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(54) **Title:** *PUFA SUPPLEMENTS*

(57) **Abstract**

Edible formulations, such as polyunsaturated fatty acids (PUFAs) such as pharmaceutical compositions or nutritional supplements, are disclosed comprising arachidonic acid (ARA). They are adapted to deliver from 150 mg to 1 g per day of ARA and may contain other PUFAs, for example docosahexaenoic acid (DHA). The DHA dosage is from 400 to 600 mg per day, and the ratio of ARA:DHA may be from 1:5 to 5:1. Pharmaceutical compositions comprising ARA and DHA at a ratio of ARA:DHA of 1:1 to 1:2 are also disclosed, as are foodstuffs comprising 0.1 to 5 % ARA. Such formulations can be used to increase ARA levels *in vivo*, for example in pregnant women or for people who have diseases or conditions associated with low ARA levels.

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PUFA SUPPLEMENTS

This invention relates to the provision of polyunsaturated fatty acids (PUFAs) in the diet of humans and animals. More specifically it relates to the provision of polyunsaturated fatty acids of the n-6 and the n-3 families, and in particular the n-6 fatty acid arachidonic acid (ARA) and the n-3 fatty acid docosahexaenoic acid (DHA), and ratios thereof in balanced amounts.

The invention is in part based on the finding that an optimal balance of the n-6 and n-3 families can play a significant role in health and the prevention of chronic diseases. The main reason for this is that the two families compete for the same enzyme(s) for the formation of the long-chain members from their C18 precursors. As a consequence, and this occurs in prior art compositions, a surplus of member(s) of one family tends to depress the amount of the other family. Moreover, the members of the two families can in some circumstances have adverse effects on essential functions in the body, such as blood clotting and the immune response.

Introduction

It is technologically relatively easy to provide the C18 n-6 fatty acid linoleic acid in the diet, since this fatty acid is abundantly present in common vegetable oils, such as corn oil and soy oil. There are also plant oils available that contain the C18 n-3 fatty acid α-linolenic acid, for instance rape seed oil, but these are much less readily used due to their lower stability. This usually leads to a surplus of the n-6 family over the n-3 family in the modern diet.

It has therefore been argued that n-3 fatty acids should be supplemented in many cases where a relative depletion is suspected. Generally this cannot be achieved by providing the C-18 precursor, since the efficiency of its conversion to C20 and C22 derivatives is low. Therefore, the consensus is that the C20 and C22 n-3 fatty acids (EPA and DHA) should be provided themselves.

In many cases the rationale behind this supplementation is to attenuate the action of the long-chain n-6 fatty acid ARA. It has been shown that the addition of the n-3 PUFAs, either derived from fish oil or from microbial (algae) oils does indeed lead to lower ARA levels. In the case of fish oil this occurs in spite of the fact that fish oil

contains low amounts of ARA.

This depression of the ARA content is not always desirable. The invention thus seeks to provide preparations that may enhance the DHA and/or EPA status of animals, without adversely affecting ARA levels, or, conversely, enhance ARA without affecting the DHA and/or EPA status.

The use of preparations containing both ARA and n-3 PUFAs has been described before in the provision of PUFAs to infant formula. The rationale behind this is that human breast milk contains appreciable amounts of ARA and DHA which are considered useful to the developing infant.

In contrast, for adult nutrition there is no such natural source of PUFAs, although both ARA and DHA can be found as components of the human diet. However, for a number of reasons these PUFA levels appear to be sub-optimal. Furthermore, different populations have different levels of these PUFAs and this can affect the suitable dosage. As there is no model from nature, the relative amounts of PUFAs to be used needs to be determined and the present invention seeks to address this problem and provide various formulations and proportions of the PUFAs for certain applications.

Prior Art

M. Makrides *et al*, European Journal of Chemical Nutrition 50:352-357 (1996) refers to a study to assess the effect of varying the internal intake of DHA (from 0 to 1.3g DHA/day) on breast milk fatty acids. DHA in the diet fed to lactating mothers had a strong specific and dose-dependent effect on breast milk DHA but did not affect ARA levels. This study used algae oils available from Martek Corporation, USA, under the brand name NEUROMINS™.

WO-A-92/12711 (Martek) refers to oil blends containing ARA and DHA, for example an ARA:DHA ratio of 3:1 to 2:1, in particular to provide levels of these PUFAs in infant formula in amounts comparable to human breast milk (which has an ARA level of 0.5 to 0.6%).

A number of PUFA-containing compositions are currently marketed. EFANATAL™ are capsules, two capsules to be taken per day to give a daily intake of DHA (125mg), ARA (8.6mg) and GLA (40mg). The capsules contain an oil that is primarily based on fish oil. The Applicant has found that this decreases *in vivo* ARA levels, because the DHA content relative to the ARA content in the capsules is too high. Thus this product is in fact an ARA lowering, rather than ARA increasing, composition

despite the fact that it contains ARA. A comparison between this product and those of the invention is provided later.

EFAMARINE™ is also capsules, containing primarily fish and evening primrose oils, of which two are to be taken per day to give a daily intake of EPA (34mg), DHA (22mg) and GLA (68mg).

EFALEX™ is an oil blend, where a teaspoon (5ml) is intended to be taken twice a day, each teaspoon giving DHA (100mg), GLA (21mg), ARA (8mg) and thyme oil (6mg).

Summary of the Invention

A first aspect of the present invention relates to an edible formulation comprising ARA in an amount adapted to deliver a dosage (of ARA) of from 150mg to 1g per day.

Preferably the formulation is adapted to deliver from 200 to 900mg per day ARA, such as from 200 to 700mg per day, optimally from 250 to 400 or 500mg per day.

Edible formulations include dietary supplements and (pharmaceutical) formulations and preparations, such as tablets, pills and capsules. They additionally include (solid or liquid) foodstuffs, for example dairy products (margarine, butter, milk, yoghurt), bread, cakes; drinks such as beverages (tea, coffee, cocoa, chocolate drinks), fruit juices, soft (e.g. fizzy) drinks; confectionery; oily foods (snacks, salad dressing, mayonnaise), soups, sauces, carbohydrate-rich foods (rice, noodles, pasta), fish-containing foods, baby foods (such as infant formula, either as a liquid or powder), pet food, and ready prepared or microwaveable foods.

The ARA can be from any suitable source. It may be from a natural (e.g. vegetable or marine) source, or it may be from a microbial source or from a microorganism, such as fungus, bacterium or a yeast.

Suitable fungi are of the order *Mucorales*, for example *Mortierella*, *Pythium* or *Entomophthora*. The preferred source of ARA is from *Mortierella alpina or Pythium insidiosum*. Suitable commercially available ARA oils include those from DSM/Gist-brocades, Wateringseweg, P.O. Box 1, 2600 MA, Delft, The Netherlands under the trade mark OPTIMAR™ and from Martek Corporation, 6480 Dobbin Road, Columbia, MD 21045, USA, under the trade mark ARASCO™.

In addition to the ARA, one or more additional PUFAs may be provided. This may be another n-6 PUFA in addition to ARA (such as a C18, C20 or C22 fatty acid) or it may be a n-3 fatty acid (for example, a C18, C20 or C22 fatty acid) and in particular EPA and/or DHA. Each PUFA that may be used in the invention may be in the form

of a free fatty acid, fatty acid ester (e.g. methyl or ethyl ester) as a phospholipid or as a triglyceride.

If the formulation comprises an n-3 fatty acid, it is preferred that this is EPA or DHA. If it is DHA, then the formulation is preferably adapted to deliver the same dosage as specified for ARA, such as from 400 to 600mg per day DHA. Alternatively, or in addition, if the formulation comprises EPA, then it is preferably adapted to deliver a dosage of from 150mg to 1g per day EPA, such as from 250 to 500mg of EPA per day.

If the formulation is to be taken (eaten or ingested) once a day then it can contain from 150mg to lg of ARA. If twice a day then the formulation can have 75mg to 0.5g of ARA, for three times a day a content of 50mg to 330g ARA, and so on, pro rata, for more frequent administrations. The same calculations can be applicable for other PUFAs that may be present, such as DHA.

If the formulation comprises more than one PUFA then the amount of each PUFA can be expressed relatively, as a ratio. For example, if an n-3 PUFA is additionally provided, then the ratio of ARA:n-3 PUFA (such as DHA or EPA) can be from 1:5 to 5:1, preferably from 2:1 to 1:3, optimally from 1:1 to 1:2. The relative amounts of the PUFAs can be balanced so that PUFA levels are supplemented, increased (or at least not decreased significantly) bearing in mind the condition of the individual.

Preferably the PUFA is present in an oil. This may be a pure oil, a processed (e.g. chemically and/or enzymatically treated) or concentrated oil. This oil may comprise from 10 to 100% of the PUFA, but the content may be from 20 to 45%, optimally from 30 to 45% of the desired PUFA, for example ARA, if a microbial oil. Of course, this oil may contain one or more PUFAs within these percentage concentrations. The oil may be a single oil derived from a single cell or a microbial source, or it may be a blend or mixture of two or more oils from these or other (e.g. vegetable or marine) sources. The oil may contain one or more antioxidants (e.g. tocopherol, vitamin E, palmitate) for example at a concentration of from 50 to 800ppm, such as 100 to 700ppm. Suitable processes for preparing PUFAs are described in International patent application numbers PCT/EP97/01446 (WO-A-97/36996), PCT/EP97/01448 (WO-A-97/37032), and PCT/US92/00517 (WO-A-92/13086).

A second aspect of the invention relates to a (pharmaceutical) composition comprising ARA and DHA at a ratio of ARA:DHA of from 1:1 to 1:2. This ratio of PUFAs has been found to provide a good balance, and can increase *in vivo* DHA levels without ARA levels being suppressed due to a too high DHA content. The DHA can be

from a natural (e.g. marine) source or from a microbial source (e.g. from an algae).

A third aspect relates to an edible formulation (eg. a foodstuff) comprising from 0.1 to 3 or 5% ARA. Preferably, the amount is from 0.5 to 1.5 or 2%, optimally from 0.3 to 0.8%. Suitable foodstuffs have already been discussed in relation to the first aspect. Preferred methods of preparing infant formula are disclosed in International application numbers PCT/EP97/01447 (WO-A-97/35487) and PCT/EP97/01449 (WO-A-97/35488).

Suitable formulations can include oils, for example to be taken orally. The oil may be taken as such, or it may be encapsulated, for example in a shell, and may thus be in the form of capsules. The shell or capsules may comprise gelatin and/or glycerol. The formulation may contain other ingredients, for example flavourings (e.g. lemon or lime flavour).

The invention has found use in improving PUFA levels in normal, healthy, well fed individuals (who would normally not be expected to benefit if on an adequate diet). However it can also be used with individuals with low PUFA level(s) or deficiencies.

Thus, a fourth aspect of the present invention relates to the use of ARA (eg. as a dietary or nutritional supplement or for the manufacture of a medicament) for a woman who is:

a. pregnant and at an age of from 15 to 20;

b. pregnant and at an age of from 40 to 60, such as from 50 to 55;

c. pregnant with her fourth, fifth or subsequent child;

d. pregnant with twins, triplets or quadruplets;

e. pregnant and is from 1 to 3 months into her pregnancy;

f. pregnant as a result of *in vitro* fertilisation (IVF) or who is undergoing IVF treatment (which includes enrolling in or participating in an IVF procedure) but not yet pregnant;

g. pregnant at from 20 or more weeks of gestation;

h. pregnant and is malnourished, poorly or marginally nourished, suffering from malnutrition or malabsorption or deficient in one or more essential fatty acids (such as a PUFA);

i. trying to become pregnant;

j. pregnant, for promoting the intra-uterine growth or health of a foetus; or

k. lactating, for increasing the level of ARA or EPA in the woman's breast milk.

In the case of (h) these conditions are relatively rare in Western Europe, but may be

found in women in Africa or some Asian countries (eg. Pakistan).

For pregnant women, the benefit to the foetus in (j) has not always been predictable or immediately apparent due to the variance in individuals in the transport of fluid between the mother and foetus. The placenta to foetus connection (the umbilical cord) can vary in size and physiological condition and so in the past the supplementation of the mother with PUFAs has not necessarily indicated that the foetus will receive these PUFAs and so benefit also.

A fifth aspect relates to the use of ARA (as a dietary or nutritional supplement) for a human (male or female) over 50 years old, preferably over 65 years old.

A sixth aspect relates to the use of ARA (as a dietary or nutritional supplement) for a non-human mammal which is pregnant or lactating.

The ARA is preferably ingested at from 150 to 700mg per day, optimally from 250 to 500mg per day.

A seventh aspect of the present invention relates to the use of ARA for the manufacture of a medicament for (assisting in) the prophylaxis, prevention, amelioration or treatment of a disease or condition associated with an abnormal or low level of an n-3 or n-6 PUFA, for example in the blood. The invention therefore finds use in subjects that have low levels of ARA, for example for those that cannot or cannot effectively convert linoleic acid (LA) to ARA. Therefore, suitable patients may have a malfunctioning, inefficient or deficiency in Δ6-desaturase.

A (mouse) model of PUFA deficiency has been established and used to mimic the effects of malnourishment. This model has shown the beneficial effects of the formulations of the invention, including during pregnancy, for both the mother and foetus. It has also allowed simulation of poor placental transfer and intra-uterine growth retardation, and shown the benefits of supplementation with formulations of the invention in the individuals mentioned in the various aspects of the invention (and the foetus if pregnant).

The Applicant has found that certain diseases or conditions, in particular neuronal diseases, are associated with low levels of *in vivo* PUFAs, in particular low levels of ARA in the blood. It is therefore thought that the administration of ARA, or a balance of the PUFAs, will be able to assist in the prophylaxis, prevention, amelioration or treatment of these diseases or conditions. The diseases in question include: neuronal disease, such as schizophrenia, cystic fibrosis, idiopathic immunoglobulin A nephropathy, multiple sclerosis, retinitis pigmentosis, Usher's syndrome, celiac disease, macular degeneration,

Parkinsons' disease, osteoporosis, Alzheimer's disease or phenylketonuria.

An eighth aspect relates to the use of ARA, optionally with DHA, for promoting lactation and/or reproductive efficiency or success or fertility in a human or non-human female mammal.

A ninth aspect of the present invention relates to the use of ARA and DHA (in an edible formulation) at an ARA:DHA ratio that increases the ARA level in blood. Preferably the ratio of ARA:DHA is from 1:5 to 5:1, such as from 1:1 to 1:2.

The invention is particularly application to those people that have low ARA levels, for example a diabetic, alcoholic, drug abuser, smoker or a subject having an abnormal or low immune level or who is immunocompromised.

The use of the fourth to ninth aspects include methods of administration of the ARA (and optionally DHA), either as such or in a formulation, to a subject (individual, human or animal) where that subject is in need of, or will benefit from, the administration, or those uses in the manufacture of a medicament for the purposes specified. Formulations may exclude GLA and/or DGLA if necessary.

The dose or amount of ARA (and DHA, if present) is preferably such that it increases either an essential fatty acid (EFA) sufficiency index (defined as the level of 20:4 n-6 (ARA) divided by the level of 20:3 n-9 fatty acid (mead acid)) and/or an EFA balance index (defined as the level of 22:6 n-3 (DHA) divided by the level of 22:5 n-6). Here, levels include those in the blood (eg. in red blood cells), brain, placenta, liver, intestine, plasma or foetus.

Preferred features and characteristics of one aspect of the invention are equally applicable to another aspect *mutatis mutandis.*

The following Examples are provided to merely illustrate the invention, and are not to be construed to be limiting.

Examples 1 to 3: Preparation of a composition containing balanced proportions of PUFAs.

This example describes the blending of n-6 and n-3 oils so that they can be included in a single capsule.

The composition was prepared by combining one n-6 PUFA-rich oil with three different n-3 PUFA-rich oils. The n-6 PUFA-rich oil was derived from the fermentation of the filamentous fungus *Mortierella alpina*, and contained approximately 40% ARA as the major fatty acid. For the n-3 PUFA-rich oil the three different sources were: a

high-EPA (above 45%) low-DHA (about 10%) fish oil (from Pronova, Norway under the trade name EPAX™ , product no. EPAx4510TG), a high-DHA (above 50%) low-EPA (about 20%) fish oil (also from Pronova under the same brand name, product no. EPAx2050TG), and an oil derived from fermentation of the unicellular alga *Crypthecodinium cohnii* which contains 40% DHA as major fatty acid but is virtually devoid of EPA (from Martek Corporation, Columbia, United States of America under the trade name DHASCO™).

The oils were mixed in appropriate quantities to give the desired amounts and proportions of n-3 and n-6 PUFAs. Here the ARA:DHA ratio for the three blends (Examples 1 to 3) was 1:1. During this procedure, the oxidation-sensitive oils were protected from environmental oxygen by a blanket of oxygen-free nitrogen gas. Subsequently, the oils were used to prepare soft-gel gelatin capsules, where each capsule had 400mg ARA and 400mg DHA.

Example 4: Provision of balanced PUFAs to pregnant women during the early or latter stages of pregnancy.

This Example concerns the trial of pregnant women that are supplemented with ARA and DHA either between weeks 6 and 15 or between weeks 20 and 25 during pregnancy until delivery (birth). The ARA source was a triglyceride oil containing 38% ARA available from DSM/Gist-brocades, Delft, The Netherlands, under the trade name OPTIMAR™. This is an oil produced by the fungus *Mortierella alpina*. For DHA either a DHA-rich fish oil of food grade or an algae-derived oil obtained from Martek Corporation under the trade mark DHASCO™ was employed.

Maternal supplementation of ARA and DHA during pregnancy was therefore studied to see if the fatty acid status of the mother measured at birth and subsequently during lactation compared with the controlled group that received no supplementation. The measurements included maternal erythrocyte ARA and DHA values, ARA and DHA content of the umbilical arteries and venous vessel wall, ARA and DHA content of breast milk.

The study was a case controlled study involving 10 pregnant women. One experimental group (of five women) received one or more gelatin capsule (each of 250mg ARA) oil per day (containing 38% ARA) and one capsule (each of 500mg DHA) oil per day (containing 25% DHA). The control group received the same amount of placebo gelatin capsules to overcome differences in daily calorie intake. The vitamin E intake of

the experimental and controlled groups was equal, and the capsules were taken during breakfast.

Blood samples were taken at the beginning of the trial and at the end of gestation. Red blood cell fatty acids were measured (as phospholipids) using capillary gas chromatography with flame ionisation.

It was found that the supplemented women had significantly higher levels of both DHA and ARA in the red blood cells during pregnancy and at the time of birth. Remarkably, these higher levels persisted during the lactation period, being apparent both in the red blood cells of the mothers and their breast milk. The ARA level in breast milk was found to have risen to from 0.8 to 1.0% ARA. In addition the ARA levels in the blood of the newly born children was found to be higher than the control group. This finding is of major significance for mothers and their children under marginal nutritional conditions.

Example 5: Provision of balanced PUFAs to elderly people.

The Applicant perceives a need to enhance the n-3 PUFA status of the population, not in the least in the elderly population, where diseases such as Parkinson's disease and Alzheimer's disease have been found to be associated with a low PUFA status. This is thought to be partly due to inefficient or deficient Δ6-desaturase enzyme. However care is needed, especially in older people, since a decrease in ARA levels could exert a negative effect on the immune system.

A formulation was prepared according to Example 1, containing n-3 and n-6 PUFAs in a ratio of DHA:ARA of 2:1. The capsules were given to a group of healthy, elderly men and women (at least 65 years of age), at a dosage of 1 g n-3 PUFAs per day.

After one month the PUFA status of the red blood cells of the subjects was assessed. It was found that in all cases the levels of DHA had increased, whereas the levels of ARA had remained constant, or showed a slight increase in some cases. Thus it was possible to enhance the n-3 PUFA status of patients, without compromising the ARA status, by the use of a balanced formulation.

Example 6: Provision of PUFAs to pregnant women.

Two types of PUFA-containing capsules were prepared. The first contained ARA, at 500mg per capsule. These were to be taken one a day. The ARA was provided as a microbial oil, obtained from DSM/Gist-brocades, Delft, The Netherlands, under the

trade name OPTIMAR™. These capsules had a gelatin coat, and contained 20mg of vitamin E. Similar capsules were also prepared having the same amount (500mg) of DHA, being present as a microbial oil obtained from Martek Corporation, Columbia, United States of America (under the trade name DHASCO™). These capsules were also designed to be taken one per day.

Trials were conducted with pregnant women ingesting either one ARA capsule per day, or one ARA and one DHA capsule per day. The women chosen for the study were those that had been found to have relatively low levels of ARA in the blood. A number of women who were pregnant were therefore tested for *in vivo* ARA blood levels and permission was obtained to take part in the study. The first group of women were teenagers of from 15 to 20 years of age. For all these women, this was their first pregnancy. Due to early maturation they were found to benefit from both ARA and ARA plus DHA supplementation in their diet. Both regimes increased *in vivo* ARA levels.

A second group of women, also pregnant, were studied, these being from age 40 to 50. During pregnancy it was also found their *in vivo* blood levels were increasing under both supplementation regimes. Half of the women chosen in this study were having their fourth child.

Three women each pregnant with twins were chosen for supplementation with one ARA capsule and one DHA capsule per day. Their ARA *in vivo* levels were found to be relatively low, probably because the ARA from the blood of the mother was being absorbed and consumed by both foetuses. These women were supplemented with the ARA and DHA capsules and the ARA levels in the blood were found to increase.

Example 7: Provision of ARA and DHA to subjects with low PUFA content.

The same capsules were used as described in Example 6, except this time the ARA capsules contained only 250mg ARA. These capsules could be taken once or twice daily, according to the subject and their condition.

A number of people were chosen for this study due to their relatively low content of PUFAs in the blood. The reason for the low PUFA content was not always immediately evident. However, it has been found that a number of diseases or adverse conditions lead to low PUFA levels, and it was therefore postulated that providing either a correct dosage of ARA, or a balance of ARA:DHA, the *in vivo* ARA levels could be increased, which might moderate some of the symptoms of the condition. Some of the

conditions were thought to result in a poor efficiency in conversion of a precursor to ARA itself, for example a defect or deficiency with the enzyme Δ6-desaturase. Those conditions that were found by the Applicant to often give rise to low PUFA levels included cystic fibrosis, multiple sclerosis, celiac disease and osteoporosis. In addition, patients who were being treated for alcoholism, addiction to drugs or who were immunocompromised (AIDs patients) were also found to have low levels of PUFAs.

A study was therefore made where either one or two ARA capsules were taken daily, to give an ARA:DHA content of either 1:1 or 1:2. In almost all cases those subjects who were taking these capsules (for at least 3 weeks) were all found to have, at the end of the trial, increased *in vivo* ARA blood levels.

Example 8: Provision of PUFAs in infant formula.

Both solid (powdered) and liquid infant formula baby food was prepared containing 0.5% ARA and 0.5% DHA. This formula was fed to babies regularly in their first three months by mothers who had decided not to breast feed their children. As a control, the *in vivo* ARA blood levels of these children were compared to those that were being breast fed over the same time period. It was found that in the infants being bottle fed that their ARA levels were comparable to those being breast fed.

Comparative Example 9 and Example 10

A number of breast feeding women were chosen for a comparative trial. One group of women were fed two EFANATAL™ capsules per day (to give a daily intake of DHA 125mg, ARA 8.6mg and GLA 40mg). For comparison, a second group of women were given similarly prepared capsules (with a gelatin/glycerol shell) containing 150mg ARA per capsules (to give a daily ARA intake of 300mg ARA,2 capsules per day). In this second group a third capsule was also taken, one per day, which contained DHA at 500mg per capsule.

The ARA levels in the lactating women in both groups, after child birth, was compared. Also compared was the level of ARA in the mothers breast milk.

In the first EFANATAL™ group the ARA levels were found to have decreased markedly in the blood, and to a lesser extent in the breast milk, only two weeks after the trial involving consumption of EFANATAL™ had begun. In contrast those women taking the two capsules of ARA and one capsule of DHA per day were found to have the ARA levels in their blood increase, and the breast milk levels also increased to above

0.7%.

Example 11: *Amelioration of fatty acid deficiency in mouse pregnancy through* supplementation with ARA and DHA.

A major problem during the pregnancy of humans and non-human mammals is the occurrence of intra-uterine growth retardation. This condition is associated with significant health risks for the infant after birth that may continue into adult life. The condition can develop even during pregnancy of an apparently healthy woman and is difficult to predict. It is generally assumed that it is caused by poor functioning of the placental interchange, for instance because the placenta is too small or in poor physiological condition.

This unpredictability has obstructed the development of a reliable animal model for this condition. In principle one could simulate a poor placental function by decreasing the blood flow through the umbilical vein, for instance by restricting its diameter by a clamp. The problem with this method is that it requires surgery of the pregnant animal, which can adversely affect both the foetus and the mother, and it is difficult to achieve a uniform decrease of the blood flow in this way. Therefore a different model has been developed. A poor placental function translates into a decreased supply of essential fatty acids (EFAs) to the foetus. In the 'natural' condition this is caused by a decreased blood flow, at an otherwise normal physiological concentration in the blood of the healthy mother. In the present example we have simulated this condition by decreasing the concentration of the essential fatty acids in the blood of the mother, but having a normal flow through the placenta. For this purpose an early phase of fatty acid deficiency in pregnant mice was induced. In this phase the deficiency was expressed in biochemical parameters, but functional defects were not apparent. Thus it was ensured that while the pregnancy proceeded in the normal way the supply of essential fatty acids to the foetus was restricted.

In the trial 40 female mice, 8-10 weeks of age, were fed a regular mouse chow diet for 1 week. Subsequently they were divided into 8 experimental groups: RD 1 to 4 and EFAD 1 to 4. The RD groups continued to receive a regular chow diet, containing 6.5% of fat. The EFAD groups received an essential fatty acid deficient diet. The numbers 1 to 4 indicate various lipid supplements, according to Table 1. ARA was from DSM, Delft, and DHA from Pronova (fish oil) as described in previous Examples.

Table 1: Amounts of lipid supplements as percentage of total dietary lipids. The diets contained between 3.8% of 5.6% (g/g) lipids.

RD or EFAD	MCT (Medium-Chain Triglycerides)	ARA (Arachidonic Acid Oil)	DHA (Docosahexaenoic Acid Oil)
1	19	0	0
2	15	4	0
3	4	0	15
4	0	4	15

The fatty acid composition of the RD (regular diet) and the EFAD (essential fatty acid deficient) diets as well as the oil supplements are given in Table 2.

Table 2: Fatty acid composition of lipid fractions, expressed as g% of total fatty acids.

Fatty Acid	RD lipid	EFAD lipid	MCT	ARA oil	DHA oil
8:0-12:0			100.00		
14:0	0.10			1.90	3.60
16:0	10.00	44.78		16.14	19.50
17:0	0.10				
18:0	4.00	54.73		12.10	5.11
20:0	0.30			0.85	0.34
22:0	0.30			1.48	0.29
24:0	0.20			1.55	0.18
18:3ω3	7.50				0.58
18:4ω3					0.96
20:4ω3					0.39
20:5ω3					6.52
22:5ω3					1.33
22:6ω3(DHA)					25.08
18:2ω6	55.00			7.01	1.74
18:3ω6				3.24	0.20

20:2ω6				0.38	0.30
20:3ω6				3.85	0.11
20:4ω6 (ARA)				37.64	2.15
22:4ω6					0.41
22:5ω6					8.32
16:1ω7					6.00
18:1ω7				0.45	2.77
18:1ω9	22.50	0.50		13.01	12.60
20:1ω9				0.36	0.96
22:1ω9					0.12
20:3ω9				0.04	
24:1ω9					0.46

Two additional control groups were included. One group (RD 0) did not receive any lipid supplement. The second group received the same diet as RD 0, but served as a non-pregnant (NP) outgroup. The animals had unrestricted access to the diets.

The experimental groups were treated according to the time schedule shown below.

Table 3: Time schedule of treatments.

Day	Treatment
day - 3	Intraperitoneal injection of 5 IU Folligonan (FSH) IP (all groups except NP). Regular diet replaced by experimental diets
day - 1	Intraperitoneal injection of 5 IU Chorulon (hHCG) IP (all groups except NP). Male mice introduced into the cages (all groups except NP)
day 0	Males removed
day 15	Animals killed by heart puncture under halothane anaesthesia (4-6% in N_2O/O_2

The hormone treatment with Folligonan™ and Chorulon™ (from Organon, the Netherlands) induced super-ovulation in the females. This procedure, combined with the short exposure to the males, gave a reasonable probability of pregnancy, but no guarantee. The fatty acid composition of various tissues or sections of both the pregnant

mice and their foetuses was determined by gas chromatography. The fractionation, homogenisation and extraction of the various tissues was performed by methods known in the art.

On average, the animals consumed 3.9g of the diets per day, without significant differences between the various RD and EFAD groups. The dietary dosage of PUFAs is shown in Table 4.

Table 4: Dietary dosage of ARA and DHA, expressed as a percentage of the lipid fraction and as mg intake per day.

No.	Diet	ARA		DHA	
		% of lipid	mg/day	% of lipid	mg/day
0	RD	0	0	0	0
1	RD+MCT	0	0	0	0
2	RD+ARA/MCT	1.29	2.7	0	0
3	RD+DHA/MCT	0.30	0.5	3.30	5.1
4	RD+ARA/DHA	1.63	2.5	3.25	5.0
1	EFAD+MCT	0	0	0	0
2	EFAD+ARA/MCT	1.11	2.4	0	0
3	EFAD+DHA/MCT	0.34	0.5	3.73	5.9
4	EFAD+ARA/DHA	1.58	2.3	3.27	4.8

First it was checked whether the EFAD indeed induced a biochemically relevant essential fatty acid deficiency in the blood of the female mice. There were few differences in the blood levels of various fatty acids between pregnant and non-pregnant mice of the same dietary group as seen in the comparison with RD0 and NP (data not shown). Therefore these two groups were compared, to increase the statistical power of the comparison, except in the cases where there was a significant difference between pregnant and non-pregnant animals. In those cases, the values for the pregnant individuals was used. The results are shown in Table 5.

Table 5: Levels of essential fatty acids (EFAs) in red blood cells of female mice.

PUFA (ratio)	RD+MCT	EFAD+MCT
18:3 n-3	0.19 ± 0.02	0.05 ± 0.01
20:5 n-3	0.24 ± 0.02	0.09 ± 0.03
22:6 n-3 (DHA)	6.38 ± 0.25	4.53 ± 0.28
18:2 n-6	8.43 ± 0.08	3.00 ± 0.12
20:4 n-6 (ARA)	17.23 ± 0.44	18.87 ± 0.85
EFA sufficiency index: 20:4 n-6/20:3 n-9	63	11
EFA balance index: 22:6 n-3/ 22:5 n-6	11	4

The EFAD caused a marked decrease in the level of essential fatty acids, with the exception of arachidonic acid. However, in spite of the maintenance of the level of arachidonic acid, there was a marked (n-6) essential fatty acid deficiency. This is clearly seen in the EFA sufficiency index, the ratio between the level of arachidonic acid (20:4 n-6) and its non-essential analogue mead acid (20:3 n-9). This latter fatty acid accumulates only if there are insufficient essential fatty acids as substrates for normal biosynthesis: in that case the non-essential fatty acid oleic acid (18:1 n-9) is elongated and desaturated instead, leading to the formation of n-9 analogues of the physiological PUFAs. It is clear from Table 5 that this EFA sufficiency index dropped dramatically in the EFAD-fed mice.

Another index indicates the correct balance of n-3 and n-6 essential fatty acids. This EFA balance index is the ratio between DHA (22:6 n-3) and arachidonic acid (22:4 n-6). This index also strongly decreased in the EFAD group.

So these data shown that the EFAD diet indeed induced a clear biochemical EFA deficiency, as was intended.

It was then checked whether the addition of arachidonic acid and/or DHA to the diet would lead to alleviation of this deficiency in the red blood cells of the female mice. First the control data of the fatty acid sufficient (RD) mice are presented in Table 6.

Table 6: Effect of PUFA supplementation on essential fatty acids in red blood cells of fatty acid-sufficient female mice. Fatty acid data expressed as percentage of the RD-group.

	RD	RD+ARA/MCT	RD+DHA/MCT	RD+ARA/DHA
18:3 n-3	100%	93%	74%	82%
22:6 n-3 (DHA)	100%	85%	131%	132%
18:2 n-6	100%	84%	94%	100%
20:4 n-6 (ARA)	100%	107%	79%	93%
20:4 n-6/20:3 n-9	63	59	59	67
22:6 n-3/22:5 n-6	11	9	15	14

It was found that addition of the supplements with either ARA or DHA depressed the levels of the other PUFA. In contrast, the combined supplement allowed the enhancement of the PUFA status, even in fatty acid sufficient mice. The supplement used caused a slight depression of the ARA status, causing an increase of the EFA balance index. This could be due to the ratio chosen, with DHA:ARA approximately at 2:1. Surprisingly, the EFA-sufficiency index was also enhanced by the supplement, even though these mice were apparently not fatty acid deficient.

It was then investigated whether the supplementation with PUFAs led to an improvement in the essential fatty acid status in the blood cells of the EFAD-fed animals.

Table 7: Effect of PUFA supplementation on essential fatty acids in red blood cells of fatty acid-deficient female mice. Fatty acid data are expressed as a percentage of the RD-group.

	EFAD	EFAD+ARA/MCT	EFAD+ DHA/MCT	EFAD+ ARA/DHA
18:3 n-3	24%	37%	25%	34%
22:6 n-3 (DHA)	71%	70%	174%	171%
18:2 n-6	36%	42%	49%	44%
20:4 n-6 (ARA)	109%	121%	70%	90%
20:4 n-6/20:3 n-9	11	43	43	67
22:6 n-3/22:5 n-6	4	5	16	16

Table 7 shows that the EFAD-mice responded quite strongly to the PUFA-supplement, especially in their DHA status. While there are no indications that supplementation with ARA depresses the DHA status, the converse is clearly true: the addition of the DHA supplement caused a clear depression of the ARA status. It is also clear that the addition of PUFAs specifically restored PUFA levels, with the levels of the C-18 fatty acids being much less affected. Interestingly, the combined supplement was the only one that caused full restoration of the EFA sufficiency index.

Finally it was investigated whether the enhancement of the PUFA status in the blood of the mother would lead to an improved status of the fetus. To this end we chose the head of the foetus as the most relevant compartment: the growth of the brain (and other neural tissue) is quantitatively the most important process depending on the provision of PUFAs.

The data for the foetuses of the RD-fed mothers are shown in Table 8.

Table 8: Effect of PUFA supplementation of EFA-sufficient mothers on essential fatty acids in mice foetus heads. Fatty acid data in the RD-group is expressed as mol-percent. Fatty acid data for the experimental groups is expressed as percentage of the RD-group.

	RD	RD+ARA/MCT	RD+DHA/MCT	RD+ARA/DHA
22:6 n-3 (DHA	5.89	101%	135%	125%
20:4 n-6 (ARA)	11.87	103%	93%	102%
20:4 n-6/20:3 n-9	27	30	28	40
22:6 n-3/22:5 n-6	8	6	18	13

The data show that the supplements caused modest changes in the concentrations of PUFAs in the heads of foetuses of the RD-fed mice. Surprisingly, there was a marked improvement in the EFA sufficiency index for the combined supplement, as opposed to the separate supplements. In addition, both DHA-containing supplements caused a significant increase in the EFA balance index.

Table 9: Effect of PUFA supplementation of EFA-deficient mothers on essential fatty acids in mouse foetus heads. Fatty acid data expressed as percentage of the RD-group. The EFAD+AA/MCT group did not contain pregnant females.

	EFAD	EFAD+ARA/MCT	EFAD+DHA/ MCT	EFAD+ARA/ DHA
22:6 n-3 (DHA)	61%	-	160%	146%
20:4 n-6 (ARA)	98%	-	76%	83%
20:4 n-6/20:3 n-9	9	-	14	17
20:6 n-3/22:5 n-6	2	-	33	24

The fatty acid deficiency of the foetuses was even more severe than that of the mothers. The PUFA-supplements led to a marked improvement of the EFA sufficiency index, almost restored to the RD-level. This was probably due to the relatively low dosage of arachidonic acid in the supplement, since the EFA balance index is even higher than in the foetuses of the RD-fed mothers. This implies that the PUFAs are efficiently incorporated into the foetus head. Indeed the inclusion of arachidonic acid in the supplement increases its concentration, although not up to the RD- level. This emphasises the need to balance the supplementation. The appropriate balance can then be assessed experimentally.

CLAIMS

1. An edible formulation comprising arachidonic acid (ARA) in an amount adapted to deliver a dosage of from 150mg to 1g/day ARA.

2. A formulation according to claim 1 which is adapted to deliver from 250 to 500 mg/day ARA.

3. A formulation according to claim 1 to 2 which is additionally adapted to deliver docosahexaenoic acid (DHA).

4. A formulation according to any preceding claim which is adapted to deliver a dosage of from 400 to 600 mg/day DHA.

5. A formulation according to any preceding claim wherein the ratio of ARA:DHA is from 1:5 to 5:1, such as from 1:1 to 1:2.

6. An edible formulation comprising from 150 to 700 mg ARA which is intended to be ingested once per day.

7. An edible formulation comprising from 75 to 350 mg ARA which is adapted to be ingested twice per day.

8. An edible formulation according to any preceding claim which is a food or nutritional supplement.

9. An edible formulation according to any preceding claim which is a pharmaceutical composition.

10. A pharmaceutical composition comprising ARA and DHA at a ratio of ARA:DHA at from 1:1 to 1:2.

11. A foodstuff comprising from 0.1 to 5% ARA.

12. The use of ARA as a dietary or nutritional supplement for a woman who is:

a. pregnant and at an age of from 15 to 20;

b. pregnant and at an age of from 40 to 60, such as from 50 to 55;

c. pregnant with her fourth, fifth or subsequent child;

d. pregnant with twins, triplets or quadruplets;

e. pregnant and is from 1 to 3 months into her pregnancy;

f. pregnant as a result of in vitro fertilisation (IVF) or who is undergoing IVF treatment but not yet pregnant;

g. pregnant at from 20 or more weeks of gestation;

h. pregnant and is malnourished, poorly or marginally nourished, suffering

from malnutrition or malabsorption or deficient in one or more essential fatty acids;

i. trying to become pregnant;

j. pregnant, for promoting the intra-uterine growth of health of a foetus; or

k. lactating, for increasing the level of ARA or EPA in the woman's breast milk.

13. The use according to claim 12 wherein the ARA is ingested at from 150 to 700, such as from 250 to 500, mg/day.

14. The use of ARA as a dietary or nutritional supplement for a human who is over 50 years old, preferably over 65 years old.

15. The use of ARA as a dietary or nutritional supplement for a non-human mammal which is pregnant or lactating.

16. The use of ARA for the manufacture of a medicament for assisting in the prophylaxis, prevention, amelioration or treatment of a disease or condition associated with an abnormal or low level of an n-3 or n-6 PUFA in the blood.

17. The use according to claim 16 wherein the disease or condition is a neuronal disease, such as schizophrenia, cystic fibrosis, idiopathic immunoglobulin A nephropathy, multiple sclerosis, retinitis pigmentosis, Usher's syndrome, celiac disease, macular degeneration, Parkinsons' disease, osteoporosis, Alzheimer's disease or phenylketonuria.

18. The use of ARA for promoting lactation and/or reproductive efficiency or success, or fertility in a human or non-human female mammal.

19. The use of ARA and DHA in an edible formulation at an ARA:DHA ratio that increases the ARA level in blood.

20. The use according to claim 19 wherein the ratio of ARA:DHA is from 1:5 to 5:1.

21. The use according to claim 20 wherein the ratio of ARA:DHA is from 1:1 to 1:2.

22. The use according to any of claims 19 to 21 for a person who is a diabetic, an alcoholic, a drug abuser, smoker or who is immunocompromised or has an abnormal immune level.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 99/07834

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/20 A23L1/30 A23K1/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A23L A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37200 A (SCOTIA HOLDINGS PLC) 28 November 1996 (1996-11-28) cited in the application page 4, line 4-12; claims 4,6; examples 3-5	1,3,4,11
A	WO 92 13086 A (MARTEK CORPORATION) 6 August 1992 (1992-08-06) cited in the application	1
A	EP 0 733 360 A (SCOTIA HOLDINGS PLC) 25 September 1996 (1996-09-25) page 3, column 54-56; examples 4-6	1,5,10, 16,17, 19-21

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

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PCT/EP 99/07834

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 409 559 A (EFAMOL HOLDINGS PLC) 23 January 1991 (1991-01-23) page 5, line 10-17 page 6, line 56 -page 7, line 2; claims 1-5	1-5,9, 15-22
X	WO 96 40106 A (MARTEK BIOSCIENCES CORPORATION) 19 December 1996 (1996-12-19) claims 30,31,39-41,61-64	1-7,9, 10,16, 17,19
A	DATABASE WPI Section Ch, Week 9822 Derwent Publications Ltd., London, GB; Class D13, AN 98-250984 XP002099507 & WO 98 16119 A (SUNTORY LTD), 23 April 1998 (1998-04-23) abstract	11,12
X	DATABASE WPI Section Ch, Week 9816 Derwent Publications Ltd., London, GB; Class D16, AN 98-179447 XP002099508 & WO 98 08967 A (SUNTORY LTD), 5 March 1998 (1998-03-05) abstract	14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/EP 99/07834

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9637200	A	28-11-1996	AU	5827796	A	11-12-1996
			CA	2195979	A	28-11-1996
			EP	0774962	A	28-05-1997
			FI	970298	A	24-01-1997
			JP	10503531	T	31-03-1998
			NO	970317	A	24-01-1997
			ZA	9604215	A	04-12-1996
WO 9213086	A	06-08-1992	AU	661674	B	03-08-1995
			AU	1235592	A	27-08-1992
			BR	9205519	A	01-03-1994
			CA	2101273	A	25-07-1992
			DE	568608	T	22-04-1999
			EP	0568608	A	10-11-1993
			IL	100732	A	29-06-1995
			JP	11151075	A	08-06-1999
			JP	6505384	T	23-06-1994
			MX	9200301	A	01-08-1992
			NZ	241358	A	27-09-1994
			OA	9909	A	15-09-1994
			US	5658767	A	19-08-1997
			ZA	9200454	A	28-10-1992
EP 733360	A	25-09-1996	AT	144706	T	15-11-1996
			AU	666782	B	22-02-1996
			AU	5183093	A	09-06-1994
			AU	5232996	A	18-07-1996
			CA	2109777	A	27-05-1994
			CN	1104494	A	05-07-1995
			DE	69305723	D	05-12-1996
			DE	69305723	T	03-04-1997
			DK	599576	T	25-11-1996
			EP	0599576	A	01-06-1994
			ES	2093935	T	01-01-1997
			GR	3021692	T	28-02-1997
			HK	114297	A	29-08-1997
			JP	6199663	A	19-07-1994
			NO	934266	A	27-05-1994
			NZ	250265	A	24-06-1997
			SG	47838	A	17-04-1998
			US	5516800	A	14-05-1996
			ZA	9308835	A	02-08-1994
EP 409559	A	23-01-1991	AT	116849	T	15-01-1995
			AU	625705	B	16-07-1992
			AU	5911590	A	24-01-1991
			CA	2021000	A	22-01-1991
			DE	69015910	D	23-02-1995
			DE	69015910	T	08-06-1995
			DK	409559	T	27-03-1995
			ES	2066134	T	01-03-1995
			GR	3015722	T	31-07-1995
			HK	109395	A	14-07-1995
			IE	64910	B	20-09-1995
			JP	3066616	A	22-03-1991
			NZ	234528	A	24-06-1997
WO 9640106	A	19-12-1996	AU	6252196	A	30-12-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 99/07834

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640106 A		EP 0831805 A	01-04-1998
WO 9816119 A	23-04-1998	JP 10191886 A	28-07-1998
		AU 4471997 A	11-05-1998
		EP 0956774 A	17-11-1999
WO 9808967 A	05-03-1998	JP 10070992 A	17-03-1998
		AU 4031197 A	19-03-1998
		CN 1232507 A	20-10-1999
		EP 0957173 A	17-11-1999

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权利要求书2页 说明书17页 附图页数0页

[54]发明名称 PUFA增补剂

[57]摘要

公开了包含花生四烯酸(ARA)的食用制剂,例如多不饱和脂肪酸(PUFA),例如药物组合物或营养增补剂。它们适合于释放150mg至1g/天的ARA,并且可以含有其它PUFA,例如二十二碳六烯酸(DHA)。DHA剂量为400至600mg/天,ARA:DHA之比可以是1:5至5:1。还公开了包含ARA和DHA的药物组合物,ARA:DHA之比为1:1至1:2,它们作为包含0.1至5%ARA的食品。这些制剂可用于增加体内ARA水平,例如用于妊娠妇女或患有与低ARA水平有关的疾病或病症的人。

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权 利 要 求 书

1. 包含花生四烯酸(ARA)的食用制剂，其量适合于释放 150mg 至 1g/天的 ARA 剂量。

2. 根据权利要求 1 的制剂，适合于释放 250 至 500mg/天的 ARA。

3. 根据权利要求 1 或 2 的制剂，另外适合于释放二十二碳六烯酸(DHA)。

4. 根据任意前述权利要求的制剂，适合于释放 400 至 600mg/天的 DHA 剂量。

5. 根据任意前述权利要求的制剂，其中 ARA:DHA 之比为 1:5 至 5:1，例如为 1:1 至 1:2。

6. 包含 150 至 700mg ARA 的食用制剂，打算每天摄取一次。

7. 包含 75 至 350mg ARA 的食用制剂，适合于每天摄取两次。

8. 根据任意前述权利要求的制剂，它是食品或营养增补剂。

9. 根据任意前述权利要求的制剂，它是药物组合物。

10. 包含 ARA 和 DHA 的药物组合物，ARA:DHA 之比为 1:1 至 1:2。

11. 包含 0.1 至 5% ARA 的食品。

12. ARA 作为妇女饮食或营养增补剂的用途，该妇女是:

a. 妊娠的，年龄为 15 至 20 岁;

b. 妊娠的，年龄为 40 至 60 岁，例如 50 至 55 岁;

c. 妊娠的，怀有第四、第五个或以上的孩子;

d. 妊娠的，怀有双胞胎、三胞胎或四胞胎;

e. 妊娠的，妊娠 1 至 3 个月;

f. 因体外受精(IVF)而妊娠，或者正在接受 IVF 治疗但还没有妊娠;

g. 妊娠的，妊娠 20 周或以上;

h. 妊娠的，并且是营养不良的、营养低劣或勉强的，患有营养不良或吸收障碍，或缺乏一种或多种必需脂肪酸;

i. 试图妊娠的;

j. 妊娠的，用于促进子宫内生长或胎儿健康；或者

k. 哺乳的，用于增加妇女母乳的 ARA 或 EPA 水平。

13. 根据权利要求 12 的用途，其中摄取 ARA 150 至 700、例如 250 至 500mg/天。

14. ARA 作为 50 岁以上、优选为 65 岁以上的人的饮食或营养增补剂的用途。

15. ARA 作为妊娠或哺乳期的除人以外的哺乳动物的饮食或营养增补剂的用途。

16. ARA 在药物制备中的用途，该药物有助于预防、防止、改善或治疗与血液中的异常或低水平 n-3 或 n-6 PUFA 有关的疾病或病症。

17. 根据权利要求 16 的用途，其中该疾病或病症是神经元疾病，例如精神分裂症、囊性纤维变性、自发性免疫球蛋白 A 肾病、多发性硬化、色素性视网膜炎、Usher 综合征、乳糜泻、黄斑变性、帕金森氏病、骨质疏松症、阿耳茨海默氏病或苯丙酮尿症。

18. ARA 用于促进人或除人以外的雌性哺乳动物的泌乳和/或生殖效率或成功性、或生育力的用途。

19. ARA 与 DHA 的用途，在食用制剂中按一定 ARA:DHA 比例增加血液中的 ARA 水平。

20. 根据权利要求 19 的用途，其中 ARA:DHA 之比为 1:5 至 5:1。

21. 根据权利要求 20 的用途，其中 ARA:DHA 之比为 1:1 至 1:2。

22. 根据权利要求 19 至 21 任一项的用途，用于糖尿病患者、嗜酒者、药物滥用者、吸烟者或者免疫受损或具有异常免疫水平的人。

01.05.15

说　明　书

PUFA 增补剂

本发明涉及在人和动物饮食中供应多不饱和脂肪酸(PUFA)。更具体地，本发明涉及供应 n-6 与 n-3 族多不饱和脂肪酸，特别是 n-6 脂肪酸花生四烯酸(ARA)和 n-3 脂肪酸二十二碳六烯酸(DHA)，二者比例保持平衡。

本发明在部分程度上基于下列发现，n-6 与 n-3 族的最佳平衡能够对健康以及慢性疾病的预防都起到重要作用。其主要原因是这两族脂肪酸竞争相同的用于从其 C18 前体生成长链成员的酶。其结果是，一族成员过剩趋向降低另一族的量，现有技术组合物会出现这种情况。而且，这两族成员在有些情况下可能对身体的重要机能具有不利影响，例如血液凝固和免疫应答。

前言

在饮食中提供 C18 n-6 脂肪酸亚油酸在技术上是相对容易的，因为这种脂肪酸富含在一般的植物油中，例如玉米油和大豆油。含有 C18 n-3 脂肪酸α-亚麻酸的植物油也是可以利用的，例如菜子油，但是由于稳定性较低，使用起来不太容易。这通常导致在现代饮食中 n-6 族相对 n-3 族过剩。

因此，关于在很多怀疑有相对缺乏的情况下应当补充 n-3 脂肪酸存在分歧。一般，这一点不能通过提供 C-18 前体来实现，因为它转化为 C20 与 C22 衍生物的效率是很低的。因此，一致的意见是，应当提供 C20 与 C22 n-3 脂肪酸(EPA 和 DHA)本身。

在很多情况下，这种补充作用背后的原理是削弱长链 n-6 脂肪酸 ARA 的作用。已经显示，加入从鱼油或微生物(藻类)油衍生的 n-3 PUFA 的确降低 ARA 水平。在鱼油的情况下，即使鱼油含有少量 ARA 也是如此。

这种 ARA 含量的降低不总是可取的。本发明因此寻求提供这样的制

剂，它们可以提高动物的 DHA 和/或 EPA 状态，并且不会对 ARA 水平产生不利影响，或者相反，增加 ARA 且不影响 DHA 和/或 EPA 状态。

以前已经描述过含有 ARA 与 n-3 PUFA 的制剂在供应婴儿配方 PUFA 中的用途。其背后的原理是人母乳含有适量 ARA 和 DHA，据认为它们对婴儿发育是有用的。

相形之下，关于成人营养品，还没有这样的天然 PUFA 来源，尽管 ARA 和 DHA 都可以作为人饮食中的成分找到。不过，由于多种原因，这些 PUFA 水平似乎是次优的。此外，不同人群具有不同的 PUFA 水平，这一点可能影响适合的剂量。由于自然界没有模型，需要确定所用 PUFA 的相对量，本发明寻求解决这一问题，提供 PUFA 的各种制剂和比例，适合于某些应用。

现有技术

M. Makrides 等《欧洲化学营养杂志》50: 352-357 (1996)涉及评价改变 DHA 的内部摄取（从 0 至 1.3g DHA/天）对母乳脂肪酸的作用的研究。哺乳期母亲饮食中的 DHA 对母乳 DHA 具有强特异性和剂量依赖性作用，但是不影响 ARA 水平。该研究采用藻类油，可从 Martek Corporation, USA 得到，商标名为 NEUROMINS™。

WO-A-92/12711 (Martek)涉及含有 ARA 与 DHA 的油掺合物，例如 ARA:DHA 之比为 3:1 至 2:1，特别涉及在婴儿配方中提供这些 PUFA，其水平相当于人母乳（ARA 水平为 0.5 至 0.6%）。

目前，大量含有 PUFA 的组合物市场有售。EFANATAL™是胶囊剂，每天服用两粒，每日摄取 DHA 125mg、ARA 8.6mg 和 GLA 40mg。胶囊含有一种油，其主要基于鱼油。申请人已经发现，它降低体内 ARA 水平，因为在胶囊中 DHA 含量相对 ARA 含量而言太高了。因此这种产品事实上是一种降低 ARA、而不是增加 ARA 的组合物，尽管它含有 ARA。下文提供这种产品与本发明产品之间的比较。

EFAMARINE™也是胶囊剂，主要含有鱼油和月见草油，每天服用两粒，每日摄取 EPA 34mg、DHA 22mg 和 GLA 68mg。

EFALEX™是油掺合物，每天两次服用一茶匙(5ml)，每茶匙提供 DHA

100mg、GLA 21mg、ARA 8mg和百里香油6mg。

发明概述

本发明在第一方面涉及包含ARA的食用制剂，其量适合于每天释放150mg至1g的(ARA)剂量。

优选地，该制剂适合于每天释放200至900mg的ARA，例如200至700mg/天，最佳为250至400或500mg/天。

食用制剂包括饮食增补剂和（药物）制剂和制品，例如片剂、丸剂和胶囊剂。它们另外包括（固体或液体）食品，例如乳制品（人造奶油、奶油、乳、酸奶）、面包、蛋糕；饮品，例如饮料（茶、咖啡、可可、巧克力饮料）、果汁、软饮料（例如泡沫饮料）；甜食；油性食品（小吃、色拉调味品、蛋黄酱）、汤、调味汁、富含碳水化合物的食品（米饭、面条、意大利面食）、鱼类食品、婴儿食品（例如婴儿配方，呈液体或粉末）、宠物食品和速食品或微波食品。

ARA可以来自任何适当的来源。它可以来自天然（例如植物或海产）来源，或者可以来自微生物来源，例如真菌、细菌或酵母。

适合的真菌是毛霉目，例如被孢霉属、腐霉属或虫霉属。优选的ARA来源是高山被孢霉或Pythium insidiosum。适合的商业上可得到的ARA油包括来自DSM/Gist-brocades, Wateringseweg, P.O.Box 1, 2600 MA, Delft, The Netherland的OPTIMAR™和来自Martek Corporation, 6480 Dobbin Road, Columbia, MD 21045, USA的ARASCO™。

除了ARA以外，还可以提供一种或多种其它PUFA。这可以是除ARA以外的另一种n-6 PUFA（例如C18、C20或C22脂肪酸），或者它可以是一种n-3脂肪酸（例如C18、C20或C22脂肪酸），特别是EPA和/或DHA。每种可以用在本发明中的PUFA可以是游离脂肪酸、脂肪酸酯（例如甲基或乙基酯）的形式，后者例如磷脂或甘油三酯。

如果制剂包含一种n-3脂肪酸，优选地它是EPA或DHA。如果它是DHA，那么制剂优选地适合于释放与ARA相同的剂量，例如每天400至600mg的DHA。或者，或另外，如果制剂包含EPA，那么它优选地适合

于每天释放 150mg 至 1g 的 EPA，例如 250 至 500mg EPA/天。

如果制剂每天服用（食入或摄取）一次，那么它可以含有 150mg 至 1g ARA。如果每天两次，那么制剂可以含有 75mg 至 0.5g ARA，每天三次则含量为 50mg 至 330g ARA 等，更频繁给药则按此比例。同样的计算结果也适用于可能存在的其它 PUFA，例如 DHA。

如果制剂包含一种以上 PUFA，那么每种 PUFA 的量可以用相对比例表示。例如，如果另外提供一种 n-3 PUFA，那么 ARA:n-3 PUFA（例如 DHA 或 EPA）之比可以是 1:5 至 5:1，优选为 2:1 至 1:3，最佳为 1:1 至 1:2。可以对 PUFA 的相对量进行平衡，以便补充、增加（或者至少不显著降低）PUFA 水平，因个体条件而异。

优选地，PUFA 以油的形式存在。它可以是纯净的油、精制（例如化学和/或酶学处理）或浓缩的油。这种油可以包含 10 至 100% PUFA，但是含量也可以是 20 至 45%，最佳为 30 至 45%的所需 PUFA，例如 ARA，如果是微生物油的话。当然，这种油可以含有在这些百分浓度内的一种或多种 PUFA。油可以是从单一细胞或微生物来源衍生的单一的油，或者它可以是来自这些或其它（例如植物或海产）来源的两种或多种油的掺合物或混合物。油可以含有一种或多种抗氧化剂（例如生育酚、维生素 E、棕榈酸盐），其浓度例如为 50 至 800ppm，例如 100 至 700ppm。适合于制备 PUFA 的方法描述在国际专利申请 PCT/EP97/01446 (WO-A-97/36996)、PCT/EP97/01448 (WO-A-97/37032) 和 PCT/US92/00517 (WO-A-92/13086)中。

本发明在第二方面涉及包含 ARA 和 DHA 的(药物)组合物，ARA:DHA 之比为 1:1 至 1:2。已经发现 PUFA 的这种比例可提供良好的平衡，能够增加体内 DHA 水平，并且不会因 DHA 含量过高而降低 ARA 水平。DHA 可以来自天然（例如海产）来源或来自微生物来源（例如来自藻类）。

第三方面涉及包含 0.1 至 3 或 5% ARA 的食用制剂（例如食品）。优选地，含量为 0.5 至 1.5 或 2%，最佳为 0.3 至 0.8%。第一方面内容已经讨论了适合的食品。优选的制备婴儿配方的方法公开在国际申请 PCT/EP97/01447 (WO-A-97/35487) 和 PCT/EP/97/01449

(WO-A-97/35488)中。

适合的制剂可以包括油类，例如用于口服。油可以服用其本身，或者它可以例如包封在一种外壳内，因此可以是胶囊剂的形式。外壳或胶囊可以包含明胶和/或甘油。制剂可以含有其它成分，例如矫味剂（例如柠檬或酸橙矫味剂）。

本发明已经发现了在正常、健康、吃得好的个体（如果基于适当的饮食，通常预期他们不会受益）中提高PUFA水平的用途。不过，它也可以用于低PUFA水平或缺乏的个体。

因此，本发明在第四方面涉及ARA在妇女中的用途（例如用作饮食或营养增补剂或者用于药物制备），该妇女是:

a.妊娠的，年龄为15至20岁;

b.妊娠的，年龄为40至60岁，例如50至55岁;

c.妊娠的，怀有第四、第五个或以上的孩子;

d.妊娠的，怀有双胞胎、三胞胎或四胞胎;

e.妊娠的，妊娠1至3个月;

f.因体外受精(IVF)而妊娠，或者正在接受IVF治疗（包括登记或参加IVF过程）但还没有妊娠;

g.妊娠的，妊娠20周或以上;

h.妊娠的，并且是营养不良的、营养低劣或勉强的，患有营养不良或吸收障碍，或缺乏一种或多种必需脂肪酸（例如PUFA）;

i.试图妊娠的;

j.妊娠的，用于促进子宫内生长或胎儿健康；或者

k.哺乳的，用于增加妇女母乳的ARA或EPA水平。

在(h)情况下，这些条件在西欧是相当罕见的，但是在非洲或有些亚洲国家（例如巴基斯坦）妇女可以见到。

对妊娠妇女来说，(j)中对胎儿的益处不总是可预测的或立即可见的，这是由于在流体在母亲与胎儿之间的运输上存在个体差异。胎盘与胎儿的联系（脐带）可以在大小和生理条件上是各不相同的，因此在过去，为母亲补充PUFA没有必然意味着胎儿也将接受到这些PUFA，从而

同样受益。

第五方面涉及 ARA 对 50 岁以上、优选为超过 65 岁的人（男性或女性）的用途（用作饮食或营养增补剂）。

第六方面涉及 ARA 对妊娠或哺乳期的除人以外的哺乳动物的用途（用作饮食或营养增补剂）。

ARA 优选地每天摄取 150 至 700mg，最佳为每天 250 至 500mg。

本发明在第七方面涉及 ARA 在药物制备中的用途，该药物用于（有助于）预防、防止、改善或治疗与例如血液中的异常或低水平 n-3 或 n-6 PUFA 有关的疾病或病症。本发明因此发现了在具有低水平 ARA 的受治疗者中的用途，例如那些不能或不能有效转化亚油酸(LA)为 ARA 的受治疗者。因此，适合的患者可能患有Δ6-去饱和酶机能障碍、无效或缺乏。

已经建立起 PUFA 缺乏的（小鼠）模型，用于模拟营养不良的后果。这种模型已经显示出本发明制剂的有益效果，包括在妊娠期间，对母亲和胎儿都是有益的。它还可以刺激胎盘转移不足和子宫内生长迟缓，并且显示出本发明制剂补充个体（和胎儿，如果妊娠的话）营养的益处，如发明各方面所述。

申请人已经发现，某些疾病或病症、特别是神经元疾病与低水平的体内 PUFA、特别是血液中低水平的 ARA 有关。因此认为，ARA 或 PUFA 均衡给药将能够有助于预防、防止、改善或治疗这些疾病或病症。有关疾病包括：神经元疾病，例如精神分裂症、囊性纤维变性、自发性免疫球蛋白 A 肾病、多发性硬化、色素性视网膜炎、Usher 综合征、乳糜泻、黄斑变性、帕金森氏病、骨质疏松症、阿耳茨海默氏病或苯丙酮尿症。

第八方面涉及 ARA 与可选的 DHA 的用途，用于促进人或除人以外的雌性哺乳动物的泌乳和/或生殖效率或成功性或生育力。

本发明在第九方面涉及 ARA 与 DHA 的用途，（在食用制剂中）ARA:DHA 按一定比例可增加血液中的 ARA 水平。优选地，ARA:DHA 之比为 1:5 至 5:1，例如 1:1 至 1:2。

本发明特别适用于具有低 ARA 水平的那些人，例如糖尿病患者、嗜

酒者、药物滥用者、吸烟者或者具有异常或低免疫水平或免疫受损的受治疗者。

第四至第九方面的用途包括 ARA（和可选的 DHA）本身或作为制剂对受治疗者（个体，人或动物）的给药方法，其中该受治疗者需要这种给药或者将从这种给药中受益，或者包括在指定目的药物的制备中的用途。如果必要的话，制剂可以排除 GLA 和/或 DGLA。

ARA（和 DHA，如果有的话）的剂量或含量优选地是这样的，它提高必需脂肪酸(EFA)充足指数(其定义是20:4 n-6 (ARA)水平除以20:3 n-9 脂肪酸(蜜酒酸)(mead acid)水平）和/或 EFA 平衡指数（其定义是 22:6 n-3 (DHA)水平除以 22:5 n-6 水平）。这里，各水平包括在血液（例如在红细胞内）、脑、胎盘、肝、肠、血浆或胎儿中的水平。

本发明在一方面上的优选特性和特征同样适用于另一方面，不过在细节上作必要的修改。

下列实施例仅供阐述本发明，不被解释为限制性的。

实施例 1 至 3: 含有平衡比例 PUFA 的组合物的制备

本实施例描述 n-6 与 n-3 油的掺合，以便它们能够包括在单一的胶囊内。

组合物是这样制备的，将一种富含 n-6 PUFA 的油与三种不同的富含 n-3 PUFA 的油混合。富含 n-6 PUFA 的油是从丝状真菌高山被孢霉的发酵作用衍生的，含有大约 40% ARA 作为主要的脂肪酸。关于富含 n-3 PUFA 的油，三种不同的来源是：高 EPA（45%以上）低 DHA（约 10%）鱼油（来自 Pronova，Norway，商品名 EPAX™，产品编号 EPAx4510TG）、高 DHA(50%以上)低 EPA(约 20%)鱼油(来自 Pronova，相同品牌，产品编号 EPAx2050TG）和从单细胞藻 Crypthecodinium cohnii 的发酵作用衍生的油，含有 40% DHA 作为主要脂肪酸，但是事实上缺乏 EPA(来自 Martek Corporation, Columbia, United States of America，商品名 DHASCO™）。

将油按适当的量混合，得到所需量和比例的 n-3 与 n-6 PUFA。这

里，三种掺合物（实施例 1 至 3）的 ARA:DHA 之比为 1:1。在操作过程中，对氧化作用敏感的油用不含氧的氮气层保护起来，不与环境中的氧接触。随后，这些油用于制备软凝胶胶囊，每粒胶囊含有 400mg ARA 和 400mg DHA。

实施例 4: 向处于妊娠早期或晚期阶段的妊娠妇女供应平衡的 PUFA

本实施例涉及在妊娠直至分娩（生产）过程中第 6 与第 15 周之间或第 20 与第 25 周之间向妊娠妇女补充 ARA 和 DHA 的试验。ARA 来源是含有 38% ARA 的甘油三酯油，可从 DSM/Gist-brocades, Delft, The Netherlands 得到，商品名为 OPTIMAR™。这是一种由真菌高山被孢霉产生的油。关于 DHA，采用食品级富含 DHA 的鱼油或从藻类衍生的油，是从 Martek Corporation 得到的，商标名为 DHASCO™。

因此研究了母亲在妊娠期间补充 ARA 和 DHA，与没有接受补充的对照组比较在生产时和随后的哺乳期间所测量的母亲脂肪酸状态。测量包括母亲的红细胞 ARA 与 DHA 值、脐动脉与静脉血管壁的 ARA 与 DHA 含量、母乳的 ARA 与 DHA 含量。

该研究是一例涉及 10 名妊娠妇女的对照研究。一个实验组（五名妇女）每天接受一粒或多粒胶囊（每粒 250mg ARA）油（含有 38% ARA）和每天一粒胶囊（每粒 500mg DHA）油（含有 25% DHA）。对照组接受等量安慰剂胶囊，以消除每日热量摄取差异。实验组和对照组的维生素 E 摄取是相等的，胶囊在早餐期间服用。

在试验开始和妊娠结束时采集血样。利用带有火焰离子化的毛细管气相色谱法测量红细胞脂肪酸（按磷脂计）。

发现在妊娠期间和生产时，经过补充的妇女具有显著更高水平的红细胞 DHA 和 ARA。这些高水平在哺乳期间显著持续，在母亲红细胞和母乳中都明显如此。发现母乳中的 ARA 水平已经升高到 0.8 至 1.0% ARA。另外，还发现新生儿血液中的 ARA 水平高于对照组。这项发现对处于最低限度营养条件下的母亲及其孩子具有重要意义。

实施例 5: 向老年人供应平衡的 PUFA

申请人意识到提高人群 n-3 PUFA 状态的需要，尤其是老龄人群，

已经发现诸如帕金森氏病和阿耳茨海默氏病等疾病与低 PUFA 状态有关。认为其部分原因是Δ6-去饱和酶无效或缺乏。不过，需要进行护理，尤其是对老年人，因为 ARA 水平的降低可能对免疫系统施加负面影响。

按照实施例 1 制备制剂，含有 DHA:ARA 之比为 2:1 的 n-3 与 n-6 PUFA。将胶囊对健康组、老年男性和妇女（至少 65 岁）给药，剂量为每天 1g n-3 PUFA。

一个月后，评定受治疗者红细胞的 PUFA 状态。发现在所有情况下，DHA 水平都有增加，而 ARA 水平保持恒定，或者在有些情况下显示轻微增加。因此，利用平衡的制剂提高患者的 n-3 PUFA 状态而不危害 ARA 状态是可能的。

实施例 6: 向妊娠妇女供应 PUFA

制备两种类型的含 PUFA 胶囊。第一种含有 ARA，每粒 500mg。它们每天服用一粒。ARA 是以微生物油的形式提供的，从 DSM/Gist-brocades, Delft, The Netherlands 得到，商品名为 OPTIMAR™。这些胶囊具有明胶衣，含有 20mg 维生素 E。还制备了相似的胶囊，具有等量(500mg)的 DHA，是以微生物油的形式存在的，从 Martek Corporation, Columbia, United States of America 得到（商品名为 DHASCO™）。这些胶囊也被设计成每天服用一粒。

对妊娠妇女进行试验，她们每天摄取一粒 ARA 胶囊，或每天摄取一粒 ARA 和一粒 DHA 胶囊。该研究选择已经发现具有较低血液 ARA 水平的妇女。因此测试了大量妊娠妇女的体内 ARA 血液水平，得到许可后参加研究。第一组妇女是从 15 至 20 岁的少女。关于这些妇女，这是她们的第一次妊娠。由于早熟，发现她们受益于饮食中补充 ARA 和 ARA 加 DHA。这两种方案都增加了体内 ARA 水平。

所研究的第二组妊娠妇女从 40 至 50 岁。在妊娠期间发现她们的体内血液水平在两种补充方案下都增加了。该研究所选择的妇女有一半怀上的是她们的第四个孩子。

选择三名各自怀有双胞胎的妇女，每天补充一粒 ARA 胶囊和一粒 DHA 胶囊。发现她们的 ARA 体内水平相对较低，这可能因为来自母亲血

液的 ARA 被两个胎儿所吸收和消耗。向这些妇女补充 ARA 和 DHA 胶囊，发现血液 ARA 水平增加了。

实施例 7: 向低 PUFA 含量的受治疗者供应 ARA 和 DHA

使用与实施例 6 所述相同的胶囊，但是这次的 ARA 胶囊仅含有 250mg ARA。这些胶囊可以每日服用一次或两次，因患者及其条件而异。

该研究选择了大量较低血液 PUFA 含量的人。低 PUFA 含量的原因不总是直接而明显的。不过，已经发现大量疾病或不利状况导致低 PUFA 水平，因此假定提供合适剂量的 ARA 或 ARA:DHA 的平衡可以增加体内 ARA 水平，这可以减轻一些疾病症状。人们认为有些疾病会导致前体向 ARA 本身的转化效率不良，例如Δ6-去饱和酶的缺损或缺乏。申请人发现通常导致低 PUFA 水平的疾病包括囊性纤维变性、多发性硬化、乳糜泻和骨质疏松症。另外，还发现因酒精中毒、药物成瘾而接受治疗的患者或免疫受损的患者（AIDS 患者）也具有低水平的 PUFA。

因此进行了研究，每日服用一粒或两粒 ARA 胶囊，使 ARA:DHA 含量为 1:1 或 1:2。在几乎所有情况下，都发现服用这些胶囊（至少 3 周）的患者在试验结束时增加了体内 ARA 血液水平。

实施例 8: 婴儿配方 PUFA 的供应

制备固体（粉末状）和液体婴儿配方的婴儿食品，含有 0.5% ARA 和 0.5% DHA。由已经决定不对孩子母乳喂养的母亲在前三个月将该配方有规律地喂给婴儿。作为对照，比较这些孩子与在相同时间阶段内接受母乳喂养的婴儿的体内 ARA 血液水平。发现接受奶瓶喂养的婴儿的 ARA 水平相当于接受母乳喂养的婴儿。

对比例 9 和 10

选择大量进行母乳喂养的妇女进行对比试验。一组妇女每天食入两粒 EFANATAL™胶囊（每日摄取 DHA 125mg、ARA 8.6mg 和 GLA 40mg）。关于对比，对第二组妇女类似地给以所制备的胶囊（具有明胶/甘油外壳），每粒胶囊含有 150mg ARA（每日 ARA 摄取量为 300mg ARA，每天 2 粒胶囊）。在该第二组中，还服用第三粒胶囊，每天一粒，每粒含有 500mg DHA。

比较两组哺乳妇女在生产后的 ARA 水平。还比较了母乳中的 ARA 水平。

在第一个 EFANATAL™组中，涉及 EFANATAL™消耗的试验开始后仅两周即发现 ARA 水平在血液中显著降低，在母乳中有较小程度的降低。相形之下，发现每天服用两粒 ARA 胶囊和一粒 DHA 胶囊的妇女的 ARA 水平在血液中增加，母乳水平也增加到 0.7%以上。

实施例 11：通过补充 ARA 和 DHA 改善妊娠小鼠脂肪酸缺乏

人和除人以外的哺乳动物在妊娠期间的一个主要问题是子宫内生长迟缓的发生。这种状况显著危及婴儿出生后的健康，并可持续到成年阶段。即使在表面健康的妇女妊娠期间也可能发展为这种状况，是难以预知的。一般假设这是由胎盘交换功能不良所导致的，例如因为胎盘过小或生理条件不良。

这种不可预知性阻碍了这种状况的可靠动物模型的发展。原则上人们通过减少流经脐静脉的血液可以模拟不良的胎盘功能，例如用夹子限制它的直径。这种方法的问题是它需要对妊娠动物进行手术，对胎儿和母亲都可能产生不利影响，而且按照这种方式难以实现血流的均匀减少。因此一种不同的模型已经发展起来。不良的胎盘功能转化为减少对胎儿的必需脂肪酸(EFA)供应。在“自然”状况下，这是由减少血流所导致的，不同于健康母亲血液中的正常生理浓度。在本实施例中，我们已经模拟了这种状况，方法是降低母亲血液中的必需脂肪酸浓度，但是通过胎盘的血流是正常的。为此，诱导妊娠小鼠的早期脂肪酸缺乏。在此阶段，缺乏以生物化学参数表示，但是功能缺损是不明显的。因此确保了在妊娠按正常方式进行的同时，限制了对胎儿的必需脂肪酸供应。

试验中，将 40 只 8-10 周龄的雌性小鼠用规则小鼠饮食喂养 1 周。随后分成 8 个实验组：RD 1 至 4 和 EFAD 1 至 4。RD 组继续接受规则小鼠饮食，含有 6.5%脂肪。EFAD 组接受缺乏必需脂肪酸的饮食。按照表 1，数字 1 至 4 表示各种脂质增补剂。ARA 来自 DSM，Delft，DHA 来自 Pronova（鱼油），如前面的实施例所述。

表 1：脂质增补剂占总饮食脂质的百分率。饮食含有 3.8%至 5.6% (g/g)脂质。

RD 或 EFAD	MCT（中链甘油三酯）	ARA（花生四烯酸油）	DHA（二十二碳六烯酸油）
1	19	0	0
2	15	4	0
3	4	0	15
4	0	4	15

RD（规则饮食）和 EFAD（必需脂肪酸缺乏）饮食以及油增补剂的脂肪酸组成列在表 2 中。

表 2：脂质部分的脂肪酸组成，以总脂肪酸的 g%表示。

脂肪酸	RD 脂质	EFAD 脂质	MCT	ARA 油	DHA 油
8:0-12:0			100.00		
14:0	0.10			1.90	3.60
16:0	10.00	44.78		16.14	19.50
17:0	0.10				
18:0	4.00	54.73		12.10	5.11
20:0	0.30			0.85	0.34
22:0	0.30			1.48	0.29
24:0	0.20			1.55	0.18
18:3ω3	7.50				0.58
18:4ω3					0.96
20:4ω3					0.39
20:5ω3					6.52
22:5ω3					1.33
22:6ω3(DHA)					25.08
18:2ω6	55.00			7.01	1.74
18:3ω6				3.24	0.20
20:2ω6				0.38	0.30
20:3ω6				3.85	0.11
20:4ω6(ARA)				37.64	2.15
22:4ω6					0.41
22:5ω6					8.32
16:1ω7					6.00
18:1ω7				0.45	2.77
18:1ω9	22.50	0.50		13.01	12.60
20:1ω9				0.36	0.96
22:1ω9					0.12
20:3ω9				0.04	
24:1ω9					0.46

另外包括两个对照组。一个组(RD 0)不接受任何脂质补充。第二组接受与 RD 0 相同的饮食，但是充当非妊娠(NP)外类组(outgroup)。动物进食不受限制。

将实验组按照下列时间表进行处理。

表 3: 处理的时间表

天	处理
第-3 天	腹膜内注射 5IU Folligonan (FSH) IP（所有组，NP 除外）。用实验饮食代替规则饮食。
第-1 天	腹膜内注射 5IU Chorulon (hHCG) IP（所有组，NP 除外）。将雄性小鼠放入笼中（所有组，NP 除外）。
第 0 天	取出雄性小鼠。
第 15 天	在氟烷麻醉（N_2O/O_2 中 4-6%）下通过心脏穿刺杀死动物。

用 Folligonan™ 和 Chorulon™（来自 Organon, The Netherlands）进行的激素处理诱发雌性小鼠排卵过速。这种操作以及与雄性小鼠的短暂接触得到合理的妊娠可能性，但是没有保证。用气相色谱法测定妊娠小鼠及其胎儿的各种组织或切片的脂肪酸组成。利用本领域已知的方法进行各种组织的分级分离、匀化和提取。

平均来说，动物每天消耗 3.9g 饮食，各 RD 与 EFAD 组之间没有显著差异。PUFA 的饮食剂量如表 4 所示。

表 4: ARA 和 DHA 的饮食剂量，以脂质部分的百分率和每天摄取的 mg 表示。

No.	饮食	ARA		DHA	
		脂质%	mg/天	脂质%	mg/天
0	RD	0	0	0	0
1	RD+MCT	0	0	0	0
2	RD+ARA/MCT	1.29	2.7	0	0
3	RD+DHA/MCT	0.30	0.5	3.30	5.1
4	RD+ARA/DHA	1.63	2.5	3.25	5.0
1	EFAD+MCT	0	0	0	0
2	EFAD+ARA/MCT	1.11	2.4	0	0
3	EFAD+DHA/MCT	0.34	0.5	3.73	5.9
4	EFAD+ARA/DHA	1.58	2.3	3.27	4.8

首先，检查 EFAD 是否真正诱发雌性小鼠血液中的生物化学相关性必需脂肪酸缺乏。各种脂肪酸的血液水平在相同饮食组的妊娠与非妊娠小鼠之间几乎没有差异；比较 RDO 和 NP 可以看出这一点（数据没有显示出来）。因此比较了这两组，以提高比较的统计学强度，在妊娠与非妊娠动物之间存在显著差异的情况除外。在那些情况下，采用妊娠个体的数值。结果如表 5 所示。

表 5：雌性小鼠红细胞中的必需脂肪酸(EFA)水平。

PUFA（比例）	RD+MCT	EFAD+MCT
18:3 n-3	0.19±0.02	0.05±0.01
20:5 n-3	0.24±0.02	0.09±0.03
22:6 n-3(DHA)	6.38±0.25	4.53±0.28
18:2 n-6	8.43±0.08	3.00±0.12
20:4 n-6(ARA)	17.23±0.44	18.87±0.85
EFA 充足指数 20:4 n-6/20:3 n-9	63	11
EFA 平衡指数 22:6 n-3/22:5 n-6	11	4

EFAD 导致必需脂肪酸水平显著降低，花生四烯酸除外。不过，尽管花生四烯酸水平得以维持，仍然存在显著的(n-6)必需脂肪酸缺乏。这一点从 EFA 充足指数可明显看出，该指数是花生四烯酸(20:4 n-6)与其非必需类似物蜜酒酸(20:3 n-9)之比。后一种脂肪酸仅在这样的情况下蓄积，即作为正常生物合成底物的必需脂肪酸不足：在这种情况下，作为替代，非必需脂肪酸油酸(18:1 n-9)延长和去饱和，导致生理 PUFA n-9 类似物的生成。从表 5 可明显看出，该 EFA 充足指数在 EFAD 喂养小鼠中戏剧性地下降。

另一种指数表示 n-3 与 n-6 必需脂肪酸的正确平衡。该 EFA 平衡指数是 DHA (22:6 n-3)与花生四烯酸(22:4 n-6)之比。该指数在 EFAD 组也明显地降低。

因此这些数据显示，EFAD 饮食的确诱发明显的生物化学 EFA 缺乏，这正是所需要的。

然后检查向饮食中加入花生四烯酸和/或 DHA 是否减轻雌性小鼠红

细胞中的这种缺乏。首先将脂肪酸充足(RD)小鼠的对照数据列在表 6 中。

表 6: 补充 PUFA 对脂肪酸充足的雌性小鼠红细胞中的必需脂肪酸的影响。脂肪酸数据以 RD 组的百分率表示。

	RD	RD+ARA/MCT	RD+DHA/MCT	RD+ARA/DHA
18:3 n-3	100%	93%	74%	82%
22:6 n-3(DHA)	100%	85%	131%	132%
18:2 n-6	100%	84%	94%	100%
20:4 n-6(ARA)	100%	107%	79%	93%
20:4 n-6/20:3 n-9	63	59	59	67
22:6 n-3/22:5 n-6	11	9	15	14

发现加入含有 ARA 或 DHA 的增补剂降低了其它 PUFA 的水平。相形之下，组合的增补剂改善 PUFA 状态，即使对脂肪酸充足的小鼠也是如此。所用增补剂导致 ARA 状态轻微降低，引起 EFA 平衡指数升高。这可能是由所选择的比例而引起的，DHA:ARA 大约为 2:1。惊人的是，增补剂还提高了 EFA 充足指数，即使这些小鼠显然不是脂肪酸缺乏的也是如此。

然后研究补充 PUFA 是否导致 EFAD 喂养动物血细胞中的必需脂肪酸状态的改善。

表 7: 补充 PUFA 对脂肪酸缺乏的雌性小鼠红细胞中的必需脂肪酸的影响。脂肪酸数据以 RD 组的百分率表示。

	EFAD	EFAD+ARA/MCT	EFAD+DHA/MCT	EFAD+ARA/DHA
18:3 n-3	24%	37%	25%	34%
22:6 n-3(DHA)	71%	70%	174%	171%
18:2 n-6	36%	42%	49%	44%
20:4 n-6(ARA)	109%	121%	70%	90%
20:4 n-6/20:3 n-9	11	43	43	67
22:6 n-3/22:5 n-6	4	5	16	16

表 7 显示，EFAD 小鼠对 PUFA 增补剂的反应相当强烈，尤其是它们的 DHA 状态。没有迹象表明补充 ARA 会降低 DHA 状态，不过反之的确

正确：加入 DHA 增补剂导致 ARA 状态明显降低。另外明显的是，加入 PUFA 特定地恢复 PUFA 水平，而 C-18 脂肪酸水平很少受影响。有趣的是，组合的增补剂是唯一导致 EFA 充足指数完全恢复的。

最后研究提高母鼠血液中 PUFA 状态是否会改善胎儿的状态。为此我们选择胎儿头部作为最相关的隔室：定量地说，脑（和其它神经组织）的生长是依赖于 PUFA 供应的最重要的过程。

RD 喂养的母鼠胎儿数据如表 8 所示。

表 8：向 EFA 充足的母鼠补充 PUFA 对小鼠胎儿头部中必需脂肪酸的影响。RD 组的脂肪酸数据以 mol－百分率表示。实验组的脂肪酸数据以 RD 组的百分率表示。

	RD	RD+ARA/MCT	RD+DHA/MCT	RD+ARA/DHA
22:6 n-3(DHA)	5.89	101%	135%	125%
20:4 n-6(ARA)	11.87	103%	93%	102%
20:4 n-6/20:3 n-9	27	30	28	40
22:6 n-3/22:5 n-6	8	6	18	13

数据显示，增补剂导致 RD 喂养的小鼠胎儿头部中的 PUFA 浓度发生适度改变。惊人的是，组合的增补剂显著改善 EFA 充足指数，这一点与单独的增补剂相反。另外，两种含有 DHA 的增补剂都导致 EFA 平衡指数显著提高。

表 9：向 EFA 缺乏的母鼠补充 PUFA 对小鼠胎儿头部中的必需脂肪酸的影响。脂肪酸数据以 RD 组的百分率表示。EFAD+AA/MCT 组不包含妊娠的雌性小鼠。

	EFAD	EFAD+ARA/MCT	EFAD+DHA/MCT	EFAD+ARA/DHA
22:6 n-3(DHA)	61%	-	160%	146%
20:4 n-6(ARA)	98%	-	76%	83%
20:4 n-6/20:3 n-9	9	-	14	17
20:6 n-3/22:5 n-6	2	-	33	24

胎儿的脂肪酸缺乏甚至比母鼠更为严重.PUFA 增补剂显著改善 EFA 充足指数，几乎恢复至 RD 水平。这可能是由于增补剂中的花生四烯酸

剂量相对较低，因为 EFA 平衡指数甚至更高于 RD 喂养的母鼠胎儿。这暗示了 PUFA 有效地结合到胎儿头部中。事实上，在增补剂中包含花生四烯酸增加了其浓度，尽管尚未达到 RD 水平。这一点强调了平衡补充的必要性。适当的平衡能够通过实验方法加以评价。