Lipid Peroxidation and 8-Hydroxydeoxyguanosine Formation in Rats Fed Fish Oil with Different Levels of Vitamin E

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Summary This study was conducted to investigate the protective role of vitamin E against the formations of lipid peroxides in plasma and tissues and of 8-hydroxydeoxyguanosine (8-OHdG) in livers of rats fed fish oil. Six-week-old male Sprague-Dawley rats were divided into four groups and fed experimental diets for 8 weeks. Three fish oil (F) groups were fed a menhaden fish oil and soybean oil (9:1) mixture as 10% (wt/wt) of their diet. These three groups (FO, FI, and FII) were provided with ≤3, 45, and 209 IU of vitamin E/kg diet, respectively. The SI group was fed soybean oil with ≤45 IU of vitamin E/kg diet. The FO group had the highest levels of thiobarbituric acid-reactive substances (TBARS) in plasma (per milligram lipid), and liver, lung, heart, and kidney. The FI group had higher levels of TBARS than the SI group in plasma and tissues except the lung. In liver, the TBARS levels of the FII group were also higher than those of SI group, but in other tissues, similar levels were observed in the FII and SI groups. Plasma level of vitamin E was lowest in F0 group and vitamin E levels were generally lower in F groups than in SI group. These levels were expressed as vitamin E per milliliter of plasma. However, plasma E levels were similar when expressed per milligram plasma lipid. The liver 8-OHdG concentration tended to decrease as

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Abbreviations: BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; TBARS, thiobarbituric acid-reactive substances; TBHQ, tertiary butylhydroquinone.
dietary vitamin E increased in the F groups, but there was no difference in the level of 8-OHdG between the FI and SI groups. These results suggest that vitamin E should be supplemented in fish oil feeding to prevent the enhanced lipid peroxidation and the formation of 8-OHdG in the body.

Key Words  fish oil, vitamin E, plasma and tissue TBARS, liver 8-OHdG

Many studies have shown that long chain n-3 polyunsaturated fatty acids in fish oil may decrease the risk of development of cardiovascular disease and several other chronic illnesses (1). Other studies have indicated that docosahexaenoic acid (DHA) is essential for normal function of the retina and brain (2–4). These studies have been utilized in establishing daily allowance of n-3 fatty acids (1, 5) and in shaping nutrition policy (6,7). All of this information and actions have already stimulated consumption of fish or fish oil capsules in the general public (8) and have led to considerable progress in utilizing fish oil in preterm infant formula (9, 10) and processed foods (6).

However, due to its high unsaturation, fish oil can be rapidly oxidized to produce lipid peroxide. Several studies have shown increases in serum or plasma and tissue contents of thiobarbituric acid-reactive substances (TBARS) in animals (11–15) and humans (16, 17) fed fish oil or fish oil concentrates. In some cases (11, 12, 17), plasma or tissue vitamin E levels were decreased concomitantly. Process of lipid peroxidation involves the formation of free radicals, which can attack other tissue components than lipids. One of the candidates is nucleic acid. It has been reported that guanine in DNA undergoes oxidation to 8-hydroxyguanine by oxygen free radical (18). If this occurs, adenine replaces cytosine for pairing with 8-hydroxyguanine during DNA replication and mutation results (18).

Therefore, in the present study we investigated 8-hydroxydeoxyguanosine (8-OHdG) formation in the liver along with lipid peroxide formation and vitamin E status in plasma, liver, lung, heart, and kidney in rats fed fish oil with three different levels of dietary vitamin E. To compare the effect of fish oil with that of n-6 polyunsaturated oil, a group of animals was fed soybean oil with one level of dietary vitamin E.

MATERIALS AND METHODS

Animals and diets. Six-week-old male Sprague-Dawley rats (Experimental Animal Management Division, National Institute of Health, Seoul, Korea) were divided into four groups of eight animals each, according to the type of oil and the level of vitamin E in the diet. For 8 weeks, each group was fed one of four respective diets, the compositions of which are shown in Table 1. Starch and glucose were purchased from Je-il Feed Co. (Seoul, Korea). Vitamin-free casein, mineral mix, cellulose, choline, inositol, DL-methionine were purchased from Teklad Test Diets (Madison, WI, U.S.A.) and DL-α-tocopheryl acetate was ob-
Table 1. Compositions of experimental diets.

| Vitamin E (IU/kg)  | SI | F0 | FI | FII |
|--------------------|----|----|----|-----|
| ≤45                | 3  | 45 | 209|     |

Component (g/kg)

| Starch             | 190|
| Glucose            | 240|
| Mineral mix (AIN-76A) | 40 |
| Vitamin E-free vitamin mix | 10 |
| Cellulose          | 40 |
| Choline            | 0.5|
| Inositol           | 0.5|
| DL-Methionine      | 2.2|
| Soybean oil        | 100|
| Menhaden oil       | 90 |
| DL-α-Tocopheryl acetate | 0.019 |

1 Without DL-α-tocopherol supplementation, vitamin E contents of SI and fish oil diets (F0, FI, FII) were calculated to be ≤26 and ≤3 IU, respectively, since active tocopherol contents of soybean and menhaden oils were ≤17.4 and ≤0.5 mg/100 g oil. 2 Vitamin E-free vitamin mix (per kg) contained thiamin-HCl, 0.6 g; riboflavin, 0.6 g; pyridoxin-HCl, 0.7 g; nicotinic acid, 3.0 g; Ca-pantothenate, 1.6 g; folic acid, 0.2 g; biotin, 0.02 g; vitamin B12, 0.001 g; retinyl acetate, 4.0 × 10^5 IU; ergocalciferol, 1.6 × 10^5 IU; menadione, 0.075 g; glucose 992.5 g.

Sample preparation. At the end of feeding period, the rats were fasted for 16 h before sacrifice. Under ether anesthesia, blood was drawn by heart puncture and plasma was prepared with heparin. Liver, heart, lung, and kidney were immediately removed, washed in saline and rapidly frozen in liquid nitrogen. Plasma and tissue were stored at −60°C until analysis.
Table 2. Fatty acid compositions of dietary oils (%).

| Fatty acid | Soybean oil | Menhaden oil (9) + soybean oil (1) |
|------------|-------------|----------------------------------|
| 14:0       | 0.1         | 7.3                              |
| 15:0       | ND¹         | 0.4                              |
| 16:0       | 10.8        | 16.7                             |
| 16:1       | 0.2         | 9.9                              |
| 18:0       | 3.6         | 4.5                              |
| 18:1       | 20.5        | 14.8                             |
| 18:2 (n-6) | 55.7        | 10.7                             |
| 18:3 (n-3) | 7.3         | 3.8                              |
| 18:4 (n-3) | ND          | 4.2                              |
| 20:5 (n-3) | ND          | 12.6                             |
| 22:1       | ND          | 1.4                              |
| 22:5 (n-3) | ND          | 1.6                              |
| 22:6 (n-3) | ND          | 8.8                              |
| Total PUFA | 63.0        | 41.7                             |
| n-3/n-6    | 0.13        | 2.90                             |
| Peroxidizability index² | 70.3        | 131.7                            |
| Peroxide value³  | 0.75        | 2.90                             |

¹Not detected. ²Peroxidizability index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5). ³Peroxide values were determined according to the American Oil Chemists' Society method (20).

**TBARS assays.** Thiobarbituric acid-reactive substances (TBARS) in plasma and tissue were measured by the methods of Yagi (21) and Hu et al. (14), respectively, using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co.) as a standard. Plasma total lipids were measured by the method of Frings and Dun (22).

**Vitamin E analysis.** Plasma vitamin E were extracted with hexane and determined by HPLC (23) using (+)-d-tocopheryl acetate (Sigma Chemical Co.) as internal standards. A C-18 microBondapak (Waters, Milford, MA) 300 × 8 mm stainless steel column was used for analysis (Waters, HPLC 510) with mobile phase of methanol/H₂O (95:5, vol/vol) and UV detection at 280 nm. Tissue vitamin E was determined by the method of Kayden et al. (24). For this purpose, 10% tissue homogenate in saline was saponified with KOH in the presence of 2% pyrogallol and extracted with hexane. Total tocopherol in hexane phase was reacted with ferric chloride and dipyridyl and optical density was measured at 520 nm.

**8-Hydroxydeoxyguanosine determination.** DNA was extracted from liver by a standard method with slight modification (25). About 100 mg of frozen tissue was digested with 1 mg/ml proteinase K (Sigma Chemical Co.) in 50 mM Tris buffer (pH 8.0) containing 100 mM NaCl, 20 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS) at 37°C for 2–4 h depending on tissue weight. After complete digestion, the samples were extracted with phenol, phenol/chloroform (1:1), and chloroform sequentially. The last aqueous phase was treated with 2 volumes of cold ethanol and precipitated by centrifugation. The precipitates were dissolved in the

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same Tris buffer without SDS and treated with 0.1 mg/ml RNAase (Sigma, R4875), which was digested by 0.2 mg/ml of proteinase K. This procedure was followed by phenol-chloroform extraction and ethanol precipitation as described above. Final nucleic acid pellet was dissolved in H₂O and the concentration of DNA was measured at 260 nm. Only samples showing an absorbance ratio of 260 nm/280 nm higher than 1.7 were used for 8-OHdG quantitation. One to 5 µg of DNA in a volume of 100 µl were digested with 200 µg/ml nuclease P₁ (Sigma, N8630) in 20 mM potassium acetate (pH 4.8) into single nucleic acid and phosphate groups were removed by treatment of 200 µg/ml alkaline phosphatase in 25 mM Tris (pH 7.5). Contents of 8-OHdG and dG in the samples were simultaneously analyzed using HPLC with an electrochemical detector (ECD; ESA model 5100A) and a UV detector (26). A reverse-phase C₁₈ column (4.6 mm × 25 cm) was used with a mobile phase of 10 mM Na₂HPO₄ (pH 8.0) containing 8% methanol and a flow rate of 0.8 ml/min. The amounts of four nucleic acids were detected by UV absorbance at 260 nm and the amount of 8-OHdG was measured by ECD. The ratio 8-OHdG versus total guanine was calculated by 8-OHdG (from Dr. H. Kasai, National Cancer Center Research Institute, Tokyo) and guanine standards (Sigma, G0381).

**Determination of fatty acid composition.** Total lipids from 0.5 to 1 g of liver extracted according to Folch et al. (27) and were subjected to thin-layer chromatography (TLC) on Silica Gel G 60 plates (Merck, Darmstadt, Germany) using a solvent system composed of petroleum ether (b.p. 30–60°C)/diethyl ether/acetic acid (85:15:1 by vol) (24) to separate phospholipids from triacylglycerol and cholesterol. About 200 mg of the liver phospholipids containing 10 mg of heptadecanoic acid as an internal standard were methylated with BF₃/methanol (14% wt/wt) reagent (28). The fatty acid composition of the methyl esters was determined by gas chromatography (Hewlett Packard, North Hollywood, CA, Model 5890) using a stainless steel column (2 m × 3 mm i.d.) packed with 20% DEGS on Chromosorb W (60–70 mesh) (Gasukuro Kogyo Inc., Tokyo, Japan) and helium as a carrier gas at a flow of 30 ml/min. Gas chromatography was performed isothermally at 200°C.

**Statistical analysis.** Data were analyzed by ANOVA and treatment differences were evaluated by Tukey’s test (29).

### RESULTS

**Growth performance and organ weights**

The average food intake per day did not vary much among the four experimental groups, but the food intake in the F0 group became lower than the other three groups after the 5th week when the F0 group began to lose weight, while the other three groups kept gaining, albeit slowly. Final body weights in the F0 group reached to 66–76% of the other groups. However, liver weights of the F0 group were not small and heart, lung, and kidney weights were rather bigger in the F0
Table 3. Contents of $\alpha$-tocopherol in plasma and tissues of rats fed four experimental diets.1

| Dietary group | SI  | F0  | FI  | FII |
|---------------|-----|-----|-----|-----|
| Vitamin E (IU/kg diet) | $\leq 45$ | 3.0 | 45.0 | 209.0 |
| Plasma (µg/ml) | 5.0±0.5a | 0.12±0.06b | 4.2±0.6bc | 3.4±0.3c |
|                  | 3.21±0.26a | 0.10±0.04b | 5.1±0.7c | 4.0±0.7bc |
| Liver (µg/g tissue) | 25.6±1.8a | 22.5±2.5b | 29.6±3.5c | 33.8±3.2d |
| Lung (µg/g tissue) | 21.5±1.7a | 9.0±1.5b | 14.3±2.7c | 18.7±2.9bc |
| Heart (µg/g tissue) | 13.5±1.2a | 6.7±0.8b | 12.1±1.1a | 16.2±3.5a |
| Kidney (µg/g tissue) | 7.0±0.6a | 3.7±0.5b | 7.4±0.6a | 8.1±1.0a |

1 Values are mean±SEM ($n=6$--8). Means in the same row not sharing a common superscript letter are significantly different by Tukey’s test ($p<0.05$).

group compared with the other groups, as per unit body weight.

**Vitamin E levels in plasma and tissue**

Plasma vitamin E levels measured as $\alpha$-tocopherol content were extremely low in the F0 group (Table 3). They were lower even in the FII group than in the SI group, when expressed per milliliter of plasma, but the plasma vitamin E levels in the SI group were not different from the FII group when expressed per milligram of total plasma lipid. Vitamin E levels in tissues were lowest in the F0 group. The vitamin E levels in the FI group were higher in the liver but lower in the lung compared with those in the SI group. However, the vitamin levels were not different in the heart and kidney of the SI and FI groups. Dietary supplementation of vitamin E up to 209 IU (FII group) increased the vitamin level in the liver but not in other tissues.

**TBARS contents in plasma and tissue**

As shown in Table 4, the plasma levels of TBARS (per milliliter) in the F groups was not elevated compared with the SI group. The levels of TBARS were lower in the FI and FII groups than in the SI group. These differences were, however, changed when expressed per milligram of total plasma lipid because of the reduction in total lipids in the F groups (0.83 mg/ml compared with 1.56 mg/ml in the SI group). The F0 group had the highest levels of plasma TBARS. The FI group also had higher levels of plasma TBARS than the SI group. Supplementation of vitamin E up to $\leq 209$ IU in the FII group decreased the level of plasma TBARS to that of the SI group. In all tissues measured, the TBARS levels were or tended to be the highest in the F0 group. In the liver, the levels of TBARS were significantly increased not only in the F0 group, but also in the FI and FII groups, as compared with the SI group. In heart and kidney of the FI and FII groups, the
Table 4. Contents of TBARS in plasma and tissue of rats fed four experimental diets.1

| Dietary group | SI (≤45) | F0 | FI | FII | 209 |
|---------------|---------|----|----|----|-----|
| Vitamin E (IU/kg diet) | 5.47±0.48a | 5.65±0.63a | 4.19±0.21b | 3.27±0.19c |
| (MDA nmol/ml) | 3.52±0.22a | 5.32±0.43b | 4.95±0.30b | 3.75±0.13a |
| (MDA nmol/mg lipid) | 147±15a | 571±40b | 380±70c | 372±34c |
| Liver (MDA nmol/g tissue) | 20.5±2.2a | 22.4±2.2a | 13.5±1.7b | 12.4±1.1b |
| Lung (MDA nmol/g tissue) | 27.1±1.9a | 81.9±14.4b | 29.6±4.3a | 22.1±2.5a |
| Heart (MDA nmol/g tissue) | 131.1±15.5a | 237.3±10.8b | 127.8±6.5a | 130.8±5.4a |
| Kidney (MDA nmol/g tissue) | 147±15a | 571±40b | 380±70c | 372±34c |

1Values are mean±SEM (n=8). Means in the same row not sharing a common superscript letter are significantly different by Tukey’s test (p<0.05).

levels of TBARS were in the same range as those of the SI group. However, in lung, the TBARS levels of the F0 group were not different from the SI group, and the levels of the FI and FII groups were lower than the SI group. Except in the liver, the TBARS levels in the F groups were decreased to a level comparable to those of the SI group by supplementation of vitamin E (≤209 IU).

8-Hydroxydeoxyguanosine contents in liver

As shown in Fig. 1, 8-OHdG contents in the liver tended to decrease as dietary

![Fig. 1. Contents of 8-hydroxydeoxyguanosine in livers of rats fed four experimental diets. Values are means±SEM (n=4–6). Means not sharing a common letter are significantly different by Tukey’s test (p<0.05).](image-url)
vitamin E increased in the F groups, but there was no difference in 8-OHdG contents between the F0 and FI groups, although the FII group had significantly lower levels of the 8-OHdG than the F0 and FI groups. The 8-OHdG content of the SI group was not different from those of the FI group.

Fatty acid composition of liver phospholipids

The fatty acid composition of liver phospholipids from the four experimental groups differed mainly in the dietary oil fed and were not affected by dietary levels of vitamin E in the three F groups (Table 5). Comparison of the fatty acid composition of liver phospholipids of the F groups with that of the SI group showed an increase in 16:0 fatty acid contents and reductions of 18:0 fatty acid. Arachidonic (20:4n-6), but not linoleic acid (18:2n-6) in the SI group was replaced partly by the n-3 PUFA 20:5 and 22:6, and partly by monounsaturated fatty acids, 16:1 and 18:1n-9. In the F groups, there were small decreases in total PUFA and virtually no change in the peroxidizability index, but 2.8- to 3.2-fold increases in the

Table 5. Fatty acid composition of liver phospholipid of rats fed four experimental diets (mol %). 

| Dietary group | SI     | F0     | FI     | FII    |
|---------------|--------|--------|--------|--------|
| Vitamin E (IU/kg diet) | ≤45 | 3 | 45 | 209 |
| Fatty acid |        |        |        |        |
| C14:0         | 0.26±0.15 | 0.32±0.17 | 0.29±0.16 | 0.36±0.16 |
| C16:0         | 21.5±1.6b | 25.0±3.9b | 30.3±3.3b | 28.4±3.5b |
| C16:1         | 0.02±0.03ab | 1.20±0.78b | 1.19±0.96b | 0.30±0.14ab |
| C18:0         | 31.8±3.4ab | 26.1±5.7ab | 23.5±1.0ab | 24.4±2.5ab |
| C18:1n-9      | 3.99±2.11ab | 8.82±3.20b | 9.38±0.87b | 9.14±1.85b |
| C18:2n-6      | 10.73±2.25 | 10.63±2.84 | 9.07±1.32 | 10.82±1.68 |
| C18:3n-3      | 0.18±0.13 | 0.19±0.11 | 0.15±0.13 | 0.21±0.10 |
| C20:3n-6      | 0.12±0.08a | 0.01±0.01ab | 0.02±0.02ab | 0.02±0.02b |
| C20:3n-3      | 0.00±0.00a | 0.08±0.03b | 0.06±0.02b | 0.07±0.04b |
| C20:4n-6      | 27.3±2.4a | 14.1±2.1b | 14.3±2.0b | 14.3±2.2b |
| C20:5n-3      | 0.12±0.09a | 5.13±2.60b | 4.75±2.20b | 3.85±0.96b |
| C22:0         | 0.35±0.16a | 0.69±0.19b | 0.58±0.12ab | 0.62±0.08b |
| C22:5n-3      | 0.19±0.07a | 0.22±0.15a | 0.62±0.25b | 0.41±0.21ab |
| C22:6n-3      | 3.48±0.98a | 7.40±3.23b | 5.85±2.00b | 7.24±2.76b |
| Total PUFA    | 42.1 | 37.8 | 34.8 | 36.4 |
| Total n-6     | 38.1 | 24.8 | 23.4 | 25.1 |
| Total n-3     | 4.0 | 13.0 | 11.4 | 11.8 |
| n-3/n-6       | 0.10 | 0.53 | 0.49 | 0.47 |
| Peroxidizability index | 112 | 112 | 104 | 107 |

1 Values are mean±SEM (n=8). Means in the same row not sharing a common superscript letter are significantly different by Tukey's test (p<0.05). 2 Peroxidizability index is the same as described in Table 2.
DISCUSSION

Accumulation of lipid peroxide was most pronounced in liver among several tissues used in this study and the levels of the peroxide remained increased in fish oil-fed animals even with excess dietary vitamin E (≤ 209 IU/kg) and hence with a high tissue level of vitamin E. This result is consistent with the reports showing the limited role of vitamin E and other antioxidants on the prevention of lipid peroxidation in fish oil capsules (30) and fish oil diets (31) and in tissues of animals fed the diets (31). Therefore, one possible reason for high TBARS levels in the liver of the fish oil groups could be the intake of dietary lipid peroxides which were higher in fish oil (2.90 mEq/kg vs 0.75 mEq/kg in soybean oil in Table 2). These preformed peroxides may not be effectively suppressed by vitamin E in the tissue. Absorption of lipid peroxides has been argued (32, 33) but once absorbed, they accumulate mainly in the liver (34). According to Fritsche and Johnson (19), fish oils used in most animal experiments contain lipid peroxide at the level of 6–22 mEq/kg. However, the results of Choi and Jin (13) showed that the lipid peroxide level of livers of rats fed sardine oil was increased, compared with soybean oil-fed animals when lipid peroxide levels were the same and lower (about 0.2 mEq/kg) in the two dietary oils. Comparison of the fatty acid compositions of liver phospholipid from fish oil- and soybean oil-fed animals (Table 5) suggested that the total n-3 contents seemed to be one of the most important factors influencing the formation of tissue lipid peroxide in the fish oil groups (Table 4). Total α-tocopherol content of liver in the present study was not decreased by replacing soybean oil (SI) with fish oil (FI). However, we recently observed that liver microsomal vitamin E contents of fish oil-fed rats were lowered to about 60% of soybean oil-fed rats, when dietary vitamin E levels were the same (35). The liver is one of the major tissues that store vitamin E. It is interesting to find out if there is any change in the rate of vitamin E mobilization to membrane fractions. Unlike in the liver, the large amount of dietary vitamin E transported to lung, heart, and kidney appears to be consumed effectively to prevent accumulation of lipid peroxides in the FII group (Table 4 and 5). In the lung, fish oil had no effect on increasing the lipid peroxide level when its vitamin E level was identical with that of soybean oil. It may be related to several adaptive responses to oxidative stress better recognized in lung than other tissue (36). In humans, serum vitamin E levels were often decreased after fish oil intake (16,17,37), but the decreases were little or none when vitamin E was supplemented (17,38). In the present study, vitamin E levels in plasma and tissue (except lung), were not lowered by fish oil feeding, compared with soybean oil with an identical level of dietary vitamin E. These results are in accordance with those of Leibovitz et al. (15) who reported no difference in liver and kidney vitamin E levels between menhaden oil- and corn oil/lard-fed rats.

Liver 8-OHdG contents were not proportional to TBARS levels in the tissue,
but were reduced by a high level of dietary, and hence of tissue, vitamin E in fish oil-fed animals. Recently, Umegaki et al. (39) reported that liver 8-OHdG contents were not significantly influenced by dietary vitamin E levels, regardless of PUFA contents in diet, although animals fed low vitamin E tended to have higher 8-OHdG contents. The dietary fats they used were lard, soybean oil, and safflower oil, all of which were less peroxidizable than fish oil. Liver TBARS and 8-OHdG levels in the present study were generally much higher range than observed in the study of Umegaki et al. (39). On the other hand, Fraga et al. (40) reported that the level of 8-OHdG in sperm DNA was increased significantly by low vitamin C intake which resulted in a decrease in the vitamin content in seminal fluid, emphasizing the preventive role of vitamin C as an antioxidant against the formation of 8-OHdG. Further studies are needed to determine the whole body status and tissue utilization of vitamin E and other physiological antioxidants to protect against lipid peroxidation and 8-OHdG formation in fish oil-fed animals.

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