Administration of DHA-PS to Aged Mice Was Suitable for Increasing Hippocampal PS and DHA Ratio

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Abstract: Docosahexaenoic acid (DHA) and phosphatidylserine (PS) are major components of the brain and play important roles functionally and structurally. Aging is associated with impairments in biological functions. According to the results of animal tests it has been shown that the loss in brain PC, PS and DHA due to aging leads to a variety of nervous deficits. In the present study, young mice and aged mice were fed a test or control diet for four weeks, and the authors examined the effects of DHA and/or PS administration (control diet group, Soy-PL diet group, Soy-PS diet group, DHA-PL diet group and DHA-PS diet group). At the end of the feeding period, the final ages were 12 (young mice) and 73 (aged mice) weeks. Hippocampal PS ratios and DHA concentrations in aged control mice were found to be lower than those in young control mice. Hippocampal PS ratios and DHA concentration in aged mice were increased with administration of PS and DHA, respectively. Authors found DHA-PS diet could increase both DHA and PS in hippocampus of aged mice.

Key words: docosahexaenoic acid, phosphatidylserine, hippocampus, administration, DHA-PS, aging

1 INTRODUCTION

Phospholipids (PLs) are a major component of the brain. Compared to other organs, the brain has an unusually high lipid content, some two-thirds of which consists of PLs. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) account for about 80% of total brain PLs, while phosphatidylserine (PS) and sphingomyelin (SM) account for 10-20%². PLs play important roles functionally and structurally, for example, maintaining cell membrane structure, and maintaining membrane-binding enzyme and receptor activities²,³. Aging is associated with impairments in a variety of biological functions, particularly conflictive functions. In the aging brain, lipid composition and content changes, and this is thought to reduce of membrane fluidity, loss of enzymatic activity, and decreased efficiency of signal transduction and transport mechanisms. Because PLs such as PC, PE and PS have polar bases, changes in PL composition directly affect cell membrane structure and fluidity. All over the body, PS is a cellular constituent, but there are differences regarding its location and function in brain membranes. Due to its structure and localization in the brain, PS is expected to suppress a decline in brain function; even improve it. Pepeu et al.⁵ reported that PS reverses acetylcholine release in the brains of aged rats. Sakai et al.⁶ reported that PS antagonizes the amnesic effects of scopolamine in rats. Furushiro et al.⁷ reported that PS improves cycloheximide-induced impairment of passive avoidance learning in mice. Clinical investigations have also shown that PS treatment improves age-related cognitive decline and Alzheimer’s disease⁸-¹³. In addition, PS supplementation may have beneficial effects on memory by allowing neurons in neuron networks to continue effectively communicating with one another, allowing existing memories to be retained and new memories to be formed¹⁴. The effects of PS on brain glucose metabolism have been studied using positron emission tomography (PET). It was found that PS increases glucose metabolism in defined cortical and subcortical structures of the brain in Alzheimer’s disease¹⁵. Docosahexaenoic acid (C22:6 n-3; DHA) is the major polyunsaturated fatty acid in neuronal membranes¹⁶,¹⁷. DHA is localized in the cell membrane PLs of the brain,

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Accepted December 18, 2009 (received for review April 27, 2009)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/

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retina and sperm. Numerous reports have documented that DHA plays an important role in the viability, maturity, fertility and function of these cells\(^1\). Animals subjected to a chronic dietary deficiency of DHA are unable to maintain central nervous system activity\(^2\), \(^3\) and suffer from impaired attention and learning ability\(^4\). DHA loss in brain membranes may contribute to cholinergic dysfunction in the hippocampus, as DHA is involved in cholinergic-stimulated signal transduction at the synapses in rat brain\(^5\). Supplementation with DHA increased cerebral choline and acetylcholine levels and improved passive avoidance performance in stroke-prone spontaneously hypertensive rats\(^6\). A DHA-enriched diet enhanced maze-learning ability and brain functions in aged mice\(^7\), \(^8\).

DHA concentration in brain cell membranes decreases with age\(^9\), \(^10\). DHA levels among PLs in the gray matter and hippocampus also decrease in Alzheimer’s disease\(^1\). This suggests that changes in fatty acid composition in the brain affect functional ability and specific memorization. DHA administration increases DHA concentration in the brain and improves brain functions modified by aging or Alzheimer’s disease. Suzuki et al.\(^1\) reported that synaptic membrane fluidity and learning ability in elderly mice decreased by aging recover to a comparable level as in young mice with long-term dietary sardine oil. DHA administration also improved learning ability in Alzheimer’s disease model rats\(^1\). DHA-enriched PL diet restored DHA concentration in brain PLs and enhanced acetylcholine release\(^1\). One-year administration of DHA supplementation to patients with moderate dementia in cerebrovascular disease improved their dementia scores\(^1\).

The aim of the present study was to study lipid contents and fatty acid profiles in the hippocampus of young (age, 12 weeks) and aged (age, 73 weeks) mice. The authors examined the effects of DHA and/or PS administration (control diet group, Soy-PL diet group, Soy-PS diet group, DHA-PL diet group and DHA-PS diet group). In previous reports, animal brains from pigs or cows have been used\(^1\), \(^1\) as a source of PS. However, these sources are associated with an increased risk of viral infection. Therefore, the authors used DHA-enriched PL extracted from salmon roe containing approximately 30% DHA. The Soy-PS and DHA-PS used in the present study were synthesized from soybean PL and salmon roe PL by base-exchange reaction with phospholipase D, respectively.

## 2 EXPERIMENTAL

### 2.1 Animals

The use of animals in all of our experiments was in accordance with guidelines of the Tsukuba Corporate Research Laboratory of NOF Corp. Young mouse group: Male ICR mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) at 7 weeks of age. After a 1-week acclimation period, 50 healthy mice were selected and divided into five groups (n=10). Aged mouse group: Male ICR mice were obtained from Japan SLC Inc. at 68 weeks of age. After a 1-week acclimation period, 50 healthy mice were selected and divided into five groups (n=10).

### 2.2 Feeding schedule

Young mice and aged mice were fed a test or control diet for 4 weeks. At the end of feeding, the final ages were 12 (young mice) and 73 (aged mice) weeks. All mice were fed MF diet (certified diet) (Oriental Yeast, Chiba, Japan) before coming to our laboratory.

### 2.3 Diets

Low-fat (basal) diet was obtained from Clea Japan, Inc. (Yokohama, Japan). Its composition was as follows (wt%): milk casein, 26.1; corn starch, 48.4; crystallized cellulose.

### Table 1 Fatty Acid Composition of Lipids in Test Diets and Control Diet.

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>Control</th>
<th>Soy-PL</th>
<th>Soy-PS</th>
<th>DHA-PL</th>
<th>DHA-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>16:0</td>
<td>11.6</td>
<td>17.8</td>
<td>18.9</td>
<td>15.8</td>
<td>16.1</td>
</tr>
<tr>
<td>18:0</td>
<td>2.1</td>
<td>3.2</td>
<td>3.4</td>
<td>4.4</td>
<td>4.6</td>
</tr>
<tr>
<td>18:1</td>
<td>25.7</td>
<td>12.2</td>
<td>15.6</td>
<td>12.6</td>
<td>13.3</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>59.7</td>
<td>60.7</td>
<td>58.1</td>
<td>30.8</td>
<td>29.6</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>0.6</td>
<td>5.4</td>
<td>3.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.0</td>
<td>6.8</td>
</tr>
<tr>
<td>SUM</td>
<td>99.9</td>
<td>99.5</td>
<td>99.8</td>
<td>93.3</td>
<td>93.5</td>
</tr>
</tbody>
</table>

n.d.= not detected
Administration of DHA-PS Increased Hippocampal PS and DHA Ratio

10.6; cellulose powder, 3.2; sugar, 2.1; potato α-starch, 1.1; and mineral mixture, 7.4. Each diet contained 94.0% basal diet and 1.2 wt% of safflower oil, to supply essential fatty acids such as linoleic acid (LA; 18:2, n-6). Control diet contained 4,8 wt% test lipids. Soy-PL was produced by Tsuru-Lecithin Industrial Co. (Mie, Japan). Soy-PS, DHA-PL and DHA-PS were products of NOF Corp. (Tokyo, Japan). DHA-PL was extracted from salmon roe with ethanol, and was then fractionated in cold acetone. Soy-PS and DHA-PS were converted from Soy-PL and DHA-PL with phospholipase D (PLD) as a catalyst, respectively. Fatty acid composition of the diets is shown in Table 1. All lipids included 0.2 wt% α-tocopherol as an antioxidant. All diets were divided into small packages and stored at 4˚C. To minimize lipid peroxidation, all diets were provided to the mice every day. PL contents of the test diets were as follows (wt%): Soy-PL; PC 65%, PE 20%, TG 10%, others 5%, Soy-PS; PS 63%, PC 10%, PE 15%, TG 7%, others 5%, DHA-PL; PC 65%, PE 20%, TG 10%, others 5%, DHA-PS; PS 68%, PC 10%, PE 10%, TG 7% others 5%.

2.4 Analytical procedures

After the feeding period, all mice were sacrificed under diethyl ether anesthesia. Serum and the excised hippocampus were stored at −80˚C until lipid analysis. Lipids were extracted using the method of Folch. PS contents were analyzed by high-pressure liquid chromatography (HPLC) using a LC Model I, (Waters, Tokyo, Japan) equipped with a DEVELOSIL model 60-5 HPLC-column (259 mm × 4.6 mm i.d.) (Nomura-chemicals, Tokyo, Japan). Mobile phase was acetonitrile/methanol/phosphoric acid (780509, v/v/v) at a flow rate of 1.5 ml/min. All solvents were HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Column oven temperature was 40˚C, and detection was performed at 220 nm. Zephiramine (Wako, Japan) was used as an internal standard to quantify PS weight. As differences in fatty acid composition affect area response, the calibration curve required Soy-PS (Sigma-Aldrich, MO, USA) as a standard PS.

Fatty acid compositions were analyzed by gas-liquid chromatography of the esters prepared by transmethylation with BF3/methanol. An Agilent 6890A series gas chromatograph (Yokogawa Analytical Systems, Musashino, Japan) equipped with flame ionization detector (FID) and DB-WAX capillary column (30 m × 0.25 mm i.d.) (J & W Scientific, CA, USA) was used. Column temperature was raised from 150 to 210˚C at 5˚C/min. Both injector and detector temperatures were 250˚C. 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).

2.5 Data analysis

Data are expressed as means ± SD of ten mice fed each diet. Data were analyzed by two-way analysis of variance (ANOVA) with both diet and age as factors, and by Bonferroni’s test. A p value of <0.05 was considered to be statistically significant.

Table 2 Fatty Acid Composition of Serum Lipids.

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>diet</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:4 (n-6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.32 ± 2.13</td>
<td>16.99 ± 3.11</td>
<td></td>
</tr>
<tr>
<td>DHA-PS</td>
<td>8.21 ± 1.89*</td>
<td>6.23 ± 2.23*</td>
<td></td>
</tr>
<tr>
<td>DHA-PL</td>
<td>7.33 ± 1.49*</td>
<td>7.15 ± 1.98*</td>
<td></td>
</tr>
<tr>
<td>Soy-PS</td>
<td>16.62 ± 3.26</td>
<td>15.36 ± 3.66</td>
<td></td>
</tr>
<tr>
<td>Soy-PL</td>
<td>17.87 ± 2.13</td>
<td>16.13 ± 3.14</td>
<td></td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.04 ± 0.12</td>
<td>0.05 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>DHA-PS</td>
<td>7.81 ± 1.33*</td>
<td>6.04 ± 1.52*</td>
<td></td>
</tr>
<tr>
<td>DHA-PL</td>
<td>8.04 ± 1.06*</td>
<td>7.16 ± 2.01*</td>
<td></td>
</tr>
<tr>
<td>Soy-PS</td>
<td>0.07 ± 1.18</td>
<td>0.08 ± 0.97</td>
<td></td>
</tr>
<tr>
<td>Soy-PL</td>
<td>0.14 ± 0.99</td>
<td>0.11 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.36 ± 0.61</td>
<td>3.09 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>DHA-PS</td>
<td>16.56 ± 2.19*</td>
<td>17.08 ± 2.96*</td>
<td></td>
</tr>
<tr>
<td>DHA-PL</td>
<td>19.03 ± 1.67*</td>
<td>18.63 ± 1.67*</td>
<td></td>
</tr>
<tr>
<td>Soy-PS</td>
<td>4.43 ± 1.04</td>
<td>3.79 ± 1.45</td>
<td></td>
</tr>
<tr>
<td>Soy-PL</td>
<td>3.49 ± 1.93</td>
<td>3.75 ± 1.81</td>
<td></td>
</tr>
</tbody>
</table>

*) significant difference from young control diet group (p<0.01)
#) significant difference from aged control diet group (p<0.01)
3. RESULTS

3.1 Mouse growth

Final body weights, and therefore total growth, did not differ among the mice fed each diet within the same generation (data not shown). No side-effects were observed.

3.2 Serum fatty acid profiles

Serum fatty acid profiles are shown in Table 2. The fatty acid profiles were not affected in aging. DHA and eicosapentaenoic acid (C20:5 n-3; EPA) contents in serum from the DHA-PS and DHA-PL diet groups were higher when compared to the control, Soy-PS and Soy-PL diet groups in both generations. Arachidonic acid (C20:4 n-6; AA) contents in serum from the DHA-PS and DHA-PL diet group were lower than when compared to the control. DHA, EPA and AA contents in serum from Soy-PS and Soy-PL were almost the same as those in the control diet group in both generations.

3.3 Hippocampal lipid proportions

The ratio of PS weight to total lipid weight extracted from hippocampus (%) is shown in Fig. 1. The PS ratio in the aged control diet group was significantly lower than that in young control mice. In young mice, the PS ratios in the DHA-PL, Soy-PS and Soy-PL diet groups tended to be higher than those in the control diet group, but the differences were not significant. PS ratios in the aged DHA-PS and Soy-PS diet groups were significantly higher than in the aged control group. In the DHA-PL and Soy-PL diet groups, aged mice had the same PS ratios as in the aged control diet group.

3.4 Hippocampal fatty acid profiles

Hippocampal fatty acid profiles are shown in Fig. 2. In the control diet group, both AA and DHA contents decreased with aging. In both generations, DHA contents in the DHA-PL and DHA-PS diet groups were higher than those in the control diet groups, significantly. Consequently, AA contents in the DHA-PL and DHA-PS diet groups were lower than those in control diet group, significantly. AA and DHA contents in the Soy-PL and Soy-PS were almost the same as those in control diet.

4 DISCUSSION

Aging is associated with impairments in various biological functions, such as cognitive function, learning ability, memory and behavior. In animal studies, the declines in learning ability and memory have been evaluated using an 8-arm radial maze, a T maze and the light/dark discrimination test \(^{34-36}\). It has been shown that the loss in brain
PC, PS and DHA due to aging leads to a variety of nervous deficits in animals. Dysfunction of the central nervous system is attributable to biochemical and structural changes in brain membranes with aging. One of the functions of PS is to stabilize and activate enzymes anchored in the membrane matrix, such as Na+/K+-ATPase and Protein kinase C (PKC). PS plays important roles in enhancing the activities of membrane-bound enzymes involved in neurotransmitter release and signal transduction. The administration of PS induces large increases in acetylcholine release in the aged brain.

In this report, the hippocampal PS ratio decreased with age, and administration of Soy-PS or DHA-PS to aged mice was found to be effective in restoring the hippocampal PS ratio. However, the authors did not examine the mechanisms of PS incorporation into the hippocampus. The pathway of PS incorporation into the hippocampus is thought to be as follows: (i) PS may be incorporated into the brain through the blood-brain barrier (BBB) as LysoPS; and (ii) PS was hydrolyzed to supply free serine, which is a substrate of base-exchange enzymes in the brain.

Dendritic spinal density of CA1 pyramidal neurons in the hippocampus and morphometric characteristics of the cholinergic neuronal population of the septal complex decreased significantly in aged rats. Long-term PS administration recovered spinal density in aged rats to comparable levels as in young control rats. In aged rats, acetylcholine and choline outputs were lower than in young rats, while PS administration reversed the brain transmitter level decreases associated with aging. The changes in serum and hippocampal fatty acid profiles suggest that DHA in diet was incorporated into the hippocampus. It has been reported that DHA concentration affects PS synthesis, and that the hippocampal PS ratio in rats fed a DHA-deficient diet is significantly lower than in those fed a DHA-supplemented diet. In addition, the proportion of hippocampal PS in 2-month-old rats on a diet containing fish oil was greater than in aged control rats. These results indicate that PS synthesis in neuronal cells is sensitive to changes in DHA levels and that DHA is a modulator of PS synthesis.

In the present study, the hippocampal PS ratio in the aged DHA-PL diet group did not increase. Exogenous L-serine is generally required for synthesis of PS from PE or/and PC by base-exchange enzymes. Serine incorporation into PS decreases with age in the hippocampus. The reduced serine incorporation may be due to reduced uptake into cells with age. The serine content may be insufficient for PS synthesis in aged mice, the PS ratio did not increase in the aged DHA-PL diet group.

The PS ratio in the young DHA-PL, Soy-PS and Soy-PL diet groups tended to be higher than in the young control diet group, but the difference was not significant. However, supplementation of lipids such as DHA, PS, PC and PE is thought to be effective at increasing hippocampal PS contents in young mice, but the increase in hippocampal PS was suppressed. The authors believe that there is a feedback control system for brain homeostasis in young mice. Kuge et al. reported that PS synthesis in cultured mammalian cells is inhibited by exogenous PS. In contrast, the hippocampal PS ratio of aged DHA-PS and Soy-PS diet groups was higher when compared to the young control diet group. In aged mice, the feedback control for brain homeostasis may have deteriorated.

The control, Soy-PS and Soy-PL diets were n-3 PUFA-deficient and n-6 PUFA (mainly 18:2 linoleic acid; LA)-sufficient. As lipids in the diet affected serum and hippocampal fatty acid profiles, in both generations, n-3 PUFA contents did not increase, while AA contents increased. In contrast, DHA contents increased and AA contents decreased in the DHA-PS and DHA-PL diet groups. The hippocampal AA was replaced with exogenous DHA in both generations.

Serum and hippocampal fatty acid profiles did not differ between diet-matched young and aged groups. The results show that aging influences neither the continuous processes of DHA absorption, transportation and incorporation from the intestinal tract to hippocampus, nor the conversion of AA from LA.

The authors did not confirm whether aging affected memory or learning. As the PS and DHA contents of the aged control diet group decreased, the authors speculate that nerve activities decreased, as indicated in previous reports.

DHA contents decrease in the brain with age. DHA contents in the brains of aged rats are significantly lower than in young rats. DHA administration increases DHA concentrations in the brain and improves brain functions that are modified by age or Alzheimer’s disease. DHA administration also improves learning ability in Alzheimer’s disease model rats. DHA-enriched-PL diet restored DHA concentrations in the brain and enhanced acetylcholine release. Hippocampal DHA concentration has been found to be markedly lower in patients with Alzheimer’s disease; however, one-year administration of DHA supplementation to cerebrovascular disease patients with moderate dementia reportedly improves dementia scores.

5 CONCLUSION
The authors conclude that PS supplementation may be a favorable method to increase the proportion of PS in the aged brain. Valzelli et al. reported that the intracerebral injection of bovine brain PS increased PS contents in the mouse brain and improved learning behaviors. DHA administration increases DHA concentrations in the aged brain. DHA-PS diet could increase both aged hippocampal DHA and PS. The authors thus believe that administration...
of DHA-PS improves the nerve functions in mice that are lost with age.

References
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Administration of DHA-PS Increased Hippocampal PS and DHA Ratio


