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Essentiality of arachidonic acid intake in murine early development

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ABSTRACT

We previously reported the importance of long-chain polyunsaturated fatty acid (LC-PUFA (> C20)) intake, including arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), for growth. This follow-up study focuses on ARA using a novel artificial rearing model during the lactation period in delta-6-desaturase knockout (D6D-KO) mice. Newborn D6D-KO male mouse pups were separated from dams within 48 hours and fed artificial milks containing 18-C essential fatty acids (EFAs) (16–17% LA, 3.8–4.1% ALA) with or without 1.2% ARA. After weaning, mice were maintained on similar diets: 15% LA, 2.3–2.4% ALA with or without 1.9% ARA. As a reference group, new born wild type (WT) male mouse pups were maintained by artificial milk and diet containing LA and ALA without ARA. Aspects of brain function were measured behaviorally (motor activity and rota-rod test) when mice were age 9 weeks. Body weight in the KO-Cont group was significantly lower (approximately 30%) than in the WT-Cont group, but this decrease was ameliorated by providing ARA in the KO-ARA group. The motor activity and coordination in the KO-Cont group decreased markedly compared to the WT-Cont group. The KO-ARA group had a tendency toward deteriorated motor coordination, although the motor activity was significantly enhanced compared to the KO-Cont group. In KO-ARA group brains, the level of ARA was increased and DHA decreased compared to WT-Cont. These results suggest that intake of LA and ALA only is insufficient to support healthy growth, and that ARA is also required, at least during the lactation period. These findings also suggested that continued intake of relatively high levels of ARA and without supplemental DHA during development led to an increased motor activity above that of WT animals. These studies indicate that both ARA dose and proper combination with DHA must be delineated to define optimal growth and behavioral function.

1. Introduction

Two families of polyunsaturated fatty acids (PUFAs), n-6 and n-3 fatty acids, are essential fatty acids, as they cannot be biosynthesized and therefore must be ingested in the diet. Although a lack of n-6 fatty acids causes growth disorders and dermopathy [1,2], linoleic acid (LA, 18:2n-6) and arachidonic acid (ARA, 20:4n-6) are contained in many types of foods, especially animal fats and vegetable oils, and therefore such deficiencies rarely occur. α-Linolenic acid (ALA, 18:3n-3), an n-3 fatty acid, is contained in perilla oil and linseed oil, and its metabolites eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are abundant in fish and seafood [3]. DHA can pass through the blood-brain barrier [4] and the blood-retinal barrier to accumulate in these organs, thereby improving brain function (e.g. learning ability), mental condition, and visual function [5–7]. It has also been reported to have preventive and therapeutic effects on cardiovascular diseases [8]. However, n-3 fatty acid–rich foods are relatively uncommon, and thus, resulting impairment of brain function by deficiency of n-3 fatty acids is a concern. Dietary LA is converted to ARA through a reaction catalyzed by delta-6-desaturase (D6D) and chain elongating enzymes, and then to the n-6 end product docosapentaenoic acid (DPAn-6, 22:5n-6) via the reactions catalyzed by chain elongating enzymes, D6D and β-oxidation [9]. Similar fatty acid–metabolizing enzymes convert dietary ALA to EPA and DHA. On the other hand, it has been reported that DPAn-6 and DHA may be synthesized by direct delta-4-desatuation in human

Abbreviations: D6D, delta-6-desaturase; KO, knock-out; PUFA, polyunsaturated fatty acid; LA, linoleic acid; ARA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid

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cells [10]. However in human infants, the activities of these desaturases are still too low to sufficiently convert LA and ALA to ARA and DHA, respectively, requiring direct intake of ARA and DHA [11]. Infants undergo marked growth and development of the body and the brain, and several studies have demonstrated the efficacy of ARA and DHA intake in infancy [12–14]; in fact, breast milk is rich in these fatty acids [15–17].

PUFAs are necessary to maintain biological functions throughout all life stages, but its intake is likely to be particularly essential in infancy. Yet, the European Food Safety Authority (EFSA) recently expressed the opinion that there is no need to add ARA and EPA to infant and follow-on formulae [18]. The effect of PUFA in infants has been examined only through administration of ARA plus DHA [13,14], and through administration of DHA only, and the effect of PUFA intervention in infancy after the initial growth period have been studied only rarely. Thus, a careful evaluation of the EFSA claim using carefully controlled diets in infants is necessary.

D6D knockout mice (D6D-KO mice), cannot convert either n-6 or n-3 fatty acids to their more unsaturated metabolites [9,19,20] and thus serve as an ideal model for investigating requirements for various PUFAs [21]. In this study, we examined the importance of ARA in infancy using a D6D-KO mouse model and an artificial feeding technique from the second day of life.

2. Materials and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Azabu University (No. 1410072).

2.1. D6D-KO mice

D6D-KO mice were back-crossed to the C57BL/J background were transferred from the University of Illinois. Heterozygous male and female fed a custom pelleted diet (termed “MF”, Oriental Yeast Co., Ltd., Tokyo, Japan, Table 1), were mated, and resultant male WT and homozygous KO were used for the study. Tail DNA genotyping by PCR was performed to confirm D6D KO status in mice [9]. DNA was extracted from tails of 2-day-old mice using a tissue PCR kit (Extract-N-Amp™, Sigma-Aldrich, Tokyo, Japan), and PCR was performed using a thermal cycler (T100™, Bio-Rad Laboratories K.K., Tokyo, Japan). After DNA genotyping, both homozygous (D6D-KO, −/−) and wild type (WT, +/+) mice were used in this study. The study design of this experiment is shown as Fig. 1. Newborn male pups of D6D-KO mice were separated from their dams within 48 h, and were fed artificial milks containing 18-C EFAs (LA and LA) with or without ARA. The fat content of two experimental milks (Control and ARA) is approximately 16% and their fatty acid compositions are shown in Table 2. After weaning, mice were housed in an artificially regulated environment at 23 ± 3 °C, 55 ± 10% humidity, under a 12-h light/dark cycle (lights on between 07:00 and 19:00), and fed a similar diet with or without ARA as during lactation (Table 3). The fatty acid compositions of these artificial milks and diets are shown in Table 4. As reference group, new born WT male mouse pups were maintained by artificial milk and diet containing LA and ALA without ARA (WT-Cont). When mice were age 9 weeks, motor coordination and spontaneous motor activity were assessed. At age 10 weeks, blood and brain tissue was collected and fatty acid composition was analyzed using gas chromatography (GC) (Fig. 1).

Table 1
Composition of custom pelleted diet.[1]

	Amount (g/100g diet)
	Pelleted diet
Casein, vitamin free	23.1
Carbohydrate:	55.3
Cellulose	2.8
Mineral-Salt mix	5.8
Fat	5.1
Fatty acid composition (% of total fatty acids)	
Saturates	**19.6**
Monounsaturates	**28.2**
18:2n-6	42.0
20:4n-6	0.13
Total n-6 FAs	**42.3**
18:3n-3	3.9
20:5n-3	1.5
22:6n-3	1.3
Total n-3 FAs	**6.3**
Total FA (μg/mg)	47.0

[1] Pelleted chow is made by Oriental Yeast Co., Ltd., Tokyo, Japan.

2.1.1. Artificial mouse milk

Artificial milk formula was developed based on the methods of Yajima et al. and Hussein et al. [22,23], with slight modifications of fat content (Table 2). Casein and whey protein were used as protein sources and lactose was used as the carbohydrate. Fat was adjusted to 16% by mixing several oils. For complete dissolution, ingredients were mixed in the order described by Yajima et al. [22] using a sonicator (Ultrasonic Processor S-4000, Misonix, Inc., Farmingdale, NY, USA). Milk was homogenized twice under high pressure (800–1000 bar) using a high-pressure homogenizer (Panda PLUS 2000, Niro Soavi S.p.A., Parma, Italy) resulting in emulsified, sterilized, and smoothed milk. The homogenized milk was stored at −80 °C.

2.1.2. Artificial rearing system

The artificial rearing procedure used a hand-feeding technique with specially constructed nursing bottles [24], (Fig. 2). Pups were separated from their dams on postnatal day 2 and fed artificial milk by hand using a nursing bottle every 3 h (5 times/day). Pups were capable of suckling from silicon nipples connected to the nursing bottles. Pups were placed in a cage with an ovariectomized foster mother for maternal care and warmth except at feeding times. From day 14, pups were fed artificial milk from a nursing bottle in combination with a pelleted diet. Infant diets were made by mixing a crushed control diet and their respective artificial milks. Pups in all groups were weaned to the pelleted diet at day 21. Pups were housed in an artificially regulated environment at 23 ± 3 °C, 55 ± 10% humidity after a 12-h light/dark cycle was used (lights on between 07:00 and 19:00).

2.2. Behavioral experiments

2.2.1. Motor activity test

Motor activity was measured using cages (19 × 30 × 13 cm) equipped with running wheels (Wireless Low Profit Running Wheel, ENV-044 wheel, and SOF-860 software, Neuro-science Co., Ltd., Tokyo, Japan). Mice were assessed individually at the same time of day by recording the number of wheel rotations over a 30 min period (08:00–11:00) [25].

2.2.2. Motor coordination test

The motor coordination test was carried out using a rota-rod setup (Rota-Rod Treadmill, ENV-575M, Neuro-science Co., Ltd., Tokyo, Japan). Mice were allowed a trial run of 5 min on the rotor, set at 4 rpm, on the day preceding the measurement. On the following day, motor coordination ability was assessed with rotational speed set to increase from 4 rpm to 40 rpm over 5 min. The