An Assessment of Docosahexaenoic Acid (DHA) Intake with Special Reference to Lipid Metabolism in Rats

Morio Saito,* Kazuhiro Kubo,** and Sachie Ikegami
Division of Food Science, The National Institute of Health and Nutrition, Tokyo 162, Japan
(Received January 23, 1996)

Summary In order to determine the appropriate intake of docosahexaenoic acid (22:6n-3, DHA), the potential changes in lipid peroxidation and antioxidant defense in serum and tissue as well as the changes in serum lipid levels were examined in rats by giving them diets containing graded levels of purified DHA (0, 1.0, 3.4 and 8.7 energy % in the diets) for 2 weeks. Serum \( \alpha \)-tocopherol concentration decreased slightly but significantly even at the 1.0 energy %. Liver lipid peroxide levels as assessed by the thiobarbituric acid (TBA) value and chemiluminescence intensity augmented at the 3.4 energy % and more, and the \( \alpha \)-tocopherol content significantly decreased in response to the increase in lipid peroxide levels. In the kidney, a slight but significant increase in TBA value was observed even at 1.0 energy % and higher. All the serum lipid levels as analyzed by total cholesterol, HDL-cholesterol, triacylglycerol (TG) and phospholipids (PLs) decreased as the dietary DHA level increased. These experimental results suggest that the dietary intake of DHA should be less than 1 energy % to avoid promoting deleterious influences such as serum and tissue lipid peroxidation and to ameliorate serum lipid levels.

Key Words assessment of docosahexaenoic acid intake, docosahexaenoic acid, docosahexaenoic acid intake, lipid peroxidation, vitamin E, serum lipid, fatty acid profile, rat

Diets recommended for the general public to prevent heart disease, cancer and diabetes involve reducing total fat and cholesterol and replacing saturated (S) fat with polyunsaturated (P) fats of both the n-3 and n-6 types. Consumption of

\(^1\) n-3 fatty acid intake and lipid peroxidation (2).

*To whom all correspondence should be addressed.

**Present address: Department of Agricultural Chemistry, Tokyo University of Agriculture, Tokyo 156, Japan

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; PI, peroxidizability index; TBA, thiobarbituric acid; MDA, malondialdehyde; VE, vitamin E; PL, phospholipid; TG, triacylglycerol

195
marine oils, predominantly the n-3 type, has attracted intense interest due to the low incidence of cardiovascular diseases, atherosclerosis and some inflammatory diseases (1,2). The prophylactic effect of marine oil is attributed to its considerably high content of n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20: 5n-3, EPA) and docosahexaenoic acid (22: 6n-3, DHA) (1, 2). More recently, studies with nonhuman primates and human newborns have shown that DHA is essential for the normal functional development of the retina and brain, particularly in premature infants (2, 3). Consequently, fish oils and the concentrates of n-3 PUFAs from fish oils have been dispensed in many countries as medicinal and/or nutritional supplements (4, 5). These products are sometimes consumed in excess by individuals who expect positive health benefits.

However, fish oil intake induces substitution of membrane n-6 PUFAs with potentially unstable n-3 PUFAs even from a normal level of dietary fish oils, which generally increases the susceptibility of cellular membranes to lipid peroxidation (6-10) and increases the need for α-tocopherol, a membranous lipid-soluble antioxidant (7, 9-11). Hence, dietary supplementation with n-3 PUFA capsules with only a low amount of α-tocopherol might weaken the normal antioxidant defenses (11). Nevertheless, the potential harmful influences of fish oils and fish oil products have been overlooked because of a number of health benefits from n-3 PUFAs as mentioned above.

Accordingly, in this study, to determine the appropriate intake of DHA from the viewpoint of safety and physiological efficacy, the potential changes in lipid peroxidation and antioxidant defense in serum and tissue as well as the changes in serum lipid levels were examined using purified DHA in rats.

MATERIALS AND METHODS

Animal and diets. Male Sprague-Dawley rats, 4 weeks of age, weighing 75-85 g, were housed individually in stainless steel wire-bottomed cages at a constant temperature of 22±1°C and humidity of 50-60% with a 12-h light-dark cycle. The compositions of experimental diets based on AIN-76 purified diet for rats (12) are shown in Table 1. The energy density of all the diets was 416 kcal/100 g (1.74 MJ/100 g), where the Atwater energy factors were used. The vitamin E (VE) content of all the diets was adjusted to 20 IU/100 g by analyzing the content of VE homologues in dietary lipids (13) and then by adding all-rac-α-tocopheryl acetate to the diets. The relative biological activities for RRR-α-, RRR-β-, RRR-γ- and RRR-δ-tocopherols were taken as 100 : 25 : 5 : 0.1 in the calculation (14). The lipid content of the diets was 10 wt % and the fat energy % 21.6%. DHA levels of the diets were made up into 0, 1.0, 3.4 and 8.7 energy %, respectively, by combining olive oil, safflower oil and DHA concentrate, the latter of which was composed of 83% of the ethyl ester form of DHA. The fatty acid composition of dietary lipids is indicated in Table 2. The control lipid devoid of DHA contained about 41% linoleic acid, which was comparable to the DHA level of 8.7 energy % diet. Also,
Table 1. Composition of experimental diets given to rats (g/100 g diet).  

<table>
<thead>
<tr>
<th>DHA level (energy %)</th>
<th>0%</th>
<th>1.0%</th>
<th>3.4%</th>
<th>8.7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic components²,³</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Lipid</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Olive oil</td>
<td>5.00</td>
<td>8.98</td>
<td>7.32</td>
<td>4.00</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>5.00</td>
<td>0.50</td>
<td>0.63</td>
<td>0.88</td>
</tr>
<tr>
<td>DHA concentrate⁵</td>
<td>0</td>
<td>0.52</td>
<td>2.05</td>
<td>5.12</td>
</tr>
</tbody>
</table>

¹The Atwater energy factors were used for the energy % calculation.
²The basic components of the diets given to all the groups were: casein, 20.0 g; DL-methionine, 0.3 g; cornstarch, 15.0 g; sucrose, 22.5 g; glucose, 22.5 g; cellulose powder, 5.0 g; AIN-76 mineral mixture, 3.5 g; AIN-76 vitamin mixture, 1.0 g; choline bitartrate, 0.2 g.
³Vitamin E content of all the diets was adjusted to 20 IU/100 g diet.
⁴Fat energy % is 21.6%.
⁵The purity of DHA concentrate (ethyl ester form) is 83%.

Table 2. Fatty acid composition of dietary lipids given to rats (%) ¹

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>DHA level (energy %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>16:0</td>
<td>8.8</td>
</tr>
<tr>
<td>16:1 (n-7)</td>
<td>0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>2.8</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>45.9</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>41.4</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>0.5</td>
</tr>
<tr>
<td>22:1 (n-11)</td>
<td>—</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>—</td>
</tr>
<tr>
<td>22:3 (n-3)</td>
<td>—</td>
</tr>
<tr>
<td>22:5 (n-3)</td>
<td>—</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>—</td>
</tr>
<tr>
<td>n-6 (%)</td>
<td>41.4</td>
</tr>
<tr>
<td>n-3 (%)</td>
<td>0.5</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>82.8</td>
</tr>
<tr>
<td>Double bond index²</td>
<td>1.31</td>
</tr>
<tr>
<td>Peroxidizability index³</td>
<td>42.4</td>
</tr>
</tbody>
</table>

¹Values of fatty acids less than 0.3% are not shown.
²Double bond index expresses mean double bond number and is the sum of the fraction of each fatty acid × the number of double bonds in that acid.
³Peroxidizability index; see test.
each test lipid was prepared to supply at least 2 energy % of linoleic acid as an essential fatty acid of the n-6 type, where the composition of linoleic acid was a little more than 9%. The degree of unsaturation of dietary lipids was presented as the double bond index (15), and of peroxidizability as the peroxidizability index (PI) (16). PI was calculated according to the following equation: PI = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

**Feeding trial.** After being fed the basal diet containing 5% olive oil for 3 days, 6–7 rats of each group were maintained on the experimental diets for 14 days. Food and water were available *ad libitum.* To prevent autoxidation of DHA concentrate in the diets, each diet was prepared beforehand without adding the concentrate and stored at −20°C. DHA concentrate stored at −80°C was mixed with the diet every day immediately before feeding. Furthermore, the diets were given to rats in the evening and removed the next morning. After fasting overnight, the rats were killed by cardiac puncture and the tissues were promptly excised, washed with isotonic saline, weighed, and the liver was perfused with ice-cold isotonic saline via the portal vein. The tissues were stored at −80°C until analysis. Serum was separated by centrifugation at 2,700×g for 15 min at 4°C.

**Analytical procedures.** The serum TBA value was determined by the method of Yagi (17) with minor modification, where butylated hydroxy toluene was added to the reaction mixture as an antioxidant at a final concentration of 0.36 mM. Serum water-soluble fluorescent substances were determined by the method of Tsuchida et al. (18). The value was expressed in terms of relative fluorescence intensity against a quinine sulfate solution (0.1 μg/ml of 0.1 N sulfuric acid) as 100. Liver and kidney TBA values were also measured (19) with minor modification, in which butylated hydroxy toluene was added to the reaction mixture at a final concentration of 0.45 mM. The TBA value is expressed in terms of malondialdehyde (MDA) equivalent. Liver and kidney chemiluminescence intensities of the homogenates were determined according to the method of Miyazawa et al. (20). A synchronous, single photon-counting apparatus, Chemiluminescence Analyzer OX-7 equipped with a Hamamatsu R878 photomultiplier with a spectral response range of 300–650 nm (Tohoku Electronic Industries Co., Sendai), was used. Liver microsomes were prepared (21) and microsomal lipofuscin content was determined by the method of Fletcher et al. (22). Quinine sulfate at a concentration of 0.1 μg/ml of 0.1 N sulfuric acid was used as the standard for fluorescence intensity analysis. The level of lipofuscin was expressed in terms of the quinine sulfate equivalent. α-Tocopherol levels in serum, liver and kidney were analyzed by HPLC as described previously (13).

Liver microsomal drug-metabolizing enzyme activities, as analyzed by the contents of cytochromes P-450 and b5, and the activities of aminopyrine N-demethylase and aniline hydroxylase, were measured as described in previous papers (21, 23). Protein content was measured by the method of Lowry et al. (24). Tissue lipids were extracted according to the method of Folch et al. (25). Fatty
acid composition of dietary, serum and total tissue lipids was determined as previously described (23). Total serum cholesterol (Determiner TC 555, Kyowa Medex Co. Ltd., Tokyo), HDL-cholesterol (Determiner HDL, Kyowa Medex), phospholipids (PLs, Determiner PL, Kyowa Medex) and TG (Nescauto TG-A, Nihonshoji Co. Ltd., Osaka) were determined by the use of standard clinical chemistry kits available commercially.

Statistical analysis. Statistical significance of differences between mean values was assessed by analysis of variance (ANOVA) coupled with Duncan’s multiple-range test at the 5% level of significance (26).

RESULTS AND DISCUSSION

Rats consumed 14.7-15.7 g of diet a day and gained 6.0-6.6 g body weight a day for 14 days. Food intake and body weight gain of all the groups were not significantly different although the body weight gain of 3.4 energy % DHA group tended to be higher. Liver weight was not significantly different among all the groups although the relative liver weight (g/100 g body weight) was significantly lower in the 3.4 energy % DHA group than those of other dietary groups. This was probably due to the higher body weight gain in the 3.4 energy % DHA group. Kidney weight was slightly but significantly higher in the 3.4 energy % of DHA than in the control although the relative weight was not significantly different among all the groups. This may also be due to the higher body weight gain in the 3.4 energy % DHA group. There were no significant differences in the heart weights among the dietary groups.

The serum TBA value increased significantly at the highest level of dietary DHA (8.7 energy %) (Fig. 1). Water-soluble fluorescent substance varied within a very small range and the variation was negligible. The serum α-tocopherol concentration decreased slightly but significantly even at the lowest level of dietary DHA (1.0 energy %).

As shown in Fig. 2, liver lipid peroxide levels as assessed by TBA value and chemiluminescence intensity augmented at the 3.4 energy % and more, and the α-tocopherol content significantly decreased in response to the increase in lipid peroxide levels. The liver microsomal lipofuscin level, however, did not change significantly even at the highest dose of DHA.

In the kidney, a slight but significant increase in TBA value was observed at the 1.0 energy % of DHA and higher, but the α-tocopherol content decreased significantly only at the highest level of dietary DHA (Fig. 3). The kidney chemiluminescence value was highest at the highest dose of DHA although no definite tendency was recognized in the values due to the wide variation of the data.

From the results of serum and tissue lipid peroxide levels and the antioxidant defense as assessed by the α-tocopherol level, the intake of DHA increased susceptibility of tissues to lipid peroxidation and augmented the requirement for α-tocopherol as was observed in fish oil intake (6–11). However, the serum GOT
Fig. 1. Influences of graded levels of dietary DHA on TBA value (●-●, μmol MDA/liter), water-soluble fluorescent substance (WSF) level (●-●, relative fluorescence intensity) and α-tocopherol (VE) concentration (○-○, μM) in serum of rats. All data are given as M±SD of 6-7 animals. Mean values within the same analytical item not followed by a common letter are significantly different (p<0.05).

Fig. 2. Influences of graded levels of dietary DHA on TBA value (●-●, nmol MDA/g), chemiluminescence intensity (■-■, count/30s), microsomal lipofuscin content (□-□, ng/mg protein) and α-tocopherol content (○-○, μg/g) in liver of rats. All data are given as M±SD of 6-7 animals. Mean values within the same analytical item not followed by a common letter are significantly different (p<0.05).

and GPT activities did not increase significantly after the ingestion of DHA, even at the highest level (data not shown). Therefore, the intake of DHA seemed not to promote parenchymal tissue cell damages in a current experimental condition, particularly in a short period of feeding. A longer feeding trial is in progress to
An Assessment of Docosahexaenoic Acid Intake

Fig. 3. Influences of graded levels of dietary DHA on TBA value (○—○, nmol MDA/g), chemiluminescence intensity (■—■, count/30 s) and α-tocopherol content (□—□, μg/g) in kidney of rats. All data are given as M±SD of 6–7 animals. Mean values within the same analytical item not followed by a common letter are significantly different (p<0.05).

Fig. 4. Influences of graded levels of dietary DHA on serum lipid concentrations (μM) in rats. Their symbols are as follows: total cholesterol, ■—■; HDL-cholesterol, □—□; triacylglycerol, ○—○; phospholipid, ●—●. All data are given as M±SD of 6–7 animals. Mean values within the same analytical item not followed by a common letter are significantly different (p<0.05).

confirm the effect of feeding period.

All the serum lipid levels as analyzed by total cholesterol, HDL-cholesterol, TG and PLs decreased as the dietary DHA level increased although the TG concentration did not decrease significantly, and the lipid levels almost leveled off at the 3.4 energy % (Fig. 4). Dietary DHA effectively diminished the serum total cholesterol concentration even at the 1.0 energy % but not the TG. This effective diminution of serum total cholesterol by dietary DHA was also observed elsewhere.
202 M. SAITO et al.

(27, 28). The HDL-cholesterol concentration also decreased through DHA intake but the changes in the ratio of HDL-cholesterol to total cholesterol (0.67, 0.67, 0.61 and 0.63 for 0, 1.0, 3.4 and 8.7 energy % DHA groups, respectively) were negligible due to the concomitant decrease in total cholesterol concentration.

Liver microsomal drug-metabolizing enzyme activities as measured by cytochrome P-450 content and aminopyrine N-demethylase and aniline hydroxylase activities (Table 3) considerably increased at the level of 3.4 energy % and higher, which was consistent with our previous results (29). Liver microsomal drug-metabolizing enzymes are generally induced when xenobiotics are ingested, leading to an alteration in oxidative detoxification of xenobiotics (30, 31). Therefore, these results show that the higher intake of highly unsaturated DHA, 3.4 energy % and above, may promote oxidative detoxification through stimulation of the liver microsomal drug-metabolizing enzyme system.

Fatty acid profiles of the serum, liver microsomes and heart lipids were analyzed (Fig. 5). The serum lipid showed compositions as a function of dietary lipids as observed particularly in the compositional changes in oleic, linoleic, arachidonic and docosahexaenoic acids. The incorporation of DHA increased and almost a constant level of arachidonic acid was retained in liver microsomal lipids. A characteristic fatty acid profile was noticed in the heart lipid, where the incorporation of DHA was higher than that of serum and liver microsomal lipids even at the 1.0 energy %, which was accompanied by a low level of linoleic acid as seen in the liver microsomal lipids. Similar results have also been obtained elsewhere when DHA is fed to animals (32, 33). The low level of linoleic acid and high level of DHA in heart lipids, particularly in mitochondrial cardiolipin, are believed to be associated with functional disorders of the heart mitochondria (34).

Table 3. Influences of graded levels of dietary DHA on liver microsomal drug-metabolizing enzyme activities in rats.

<table>
<thead>
<tr>
<th></th>
<th>DHA level (energy %)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>1.0%</td>
<td>3.4%</td>
<td>8.7%</td>
<td></td>
</tr>
<tr>
<td>Microsomal protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td>21.0±2.2a</td>
<td>22.8±1.5ab</td>
<td>24.4±1.7bc</td>
<td>26.1±2.1c</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td>0.79±0.07ab</td>
<td>0.67±0.07c</td>
<td>0.90±0.17ad</td>
<td>0.99±0.05d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.5±1.3a</td>
<td>15.3±1.7a</td>
<td>22.5±3.9b</td>
<td>25.8±2.6c</td>
<td></td>
</tr>
<tr>
<td>Aminopyrine N-demethylase</td>
<td>10.4±0.8ab</td>
<td>9.6±1.2a</td>
<td>11.2±1.0b</td>
<td>11.4±0.9b</td>
<td></td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td>217±22a</td>
<td>219±31a</td>
<td>266±22b</td>
<td>296±27c</td>
<td></td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td>0.80±0.13a</td>
<td>0.85±0.18ab</td>
<td>0.98±0.08bc</td>
<td>1.04±0.10c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.8±3.6a</td>
<td>19.3±4.6ab</td>
<td>23.0±2.6bc</td>
<td>27.0±3.3c</td>
<td></td>
</tr>
</tbody>
</table>

All data are given as M±SD of 6–7 animals. Mean values within the same row that are not followed by a common letter are significantly different (p < 0.05).
As recognized in this study, feeding DHA to rats increased the serum and tissue DHA levels and decreased n-6 PUFA, linoleic acid, implying a substitution of tissue membrane fatty acids with the potentially unstable DHA. Such changes in the fatty acid profiles, particularly in the hearts, are reported to be associated with functional disorders of the heart mitochondria as described above. Moreover, the
higher intake of DHA overwhelmed the normal antioxidant defenses, and thus, the serum and tissue lipid peroxidation increased significantly followed by decrease in the $\alpha$-tocopherol levels. These phenomena are generally recognized in fish oil ingestion in animals and humans (6-11). On the other hand, DHA had a hypolipidemic effect as observed in the total cholesterol in the circulating blood even at the lowest dose of DHA, 1.0 energy %, in rats.

Taking all the results obtained herein into consideration from the viewpoint of safety and physiological efficacy, the recommended dietary intake of DHA is less than 1 energy % to avoid promoting detrimental in vivo lipid peroxidation, to ameliorate serum lipid levels, and to avoid disturbing homeostatic and physiological functions of tissues which might interfered with at higher intake of DHA.

According to the National Nutrition Survey in Japan in 1993, the energy intake and fat energy % were 2,034 (kcal/day/capita) and 25.7%, respectively. The total fat intake, therefore, is 58.1 g ($2,034 \times 0.257 \times 0.9$) and the total fatty acid intake 52.3 g ($58.1 \times 0.9$). Rather limited data including personal communications are currently available regarding the ratios of intake of saturated (S), monounsaturated (M) and polyunsaturated (P) fatty acids, and that of $n$-6 and $n$-3 PUFAs calculated from the National Nutrition Survey in Japan (35). The ratios of S : M : P, 1.1 : 1.3 : 1.0, and of $n$-6 : $n$-3, 4.3 : 1, may be considered to reflect the current status. So, the intake (g) of S : M : P is 16.9 : 20.0 : 15.4, and that of $n$-3 PUFAs 2.9 g ($15.4 \times 1/5.3$). In addition, it may be postulated to calculate the daily intake of EPA and DHA as follows: the total rate of EPA and DHA in the total $n$-3 PUFAs is about 35%, of which 15% is EPA and 20% DHA, indicating that the intake of EPA is 0.44 g ($2.9 \times 0.15$) and of DHA 0.58 g.

The amount of fatty acid equivalent to 1 energy % calculated from the National Nutrition Survey in Japan in 1993 is about 2 g. If it would be possible to extrapolate the result obtained herein in rats to humans, the daily intake of DHA plus EPA in Japanese is less than 1 energy % of daily total energy intake. Moreover, an estimated minimal daily requirement of $\alpha$-linolenic acid and of long-chain $n$-3 PUFAs is reported to be 0.2–0.5% and 0.1–0.2%, respectively, of total energy intake (36, 37). Accordingly, Japanese take enough DHA and EPA as essential fatty acids in our daily meals.

The technical assistance of Misses Fumi Uetsuji, Megumi Ueno and Mihoko Yazawa is greatly appreciated. The authors thank Maruha Corporation, Japan, for the generous gift of purified DHA.

REFERENCES

An Assessment of Docosahexaenoic Acid Intake


An Assessment of Docosahexaenoic Acid Intake

467–472.
