Published in final edited form as:

Metabolism. 2013 April ; 62(4): 499–508. doi:10.1016/j.metabol.2012.10.003.

The Effect of varying ratios of docosaheaxaenoic Acid and arachidonic acid in the prevention and reversal of biochemical essential fatty acid deficiency in a murine model

Hau D. Le^{1,*}, Erica M. Fallon^{1,*}, Brian T. Kalish¹, Vincent E. de Meijer¹, Jonathan A. Meisel¹, Kathleen M. Gura², Vania Nose³, Amy H. Pan¹, Bruce R. Bistran⁴, and Mark Puder^{1,**}

¹Department of Surgery and the Vascular Biology Program, Boston Children's Hospital and Harvard Medical School

²Department of Pharmacy, Boston Children's Hospital

³Department of Pathology, University of Miami and Miller School of Medicine, Miami, FL

⁴Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA

Abstract

© 2012 Elsevier Inc. All rights reserved.

**Corresponding author: Mark Puder, MD, PhD, 300 Longwood Ave, Fegan 3; Boston, MA 02115, Phone: 617-355-1838, Fax: 617-730-0477; mark.puder@childrens.harvard.edu.

*HDL and EMF contributed equally to this work.

AUTHOR CONTRIBUTIONS

1. Hau D. Le : substantial contributions in study conception, design, and conduct; acquisition of data, analysis and interpretation of data; drafting manuscript; final approval of version to be published.
2. Erica M. Fallon : substantial contributions in study design and conduct; acquisition of data and interpretation; drafting manuscript; final approval of version to be published.
3. Brian T. Kalish : substantial contributions in study design and conduct; acquisition of data and interpretation; drafting manuscript; final approval of version to be published.
4. Vincent E. de Meijer: substantial contributions in study design and conduct; acquisition of data and interpretation; reviewing and revising manuscript draft; final approval of version to be published.
5. Jonathan A. Meisel : substantial contributions in study design and conduct; acquisition of data and interpretation; reviewing and revising manuscript draft; final approval of version to be published.
6. Kathleen M. Gura: substantial contributions in study conception, design and conduct; data interpretation; reviewing and revising manuscript draft; final approval of version to be published.
7. Vania Nose: substantial contributions in data interpretation; final approval of version to be published.
8. Amy H. Pan: substantial contributions in acquisition of data and interpretation; final approval of version to be published.
9. Bruce R. Bistran: substantial contributions in study conception, design and conduct; data interpretation; reviewing and revising manuscript draft; final approval of version to be published.
10. Mark Puder: substantial contributions in study conception, design and conduct; data interpretation; reviewing and revising manuscript draft; final approval of version to be published.

DISCLOSURE

DHA (87.4% DHA, 12.6% sterols) was provided by Martek (Columbia, MD).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Objective—Essential fatty acids (EFA) are necessary for growth, development, and biological function, and must be acquired through the diet. While linoleic acid (LA) and alpha-linolenic acid (ALA) have been considered the *true* EFAs, we previously demonstrated that docosahexaenoic acid (DHA) and arachidonic acid (AA) taken together as the sole source of dietary fatty acids can prevent biochemical essential fatty acid deficiency (EFAD). This study evaluates the effect of varying dietary ratios of DHA:AA in the prevention and reversal of biochemical EFAD in a murine model.

Methods—Using a murine model of EFAD, we provided mice with 2.1% of daily caloric intake in varying DHA:AA ratios (1:1, 5:1, 10:1, 20:1, 200:1, 100:0) for 19 days in association with a liquid high-carbohydrate fat-free diet to evaluate the effect on fatty acid profiles. In a second experiment, we evaluated the provision of varying DHA:AA ratios (20:1, 200:1, 100:0) on the reversal of biochemical EFAD.

Results—Mice provided with DHA and AA had no evidence of biochemical EFAD, regardless of the ratio (1:1, 5:1, 10:1, 20:1, 200:1, 100:0) administered. Biochemical EFAD was reversed with DHA:AA ratios of 20:1, 200:1, and 100:0 following 3 and 5 weeks of dietary provision, although the 20:1 ratio was most effective in the reversal and stabilization of the triene:tetraene ratio.

Conclusion—Provision of DHA and AA, at 2.1% of daily caloric intake in varying ratios can prevent biochemical evidence of EFAD and hepatic steatosis over the short-term, with a ratio of 20:1 DHA:AA most effectively reversing EFAD.

Keywords

omega-3; polyunsaturated fatty acid; triene:tetraene ratio; dietary lipid; mouse

Introduction

Fatty acids (FA) are major cellular components that form integral parts of the cell membrane and serve as principal constituents of phospholipids, triglycerides, and cholesterol esters. In mammalian cells, there are three principal types of unsaturated FA: omega-3 (n-3), omega-6 (n-6), and omega-9 (n-9), of which the n-3 and n-6 FAs are considered the families of essential fatty acids (EFAs). These polyunsaturated fatty acids (PUFAs) are necessary for growth, development, and biological function, and must be acquired through exogenous sources because mammals cannot synthesize them from simple carbon precursors. They are usually provided as linoleic acid (LA, 18:2 n-6 FA) and alpha-linolenic (ALA, 18:3 n-3 FA). Essential fatty acid deficiency (EFAD) typically occurs when less than 1-2% of total calories are provided from these EFAs, and can produce dermatitis, alopecia, and/or growth retardation[1].

Traditionally, LA and ALA have been considered *essential* since all downstream FAs can be synthesized from these two 18-carbon precursors. LA is converted to γ -linolenic, dihomo- γ -linolenic acid, and then arachidonic acid (AA), the most biologically active compound. AA downstream products include the 4-series leukotrienes and the 2-series prostanoids, prostaglandins, prostacyclins, and thromboxanes. The homologous downstream products of ALA are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), with the downstream products of EPA being the 5-series leukotrienes and the 3-series prostanoids along with resolvins from both EPA and DHA and neuroprotectins from DHA. While these downstream products collectively mediate numerous physiological and biochemical processes, AA products are thought to be relatively pro-inflammatory while EPA and DHA products are thought to be relatively anti-inflammatory.

In a recent study, we demonstrated that supplementation with DHA and AA alone (without ALA and LA) prevents biochemical and pathophysiologic evidence of EFAD, suggesting that DHA and AA can alternatively be considered the *true* EFAs[2]. However, the optimal ratio of these very long chain n-3 to n-6 FAs to promote growth and brain development, but prevent EFAD, has yet to be determined. Previous recommendations suggest a ratio between 20:1 to 10:1 based on the ratio of EPA plus DHA to AA in fish oil, when fish oil is provided in amounts that prevent EFAD. However, recent studies recommend a higher proportion of n-3 to n-6 FAs to promote the beneficial effects of n-3 FAs[2-4]. The objective of the current study is to evaluate the effect of varying DHA to AA ratios on murine FA profiles, with the aim of determining optimal biochemical ratios to implement clinically in the development of new intravenous lipid emulsions.

Methods

Experiments on EFAD prevention and reversal were performed on 4-6 week-old C57Bl6 male mice and the growth experiment used 6 week-old C57Bl6 female mice (Jackson Laboratories, Bar Harbor, ME). The animals were housed in groups of 5 within a barrier room with a 12-hour light cycle and acclimated to the environment for at least 72 hours prior to initiation of the experiments. During this time, the animals were fed rodent chow (Prolab Isopro, RMH 3000 #25; Prolabs Purina; Richmond, IN) which contained 14% fat, 26% protein, and 60% carbohydrate by calories, and had access to water *ad libitum*. Animals were switched to experimental diets at the initiation of the experiment. Cages were changed and animals were weighed twice weekly following the start of the experiment. The animals receiving the liquid diet had wire-bottom cages and no bedding. The percent change in body weight between study start and study end was calculated for all animals. All experiments were performed in duplicate.

Hydrogenated coconut oil (HCO) and AA (98% grade) were purchased from Cayman Chemical (Ann Arbor, MI). Esterified DHA (87.4% DHA, 12.6% sterols) was provided by Martek (Columbia, MD). HCO, AA and DHA were stored at -60°C. Prior to gavage, the HCO was warmed to 40° Celsius, mixed in methylcellulose vehicle, and then administered in suspension in a liquid state.

The animal protocol (#09-10-1510R) complied with the NIH Animal Research Advisory Committee guidelines and was approved by the Boston Children's Hospital Animal Care and Use Committee.

a. Prevention of EFAD

After the acclimation period, experimental animals (n = 5 per group) were fed a liquid, fat-free, high carbohydrate diet (HCD) *ad libitum* for 19 days, a duration known to cause severe hepatic steatosis and biochemical evidence of EFAD [5]. The HCD contained 20% dextrose, a mixture of 2% essential and nonessential amino acids (TrophAmine, B. Braun Medical, Irvine, CA), 30 mEq/L sodium, 20 mEq/L potassium, 15 mEq/L calcium (as gluconate), 10 mEq/L magnesium, 10 mM phosphate, 5 mEq/L acetate, 30 mEq/L chloride, 0.2% pediatric trace elements (American Reagent, Shirley, NY), and 0.5% pediatric multivitamins (Hospira, Inc., Lake Forest, IL). In addition to the HCD, experimental mice received a lipid mixture via orogastric gavage daily at the same time each morning. The lipid mixture contained DHA and AA, in ratios of 1:1, 5:1, 10:1, 20:1, 200:1 (Table 1), which provided approximately 2.1% of daily caloric intake. HCO was added to bring the total fat calories within the mixtures to 5%. In addition to the aforementioned ratios, there were two additional groups of animals: one received a lipid mixture containing only DHA (100:0 DHA:AA) and the other received a lipid mixture containing only HCO (EFA-deficient).

Control animals (n = 5) were fed an AIN-93M-based purified rodent diet (Dyets Inc., Bethlehem, PA), containing 140 g/kg casein, 1.8 g/kg L-cystine, 100 g/kg sucrose, 465.9 g/kg cornstarch, 155 g/kg dextrose, 40 g/kg soybean oil, 0.8 mg/kg t-butylhydroquinone, 50 g/kg cellulose, 35 g/kg mineral mix, 10 g/kg vitamin mix, and 2.5 g/kg choline bitartrate) with access to water *ad libitum*. The FA composition of the soybean oil included: 6.42% ALA (18:3 n-3), 51.3% LA (18:2 n-6), 21.9% oleic acid (18:1 n-9), 10% palmitic acid (16:0), 3.9% stearic acid (18:0), 0.29% arachidic acid (20:0), and 0.32% behenic acid (22:0).

At the end of the 19-day experiment, animals were fasted for 4 hours and then anesthetized with 300 μ l of 2.5% tribromoethanol (Sigma-Aldrich Corporation, St. Louis, MO) via intraperitoneal injection. Approximately 600 μ l of blood was collected from each mouse via retro-orbital puncture and centrifuged at 4°C at 1,000 g for 10 minutes. The serum was aspirated and alanine aminotransferase (ALT) was measured by the Clinical Laboratory at Boston Children's Hospital. Serum ALT levels were measured as markers of hepatocellular injury. The remaining serum was stored at -80°C and used for serum FA analysis.

Mice were euthanized and livers excised. A designated lobe of the liver was fixed in 10% formalin overnight, washed with chilled 70% ethanol within phosphate buffered saline solution, and embedded in paraffin. The specimens were then sliced and stained with hematoxylin and eosin (H&E). Another portion of the liver was embedded in Tissue-Tek medium (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. It was then sectioned and stained with Oil Red O to detect lipid accumulation. The remaining portion of liver was immediately snap-frozen in liquid nitrogen and used for liver FA analysis.

b. Reversal of EFAD

To investigate the reversibility of EFAD following the administration of AA and DHA, five groups of mice (n=5 each) were fed a HCO (EFA-deficient) diet for 4 weeks, a duration sufficient to cause EFAD. This customized rodent diet differed from the HCD diet used in the prevention study to comply with the animal regulatory policy, as the duration of these experiments was longer. This diet is based on AIN-93M purified rodent diet in which soybean oil was replaced with 5% HCO. After 4 weeks, mice in each group were placed on one of the following diets: Chow (control), EFA-deficient (HCO-R), 20:1 DHA:AA (20:1-R), 200:1 DHA:AA (200:1-R), and 100:0 DHA:AA (100:0-R; Table 1). At 3 and 5 weeks following initiation of the experimental diets, mice were fasted for 4 hours and approximately 100 μ l of blood was obtained via retro-orbital puncture to evaluate serum FA analysis.

c. Evaluation of Diet on Growth

Three groups of 6 week-old female mice (F0 generation) were placed on either the control, HCO, or 20:1 DHA:AA diet for 4 weeks. After 4 weeks, they were bred and offspring (F1 generation) were weaned on day-of-life 21. Five F1 male offspring were kept on the same diet as their mother, and growth was monitored from wean to 8 weeks-old.

d. Analysis of Serum FA Composition

A comprehensive serum FA analysis was performed on serum samples from the prevention and reversal experiments as well as in liver samples from mice in the prevention experiment. Serum was prepared and total lipids were extracted based on the methods of Folch[6]. Briefly, chloroform and methanol (Fisher, Fair Lawn, NJ) were added at a ratio of 2:1 followed by a potassium chloride (Aldrich, Milwaukee, WI) salt wash to isolate the total lipid fraction. Plasma total lipids were extracted from 40 to 150 μ l of plasma. Tricosanoic free FA (Sigma, St. Louis, MO) was added to each sample as an internal standard. The plasma and liver total lipids were saponified with 0.5 N methanolic sodium hydroxide

(Sigma, St. Louis, MO) and the FAs were converted to methyl esters with 14% BF_3 /methanol (Sigma-Aldrich, St. Louis, MO) at 100°C for 30 minutes[7]. Butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO) was added before saponification and all samples were purged with N_2 throughout the process to minimize oxidation. FA methyl esters were analyzed by gas liquid chromatography using a Hewlett Packard 6890 equipped with a flame ionization detector. Peaks were identified by comparison of retention times with external FA methyl ester standard mixtures from NuCheck Prep (Elysian, MN). The FA profiles were expressed as percentage of the total FA (weight percent).

e. Determination of EFAD

The triene-tetraene (T:T) ratio is a biochemical marker used to characterize EFAD, determined when the ratio of serum Mead acid (MA, 20:3 n-9) to AA (20:4 n-6) is greater than 0.2. Another marker of EFAD is the EFA index, defined as the (n-3 + n-6) to (n-7 + n-9) ratio. These ratios are widely accepted biochemical markers to diagnose EFAD in both animals[8-10] and humans[11,12]. In the presence of EFAD, animals will elongate and desaturate oleic acid to produce MA (20:3n-9), and therefore MA levels can indirectly reflect EFAD. In addition, the 16:1/16:0 and 18:1/18:0 desaturation indices are surrogates of the activity of stearoyl-CoA desaturase-1, the rate-limiting enzyme in the synthesis of monounsaturated FA and indicative of increased lipogenesis. When there is a decrease in dietary EFAs, desaturation indices increase.

f. Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). The group means were compared using the analysis of variance (ANOVA) for differences with *post hoc* analysis using Tukey's test. $P < 0.05$ was considered statistically significant. All statistical tests were performed using SPSS version 17.0 (SPSS Inc. Chicago, IL) and all figures were created using Prism 5.01v Software (GraphPad Software Inc, La Jolla, CA).

Results

Prevention of EFAD

All animals survived during the study period. No animals displayed signs of morbidity that are typically associated with EFAD. Animals that received HCO gained less weight than the control mice, but the difference was not statistically significant. Only mice in the 5:1 group gained more weight than the control mice ($P < 0.05$).

a. FA Analysis—Table 2 summarizes the composition of major FAs in the serum and liver of mice in the 19-day prevention experiment. Data are expressed as mean of percentage mole $\pm$ SD.

Linoleic acid and α -linolenic acid: LA (18:2 n-6) and ALA (18:3 n-3) are the previously characterized EFAs. These levels in the serum and liver of experimental groups were significantly lower compared to the control group ($P < 0.01$).

Arachidonic acid: The mean percentage of AA (20:4 n-6) was 14.89% in the serum and 8.84% in the liver of control mice after 19 days. In the HCO group, the serum and liver concentrations were significantly lower at 9.06% and 2.28%, respectively, compared to the control animals ($P < 0.01$). In the 1:1 group, mice received 1.05% of their daily calories as AA; on analysis, the serum and liver levels of AA were significantly higher (22.05% and 10.70%, respectively), compared to the control group ($P < 0.01$ and $P < 0.05$, respectively). As the amount of AA decreased in the diets (5:1, 10:1, 20:1, 200:1), the serum and liver levels of AA steadily decreased and reached statistical significance in the 20:1 and 200:1 groups,

when compared to the control group ($P<0.01$). Mice that received no AA and 2.1% of daily caloric intake as DHA had the lowest serum and liver AA concentrations of 3.61% and 1.85%, respectively.

Eicosapentaenoic acid: The mean percentage of EPA (20:5 n-3) was 0.52% in the serum and 0.20% in the liver of control mice after 19 days. The serum and liver concentrations of EPA showed a generalized upward trend, which paralleled the increasing concentration of DHA in the experimental diets. This increase reached statistical significance in the 5:1, 10:1, 20:1, 200:1, and 100:0 groups in serum ($P<0.01$). Mice in the 200:1 group had the highest serum and liver concentration of EPA at 8.76% and 3.67%, respectively.

Docosahexaenoic acid: The mean levels of DHA (22:6 n-3) were 5.03% in serum and 3.57% in liver of the control mice after 19 days. There was no statistical difference in the serum and liver DHA levels of HCO mice compared to the control mice. However, serum and liver DHA levels in 1:1, 5:1, 10:1, 20:1, 200:1 and 100:0 groups were all significantly higher than those of control mice ($P<0.01$).

b. Evidence of EFAD—Table 3 summarizes the indices and measurements used to assess EFAD in both the serum and liver of mice included in the 19-day prevention experiment. Data are expressed as mean of percentage mole $\pm$ SD.

T:T ratios: Mice did not have biochemical evidence of EFAD after 19 days on the control diet, with serum and liver T:T ratios well below 0.2, which defines biochemical EFAD. Mice in the HCO group (EFA-deficient) had severe EFAD, with serum and liver T:T ratios of 0.74 ± 0.15 and 0.68 ± 0.02 , respectively. All other experimental groups had serum and liver T:T ratios well below 0.2 (range 0.003 – 0.06). The serum T:T ratios of the experimental groups were not significantly different compared to the control mice.

EFA index: Serum and liver EFA indices of control mice were 1.88 ± 0.09 and 0.77 ± 0.13 , respectively. The serum EFA index of HCO mice was significantly lower than that of control mice (0.83 ± 0.25 versus 1.88 ± 0.09 , $P<0.01$). The liver EFA index of HCO mice (0.15 ± 0.01) was also much lower than that of control mice; however the difference was not statistically significant ($P=0.13$). The serum and liver EFA indices of 1:1, 5:1, 10:1, 20:1, 200:1 and 100:0 groups were comparable to those of the control group, with the exception of the serum in the 5:1 group (2.73 ± 0.61) which was the highest index among all groups, and significantly varied from that of control mice ($P<0.05$).

Desaturation indices: The serum 18:1/18:0 and liver 16:1/16:0 and 18:1/18:0 indices in the HCO group were significantly higher than those of the control group, indicating an increase in the synthesis of monounsaturated FAs. In the experimental groups, only liver 16:1/16:0 of the 200:1 and 100:0 groups were significantly higher than the control group ($P<0.01$).

Mead acid: The HCO group had significantly higher serum and liver MA levels of 6.64% and 1.55%, respectively, compared to control levels of 1.04% and 0.59%, respectively ($P<0.01$). The serum and liver MA levels in the 1:1, 5:1, 10:1, 20:1, 200:1, and 100:0 groups were all significantly lower compared to the control group ($P<0.01$).

c. Histology and serum liver enzymes—Figure 1A and 1B show representative H&E and Oil Red O stains of mouse livers in the 19-day experiment. Control mice showed normal hepatic architecture with no evidence of hepatic steatosis. Liver sections from the HCO mice exhibited diffuse macro- and micro-vesicular steatosis. Liver sections from all the other

groups showed well-preserved hepatic architecture and minimal evidence of hepatic steatosis.

There was no statistical difference between mean ALT values of the 1:1, 5:1, 10:1, 20:1 and 200:1 groups compared to the control group (Figure 2). The mean ALT value of the HCO group was higher compared to the control mice, but did not reach statistical significance.

Reversal of EFAD

All animals survived during the study period. No animal displayed signs of morbidity associated with EFAD (e.g., dermatitis, growth retardation, alopecia). All animals gained weight and there was no significant difference in weight gain among groups at the end of 4 weeks on the HCO diet, or at 3 and 5 weeks after switching to the experimental diets (20:1, 200:1, 100:0 DHA:AA; data not shown).

a. FA Analysis—Table 4 summarizes the composition of major FAs in the serum of mice within the reversal experiment at 3 and 5 weeks following initiation of the experimental diets. Data are expressed as mean of percentage mole $\pm$ SD.

LA and ALA: Serum LA and ALA levels were 23.63% and 0.51% at 3 weeks and 20.79% and 0.60% at 5 weeks in control mice. The serum LA and ALA levels were significantly lower in the HCO-R, 20:1-R, 200:1-R and 100:0-R groups at both 3 and 5 weeks ($P<0.01$), as compared to the control group.

AA: Serum AA concentrations of control mice were 14.34% and 13.70% at 3 and 5 week points, respectively. In the HCO-R group, the serum AA levels significantly decreased to 5.17% and 6.98%, compared to the control mice, at the 3 and 5 week points, respectively ($P<0.01$). Moreover, serum AA levels of 20:1-R, 200:1-R and 100:0-R groups were also significantly lower after 3 and 5 weeks of reversal compared to the control group (all $P<0.01$). The lowest serum AA levels at 3 and 5 weeks were seen in the 100:0-R group at 2.90% and 3.49%, respectively.

EPA: Serum EPA concentrations were 0.57% and 0.40% in control mice at the 3 and 5 week points, respectively. These levels decreased in the HCO-R group but were not statistically significant, as compared to the control levels. Mice in the 20:1-R, 200:1-R, and 100:0-R groups had significantly higher serum EPA after 3 and 5 weeks of reversal compared to levels in the control group ($P<0.01$). This indicates a retro-conversion of DHA to EPA in these animals.

DHA: Serum DHA concentrations were 4.54% and 7.28% at the 3 and 5 week points, respectively in the control group. Similar to EPA, these levels decreased in the HCO-R group there was no significant difference from those in the control group. Serum DHA levels in the 20:1-R, 200:1-R and 100:0-R groups both increased significantly after 3 and 5 weeks of dietary reversal.

b.Evidence of EFAD—Table 5 summarizes the indices and measurements to assess EFAD in serum of mice in the reversal experiment. Data are expressed as mean of percentage mole $\pm$ SD.

T:T ratios: The T:T ratios of control mice were 0.01 ± 0.00 and 0.01 ± 0.00 , respectively, at the 3 and 5 week points, well below the value of 0.2 that indicates biochemical EFAD. Mice that remained on the HCO-R diet developed severe biochemical EFAD with mean T:T ratios of 1.29 ± 0.24 and 0.89 ± 0.22 at the 3 and 5 week points, respectively. Mice treated with 20:1-

R, 200:1-R, and 100:0-R diets had T:T ratios of 0.02 ± 0.00 and 0.02 ± 0.01 , 0.06 ± 0.01 and 0.12 ± 0.06 , and 0.10 ± 0.02 and 0.13 ± 0.03 at the 3 and 5 week periods, respectively (Figure 3). The T:T ratios in the 20:1-R group were stable from 3 to 5 weeks, whereas the ratios in the 200:1-R and 100:0-R groups increased over time.

EFA index: Serum EFA indices of control mice at 3 and 5 weeks were 2.83 ± 0.22 and 2.76 ± 0.36 , respectively, compared to 0.56 ± 0.08 and 0.54 ± 0.10 , respectively, in the HCO-R group ($P < 0.01$). Serum EFA indices of the 200:1-R and 100:0-R groups were significantly lower than those of control mice at 3 weeks ($P < 0.01$). At 5 weeks, serum EFA indices of the 20:1-R, 200:1-R and 100:0-R groups were all significantly lower than the control group ($P < 0.01$).

Desaturation indices: The 16:1/16:0 and 18:1/18:0 desaturation indices were significantly higher in HCO-R mice compared to control mice ($P < 0.01$), indicating an effort to convert saturated FAs to monounsaturated FAs to preserve the total number of double bonds and reflecting increased lipogenesis.

MA: The MA in control mice after 3 and 5 weeks was 0.19% and 0.13%, respectively, as compared to 6.53% and 5.98% in the HCO-R group ($P < 0.01$). There were no significant differences between experimental and control groups, although MA levels in the 20:1-R group for both time-points (0.10 ± 0.04 and 0.09 ± 0.03 , respectively) were closest to control values. The values in the 200:1-R and 100:0-R groups were 0.29 ± 0.10 , 0.52 ± 0.30 and 0.29 ± 0.07 , 0.45 ± 0.16 at 3 and 5 weeks, respectively.

Evaluation of Diet on Growth

F1 male mice in the HCO and 20:1 DHA:AA groups weighed significantly less than the control mice at the time of wean (21 days-old). However, from day-of-life 21 until 8 weeks-old, 20:1 DHA:AA animals gained significantly more weight than HCO animals and became indistinguishable from control animals at 8 weeks-old whereas HCO animals remained significantly different from control animals at all time points (Figure 4). There was no evidence of dermatitis or alopecia in any animal, regardless of diet group.

DISCUSSION

The development of EFAD is rare in the general healthy population, but of significant concern for neonates and infants dependent on total parenteral nutrition, as well as in adults and children with severe malabsorption syndromes. We have previously shown that the provision of n-3 FAs found in fish oil as the sole source of fat prevented EFAD and hepatic steatosis in a murine model[9,13,14]. We have additionally shown that purified fish oil without LA and ALA containing approximately 90 - 95% EPA and DHA in similar amounts to those in the present study prevents the development of biochemical evidence of EFAD over the short-term[18]. Furthermore, we have not observed any clinical or biochemical evidence of EFAD in neonates and infants who have been maintained exclusively on a fish oil-based lipid emulsion (without having missed any doses) at our institution[11,15-17].

To more closely evaluate the *essentiality* of FA, our laboratory initially performed experimentation on mice to determine whether exclusive supplementation of DHA and AA, downstream products in the n-3 and n-6 pathways, could prevent EFAD and inhibit or attenuate hepatic steatosis. We found that the provision of DHA and AA at 2.1% of calories for 19 days resulted in normal liver histology without biochemical evidence of EFAD, and persisted when observed after nine weeks without the development of biochemical and clinical EFAD [2]. These results suggest that DHA and AA even without EPA can

alternatively be considered the *true* EFAs[2], and therefore supplementation of these FA alone could be sufficient to prevent EFAD, similar to the traditionally known LA and ALA. An additional advantage of using DHA plus AA or EPA and DHA plus AA (as *true* EFAs) is the ability to achieve lower AA levels that may favorably influence the level of inflammation[19].

In the aforementioned study[2], the ratio of DHA to AA chosen was 20:1, in order to mimic the FA ratio of EPA plus DHA to AA of cold water fish. However, it is unknown whether this ratio is optimal in terms of avoiding EFAD and maximizing the degree of anti-inflammation. In the current study, we evaluated varying ratios of DHA to AA and demonstrated that the provision of DHA and AA at 2.1% of daily caloric intake for 19 days, regardless of the ratio (1:1, 5:1, 10:1, 20:1, 200:1, 100:0; DHA to AA), prevented biochemical EFAD and hepatic steatosis. However, it is acknowledged that over time, the absence of AA altogether (100:0) or perhaps when provided in very small amounts (200:1) would ultimately produce clinical EFAD, even though 19 days was an insufficient study period to accomplish this.

Biochemically, EFAD is characterized by decreased AA and increased MA levels. Oleic acid is only converted to MA under conditions of low ALA and LA as a means of maintaining the steady-state number of double bonds in the cell membrane. The T:T ratio describes the ratio of MA to AA, and a ratio greater than 0.2 defines biochemical EFAD. This study underscores an important caveat in using the T:T ratio as a biochemical marker of EFAD, as animals were fed little or no AA and in amounts substantially less than the usual requirements for LA. However, elevated T:T ratios (>0.2) were not observed. In fact, in evaluating the prevention of EFAD, animals fed with ratios of 1:1, 5:1, 10:1, 20:1, 200:1, and 100:0 DHA to AA had low levels of MA relative to AA, resulting in a low T:T ratio in both the serum and liver samples analyzed. Animals fed 200:1 and 100:0 DHA to AA were found to have the highest T:T ratios among groups, although ratios remained below 0.2. These findings suggest that if the dietary period were prolonged beyond 19 days, the development of EFAD would likely occur in the 200:1 and 100:0 groups. Furthermore, the increase in T:T levels in these groups was entirely due to the relative decrease in AA, as opposed to increased levels of MA. This is further confirmed by their high levels of serum and liver EFA indices, suggesting early evidence of EFAD. The animals in these groups did not display any signs of EFAD (e.g., growth retardation, dermatitis, alopecia).

If the number of double bonds of FAs in cell membranes is an important determinant of MA production, then it is possible that T:T ratios may be a less sensitive indicator of the development of EFAD under these conditions, since EPA and DHA may impede the production of MA by virtue of their multiple double bonds and/or their inhibition of delta-6 and delta-5 desaturases that limit the production of MA. However, our previous study has demonstrated that prolonged dietary intake of DHA and AA in this amount and at a 20:1 ratio prevents the development of biochemical and clinical evidence of EFAD[2].

While the beneficial effects of n-3 FA are well-known, the use of high ratios of DHA to AA to reverse EFAD should be cautioned. In the 200:1-R group, the T:T ratio rose from 0.06 ± 0.01 to 0.12 ± 0.06 between 3 and 5 weeks following reversal of EFAD whereas in the 100:0 group, the T:T ratio was 0.10 ± 0.02 at 3 weeks and 0.13 ± 0.03 at 5 weeks. With an increase in the ratio of DHA to AA, the T:T ratio more closely approached 0.2 (biochemical EFAD) over time which may underestimate the time course of EFAD development, as defined above. In contrast, the 20:1 group demonstrated steady T:T ratios of 0.02 ± 0.00 and 0.02 ± 0.01 between 3 and 5 weeks. This is an important finding, suggesting this formulation of 20:1 DHA to AA may optimally balance the benefits of DHA and derived EPA with the metabolic demands for AA. Over a longer period of time, the animals in the 200:1 and 100:0

groups would invariably develop clinical EFAD. In clinical situations over the short-term, this may be of less concern.

The long-term effects of providing DHA in conjunction with extremely minimal to no AA have not been studied. AA is important for the synthesis of eicosanoids, which play important roles in cellular signaling, inflammation, and vasomodulation. An intact pathway between LA and AA is therefore important to maintain adequate levels of AA and the downstream eicosanoids. Although there is an absolute requirement for LA to maintain skin integrity[20,21], it has been shown that AA can be retroconverted to LA[22] similar to the retroconversion of DHA to EPA. Since there is no evidence that animals can convert n-3 to n-6 FA and vice versa, it is foreseeable that some amount of n-6 FA are required to sustain certain biological functions in animals. Based on our previous work and the results of the present study, AA potentially can replace LA as the sole source of dietary n-6 FAs without detrimental consequences in the short-term. However, long-term studies of the effect of DHA alone or with very small amounts of AA are necessary and important to more thoroughly understand the role AA in both animals and humans.

This study demonstrates that the downstream products of ALA and LA, specifically DHA and AA, can prevent and reverse biochemical indicators of EFAD. This is an important scientific step in defining the necessary content of new lipid emulsions. The data presented in this study furthers translational efforts to create lipid emulsions with ideal proportions of n-3 and n-6 FA to promote growth, brain development, and maintain fertility by achieving the maximal benefits of n-3 FA with minimal, yet essential quantities of n-6 FA while fostering the anti-inflammatory effects of lower AA levels in membrane phospholipids. While there are many components within a lipid emulsion, this work provides further insight into the building blocks necessary to create an emulsion. Nonetheless, additional experiments will be necessary to determine the effects of varying DHA to AA ratios on physiologic function.

Acknowledgments

FUNDING

HDL and EMF were recipients of the Joshua Ryan Rappaport Fellowship. EMF was supported by the National Institute of Diabetes And Digestive and Kidney Diseases (grant F32DK083880). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes And Digestive and Kidney Diseases or the National Institutes of Health. BTK was supported by a Howard Hughes Medical Institute Research Fellowship. HDL, EMF, BTK, VEM, JAM, AHP, and MP were supported by a Translational Research Program Grant, the Surgical Research Foundation, and the Vascular Biology Program at Boston Children's Hospital in Boston, MA.

References

1. Aaes-Jorgensen E, Leppik EE, Hayes HW, et al. Essential fatty acid deficiency. II. In adult rats. *J Nutr.* 1958; 66:245–259. [PubMed: 13599066]
2. Le HD, Meisel JA, de Meijer VE, et al. Docosahexaenoic acid and arachidonic acid prevent essential fatty acid deficiency and hepatic steatosis. *JPEN J Parenter Enteral Nutr.* 2011;110.1177/0148607111414580
3. Simopoulos AP. Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain. *Mol Neurobiol.* 2011; 44:203–215. [PubMed: 21279554]
4. Simopoulos AP. Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Rev Nutr Diet.* 2003; 92:1–22. [PubMed: 14579680]
5. Alwayn IP, Javid PJ, Gura KM, et al. Do polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREPB-1 suppression or by correcting essential fatty acid deficiency. *Hepatology.* 2004; 39:1176–1177. [PubMed: 15057926]

6. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226:497–509. [PubMed: 13428781]
7. Morrison WR, Smith LM. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride--methanol. *J Lipid Res.* 1964; 5:600–608. [PubMed: 14221106]
8. Lukovac S, Los EL, Stellaard F, et al. Effects of essential fatty acid deficiency on enterohepatic circulation of bile salts in mice. *Am J Physiol Gastrointest Liver Physiol.* 2009; 297:G520–531. [PubMed: 19608735]
9. Strijbosch RA, Lee S, Arsenault DA, et al. Fish oil prevents essential fatty acid deficiency and enhances growth: clinical and biochemical implications. *Metabolism.* 2008; 57:698–707. [PubMed: 18442636]
10. Saether T, Tran TN, Rootwelt H, et al. Essential fatty acid deficiency induces fatty acid desaturase expression in rat epididymis, but not in testis. *Reproduction.* 2007; 133:467–477. [PubMed: 17307914]
11. Puder M, Valim C, Meisel JA, et al. Parenteral fish oil improves outcomes in patients with parenteral nutrition-associated liver injury. *Ann Surg.* 2009; 250:395–402. [PubMed: 19661785]
12. Aldamiz-Echevarria L, Vallo A, Aguirre M, et al. Essential fatty acid deficiency profile in patients with nephrotic-range proteinuria. *Pediatr Nephrol.* 2007; 22:533–540. [PubMed: 17123116]
13. Alwayn IP, Gura K, Nose V, et al. Omega-3 fatty acid supplementation prevents hepatic steatosis in a murine model of nonalcoholic fatty liver disease. *Pediatr Res.* 2005; 57:445–452. [PubMed: 15659701]
14. Meisel JA, Le HD, de Meijer VE, et al. Comparison of 5 intravenous lipid emulsions and their effects on hepatic steatosis in a murine model. *J Pediatr Surg.* 2011; 46:666–673. [PubMed: 21496535]
15. Le HD, de Meijer VE, Zurakowski D, et al. Parenteral fish oil as monotherapy improves lipid profiles in children with parenteral nutrition-associated liver disease. *JPEN J Parenter Enteral Nutr.* 2010; 34:477–484. [PubMed: 20852175]
16. Le HD, de Meijer VE, Robinson EM, et al. Parenteral fish-oil-based lipid emulsion improves fatty acid profiles and lipids in parenteral nutrition-dependent children. *Am J Clin Nutr.* 2011; 94:749–758. [PubMed: 21775562]
17. de Meijer VE, Le HD, Meisel JA, et al. Parenteral fish oil as monotherapy prevents essential fatty acid deficiency in parenteral nutrition-dependent patients. *J Pediatr Gastroenterol Nutr.* 2010; 50:212–218. [PubMed: 20038849]
18. Ling PR, Puder M, Bistran BR. Purified fish oil eliminating linoleic and alpha linolenic acid meets essential fatty acid requirements in rats. *Metabolism.* 2012 Apr 16. Epub ahead of print.
19. Ling PR, Boyce P, Bistran BR. Role of arachidonic acid in the regulation of the inflammatory response in TNF-alpha-treated rats. *JPEN J Parenter Enteral Nutr.* 1998; 22:268–275. [PubMed: 9739028]
20. Hansen HS, Jensen B. Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. *Biochim Biophys Acta.* 1985; 834:357–363. [PubMed: 3922424]
21. Zheng Y, Yin H, Boeglin WE, et al. Lipoxygenases mediate the effect of essential fatty acid in skin barrier formation: a proposed role in releasing omega-hydroxyceramide for construction of the corneocyte lipid envelope. *J Biol Chem.* 2011; 286:24046–24056. [PubMed: 21558561]
22. Hansen HS, Jensen B, von Wettstein-Knowles P. Apparent in vivo retroconversion of dietary arachidonic to linoleic acid in essential fatty acid-deficient rats. *Biochim Biophys Acta.* 1986; 878:284–287. [PubMed: 3092868]

Abbreviations

AA	arachidonic acid
ALA	alpha-linolenic acid

ALT	alanine aminotransferase
ANOVA	analysis of variance
DHA	docosahexaenoic acid
EFA	essential fatty acids
EFAD	essential fatty acid deficiency
EPA	eicosapentaenoic acid
FA	fatty acid
H&E	hematoxylin and eosin
HCD	high carbohydrate diet
HCO	hydrogenated coconut oil
LA	linoleic acid
MA	Mead acid
PUFA	polyunsaturated fatty acid
SD	standard deviation
T:T	triene-tetraene

Figure 1. H&E (1A) and Oil Red O (1B) stains of mouse livers in the 19-day prevention experiment. Control mice showed normal hepatic architecture without evidence of hepatic steatosis. Liver sections from the HCO mice exhibited diffuse macro and microvesicular steatosis. Liver sections from all the other groups showed well-preserved hepatic architecture with minimal evidence of hepatic steatosis.

Figure 2.

Serum ALT levels in the 19-day prevention experiment. There was no difference between mean ALT values of the 1:1, 5:1, 10:1, 20:1 and 200:1, and 100:0 groups compared to that of the control group.

Figure 3. T:T ratio measured at 3 and 5 weeks in the reversal experiment. The dotted line represents 0.2, the T:T ratio at which EFAD is biochemically defined.

Figure 4. Growth (in grams) of F1 male mice in the control, HCO, and 20:1 DHA:AA diet groups from wean to 8 weeks-old.

Table 1

The percentage of HCO, DHA, and AA in each lipid mixture

Group	HCO (%)	DHA (%)	AA (%)
Control, Control-R	-	-	-
HCO, HCO-R	5:00	0.00	0.00
1:1	2.90	1.05	1.05
5:1	2.90	1.75	0.35
10:1	2.90	1.90	0.19
20:1; 20:1-R	2.90	2.00	0.10
200:1, 200:1-R	2.90	2.09	0.01
100:0, 100:0-R	2.90	2.10	0.00

The prevention experiment has 8 groups: Control, HCO, 1:1, 5:1, 10:1, 20:1, 200:1 and 100:0.

The reversal experiment has 5 groups: Control-R, HCO-R, 20:1-R, 200:1-R and 100:0-R.

HCO, hydrogenated coconut oil; DHA, docosahexaenoic acid; AA, arachidonic acid; -R, reversal.

Table 2

Fatty acid composition of serum and liver in the prevention experiment

Mole (%)	Control	HCO	1:1	5:1	10:1	20:1	200:1	100:0
<i>Serum</i>								
16:0	21.75±0.89	23.60±0.33	27.44±0.57 [†]	27.63±1.26 [†]	26.33±0.61 [†]	23.55±2.10	28.20±1.56 [†]	28.26±1.23 [†]
16:1	5.19±0.35	7.22±0.90 [†]	5.48±0.58	5.30±0.91	5.35±0.49	5.05±0.51	6.15±0.80	5.99±0.91
18:0	10.07±0.40	9.67±1.01	10.20±0.37	10.03±0.85	9.46±0.23	11.19±1.94	9.48±0.34	8.98±0.95
18:1 n-9	15.70±0.38	19.66±4.04 [*]	14.07±1.04	13.49±1.73	14.70±1.53	22.70±5.16 [*]	15.54±1.02	17.48±2.14
18:1 n-7	4.22±0.38	4.93±0.66	1.58±0.42 [†]	1.28±0.21 [†]	1.60±0.28 [†]	2.67±1.19 [†]	1.63±0.27 [†]	1.59±0.26 [†]
18:2 n-6	16.22±0.73	7.46±1.50 [†]	1.31±0.34 [†]	3.37±0.47 [†]	3.21±1.09 [†]	2.96±0.72 [†]	4.68±1.13 [†]	5.66±1.74 [†]
18:3 n-6	0.30±0.04	0.19±0.10 [†]	0.09±0.01 [†]	0.02±0.01 [†]	0.03±0.01 [†]	0.03±0.03 [†]	0.02±0.02 [†]	0.04±0.05 [†]
18:3 n-3	0.24±0.04	0.08±0.03 [†]	0.03±0.00 [†]	0.02±0.02 [†]	0.05±0.02 [†]	0.09±0.03 [†]	0.06±0.05 [†]	0.04±0.03 [†]
20:3 n-9	1.04±0.25	6.64±0.59 [†]	0.07±0.07 [†]	0.06±0.05 [†]	0.09±0.02 [†]	0.18±0.02 [†]	0.10±0.03 [†]	0.12±0.06 [†]
20:3 n-6	2.34±0.23	1.98±0.31 [*]	0.14±0.12 [†]	0.16±0.10 [†]	0.25±0.05 [†]	0.30±0.14 [†]	0.27±0.08 [†]	0.32±0.10 [†]
20:4 n-6	14.89±0.60	9.06±0.90 [†]	22.05±1.06 [†]	15.91±3.04	12.08±1.56	7.51±1.62 [†]	4.09±0.61 [†]	3.61±0.42 [†]
20:5 n-3	0.52±0.06	0.28±0.08	1.62±0.19	4.98±0.23 [†]	6.48±0.72 [†]	4.58±1.66 [†]	8.76±0.84 [†]	8.24±0.96 [†]
22:4 n-6	0.09±0.05	0.10±0.02	0.16±0.08	0.05±0.03	0.00±0.00 [*]	0.04±0.03	0.01±0.02 [*]	0.00±0.00 [*]
22:5 n-6	0.42±0.05	0.74±0.12 [†]	0.05±0.03 [†]	0.00±0.00 [†]	0.00±0.00 [†]	0.02±0.02 [†]	0.00±0.00 [†]	0.10±0.22 [†]
22:5 n-3	0.19±0.02	0.11±0.02	0.39±0.07 [*]	0.41±0.05 [†]	0.54±0.08 [†]	0.54±0.14 [†]	0.61±0.02 [†]	0.68±0.21 [†]
22:6 n-3	5.03±0.33	5.47±0.17	13.16±1.27 [†]	15.20±1.03 [†]	17.93±0.91 [†]	16.65±3.13 [†]	18.50±1.27 [†]	16.34±1.47 [†]
<i>Liver</i>								
16:0	23.84±0.66	24.79±0.71	26.61±1.43 [†]	25.08±1.02	26.63±1.29 [†]	26.03±0.86 [*]	25.40±0.77	25.25±1.11
16:1	7.25±1.10	10.51±0.34 [†]	9.16±1.57 [*]	9.23±0.75 [*]	9.70±0.51 [†]	6.51±0.64	10.31±0.62 [†]	10.32±0.50 [†]
18:0	8.81±0.80	4.75±0.26 [†]	10.04±1.03	8.41±0.97	7.97±0.69	6.85±1.32 [*]	7.24±0.74	6.69±0.49 [*]
18:1 n-9	26.05±1.88	38.80±1.03 [†]	21.31±2.38	23.02±1.86	24.77±2.35	28.85±9.65	24.13±1.70	26.40±1.80
18:1 n-7	5.64±0.29	6.62±0.66	2.50±0.39 [†]	2.22±0.31 [†]	3.09±0.35 [†]	3.33±1.74 [†]	2.67±0.21 [†]	2.75±0.67 [†]
18:2 n-6	10.59±0.80	2.75±0.05 [†]	1.78±0.55 [†]	3.93±0.14 [†]	2.93±0.87 [†]	2.47±0.77 [†]	3.95±0.98 [†]	4.44±0.99 [†]
18:3 n-6	0.14±0.04	0.05±0.01 [†]	0.02±0.00 [†]	0.01±0.00 [†]	0.01±0.00 [†]	0.02±0.01 [†]	0.01±0.00 [†]	0.01±0.00 [†]

Mole (%)	Control	HCO	1:1	5:1	10:1	20:1	200:1	100:0
18:3 n-3	0.24±0.07	0.03±0.01 [†]	0.03±0.01 [†]	0.16±0.03	0.12±0.03 [†]	0.12±0.06 [†]	0.20±0.03	0.22±0.06
20:3 n-9	0.59±0.09	1.55±0.11 [†]	0.05±0.02 [†]	0.04±0.01 [†]	0.07±0.02 [†]	0.08±0.07 [†]	0.08±0.01 [†]	0.11±0.03 [†]
20:3 n-6	1.30±0.13	0.41±0.04 [†]	0.22±0.05 [†]	0.17±0.02 [†]	0.16±0.02 [†]	0.15±0.05 [†]	0.20±0.06 [†]	0.21±0.04 [†]
20:4 n-6	8.84±1.08	2.28±0.24 [†]	10.70±1.35 [*]	6.28±1.26 [†]	4.60±0.60 [†]	3.36±0.48 [†]	2.26±0.59 [†]	1.85±0.22 [†]
20:5 n-3	0.20±0.05	0.03±0.01	1.06±0.15	2.40±0.09 [†]	2.49±0.20 [†]	1.99±1.08 [†]	3.67±0.45 [†]	3.27±0.50 [†]
22:4 n-6	0.16±0.04	0.05±0.01 [*]	0.40±0.09 [†]	0.14±0.02	0.09±0.01	0.08±0.02	0.04±0.01 [†]	0.04±0.01 [†]
22:5 n-6	0.27±0.07	0.19±0.04 [*]	0.08±0.02 [†]	0.04±0.01 [†]	0.03±0.01 [†]	0.01±0.02 [†]	0.04±0.01 [†]	0.04±0.01 [†]
22:5 n-3	0.15±0.05	0.04±0.01	0.59±0.12 [*]	0.93±0.15 [†]	0.88±0.08 [†]	0.87±0.40 [†]	1.18±0.13 [†]	1.10±0.18 [†]
22:6 n-3	3.57±0.89	1.53±0.11	12.75±1.62 [†]	14.82±1.13 [†]	13.30±0.82 [†]	16.94±8.47 [†]	15.39±1.17 [†]	13.98±2.58 [†]

The group means were compared using analysis of variance (ANOVA) for difference with *post hoc* analysis using Tukey's test.

^{*},[†] indicate statistically significant differences with *P*<0.05 and *P*<0.01, respectively, compared to the control group.

HCO, hydrogenated coconut oil.

Table 3
Indices and measurements of essential fatty acid deficiency in serum and liver in the prevention experiment

	Control	HCO	1:1	5:1	10:1	20:1	200:1	100:0
Serum T:T ratio	0.07±0.02	0.74±0.15 [‡]	0.003±0.003	0.004±0.003	0.007±0.002	0.02±0.03	0.02±0.01	0.03±0.01
Serum EFA index	1.88±0.09	0.83±0.25 [‡]	2.46±0.30	2.73±0.61 [*]	2.47±0.37	1.36±0.62	2.13±0.27	1.83±0.31
Serum (DHA+EPA)/AA	0.37±0.02	0.64±0.07	0.67±0.07	1.30±0.23	2.04±0.22 [‡]	2.91±0.69 [‡]	6.78±0.94 [‡]	6.87±0.91 [‡]
Serum 16:1/16:0	0.23±0.02	0.31±0.04	0.20±0.02	0.19±0.02	0.19±0.01	0.22±0.03	0.21±0.04	0.21±0.03
Serum 18:1/18:0	1.56±0.08	2.08±0.58 [‡]	1.40±0.15	1.36±0.25	1.56±0.19	2.12±0.77	1.97±0.37	1.64±00.08
Liver T:T ratio	0.07±0.01	0.68±0.02 [‡]	0.004±0.002 [‡]	0.007±0.003 [‡]	0.02±0.006 [‡]	0.03±0.02 [‡]	0.04±0.01 [*]	0.06±0.01
Liver EFA index	0.77±0.13	0.15±0.01	1.15±0.25	1.13±0.17	0.87±0.13	1.00±0.73	0.98±0.12	0.85±0.16
Liver (DHA+EPA)/AA	0.42±0.09	0.69±0.09	1.25±0.10	2.59±0.52	3.11±0.33	5.13±1.98 [‡]	7.90±2.82 [‡]	8.32±2.21 [‡]
Liver 16:1/16:0	0.30±0.05	0.42±0.01 [‡]	0.36±0.02	0.37±0.04	0.34±0.06	0.25±0.03	0.41±0.01 [‡]	0.41±0.03 [‡]
Liver 18:1/18:0	2.99±0.43	8.19±0.62 [‡]	2.15±0.40	2.78±0.55	3.14±0.52	5.54±2.18	3.38±0.28	3.98±0.53

The group means were compared using analysis of variance (ANOVA) for difference with *post hoc* analysis using Tukey's test.

^{*}, [‡] indicate statistically significant differences with $P<0.05$ and $P<0.01$, respectively, compared to the control group.

HCO, hydrogenated coconut oil; T:T ratio, triene-tetraene ratio; EFA index, essential fatty acid index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid.

Table 4

Fatty acid composition of serum after 3 and 5 weeks of dietary reversal

Mole (%)	Control	HCO-R	20:1-R	200:1-R	100:0-R
<i>3 week</i>					
16:0	22.50±1.71	17.97±0.27 [†]	30.81±1.50 [†]	24.59±1.36	23.08±0.82
16:1	4.21±0.65	11.78±0.58 [†]	4.05±0.58	8.70±0.66 [†]	9.55±1.13 [†]
18:0	9.08±0.56	8.88±0.30	13.40±1.14 [†]	9.54±0.22	9.82±0.62
18:1 n-9	13.19±0.69	21.29±1.37 [†]	11.30±0.96 [*]	16.16±1.24 [†]	16.31±1.49 [†]
18:1 n-7	2.38±0.15	5.32±0.46 [†]	0.96±0.17 [†]	2.04±0.44	2.58±0.86
18:2 n-6	23.63±0.72	8.29±0.60 [†]	8.63±0.55 [†]	9.20±0.52 [†]	8.77±0.35 [†]
18:3 n-6	0.15±0.07	0.32±0.15 [*]	0.00±0.00 [†]	0.00±0.00 [*]	0.00±0.00 [*]
18:3 n-3	0.51±0.08	0.16±0.16 [†]	0.05±0.02 [†]	0.15±0.09 [†]	0.05±0.02 [†]
20:3 n-9	0.19±0.05	6.53±0.39 [†]	0.10±0.04	0.29±0.10	0.29±0.07
20:3 n-6	1.50±0.12	1.46±0.13	0.19±0.05 [†]	0.41±0.15 [†]	0.42±0.08 [†]
20:4 n-6	14.34±1.54	5.17±0.93 [†]	6.04±0.53 [†]	4.52±0.84 [†]	2.90±0.32 [†]
20:5 n-3	0.57±0.08	0.17±0.02	3.89±0.50 [†]	6.17±1.23 [†]	6.91±1.29 [†]
22:4 n-6	0.24±0.21	0.13±0.04	0.02±0.02	0.00±0.00	0.38±0.32
22:5 n-6	0.16±0.06	0.76±0.06 [†]	0.00±0.00 [†]	0.01±0.01 [†]	0.00±0.00 [†]
22:5 n-3	0.19±0.03	0.03±0.01	0.46±0.07 [†]	0.50±0.09 [†]	0.25±0.19
22:6 n-3	4.54±0.57	2.17±0.15	14.54±0.82 [†]	14.32±2.06 [†]	13.80±1.89 [†]
<i>5 weeks</i>					
16:0	24.18±0.79	22.80±0.92	29.19±4.12	21.19±7.92	24.96±1.59
16:1	4.81±0.54	7.73±1.67	7.51±0.83	9.86±2.70 [†]	6.80±0.98
18:0	7.57±0.29	6.62±0.42	14.89±9.82	7.74±3.36	6.68±0.99
18:1 n-9	13.52±0.81	24.82±1.20 [†]	15.60±1.86	15.74±7.78	19.58±2.51
18:1 n-7	2.42±0.32	7.34±0.80 [†]	1.12±0.24 [*]	3.70±1.15 [*]	2.96±0.73
18:2 n-6	20.79±0.67	8.29±0.65 [†]	7.00±1.18 [†]	4.29±3.52 [†]	6.37±1.04 [†]
18:3 n-6	0.44±0.02	0.18±0.05 [†]	0.00±0.00 [†]	0.02±0.03 [†]	0.01±0.02 [†]

Mole (%)	Control	HCO-R	20:1-R	200:1-R	100:0-R
18:3 n-3	0.60±0.15	0.03±0.01 [†]	0.09±0.02 [†]	0.09±0.08 [†]	0.02±0.02 [†]
20:3 n-9	0.13±0.07	5.98±0.48 [†]	0.09±0.03	0.52±0.30	0.45±0.16
20:3 n-6	1.25±0.07	1.30±0.19	0.16±0.06 [†]	0.33±0.20 [†]	0.32±0.09 [†]
20:4 n-6	13.70±1.23	6.98±1.44 [†]	5.01±0.85 [†]	4.33±0.61 [†]	3.49±0.57 [†]
20:5 n-3	0.40±0.08	0.03±0.01	3.88±0.68 [†]	2.27±1.32 [†]	4.25±0.82 [†]
22:4 n-6	0.09±0.01	0.06±0.03	0.00±0.00 [†]	0.01±0.02 [†]	0.01±0.01 [†]
22:5 n-6	0.18±0.06	0.65±0.08 [†]	0.00±0.00 [†]	0.05±0.04 [†]	0.02±0.03 [†]
22:5 n-3	0.42±0.03	0.00±0.00 [†]	0.38±0.11	0.55±0.11	0.67±0.14
22:6 n-3	7.28±0.76	3.69±0.53	11.12±1.78 [*]	19.58±3.40 [†]	21.18±3.24

The group means were compared using analysis of variance (ANOVA) for difference with *post hoc* analysis using Tukey's test.
^{*},[†] indicate statistically significant differences with *P*<0.05 and *P*<0.01, respectively, compared to the control group.
HCO, hydrogenated coconut oil.

Table 5

Indices and measurements of essential fatty acid deficiency in serum after 3 and 5 weeks of dietary reversal

	Control	HCO-R	20:1-R	200:1-R	100:0-R
<i>3 weeks</i>					
T:T ratio	0.01±0.00	1.29±0.24 [‡]	0.02±0.00	0.06±0.01	0.10±0.02
EFA index	2.83±0.22	0.56±0.08 [‡]	2.60±0.23	1.88±0.31 [‡]	1.71±0.32 [‡]
(DHA+EPA)/AA	0.36±0.04	0.46±0.06	0.72±0.04	4.64±1.03 [‡]	7.24±1.40 [‡]
16:1/16:0	0.19±0.03	0.66±0.03 [‡]	0.13±0.03	0.35±0.04 [‡]	0.42±0.06 [‡]
18:1/18:0	1.46±0.13	2.40±0.20 [‡]	0.85±0.14 [‡]	1.70±0.12	1.81±0.40
<i>5 weeks</i>					
T:T ratio	0.01±0.00	0.89±0.22 [‡]	0.02±0.01	0.12±0.06	0.13±0.03
EFA index	2.76±0.36	0.54±0.10 [‡]	1.70±0.51 [‡]	1.90±1.23 [‡]	1.60±0.40 [‡]
(DHA+EPA)/AA	0.56±0.04	0.54±0.04	0.85±0.05	5.15±1.43 [‡]	7.44±1.59 [‡]
16:1/16:0	0.20±0.02	0.34±0.08 [‡]	0.26±0.07	0.67±0.30 [‡]	0.27±0.05
18:1/18:0	1.79±0.12	3.77±0.39 [‡]	1.53±0.73	2.48±1.30	3.00±0.67

The group means were compared using analysis of variance (ANOVA) for difference with *post hoc* analysis using Tukey's test.

^{*, ‡} indicate statistically significant differences with $P<0.05$ and $P<0.01$, respectively, compared to the control group.

HCO, hydrogenated coconut oil; T:T ratio, triene-tetraene ratio; EFA index, essential fatty acid index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid.