Optimizing DHA levels in piglets by lowering the linoleic acid to α-linolenic acid ratio

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Abstract

We examined the effect of altering the linoleic acid (LA, 18:2n-6) to α-linolenic acid (ALA, 18:3n-3) ratio in the dietary fats of 3 day old piglets fed formula for 3 weeks. The LA-ALA ratios of the experimental formulas were 0.5:1, 1:1, 2:1, 4:1, and 10:1. The level of LA was held constant at 13% of total fats while the level of ALA varied from 1.3% (10:1 group) to 26.8% (0.5:1 group). Incorporation of the n-3 long chain PUFA EPA and 22:5n-3 into erythrocytes, plasma, liver, and brain tissues was linearly related to dietary ALA. Conversely, incorporation of DHA into all tissues was related to dietary ALA in a curvilinear manner, with the maximum incorporation of DHA appearing to be between the LA-ALA ratios of 4:1 and 2:1. Feeding LA-ALA ratios of 10:1 and 0.5:1 resulted in lower and similar proportions of DHA in tissues despite the very different levels of dietary ALA (1.3% vs. 26.8% of total fats, respectively). These results are relevant to term infant studies in that they confirm our earlier findings of the positive effect on DHA status by lowering the LA-ALA ratio from 10:1 to 3:1 or 4:1, and they predict that ratios of LA-ALA below 4:1 would have little further beneficial effect on DHA status.—Blank, C., M. A. Neumann, M. Makrides, and R. A. Gibson. Optimizing DHA levels in piglets by lowering the linoleic acid to α-linolenic acid ratio. J. Lipid Res. 2002. 43: 1537–1543.

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DHA is found in high concentrations in neural tissue including brain cortex and the retina. The proportion of DHA in brain cortex is partly dependent on diet, because cortical DHA is higher in breastfed infants when compared with those fed standard formula (1, 2). A number of clinical trials have demonstrated that preterm and term infants fed breast milk or formula supplemented with DHA perform better at a range of neurological and visual tests (3, 4) than their counterparts fed unsupplemented formulas, although some trials have demonstrated no effect of supplementation (5–8). It has been hypothesized that this may be explained by the fact that placebo groups had inadequate precursor α-linolenic acid (ALA, 18:3n-3), and hence, insufficient endogenous synthesis of DHA occurred (9). This line of thought is based on the premise that increased ALA will result in improvements in DHA synthesis and hence tissue DHA. Infant studies allow a limited range of ALA levels to be tested as regulatory committees limit the linoleic acid (LA)-ALA ratio to between 3:1 and 10:1 (10). Thus, we planned a study in formula fed piglets, as they are accepted models of human metabolism.

As linoleic acid (LA, 18:2n-6) must be added to all formulas to provide essential n-6 fatty acids, testing the effect of increasing the dietary supply of ALA also results in changing the ratio of LA to ALA in the diet. Several different means of altering the LA-ALA ratio have been studied in human and animal models. Most studies have altered both the LA and ALA levels in order to attain a range of LA-ALA ratios (11–15), and there has been little consistency in the percentage of total PUFA of the diet in the various studies. Some investigators have kept the LA constant and varied the level of ALA (16–18), others have kept the ALA constant while varying the LA level (19). Thus, there was a need for a systematic dose-response study over a broad range of ALA intakes.

We chose to examine the effect of altering the LA-ALA ratio in the diet of newborn piglets, as the timing of the brain growth spurt is similar to that reported for humans. In this experiment we tested LA-ALA ratios ranging from 10:1 (found in many infant formulas) through to 0.5:1 by keeping the LA level constant and varying the ALA concentration as a first step in elucidating the regulatory steps in the conversion of LA and ALA into long chain polyunsaturated fatty acids (LCPUFA). The primary endpoint in the study was DHA status in a range of tissues including brain, plasma, erythrocytes, and liver. A secondary aim was...
to assess the value of plasma or erythrocyte DHA as predictors of brain DHA.

**EXPERIMENTAL PROCEDURES**

**Animals and diets**

Newborn female piglets (Pure Large White) of normal term gestation (116–118 days) were housed and cared for by the Northfield Research Piggery Unit (Adelaide). They were kept in individual pens in a thermo-neutral environment (room temperature 26°C) with a 12 h day to 12 h night cycle. The piglets were allowed to suckle colostrum from their natural mothers for the first 36–72 h, as immunoglobulins in the colostrum provide passive immunity to the piglet. They also received 100 mg of an iron-dextran complex (Ferrojet) intramuscularly on day 3 post-partum (standard procedure). Piglets were sequentially assigned to one of five feeding groups with nonlitter mates being within a study group (five per group). Piglets were fed their assigned study formula ad libitum for a total period of 15 days (normal suckling period 28 days). Growth and formula intakes of individual piglets were monitored during the study period.

All nutritional requirements for the growing piglets were provided using a specific pig milk replacer (PMR) (Wombaroo Food Products, Australia). To enable the manipulation of the fat composition, the PMR powder contained only 3.5% fat (50% of total required fat) as milk fat and the other 3.5% of fat was provided by the addition of our vegetable oil blends. Vegetable oils were purchased from local supermarkets. Six different types of vegetable oils (Canola, Flaxseed, Safflower, Sunflower, Sunola, and Soya Bean) containing no LCPUFA were used to achieve five different LA-ALA ratios, using up to three types of oil for one blend. The LA-ALA ratios of the experimental formulas were 0.5:1, 1:1, 2:1, 4:1, and 10:1. The levels of LA were kept constant at 13% of total saturates was maintained constant at around 36% with a decrease of the amount of total monoenes, while the concentration of total PUFA was 40.7 ± 0.2.

**TABLE 1. Macronutrient content and major fatty acid components in experimental formulas**

| LA-ALA Ratio | Protein (g/l) | Carbohydrate (g/l) | Energy (kJ/l) | Fat (g/l) | Total saturates | Total monoenes | 18:2n-6 | Total n-6 | 18:3n-3 | Total n-3 | Total PUFA | LA-ALA |
|--------------|--------------|--------------------|--------------|-----------|----------------|----------------|--------|----------|--------|----------|-----------|--------|
| 0.5:1        | 56           | 45                 | 4051         | 64.3 ± 0.9| 36.4 ± 0.1    | 21.0 ± 0.2     | 13.7 ± 0.0 | 13.8 ± 0.0 | 26.8 ± 0.3 | 26.9 ± 0.3 | 40.7 ± 0.2 | 0.5     |
| 1:1          | 56           | 45                 | 3956         | 61.1 ± 0.9| 35.7 ± 0.0    | 36.1 ± 0.2     | 13.2 ± 0.0 | 13.5 ± 0.0 | 12.6 ± 0.0 | 12.8 ± 0.0 | 26.2 ± 0.0 | 1.0     |
| 2:1          | 56           | 45                 | 3844         | 58.7 ± 1.3| 35.7 ± 0.1    | 42.2 ± 0.1     | 12.7 ± 0.0 | 13.0 ± 0.0 | 6.3 ± 0.0  | 6.4 ± 0.0  | 19.4 ± 0.0 | 2.0    |
| 4:1          | 56           | 45                 | 3851         | 58.9 ± 1.0| 35.7 ± 0.0    | 45.0 ± 0.0     | 13.6 ± 0.0 | 13.8 ± 0.0 | 3.2 ± 0.0  | 3.7 ± 0.0  | 17.5 ± 0.0 | 4.3     |
| 10:1         | 56           | 45                 | 3885         | 59.8 ± 0.6| 36.0 ± 0.0    | 47.2 ± 0.0     | 13.3 ± 0.0 | 13.3 ± 0.0 | 1.5 ± 0.0  | 1.6 ± 0.0  | 14.9 ± 0.0 | 10.2   |

**Tissue collection**

On day 16 of the experiment, the animals were anaesthetized with an intramuscular injection of Ketasol/Xylazine (50 mg/kg) and blood sampled (9 ml) by intracardiac puncture. The animals were then immediately sacrificed by an intracardiac injection of Lethabarb (50 mg/kg). The animals were weighed, and snout-rump length was taken.

Blood samples were added to tubes containing 1ml of 3.8% sodium citrate as the anticoagulant. Erythrocytes were removed from plasma after centrifugation (5 min at 1,000 g) and washed in cold isotonic saline three times. From each piglet, one cerebral hemisphere was homogenized, and a 0.25 g sample was taken for fatty acid analysis. Liver samples (0.5 g) were homogenized in 3 ml of isotonic saline prior to fatty acid analysis. Erythrocyte lipid extractions were performed within 12 h of sampling, while all other tissue samples were stored at −20°C for up to 2 months before fatty acid analysis.

This study was approved by the South Australian Research and Development Institute Animal Ethics Committee and Flinders Medical Centre/University Animal Ethics Committee.

**Lipid extraction**

Total lipids were extracted from erythrocytes with chloroform-isopropanol (2:1, v/v (20) and from brain, plasma, liver, and experimental formula with chloroform-methanol (2:1, v/v) (21). Lipid extracts were separated by TLC into phospholipid, triglyceride and cholesterol ester classes on silica gel plates (Silica gel 60H Merck Darmstadt Germany). The solvent system for all TLC was petroleum spirit-diethyl ether-acetic acid (180:30:2, v/v/v). Lipid classes were visualized with fluorescein 5-isothiocyanate against TLC standard 18:3 (Nuchek Prep, Ely, street, MN). All solvents contained the antioxidant butylated hydroxy anisole at 0.005% (w/v).

**Fatty acid methylation**

All lipid fractions were transesterified by methanolysis (1% Na2SO4 in methanol) for 3 h at 70°C. After cooling, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane and transferred into gas chromatography vials containing anhydrous Na2SO4.

**Gas chromatographic analysis of FAME**

FAMEs were separated and quantified with a Hewlett-Packard 5880 gas-liquid chromatograph using a capillary column equipped...
with flame ionization detection and Hewlett-Packard ChemStation data system. Separation was achieved on a 50 m × 0.33 mm ID. BPX-70 column (SGE, Melbourne, Australia). Helium was the carrier gas at a column flow rate of 35 cm per second. The inlet split ratio was set at 30:1. The oven temperature at injection was set at 140 °C and programmed to rise to 220 °C at 5 °C per minute. The injector and detector temperatures were set at 250 °C and 300 °C, respectively. FAMEs were identified by comparison of retention times to authentic lipid standards (NuChek Prep, Elysian, MN).

Statistical analysis
All data are expressed as group mean ± SD. One-way ANOVA followed by the Student-Newman-Keuls test was used to identify diet effects among the experimental groups. A probability of 0.05 was assumed to represent statistical significance. All analyses were performed using SPSS for Windows 6.0 (SPSS Inc., Chicago, IL). Curve fitting was performed using the Sigma plot (Version 4.1, Jandel Scientific Corp., Corte Madera, CA) curve-fitting program.

RESULTS

Animals
The formula and energy intake among the groups were not different. There were also no differences in body, brain, and liver weight or snout-rump length between dietary groups (Table 2). Over the 15 day feeding period, the piglets increased their body weight on average by 2.5 kg, brain weight by 8 g, liver weight by 110 g, and their snout-rump length by 10 cm (data not shown).

Tissue fatty acid analysis
The phospholipid fatty acid composition from plasma and brain of piglets fed each of the five experimental formulas is shown in Tables 3 and 4, respectively. Total saturates in plasma and brain phospholipids were greater in the group fed the lowest LA-ALA ratio (and highest total PUFA content) compared with all other groups. There was an inverse relationship between the proportion of monoenes in the plasma and the PUFA in the diet, but this was not seen in brain phospholipids. The fatty acid changes in erythrocyte and liver tissues followed the same general pattern as seen in plasma phospholipids (data not shown). The diet-induced shifts in the fatty acid composition were greatest in liver, followed by plasma, erythrocytes, and then brain.

The effect of diet on some individual polyunsaturated fatty acids in the plasma phospholipid fractions are highlighted in Fig. 1A–C. Overall, plasma phospholipid LA

| TABLE 2. Animal characteristics in the different experimental groups* |
|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Experimental Feedings Groups | LA-ALA Ratio | 0.5:1 | 1:1 | 2:1 | 4:1 | 10:1 |
| Birth weight (kg) | 1.8 ± 0.2 | 1.7 ± 0.2 | 1.8 ± 0.2 | 1.7 ± 0.2 | 1.7 ± 0.1 |
| Final body weight (kg) | 4.2 ± 0.5 | 4.5 ± 0.5 | 4.5 ± 0.6 | 4.4 ± 0.4 | 4.5 ± 0.6 |
| Brain weight (g) | 35.5 ± 2.1 | 36.5 ± 3.2 | 33.9 ± 1.9 | 35.8 ± 2.8 | 34.4 ± 2.5 |
| Liver weight (g) | 199 ± 21 | 194 ± 32 | 198 ± 31 | 199 ± 23 | 180 ± 31 |
| Snout-rump length (cm) | 52.2 ± 2.9 | 52.1 ± 1.3 | 52.9 ± 2.1 | 53.0 ± 1.4 | 52.7 ± 1.0 |

* Data are the means ± SD of values obtained from five animals.

| TABLE 3. Fatty acid composition of plasma phospholipids from piglets fed experimental formulas varying in LA-ALA ratio for 15 days |
|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Experimental Formula | LA-ALA Ratio | 0.5:1 | 1:1 | 2:1 | 4:1 | 10:1 |
| Fatty acid* | | | | | | |
| Total saturates | 44.83 ± 0.83a | 42.45 ± 1.09b | 43.15 ± 1.45b | 42.87 ± 0.94b | 42.21 ± 0.83b |
| 18:1n-9 | 7.89 ± 0.43a | 12.73 ± 0.61b | 13.89 ± 1.02b,c | 15.04 ± 1.95b | 17.29 ± 1.11b,c |
| 20:3n-9 | 0.13 ± 0.06a | 0.18 ± 0.04b | 0.23 ± 0.07b | 0.22 ± 0.02b | 0.34 ± 0.09b |
| Total monoenes | 9.57 ± 0.45a | 15.10 ± 0.75b | 16.77 ± 1.19bc | 17.27 ± 1.97bc | 18.72 ± 1.19b,c |
| 18:2n-6 | 18.44 ± 1.20a | 18.37 ± 1.78b | 17.14 ± 0.70b,a | 17.26 ± 1.66b | 17.50 ± 1.43b,a |
| 18:3n-6 | 0.09 ± 0.00 | 0.14 ± 0.01 | 0.13 ± 0.04 | 0.13 ± 0.04 | 0.12 ± 0.05 |
| 20:3n-6 | 0.54 ± 0.14a,b | 0.68 ± 0.09b,c | 0.86 ± 0.18b,c | 0.84 ± 0.16b,c | 0.96 ± 0.27b,c |
| 20:4n-6 | 3.31 ± 0.27a | 5.12 ± 0.92b | 7.27 ± 0.94b | 9.12 ± 1.10b,c | 10.63 ± 0.94b,c |
| 22:4n-6 | 0.44 ± 0.12a | 0.22 ± 0.14 | 0.37 ± 0.18 | 0.25 ± 0.03 | 0.39 ± 0.06 |
| 22:5n-6 | nd | 2.2 ± 0.5 | nd | nd | 0.14 ± 0.06b |
| Total n-6 | 22.87 ± 2.09a | 24.57 ± 1.19b | 25.68 ± 0.63b | 27.75 ± 0.63b,c | 30.14 ± 0.56b,c |
| 18:3n-3 | 6.23 ± 0.85a | 3.71 ± 0.95b | 1.61 ± 0.26b | 0.81 ± 0.17b,c | 0.40 ± 0.06b,c |
| 20:5n-3 | 7.09 ± 1.41a | 3.29 ± 0.90b,c | 2.14 ± 0.73b,c | 0.76 ± 0.10bc | 0.39 ± 0.02bc |
| 22:5n-3 | 2.97 ± 1.41a | 2.76 ± 0.51b | 2.09 ± 0.50b | 1.74 ± 0.07b,c | 1.25 ± 0.26b,c |
| 22:6n-3 | 4.33 ± 0.95a | 5.69 ± 1.09b,c | 6.36 ± 1.00b,c | 6.71 ± 1.63b,c | 4.84 ± 0.75b,c |
| Total n-3 | 20.83 ± 1.63a | 15.45 ± 1.33b | 12.24 ± 0.80b | 10.23 ± 1.53b | 6.85 ± 0.62b |

Data are the means ± SD values obtained from five animals. Values in rows without a common superscript are significantly different at P < 0.05.

* Data represent percent, relative to total fatty acids. nd, not detected.
and ALA reflect dietary levels. Thus the proportion of plasma phospholipid LA remained relatively constant at 18%, whereas ALA increased from 0.4 to 6.2% as the LA-ALA ratios decreased (Fig. 1A). There was also an increase in EPA from 0.4 to 7.1% as the LA-ALA ratio was decreased. This was matched by an almost linear decrease of arachidonic acid (AA) from 10.6% to 3.3% with a decreasing LA-ALA ratio (Fig. 1B). The 22 carbon homologs displayed a different pattern. There was a linear increase in plasma phospholipid 22:5n-3 as the LA-ALA ratio was decreased. In contrast, DHA increased as the ratio was decreased from 10:1 to reach a maximum when the LA-ALA ratio was between 4:1 and 2:1, but then decreased again as the LA-ALA ratio was further decreased to 0.5:1 (Fig. 1C).

The curvilinear relationship between dietary LA-ALA ratios and tissue DHA was also found in other tissues (Fig. 2). The rate of change from lowest to highest DHA phospholipid was different amongst the four tissues, being lower in brain and erythrocytes but greater in plasma and liver.

In brain tissue, although the overall change was small, the proportion of DHA was reflective of DHA concentrations seen in other tissues. (Table 4). 22:5n-3 increased from 0.37 to 1.71% as the LA-ALA ratio was decreased from 10:1 to 0.5:1. This corresponded with a concurrent decrease in the n-6 metabolites AA, 22:4n-6, and 22:5n-6. In contrast to plasma, LA, and EPA were not significantly incorporated into brain tissues of piglets.

There was a strong correlation between brain AA and erythrocyte AA ($r^2 = 0.85$, $P < 0.001$), as well as brain AA and plasma AA ($r^2 = 0.91$, $P < 0.001$) (Fig. 3A, B). However, the relationship between brain DHA and erythrocyte DHA was weak ($r^2 = 0.18$, $P < 0.05$), and no correlation was seen between brain DHA and plasma DHA ($r^2 = 0.03$, n.s.) (Fig. 3C and D). There was a modest correlation between the proportion of erythrocyte and plasma DHA ($r^2 = 0.35$, $P < 0.01$).

**DISCUSSION**

The most significant finding of this study is the complex response of DHA to changes in dietary ratios of LA to ALA. While reducing the LA-ALA ratio from 10:1 to about 2:1 resulted in an increase in the proportion of DHA in all tissues examined, further decreases in the ratio actually resulted in a decrease in the observed proportion of DHA, while at the same time, all other n-3 LCPUFAs continued to increase. Thus, ratios of 10:1 and 0.5:1 resulted in similar proportions of DHA in all tissues despite the very different levels of dietary ALA. These results are relevant to infant studies in two ways. First, they confirm that the positive effect on DHA status seen in infants fed formula with LA-ALA in the range of 3:1 or 4:1 (12, 22, 23) was maximal. Secondly, we think that the results predict that ratios of LA-ALA below 4:1 would have little further beneficial effect on DHA status. It should also be noted that none of the dietary treatments in our infant trials (12, 23) achieved an effect on DHA status. It should also be noted that none of the dietary treatments in our infant trials (12, 23) achieved an effect on DHA status. It should also be noted that none of the dietary treatments in our infant trials (12, 23) achieved an effect on DHA status.

We chose to hold LA levels of the diets constant while altering the level of ALA in the belief that this was better than changing two variables at once. This approach has been used by other workers but has the disadvantage that the total PUFA content of the diet increases as the level of...
dietary ALA is increased. In our case, the total PUFA content varied from about 15 to 40% of the total fatty acids. Thus, it could be that some of our results might be explained in terms of total PUFA rather than LA-ALA ratio. Arbuckle, MacKinnon, and Innis (14) demonstrated by regression analysis that the variance in DHA was better explained by ALA content rather than LA-ALA ratio, but our data highlight a complex nonlinear relationship between ALA and DHA. Lands, Morris, and Libelt (24) commented on the difficulty of fitting DHA into their n-3 prediction equations because tissue levels of DHA were lowered with increasing dietary ALA. This complex relationship between dietary LA and ALA with tissue DHA precludes simple linear regression analysis.

In this study we explored a range of LA-ALA ratios from 10:1, which is about the most common ratio in infant formulas, through to 0.5:1, which is essentially the ratio found in flaxseed oil. Woods, Ward and Salem (15) investigated an even wider range in rat pups, lowering the LA-ALA ratio to 1:12 by the use of ALA ethyl esters and fixing the total PUFA at 30% total fatty acids. They reported a small linear effect of ALA on brain DHA but no effect on the proportion of retinal DHA, leading them to hypothesize the need for preformed DHA to meet retinal requirements. On the other hand, Bowen, Wierzbicki, and Clandinin (25) varied the LA-ALA ratio from 22:1 to 1:1, but the total PUFA also varied in a nonlinear way from 19 to 36% of total fatty acids. They did, however, report a curvilinear response of dietary ALA on the proportion of neuronal cell phosphatidyl serine and phosphatidyl inositol DHA that was similar to trends seen in our data.

The implications of how the linear response of tissue EPA and 22:5n-3 to dietary ALA contrast the curvilinear response of tissue DHA deserves special comment. It is clear that the synthesis of both EPA and 22:5n-3 follow simple zero-order kinetics, whereas the situation is more complex.
complex for the steps from 22:5\textit{n}-3 to DHA. The pathway delineated by Voss et al. (26) (Fig. 4) highlights the fact that the synthesis of EPA and 22:5\textit{n}-3 from ALA requires only one pass of the \( \Delta^6 \)-desaturase that may explain the simple kinetics for these compounds. However, the synthesis of DHA from 22:5\textit{n}-3 requires a second pass at the \( \Delta^6 \)-desaturase after it is elongated to 24:5\textit{n}-3. Thus 24:5\textit{n}-3 would be in direct competition with 18 carbon fatty acids for access to the \( \Delta^6 \)-desaturase, which may explain the complex kinetics between dietary ALA and tissue DHA (26). This explanation is also consistent with a study demonstrating preferential desaturation of ALA over 24:5\textit{n}-3, where increasing concentrations of ALA inhibited 24:5\textit{n}-3 conversion to DHA by up to 81\%, whereas similar levels of 24:5\textit{n}-3 only inhibited ALA conversion to 18:4\textit{n}-3 by up to 27\% (27).

The current experiment gives important information regarding the usefulness of erythrocyte fatty acids as markers of brain fatty acid composition. The data show that while there is a strong relationship between both plasma and erythrocyte AA and brain AA, the relationship be-
between erythrocyte DHA and brain DHA is weak \( (r^2 = 0.18) \), and that between plasma DHA and brain DHA is nonexistent. This is due in part to the very small change in the proportion of brain DHA across all groups. In post-mortem examination of term infants (2), we reported a similar weak relationship between erythrocyte and brain cortex DHA \( (r^2 = 0.16) \), which is striking. Combined with the piglet data, we suggest that attempts to use erythrocyte DHA as a marker of brain DHA status have limited usefulness.

Based on our data and the work of others, it appears that increasing ALA above current levels in infant formulas is only a partial solution to increasing the proportion of tissue DHA. In this study the relatively small increase in dietary ALA from 1.3 to 3.2% \((10:1 \text{ group compared with } 4:1 \text{ group})\) successfully increased plasma DHA from 4.8 to 6.7%, while at the same time only decreased plasma AA from 10.6 to 9.1% and increased plasma EPA from 0.39 to 0.76%. The implications for dietary recommendations for infants is that variations in dietary LA:ALA ratios will have a modest impact on DHA status, with ratios at the lower limit of recommendations having an advantage over those at the higher end. Although, if high DHA status is desirable, this can best be achieved through supplementation with high-DHA oils, and this is the strategy chosen by many infant formula manufacturers around the world.

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