4) 20 mg/kg SQ-PS-treated aged group (AG-SQ-PS20, n = 7); and 5) 50 mg/kg SQ-PS-treated aged group (AG-SQ-PS50, n = 7) and 6) 50 mg/kg Soy-PS-treated aged group (AG-Soy-PS50, n = 7). The daily doses were determined based on previous studies by Lee et al. (2010). The SQ-PS and Soy-PS used in this study were manufactured and kindly provided by Doosan Co. Glonet BU (Youngin-si, Korea). The applied SQ-PS formula contains 92% PS, 1% PC, and 5% phosphatidic acid (PA). In the case of Soy-PS, it contained 90% PS, 2% PC, and 6% PA.

Fatty acid compositions of the standard chow diets for the rats and phospholipids such as SQ-PS and Soy-PS are shown in Table 1 and 2, respectively. Because total fat content of the standard chow diet is 5% per 100 g diet, and DHA and EPA contents are 0.48% and 0.41% of total fatty acids in chow diet (Table 1), 0.024 g DHA and 0.021 g EPA in 100 g diet are daily supplied. In the case of the AG-SQ-PS50 (50 mg PS/kg body weight) group, orally administered amount of squid PS is 10 mg per day per rat weighing 200 g. Because 40.7 g DHA and 13.5 g EPA are included in 100 g squid PS (Table 2), 4.07 mg DHA and 1.35 mg EPA are daily administered to each rat weighing 200 g. In summary, less than 0.024 g DHA and 0.21 g EPA might be ingested in the standard diet group as a control, respectively, and 4.07 mg DHA and 1.35 mg EPA were orally administered to the rats in the SQ-PS diet groups. The PS was homogenously suspended in saline and orally administered to the subjects once a day for seven consecutive days. MWM testing was performed 30 min subsequent to every PS treatment on each of the seven days.

2.3. Morris water maze test

2.3.1. Morris water maze apparatus

The MWM test was performed using a polypropylene circular pool (painted white internally, 2.0 m in diameter and 0.35 m high). The pool contained water, which was maintained at a temperature of 22 ± 2 °C. During MWM testing, a platform 15 cm in diameter was located 1.5 cm below the water in one of four sections of the pool and was approximately 50 cm from the sidewalls. The pool was divided into four quadrants of equal area and was surrounded by several external cues. A digital camera mounted to the ceiling above the pool was connected to a computerized recording system equipped with a tracking program (S-MART: PanLab Co., Barcelona, Spain) which permitted on- and off-line automated tracking of the paths taken by the rat.

2.3.2. Hidden platform trial for acquisition test

The animals received three trials per day. The rats were trained to find the hidden platform, which remained in a fixed location throughout the test. The trials lasted for a maximum of 180 s, with the time it took to find the submerged platform recorded each time. The animals were tested in this way three trials per day for six days, and received a 60 s-probe trial on the seventh day. Finding the platform was defined as staying on it for at least 4 s before the acquisition time of 180 s ended. Because of rats that crossed the platform without stopping (jumping immediately into the water) were left to swim. After staying on the platform for 10 s, the rats were gently picked up using a steel spatula, returned to its home cage, and allowed to warm up and dry off under a 125-W heat lamp. If the rat failed to find the platform in the allotted time it was placed onto the platform for 20 s and assigned a latency of 180 s. The water was stirred in between each trial to remove olfactory traces of previous swim patterns. The entire procedure took place over seven consecutive days and each animal had three training trials per day with a 30–40 min interval.

| Table 1 |
| Fatty acid composition of lipids in standard chow diet used in this study. |
|---|---|
| Fatty acid | Content (%) |
| Palmitic acid (C16:0) | 20.51 |
| Stearic acid (C18:0) | 8.37 |
| Oleic acid (C18:1) | 32.04 |
| Linoleic acid (C18:2) | 28.76 |
| Linolenic acid (C18:3) | 2.45 |
| EPA (C20:5) | 0.41 |
| DHA (C22:6) | 0.48 |
| Other fatty acids | 6.98 |
| SDM | 100 |

The values were expressed in % of total fatty acid in chow diet.
the experimental groups. Hippocampal area cells were obtained according to the stereotactic atlas of Paxinos and Watson (1986). The cells were counted in three sections per rat within the hippocampal area.

2.6. Acetylcholinesterase (AchE) immunohistochemistry

For AchE immunohistochemistry, the sections were washed in PBS and incubated in a solution with 25 mg of acetylthiocholine iodide for 1 h. The solution was composed of 32.5 ml of 0.1 M sodium hydrogen phosphate buffer (NaH₂PO₄·H₂O, pH 6.0), 2.5 ml of 0.1 M sodium citrate, 5 ml of 30 mM copper sulfate, 5 ml of 5 mM potassium ferricyanide, and 5 ml of distilled water. The color of the mixing solution was green. The densities of stained nuclei of the hippocampal cells were measured using a Scion image program (Scion Co., Frederick, MD, USA). The sections were viewed at 200× magnification and the number of cells within 100 × 100 mm² grids was counted by observers blind to the experimental groups. Hippocampal area cells were obtained according to the stereotactic atlas of Paxinos and Watson (1986). The cells were counted in three sections per rat within the hippocampal area.

2.7. Total RNA preparation and RT-PCR analysis

For RT-PCR analysis, the hippocampus from each of four rats in each group were just randomly selected after behavioral test and isolated. After decapitation, the brain was quickly removed and stored at −80 °C until use. The total RNA was isolated from the brain sample using a TRIzol® reagent (Invitrogen Co., Carlsbad, CA, USA) and RNA was extracted according to the supplier's instruction. Complementary DNA was synthesized from total RNA with a reverse transcriptase (Takara Co., Shiga, Japan). Choline transporter (CHT) and muscarinic acetylcholine receptor type 1 (mACHR-M1) mRNA expression levels were determined by the reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA). All primers were designed using published mRNA sequences and primer design software (Primer 3; The Whitehead Institute for Biomedical Research, Cambridge, MA, USA; www.genome.wi.mit.edu) offered through their website. The following sequences were used: for GAPDH (409 bp), (forward) 5′-ATC CCA TCA CCA CCT TCT AG-3′ and (reverse) 5′-CCT GCT TCA CCA CCT TCT TG-3′; for CHT1 (90 bp), (forward) 5′-CTG ACC GCC TTC CCA TAG AT-3′ and (reverse) 5′-AGG TGG CCT GTT TGC AA-3′; for mACHR-M1 (315 bp), (forward) 5′-CCT CCC AAA AGC TCC CCA-3′ and (reverse) 5′-TGT CCC GGA AGG CCT TGC-3′. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide after which the density of each band was evaluated using an image-analyzing system (I-Max™, CoreBio System Co., Seoul, Korea). Complementary DNA expression levels were determined by calculating the relative density of each CHT1 or mACHR-M1 band to GAPDH.

2.8. Sample preparation and measurement of fatty acid profile

After behavioral tests for 1 week, the rat were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), the hippocampus was separated as previously described (Hashimoto et al., 2002). The tissues were stored at −80 °C by flash-freezing in liquid N₂ until use. Total lipid was

<table>
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Table 2

Fatty acid composition of phosphatidylserine originated from squid (SQ-PS) and soybean (Soy-PS).

**Notes:**
- EPA: Eicosapentaenoic acid.
- DHA: Docosahexaenoic acid. The values were expressed in g/100 g SQ-PS or Soy-PS.
extracted from hippocampus with mixture solvent (chloroform, methanol, Distilled water, 65/35/4, v/v/v) according to previously described (Folch et al., 1957), and fatty acid composition was analyzed by gas–liquid chromatography (Ohkubo and Tanaka, 2010). The esters prepared by transmethylization with BF3/methanol. An Agilent 7890A series gas chromatograph equipped with flame ionization detector (FID) and DB-225 capillary column (30 μm × 0.25 mm i.d.) (Agilent & W Scientific, CA, USA) was used. Column temperature was raised from 175 to 220 °C at 2.5 °C/min. Injector and detector temperatures were 250 °C and 260 °C, respectively. Carrier gas was helium, and hydrogen and air were supplied to the FID. Fatty acids were identified by comparing retention times with the lipid standard (Sigma-Aldrich). The same protocol as above was used for analyzing fatty acid composition of the rat diet.

2.9. Statistical analyses

The experimental results are expressed as the mean ± standard error (SE). The behavioral data were calculated and analyzed by repeated measures analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS Inc., Chicago, IL, USA). The statistical significance among groups was further analyzed using Tukey’s post-hoc test. Immuno-histochemical data and PCR analysis were also analyzed by a one-way ANOVA followed by Tukey’s post-hoc test. Statistical significance was set at p < 0.05.

3. Results

3.1. Effect of SQ-PS during the visible platform trial of Morris water maze test

To exclude the possibility of impairing the vision of the subjects and altering the motivation to escape the water due to SQ-PS administration, a cued version of the MWM test was performed and the swimming time to reach the visible platform was measured (Fig. 1C). When trained to a visible platform there were no significant differences between groups [F(5,44) = 0.291; p = 0.881]. The latency to find the visible platform did not differ among groups on the first, second, or third trials (Fig. 1C). On the second and third trials, the latency was markedly reduced in all groups, as compared to the first trial. All rats, irrespective of grouping, were able to locate the visible platform more rapidly as the trials progressed.

3.2. Effect of SQ-PS during the hidden platform trial of the Morris water maze test

Rats in the Young group rapidly learned the location of the submerged hidden platform and reached it within 20 s on Day 6 of the trials (Fig. 1C). The Aged group showed a marked retardation in escape latency, probably due to memory deficits resulting from age-induced impairments of learning and memory. Analysis of the training data by repeated-measures ANOVA showed that escape latency differed significantly among the groups when the times were averaged over all sessions [F(5,40) = 9.216, p < 0.001]. During the experiment, escape latency decreased over time [F(5,200) = 142.076, p < 0.001], and there was a significant interaction between experimental groups and time [F(25,200) = 2.393, p < 0.05]. Tukey’s post-hoc test revealed that rats in both the AG-SQ-PS20 and AG-SQ-PS50 groups had significantly reduced swimming latency compared with subjects in the control group (AG-SQ-PS20 group: p < 0.01 on Days 5 and 6; AG-SQ-PS50 group: p = 0.01 on Days 5 and 6; Fig. 1C). The Aged group was not significantly different from other groups in terms of mean swimming speed, as calculated by dividing the total swim distance by the latency [F(5,44) = 0.860, p = 0.517] (Fig. 1E). Total distance traveled in each group was closely associated with escape latency in this task (data not shown). Based on these results, the SQ-PS-treated aged rats showed greater improvements in acquisition during the hidden-platform trial and reached the platform quicker than the saline-treated aged rats.

3.3. Effect of SQ-PS during the probe trial of the Morris water maze test

To examine the spatial memory of rats, performance in the probe test on Day 7 was analyzed by comparing the percentage of time spent swimming to the expected position of the platform (Fig. 1D). The time spent swimming around was significantly reduced in the rats that swam to the target area where the platform had been located [F(5,44) = 5.213, p = 0.01]. The aged rats displayed severely impaired spatial performance in the MWM (p < 0.01). Rats in the 20 mg/kg (p < 0.05) and 50 mg/kg SQ-PS-treated (p < 0.01) groups spent more time around the platform area than did those in the Aged group. SQ-PS treatment significantly attenuated the age-induced deficit of learning and memory demonstrated in the water maze. Thus, SQ-PS-treated rats showed a significant amelioration in the memory retention test because they spent more time in the quadrant where the platform was formerly located and swam over the former location of the platform more frequently. Our results have shown that swimming latency time in the AG-SQ-PS20 group was similar to that of the AG-Soy-PS50 group.

3.4. Effect of SQ-PS during the open field test

Analysis of performance in the open-field test by a parametric one-way ANOVA showed no significant differences between groups in terms of memory deficit-related locomotor activity [F(2,24) = 0.870, p = 0.439] or the total number of line crossings [F(2,24) = 1.857, p = 0.190] (Fig. 2). This indicates that SQ-PS administration did not affect psychomotor function as measured by performance in the MWM test.

3.5. Effect of SQ-PS on hippocampal choline acetyltransferase

Following the behavioral tasks, brain tissue samples from the subjects were analyzed using immunohistochemistry to investigate the effect of SQ-PS administration on neuronal cell loss due to aging. ChAT immunoreactivity analysis in the CA1 and CA3 areas of the hippocampus are shown in Fig. 3. Comparison of the numbers of ChAT-immunoreactive neurons using a one-way ANOVA revealed a significant difference among the groups [F(5,71) = 3.957, p < 0.01; CA1 and F(5,71) = 4.104, p < 0.01; CA3]. The brains of the Aged group showed significant neuronal cell loss in the CA1 area of the hippocampus, as compared to the Young group (p < 0.01). The number of ChAT-immunoreactive neurons significantly increased in hippocampal region CA1 in the AG-SQ-PS20 (p < 0.05) and the AG-SQ-PS50 groups (p < 0.01), as compared to the Aged group (Fig. 4). The number of ChAT-immunoreactive neurons significantly increased in hippocampal area CA3 in the AG-SQ-PS20 (p < 0.05) and the AG-SQ-PS50 groups (p < 0.05), as compared to the Aged group (Fig. 4). Losses of ChAT-immunoreactivity in aged rats were significantly restored by the SQ-PS administration, and the number of ChAT-immunonegative neurons was closely similar to that of the Young group. Our results have shown that ChAT activity in the hippocampus of the AG-SQ-PS20 group was similar to that of AG-Soy-PS50 group.

3.6. Effect of SQ-PS on hippocampal acetylcholinesterase

The density of AChE-immunopositive fibers in the CA1 and CA3 area of the rat hippocampus was significantly reduced in the Aged group, as compared to the Young group (Fig. 3). Comparison of the numbers of AChE-positive neurons density using a one-way ANOVA revealed a significant difference among the groups [(5,71) = 6.019, p < 0.001; CA1 and F(5,71) = 2.721, p < 0.05; CA3]. The AChE-reactive neuronal loss in hippocampus area CA1 due to aging was significantly restored in the AG-SQ-PS20 (p < 0.05) and the AG-SQ-PS50 groups (p < 0.01), as compared to the Aged group (Fig. 5). The density of AChE-reactive neurons in the AG-SQ-PS20 group and the AG-SQ-PS50 group was closely
Fig. 1. Schematic drawing of the MWM pool (A), the experimental schedule (B), the time to escape (latency) during acquisition trials of visible and hidden platform (C), time spent around the platform in post-training probe test (D), and swim speed (E) during the MWM test. In Fig. 1A, a small circle in each quadrant of the pool indicates the fixed location of visible platform and a closed circle indicates the hidden platform location in the acquisition trial. The rats were randomly divided into six groups as follows: 1) saline-treated normal adult group (Young group; six week old subjects, n = 8); 2) saline-treated aged group (Aged group; sham control; 15 month old subjects, n = 10); 3) 10 mg/kg SQ-PS-treated aged group (AG-SQ-PS10, n = 7); 4) 20 mg/kg SQ-PS-treated aged group (AG-SQ-PS20, n = 7); 5) 50 mg/kg SQ-PS-treated aged group (AG-SQ-PS50, n = 7) and 6) 50 mg/kg Soy-PS-treated aged group (AG-Soy-PS50, n = 7). Data were analyzed using repeated measures ANOVA followed by Tukey’s post-hoc test. **p < 0.01 and ***p < 0.001 vs. the Young group; #p < 0.05 and ##p < 0.01 vs. the Aged group. Vertical bars indicate SE.
similar to that of the Young group. The AchE-reactive neuronal loss in hippocampus area CA3 due to aging was significantly restored in the AG-SQ-PS50 group ($p < 0.05$), as compared to the Aged group (Fig. 5).

The densities of AchE-reactive neurons in the AG-SQ-PS50 group were closely similar to that of the Young group. The effect of SQ-PS on the density of AchE reactive neurons in hippocampus area CA3 was similar to that in the CA1 region. Our results have showed that the density of AchE-reactive neurons in the hippocampus of the AG-SQ-PS20 groups were similar to that of the AG-Soy-PS50 group.

3.7. Effect of SQ-PS on choline transporter and muscarinic acetylcholine receptor mRNA expression levels in the hippocampus

The effect of SQ-PS administration on ChT and mAChR-M1 mRNA expression levels in the aged rat hippocampus was investigated using RT-PCR analysis (Fig. 6). The ChT and mAChR-M1 mRNA expression levels were normalized using GAPDH mRNA as an internal control. Hippocampal ChT mRNA expression in the Aged group was significantly decreased, as compared to that of the Young group ($p < 0.01$). The
reduced expression of CHT mRNA in the Aged group was significantly restored in the AG-SQ-PS10 group \((p < 0.05)\), the AG-SQ-PS20 group \((p < 0.05)\), and the AG-SQ-PS50 group \((p < 0.05)\). The restored levels were similar to that of normal rats in the Young group. Hippocampal mACHR-M1 mRNA expression in the Aged group was also significantly decreased, as compared to that of the Young group \((p < 0.001)\). The reduced expression of mACHR-M1 mRNA in the Aged group was significantly restored in the AG-SQ-PS10 group \((p < 0.05)\), the AG-SQ-PS20 group \((p < 0.01)\), and the AG-SQ-PS50 group \((p < 0.01)\). The restored levels were similar to that of normal rats in the Young group. There was a strong statistically significant \((p < 0.05)\) relationship between CHT and mACHR-M1 mRNA expressions.

3.8. Effect of SQ-PS on fatty acid composition in the hippocampus

In the brain hippocampus of aged rats, concentration of docosahexanoic acid (DHA), an \((n-3)\) PUFA (polyunsaturated fatty acid), was remarkably reduced as compared to those in the Young group. Docosahexanoic acid (DHA), an \((n-3)\) PUFA (polyunsaturated fatty acids), was notably reduced in the Aged group. The administration of 20 mg/kg SQ-PS was remarkably improved the concentration of DHA and AA, representative PUFAs (polyunsaturated fatty acids), in the brain hippocampus. And the administration of SQ-PS also slightly enhanced AA concentration of the hippocampus in the aged rats although the difference is not statistically significant. In case of AA, there were little changes among the Young, Aged, and Soy-PS-treated groups. However, it was noticeable that the administration of 20 mg/kg SQ-PS was remarkably improved the concentration of DHA and AA, representative PUFAs (polyunsaturated fatty acids), in the brain hippocampus.

Fig. 5. The percentage \((\pm SE)\) values of the density of AchE stained nuclei in different hippocampal areas after the MWM. For immunohistochemical analysis, rats were just randomly selected into six experimental groups \((n = 3-7)\) and the tissue were counted in three sections per rat within the hippocampus area. Immunohistochemical data were analyzed via a separate one-way ANOVA followed by Tukey’s post-hoc test. \(*p < 0.05\) and \(**p < 0.01\) vs. the Young group; \#p < 0.05 and ##p < 0.01 vs. the Aged group. Vertical bars indicate SE.

4. Discussion

In the present study, a pilot experiment comparing SQ-PS to Soy-PS activity was performed to evaluate the predicted superiority of squid-derived PS as a nutritional supplement and to determine the optimal amount of SQ-PS intake for age-associated memory impairment in aged animals. Of the SQ-PS doses used in this study, it was found that 50 mg/kg resulted in the most significant memory improvement, which corresponds to previous studies \((Lee et al., 2010)\). Here, PS was prepared from squid skin lecithin, which contains abundant DHA, using enzymatic transphosphatidylidation. The DHA content in SQ-PS is 43% higher than that of BC-PS \((Chen and Li, 2008)\). A previous report demonstrated that SQ-PC and SQ-PS can easily pass across the Caco-2 cell barrier, where Soy-derived phospholipids may be blocked \((Hossain et al., 2006)\). Accordingly, oral administration of SQ-PC and its transphosphatidylated PS have considerable potential to improve memory and cognitive function by directly affecting cholinergic activity in the brain.

The MWM is a hippocampus-dependent memory task that is frequently used for examining cognitive deficits and demonstrating permanent spatial learning capability and reference memory in rodents \((Jonasson, 2005)\). The current finding that memory deficits in aged rats produce impaired behavioral performance in a MWM task agrees with findings from previous studies \((Lee et al., 2010)\). The scores for the escape test and the spatial probe test are considered to primarily reflect long-term spatial memory ability in the MWM test. Here, SQ-PS treatment shortened the escape latency without affecting swimming velocity and extended the time spent swimming in the place where the platform had previously existed \((Lee et al., 2010)\). This indicates that oral administration of SQ-PS significantly improves long-term spatial memory in aged rats.

An open-field test was also performed to rule out any confounding motor impairments, which may influence outcomes in behavioral tests of depression \((Moretti et al., 2011)\). No significant individual differences in locomotor activities were observed between groups in the open-field test, which suggests that the SQ-PS administration had no effect on motor performance. Accordingly, it was verified that the changes in behavioral performance during the MWM task are most likely due to

Fig. 6. The PCR bands (A) and their relative intensities (B) of CHT and mACHR-M1 mRNA in the hippocampus of the different experimental groups. For RT-PCR analysis, four rats in each group were just randomly selected after behavioral test. Data were analyzed via a separate one-way ANOVA followed by Tukey’s post-hoc test. **p < 0.01 and ***p < 0.001 vs. the Young group; #p < 0.05 and ##p < 0.01 vs. the Aged group. Vertical bars indicate SE.
improved memory function instead of changes in sensorimotor function, such as motor output and limb flexibility.

The hippocampus is a medial temporal lobe structure that has been implicated in the consolidation of declarative memory in humans and spatial memory in rodents (Burger et al., 2007; Miyagawa et al., 1998). Impairments in the hippocampus generally result in diminished spatial learning ability. Thus, most age-related psychosomatic disorders are associated with decreased working memory function that is directly related to alterations of hippocampal function (Casamenti et al., 1991). Here, it is demonstrated that SQ-PS treatment protects aged rats from spatial working memory deficits and attenuates the decrease in AchE and ChAT-immunoreactivity in the hippocampus. It is likely that the observed learning- and memory-related improvements of SQ-PS-treated animals in the MWM are associated with the attenuation of hippocampal cell loss, the increase in central cholinergic function, and the prevention of degeneration in the cholinergic neuronal population of the basal forebrain (Millan et al., 1988).

On the other hand, the current results show that the altered expression levels of CHT and mAChR-M1 mRNA in the hippocampus are associated with the memory deficits due to normal aging. Thus, this might also contribute to reduced changes in cholinergic markers in the hippocampus as well. It was also demonstrated that SQ-PS treatment significantly attenuates decreases in CHT and mAChR-M1 mRNA expression in the hippocampus of aged rats. Several studies have suggested an association between hippocampal CHT and mAChR-M1 expression and memory performance, particularly in water maze tests (Falkenberg et al., 1992; Schaaf et al., 2000). These findings suggest that the memory-improving activity of SQ-PS can be verified by using several behavioral paradigms, and that this type of amelioration is exerted through modulation of cholinergic neurons in the brain.

The expression and activation of AchE and ChAT regulates the dynamic concentration of ACh in cholinergic synapses in the brain. Thus, the hippocampal expression of AchE and ChAT and its correlation with the memory performance of aged rats was examined. Previous biochemical and behavioral evidence indicates that central cholinergic transmission declines with age (Gage et al., 1988) and with dementia of the Alzheimer’s type (Uabundit et al., 2010). This type of decline is closely associated with cognitive disturbances. PS enhances cognitive performance not only in aged rats but also in elderly human subjects showing varied levels of age-dependent or disease-related memory.

Fig. 7. Concentration of fatty acids, such as palmitic (C16:0, A), stearic (C18:0, B), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidonic acid (C20:4n6) and DHA (C22:6n3) in the brain hippocampus of SQ-PS-treated or Soy-PS-treated rats. **p < 0.01 vs. the Young group; ###p < 0.01 vs. the Aged group. Vertical bars indicate SE. The numbers of animals for analyzing each fatty acid composition are 3 as indicated in the bracket in the figure.
impairment (Crook et al., 1991; Kato-Kataoka et al., 2010; Lee et al., 2010). The beneficial effects of PS could be related to the increases in the central cholinergic function (Lee et al., 2000).

High proportions of PUFAs in the brain are crucial for maintaining the structure and physiological function of the central nervous system (Chang et al., 2001). A deficit of dietary DHA is known to contribute to inflammatory signaling, apoptosis and neuronal dysfunction, and is closely associated with a cognitive decline in the elderly people and in those with age-related neurological disorders (Babenko and Semenova, 2010). Also, animal studies using experimental diets lacking (n-3) PUFA demonstrated that those diets elicited a significant reduction of DHA level in cerebral membranes, which means that it is eventually associated with disturbances of neural functions such as visual acuity, attention, learning, and memory (Bourre et al., 1989). Dietary supplementation of DHA resulted in the restoration of age-related declines of cognitive functions by stimulating synthesis of new polyunsaturated phosphatidylserine species in the brain cerebral cortex and hippocampus (Babenko and Semenova, 2010). It was also reported that AA supplementation also ameliorated cognitive dysfunction caused by either organic brain damage or aging not neurodegenerative diseases like Alzheimer’s disease (Kotani et al., 2006). The purpose of this experiment was to determine whether hippocampus FA composition can be altered as a result of the repeated administration of SQ-PS, and whether the changes of FA composition can affect the age-related monoaminergic and cholinergic neurotransmission systems in the brain tissues. Our results clearly showed that the administration of SQ-PS significantly increased the concentration of DHA and AA in the hippocampal regions of the rat brains. Accordingly, it can be suggested that a positive correlation was observed between percentage of time spent swimming to the expected position of the platform in the MWM and the concentration of DHA and AA in the brain. Because exogenous phospholipids may serve as an extra supply for endogenous phospholipid turnover in the cell membranes (Claro et al., 2006), the memory-improving activity of SQ-PS can be attributed to a biochemical role played by PS. PS may elicit the fast and stable turnover of phospholipids in the membranes of brain tissues. In addition, PS is an important activator of several enzymes including protein kinase C, which plays a crucial role in synaptic plasticity and information storage (Orr et al., 1992; Van der Zee and Douma, 1997; Vance, 2003). It has also been suggested that PS influences the regulation of glutamate receptor-mediated signaling (Gnäg et al., 1996). Glutamate receptors are thought to be involved in the regulation of long-lasting potentiation of synaptic transmission believed to underlie information storage in the brain (Bliss and Collingridge, 1993). Accordingly, it is possible that PS directly acts on downstream cascades of intracellular signaling in memory consolidation processes.

In the present study, administration of Soy-PS exhibited a comparable effect of improving memory ability with that of SQ-PS in the aged rats, although SQ-PS has plenty of (n-3) PUFA such as DHA and EPA that are not detected in Soy-PS. It indicates that the contribution of DHA and EPA in SQ-PS to the maintenance of structural and physiological function of the brain was not remarkable in the aged rats. Because high proportion of (n-3) PUFA in the brain membranes is important for maintaining normal metabolism and function of the brain, DHA and EPA in SQ-PS administered may charge a certain role of maintaining brain integrity if those fatty acids are released individually from PS after intake and can be moved to the brain tissues in their intact forms. Also, since the contents of (n-3) PUFA in the brains gradually decreased with aging, those lipids should be supplied to the brain either directly from food supplements such as maternal milk and fish oil or by internal synthesis in small amounts from α-linolenic acid. In this respect, a dietary-induced depletion of (n-3) PUFA is important to investigate the effect of diet including (n-3) PUFA such as DHA and EPA on brain integrity and memory function in animal study. Accordingly, a long-term (n-3) PUFA deficient feeding over two generations was often exploited in animal studies even though severe deficiency of (n-3) PUFA is scarcely caused in human (Aid et al., 2005; Innis and de La Presa, 2001; Kimura et al., 2011). Unfortunately, we did not use (n-3) PUFA-deficient diet for feeding the rats in this study. The standard chow diet used in this study includes small but substantial amounts of (n-3) PUFA as shown in Table 1. It seems that (n-3) PUFA in the chow diet played a certain role of maintaining the basal level of brain function in the aged rats even though DHA contents in the brain hippocampus in the aged rats was noticeably reduced as compared with those in young rats in the present study.

5. Conclusion

The present study demonstrates that memory and cognitive deficits induced by age-induced hippocampal lesions are closely related to the degeneration of cholinergic neurons in the rat hippocampus. Moreover, orally administered SQ-PS significantly ameliorates learning and memory deficits through the recovery of cholinergic activity in the brain. SQ-PS treatment improved performance in the spatial memory test and protected hippocampal cholinergic neurons from age-associated destruction. The attenuation of impairments of memory and cognition by SQ-PS administration may be due to the restoration of cholinergic neurochemical activity. It is likely that SQ-PS is strongly effective in protecting against memory-related neuronal degeneration in the brain and slowing the progression of memory deficits associated with various neurodegenerative diseases.

Acknowledgments

This research was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs (Grant No. A091037) and the National Research Foundation of Korea funded by the Korean government (MEST) (2013R1A1A2008487 and 2013R1A1A2063051), Republic of Korea.

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