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Protective Effect of Supplementation of Fish Oil with High *n*−3 Polyunsaturated Fatty Acids against Oxidative Stress-Induced DNA Damage of Rat Liver in Vivo

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The present study was undertaken to know the effect of supplementation of fish oil with high *n*−3 polyunsaturated fatty acids (PUFA) on oxidative stress-induced DNA damage of rat liver in vivo. Male Wistar rats were fed a diet containing fish oil or safflower oil with high *n*−6 PUFA at 50 g/kg of diet and an equal amount of vitamin E at 59 mg/kg of diet for 6 weeks. Livers of rats fed fish oil were rich in *n*−3 PUFA, whereas those of rats fed safflower oil were rich in *n*−6 PUFA. Ferric nitrilotriacetate was intraperitoneally injected to induce oxidative stress. The degree of lipid peroxidation of the liver was assessed by the levels of phospholipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS), and the degree of oxidative DNA damage was assessed by comet type characterization in alkaline single-cell gel electrophoresis and 8-hydroxy-2′-deoxyguanosine levels. The levels of TBARS of the livers of the fish oil diet group increased to a greater extent than those of the safflower oil diet group, whereas the levels of the hydroperoxides of the livers of both diet groups increased to a similar extent. The vitamin E level of livers of the fish oil diet group was remarkably decreased. The degree of DNA damage of both diet groups was increased, but the increased level of the fish oil diet group was remarkably lower than that of the safflower oil diet group. The above results indicate that fish oil supplementation does not enhance but appears to protect against oxidative stress-induced DNA damage and suggest that lipid peroxidation does not enhance but lowers the DNA damage.

KEYWORDS: Ferric nitrirotriacetic acid; *n*−3 polyunsaturated fatty acid; *n*−6 polyunsaturated fatty acid; lipid peroxidation; DNA damage

INTRODUCTION

Epidemiological studies have shown an apparent beneficial effect of fish oil containing high *n*−3 polyunsaturated fatty acids (PUFA) on mortality from heart disease and cancer (*1*, *2*). Undesirable effects of a high fish oil intake have been nonetheless of concern, because *n*−3 PUFA are more readily oxidized under atmospheric conditions (*3*). Many earlier studies have suggested that a diet containing high fish oil enhances lipid peroxidation in the organs, blood, and urine of experimental animals and humans (references cited in refs *4* and *5*). In contrast, we have demonstrated that there are no harmful effects of fish oil feeding on in vivo lipid peroxidation of rat erythrocyte membranes (*4*) and organs (*5*), whereas the degree of lipid peroxidation of erythrocyte membranes of the fish oil diet group was remarkably higher than that of the safflower oil diet group when the in vitro oxidation was induced under atmospheric conditions (*4*). In our experiments, the assay for lipid peroxidation was conducted by avoiding the generation of artificial lipid peroxidation products during the assay. The discrepancy between the earlier and our results may be due to the difference of the assay methods for lipid peroxidation.

There are many observations suggesting that lipid peroxidation mediates DNA damage under oxidative stress. Damage to DNA concurrent with lipid peroxidation has been shown in mitochondria (*6*) and liver (*7*). DNA damage as assessed by the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) increases as the fatty acid unsaturation in the diet increases (*8*, *9*) and with glutathione depletion (*10*). In vitro studies have shown that peroxidized lipids or unsaturated fatty acids mediate DNA damage as assessed by DNA chain breaking (*11*−*15*) and 8-OHdG formation (*16*−*19*). On the other hand, there are contrary observations on the relationship between lipid peroxidation and DNA damage. There are reports suggesting that lipid peroxidation does not mediate but prevent DNA damage in the in vitro studies (*20*, *21*). There is also a report showing that lipid peroxidation and DNA damage induced by oxidative stress are independent events (*22*).

The aim of the present study was to know the effects of fish oil supplementation to rats on the DNA damage of liver caused

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by in vivo oxidative stress. Rats had been fed *n*−3 PUFA-rich fish oil diet or *n*−6 PUFA-rich safflower oil diet (control), and ferric nitrilotriacetate (Fe-NTA), a known inducer of oxidative stress (*23*−*28*), was injected intraperitoneally. It was found that the degree of lipid peroxidation of the fish oil diet group was higher but that the degree of DNA damage in the fish oil diet group was lower than that of the safflower oil diet group. The results indicate that fish oil supplementation does not enhance but appears to protect against oxidative stress-induced DNA damage and suggest that lipid peroxidation does not enhance but lowers DNA damage.

MATERIALS AND METHODS

Materials. Pentobarbital sodium salt was obtained from Dainippon Pharmaceutical (Osaka, Japan). Bovine serum albumin (BSA), cytochrome *c* (from horse heart), alkaline phosphatase type III (EC 3.1.3.11) (from *Escherichia coli*), luminol, and Triton-X 100 were purchased from Sigma Chemical Co. (St. Louis, MO). DL-α-Tocopherol and nitrilotriacetic acid sodium salt were from Tokyo Chemical Industry (Tokyo, Japan). Thiobarbituric acid (TBA) was obtained from Nacalai Tesque (Kyoto, Japan). EDTA disodium salt was obtained from Dojindo Laboratories (Kumamoto, Japan). Phosphatidylcholine (egg yolk) (PC) was obtained from Funakoshi Co. Ltd. (Tokyo, Japan), and phosphatidylethanolamine (egg yolk) (PE) was obtained from Taiyo Kagaku Co. Ltd. (Tokyo, Japan). PC hydroperoxide (PCOOH) and PE hydroperoxide (PEOOH) were prepared just before use according to the method described elsewhere (*29*, *30*). Briefly, PC and PE were photooxidized at 10 °C for 10 h in the presence of methylene blue, and methylene blue was removed by passage through a column of Florisil. Peroxide values of PCOOH and PEOOH were estimated to be 3000 and 2700 nequiv/mg, respectively.

Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Ferric Nitrilotriacetic Acid (Fe-NTA). Fe-NTA was prepared just before use according to the method of Zainal et al. (*31*). Thus, a solution of 300 mM ferric nitrate solution in water and a solution of 600 mM nitrilotriacetic acid disodium salt in water were mixed in a volume ratio of 1:2, and the pH of the solution was adjusted to pH 7.4 by sodium carbonate.

Animals and Diets. The protocol of animal preparations for the present experiment was approved by the Ethics Committee of our institute. Eighteen 4-week-old male Wistar rats weighing 50−70 g supplied by Japan Laboratory Animals Corporation (Tokyo, Japan) were used. Three animals were housed together in a stainless steel cage in a room of controlled temperature at 23 ± 1 °C, humidity at 55 ± 5%, and lighting of 12 h dark−light cycle. Rats were allowed free access to diet. The animals were fed a normal solid diet, Clea rodent CE-2, containing 4.5% soybean oil and 70 mg/kg vitamin E (Clea Japan Corp., Tokyo, Japan) during 1 week for acclimatization. Rats were divided into two groups of nine animals each, and each group was fed a diet composed of 427.5 g of AIN-70 (corn oil free, 45.6 mg/kg vitamin E) (Oriental Yeast Co., Tokyo, Japan), 22.5 g of safflower oil or fish oil (each vitamin E at 32.5 mg/kg), and 62.5 mL of water ad libitum for 6 week. The overall content of vitamin E in both diets was equal at 59 mg/kg of dried solid diet. Diets were freshly prepared every day. Weights of rats after feeding were 300−400 g.

Safflower oil was rich in *n*−6 PUFA (*n*−6 total, 78.0%; *n*−3 total, 0.3%), and fish oil was rich in *n*−3 PUFA (*n*−6 total, 1.3%; *n*−3 total, 26.5%) (*4*). The unsaturation index (UI), as expressed by the sum of percentages of individual fatty acids times number of double bonds, of fish oil (168) was equal to that of safflower oil (168), whereas the peroxidizability index (PI), as expressed by the sum of percentages of individual fatty acids times number of active methylene groups, of fish oil (114) was higher than that of safflower oil (78). Peroxide and acid values of safflower oil were 1.1 nequiv/mg and 0.2, respectively, and those of fish oil were 1.2 nequiv/mg and 0.2, respectively.

The fatty acid composition of total lipids of the liver of rats fed the safflower oil diet was rich in *n*−6 PUFA (*n*−6 total, 46.5%; *n*−3 total, 3.4%) and that of the rats fed the fish oil was rich in *n*−3 PUFA (*n*−6 total, 13.2%; *n*-3 total, 20.5%) (*5*). The UI of the fish oil diet group (183) was similar to that of the safflower oil diet group (186), and the PI of the fish oil diet group (126) was similar to that of the safflower oil diet group (127).

Induction of Oxidative Stress by Fe-NTA. Eight rats selected randomly from each diet group were divided into two groups of four animals each. To one group was intraperitoneally injected Fe-NTA at a single dose of 10 mg of Fe/kg of body weight, and to the other group physiological saline at 1.79 mL/kg of body weight was similarly injected as control. Six hours later, pentobarbital sodium salt at 50 mg/kg of body weight was intraperitoneally injected to anesthetize animals. Livers were quickly isolated after perfusion with 0.2 mM dithiothreitol in Dulbecco's phosphate-buffered saline (DPBS). Livers were cut in 5 mL of ice-cold solution of 0.2 mM dithiothreitol in DPBS to exude liver cells for comet assay. A part of the liver was homogenized in a 17/3 volume amount of a solution of 0.2 mM dithiothreitol in DPBS using a Potter-type Teflon homogenizer under cooling. The homogenate was stored at −80 °C under nitrogen atmosphere for measurement of lipid peroxidation levels and vitamin E contents. Another part of the liver was stored for measurement of 8-OHdG. Protein content was determined according to the Lowry method (*32*) using BSA as a reference standard.

High-Performance Liquid Chromatography (HPLC) Chemiluminescence Determination of PEOOH and PCOOH in Total Lipids of Liver Homogenate. Levels of PCOOH and PEOOH in the total lipid fractions of liver homogenate were determined according to an HPLC chemiluminescence method (*29*, *30*). The lipid fraction was obtained from 2.0 mL of homogenate in DPBS by extraction with 12 mL of chloroform/methanol (1:2, v/v) according to the method of Bligh and Dyer (*33*). The lower organic phase was dried by purging nitrogen gas and redissolved into 2.5 mL of a mixture of chloroform/methanol (2:1, v/v) and 0.5 mL of 0.05 M KCl. The mixture was centrifuged to obtain 1.6 mL of lower phase, which was evaporated to dryness and redissoved into 100 μL of chloroform. A 10 μL portion of this extract was subjected to HPLC. HPLC was carried out by using a Hitachi 655 liquid chromatograph (Tokyo, Japan) equipped with a column of YMC A-012 S-5 120 A SIL (4.6 mm i.d. × 250 mm) (YMC Co., Kyoto, Japan) and a mobile phase composed of chloroform/methanol (1:9, v/v) at a flow rate of 0.8 mL/min. A solution composed of 10 μg/mL cytochrome *c* and 1 μg/mL luminol in 50 mM borate buffer (pH 9.3) was mixed with the eluate at a flow rate of 0.7 mL/min, and chemiluminescence generated was detected using a JASCO 825-CL Intelligent CL detector (Tokyo, Japan). The chemiluminescent peaks due to PEOOH and PCOOH exhibited retention times of 5 and 8 min, respectively. The amounts (nanoequivalents) of PCOOH and PEOOH were determined by comparing their peak areas with a calibration curve of the peak areas of the standard solutions of PCOOH and PEOOH (0−100 nequiv).

Thiobarbituric Acid-Reactive Substances (TBARS) in Liver Homogenate. TBARS in liver homogenate were determined according to the method described elsewhere (*34*−*38*). To the liver homogenate (200 μL) in DPBS were added 650 μL of a mixture composed of 0.20 mL of 5.2% sodium dodecyl sulfate (SDS) in water, 50 μL of 0.8% butylated hydroxytoluene (BHT) solution in glacial acetic acid, 1.50 mL of 0.8% TBA solution in water, and 1.70 mL of water with or without EDTA at a final concentration of 2 mM, and finally 150 μL of 20% acetate buffer (pH 3.5) (total volume = 1.0 mL). The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 60 min. The mixture was extracted with 1.0 mL of a mixture of 1-butanol/pyridine (15:1, v/v). Absorbance at 532 nm of the extract was measured. The amount of red pigment reflecting TBARS was calculated using a molecular extinction coefficient of pigment of 156000 M^{-1} cm^{-1}.

Vitamin E Levels in Liver Homogenate. Vitamin E levels of the liver homogenate were determined according to the method described elsewhere (*39*). To 0.5 mL of the liver homogenate were added 0.5 mL of 6% pyrogallol solution in ethanol and 1.0 mL of ethanol. After the mixture had been heated at 70 °C for 2 min, 0.2 mL of 60% KOH solution was added, and the mixture was heated at 70 °C for 30 min. Vitamin E was extracted by the addition of 2.5 mL of water and 5.0 mL of *n*-hexane and subsequent centrifugation at 1500*g* for 5 min. The upper phase (4.0 mL) was collected and evaporated to dryness to

be redissolved into 0.2 mL of methanol. HPLC was carried out by using an Inertsil ODS-2 column (4.6 mm i.d. × 250 mm) (GL Sciences Inc., Tokyo, Japan) by injection of 10 μL of the sample solution in methanol, and the column was eluted with a mobile phase composed of methanol/water (98:2, v/v) at a flow rate of 1.4 mL/min. A fluorescent peak was detected at 292/335 nm with a Shimadzu RF-535 fluorescence spectromonitor (Osaka, Japan). The peak due to vitamin E appeared at a retention time of 17−19 min. The amount of vitamin E in the sample was estimated by comparing the peak area with those of the calibration curve of the standard DL-α-tocopherol.

Alkaline Single-Cell Gel Electrophoresis (Comet Assay) of Liver Cells. A comet assay was performed according to the method of Singh et al. (*40*). Briefly, a fully frosted microscopic slide glass was coated with 110 μL of a solution of 0.5% normal-melting-point agarose (SeaKem ME agarose, FMC Bio-Products, Rockland, ME) in DPBS; subsequently, a mixture of 10 μL of the liver cell suspension and 75 μL of a solution of 0.5% low-melting-point agarose (SeaPlaque GTG agarose, FMC Bio-Products) in DPBS and, finally, 75 μL of the solution of low-melting-point agarose were overlayed. Each slide was kept at 4 °C for 1 h in a lysing solution composed of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl buffer (pH 10.0), 1% sodium sarcosinate, 1% Triton X-100, and 10% dimethyl sulfoxide. The slide glass was then kept at 4 °C for 15 min in an electrophoresis buffer composed of 0.3 M NaOH and 1 mM EDTA to allow unwinding of DNA and expression of alkali labile sites. Electrophoresis was performed at a constant voltage of 30 V and a constant current of 300 mA for 20 min. The slide glass was neutralized with 0.4 M Tris-HCl buffer (pH 7.4) and stained with 50 μL of a solution of 50 μg/mL ethidium bromide in water.

Nuclei of cells were observed and converted to digital images under fluorescent microscope model DMIRB-3 (Leica Microsystems Co., Ltd., Tokyo, Japan) equipped with a rhodamine filter and a charge-coupled device camera model MicroMAX782Y (Princeton Instruments, Inc., Trenton, NJ). Comets were classified into five types: type 1, unchanged nuclei without a tail; type 2, nuclei with a tiny tail; type 3, nuclei with a dim tail; type 4, nuclei with a clear tail; and type 5, tail alone (*41*). The number of nuclei of types 1−5 amounting to 100 nuclei were counted. Tail DNA% in total DNA was obtained by estimating the DNA tail in each comet using NIH image version 1.61 (U.S. National Institutes of Health).

8-OHdG in Liver Homogenate. DNA of liver homogenate was extracted using a DNA Extractor WB kit due to the sodium iodide method (*42*) (Wako) according to the protocol. Liver was homogenized in 17/3 volumes of kit Lysis Solution using a Potter-type Teflon homogenizer. Nuclei were suspended in 2.0 mL of kit Enzyme Reaction Solution, and 100 μL of 17 mg/mL kit Protease (proteinase K) was added. The mixture was incubated at 37 °C for 1 h under nitrogen atmosphere. Kit NaI Solution (7.6 M NaI) and 2-propanol were added to precipitate DNA at −80 °C. DNA was washed with kit Washing Solution A (40% 2-propanol) and kit Washing Solution B (ethanol). Two tubes of DNA were combined and precipitated by the addition of ethanol. The concentration of DNA was determined by absorbance at 260 nm after dissolving into water.

DNA was hydrolyzed according to the method of Sato et al. (*43*). All of the operations were carried out under an atmosphere of nitrogen to avoid artificial oxidation. A 150 μL aliquot of 3 mg/mL DNA solution in water was heated at 95 °C for 5 min and quickly cooled to 0 °C to denature the DNA. To this were added 4.5 μL of 1 M sodium acetate buffer (pH 4.8) and 22.5 units of nuclease P_1, and the mixture was incubated at 37 °C for 1 h. To the mixture were added 27 μL of 1 M Tris-HCl (pH 7.5) and 13.5 units of alkaline phosphatase type III, and this mixture was incubated at 37 °C for 1 h. The sample was stored at −80 °C for determination of 8-OHdG.

8-OHdG was determined by using a competitive enzyme-linked immunosorbent assay (ELISA) kit, 8-OHdG Check (Japan Institute for the Control of Aging, Shizuoka, Japan) according to the protocol (*44*). In the well of an 8-HOdG microtiter plate (8-HOdG labeled) were placed 50 μL of a sample or a kit Standard 8-OHdG Solution (0.5, 2, 8, 20, 80, or 200 ng/mL) and 50 μL of primary antibody (anti 8-OHdG monoclonal antibody N45.1), and the plate was incubated at 37 °C for 1 h. After the well was washed with kit Washing Solution, 100 μL of secondary antibody (enzyme-labeled-anti mouse IgG) was placed, and the plate was incubated at 37 °C for 1 h. After the well was washed with kit Washing Solution, 100 μL of kit Chromatic Solution (3,3′,5,5′-tetramethylbenzidine in hydrogen peroxide) was added, and the plate was incubated at room temperature for 15 min in the dark. Three minutes after the addition of 100 μL of kit Reaction Terminating Solution (1 M phosphoric acid), absorbance at 450 nm was measured using a microplate reader model Benchmark (Bio-Rad, Tokyo, Japan). Calibration curves were established by plotting absorbance at 450 nm versus the logarithmic concentration of the kit Standard 8-OHdG Solution.

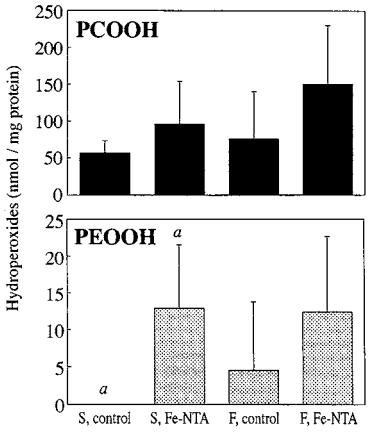

Figure 1. PCOOH and PEOOH levels in liver homogenate of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. Data are expressed as the mean ± SD ($n = 4$). [a] $P < 0.03$.

Statistical Analysis. Data were analyzed by Student's *t* test.

RESULTS

Groups of male Wistar rats, eight animals each, were fed diets containing safflower oil or fish oil, respectively, at 50 g/kg of diet and an equal amount of vitamin E of 59 mg/kg of diet for 6 weeks. The susceptibility of fish oil to lipid peroxidation induced by 2,2′-azobis(2,4-dimethylvaleronitrile) at 37 °C for 2 h in *tert*-butyl alcohol/methanol (3:1. v/v) was much higher than that of safflower oil: TBARS of fish oil and safflower oil increased to 100 and 4 nmol/mg of oil, respectively. Total liver lipids of the safflower oil diet group were *n*−6 PUFA-rich and those of the fish oil diet group *n*−3 PUFA-rich (*5*). Each diet group of rats was divided into two groups, four animals each, and Fe-NTA was injected intraperitoneally to rats of one group to induce oxidative stress; physiological saline was given to the rats of the other group as a control. Livers were quickly isolated for analysis of the degree of lipid peroxidation and DNA damage. The degree of lipid peroxidation was assessed by the increase in the levels of phospholipid hydroperoxides, PCOOH and PEOOH, by an HPLC chemiluminescence method (*29*, *30*) and also by the increase in the levels of TBARS in the assays in the presence of BHT with and without EDTA (*34*−*38*). Incorporation of BHT in the TBA assay can prevent artificial lipid peroxidation, and incorporation of EDTA can prevent coloration due to alkenal/alkadienal derivatives and allow the determination of malonaldehyde derivatives exclusively. The degree of DNA damage was assessed by the comet type classification and the tail DNA% in total comet DNA in the alkaline single-cell gel electrophoresis (comet assay) (*40*) in addition to the increase in the levels of 8-OHdG (*44*).

The PCOOH and PEOOH levels in the control liver homogenates of both diet groups were similarly low, but the levels, especially those of PEOOH, increased by the oxidative stress (**Figure 1**). However, no significant differences in the increased

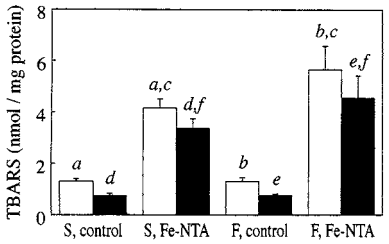

Figure 2. TBARS levels of liver homogenate of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. TBA assay was performed in the presence of BHT and with (■) and without EDTA (□). Data are expressed as the mean ± SD ($n = 4$). $^{a,b,d,e}P < 0.001$; $^{c}P < 0.03$; $^{f}P < 0.05$.

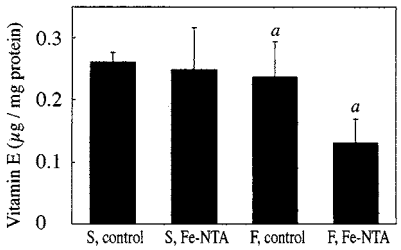

Figure 3. Vitamin E levels of liver homogenate of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. Data are expressed as the mean ± SD ($n = 4$). $^{a}P < 0.03$.

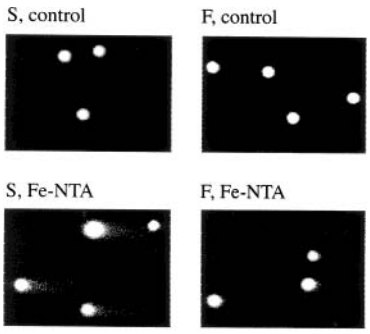

Figure 4. Representative comet images of liver cells of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively.

levels of these hydroperoxides were observed between the two diet groups. The TBARS levels in the liver homogenates of both diet groups were increased by the oxidative stress (**Figure 2**). The increased TBARS levels obtained by both assays with and without EDTA of liver homogenate of the fish oil diet group were significantly higher than those of the safflower oil diet group. Although the increased levels of the lipid hydroperoxides of both diet groups were similar, the increased levels of TBARS of the fish oil diet group were higher than those of the safflower oil diet group, indicating that the degree of lipid peroxidation of liver of the fish diet group was higher than that of the safflower oil diet group. In accordance with this observation, the vitamin E level of liver homogenate of the fish oil diet group was markedly decreased as compared with that of the safflower oil diet group (**Figure 3**). The above results indicate that the degree of lipid peroxidation of the liver of the fish oil diet group induced by the oxidative stress was higher than that of the safflower oil diet group.

Representative comet images of liver cells of both diet groups are shown in **Figure 4**, and the results obtained by the quantitative estimation of the number of comet types are shown

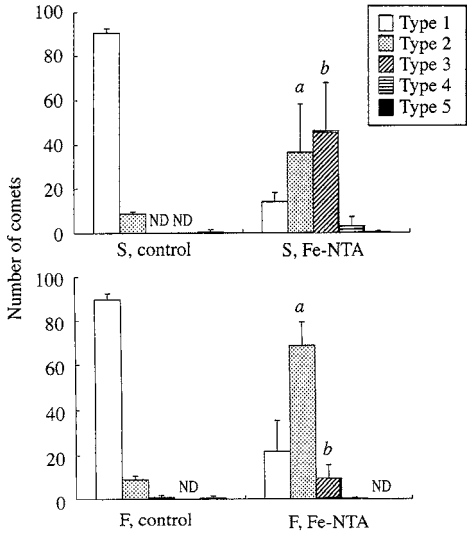

Figure 5. Comet classification of liver cells of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. Data are expressed as the mean ± SD ($n = 4$). $^{a}P < 0.04$; $^{b}P < 0.02$.

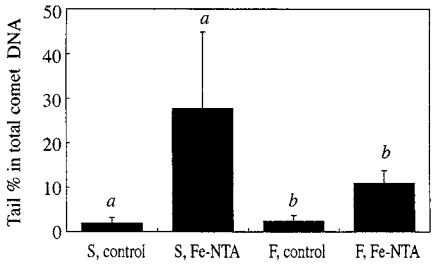

Figure 6. Tail DNA% in total DNA in comet assay of liver cells of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. Data are expressed as the mean ± SD ($n = 4$). $^{a}P < 0.03$; $^{b}P < 0.002$.

in **Figure 5**. In the control liver cells of both diet groups (S, control; F, control), type 1 comets without tail were predominant. After oxidative stress of the safflower oil diet group (S, Fe-NTA), type 3 comets with a dim tail became predominant. In contrast, after oxidative stress of the fish oil diet group (F, Fe-NTA), type 2 comets with a tiny tail prevailed over type 3 comets. Whereas the number of type 3 comets was the largest at 45% in Fe-NTA-treated liver cells of the safflower oil diet group, the number of type 2 comets was the largest at 65% in Fe-NTA-treated liver cells of the fish oil diet group. Quantitative analysis of tail DNA% in total DNA gave the same results (**Figure 6**). After oxidative stress, tail DNA% in total DNA of comets of the safflower oil diet group was estimated to be 30%, whereas that of the fish oil diet group was estimated to be only 9%.

The 8-OHdG levels of the control liver homogenate of both diet groups were low but increased after oxidative stress (**Figure 7**). Whereas the 8-OHdG level of the safflower oil diet group was estimated to be $\sim 50/10^7$ dG, that of the fish oil diet group was $\sim 25/10^7$ dG. The results obtained by the comet assay and the 8-OHdG level indicate that the degree of DNA damage to the liver of the fish oil diet group induced by Fe-NTA oxidative stress was lower than that of the safflower oil diet group.

DISCUSSION

It is known that Fe-NTA generates hydroxyl radical upon reaction with hydrogen peroxide (*26*−*28*). Fe-NTA causes iron-

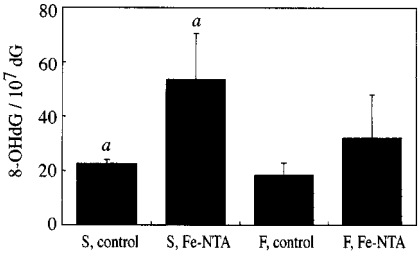

Figure 7. 8-OHdG levels of liver homogenate of rats treated with intraperitoneal injection of Fe-NTA. S and F indicate liver of safflower oil and fish oil diet groups, respectively. Data are expressed as the mean ± SD ($n = 4$). [a] $P < 0.02$.

induced free radical injury and cancer in rodent kidney (*45*), and a high incidence of renal cell carcinoma is prevalent in mice and rats following repeated injection of Fe-NTA (*46*, *47*). In vitro oxidative stress induced by Fe-NTA increased both the extent of lipid peroxidation and 8-OHdG levels of normal rat liver hepatocytes (*48*).

In the present study, the effects of fish oil and safflower oil supplementation on the Fe-NTA oxidative stress-induced rat liver DNA damage were compared. The increased levels of primary lipid peroxidation products, PCOOH and PEOOH, were not significantly different between livers of the two diet groups. This may be due to the instability of the hydroperoxides in liver, because in vitro oxidation of rat liver microsomes causes a rapid increase in the levels of the hydroperoxides, which disappear shortly thereafter (*49*). In contrast, increased levels of secondary lipid peroxidation products, TBARS, were significantly different between the two diet groups. The levels of TBARS monitored in the assay with BHT reflect those in situ, because formation of artificial TBARS during the assay is prevented. By monitoring TBARS in the assay with and without EDTA, the degree of lipid peroxidation was increased to a significantly greater extent in the livers of the fish oil diet group than in the safflower oil diet group. Markedly higher consumption of vitamin E in the livers of rats fed the fish oil diet than in the safflower oil diet group supported the conclusion that the higher degree of lipid peroxidation had occurred in livers of the fish oil diet group. The Fe-NTA-induced DNA damage as assessed by comet assay and 8-OHdG levels of livers of rats fed the fish oil diet group was remarkably lower than that of livers of the safflower oil diet group. It was clearly demonstrated that DNA damage in the livers of the fish oil diet group was lowered as compared to the damage in livers of the safflower oil diet group.

The results obtained here may be significant for two reasons. The first is the effect of lipid peroxidation on oxidative stress-induced DNA damage. It has been shown that vitamin E should be supplemented during fish oil feeding to prevent lipid peroxidation and 8-OHdG formation in the body (*50*). In the present study, under conditions when an adequate amount of vitamin E was supplied, increased lipid peroxidation did not mediate but lowered oxidative stress-induced DNA damage. Although it has been shown that lipid peroxidation products give damaging effects on DNA (*11*−*19*), the damaging potencies of the products may be lower than those of the direct oxidative stress due to reactive oxygen species. It is conceivable that the potency of oxidative stress induced by Fe-NTA may be attenuated by lipid peroxidation, and as a consequence DNA damage may be lowered. In another in vitro experiment, we found that strand cleavage of supercoiled plasmid DNA induced by oxidative stress due to reactive oxygen species was suppressed by low-density lipoprotein, during which lipid peroxidation was induced as assessed by TBARS formation, and the lipoprotein peroxidized beforehand could not mediate DNA damage under the same conditions (*51*). Moreover, our present results were consistent with those obtained by Hu and Shih, who have demonstrated that, in the Fe(II) ion-induced oxidation of rat liver nuclei, ascorbic acid inhibited lipid peroxidation but enhanced DNA damage (*20*) and that chain-breaking antioxidants inhibited lipid peroxidation but not DNA damage (*21*), suggesting that lipid peroxidation does not mediate but prevents DNA damage. These in vitro data would support the idea that lipid peroxidation does not enhance but lowers oxidative stress-induced DNA damage.

On the basis of the established concept, PUFA is converted by oxidative stress and molecular oxygen into reactive peroxy radicals, which are in turn transformed into less active hydroperoxides and TBARS by the chain-breaking antioxidant system composed of vitamin E. From the results obtained here, it is suggested that the increase in the levels of hydroperoxides and TBARS formed as a consequence of scavenging oxidative stress may result in the decrease in the levels of oxidative stress-induced DNA damage. Hence, PUFA can be regarded as an antioxidant defense system linked to the vitamin E antioxidant system for the prevention of oxidative stress-induced DNA damage.

The second point is the higher protective role of supplementation of fish oil with high $n-3$ PUFA against oxidative stress-induced DNA damage. If a sufficient amount of vitamin E is supplied, supplementation of the diet with fish oil may not mediate but actually offer some protection against oxidative stress-induced DNA as compared with the other types of dietary PUFA.

In conclusion, dietary fish oil supplementation resulted in the occurrence of lower levels DNA damage than did dietary safflower oil supplementation in rats exposed to oxidative stress. Elevated lipid peroxidation in rat liver in vivo did not enhance but appeared to protect against oxidative-stress induced DNA damage.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; DPBS, Dulbecco's phosphate-guffered saline; ELISA, enzyme-linked immunosorbent assay; Fe-NTA, ferric nitrilotriacetic acid; HPLC, high-performance liquid chromatography; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PC, phosphatidylcholine; PCOOH, PC hydroperoxide; PE, phosphatidylethanolamine; PEOOH, PE hydroperoxide; PI, peroxidizability index; PUFA, polyunsaturated fatty acid; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TBARS, TBA-reactive substances; UI, unsaturation index.

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