The Use of Docosahexaenoic Acid Supplementation to Ameliorate the Hyperactivity of Rat Pups Induced by in utero Ethanol Exposure

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Abstract

It has been demonstrated that *in utero* ethanol (EtOH) exposure induces hyperactive behavior and learning disturbances in offspring. In order to investigate the effects of docosahexaenoic acid (DHA) on these neurobehavioral dysfunctions of rat pups induced by *in utero* EtOH exposure, pregnant Wistar rats were divided into four treatment groups depending on the type of oil added to the diet and drinking water as follows; (a) 5% safflower oil with tap water (TW/n-6), (b) 3% safflower oil and 2% DHA with tap water (TW/n-3), (c) 5% safflower oil with 10%-EtOH (ET/n-6), (d) 3% safflower oil and 2% DHA with 10%-EtOH (ET/n-3) at gestational day (GD) 7.

10%-EtOH was administered to dams in ET/n-6 and ET/n-3 groups from GD 7 to the pups' weaning (postnatal week 4), and all pups were fed with the same diet that was given to their dams during the entire examination period. The open-field test and the water E-maze test were conducted for all pups, and a spontaneous motor activity test and the Sidman electric shock avoidance test were performed for some of male pups. Amounts of monoamine metabolites in striatum were then determined, and fatty acid analyses of total brain lipids were performed.

The male pups in the ET/n-6 group showed significantly more rearing and square-crossing movements in the open-field test, and significantly higher spontaneous motor activity during the dark period in the daily cycle compared to the males in the TW/n-6 group. The male pups in the ET/n-3 group showed fewer of these behaviors in the open-field test compared to the ET/n-6 group males, and a normal pattern of spontaneous motor activity.

Learning disturbance induced by *in utero* EtOH exposure was not observed in the E-shaped water maze, but was observed in the avoidance rates in the Sidman electric shock avoidance test. However, there was no significant modifying effect of DHA on the avoidance rates in EtOH exposed pups.

The analysis of the fatty acid composition of total lipids in the brains of the pups revealed high levels of DHA in the diet reflected an increased level of brain DHA and caused a decreased level of the brain arachidonic acid. Retroconversion from DHA to eicosapentaenoic acid was also observed. However, there was no significant effect of DHA on the levels of monoamine metabolites.

These results support the hypothesis that DHA can counteract the attention deficit hyperactivity disorder.

Key words: fetal alcohol effect, docosahexaenoic acid, learning behavior, hyperactive behavior, monoamine metabolites

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Introduction

Fetal alcohol syndrome (FAS) is characterized as follows^{1,2)}:

(a) prenatal and/or postnatal growth deficiency, (b) characteristic craniofacial abnormalities—at least two of the following signs must be present: head circumference below the third percentile; microphthalmia and/or short palpebral fissures; poorly developed philtrum, thin upper lip and flattening of the maxillary area, (c) central nervous system (CNS) dysfunction: signs of neurological abnormality, developmental delay or intellectual impairment. Most children with fetal alcohol effects (FAE; incomplete expression of the FAS; Rosett et al.³⁾) show mild behavioral disturbances such as attention deficit and hyperactive behavior, or mental retardation. Under the proper nutrition and care conditions, the growth deficiency may be normalized and the craniofacial abnormalities may dissipate. The microcephaly, however, persists and mental deficiency remains during the lifelong period⁴⁾.

The latest conservative estimate for the incidence of FAS in the Western world is 1.02 per 1000 live births, and the estimate for annual costs related to FAS for one year is \$249.7 million in the U.S.A. The incidence of FAS among mentally handicapped persons was estimated as 23 cases per 1000 from the studies in five countries (Belgium, Chile, Sweden, U.S.A. and Japan). The cost for FAS patients requiring 24-hr residence care due to mental retardation accounts for almost 60% of the estimated total cost⁵⁾. To prevent FAS, multilevel interventions including community education, therapeutic interventions with the alcohol-abusing mother, parenting education, and early identification and intervention with the alcohol-affected child have been tried, but have not achieved the purpose of lessening the incidence of FAS⁶⁾. At present there are no specific pharmacological treatments which are recommended specifically for children showing CNS dysfunction following fetal EtOH exposure⁷⁾. If an effective pharmacotherapeutic or nutritional interventions for such children can be identified, the cost of their care may be reduced.

To understand the mechanism of such CNS dysfunctions from the viewpoint of neurochemical changes, we gave 5%-, 10%-, and 20%-EtOH as drinking water to pregnant Wistar rats, and investigated the dose-effect relationship between the concentrations of EtOH and the learning ability using the Sidman avoidance test or by evaluating the levels of monoamines and metabolites in the brains of their offspring. We observed that EtOH had no effect on learning ability but that the concentrations of EtOH had a dose-effect relationship with the levels of monoamines and metabolites, and suggested that moderate *in utero* EtOH exposure might cause neurochemical changes, though significant learning disturbance was not observed⁸⁾. We had developed a unique rat strain Tokai High Avoider (THA) from the JCL-Wistar rat by selective mating of brother-sister rats shown a high-avoidance rate (95%) and small individual differences in the lever-press Sidman avoidance test. We applied the same schedule on pregnant THA rats. EtOH-exposed THA offspring showed high pre- and postnatal mortality, delay of growth spurt, brain weight reduction, and individually different learning disturbances that were not apparent in the Wistar rats⁹⁾. Comparing these two results, we proposed that the THA rat may have higher susceptibility to the effects of *in utero* exposure than the Wistar rats. A better understanding of the mechanisms will lead us to develop

interventions at both the biological and behavioral levels for ameliorating the CNS dysfunction induced by prenatal EtOH exposure.

Docosahexaenoic acid (DHA, 22:6n-3), one of the major polyunsaturated fatty acids (PUFA) of phospholipids, is synthesized principally from α -linolenic acid (ALA, 18:3n-6). It has been reported that ALA or DHA deprivation during the pre- and postnatal period caused the disability of learning, hyperactivity and unstable emotionality in the mouse and rat pups^{10, 11, 12, 13)}. On the other hand there is some findings of a decrease in the levels of arachidonic acid (AA, C20:4n-6) and of DHA in the brain phospholipids of adult animals chronically exposed to EtOH¹⁴⁾. These findings may be related to the inhibition by EtOH of both delta-6 and delta-5 desaturase enzymes¹⁵⁾. These facts suggest the hypothesis that *in utero* EtOH exposure may reduce levels of DHA in the developing brain and the deficit of DHA may cause CNS dysfunction in pups. To investigate this hypothesis a few researchers examined the fatty acid composition of brain phospholipids in pups or fetuses exposed to EtOH during the pre- and postnatal period^{16, 17, 18)}. A recent report showed that the fish oil (rich in n-3 fatty acids) supplementation to pregnant mice counteracted only some of the effects of EtOH, by increasing pup body weight and enhancing sensory development in the pups. But the fish oil supplementation failed to make a significant effect on the number of trials with errors during the acquisition and reversal learning phase in the water T-maze¹⁹⁾. The report written by Wainwright et. al.¹⁹⁾ is the only one about the effects of prenatal EtOH and n-3 fatty acid supplementation.

Thus, the purpose of the present study was to determine the preventive effects of dietary DHA on the behavior and learning ability of rat offspring with *in utero* EtOH exposure.

Materials and methods

Animals

Nine weeks aged pregnant Wistar rats were obtained from Charles River Japan (Yokohama, Japan) on gestational day (GD) 4 and maintained under standard laboratory housing conditions with a 12-h light/dark cycle (light cycle 8:00-20:00), at a temperature of $22 \pm 2^\circ\text{C}$, and a humidity of $55 \pm 10\%$.

The pregnant rats were divided into four groups according to their diets, based on the content of 5% oil by weight added to a fat-free basal diet (Oriental Yeast Co., Tokyo, Japan), with or without 10% ethanol (EtOH). The components of the 5% oil were 5% safflower oil alone, or 3% safflower oil and 2% DHA (95% DHA-ethylester with 0.03% Vitamin E, Kanagawa Kagaku Co., Kanagawa, Japan) mixture. The groups were defined as follows. (a) The basal diet with 5% safflower oil and tap water was administered (TW/n-6 group; n=3 dams). (b) The basal diet with 3% safflower oil and 2% DHA, and tap water (TW/n-3 group; n=3). (c) The basal diet with 5% safflower oil and 10%-EtOH (ET/n-6 group; n=5). (d) The basal diet with 3% safflower oil and 2% DHA, and 10%-EtOH (ET/n-3 group; n=4). The diets were freshly prepared every week and stored at -20°C under nitrogen atmosphere; the food was renewed every day, and the diets containing DHA were used within one week after preparation.

The experimental diets were fed to dams from GD 7. Their litters of each dam were adjusted to include eight pups (basically 4 males and 4 females) on postnatal day (PND) 4, and they were

fed the same diets as that which was given to their dams, until the day for measurement of neurotransmitters. 10%-EtOH was administered from GD7 to two days before weaning during postnatal week (PNW) 4. After weaning, all pups (61 males and 30 females) were tested in the open-field and in the water maze test. The spontaneous motor activity of 18 male pups was measured during PNW 5. The Sidman avoidance test was conducted for all male pups from PNW 5 to 6, and then a fatty acid analysis of total brain lipids was conducted for 32 male pups during PNW 7. For the other 23 male pups, the amounts of neurotransmitters released from the striatum were measured after PNW 10 after the neurochemical function had matured. The outline of the experimental schedule is illustrated in Fig. 1.

Behavioral testing.

(1) Open-field test

The open-field was a 100 × 100 cm open black acrylic box with walls 41 cm high. One side wall adjoined a starting box with the dimensions 17.5 × 25 cm. The floor of the field was divided into 16 equal squares by grids. The latency time for walking out from the starting box into the field, the numbers of crossed squares, rearing movements and fecal pellets were observed as indicators of activity or exploratory tendencies, and recorded. The numbers of water puddles, washing and grooming behaviors were counted as indicators of emotionality on each pups, for 3 min in the open field.

(2) Measurement of spontaneous motor activity

The activity monitor apparatus (MK-Animex SE-type, Muromachi Science Co., Tokyo) was used for the measurement of spontaneous motor activity. The number of vertical and horizontal movements was measured for 36 hours.

Learning testing

(1) Water maze test

The apparatus consisted of an E-shaped water alley of a black acrylic box with three arms 60 cm long and a cross-bar 105 cm long; each was 45 cm deep and 15 cm wide. The E-maze was filled with water at $23 \pm 1^\circ\text{C}$ so that the water was 24 cm deep. A platform covered with rubber was placed on the end of the right or left arm. The position of the platform was randomly assigned to the rat. The rat was released at the end of the central arm of the E-maze to allow for swimming.

A correct response was defined as swimming directly to the platform. Turning to the opposite direction or returning to the start arm were considered incorrect responses. Each rat was given 10 trials per day on the 1st, 2nd and 3rd trial days with the setting of the platform on the same side. On the 4th trial day, the platform was set on the reverse side, and 10 trials were performed. Each trial was terminated when the rat failed to reach the platform within 3 min. The intertrial interval was 45 sec. The number of incorrect responses and the swimming time to the platform were recorded.

(2) Sidman avoidance test

The schedule of the Sidman electric shock avoidance test was as follows; shock-shock interval, 5 sec; response-shock interval, 30 sec; shock intensity, 100 V and 3 mA for 0.5 sec. All avoidance test sessions were performed for 60 min/day for 10 consecutive days. We employed the data from the latter half of each test for the evaluation of learning ability to rule out the warm-up effect observed in the earlier half of the session. The avoidance rate was calculated as:

$$A = \{(S-G)/S\} \times 100 (\%),$$

where A is the avoidance rate, S is the total number of electric

Fig.1 The outline of this experimental schedule.

EtOH: ethanol, GD: gestational day, PNW: postnatal week

TW/n-6: 5% safflower oil with tap water was administered to dams and their pups

TW/n-3: 3% safflower oil and 2% DHA with tap water

ET/n-6: 5% safflower oil with 10%-EtOH

ET/n-3: 3% safflower oil and 2% DHA with 10%-EtOH

stimuli when no response would occur, and G is the total number of electric stimuli given to the rat in a test session.

Fatty acid analyses

The rats were sacrificed by deep etherization, and their whole brains were removed and stored at -80°C until the fatty acid analysis. The brains were homogenized in chloroform-methanol, and lipids were extracted according to the method of Folch. The portion of lipids was methylated by 0.4M potassium methoxy-methanol and 14 wt%-boron fluoride in methanol. The methyl esters were extracted with hexane, and the fatty acid composition of the extract was analyzed on a gas chromatograph (model GC17A, Shimadzu, Kyoto) with a $30\text{mm} \times 0.25\text{mm} \times 0.25\text{ }\mu\text{m}$ capillary column (Omegawax, Supelco).

Measurement of neurotransmitters in the brain by microdialysis

The 3-mm tip housing the dialysis membrane of the probe was inserted into the striatum of the right hemisphere of the rat under 2.5% halothane, and fixed with dental resin. Twenty-four hours after the probe was inserted, the measurement of neurotransmitters was performed. Physiological saline was perfused at $2\text{ }\mu\text{L}/\text{min}$ for measurement of the monoamine metabolites 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole acetic acid (5-HIAA). The perfused solution was pooled, collected every 20 min, and quantified by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD, Eicom Corp., Kyoto, Japan).

Statistical analyses

SPSS program package was applied for statistical analyses. One-way ANOVA was performed among the groups to analyze the effects of EtOH intake and/or DHA administration. The Bonferroni/Dunn and Fisher's PLSD tests were applied for post hoc multiple comparisons among the groups.

Results

EtOH intake and body weight of dams

The EtOH intake of the dams in the ET/n-6 and ET/n-3 groups was calculated from water consumption as 8.9 ± 1.1 and $9.2 \pm 1.3\text{ g/kg/day}$, respectively. Diet consumptions of dams in the ET/n-6 and ET/n-3 groups were found to be significantly lower (maximally 18%) than those in the TW/n-6 group during pregnancy, but there was no significant difference of diet consumption among the groups from PNW 2 to weaning. The DHA ingestion by the dams in the TW/n-3 and ET/n-3 groups until weaning was estimated from their diet consumption as 5.8 g/kg/day and 6.1 g/kg/day , respectively. No significant differences were found in the dams' body weights among each of the four groups (data not shown).

Outcome of pregnancy, growth and development of pups

The number of live litters per dam on PND 3 in the ET/n-3 group was significantly lower than the value in the ET/n-6 group (Table 1). One pup died before the end of PNW 2 in the ET/n-6 and the ET/n-3 groups. Body weights of the pups on PND 3 are also shown in Table 1. Body weights of the ET/n-3 group male pups were significantly higher than the corresponding values in the ET/n-6 group. Among the female pups the body weights in the TW/n-3 and ET/n-6 groups were significantly lower than

those in the TW/n-6 group. Body weights of the ET/n-3 group females were significantly higher than the values in the ET/n-6 group. On PND 4, litters of eight pups (basically 4 males and 4 females) from each dam were selectively maintained; other pups were removed to achieve average growth. After the selection, there was no significant difference in the body weights of the pups among the groups.

Open-field test

The behaviors of the male pups in the open-field are summarized in Table 2. A significant increase in the number of rearing movements was observed [$F(3, 57) = 2.861$, $p < 0.05$]. The number of squares crossed was also significantly increased in ET/n-6 and ET/n-3 against TW/n-6 and TW/n-3 respectively [$F(3, 57) = 3.348$, $p < 0.05$]. The numbers of rearing movements and squares crossed in the ET/n-6 group were both significantly higher than those in the TW/n-6 group ($p < 0.05$), and the numbers in the ET/n-3 group were significantly lower than those in the ET/n-6 group ($p < 0.05$). The number of face washing movements was significantly reduced according to diet and EtOH intake [$F(3, 57) = 6.973$, $p < 0.05$], and the number of washing movements in the ET/n-6 group was significantly lower than that in the TW/n-6 group. There were no significant differences in any of these behaviors among the groups of female pups (data not shown).

Spontaneous motor activity

The spontaneous motor activities of male pups for the latter 24 h of the 36 h measurement period are shown in Fig. 1. Repeated two-way ANOVA showed a non-significant correlation but a tendency between the diet or EtOH intake and on increase of activity [$F(3, 14) = 2.931$, $p = 0.07$]. The 24 h activity levels in the TW/n-3 and ET/n-3 groups were significantly higher than those of the TW/n-6 group ($p < 0.05$).

Water maze test

Significantly longer swimming times in the TW/n-3 group male pups compared to the other groups were observed in just the 1st trial of the reversal learning trial on the 4th experimental day [$F(3, 26) = 4.216$, $p < 0.05$], but there were no significant

Table 1 Number of live litters per dam and the body weight of pups on postnatal day 3.

Group (no. of dams)	Number of live litters	Body weight of pups (g)	
		Male (no. of pups)	Female (no. of pups)
TW/n-6 (n=3)	13.3 ± 0.6	9.15 ± 1.11 (n=18)	8.89 ± 0.67 (n=22)
TW/n-3 (n=3)	12.3 ± 1.5	8.63 ± 0.97 (n=17)	$8.03 \pm 0.99^{**}$ (n=20)
ET/n-6 (n=5)	14.8 ± 2.4	8.61 ± 0.94 (n=35)	$8.14 \pm 1.15^{**}$ (n=39)
ET/n-3 (n=4)	$11.1 \pm 3.4^{+}$	$9.32 \pm 1.05^{++}$ (n=24)	$8.84 \pm 1.05^{++}$ (n=21)

Values are mean $\pm$ SD. **: Significantly different from TW/n-6 at $p < 0.01$.

Significant differences compared with ET/n-6 are $^{+}p < 0.05$ and $^{++}p < 0.01$.

Table 2 Open-field behavior of male rat pups

Group	Exploration		Emotionality	
	Rearing	Square crossing	Washing	Grooming
TW/n-6 (n=12)	17.8 ± 8.2	17.9 ± 14.6	2.6 ± 1.1	1.1 ± 1.2
TW/n-3 (n=12)	16.4 ± 9.1	18.9 ± 14.7	2.2 ± 1.9	0.6 ± 0.7
ET/n-6 (n=20)	$26.7 \pm 15.9^{*}$	$34.9 \pm 24.8^{*}$	$1.1 \pm 1.5^{**}$	0.5 ± 0.8
ET/n-3 (n=17)	$18.3 \pm 12.2^{+}$	$20.4 \pm 12.2^{+}$	1.5 ± 1.6	0.6 ± 0.9

Values are mean $\pm$ SD. (): number of pups.

Significant differences compared with TW/n-6 are $^{*}p < 0.05$ and $^{**}p < 0.01$.

Significant differences compared with ET/n-6 are $^{+}p < 0.05$.

differences in the swimming times among the four groups on the 1st, 2nd or 3rd experimental days. The number of errors made by the pups in the TW/n-3 group was significantly higher than the numbers in the other groups in the 1st trial on the 4th experimental day [$F(3, 26)=3.690$, $p<0.05$]. There were no significant differences in the number of errors among the four groups on the 1st, 2nd or 3rd experimental days.

For the female pups, there was no significant difference in the swimming time or in the number of errors among the groups.

Sidman avoidance test

The avoidance rates in the Sidman electric shock avoidance test are shown in Fig. 2. The repeated two-way ANOVA showed a significant correlation between the diet and EtOH intake, and avoidance rates of the male rats [$F(27, 55) = 2.065$, $p<0.01$]. The post-hoc multiple comparison showed that the avoidance rates in the ET/n-6 and ET/n-3 groups were significantly lower than those in the TW/n-6 group ($p<0.05$), and that the ET/n-6 group rate ($p<0.05$). There was no significant difference in avoidance rates between the TW/n-3 group and the ET/n-3 group.

Brain fatty acid composition

The whole brain weights (except olfactory bulbs) of the male pups on PNW 7 were as follows TW/n-6 group; 1841.2 ± 38.2 (mean $\pm$ SD) mg, TW/n-3 group; 1834.5 ± 51.4 mg, ET/n-6 group; 1839.7 ± 89.4 mg, and ET/n-3 group; 1817.1 ± 43.2 mg. There were no significant differences among each group.

The fatty acid composition (%) in total lipids in the brains of the male pups is shown in Table 3. There were no significant differences in the saturated fatty acids composition among each group. In the n-3 series, the levels of both eicosapentaenoic acid (EPA, C20:5n-3) and DHA in the TW/n-3 and ET/n-3 groups were significantly higher than those in the TW/n-6 and ET/n-6

groups, respectively. On the other hand, in the n-6 series, the levels of both AA and C22:4 in the TW/n-3 and ET/n-3 groups were significantly lower than the levels in the TW/n-6 and ET/n-6 groups, respectively. The levels of both linoleic acid (LA, C18:2n-6) and dihomo- γ -linolenic acid (DGLA, 20:3n-6) in the TW/n-3 and ET/n-3 groups were significantly higher than the levels in the TW/n-6 and ET/n-6 groups, respectively. The level of AA in the ET/n-6 group was higher than the level in the TW/n-6 group (p value between these two groups was 0.03 by the Student's t -test). In the n-9 series, the levels of C18:1 in the

Fig. 2 Spontaneous motor activity of male rat pups over a 24-hour observation period.

Values are mean $\pm$ SD for every 3 hours. $\blacksquare$: dark period, $\square$: TW/n-6 (n=4), $\diamond$: TW/n-3 (n=4), $\circ$: ET/n-6 (n=5), $\triangle$: ET/n-3 (n=5)

*: Significant difference against TW/n-6 ($p<0.05$).

+: p value between ET/n-6 and ET/n-3 is 0.07.

Table 3 Fatty acid composition (%) of total lipids in the brains of the male rats.

Fatty acid	Group			
	TW/n-6 (n=6)	TW/n-3 (n=6)	ET/n-6 (n=13)	ET/n-3 (n=7)
C12	<0.01	<0.01	<0.01	<0.01
C14	0.60 ± 0.10	0.61 ± 0.03	0.63 ± 0.11	0.66 ± 0.07
C14: 1n-5	<0.01	<0.01	<0.01	<0.01
C16	17.86 ± 0.62	17.83 ± 0.37	18.03 ± 0.42	18.00 ± 0.92
C18	21.86 ± 0.21	21.52 ± 0.18	21.92 ± 0.22	21.64 ± 0.50
C18: 1n-9	19.85 ± 0.28	21.31 ± 0.31^a	19.56 ± 0.46^b	20.70 ± 1.10^c
C18: 2n-6 (LA)	0.99 ± 0.03	1.29 ± 0.05^a	0.98 ± 0.03^b	$1.35 \pm 0.10^{a,c}$
C18: 3n-6 (ALA)	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
C18: 3n-3	<0.01	<0.01	<0.01	<0.01
C20	1.19 ± 0.07	1.06 ± 0.05	1.15 ± 0.05	1.01 ± 0.16
C20: 1n-9	2.67 ± 0.25	2.55 ± 0.13	2.65 ± 0.13	2.41 ± 0.40
C20: 2n-6	0.48 ± 0.02	0.37 ± 0.02^a	0.48 ± 0.02^b	$0.38 \pm 0.05^{a,c}$
C20: 3n-9	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
C20: 3n-6 (DGLA)	0.58 ± 0.03	1.07 ± 0.03^a	0.59 ± 0.03^b	$1.08 \pm 0.05^{a,c}$
C20: 4n-6 (AA)	9.03 ± 0.17	6.21 ± 0.11^a	9.26 ± 0.21^b	$6.50 \pm 0.62^{a,c}$
C20: 5n-3 (EPA)	<0.01	<0.01	<0.01	<0.01
C22	1.35 ± 0.09	1.25 ± 0.06	1.29 ± 0.07	1.18 ± 0.19
C22: 1n-9	0.33 ± 0.04	0.29 ± 0.01	0.32 ± 0.01	0.30 ± 0.05
C22: 4n-6	4.90 ± 0.10	1.94 ± 0.04^a	4.98 ± 0.13^b	$2.02 \pm 0.14^{a,c}$
C22: 5n-3	0.07 ± 0.01	0.55 ± 0.03^a	0.07 ± 0.01^b	$0.53 \pm 0.04^{a,c}$
C22: 6n-3 (DHA)	11.58 ± 0.50	15.71 ± 0.31^a	11.59 ± 0.49^b	$16.06 \pm 0.73^{a,c}$
C24	2.70 ± 0.08	2.52 ± 0.13	2.60 ± 0.16	2.55 ± 0.40
C24: 1n-9	3.84 ± 0.16	3.60 ± 0.16	3.77 ± 0.17	3.35 ± 0.57

Values are mean $\pm$ SD. (): number of pups.

Significant differences, respectively; a: against TW/n-6 at $p<0.001$, b: against TW/n-3 at $p<0.001$, c: against ET/n-6 at $p<0.001$.

LA: linoleic acid, ALA: α -linolenic acid, DGLA: dihomo- γ -linolenic acid,

AA: arachidonic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid

TW/n-3 and ET/n-3 groups were significantly higher than the levels in the TW/n-6 and ET/n-6 groups, respectively.

Neurotransmitters in the brain

Released amount of monoamine metabolites from the striatum of the pups' brains are shown in Table 4. The amount of 5-HIAA from the ET/n-6 group was significantly lower than that from the TW/n-6 group ($p < 0.05$). There were no significant differences in the amounts of HVA and DOPAC among each of the groups. The amount of HVA from the ET/n-3 group tended to be higher than that from the ET/n-6 group (p value between their data was 0.08 by the Student's t -test).

Discussion

There is an agreement that the most important problem in EtOH teratogenesis is CNS dysfunction but there are no specific pharmaceutical treatments. The purpose of this study was to investigate preventive therapeutical effects of dietary DHA administration on the behavior and learning abilities of rat offspring with *in utero* EtOH exposure.

The only previous report concerned with this issue was written by Wainwright et al.¹⁹⁾ They fed to pregnant mice a liquid diet, containing 25% (kcal) EtOH or supplemented maltose dextrin substituted isocalorically for ethanol. Both diets also contained 20% oil; this was either of two mixtures, one comprised of LA, and the other containing an equivalent amount of LA and fish oil (rich in EPA and DHA). Those combined diets were administered from GD 7 to 17, and then all the groups were fed lab chow. They also investigated pup body weight, sensory and motor development, and water T-maze learning in the pups. We have made several comparisons with their results.

In this study there was no significant decline of body weight in only the male pups of the ET/n-6 and ET/n-3 group. Therefore, the lesser diet consumption of the dam in these groups was considered to have no effect on the growth of these male pups. Pup body weight in the ET/n-3 group was higher than that in the ET/n-6 group in both male and female pups. Wainwright et al.¹⁹⁾ reported that the n-3 supplementation increased maternal weight gain and pup body weight, which counteracted the EtOH-induced decreases in these weights. Our result was consistent with that of theirs.

In the open-field test, the significant increases in the number of rearing and squares crossing in the ET/n-6 group compared with the TW/n-6 group, suggested an increase of activity following perinatal EtOH exposure. Significantly less washing and grooming in the ET/n-6 group was observed in comparison with the value in the TW/n-6 group; this is understandable as the expression of unstable emotionality of the pups induced by perinatal EtOH exposure. Several experimental studies using animal models had reported that the offspring from dams ingested with EtOH by a liquid diet or intubation during pregnancy showed an increase of behavioral activity in the open-field test^{20, 21)}. Our findings are consistent with the results of these previous reports.

The spontaneous motor activity of the pups in the groups except the ET/n-6 group over a 24 h period showed the same two peaks of activity during the dark period 23-2 h and 5-8 h as is usually observed. These peaks at the beginning and the end of the dark period were related to water and diet intake activity. It is thought that no peaks of activity observed in the ET/n-6 group

Table 4 Levels of extracellular monoamine metabolites in the rat striatum

Group	DOPAC ($\mu\text{g}/40 \mu\text{l}/20\text{min}$)	5-HIAA ($\mu\text{g}/40 \mu\text{l}/20\text{min}$)	HVA ($\mu\text{g}/40 \mu\text{l}/20\text{min}$)
TW/n-6 (n=5)	164.18 $\pm$ 47.30	83.64 $\pm$ 20.80	107.24 $\pm$ 34.79
TW/n-3 (n=6)	186.81 $\pm$ 62.36	72.26 $\pm$ 21.40	117.34 $\pm$ 46.78
ET/n-6 (n=6)	143.52 $\pm$ 44.76	49.03 $\pm$ 13.51*	74.73 $\pm$ 27.24
ET/n-3 (n=6)	156.45 $\pm$ 45.48	60.71 $\pm$ 14.07	106.25 $\pm$ 29.14

Values are mean $\pm$ SD. (): number of pups. DOPAC: 3, 4-dihydroxyphenyl acetic acid, HVA: homovanillic acid, 5-HIAA: 5-hydroxyindole acetic acid.

*: Significant difference between TW/n-6 and ET/n-6 group at $p < 0.05$.

was the result of continuous hyperactivity throughout the dark period.

Several investigators have examined the effect of DHA on both animal and human behavior. Nakashima et al.¹³⁾ fed semi-purified diets supplemented with a high linoleate (n-6) (safflower) oil or a high α -linolenate (n-3) (perilla) oil to mouse dams and offspring from dams' weaning to pups' 6 weeks of age, and conducted an elevated plus maze test to evaluate the anxiety and dark preference. They found that the total number of entries into the open- and enclosed-arms for 5 min was significantly higher in the safflower oil group than in the perilla oil group, and they suggested that the dietary α -linolenate/linoleate balance caused the behavioral changes. Hamazaki et al.²²⁾ conducted an interventional study in which 41 university students took either DHA-rich oil capsules containing 1.5-1.8 g DHA/day or control oil capsules for 3 months in a double-blind fashion. The students were examined using such a psychological test as the picture-frustration (P-F) study at the start and the end of the interventional study. It was found that the DHA intake prevented extraaggression from increasing at times of mental stress such as final exams, according to the results of the P-F study.

In this study, the numbers of rearing and squares crossing in the ET/n-3 group were significantly lower than the values in the ET/n-6 group in the open-field test. And the ET/n-3 group tended to be less active than the ET/n-6 group in the spontaneous motor activity of the pups. From these findings and from the results of these two previous reports about DHA, it is noted that DHA may stabilize the emotionality and reduce the activity of the pups.

In the Sidman test, the avoidance rates in both the ET/n-6 and ET/n-3 groups were significantly lower than that in the TW/n-6 group, but there was no significant difference in avoidance rates between the ET/n-6 and ET/n-3 groups. Deficits in several learning tasks (discrimination learning, active and passive avoidance, and operant learning) by prenatal EtOH exposure in the animal study have been reported²⁰⁾. Reduction of the avoidance rates in the ET/n-6 and ET/n-3 groups observed in this study was consistent with the results of these previous reports. Many studies have reported that the supplementation of n-3 fatty acids through the prenatal and preweaning developmental periods indicates a good effect on learning ability¹⁰⁾.

Wainwright et al.¹⁹⁾ observed that EtOH significantly reduced the number of errors in acquisition learning, but observed the opposite effect in reversal learning in the water T-maze for pups on PND 27. They also observed that the n-3 oil had no significant effect against these EtOH effects. In our study there was no significant effect of EtOH or DHA in the water maze test. It is thought that *in utero* EtOH exposure affected the

Fig. 3 Avoidance rate of male rat pups in the latter half of the 60min Sidman electric shock avoidance test.

Values are mean $\pm$ SD. $\square$: TW/n-6 (n=12),

$\diamond$: TW/n-3 (n=12), $\circ$: ET/n-6 (n=20), $\triangle$: ET/n-3 (n=17)

Significant difference compared with TW/n-6 group are * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 4 Pathway of the synthesis of polyunsaturated fatty acid.

LA: linoleic acid, ALA: α -linolenic acid,

GLA: γ -linolenic acid, DGLA: dihomogamma-linolenic acid,

AA: arachidonic acid, EPA: eicosapentaenoic acid,

DHA: docosahexaenoic acid,

Δ^5 : Δ^5 -desaturase, Δ^6 : Δ^6 -desaturase.

pups' learning ability but DHA did not prevent this affection.

The pathway of the synthesis of polyunsaturated fatty acid is shown in Fig. 4. Regarding the brain fatty acid composition in the present study, it is thought that the supplemental DHA was reversely converted to EPA in the n-3 series, and that delta-5 and delta-6 desaturase were consumed. Because the antagonistic blockade of these desaturases in the n-6 series inhibited the conversion of LA to C18:3 and the conversion of DGLA to AA, the levels of LA and DGLA was higher and the level of AA was lower in the TW/n-3 and ET/n-3 groups than each value in TW/n-6 and ET/n-6 respectively. In adult animals, EtOH might inhibit both delta-6 and delta-5 desaturase enzyme activities and decrease the levels of AA and DHA in the brain phospholipids²³. With the hypothesis that EtOH exposure *in utero* reduced the supply of AA and DHA which are critical to the developing brain in the fetus, a few researchers analyzed the fatty acid composition of brain phospholipids of pups. Wainwright et al.¹⁶ performed

the same experimental procedures as the previous work to evaluate the effect of the supplemental DHA on learning disability. They determined the fatty acid composition of the brain membrane phospholipids in pups on PND 3 and 13. On both PNDs they observed that n-3 supplementation increased n-3/n-6 and DGLA/AA ratio, and the increase of the DGLA/AA ratio indicated the inhibition of delta-5 desaturase activity, and observed that n-3 supplementation decreased the levels of 22:5n-6 while increasing those of DHA with the sum of these two fatty acids remaining unchanged. They observed that EtOH decreased n-3/n-6 ratio in the phosphatidylcholine (PC) fraction on the both PNDs, and decreased levels of 22:5n-6 and DHA on PND 3. Duffy et al.¹⁷ gave EtOH as a liquid diet from 3 weeks before mating to the PND 10 or 20 to female Wistar rats, and observed a decrease in 18:1n-9 content of PC on PND 10, and an increase in the AA content of phosphatidylserine on PND 20 in the pups. Burdge et al.¹⁸ gave female guinea pigs 6g/kg/day EtOH both before mating and throughout pregnancy, and observed a decreased DHA concentration in both PC and phosphatidylethanolamine fractions in the fetal brain at both 40 and 68 (at term) days gestation. In the present study, such inhibition in the pathway of n-6 series reflected by the DHA supplementation is consistent with the observation of Wainwright et al.¹⁶ However, EtOH did not decrease the level of DHA in the pup brain phospholipids in our study, and this finding is inconsistent with the results of Wainwright et al.¹⁶ and Burdge et al.¹⁸ The increase in the level of AA in the ET/n-6 pup brain on PNW 7 may be an effect of EtOH and is consistent with the result of Duffy et al.¹⁷ Furthermore the continual increase of AA for 3 weeks even after the end of EtOH intake is a new finding.

As for the levels of monoamine metabolites in the present study, the decrease of 5-HIAA in the striatum of pup brain is obvious following fetal alcohol exposure, but no significant preventive effect of DHA on the levels of monoamine metabolites was detected. However, a trend of the increase in the level of HVA in ET/n-6 than ET/n-3 may be the effect of DHA administration. The changes of neurotransmitters in the pup brain associated with in utero EtOH exposure were reported^{24, 25}. A decrease of 5-HIAA is consistent with our and previous reports

Several studies have been reported as possible candidates for the explanation of the mechanisms which cause the effects of dietary DHA on the learning ability and behavior in rats²⁶. However, further investigation is needed to clarify the mechanism underlying the beneficial effect of DHA on the behavioral deficits caused by in utero EtOH exposure.

As mentioned above, Wainwright et al.¹⁹ reported that the n-3 supplementation increased maternal weight gain and pup body weight, and accelerated sensorimotor development. It, also, counteracted the EtOH-induced decreases in these weights and the retardation of development. From our experiment, DHA in the diet may prevent growth reduction, and moreover improve behavioral anomalies caused by in utero EtOH exposure.

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