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Dietary docosahexaenoic acid-induced production of tissue lipid peroxides is not suppressed by higher intake of ascorbic acid in genetically scorbutic Osteogenic Disorder Shionogi/Shi-od/od rats

Seiji Sekine[1,2], Kazuhiro Kubo[1], Tadahiro Tadokoro[2], Akio Maekawa[2] and Morio Saito[1]*

[1]*Division of Food Science, Incorporated Administrative Agency, National Institute of Health and Nutrition, Toyama, Shinjuku-ku, Tokyo 162-8636, Japan*
[2]*Laboratory of Nutritional Biochemistry, Department of Applied Biology and Chemistry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-0054, Japan*

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In previous studies, we showed that docosahexaenoic acid (DHA) ingestion enhanced the susceptibility of rat liver and kidney to lipid peroxidation, but did not increase lipid peroxide formation to the level expected from the relative peroxidizability index (P-index) of the total tissue lipids. The results suggested the existence of some suppressive mechanisms against DHA-induced tissue lipid peroxide formation, as increased tissue ascorbic acid (AsA) and glutathione levels were observed. Therefore, we focused initially on the role of AsA for the suppressive mechanisms. For this purpose, we examined the influence of different levels of dietary AsA (low, moderate, high and excessive levels were 100, 300 (control), 600 and 3000 mg/kg diet respectively) on the tissue lipid peroxide and antioxidant levels in AsA-requiring Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed DHA (6·4 % total energy) for 32 or 33 d. Diets were pair-fed to the DHA- and 100 mg AsA/kg diet-fed group. We found that the lipid peroxide concentrations of liver and kidney in the DHA-fed group receiving 100 mg AsA/kg diet were significantly higher or tended to be higher than those of the DHA-fed groups with AsA at more than the usual control level of 300 mg/kg diet. Contrary to this, the liver α-tocopherol concentration was significantly lower or tended to be lower in the DHA and 100 mg AsA/kg diet-fed group than those of the other DHA-fed groups. However, tissue lipid peroxide formation and α-tocopherol consumption were not suppressed further, even after animals received higher doses of AsA. The present results suggest that higher than normal concentrations of tissue AsA are not necessarily associated with the suppressive mechanisms against dietary DHA-induced tissue lipid peroxide formation.

Docosahexaenoic acid: Peroxidizability index: Ascorbic acid: Osteogenic Disorder Shionogi/Shi-od/od rats

Docosahexaenoic acid (22 : 6*n*-3; DHA) and eicosapentaenoic acid (20 : 5*n*-3; EPA) are the predominant *n*-3 polyunsaturated fatty acids (PUFA) in fish oils. Consumption of fish oils is particularly associated with low incidence of atherosclerosis and cardiovascular diseases, and this prophylactic effect of fish oil ingestion is attributed to *n*-3 PUFA such as EPA and DHA (Dyerberg, 1986; Herold & Kinsella, 1986; Harris, 1989; Simopoulos, 1991; Kristensen *et al.* 2001; Schmidt *et al.* 2001). However, DHA is very prone to lipid peroxidation due to its unstable chemical structure with six double bonds. It has been reported that the relative reaction rate constants of peroxidation were 1, 2, 3, 4 and 5 against PUFA in which the number of methylene groups among double bonds was 1, 2, 3, 4 and 5 respectively (Cosgrove *et al.* 1987). The peroxidizability index (P-index) is therefore calculated according to the following equation (Cosgrove *et al.* 1987): P-index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (%pentaenoic × 4) + (% hexaenoic × 5).

In previous studies, we showed that DHA ingestion (Saito *et al.* 1996; Kubo *et al.* 1997, 1998, 2000; Saito & Kubo, 2003), similar to fish oil ingestion (Hammer & Wills, 1978; Kobatake *et al.* 1983; Mouri *et al.* 1984; Song *et al.* 2000), enhanced the susceptibility of rat liver and kidney to lipid peroxidation and increased the requirement for vitamin E. The enhancement was a function of dietary DHA levels and was thought to be attributable to the substitution of membrane fatty acids with highly unsaturated DHA, which is very susceptible to lipid peroxidation.

However, we found in previous rat studies (Kubo *et al.* 1998, 2000) that dietary DHA-induced tissue lipid peroxide

Abbreviations: AsA, ascorbic acid; DHA, docosahexaenoic acid; EC, excessive-vitamin C; EPA, eicosapentaenoic acid; HC, high-level ascorbic acid; LC, low-level ascorbic acid; MC, moderate- and control-level ascorbic acid; ODS rats, Osteogenic Disorder Shionogi/Shi-od/od rats; P-index, peroxidizability index; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substance.

* **Corresponding author:** Dr Morio Saito, fax +81 3 3203 7584, email msaito@nih.go.jp

formation did not increase to the level expected from the P-index of the total tissue lipids. This phenomenon was especially prominent in the liver. Moreover, the suppression of lipid peroxide formation below the P-index was also seen with EPA ingestion, but not with α-linolenic acid ingestion (Saito & Kubo, 2003). Therefore, in the present study, we focused on the mechanism of the suppression by DHA ingestion. With regard to this suppressive mechanism, we also found (Kubo *et al.* 1997; Saito & Kubo, 2003) that the tissue ascorbic acid (AsA) and glutathione levels were increased in the rats fed DHA, and assumed that the increase in these levels might potentiate reductive-recycling of vitamin E, leading to an augmentation of the antioxidant activity of vitamin E. In addition, AsA is known to be a potent quencher of superoxide and other reactive oxygen species, namely singlet oxygen, hydroxyl radical and peroxyl radical. Many studies (Wartanowicz *et al.* 1984; Kimura *et al.* 1992; Chakraborty *et al.* 1994) have already reported that AsA suppressed lipid peroxidation *in vivo*. Moreover, glutathione is a necessary substrate for the antioxidant enzymes glutathione *S*-transferases and glutathione peroxidases. Therefore, we presumed that the increase in dietary DHA-induced tissue AsA and glutathione levels could be part of the mechanism for suppressing tissue lipid peroxide formation below the level expected from the P-index of the total tissue lipid.

A study to clarify the suppressive mechanisms against dietary DHA-induced lipid peroxide formation is thought to be important for reasons of safety, but also to define a means of efficiently enhancing the physiological efficacy of *n*-3 PUFA.

In the present study, we focused in particular on the influence of dietary AsA levels on reducing dietary DHA-induced tissue lipid peroxidation. For this purpose, we fed graded levels of AsA together with DHA to Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats. The ODS rat lacks l-gulono-γ-lactone oxidase, an enzyme that catalyses the terminal step of AsA biosynthesis (Kawai *et al.* 1992).

Materials and methods

Animals and diets

The experimental procedures used in the present study met the guidelines of the Animal Handling Committee for the Independent Administrative Institution, National Institute of Health and Nutrition (Tokyo, Japan).

Male ODS rats (CLEA Japan, Tokyo, Japan), 6 weeks of age and weighing 120–150 g, were housed individually in stainless-steel wire-bottomed cages kept at a constant temperature of 22 ± 1°C and humidity of 50–60 % with a 12 h light–dark cycle. The composition of the experimental diets, based on the AIN-76 purified diet for rats (American Institute of Nutrition, 1977, 1980), is shown in Table 1. DHA ethyl esters (purity 920 g/kg) prepared from the orbital fat of tuna were donated by Maruha Corporation (Tsukuba, Japan). To prevent the autoxidation of DHA in the diets, the diet was prepared beforehand without adding DHA and was stored at − 20°C until use.

Table 1. Composition of the experimental diets (g/kg diet) and fatty acid composition (g/100 g fatty acids) of dietary lipids*

	Control	DHA-fed groups
DHA (% total energy)	0	6·4
LA (% total energy)	8·6	2·3
Basic components†	900·0	900·0
Test lipids‡	100·0	100·0
Safflower oil	40·0	0
High-oleic safflower oil	60·0	65·0
DHA concentrate‖	0	35·0
Fatty acid		
16 : 0	5·5	3·4
18 : 0	2·3	1·5
18 : 1*n*-9	50·0	50·3
18 : 2*n*-6 (LA)	40·3	10·9
18 : 3*n*-3	0·5	0·2
20 : 4*n*-6	0	0·6
20 : 5*n*-3 (EPA)	0	0·9
22 : 5*n*-3	0	0·6
22 : 6*n*-3 (DHA)	0	30·5
Others	1·4	1·1
Polyunsaturated fatty acids	40·8	43·7
Double-bond index§	1·30	2·66
Peroxidizability index§	41·3	171·6

DHA, docosahexaenoic acid; LA, linoleic acid; EPA, eicosapentaenoic acid.

* The energy density of all diets was 17·4 MJ (4160 kcal)/kg diet, using Atwater energy factors for energy calculation (Atwater, 1902). The vitamin E content of the experimental diets as *RRR*-α-tocopherol equivalent was 70 mg/kg diet. The ascorbic acid content of the control LA diet (DHA-free) was 300 mg/kg diet and those of the test diets (DHA-added) were 100, 300, 600 and 3000 mg/kg diet.

† The basic components of the diet given to all the groups were as follows (g/kg): casein 200·0, DL-methionine 3·0, maize starch 150·0, sucrose 225·0, glucose 225·0, cellulose powder 50·0, AIN-76 vitamin mixture (American Institute of Nutrition, 1977, 1980) 10·0, AIN-76 mineral mixture (American Institute of Nutrition, 1977) 35·0, choline bitartrate 2·0.

‡ Fat provided 21·6 % total energy.

§ Calculated from the fatty acid compositions of the dietary lipids: For details, see p. 387.

‖ DHA ethyl esters prepared from the ethyl esters of the orbital fat of tuna were used (Maruha Corporation, Tsukuba, Japan), the purity was 920 g/kg.

The DHA was stored at − 80°C and was mixed with the diet every day immediately before feeding. In a previous study (Kubo *et al.* 2000), we showed that dietary DHA increased tissue lipid peroxidation and decreased α-tocopherol content in the DHA-fed group administered a diet containing 54 mg vitamin E/kg. However, dietary vitamin E was unable to suppress lipid peroxidation, even after ingestion of a high level (402 mg/kg diet) of vitamin E. We therefore set the dietary vitamin E level as 70 mg *RRR*-α-tocopherol equivalent/kg diet. The AsA content of the control linoleic acid diet was 300 mg/kg diet, and the contents of the test diets (DHA-fed groups) were 100 (low-level AsA, LC), 300 (moderate- and control-level AsA, MC), 600 (high-level AsA, HC) and 3000 (excessive-level AsA, EC) mg/kg diet. The dietary addition of 300 mg AsA/kg was enough to prevent vitamin C deficiency and to achieve maximum growth in the ODS rats (Horio *et al.* 1985). Therefore, the dietary AsA level of the control group was 300 mg AsA/kg diet. The fatty acid composition (g/100 g fatty acids) of dietary lipids is also shown in Table 1. The proportion of total PUFA was at almost the same level in the control linoleic acid and four DHA-fed groups. The degree of unsaturation of dietary lipids is presented as the double-bond index (Pietrangelo *et al.* 1990) and P-index (Cosgrove

et al. 1987). The P-index was calculated according to the following equation: P-index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

After the rats were fed a basal diet containing 50 g high-oleic safflower oil/kg diet for 5 d, six rats from each group were fed the experimental diets for 33 d in the LC group or 32 d in all the other groups. Water was consumed *ad libitum*. Diets were pair-fed to the LC group. During the supplementation period, each diet was made available to the rats in the evening and was removed the next morning. After being deprived of food overnight, the rats were killed by cardiac puncture. The tissues were promptly excised, washed with isotonic saline (9 g NaCl/l) and weighed. The liver was then perfused with ice-cold isotonic saline via the portal vein. Samples of liver, kidney and testis were stored at − 80°C until used for the analysis. Sodium citrate was used as an anticoagulant, and the plasma was separated by centrifugation at 2700 ***g*** for 15 min at 4°C.

Determination of lipid peroxides

When lipid peroxidation occurs, polyunsaturated lipids are initially oxidized to primary products of lipid peroxidation such as lipid hydroperoxides via conjugated diene formation. The primary products then decompose to secondary products such as aldehydes. Aldehydes react with amino compounds, and produce the end products of lipid peroxidation, such as lipofuscin. For the present study, we determined the primary products of lipid peroxidation (i.e. plasma lipid hydroperoxides, tissue conjugated dienes and chemiluminescence emission), the secondary products (i.e. plasma and tissue thiobarbituric acid-reactive substances (TBARS)) and the endproducts (i.e. plasma water-soluble fluorescent substance and liver microsomal lipofuscin). The plasma lipid hydroperoxide concentration was determined using a lipid hydroperoxide assay kit (Determiner LPO; Kyowa Medex Co., Ltd, Tokyo, Japan). The determination utilized haemoglobin as the catalyst of the reaction, in which lipid hydroperoxide and a Methylene Blue-derivative react, and equimolar Methylene Blue is produced. The Methylene Blue was measured at 675 nm. Conjugated dienes in the liver, kidney and testis were determined by the method of Hu *et al.* (1989) and Rao & Recknagel (1968). The conjugated dienes were measured at 233 nm using an extinction coefficient of 27 000 $(\text{mol/l})^{-1} \cdot \text{cm}^{-1}$.

The tissue chemiluminescence intensities of the homogenates were determined according to the method of Miyazawa *et al.* (1984). The light emitted from the homogenates is due mainly to singlet molecular oxygen and/or excited carbonyl compounds resulting from the breakdown of lipid peroxyradicals (Boveris *et al.* 1981; Miyazawa *et al.* 1984), which are produced in the early stage of peroxidation.

The plasma TBARS were determined by the method of Yagi (1976). As an antioxidant, butylated hydroxytoluene was added to the reaction mixture at a final concentration of 0·36 mM. The tissue TBARS were measured according to the method of Ohkawa *et al.* (1979) with a minor modification, in which butylated hydroxytoluene was added to the reaction mixture at a final concentration of 0·45 mM. TBARS are expressed in terms of the malondialdehyde equivalent. This method measures primarily malondialdehyde, alkenals and alkadienals produced at acidic and heating conditions of the assay.

The plasma water-soluble fluorescent substances were analysed by the method of Tsuchida *et al.* (1985). Liver microsomes were prepared (Saito & Yamaguchi, 1988) and the microsomal lipofuscin concentration was determined by the method of Fletcher *et al.* (1973).

Determination of antioxidants

α-Tocopherol concentrations in the test lipids, plasma and tissues were analysed by HPLC as described by Saito *et al.* (1992). AsA levels in the tissues were analysed by the method of Roe *et al.* (1948). Non-protein sulfhydryl (mostly glutathione) levels in the tissues were analysed by the method of Beutler *et al.* (1963). Dehydroascorbic acid reductases are primarily categorized as glutathione-dependent glutaredoxin and NADPH-dependent thioredoxin reductase. Glutaredoxin and thioredoxin reductase activities were determined as follows. Liver homogenate (250 g/kg) was prepared in ice-cold 0·1 M-potassium phosphate buffer, pH 6·85. The homogenate was centrifuged at 20 000 ***g*** for 15 min at 4°C, and the supernatant fraction was further centrifuged at 105 000 ***g*** for 60 min at 4°C. The resultant cytosolic fraction was used for the glutaredoxin (Wells *et al.* 1995) and thioredoxin reductase (Holmgren & Björnstedt, 1995) assays. One unit of glutaredoxin activity was defined as the amount of enzyme that reduced 1 μmol dehydroascorbic acid in 1 min at 30°C. One unit of thioredoxin reductase activity was defined as 1 μmol 5′-thionitrobenzoic acid formed per min.

The cytosolic protein content was measured by the method of Lowry *et al.* (1951).

Plasma aspartate aminotransferase and alanine aminotransferase assays

The activities of aspartate aminotransferase and alanine aminotransferase in the plasma were determined using a clinical enzyme assay kit (Wako Pure Chemical Ind., Osaka, Japan) by the method of Reitman & Frankel (1957).

Fatty acid composition analysis

Total lipids in the liver were extracted according to the method of Folch *et al.* (1957). Total lipids in the kidney and testis were extracted according to the method of Bligh & Dyer (1959). Fatty acid methyl esters of dietary lipids and total tissue lipids were prepared according to Kubo *et al.* (2000). The methyl esters were analysed by GLC with a flame ionization detector (Shimadzu GC-18A, Kyoto, Japan) by using a 25 m × 0·25 mm internal diameter HR-SS-10 capillary column (Shinwa Chemical Industries Ltd, Kyoto, Japan). The temperature of the column oven was programmed from 160 to 210°C at a rate of 2·0°C/min. Injector and detector temperatures were 250°C. The carrier gas was He.

Statistical analysis

All results were expressed as means values and standard deviations. Significant differences between the mean values of the control and MC group were evaluated by Student's *t* test (Dixon & Massey, 1983). Significant differences among the mean values of the DHA-fed groups were evaluated by ANOVA coupled with Bonferroni's multiple comparison. For clarifying differences between the increases in the relative tissue lipid peroxide levels and the relative P-index, significant differences between the relative ratios of the lipid peroxide levels to the control values and those of P-index were also compared by Student's *t* test. The limit of significance was set at $P<0{\cdot}05$.

Results

In the rats of the LC group, signs of scurvy, such as haemorrhages around the eyes and nose, did not appear during the 33 d of the experimental period. The body-weight gain, food intake, and relative kidney and testis weights did not differ significantly among the treatment groups (results not shown). Relative liver weight did not significantly differ between the control group and DHA-fed MC group (34 (SD 2) *v.* 34 (SD 1) g/kg body weight respectively). When the DHA-fed groups were compared, the relative liver weight of the LC group was significantly higher than that of EC group (36 (SD 2) *v.* 32 (SD 2) g/kg body weight respectively, $P<0{\cdot}05$).

The major PUFA compositions of total lipids in the liver, kidney and testis are shown in Table 2. In the liver, the proportions of linoleic acid (18 : 2*n*-6) and arachidonic acid (20 : 4*n*-6) were significantly higher ($P<0{\cdot}05$) in the control group than in the DHA-fed MC group, but those of EPA (20 : 5*n*-3) and DHA were higher in the DHA-fed MC group than in the control. When the proportions of those PUFA were compared among the DHA-fed groups, all proportions varied within a small range, and the variation was negligible. The double-bond index and P-index were significantly higher ($P<0{\cdot}05$) in the DHA-fed MC group than in the control group, but no significant difference was noticed among the DHA-fed groups. The liver P-index of total lipids in the DHA-fed groups was 1·6- to 1·7-fold higher than that of the control group.

In the kidney, the proportion of arachidonic acid was significantly lower ($P<0{\cdot}05$), but linoleic acid, EPA (22 : 5*n*-3) and DHA were higher, in the DHA-fed MC group than in the control group. Moreover, the proportion of EPA was comparable with that of DHA achieved by

Table 2. Major polyunsaturated fatty acid compositions (g/100 g) of total lipids in the liver, kidney and testis of Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed docosahexaenoic acid (DHA) and graded levels of ascorbic acid†

(Mean values and standard deviations for six rats per group)

	Control		DHA-fed groups							
			LC		MC		HC		EC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Liver										
18 : 2*n*-6 (LA)	12·9	1·9	8·3	0·5	8·2*	0·4	8·3	0·3	8·6	0·7
20 : 4*n*-6	13·8	1·6	3·7^{ab}	0·3	3·3^{a}*	0·3	3·4^{ab}	0·3	3·7^{b}	0·2
20 : 5*n*-3 (EPA)	0·1	0·1	3·2^{b}	0·4	2·4^{a}*	0·7	2·7^{ab}	0·4	2·6^{ab}	0·5
22 : 5*n*-3	0·3	0·1	1·4^{b}	0·2	1·2^{ab}*	0·2	1·1^{a}	0·1	1·2^{ab}	0·2
22 : 6*n*-3 (DHA)	1·9	0·7	18·4	1·2	17·6*	2·8	17·5	1·0	18·4	1·7
Double-bond index‡	1·4	0·1	1·9	0·1	1·8*	0·2	1·9	0·1	1·9	0·1
Peroxidizability index‡	77·8	6·9	132·7	7·8	122·4*	15·8	123·6	5·4	129·5	9·1
Kidney										
18 : 2*n*-6 (LA)	9·5	0·3	10·5	0·7	11·1*	0·3	11·1	0·5	11·0	0·4
20 : 4*n*-6	23·9	3·2	9·1^{a}	1·1	10·9^{b}*	0·4	10·9^{b}	0·8	10·6^{ab}	1·0
20 : 5*n*-3 (EPA)	0·1	0·1	6·9	0·8	7·6*	0·6	7·3	0·6	6·8	0·9
22 : 5*n*-3	0·2	0·1	0·5	0·1	0·6*	0·1	0·6	0·1	0·5	0·1
22 : 6*n*-3 (DHA)	1·2	0·3	6·8	1·0	7·5*	0·5	7·4	0·3	6·9	0·7
Double-bond index‡	1·6	0·1	1·6	0·1	1·8*	0·1	1·8	0·1	1·7	0·1
Peroxidizability index‡	96·9	11·3	105·0	11·1	118·0*	4·8	116·0	6·0	110·4	9·5
Testis										
18 : 2*n*-6 (LA)	11·6	4·0	6·0	2·2	7·0*	1·3	6·7	1·2	6·3	1·5
20 : 4*n*-6	7·3	2·4	6·9	2·3	5·0	1·9	5·5	2·2	6·3	2·1
20 : 5*n*-3 (EPA)	0·0	0·1	0·2	0·3	0·4	0·2	0·3	0·3	0·3	0·3
22 : 5*n*-6	9·6	3·6	8·7	3·6	8·0	3·0	8·5	3·9	9·5	3·4
22 : 6*n*-3 (DHA)	0·6	0·3	4·8	2·0	6·6*	1·1	6·1	1·4	5·4	1·4
Double-bond index‡	1·6	0·2	1·7	0·2	1·7	0·1	1·7	0·2	1·7	0·1
Peroxidizability index‡	89·0	21·3	111·8	19·1	102·8	16·2	100·1	17·9	104·6	17·5

DHA, docosahexaenoic acid; LC, low-level ascorbic acid; MC, moderate-level ascorbic acid; HC, high-level ascorbic acid; EC, excessive-level ascorbic acid; LA, linoleic acid; EPA, eicosapentaenoic acid.

[a,b] Mean values for the DHA-fed groups of each tissue within a row with unlike superscript letters were significantly different (ANOVA coupled with Bonferroni's multiple comparison, $P<0{\cdot}05$).

Mean values were significantly different from those of the control group (Student's *t* test): *$P<0{\cdot}05$.

† For details of diets and procedures, see Table 1 and p. 386.

‡ Calculated from the fatty acid compositions of the tissue total lipids: For details, see p. 387.

DHA ingestion. The variations of proportions of PUFA among the DHA-fed groups were negligible. Both the double-bond index and P-index were significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group, but no significant difference was noticed among the DHA-fed groups. The P-index of total lipids in the DHA-fed groups was 1·1- to 1·2-fold greater than the control value, and the increase was slight.

In the testis, the proportion of linoleic acid was significantly lower ($P<0·05$) in the DHA-fed MC group than in the control group, and that of arachidonic acid did not differ significantly between the control and DHA-fed MC groups. The proportion of DHA was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group, but the proportions of arachidonic acid, EPA and 22 : 5*n*-6 did not differ significantly. None of the proportions of the major PUFA differed significantly among the DHA-fed groups. The double-bond index and P-index did not differ significantly among any of the groups, even after the highly unsaturated DHA was administered to the rats. The P-index of total lipids in the DHA-fed groups was 1·1- to 1·3-fold greater than the control value, and this increase was determined to be slight.

When the liver, kidney and testis were compared, the proportion of DHA was highest in the liver of the DHA-fed groups. In the kidney, the proportion of arachidonic acid was high and was maintained at high proportions even after DHA was ingested. In the testis, highly unsaturated *n*-6 fatty acids, particularly 22 : 5*n*-6, were in high proportions, and even in the DHA-fed groups, the proportion of 22 : 5*n*-6 was higher than that of DHA.

The plasma lipid hydroperoxide concentrations did not differ significantly among any of the treatment groups (results not shown). The TBARS level was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group (Table 3), but no significant difference was noticed among the DHA-fed groups. Hence, the increased levels of dietary AsA did not affect the TBARS concentration. The water-soluble fluorescent substance level was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group, and was slightly but significantly lower ($P<0·05$) in the MC group than in the EC group when the DHA-fed groups were compared (Table 3). The plasma α-tocopherol concentration of the DHA-fed MC group was significantly lower ($P<0·05$) than that of the control group, but no significant difference was noticed among the DHA-fed groups (Table 3). The aspartate aminotransferase activity did not differ significantly in any of the treatment groups (Table 3). However, the alanine aminotransferase activity was greater in the MC group than in the control group, and was suppressed slightly in the HC and EC groups, according to the DHA-fed group comparisons (Table 3). Thus, tissue parenchymal cell injury was demonstrated in the LC and MC groups, and was suppressed by a relatively increased intake of AsA. Such injury was not paralleled by the lipid peroxide and α-tocopherol concentrations in the plasma and in the tissue, as will be described later.

The lipid peroxide levels in the liver and kidney are shown in Table 4, and the relationship between the P-index and lipid peroxide levels in the tissues expressed relative to the control values is shown in Fig. 1. In the liver, the conjugated diene level did not differ significantly among any of the treatment groups (Fig. 1(A)). The chemiluminescence intensity was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group. When the DHA-fed groups were compared, the intensity tended to be higher ($P=0·0937$) in the LC group than in the MC, HC and EC groups (Table 4 and Fig. 1(A)), although no significant difference in the intensity was noticed among the DHA-fed groups. The liver TBARS levels showed a tendency similar to that of the

Table 3. Influence of graded levels of dietary ascorbic acid on concentrations of thiobarbituric acid-reactive substances (TBARS), water-soluble fluorescent substance and α-tocopherol, and on activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma of Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed docosahexaenoic acid (DHA)†

(Mean values and standard deviations for six rats per group)

	Control		DHA-fed groups							
			LC		MC		HC		EC	
DHA (% total energy)	0		6·4		6·4		6·4		6·4	
LA (% total energy)	8·6		2·3		2·3		2·3		2·3	
AsA (mg/kg diet)	300		100		300		600		3000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TBARS (μmol MDA/l)	3·0	0·3	4·1	0·4	5·2*	1·2	4·2	0·6	4·1	0·4
Water-soluble fluorescent substance (relative fluorescence intensity)‡	3·1	0·3	4·1^{ab}	0·1	3·8^{a}*	0·2	4·2^{ab}	0·4	4·5^{b}	0·4
α-Tocopherol (μmol/l)	16·9	3·5	7·7	0·7	7·3*	1·8	7·2	1·3	7·6	1·5
AST (μkat/l)	0·34	0·06	0·40	0·08	0·36	0·02	0·37	0·05	0·35	0·02
ALT (μkat/l)	0·04	0·02	0·10^{b}	0·03	0·08^{ab}	0·01	0·06^{a}	0·01	0·06^{a}	0·02

LC, low-level ascorbic acid; MC, moderate-level ascorbic acid; HC, high-level ascorbic acid; EC, excessive-level ascorbic acid; LA, linoleic acid; AsA, ascorbic acid; MDA, malondialdehyde.

^{a,b} Mean values for the DHA-fed groups within a row with unlike superscript letters were significantly different (ANOVA coupled with Bonferroni's multiple comparison, $P<0·05$).

Mean values were significantly different from those of the control group (Student's *t* test): *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 386.

‡ The instrument was calibrated to read 100 relative fluorescence units against a quinine sulfate solution (0·128 μmol/l 50 mM-H_2SO_4).

Table 4. Influence of graded levels of dietary ascorbic acid on liver and kidney chemiluminescence intensities and thiobarbituric acid-reactive substance (TBARS) levels, and liver microsomal lipofuscin level in Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed docosahexaenoic acid (DHA)†

(Mean values and standard deviations for six rats per group)

			DHA-fed groups							
	Control		LC		MC		HC		EC	
DHA (% total energy)	0		6·4		6·4		6·4		6·4	
LA (% total energy)	8·6		2·3		2·3		2·3		2·3	
AsA (mg/kg diet)	300		100		300		600		3000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Liver										
Chemiluminescence intensity (counts per 30 s)	394·7	155·0	681·0	140·4	494·0*	95·1	543·8	121·1	523·5	129·0
TBARS (nmol MDA/g)	95·0	21·1	123·8	21·5	107·8	16·2	117·3	9·7	117·4	23·8
Microsomal lipofuscin (ng/mg protein)	10·1	1·5	10·6	2·9	10·6	1·5	9·8	1·1	11·4	11·4
Kidney										
Chemiluminescence intensity (counts per 30 s)	363·6	99·7	469·7[b]	119·8	325·3[a]	48·3	451·2[ab]	46·8	386·0[ab]	40·4
TBARS (nmol MDA/g)	133·1	4·3	172·7	24·0	178·1*	24·5	167·1	11·5	152·8	8·5

LC, low-level ascorbic acid; MC, moderate-level ascorbic acid; HC, high-level ascorbic acid; EC, excessive-level ascorbic acid; LA, linoleic acid; AsA, ascorbic acid; MDA, malondialdehyde.

[a,b] Mean values for the DHA-fed groups within a row with unlike superscript letters were significantly different (ANOVA coupled with Bonferroni's multiple comparison, $P<0·05$).

Mean values were significantly different from those of the control group (Student's *t* test): *$P<0·05$.

† For details of diets and procedures, see Table 1 and p. 386.

chemiluminescence intensity, but the values did not differ significantly among the treatment groups (Table 4 and Fig. 1(A)). The liver microsomal lipofuscin levels did not differ significantly among any of the treatment groups (Table 4 and Fig. 1(A)). When the liver lipid peroxides and P-index expressed relative to the control values were compared, only the chemiluminescence intensities in the LC and HC groups coincided with the P-index, and the other lipid peroxides in each DHA-fed group were significantly lower ($P<0·05$) than each P-index (Fig. 1(A)).

In the kidney, the conjugated diene levels did not differ significantly among any of the treatment groups (Fig. 1(B)). The chemiluminescence intensity did not differ significantly between the control and DHA-fed MC groups, but when the DHA-fed groups were compared, the intensity was significantly lower ($P<0·05$) in the MC group than in the LC group (Table 4 and Fig. 1(B)). The TBARS level was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group. When the TBARS levels of DHA-fed groups were compared, the level did not differ significantly. When the lipid peroxides and P-index expressed relative to the control values were compared, the conjugated diene levels in the MC and HC groups and the chemiluminescence intensity in the MC group were significantly lower ($P<0·05$) than each P-index (Fig. 1(B)). Contrary to this, the TBARS level in the LC group was significantly higher ($P<0·05$) than the P-index. No other significant differences between the TBARS levels and each P-index were observed.

In the testis, the conjugated diene level and TBARS level did not differ significantly among any of the treatment groups (Fig. 1(C)). The chemiluminescence intensity was significantly higher ($P<0·05$) in the DHA-fed MC group than in the control group, but no significant difference was noticed when the DHA-fed groups were compared (Fig. 1(C)). When the lipid peroxides and P-index expressed relative to the control values were compared, those values nearly coincided in each group except that the conjugated diene level of the LC group was significantly ($P<0·05$) lower than the P-index.

The tissue antioxidant levels are shown in Fig. 2. In the liver, the α-tocopherol level was significantly lower ($P<0·05$) in the DHA-fed MC group than in the control group. When the DHA-fed groups were compared, the α-tocopherol level in the LC group was significantly lower ($P<0·05$) than in the MC group. However, no significant difference was noticed among the MC, HC and EC groups (Fig. 2(A)). Therefore, a vitamin E-sparing effect by dietary AsA intake ≥ 300 mg/kg diet was observed. The AsA level was significantly lower ($P<0·05$) in the DHA-fed MC group than in the control group, but it increased with an increase in the dietary AsA levels in the DHA-fed groups. The liver glutathione levels and glutaredoxin activities (results not shown) did not differ significantly among the treatment groups. The liver thioredoxin reductase activities were 48·3 (SD 3·2), 41·1 (SD 3·6), 44·6 (SD 2·5), 46·6 (SD 1·7) and 43·3 (SD 6·3) units/mg protein in the control, LC, MC, HC and EC groups respectively. The activity was significantly lower ($P<0·05$) in the MC group than in the control, but no significant difference was noticed among the DHA-fed groups.

In the kidney, the α-tocopherol level was significantly lower ($P<0·05$) in the MC group than in the control group (Fig. 2(B)). When the DHA-fed groups were compared, the levels did not differ significantly. Hence, the α-tocopherol level was not influenced by a higher intake of AsA. The AsA level did not differ significantly between the control and MC groups, but did increase with increasing the dietary AsA levels, as shown by comparison of the DHA-fed groups (Fig. 2(B)). The glutathione level did not differ significantly among any of the treatment groups (Fig. 2(B)).

In the testis, the α-tocopherol level was significantly lower in the MC group than in the control (Fig. 2(C)). However, when the DHA-fed groups were compared, no influence of

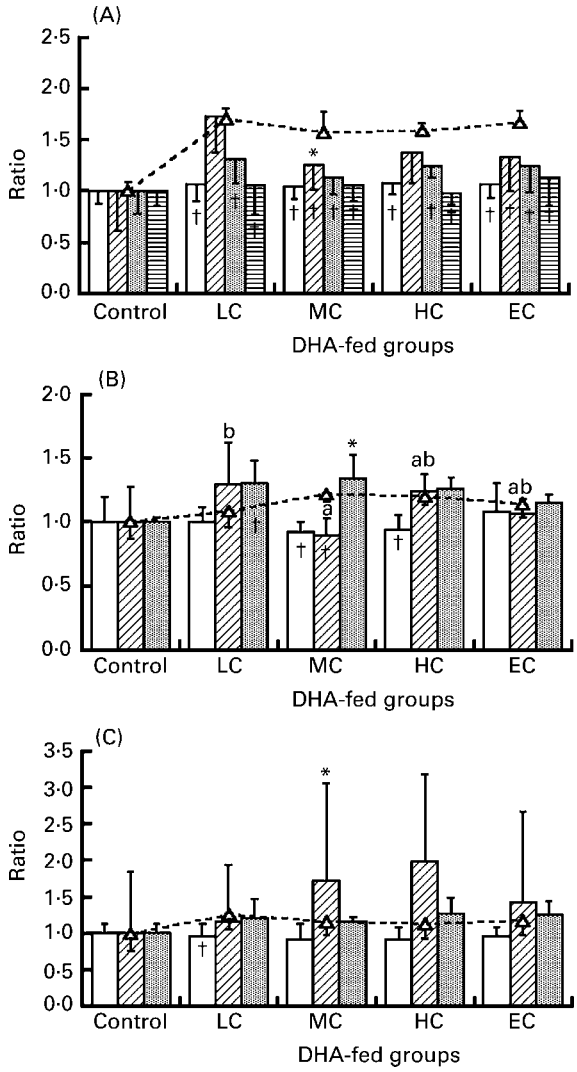

Fig. 1. Lipid peroxide levels and peroxidizability indices (P-index) of total lipids expressed relative to the control values in the liver (A), kidney (B) and testis (C) of Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed docosahexaenoic acid (DHA) and graded levels of ascorbic acid. The relative P-index was used in the present study as an indicator to determine the vulnerability of tissue lipids to oxidation. The P-index of total lipids was calculated according to the following equation (Cosgrove *et al.* 1987): P-index = (% dienoic×1)+(% trienoic×2)+(% tetraenoic×3)+(% pentaenoic×4) +(% hexaenoic×5). The ascorbic acid content of the control linoleic acid diet (DHA-free) was 300 mg/kg and those of the test diets (DHA-added) were 100 (low-level ascorbic acid, LC), 300 (moderate-level ascorbic acid, MC), 600 (high-level ascorbic acid, HC) and 3000 (excessive-level ascorbic acid, EC) mg/kg diet. □, Conjugated diene; ▨, chemiluminescence intensity; ▤, thiobarbituric acid-reactive substances; ▤, lipofuscin; —Δ—, P-index. For details of diets and procedures, see Table 1 and p. 386. Values are means with standard deviations shown by vertical bars (six per group). [a,b]Mean values with unlike superscript letters were significantly different (ANOVA and Bonferroni's multiple comparison $P<0{\cdot}05$). Mean values were significantly different from those of the control group (Student's *t* test): $^{*}P<0{\cdot}05$. Mean values for the relative ratio of lipid P-index were significantly different from those for lipid peroxide in each group (Student's *t* test): $\dagger P<0{\cdot}05$.

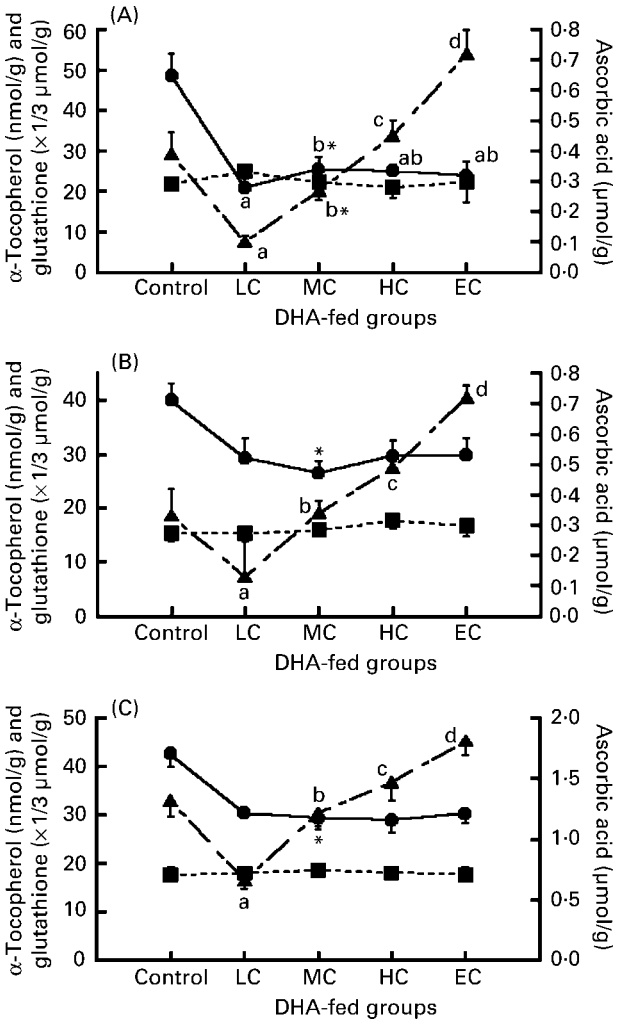

Fig. 2. Changes in α-tocopherol (•), ascorbic acid (▲) and glutathione (■) levels in the liver (A), kidney (B) and testis (C) of Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats fed docosahexaenoic acid (DHA) and graded levels of ascorbic acid. The ascorbic acid content of the control linoleic acid diet (DHA-free) was 300 mg/kg and those of the test diets (DHA-added) were 100 (low-level ascorbic acid, LC), 300 (moderate-level ascorbic acid, MC), 600 (high-level ascorbic acid, HC) and 3000 (excessive-level ascorbic acid, EC) mg/kg diet. Values are means with standard deviations shown by vertical bars (six per group). [a,b,c,d]Mean values with unlike superscript letters were significantly different (ANOVA and Bonferroni's multiple comparison, $P<0{\cdot}05$). Mean values were significantly different from those of the control group (Student's *t* test): $^{*}P<0{\cdot}05$.

dietary AsA levels was observed; these results were similar to those of the kidney (Fig. 2(B)). The AsA level did not differ significantly between the control and MC groups, but increased with increasing the dietary AsA levels, according to the DHA-fed group comparisons (Fig. 2(C)). The AsA level in the testis was higher, on the whole, than in the liver and kidney. The glutathione level did not differ significantly among any of the treatment groups (Fig. 2(C)).

Discussion

Lipid peroxidation has been implicated in a potential cause of chronic lifestyle-related diseases such as arteriosclerosis, diabetes and cancer. Therefore, clarification of the suppressive mechanisms reducing dietary DHA-induced lipid peroxide formation is important from the viewpoint of safety, and such study is also necessary to improve the physiological efficacy of *n*-3 PUFA.

In previous studies (Kubo *et al.* 2000; Saito & Kubo, 2003), we found that the production of tissue lipid peroxides was closely associated with the P-index of the total lipids in DHA-fed rats that were almost vitamin E-deficient. In rats exhibiting the vitamin E-deficient state, tissue parenchymal cell injuries were also observed. However, when the rats ingested a typical level of vitamin E at 54 mg α-tocopherol equivalent/kg diet (American Institute of Nutrition, 1977, 1980), the dietary DHA-induced lipid peroxide formation did not increase to the level that was expected from the P-index of the total tissue lipids (Kubo *et al.* 1998, 2000; Saito & Kubo, 2003). This phenomenon was especially prominent in the liver. Tissue parenchymal cell injuries were also not observed in rats with this vitamin E status (Kubo *et al.* 2000; Saito & Kubo, 2003). However, the lipid peroxide formation, particularly in the liver, was not suppressed further, even after a high level of vitamin E ingestion (Kubo *et al.* 2000). This result suggested that vitamin E exerted a limited antioxidant capacity when highly unsaturated DHA was ingested. Moreover, we found (Kubo *et al.* 1997; Saito & Kubo, 2003) that the AsA and glutathione levels in the tissues of rats concurrently increased when DHA was ingested. Accordingly, we presumed that this phenomenon could represent one of the mechanisms suppressing tissue lipid peroxide formation below the level expected from the P-index of the total tissue lipids (Kubo *et al.* 1998, 2000; Saito & Kubo, 2003). The suppression was also seen with EPA ingestion, but not with α-linolenic acid ingestion (Saito & Kubo, 2003). Therefore, in the present study, we focused on the influence of DHA ingestion and different levels of dietary AsA on tissue lipid peroxidation in genetically scorbutic ODS rats.

In previous studies, we also observed that dietary DHA-stimulated lipid peroxidation was most apparent in the liver, intermediate in the kidney, least observable in the testis (Kubo *et al.* 2000) and non-observable in the brain (Kubo *et al.* 1998). Hence, we chose the liver, kidney and testis to examine the effects of dietary AsA and DHA in the present study.

During the feeding period, no rats showed signs of scurvy, e.g. no haemorrhages around the eyes and nose were observed, and the concentrations of AsA did not reach complete depletion in the tissues analysed (Fig. 2). Therefore, none of the rats appeared to have been AsA-deficient.

The plasma α-tocopherol concentration was decreased by DHA ingestion (Table 3), similar to previous studies (Saito *et al.* 1996; Kubo *et al.* 1997, 1998, 2000). The concentrations in the DHA-fed groups were not influenced by the dietary AsA levels; this result was in agreement with the reports of Cadenas *et al.* (1996) and Smith *et al.* (1999), both employing ODS rats under non-scorbutic and normal vitamin E conditions. Therefore, no vitamin E-sparing effect by increased dietary AsA intake was observed in the plasma. Moreover, the plasma lipid peroxide levels were also not suppressed by an increase in AsA intake, although AsA intake of ≥ 600 mg/kg diet suppressed tissue parenchymal cell injuries, possibly in the liver, as seen in the decreased plasma alanine aminotransferase activity (Table 3). Therefore, these results suggested that AsA exerted a limited antioxidant capacity, and also that the highly unsaturated DHA ingestion raised the requirement for AsA as well as vitamin E.

Under an almost vitamin E-deficient state, the liver chemiluminescence intensity was associated with an increase in the liver P-index (Kubo *et al.* 2000). Under the low AsA conditions in the present study, we observed the phenomenon similar to that under an almost vitamin E-deficient state. The intensities in the DHA-fed groups with ≥ 300 mg AsA/kg diet were suppressed below the P-index (Fig. 1(A)). This phenomenon suggests an antioxidant effect of AsA in the liver. An *in vitro* study (Benzie *et al.* 1999) has indicated that antioxidant capacity of AsA was linear up to 1000 μM-ascorbate, as shown by ferric reducing–antioxidant power assay. However, the lipid peroxide levels in the DHA-fed groups were not further suppressed by the AsA intake ≥ 600 mg/kg diet. Hence, the *in vitro* study was different from our present *in vivo* study in which the higher AsA intake did not suppress further the liver lipid peroxide formation. This difference between *in vivo* and *in vitro* studies remains to be explained.

Similarly, the levels of AsA intake would be related to a reductive recycling of vitamin E and/or vitamin E-sparing effect, although there are contradictory reports that have suggested the vitamin E-sparing effect (Igarashi *et al.* 1991; Tanaka *et al.* 1997) and those that have shown evidence against it (Burton *et al.* 1990). In the present study, the α-tocopherol levels in the liver decreased with DHA ingestion, and significantly decreased even further with low AsA intake (Fig. 2(A)). Therefore, the vitamin E-sparing effect may be observable under the low AsA conditions. This phenomenon was in accordance with the reports of *in vivo* (Tanaka *et al.* 1997) and *in vitro* studies (Niki *et al.* 1984; Scarpa *et al.* 1984; Halpner *et al.* 1998*a*,*b*; May *et al.* 1998, 2000; Chepda *et al.* 2001; Mawatari & Murakami, 2001). However, the lipid peroxidation induced consumption of vitamin E was not further suppressed by a large intake of AsA, and thus, these results suggest that AsA exerted a limited antioxidant capacity and also a limited suppression of the effects of vitamin E consumption in the liver. Therefore, factors other than AsA may probably be related to the suppression of lipid peroxide formation to a level below the P-index in the liver.

In the PUFA composition of the kidney (Table 2), the proportions of *n*-6 PUFA were generally higher, and that of DHA was lower, than in the liver. In addition, high retroconversion from DHA to EPA was shown in the kidney, as already recognized (Saito *et al.* 1998). Because of these characteristic profiles of PUFA in the kidney, the increase in the P-index seemed to be modest, even after the highly unsaturated DHA was ingested. Accordingly, the increase in the lipid peroxide levels in the kidney was moderate, and nearly coincided with the increase in the P-index, irrespective of the dietary AsA levels (Fig. 1(B)). We presume, therefore, that dietary AsA in excess of normal levels is not significantly related to suppressing lipid peroxide formation in the kidney.

The proportion of *n*-6 PUFA in the testis was remarkably high even after the highly unsaturated DHA was ingested (Table 2). This phenomenon, namely preferential incorporation and/or synthesis of *n*-6 PUFA in

the testis, may regulate the increases in the P-index and lipid peroxide levels. Thus, the lipid peroxide formation nearly coincided with the P-index, regardless of the dietary AsA levels. Therefore, we thought that AsA was hardly involved in the suppression of testis lipid peroxide formation with highly unsaturated DHA ingestion.

The methods used in the present study to measure lipid peroxidation products were indirect, and so there are limitations in such measurements as compared with the direct methods using HPLC, immunological techniques, GC–MS and so on. However, Cadenas *et al.* (1996) showed that dietary vitamin E decreased lipid peroxidation in the liver and plasma as measured by TBARS, as well as by highly specific phosphatidylcholine and phosphatidylethanolamine hydroperoxides using HPLC–chemiluminescence analyser. Therefore, the TBARS levels measured in the present study may be correlated with phosphatidylcholine and phosphatidylethanolamine hydroperoxide levels. We are now establishing direct methods to measure phosphatidylcholine and phosphatidylethanolamine hydroperoxides using HPLC–chemiluminescence analyser, as well as to measure degradation products of lipid peroxidation, aldehydes, using immunological techniques. Results obtained from such direct measurements are necessary to verify our hypothesis so far proposed.

In conclusion, when highly unsaturated DHA was given to genetically scorbutic ODS rats, dietary AsA levels >300 mg/kg diet did not further suppress lipid peroxide formation and concentration of vitamin E in the liver. In addition, the higher intake of AsA also did not further suppress lipid peroxide formation in the kidney and testis. Therefore, other factors, including glutathione, but not AsA, may be related to the mechanisms that suppress DHA-stimulated liver lipid peroxide formation to the level below that expected from the P-index of the total liver lipids.

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