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Docosahexaenoic Acid Supplementation-Increased Oxidative Damage in Bone Marrow DNA in Aged Rats and its Relation to Antioxidant Vitamins

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We compared the influence of docosahexaenoic acid (DHA) supplementation on oxidative DNA damage in bone marrow between young and aged rats. As a marker of oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG) in DNA was analyzed. Young (5-week-old) and aged (100-week-old) female Wistar rats were given DHA (300mg/kg body weight/day) or vehicle (control) orally for 12 weeks. The 8-OHdG in the bone marrow in the aged DHA group was significantly higher than that in the other groups. Vitamin E concentrations, however, did not differ among the groups regardless of the DHA supplementation. Vitamin C (ascorbic acid) concentrations in the aged control group were approximately 1/2 those in the young control group. The concentrations of vitamin C tended to be higher in the young DHA group and lower in the aged DHA group when compared to their respective control groups. Changes in the concentrations of vitamin C and vitamin E in plasma were similar to those in the bone marrow. The activity of hepatic l-gulono- γ -lactone oxidase, an enzyme responsible for vitamin C synthesis, corresponded well to the concentrations of vitamin C in the bone marrow and the plasma. These results suggest that in aged rats, but not young rats, excess supplementation of DHA induces oxidative DNA damage in bone marrow and that the decrease in vitamin C synthesis in aged rats is involved in the mechanisms of DNA damage.

Keywords: Docosahexaenoic acid, aged rats, oxidative damage, DNA, vitamin C

INTRODUCTION

Docosahexaenoic acid (DHA) has beneficial hypolipidemic and antihypertensive effects (1–3), and many individuals consume diets or food supplements containing DHA to receive these beneficial effects. DHA and eicosapentaenoic acid (EPA), two typical fatty acids in fish oil, are highly unsaturated and particularly susceptible to peroxidation. Thus, high intakes of DHA and EPA may induce oxidative stress in the body. Many studies have been conducted to explore the relationship between DHA and EPA intake and oxidative stress in the body (4–9). In those

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studies dealing with DHA- and EPA-induced oxidative stress, the increase in lipid peroxidation and decrease in vitamin E levels in the plasma or liver have primarily been evaluated in young animals or healthy subjects. It has been shown that the liver is more resistant to oxidative stress than other tissue (10), and that young animals are more resistant to oxidative stress than aged animals (11,12). It is also important to note that persons who consume DHA-containing foods or supplements are often aged or unhealthy. In addition, immune function has been reported to decrease with age in animals and humans (13). Bone marrow produces immune cells and appears to be susceptible to oxidative stress (14,15). However, little is known about DHA- or EPA- induced oxidative damage in aged animals or humans, especially in bone marrow.

Accumulating evidence has indicated that oxidative damage to DNA is associated with aging and degenerative diseases (16). The increase in 8-OHdG in DNA, a marker of oxidative DNA damage, has been detected in aged rats and humans (17–20). Kaneko et al. (18) have reported finding increases in 8-OHdG levels in DNA from various tissues in rats at ages greater than 25 months. Similar findings have been reported by other investigators (19,20). These findings indicate that aged animals are more susceptible to oxidative DNA damage, and that excess DHA supplementation may accelerate the formation of oxidative DNA damage in the aged animals.

In this study, we administered low doses of DHA to young (5-week-old) and aged (100 -week-old) rats for 12 weeks and subsequently compared the oxidative damage to DNA in the bone marrow and its relation to the concentrations of antioxidant vitamins.

MATERIALS AND METHODS

Chemicals

The ethyl ester form of DHA (purity 95%, peroxide value 2 meq/kg) was obtained from Harima Chemical Ind. To prevent the oxidation of DHA, DHA was divided into several portions, sealed and kept in an nitrogen atmosphere and stored in a freezer at –80°C until use. L-gulono- γ -lactone and acid phosphatase were purchased from Sigma Chemical Co.(St Louis, MO). P1 nuclease was obtained from Seikagaku Kogyo (Tokyo), and assay kits of triglyceride and cholesterol in plasma were obtained from Kyowa Medics (Tokyo). Other chemicals and kits were obtained from Wako Pure Chemical Ind. (Osaka, Japan).

Experimental Animals

Female Wistar (Jcl:Wistar) rats purchased from Clea Japan, Inc., Tokyo, were maintained for a predetermined time, then used for the experiment as aged (100-week-old) and young (5-week-old) rats. The rats were allowed free access to a commercial rodent chow F1 (Funabashi Farm, Chiba) throughout the experiment. This F1 diet contains 7.5 mg vitamin E (alpha-tocopherol) and 0.15 mg vitamin C (ascorbic acid)/100 g diet. The aged and young rats were randomly divided into control and DHA-treated group. The rats to be supplemented with DHA were given by intragastric gavage a daily dose of DHA (300 mg/kg body weight) for 12 weeks. The unsupplemented control rats were given equal amounts of vehicle (5 g/L gum arabic) for the same period.

The rats were anesthetized with pentobarbital and sacrificed. Their blood was taken from the abdominal aorta, and organs were immediately removed. The bone marrow cells were prepared from the femurs as described previously (14). The blood was immediately centrifuged at 5,000 × g for 3 min, and the plasma was prepared. Bone marrow and plasma samples were immediately mixed with 5 volumes of 6 % metaphosphoric acid for the analysis of vitamin C, and with 10 volumes of 0.15 % butylated hydroxytoluene (BHT) for the analysis of vitamin E, respectively, then stored at –80°C for several days until analyzed (21).

All procedures were carried out in accordance with the National Institute of Health and Nutrition guidelines for the care and use of laboratory animals.

Analytical Methods

Vitamins E and C in the samples were analyzed by HPLC with an electrochemical detector (ECD) according to the method described previously (15,21). We only analyzed ascorbic acid as vitamin C, because the levels of dehydroascorbic acid, an oxidized form of ascorbic acid, were very low in plasma and tissue. The activity of l-gulono-γ-lactone oxidase (EC1.1.3.8) was analyzed principally according to the method of Kito *et al.*(22). Briefly, rat liver microsomes were incubated with 50 mM sodium citrate, 1.7 mM dithiothreitol, and 2.5 mM l-gulono- γ -lactone in 50 mM potassium phosphate buffer (pH 7.0) at 37°C for 30 min. The enzymatic reaction was stopped by the addition of a one-sixth volume of 30 % metaphosphoric acid, and the reactants were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was injected into the HPLC with ECD for the analysis of vitamin C (21).

The analysis of 8-OHdG in bone marrow was performed as follows. DNA was extracted using a DNA extraction kit (DNA Extraction WB kit No. 293–50501, Wako Pure Chemical Ind.). The isolated DNA was digested by P1 Nuclease, and acid phosphatase according to the method of Yamaguchi *et al.* (23). The 8-OHdG and deoxyguanosine (dG) content in the deoxynucleotide mixture was analyzed by HPLC (Shimadzu LC10AD) with an ECD (Coulochem II, ESA, MA) equipped with analytical cells (detector 1, 180 mV; detector 2, 380 mV) and an ultraviolet detector (Shimadzu SPD-10A, at 280 nm). The separating conditions were as follows: column, Beckman Ultrasphere ODS (4.6 × 250mm); column temperature, 23°C; mobile phase, 10 mM NaH_2PO_4 containing 8% methanol; the flow rate, 1 ml/min. The 8-OHdG levels in the DNA are expressed as the number of 8-OHdG per 10^5 dG.

BHT was added to the plasma and bone marrow samples at the final concentration of 0.005%. Total fatty acids in the samples were extracted and measured as described elsewhere (2). The fatty acid methyl esters were separated on a capillary column (25 m × 0.25 mm i.d. fused-silica column, DB-WAX/PN 122–7032, J&W Scientific, Folsom, CA) in a Model 5890 II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector. The fatty acid unsaturation index (UI) was calculated as a function of the sum of the mole percentages of the unsaturated fatty acids times the number of olefinic double bonds (2).

The triglyceride and cholesterol levels in plasma were determined using diagnostic kits. Protein levels were determined using a BCA protein assay kit (Pierce, Rockford, IL). Lipid peroxide levels were determined by the thiobarbituric acid method (24) and expressed as thiobarbituric acid reacting substances (TBARS). Sulfhydryl (SH) concentrations in the plasma were analyzed by a colorimetric method using DTNB (5,5'-dithiobis-2-nitrobenzoic acid) (25).

Statistical Analyses

The data are presented as means with standard error (SEM) for the individual groups. Statistical analyses of the data for the groups were carried out using ANOVA followed by a post hoc test of Fisher's Protected Least Significant Difference. A P value <0.05 was considered to be significant. All statistical analyses were performed using the computer program Stat View 4.5 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Body weight at the end of the experimental period did not differ between the control and DHA groups in both young and aged rats. The average weight (control group versus DHA

group) was 238 g versus 239 g for the young rats, and 380 g versus 385 g for the aged rats, respectively. The amounts of bone marrow cells obtained from the femur did not differ among the groups when assessed by the content of protein in the samples. Tables I and II show the concentrations and profiles of fatty acids in plasma and bone marrow. Total fatty acid concentrations in the bone marrow and plasma were higher in the aged than in the young rats. In the DHA-supplemented young and aged rats, the composition of DHA was higher, and that of arachidonic acid was lower when compared with their respective control rats. The unsaturation index of fatty acids in the DHA-supplemented rats was higher in the plasma and tended to be higher in the bone marrow when compared to the respective control rats.

TABLE I Total fatty acid concentrations and major fatty acid pattern in plasma of young and aged rats treated with or without DHA

	Young		*Aged*	
	control	*DHA*	*control*	*DHA*
Total (mM)	5.4 ± 0.4	5.0 ± 0.5	6.8 ± 0.8	6.8 ± 0.5[a]
Major FFA (% of total)				
16:0	19.6 ± 0.6	18.9 ± 0.8	23.0 ± 0.4[a]	23.2 ± 0.8[a]
18:0	14.4 ± 0.6	14.7 ± 0.9	12.8 ± 0.5	13.0 ± 0.6
18:1(n9)	14.3 ± 0.7	13.0 ± 1.2	14.4 ± 0.9	13.1 ± 0.9
18:2(n-6)	26.1 ± 0.8	26.1 ± 0.8	22.6 ± 0.7[a]	23.6 ± 1.2
20:4(n-6)	19.8 ± 0.9	16.7 ± 1.3	20.7 ± 1.1	16.6 ± 1.4[b]
22:6(n-3)	3.7 ± 0.1	7.2 ± 0.5[b]	4.6 ± 0.2	7.4 ± 0.3[b]
USI	177 ± 3	190 ± 4[b]	178 ± 3	186 ± 4

Data are the mean ± SEM for 9 - 11 rats.

a. age effect (young versus aged, $P<0.05$)
b. DHA effect (control versus DHA, $P<0.05$)

TABLE II Total fatty acid concentrations and major fatty acid pattern in bone marrow cells of young and aged rats treated with or without DHA

	Young		*Aged*	
	control	*DHA*	*control*	*DHA*
Total (nmol/mg prot)	481 ± 23	686 ± 52	766 ± 55	1005 ± 141[a]
Major FFA (% of total)				
16:0	28.1 ± 0.2	27.4 ± 0.1	28.8 ± 0.4	27.9 ± 0.4
18:0	20.8 ± 1.5	19.0 ± 0.3	16.7 ± 0.7[a]	16.1 ± 0.5[a]
18:1(n9)	10.4 ± 0.5	10.1 ± 0.2	14.1 ± 0.7	13.8 ± 0.6
18:2(n-6)	14.7 ± 0.5	16.5 ± 0.3	16.6 ± 0.5[a]	18.9 ± 0.6[ab]
20:4(n-6)	21.4 ± 0.9	20.1 ± 0.3	19.0 ± 1.1	15.4 ± 0.8[ab]
22:6(n-3)	3.9 ± 0.2	5.1 ± 0.1[b]	3.0 ± 0.1[a]	5.5 ± 0.2[b]
USI	153 ± 5	163 ± 1	149 ± 4	157 ± 3

Data are the mean ± SEM for 9 _ 11 rats.

a. age effect (young versus aged, $P<0.05$).
b. DHA effect (control versus DHA, $P<0.05$).

TABLE III Antioxidants in plasma and bone marrow of young and aged rats treated with or without DHA

	Young		*Aged*	
	control	*DHA*	*control*	*DHA*
Plasma				
Vitamin C(uM)	60 ± 4.4	68.7 ± 5.1[a]	52.5 ± 6.0	54.0 ± 3.4[b]
Vitamin E (uM)	16.5 ± 1.1	15.5 ± 1.0	23.7 ± 2.2[b]	23.4 ± 2.2[b]
(Lipids corrected[c])	4.82 ± 0.25	4.86 ± 0.32	5.02 ± 0.10	4.45 ± 0.48
Cholesterol (mM)	1.50 ± 0.07	1.32 ± 0.08	2.53 ± 0.15[b]	2.38 ± 0.16[b]
Triglyceride (mM)	2.00 ± 0.27	1.72 ± 0.29	2.42 ± 0.45	2.15 ± 0.24
SH (mM)	0.64 ± 0.03	0.61 ± 0.03	0.72 ± 0.05	0.66 ± 0.04
Bone marrow				
Vitamin C (nmol/mg protein)	5.23 ± 0.42	5.86 ± 0.34	4.02 ± 0.35[b]	3.78 ± 0.35[b]
Vitamin E (nmol/mg protein)	0.78 ± 0.04	0.77 ± 0.05	1.16 ± 0.06[b]	1.03 ± 0.05[b]

Data are the mean ± SEM for 9 - 11 rats.

a. DHA effect (control versus DHA, $P<0.05$).
b. age effect (young versus aged, $P<0.05$).
c. Vitamin E (uM) / (Choesterol(mM) + Triglycerode (mM)).

Figure 1 shows TBARS (an indicator of lipid peroxide) and 8-OHdG of DNA (an indicator of oxidative DNA damage) in the bone marrow. Both TBARS and 8-OHdG levels tended to be higher in the aged than in the young rats. DHA supplementation did not influence the TBARS values in either the aged or the young rats. However, the DHA supplementation significantly increased the 8-OHdG levels of DNA in the aged rats, but not the young rats.

Concentrations of vitamin C and vitamin E were analyzed in the bone marrow and plasma, and the results shown in Table III. Vitamin E concentrations in the bone marrow and plasma were higher in the aged than in the young rats, with the high concentrations in the aged rats being due to the high levels of lipids. In contrast, vitamin C concentrations in the bone marrow and plasma were higher in the young than in the aged rats. The DHA supplementation significantly increased the concentrations of vitamin C in the young rats, while such increases in vitamin C were not detected in the aged rats. Plasma SH levels did not differ between the aged and young rats regardless of DHA supplementation.

Rats synthesize vitamin C in the liver, and l-gulono- γ -lactone oxidase catalyzes the last step of the vitamin C synthesis. As shown in Fig. 2, the enzyme activity was corresponded well to the vitamin C concentrations in the plasma and bone marrow among the groups. DHA supplementation significantly increased the enzyme activity in the young rats, but such increases in enzyme activity were not detected in the aged rats.

DISCUSSION

Increases in 8-OHdG levels in DNA from the liver and lung have been detected in aged animals and humans (17-20). In these studies, it has been shown that obvious increases in 8-OHdG levels cannot be detected at certain early ages, but can be detected thereafter. In this study, 8-OHdG levels in bone marrow DNA tended to

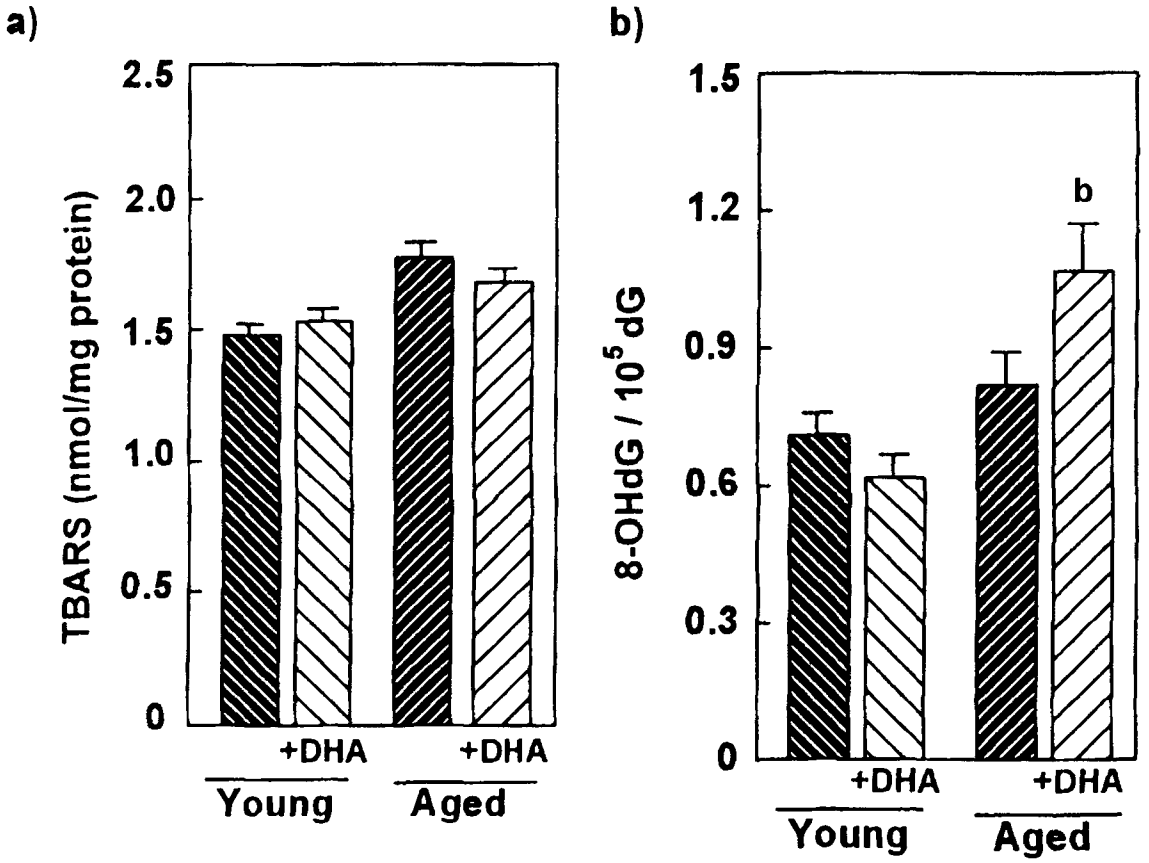

FIGURE 1 Lipid peroxide (TBARS, a)) and oxidative DNA damage (8-OHdG in DNA, b)) in marrow of young and aged rats treated with or without DHA. Young (5-week-old) and aged (100-week-old) rats received daily DHA (300 mg/kg body weight) or vehicle for 12 weeks. Data are mean ± SEM for 9 – 11 rats. b. DHA effect (control versus PHA, $P<0.05$)

be higher in aged rats than in young rats. This finding is consistent with the report of Kaneko *et al.* (18), who have shown that 8-OHdG levels in liver DNA increase in rats older than approximately 100 weeks. In the present study, DHA supplementation significantly increased 8-OHdG levels in the bone marrow DNA from the aged rats, but such changes were undetected in the bone marrow DNA from young rats. At the time of analysis, the young and aged rats were 17 weeks and 112 weeks old, respectively. These findings indicate that the bone marrow of aged rats is more susceptible to DHA- induced oxidative DNA damage than that of young rats.

The induction of oxidative damage in the body is the result of an oxidative stress that exceeds the antioxidant capacity, which is dependent on antioxidants and antioxidative enzymes. Several studies have shown that the levels of antioxidants and antioxidative enzymes are reduced in aged animals (11,12). Consistent with these findings, we also detected low vitamin C concentrations in bone marrow and plasma from the aged rats. It is also noteworthy that the activity of l-gulono- γ -lactone oxidase, which catalyzes the last step of vitamin C synthesis, was markedly lower in the aged than in the young rats. In the young rats, DHA supple-

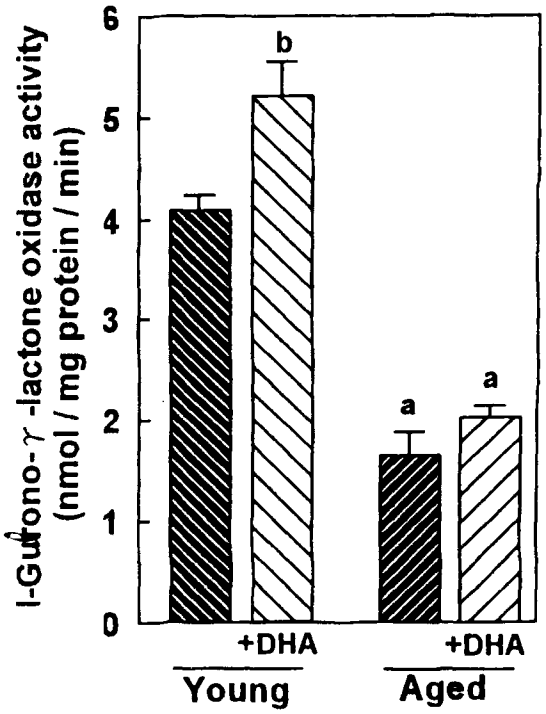

FIGURE 2 Enzyme activity of l-gurono- γ -lactone oxidase in the livers of young and aged rats treated with or without DHA. Young (5-week-old) and aged (100-week-old) rats received daily DHA (300 mg/kg body weight) or vehicle for 12 weeks. Data are mean ± SEM for 9 – 11 rats
a. age effect (young versus aged, $p<0.05$)
b. DHA effect (control versus DHA, $p<0.05$)

mentation was found to increase vitamin C concentrations in the plasma and bone marrow, indicating that DHA supplementation causes oxidative stress and that there is an adaptation against oxidative stress in the young rats. Changes in the activity of l-gulono- γ -lactone oxidase showed more clearly the relationship between DHA-induced oxidative stress and the adaptation against this stress in young rats. Those changes in vitamin C concentrations and enzyme activity due to DHA supplementation were very slight in the aged rats. These different vitamin C concentrations in responses to DHA supplementation between the aged and young rats could explain why 8-OHdG levels in bone marrow DNA were only increased in the aged DHA-treated rats. In this experiment, vitamin C was also derived from the diet, which contained 0.15 mg of vitamin C/100 g diet. The supply of vitamin C from the diet could explain why the differences in vitamin C concentrations between the plasma and bone marrow were milder than the differences in l-gulono- γ -lactone oxidase activity between the aged and young rats.

It has previously been shown in a guinea pig experiment that decreases in vitamin C and E do not cause increases in 8-OHdG in DNA (26). We have also reported that feeding a low vitamin E diet to rats or mice for 8 weeks or 50 weeks does not induce oxidative DNA damage in the liver and bone marrow (27,28). Cho *et al.* (29) have also shown the lack of effect of vitamin E deficiency on oxidative DNA damage in the liver of rats fed fish oil. Unlike this study, however those studies were not performed in aged animals. These results indicate that only the lower concentration of antioxidant vitamins is insufficient to induce the DHA-induced oxidative DNA damage in aged rats. The oxidative damage of nuclear DNA is efficiently repaired by the 8-OHdG repair enzyme, and the ubiquitous presence of the repair enzyme is demonstrated in mammalian cells (23,30). The repair of 8-OHdG in DNA has been reported to reduce with aging (20,31), resulting in an accumulation of 8-OHdG in DNA. According to these findings, it is reasonable to speculate that both the weakened antioxidant system and the reduced capacity for DNA repair were involved in the appearance of oxidative DNA damage due to DHA supplementation in the aged rats. It is also possible that different cellular composition of the bone marrow between young and aged rats is related to the different response to DHA. Further detailed study, particularly focused on the DNA repair ability, will be needed to clarify the mechanism for the increase in 8-OHdG levels in DNA in aged rats due to DHA supplementation.

Vitamin E is an important lipid-soluble antioxidant and efficiently prevents the formation of

lipid peroxides. As lipid peroxides have been shown to induce oxidative damage of DNA in an *in vitro* experiment (32), decreases in vitamin E in response to DHA treatment and sequential formation of lipid peroxides would cause oxidative DNA damage in the bone marrow of rats. However, in this study, we could detect oxidative DNA damage, but not marked decreases in vitamin E and increases in lipid peroxide levels in the DHA-supplemented aged rats. This unclear relation between lipid peroxides and oxidative DNA damage in this study suggests that 1) changes in vitamin E and lipid peroxide levels do not always accompany oxidative damage to DNA and 2) vitamin C is an important antioxidant to prevent oxidative damage to DNA. In this study, we analyzed lipid peroxide and vitamin E only in whole cells in the bone marrow, thus, clearer changes in lipid peroxide and vitamin E levels may exist in the nucleus where DNA is present. A report, showing that vitamin C efficiently prevents oxidative DNA damage in the sperm of human subjects (33) may support the importance of vitamin C against oxidative stress-induced DNA damage.

In conclusion, we have shown that 1) DHA supplementation induces oxidative DNA damage in the bone marrow of aged rats, but not of young rats, and 2) antioxidative defense systems, particularly vitamin C synthesis, are involved in the different responses to DHA-induced oxidative DNA damage between aged and young rats. Our results thus suggest that appropriate intakes of vitamin C and E could prevent DHA-induce oxidative DNA damage in the aged and enhance the beneficial effects of DHA on cardiovascular disease.

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