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Effect of Dietary Docosahexaenoic Acid on Maze-learning Ability in Aged Mice Fed N-3 Fatty Acid Deficient Diet

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Abstract The effect of docosahexaenoic acid (DHA) on maze-learning ability in aged mice was investigated. Aged mice fed a diet deficient in n-3 fatty acid received a semi-purified diet of 0.5, 1 and 2% DHA-ethyl ester (DHA-EE) for 4 months. Maze-learning ability was assessed at 3 months after the start of the experiment. The time required to reach the maze exit and the number of times that a mouse strayed into blind alleys in the maze were measured in 3 trials conducted at 4-day intervals. The time was significantly less in the DHA-EE 0.5% and DHA-EE 2% groups than in the control group ($p<0.05$). The number of times mice strayed into blind alleys in the maze was significantly lower in the DHA-EE 2% group than in the control group ($p<0.05$). Mice fed DHA showed increased DHA and decreased level of arachidonic acid (AA) in the brain. These results suggest that the intake of a 2% DHA diet improves learning ability in aged mice, which is associated with a higher DHA content in the brain.

Keywords: age, brain, docosahexaenoic acid (DHA), n-3 fatty acids, maze-learning ability

Introduction

The lipids in brain and retinal tissues of mammals contain high levels of long-chain polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) (1). AA and DHA accrue rapidly during early neural development and play a major role in the structure and function of these organs (2,3). AA is found at relatively high levels not only in the brain but also in many other tissues, while DHA is found predominately in the central nervous system (CNS). Brain AA and DHA are derived by desaturation and elongation of their precursors, linoleic and α-linolenic acids, respectively (4). In this process, linoleic and α-linolenic acids are desaturated by Δ6 desaturase prior to further metabolism. The results of previous studies describing the effect of age on Δ6 desaturase activities in liver microsomes have been inconsistent. Bordoni *et al.* (5) reported that Δ6 desaturase activity decreased progressively with increasing age. However, Maniongui *et al.* (6) suggested that the activity of desaturases may be regulated during aging to maintain a constant level of PUFA in cellular membranes. Since it is known that changes in the fatty acid profiles of membrane phospholipids of the nervous system can affect numerous cellular functions (7, 8), maintenance of membrane fatty acid composition is probably essential for the proper functioning of cells including nerve cells. Suzuki *et al.* (9) found that the percentage of AA and DHA in rat brain decreased with age but that DHA levels were elevated if aged rats were fed fish oil containing DHA.

In order to understand the function of DHA in the CNS, many studies were conducted to produce animals with lower brain DHA levels through either multiple generation experiments (10, 11) or artificial rearing method (12, 13). These studies found that rats fed an n-3 fatty acid deficient diet had a poorer learning performance in certain learning tasks including spatial task performance (10), olfactory discrimination (14), brightness discrimination (15) and shock avoidance (16). Moreover, n-3 fatty acid deficiency resulted in alternation of a- and b- wave amplitudes in the electroretinogram in guinea pigs (17) and impaired recovery time after dark adaptation in electroretinogram in primates (18). In human studies, supplementation of DHA to infant formula supported improved performance in visual acuity (19), visual recognition memory (20) and learning a means-end problem-solving task (21). However, few studies of the direct effect of DHA on learning ability during aging have been conducted. In the present experiment, our purpose was to examine the effect of various doses of dietary DHA on the maze-learning ability of aged mice fed n-3 fatty acid deficient diets. The relationship between maze behavior and brain fatty acid composition was also investigated in aged mice fed either n-3 fatty deficiency or DHA supplementation.

Materials and Methods

Animals and diets The experimental protocol was approved by the Animal Care and Use Committee of the National Food Research Institute, Japan. All mice were housed in a standard environment, with temperature maintained at 22±0.5°C, relative humidity at 65±5% and a 12 h/12 h light and dark cycle. Male Crj : CD-1 mice were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan). DHA (DHA-95E, ethyl ester derivative of all cis-4,7,10,13,16,19- DHA, 95% pure) was obtained from Harima Chemicals (Tokyo, Japan). Twenty-four mice aged 18 months old were randomly divided into 4 groups of six: i) a group fed 5 g palm oil/100 g diet (Control Group); ii) a group fed 0.5 g DHA ethyl ester/100 g diet

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plus 4.5 g palm oil /100 g diet (DHA-EE 0.5% Group); iii) a group fed 1 g DHA ethyl ester/100 g diet plus 4 g palm oil/100 g diet (DHA-EE 1% Group); iv) a group fed 2 g DHA ethyl ester/100 g diet plus 3 g palm oil /100 g diet (DHA-EE 2% Group) for 4 months. Each diet contained 5 g/100 g lipid sources and is presented in Table 1. The salt and vitamin mixtures were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) and the compositions have previously been described by Kohashi *et al.* (22). The main fatty acid composition of lipids in each diet group is presented in Table 2. In the present study, the control diet was (n-3) fatty acid deficient and all diets were deficient in α-linolenic acid. The diets were stored at -25°C and fresh supplies were given to the mice once every two days. All diets were handled so as to minimize oxidation of the fatty acids. The diet and water were given ad libitum. Body weights were measured once a week.

Maze-learning ability test To determine the maze-learning ability in the mice, a video tracking and motion analysis system (EMTEC Co., Ltd., Tama, Tokyo, Japan) was used. The analysis system, measuring the rapid real time picture acquisition, has previously been described in our previous study (23). A program for monitoring the pattern of mouse movement and the time spent getting from the maze entrance to exit was adopted. This program allowed direct recording of the X-Y coordinates of mouse movement on a computer disk file. Maze learning ability was assessed at 3 months after the start of the feeding trial. Before the learning test, the conditioning of all mice was perfected using a simple maze of three partition walls and was the training for mice to drink water. The first maze trial was done after 24 h of water deprivation so that the thirsty mice sought water which was placed outside the maze exit. Trial 2 was performed under the same conditions at 4 days after the first trial and trial 3 was conducted at a further 4 days after the second trial. The time required to reach the exit, the number of times that a mouse strayed into blind alleys and the behavior of a mouse in the maze were measured.

Measurement of lipid composition After the feeding trials, all mice were fasted for 24 hr, before being anesthetized with diethyl ether. Blood was then collected from the inferior vena cava, and the mice sacrificed by decapitation. The blood plasma was separated by centrifugation at 900 g for 20 min at 4°C. The whole brain of each mouse was rapidly removed and homogenized in ice-cold 0.32 M sucrose (9 ml/g tissue) using a Teflon-glass homogenizer. The blood plasma samples and brain homogenates were kept at -25°C until required for fatty acid analysis.

Total lipid was extracted by the method of Bligh and Dyer (24) and then transmethylated with 14 % boron trifluoride-methanol at 100°C for 60 min (25). The fatty acid methyl esters were separated by gas liquid chromatography using a 30 m × 0.25 mm i.d. capillary column (Supelcowax 10, Supelco, Bellefonte, USA) and detected by flame ionization (Shimadzu, Co., Tokyo, Japan) (20). The chromatograms were recorded and the percentage composition of individual peaks was calculated with a Chromatopac C-R6A (Shimadzu, Co., Tokyo, Japan). The fatty acid esters were identified by comparison of their retention times with authentic standards.

Table 1. Diet Composition

Ingredients	Dietary group (g/kg diet)			
	Control (Palm oil)	DHA-EE[3] 0.5%	DHA-EE 1%	DHA-EE 2%
Corn starch	488	488	488	488
Casein	200	200	200	200
Sucrose	150	150	150	150
Cellulose	50	50	50	50
Mineral mixture[1]	40	40	40	40
Vitamin mixture[2]	20	20	20	20
L-Methionine	2	2	2	2
Palm oil	50	45	40	30
DHA-EE	-	5	10	20

[1]The mineral and vitamin mixtures were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) at compositions previously described by Kohashi *et al.* (22). The mineral mixtures contained per kg: $CaHPO_4 \cdot 2H_2O$, 14.56 g; KH_2PO_4, 25.72 g; NaH_2PO_4, 9.35 g; NaCl, 4.66 g; Ca-lactate, 35.09 g; Fe-citrate, 3.18 g; $MgSO_4$, 7.17 g; $ZnCO_3$, 0.11 g; $MnSO_4 \cdot 4H_2O$, 0.12 g; $CuSO_4 \cdot 5H_2O$, 0.03 g; KI, 0.01 g.
[2]The vitamin mixtures contained per kg: retinyl acetate, 0.1 g; cholecalciferol, 0.00025 g; isomer α-tocopheryl acetate, 0.5 g; menadione, 0.52 g; thiamin·HCl, 0.12 g; riboflavin, 0.4 g; pyridoxine·HCl, 0.08 g; cyanocobalamin, 0.00005 g; ascorbic acid, 3 g; biotin, 0.002 g; folic acid, 0.02 g; calcium pantothenate, 0.5 g; ρ-aminobenzoic acid, 0.5 g; niacin, 0.6 g; inositol, 0.6 g; choline chloride, 20 g; cellulose powder, 73.1 g.
[3]DHA-EE, docosahexaenoic acid ethyl ester

Table 2. Fatty acid composition (weight %) of diet

Fatty acids	Dietary group			
	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
16:0	46.4	45.4	44.2	38.6
18:0	5.3	3.7	3.4	2.9
18:1 (n-9)	38.3	35.2	31.2	26.5
18:1 (n-7)	0.7	0.6	0.6	0.5
18:2 (n-6)	9.0	8.1	7.5	6.2
20:0	0.2	0.3	0.2	0.2
20:1 (n-9)	0.1	-	-	-
20:5 (n-3)	-	0.4	0.7	1.4
22:6 (n-3)	-	6.3	12.2	23.7
Σ SFA[1]	51.9	49.4	47.8	41.7
Σ MUFA[2]	39.1	35.8	31.8	27.0
Σ (n-6) PUFA[3]	9.0	8.1	7.5	6.2
Σ (n-3) PUFA	-	6.7	12.9	25.1

[1]SFA, saturated fatty acid
[2]MUFA, monounsaturated fatty acid
[3]PUFA, polyunsaturated fatty acid

Statistics All results were expressed as means ± the standard error of the mean (SEM), and statistical significance was determined by one-way analysis of variance (ANOVA) using the SIGMASTAT statistical program package (Jandel Co., Erkrath, Germany). When the F-test was significant, comparisons among the dietary groups were carried out using the Tukey test and the significance level was set at $p<0.05$.

Results and Discussion

Body weight and food intake The average final body weight (mean ± SEM) showed little variation among the dietary groups (control, 45.3 ± 0.50 g; DHA-EE 0.5%, 44.8 ± 0.49 g; DHA-EE 1%, 44.4 ± 0.61 g; DHA-EE 2%, 44.0 ± 0.53 g). There were no differences in body weight gain during the experimental periods when compared with the initial body weights (control, 41.8 ± 0.48 g; DHA-EE 0.5%, 40.3 ± 0.25 g; DHA-EE 1%, 40.8 ± 0.25 g; DHA-EE 2%, 40.3 ± 0.85 g). The food consumption was 4.0 ± 0.31 g/day and the differences among the dietary groups were not significant.

Effect on maze-learning ability All mice were conditioned by training to drink water which was performed using a simple maze of three partition walls before the main experiment. Then, the maze-learning ability was measured using a new and more complicated maze, which contained many blind alleys. The time required to reach the maze exit was significantly less in the DHA-EE 0.5% and DHA-EE 2% diet groups than in the control group in trial 1 ($p<0.05$, Fig. 1) and tended to be shorter in trials 2 and 3, although the difference was not significant. As for the absence of significantly decreased time for DHA-EE 1%, the study results may have been limited by the numbers of mice. In this work, six mice in each group were used for detecting the learning ability. However, Wainwright (26) has pointed out that carefully controlled behavioral experiments require more than ten animals. This suggests that mice in the control group may have learned how to find the maze exit with repeated trials, while the DHA groups took a much shorter time to reach the exit at trial 1. The number of times that a mouse strayed into blind alleys in the maze was significantly fewer in the DHA-EE 2% diet group than in the control group during trial 1 ($p<0.05$, Fig. 2) and in the DHA-EE 1% and DHA-EE 2% diet groups than in the control group during trial 3 ($p<0.05$), but the differences among the dietary groups were not significant during trial 2.

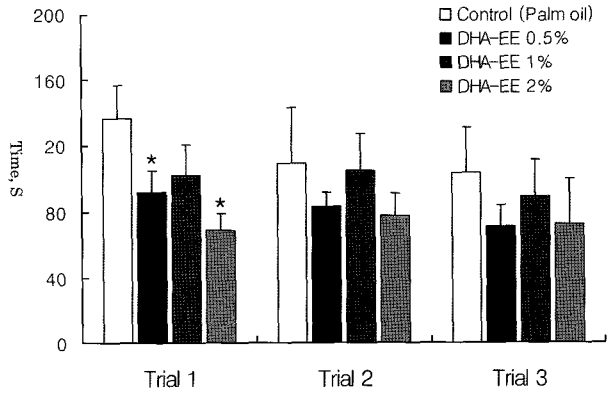

Fig. 1. Differences in the time (Time, S) required for mice to reach the maze exit. Mice aged 18 months fed either the control (palm oil), docosahexaenoic acid ethyl ester 0.5% (DHA-EE 0.5 %), docosahexaenoic acid ethyl ester 1% (DHA-EE 1%) or docosahexaenoic acid ethyl ester 2% (DHA-EE 2%) diet for 3 months. Results are expressed as means ± SEM, n=5-6. Differences were analyzed by one-way ANOVA and comparisons among the dietary groups were done using Tukey's test. Asterisks (*) indicate a statistically significant difference between the control and each experimental diet group at $p<0.05$.

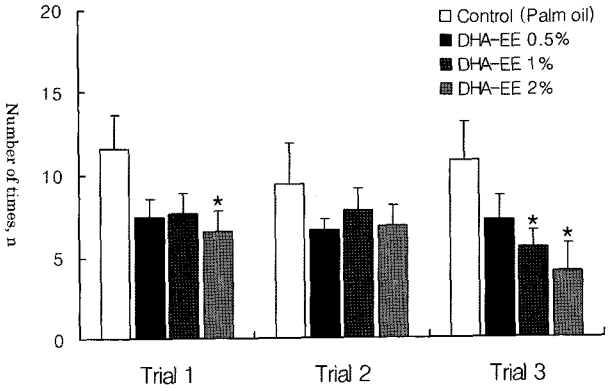

Fig. 2. Differences in the number of times (n) that a mouse strayed into blind alleys of the maze. Mice aged 18 months fed either the control (palm oil), docosahexaenoic acid ethyl ester 0.5% (DHA-EE 0.5%), docosahexaenoic acid ethyl ester 1% (DHA-EE 1%) or docosahexaenoic acid ethyl ester 2% (DHA-EE 2%) diet for 3 months. Results are expressed as means ± SD, n=5-6. Differences were analyzed by one-way ANOVA and comparisons among the dietary groups were done using Tukey's test. Asterisks (*) indicate a statistically significant difference between the control and each experimental diet group at $p<0.05$.

The improved learning ability observed after the intake of DHA is in agreement with our previous study (23) in which the learning ability of aged mice fed DHA-EE 2% for 5 months was improved, although the effect was not clear in trial 2. It may be that increased DHA and the reciprocal decreased AA in the mouse brain after ingestion of DHA are associated with improved learning ability. Suzuki *et al* (9) demonstrated that levels of DHA in the brain decrease with increasing age. In the case of Alzheimer's disease, a decreased level of DHA in brain phosphatidylethanolamine has been reported (27). Several studies demonstrated that supplementation with n-3 fatty acids improved brain function in aged animals and humans. For example, aged animals fed α-linolenic acid-rich diet had an increased learning ability and a longer mean survival time (28, 29). In humans with senile dementia, supplementation with fish oil capsules (1400 mg DHA/d) for 4 months in addition to the usual drugs improved the intellectual function (30). These studies suggest that n-3 PUFA are an essential component for maintaining and improving brain function in aged animals.

Several studies have attempted to explain the importance of DHA's role in the nervous system (31, 32). Kim *et al.* (31) demonstrated that DHA positively modulates the biosynthesis and accumulation of phosphatidylserine (PS) in neuronal membranes and that this observed effect of DHA on PS is closely related to signaling events supporting cell functions such as Raf-1 kinase translocation. Nui *et al.* (33) found that n-3 deficiency led to desensitization in visual signaling as evaluated by rhodopsin activation, rhodopsin-G protein coupling and integrated phosphodiesterase (PDE) activity. Overall, the observed DHA effect on the signaling pathway could explain the general mechanism of DHA in the CNS. Moreover, Minami *et al.* (34) reported that dietary DHA increased cerebral choline and acetylcholine

levels in the brain and improved the impairment of passive avoidance task in stroke-prone, spontaneously hypertensive rats. Zimmer *et al.* (35) suggested that DHA deficiency altered several factors of dopaminergic neurotransmission in the nucleus accumbens and that it is associated with behavioral deficits in DHA deficient rats. It may be that neurotransmitters are involved in the observed effects of DHA on learning ability, although we have had no definitive data. In our previous work, we examined the synergistic effect of dietary DHA and PC as choline precursor on the learning task but found no synergistic effect in the DHA-EE+egg-PC group (23).

Fatty acid composition of brain and plasma In the brain, the percentage of DHA significantly increased in a dose dependent manner for DHA-EE 0.5%, DHA-EE 1% and DHA-EE 2% (Table 3). Mice fed the DHA-EE 2% diet had the highest DHA but lowest AA content ($p<0.05$) of all the groups. Conversely, mice fed the control diet had the lowest DHA and highest AA levels in brain total lipids ($p<0.05$). The percentages of 18:0, 20:1(n-9) and 22:0 were higher in the control than in the DHA-EE 1% and DHA-EE 2% diet groups ($p<0.05$).

There was a striking difference in the mean percentages of both DHA and AA fatty acids in plasma among the dietary groups (Table 4). The mice fed three different doses of DHA-EE had higher levels of DHA and much reduced levels of AA compared with the control group ($p<0.05$). The control group mice had a lower level of DHA and eicosapentaenoic acid (EPA, 20:5n-3) in plasma lipids ($p<0.05$). The control group showed a higher 18:1(n-7) and lower 16:0 than the DHA-EE diet groups ($p<0.05$).

Gamoh *et al.* (36) suggested that chronic administration of DHA had a beneficial effect on the neuronal state condition by decreasing the level of lipid peroxide and that the ratio of DHA/AA in the cerebral cortex may be considered as a novel indicator of learning ability. Our previous results in adult mice suggested that a DHA level above 13.2% and a DHA/AA ratio above 3.77 would be needed in the brain to improve the mice's learning ability in our maze-learning measurement system (37). The DHA level was negatively correlated and the brain AA level positively correlated to the time spent in the maze.

In summary, the intake of DHA-EE at 2% was more effective on improving maze-learning ability in aged mice than at 0.5% and 1%, although the results did not exhibit a dose-dependent relationship with DHA level. Our results suggest that the improved learning ability that is evident in aged mice fed DHA is associated with higher brain DHA levels. Thus, adequate amounts of DHA in the brain may play an important role in maintaining the nervous function by protecting against age-related apoptosis in neural cells, in line with the suggestion by Kim *et al* (31) that adding DHA to neural cells prevents stauroporine-induced apoptosis.

Table 3. Selected fatty acid compositions (weight %) of brain in aged mice fed different diets for four months[1]

Fatty acids	Dietary group			
	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
16:0	20.9 ± 1.25	20.9 ± 1.26	22.1 ± 1.79	21.1 ± 1.31
18:0	18.2 ± 0.65^a	17.4 ± 1.60^a	15.7 ± 0.74^b	16.1 ± 0.77^b
18:1 (n-9)	18.1 ± 0.66	18.8 ± 1.53	18.2 ± 1.28	18.9 ± 0.75
18:1 (n-7)	3.6 ± 0.70	3.7 ± 0.26	3.5 ± 0.24	3.5 ± 0.09
18:2 (n-2)	0.3 ± 0.01	0.3 ± 0.04	0.4 ± 0.03	0.3 ± 0.04
20:1 (n-9)	2.4 ± 0.21^a	2.3 ± 0.19^a	1.9 ± 0.12^b	2.2 ± 0.15^a
20:1 (n-7)	0.4 ± 0.04	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.03
20:3 (n-6)	0.3 ± 0.09^b	0.5 ± 0.20^a	0.4 ± 0.08^{ab}	0.5 ± 0.11^a
20:4 (n-6)	8.2 ± 0.58^a	5.7 ± 0.68^b	4.4 ± 0.54^b	4.4 ± 0.34^b
22:0	1.9 ± 0.20^a	1.1 ± 0.40^b	0.8 ± 0.14^a	0.9 ± 0.09^b
22:4 (n-6)	0.7 ± 0.25	0.6 ± 0.56	0.8 ± 0.14^b	0.9 ± 0.09
22:5 (n-6)	-	0.3 ± 0.02	-	0.3 ± 0.08
22:5 (n-3)	-	0.3 ± 0.07	0.3 ± 0.05	0.4 ± 0.05
22:6 (n-3)	10.1 ± 0.57^b	11.8 ± 0.85^a	12.2 ± 0.78^a	13.3 ± 0.89^a

[1]The values are means ± SD of six mice per group. Values for each dietary group with different superscript roman letters in the same fatty acid are significantly different at $p<0.05$ by Tukey's pairwise comparisons.

Table 4. Selected fatty acid compositions (weight %) of plasma in aged mice fed different diets for four months[1]

Fatty acids	Dietary group			
	Control (Palm oil)	DHA-EE 0.5%	DHA-EE 1%	DHA-EE 2%
16:0	21.6 ± 2.63^b	27.9 ± 2.82^a	28.8 ± 2.88^a	26.9 ± 1.88^a
18:0	6.1 ± 0.54	6.1 ± 1.45	6.4 ± 1.77	7.3 ± 0.54
18:1 (n-9)	21.5 ± 3.28	21.8 ± 1.39	21.3 ± 2.52	20.7 ± 2.23
18:1 (n-7)	2.4 ± 0.36^a	2.0 ± 0.77^a	1.3 ± 0.41^b	1.3 ± 0.28^b
18:2 (n-2)	9.7 ± 1.29	12.4 ± 1.58	13.2 ± 3.94	10.6 ± 2.27
20:1 (n-9)	0.4 ± 0.09	0.5 ± 0.26	0.4 ± 0.30	0.3 ± 0.14
20:3 (n-6)	0.7 ± 0.11^a	0.8 ± 0.17^a	0.5 ± 0.08^b	0.6 ± 0.11^{ab}
20:4 (n-6)	19.4 ± 6.98^a	3.8 ± 0.67^b	1.8 ± 0.10^c	1.0 ± 0.80^c
20:5 (n-3)	-	3.2 ± 0.74^c	5.1 ± 0.62^b	9.1 ± 1.50^a
22:4 (n-6)	0.8 ± 0.25	-	-	-
22:5 (n-6)	-	0.3 ± 0.08	0.3 ± 0.10	0.4 ± 0.03
22:6 (n-3)	1.5 ± 0.41^c	8.0 ± 1.16^b	9.0 ± 0.55^b	12.4 ± 1.62^a

[1]The values are means ± SD of six mice per group. Values for each dietary group with different superscript roman letters in the same fatty acid are significantly different at $p<0.05$ by Tukey's pairwise comparisons.

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