Dietary Long-Chain Polyunsaturated Fatty Acids from Different Sources Affect Fat and Fatty Acid Excretions in Rats

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ABSTRACT Several sources of long-chain polyunsaturated fatty acids (LCP) have been evaluated for infant-formula supplementation. These sources differ in their chemical structure [triglyceride (TG) or phospholipid (PL)], arrangement of fatty acids on the TG or PL backbone, fatty acid composition and presence of other lipid components. All of these characteristics influence fat digestion, may affect fat and fatty acid absorption, and hence, LCP bioavailability and metabolism in infancy. The main objective of this work was to establish the influence of different dietary LCP sources on overall fat and LCP absorption in early life. We compared fat and fatty acid excretions at weaning in rats fed control diets or diets supplemented with LCP as TG or PL. Two separate experiments were conducted. In Experiment 1, weanling rats were fed for 3 wk a control diet (C1), a diet with TG from tuna and fungal oils (TF-TG) or a diet with PL from pig brain concentrate (PB-PL). In Experiment 2, weanling rats were fed for 3 wk a control diet (C2), a diet containing egg-TG (EG-TG) or a diet containing egg-PL (EG-PL).

Fat, mineral and saturated fatty acid excretions in feces were higher in rats fed TF-TG compared with those fed TF-TG diet. In Experiment 2, groups did not differ in fat and mineral excretions. However, the EG-PL group had lower fecal excretions of saturated fatty acids than the C2 and EG-TG groups. The 16:1(n-7), 18:1(n-9), 18:2(n-6) and 22:6(n-3) levels in feces were higher in the EG-PL group than in the EG-TG group. In summary, total fat and LCP excretions differed among rats fed diets supplemented with LCP from different sources.


KEY WORDS: • absorption • excretion • fat • long-chain polyunsaturated fatty acids • rats

Long-chain polyunsaturated fatty acids (LCP),1 especially arachidonic (AA) and docosahexaenoic (DHA) acids, are essential components of the central nervous system and, early in life, are involved in the development of the neurological and visual systems (1–3). Several reports have indicated that LCP status is improved in infants fed LCP-containing formulas (4,5). Moreover, accelerated visual development and enhanced cognitive development have been found in low-birth-weight infants fed human milk or LCP-supplemented formulas (3,6–12). For this reason, several international committees have proposed the use of formulas containing balanced quantities of (n-3) and (n-6) LCP, similar to those found in human milk. This recommendation is of particular importance for preterm infants (13–17).

There are several sources of LCP such as fish oils, oils from unicellular organisms or from eggs that could be used to supplement infant formula and meet such recommendations. The mentioned lipid sources differ in several aspects including the following: 1) Fatty acid composition: some sources are enriched in one LCP from the (n-6) or (n-3) series (algae or fungi); others contain variable amounts of both LCP series (egg lipids). 2) Chemical structure [triglyceride (TG) or phospholipid (PL): triglyceride or PL digestion yields different products at the intestinal level, which may in turn follow different metabolic pathways. Triglycerides are hydrolyzed by pancreatic lipase to 2-monoglycerides (2-MG) and free fatty acids (FFA) (18,19), whereas PL are attacked by pancreatic phospholipase A2 yielding lysophospholipids (lysoPL) and FFA. Ionized FFA and 2-MG enter into bile micelles, to form mixed micelles with PL, which help nonpolar lipids pass through the unstirred water layer and reach the microvillous membrane where they are absorbed. Absorbed lipids are reesterified to newly formed TG and PL in the smooth endoplasmic reticulum. Triglycerides can be synthesized via 2-MG or 3-glycerol-phosphate, although in the fed state, the 2-MG pathway predominates (20). Phospholipids are synthesized from 1-lysoPL and FFA. 3) Molecular structure: the positional distribution of each fatty acid within dietary TG determines whether fatty acids are absorbed as 2-MG or FFA, thus influencing the composition of the newly formed chylomicrons. However, little specificity is shown concerning whether FFA are reesterified to the sn-1 or sn-2 position (21). 4) Presence of other components: dietary phospholipids contain a phosphate group and a nitrogen base (mainly choline) that may interact
in several metabolic pathways (22). Moreover, some PL sources (from egg yolk or animal tissues) contain substantial amounts of cholesterol. The differences among these lipid sources (from egg yolk or animal tissues) contain substantial

Because of the above-mentioned differences, LCP from different sources may not be equally bioavailable. Therefore, the purpose of the present study was to evaluate a number of LCP sources for their influence on fat and fatty acid absorption in rats at weaning. The sources of LCP evaluated in this study are pig-brain PL (PB-PL), egg PL (EG-PL), tuna and fungal TG (TF-TG) and egg TG (EG-TG).

**MATERIALS AND METHODS**

**Animals and feeding**

Female weanling Wistar rats (Interfauna Iberica; Barcelona, Spain) were housed in individual metabolic cages in a room maintained at a constant temperature and kept on a 12-h light:dark cycle. Rats were fed their assigned experimental diet for 3 wk. Rats consumed food and water ad libitum. Fresh food was weighed and given daily. All diets had the same macronutrient composition (g/100 g): protein, 20.3 (calcium caseinate); carbohydrates, 65.7 (wheat starch/cellulose, 65.3:22.5:12); fat, 3.9 (vegetable oils); ash, 2.6; and moisture, 7.5; they differed only in their fat composition. Diets followed the AIN-76 international recommendation (23). Although there have been later recommendations (AIN-93), at the time these experiments were planned, AIN-93 recommendations had not been implemented in our laboratory. In fact, for several years, both recommendations coexisted; even now, some investigators still use diets based on the AIN-76 recommendations (24,25).

Due to the different ratios of AA/DHA present in the LCP sources used in this study, it was not possible to adjust the proportion of these fatty acids in all of the diets; therefore two separate experiments were conducted.

**Experiment 1.** Rats (n = 30) were randomly divided into three groups. One group was fed a control diet without added LCP (C1), one a diet containing LCP in form of TF-TG and EG-TG from a fish oil rich in DHA (Mochida, Tokyo, Japan) and a fungal oil rich in AA (Suntory, Tokyo, Japan), and the third group a diet containing LCP in form of a pig brain PL concentrate (PB-PL). The C1 fat consisted of a mixture of vegetable oils (olive/soybean/refined coconut, 66:5:23:10.5). Part of the vegetable oil of the C1 diet was replaced by the corresponding LCP source to reach the same concentration of AA and DHA in both supplemented diets (1 and 0.9 g/100 g total fatty acids, respectively). The fatty acid composition for the diets of Experiment 1 is given in Table 1.

**Experiment 2.** Rats (n = 30) were randomly divided into three groups. One group was fed a control diet without added LCP (C2), one a diet containing LCP in the form of TG obtained from egg yolk by a reesterification process (EG-TG), and the third group was fed a diet containing LCP in form of PL from egg yolk (EG-PL) provided by Lucas Meyer (Hamburg, Germany). Fat of the C2 diet consisted of a mixture of vegetable oils (high oleic sunflower/palm/soybean/coconut, 39:21:21:19). In the EG-TG and EG-PL diets, part of the vegetable oil was replaced by each LCP source to reach the same concentration of AA and DHA in both supplemented diets (3.3 and 0.9 g/100 g total fatty acids, respectively). In addition, a small proportion of soy oil had to be added to the supplemented diets to increase 18:3(n-3) content. The fatty acid composition for the diets of Experiment 2 is shown in Table 2.

In both experiments, feces were collected daily and stored at 20°C. Samples of seven consecutive days during wk 2 of the study (d 8 to 14) were pooled, lyophilized and analyzed for the contents of fat, calcium, magnesium, phosphorus and fatty acids. Fat apparent absorption was calculated by difference between the amount of fat ingested with the diet and that excreted in feces.

The study was approved by the Animal Care Committee at the University of Granada and conformed with the European Union Regulation of Animal Care for care and use of animals for research.

**Analytical methods**

Total fat content in feces was determined gravimetrically after treatment with hydrochloric acid to convert soap into free fatty acids and extracted with organic solvent (26). Lyophilized feces (~2 g) were treated with 20% (v/v) hydrochloric acid, then extracted with petroleum ether under reflux, dried and weighed.

Calcium and magnesium contents in feces were determined by atomic absorption spectrometry (26) and phosphorus by a spectrophotometric method after acid digestion of the organic matrix (26). Lyophilized feces (~2 g) were incinerated, dissolved in concentrated nitric acid and diluted in deionized water. An aliquot was added with lanthanum oxide to avoid the formation of phosphate salts and calcium and magnesium measured. Another aliquot was treated with concentrated sulfuric acid and diluted in deionized water; phosphorus was determined by reaction with ammonium molybdate and 1-amino-2-hydroxy-4-naphthalenesulfonic acid and absorbance measured at 690 nm (24).

Fatty acids were identified by gas-liquid chromatography using a Hewlett-Packard no. 3989 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a 60 m long x 0.32 mm i.d. capillary SP-2330 column (Supelco, Bellefonte, PA). Nitrogen at a flow rate of 1 mL/min was used as the carrier gas, and the split ratio was 1:40. Temperature programming consisted of 165°C for 3 min, an increase of 2°C/min to 195°C, held for 2 min at 195°C, an increase of 3°C/min to 211°C, held 6 min at 211°C and a decrease of 15°C/min to return to 150°C. Injector and detector temperature were 275°C. Fatty acids were identified by comparing their retention times with those of authentic standards (Sigma, St. Louis, MO) and the results were expressed as concentrations in mg/100 g lyophilized feces.

**Statistical analysis**

Data were analyzed by one-way ANOVA. Homogeneity of variances was assessed by the Levene’s test. When a significant effect was
Food intake, g/d from egg-yolk PL; EG-TG, group fed a diet supplemented with LCP from egg-yolk TG; EG-PL, group fed a diet supplemented with LCP from tuna and fungal oil TG; C2, group fed the control diet of Experiment 2; ND, not detected.

**RESULTS**

**Growth**

There were no significant differences in weight gains or final body weights among groups in either Experiment 1 or 2 (Table 3). In Experiment 1, food intake was significantly higher in the PB-PL group than in control and TF-TG groups, but the food efficiency ratio (FER) did not differ among groups. In Experiment 2, the FER was significantly greater in the EG-PL group compared with the EG-TG and control groups.

**Fat and mineral excretion**

In Experiment 1, the concentration of fat in feces was higher, and the fat apparent absorption lower in rats fed the PB-PL diet than in other two groups. Magnesium and phosphorus contents in feces were also higher in this group than in other groups (Table 4). However, Ca excretion did not differ among groups. No significant differences were found for fecal fat and mineral excretions in Experiment 2.

**Fatty acid excretion**

**Experiment 1.** Rats fed the diet supplemented with PB-PL excreted more saturated fatty acids in feces than the other groups (Table 5). The levels of the LCP 20:4(n-6), 22:4(n-6) and 22:6(n-3) in feces were also higher in the PB-PL group than in the other groups. This greater excretion of fatty acids was related to their lower absorption and is in agreement with this group’s fat apparent absorption. On the other hand, no differences were found between rats fed the control and TF-TG diets.

**Experiment 2.** Rats fed the LCP-supplemented diet from egg-yolk TG (EG-TG) excreted more saturated (16:0 and 18:0) and other fatty acids such as 16:1(n-7), 18:1(n-9), 18:2(n-6) and 22:6(n-3), than rats fed the EG-PL diet (Table 6). Controls generally excreted more of these fatty acids than the EG-PL group.

**DISCUSSION**

In the present study, the effect of the addition to the diet of several LCP sources on fat and fatty acid excretion and fat apparent absorption was investigated in rats at weaning. The LCP sources tested were tuna and fungal TG, egg-derived TG, pig-brain PL and egg PL. At the time these experiments were conducted, all of these ingredients were considered appropriate for human consumption. The PB-PL concentrate was tested in different experiments with piglets and showed good properties such as a high stability and adequate ratio AA/DHA, and positive effects on plasma and tissue composition of healthy and malnourished pigs (29,30). This source was also evaluated in a clinical trial with low-birth-weight infants (31). There currently are many concerns about the use of ingredients of animal origin in infant formulas; thus PB-PL are no longer available for infant formula supplementation. EG-TG were studied as an alternative to avoid some of the concerns surrounding EG-PL, such as the cholesterol and the phosphatidylcholine content; however, the cost of its production and the need for an additional source of (n-3) fatty acids to increase the content of 22:6(n-3) makes its use less desirable. In spite of that, the sources studied in this work differed in their fatty acid composition, chemical structure (TG or PL), ar-

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**Table 3**

Initial and final body weights, weight gain daily food intake and the food efficiency ratio (FER) in rats fed a control diet or diets supplemented with long-chain polyunsaturated fatty acids (LCP) as triglycerides (TG) or phospholipids (PL)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>PB-PL</td>
</tr>
<tr>
<td>Initial body weight, g</td>
<td>43.7 ± 2.5</td>
<td>48.1 ± 1.3</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>139.5 ± 5.3</td>
<td>152.1 ± 4.5</td>
</tr>
<tr>
<td>Weight gain, g/d</td>
<td>11.8 ± 0.2</td>
<td>13.9 ± 0.5</td>
</tr>
<tr>
<td>Food intake, g/d</td>
<td>0.42 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. For each experiment, means in a row without a common letter differ, P < 0.05.
2 C1, group fed control the diet of Experiment 1; PB-PL, group fed a diet supplemented with LCP from pig brain PL; TF-TG, group fed a diet supplemented with LCP from tuna and fungal oil TG; C2, group fed the control diet of Experiment 2; EG-PL, group fed a diet supplemented with LCP from egg-yolk PL; EG-TG, group fed a diet supplemented with LCP from egg-yolk TG.
arrangement of fatty acids on the TG or PL backbone, and presence of other components such as cholesterol and choline. Their evaluation in an experimental model is useful to better understand the influence of chemical characteristics of LCP sources on their absorption and availability. In addition, differences in chemical composition may affect the lipid environment in the intestinal lumen and hence influence overall fat absorption.

Our goal was to design the diets to contain similar amounts of LCP from both the (n-6) and (n-3) series. Further, the background fat was to be the same to avoid the possible influence of different oil mixtures on fat absorption (32). Due to the fatty acid composition of the LCP sources, we could not balance the fatty acid profiles of all of the diets. Therefore, we conducted two separate experiments. In Experiment 2, the LCP sources used were not as rich in LCP as the LCP sources used in Experiment 1 and had high AA/DHA ratios. Therefore, to obtain the target level of DHA, 30% of the control oil mixture was replaced by the corresponding LCP source. Differences in fatty acid profile between LCP sources and the control oil mixture justified the unbalanced diets in Experiment 2 (Table 2). If the recommended ratio of AA/DHA had had to be met, it would have been necessary to add another source of (n-3) LCP. We decided not to do this because the experiment gave us the unique opportunity to compare LCP from the same origin and with the same AA/DHA ratio but with different chemical forms.

In this work, fecal excretions of fat, fatty acids and minerals were measured. Fecal composition also reflects contribution from bile, microbes and intestinal cell sloughing; thus, no accurate measurements of fat absorption can be assessed. The use of a true model of fat absorption that employs a fat-free diet-fed group would overcome this issue, but limitations of our animal facilities and handling made that difficult. Nevertheless, numerous fat balance studies have been conducted in animals (33) and infants using the difference between ingestion and fecal excretion as a useful index of apparent absorption (34,35). Therefore, we calculated fat apparent absorption.

In Experiment 1, the apparent absorption of fat was impaired in the rats fed the PB-PL diet (Table 4). The excretion
of fatty acids was also significantly higher in this group (Table 5). These rats also had increased food intakes that may have compensated for the intestinal losses. However, fat intestinal losses did not lower body weight gain or affect the FER in the PB-PL group. On average, the PB-PL group consumed 2.2 g/d more food than the control and TG-PL groups. This included 0.09 g fat/d (3.35 kJ/d). They excreted 1.21 g of fat/100 g of lyophilized feces. When corrected for humidity and the amount of feces produced every day (~1.4 g), this was 0.63 kJ/d. Therefore, the excretion of fat did not equal the greater amount of food consumed by this group. Nevertheless, we contend that the increase in food intake was related to the fat intestinal losses. The slightly higher body weight in this group (P = 0.07) may have accounted for the differences in the calculations. The higher fat excretion in the PG-PL group could be related to the chemical complexity of this LCP source. Pig brain concentrate contains large amounts of lipid compounds such as cerebrosides, gangliosides, sphingolipids and lysophosphatidylcholine that could potentially affect the intestinal absorption of fat and minerals. Further, lipid digestibility of this diet would probably be higher than the apparent absorption reported in our study when endogenous lipid excetration is considered. In fact, as mentioned above, our research group has previously used pig brain PL as a source of LCP in experimental models reporting positive effects of this source on plasma and tissue composition (29,30). However, until now, fat absorption of diets containing this kind of PL has not been studied.

The fact that LCP are esterified as PL in the PB-PL diet might account for the lower fat and fatty acid absorptions in this group. However, the current literature and our own results in Experiment 2 do not support this hypothesis. Morgan et al. (35) found improved fat absorption in infants fed a diet supplemented with egg-lecithin compared with infants fed non-LCP–supplemented formula. Similar results have been reported by other authors. Camielli et al. (34) showed higher absorption of (n-3) LCP in preterm infants fed a formula containing LCP in the form of PL compared with the same formula supplemented with LCP in the form of TG and with breast-fed infants.

In Experiment 2, no differences were found in the fat and mineral excretions among the study groups. However, fewer saturated fatty acids were excreted in the feces of rats fed the diet containing LCP as EG-PL, than in either control or EG-TG groups. This could account for the higher FER in the EG-PL group compared with the other groups. There was also a greater fecal excretion of DHA in the EG-TG–fed rats compared with the EG-PL group. Taken together, these results indicate a better apparent absorption of fatty acids in the rats fed the diet containing egg PL than egg TG. This is consistent with the clinical trials mentioned above (34,35).

On the other hand, the rats fed the EG-TG diet had greater fecal excretions of saturated fatty acids in feces (16:0 and 18:0) compared with the control group. The LCP source used to supplement this diet was obtained from egg-yolk powder by release and later reesterification of fatty acids with glycerol. This procedure yields a random distribution of fatty acids in the TG structure, which would influence their absorption. The positional distribution of fatty acids in the LCP sources used in this work was determined by a stereospecific analysis (36) showing a random distribution of fatty acids in the EG-TG source (17% 16:0 and 40.65% 18:0 were at the sn-2 position; a random distribution would be represented by 33.3% fatty acid on each position). Numerous studies have reported that fatty acids located at sn-2 position of TG molecule have an absorptive advantage compared to fatty acids esterified to the outer positions of TG (37–41). After digestion, fatty acids located at sn-2 position are released mainly as 2-MG, which are very efficiently absorbed by enterocytes in the small intestine. On the contrary, free saturated fatty acids are not well absorbed from the lumen because of their strong tendency to form insoluble calcium soaps with divalent cations at the alkaline pH of the small intestine (42). For instance, palmitic acid absorption is greater in infants fed breast milk (in which 16:0 is located primarily in the sn-2 position) than in infants fed palmitolein-containing formula in which 16:0 is esterified primarily to the outer positions of TG molecules (41–43). Similar results have been found in animals (38–48) and for other fatty acids such as 18:2(n-6) and (n-3) LCP that were also better absorbed when esterified to the sn-2 position (44,45). Thus, the randomization of fatty acids likely explains the lower absorption of saturated fatty acids in the EG-TG group compared with the control group.

In summary, total fat and LCP excretions in weanling rats fed diets containing LCP from different sources depend more on the characteristics of each LCP source used for diet supplementation than on the chemical form to which the LCP are esterified, especially lipid composition and fatty acid distribution. It is important to provide LCP for formula supplementation in a form that is both available and efficient to meet infant requirements.

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LITERATURE CITED


