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To cite this version:

Catherine Calzada, Romain Colas, Nicolas Guillot, Michel Guichardant, Martine Laville, et al.. Subgram daily supplementation with docosahexaenoic acid protects low-density lipoproteins from oxidation in healthy men.. Atherosclerosis, 2010, 208 (2), pp.467-72. 10.1016/j.atherosclerosis.2009.07.049 . inserm-00416728

HAL Id: inserm-00416728

https://inserm.hal.science/inserm-00416728

Submitted on 15 Sep 2009

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Subgram daily supplementation with docosahexaenoic acid protects low-density lipoproteins from oxidation in healthy men

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4224 words (including references), 32 references

2 figures, 3 tables

Abstract

Objective : To determine the effect of supplementation with increasing doses of docosahexaenoic acid (DHA), as the only n-3 polyunsaturated fatty acid (PUFA), on low-density lipoprotein (LDL) redox status and oxidizability.

Methods : Twelve healthy men aged 53 to 65 years ingested consecutive doses of DHA (200, 400, 800 and 1600 mg/day), each dose for two weeks.

Results : The proportions of DHA increased dose-dependently in LDL phospholipids and cholesteryl esters, even after two weeks of supplementation with 200 mg/day DHA. The daily intake of 200, 400 and 800 mg DHA resulted in increased alpha-tocopherol concentrations, decreased MDA concentrations, and a longer lag time for copper-induced LDL oxidation. Supplementation with 1600 mg/day DHA had no effect on the above parameters. In plasma, concentrations of 4-hydroxy-hexenal, specifically derived from the peroxidation of n-3 fatty acids, significanty increased after 800 and 1600 mg DHA, representing 0.01% of plasma n-3 PUFAs, while 4-hydroxy-nonenal concentrations, derived from the peroxidation of n-6 fatty acids, did not change.

Conclusion : Our results clearly show that an intake of 200 to 800 mg/day DHA may have protective and antioxidant effects on LDL and could represent optimal doses for cardiovascular disease prevention in a healthy population.

Keywords : n-3 polyunsaturated fatty acids, antioxidant, peroxidation, low-density lipoproteins.

Introduction

Prospective cohort studies suggest that consumption of fish or fish oils decreases the risk of cardiovascular diseases in Western populations [1]. This is thought to be due to their content in long-chain n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Some recommendations for long-chain n-3 PUFAs intake for cardiovascular health and primary cardiovascular disease prevention have been issued by scientific organizations and government agencies (about 200-500 mg/day depending on countries [2,3]). However, there currently is no specific recommendation for DHA (or EPA) individually.

Dietary supplementation with PUFAs leads to an increased proportion of these fatty acids in plasma lipoproteins, cell and tissue lipids. Taking into account the high susceptibility of the highly unsaturated fatty acids to undergo non-enzymatic peroxidation, enrichment of low-density lipoproteins (LDL) in DHA may increase their susceptibility to oxidation and result in enhanced risk for atherosclerosis [4]. Although many studies have determined the effects of dietary n-3 PUFAs or fish oils on the susceptibility of LDL to oxidation, results were discordant showing either a detrimental, beneficial effect or an absence of effect.

In order to define the most adequate dose of DHA in a healthy population, we performed a dose-response study in healthy middle-aged men and examined the response of blood cells [5] and LDL to DHA dietary intake. Both tocopherol concentrations and lipid peroxide levels of LDL and their oxidizability in response to copper sulphate were investigated.

Methods

Study design

Twelve healthy men volunteers, aged 53 to 65 years, were recruited. Based on a pretrial power analysis, a minimum of six subjects provided 90% power to detect a 25% difference in platelet MDA and phosphatidylethanolamine DHA at $P < 0.05$. The subjects were normolipidemic, nonsmokers, did not use any medication or vitamin supplements and were asked to keep their dietary habits throughout the study. The volunteers received DHA-rich capsules (Pro-Mind, Decola, Belgium). Each capsule contained 200 mg DHA in triglycerides from algal oil, 0.125 mg DL-α-tocopherol and 0.125 mg ascorbyl palmitate. Fatty acid composition of the supplement, expressed as mol %, was 40% DHA, 1.2% 18:2 n-6, 22.2% monounsaturated fatty acids (2% 16:1 n-7 and 20.2% 18:1 n-9) and 36.6% saturated fatty acids (6.6% 12:0, 16% 14:0 and 14% 16:0). The volunteers ingested consecutive doses of 200, 400, 800 and 1600 mg/day DHA, each period lasting for two weeks, followed by an 8-week wash out (WO) period. Compliance to the study (99%) was assessed by the number of capsules returned and the measurement of DHA levels in plasma [5] and LDL. The study was approved by the local ethical committee and written informed consent was obtained from all subjects.

Blood collection

Blood was collected in ACD (acid-citrate-dextrose)-containing Vacutainer tubes after overnight fasting before the start of the intervention (0), at the end of each 2-week period and after 8-week post-supplementation (WO). The blood was immediately centrifuged at 200 g for 17 min at 20°C to get platelet-rich plasma and remaining blood was then centrifuged at 1500 g for 10 min to isolate plasma supernatant.

Low-density lipoprotein isolation

LDL (density 1.019-1.063 $g.ml^{-1}$) were separated from plasma containing 2.7 mM EDTA by potassium bromide stepwise density gradient ultracentrifugation [6] in a TLA 100.3 fixed-angle rotor of a Beckman TL-100 ultracentrifuge. The LDL fraction was dialyzed extensively against phosphate buffered saline (PBS, pH 7.2) containing 1mM EDTA. Last dialysis was performed in PBS without EDTA. The concentration of protein was immediately estimated using the Bradford assay [7].

Vitamin E determinations

Tocopherol concentrations in LDL were determined by reversed-phase high-performance liquid chromatography (RP-HPLC). Briefly, following extraction with hexane in the presence of tocol and δ-tocopherol as internal standards, tocopherol isomers were detected and quantified by fluorimetry (excitation 295 nm, emission 340 nm) [8].

Malondialdehyde measurements

Overall lipid peroxidation was evaluated by quantitation of malondialdehyde (MDA) in LDL particles according to the method of Therasse & Lemonnier [9]. Briefly, TBA-MDA adducts were separated by RP-HPLC and measured by fluorimetry (excitation 515 nm, emission 553 nm).

Fatty acid compositions of LDL phospholipids and cholesteryl esters

Following extraction with ethanol:chloroform (1:2, vol/vol), lipid classes were separated by thin-layer chromatography with hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) into phospholipids, triglycerides and cholesteryl esters [10]. The phospholipid and

cholesteryl ester zones were scrapped off, treated with trifluoride boron/methanol for 90 minutes at 100°C, and extracted twice with isooctane. Fatty acid methyl esters were separated by gas-liquid chromatography, using an HP 6890 gas chromatograph equipped with a SP 2380 capillary column (30 m x 0.25 mm ; Supelco, Bellefonte, PA, USA).

Copper-induced LDL oxidation

Aliquots of freshly dialyzed LDL (50 µg protein/mL) were oxidized in the presence of 1 µM $CuSO_4$ within the day following last dialysis. The oxidation of LDL was monitored by measuring the increased formation of conjugated dienes [11] at 234 nm every minute for 60 minutes and every 5 minutes for 8 hours in a Kontron computer-linked spectrophotometer. The lag time was graphically defined for each oxidation curve as the time from the addition of $CuSO_4$ until the intersection of the tangent with the baseline.

Hydroxy-alkenal concentrations in plasma

4-hydroxy-nonenal (4-HNE) and 4-hydroxy-hexenal (4-HHE) in their free forms were analyzed and quantified by gas chromatography-mass spectrometry (GC-MS) according to the procedure described by Van Kuijk *et al.* [12] modified as follows. Plasma samples, in the presence of deuterated d_3-4-HNE and d_3-4-HHE, were treated with *O*-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride for 30 minutes at room temperature. After acidification, pentafluorobenzyloxime derivatives were extracted with methanol/hexane and the hydroxyl groups were converted into trimethylsilylether with *N,O*-Bis(trimethylsilyl)trifluoroacetamide. The pentafluorobenzyloxime, trimethylsilylether derivatives of 4-HNE (*O*-PFB-TMS-4-HNE) and 4-HHE (*O*-PFB-TMS-4-HHE) were analyzed by negative ion chemical ionization (NICI) GC-MS as previously described [13].

Statistics

Results are expressed as the mean ± S.E.M. for the 12 subjects, except for the WO period (n=11) and plasma hydroxy-alkenal concentrations (n=7). Comparisons between groups were performed using a non parametric Friedman test. Differences between values at two-week supplementation periods or wash-out period and baseline values were determined by using a non parametric Wilcoxon post-test. A Spearman's correlation test was also used to determine correlations between parameters. Statistical significance was established at $P < 0.05$. StatView for Windows (version 5.0.0.0 ; Albacus Corp., Baltimore, MD, USA) was used for statistical analysis.

Results

Fatty acid composition of LDL phospholipids

The proportions of the main fatty acids present in LDL phospholipids are listed in Table 1. Compared to baseline, there was a dose-dependent increase in the proportion of DHA in LDL phospholipids after each supplementation period. This corresponds to an increase of 18% after 200 mg DHA for two weeks, 49% after 400 mg DHA for two additional weeks, 84% after 800 mg DHA and 114% after 1600 mg DHA. DHA proportion returned to baseline after the WO period. Ingestion of 400, 800 and 1600 mg DHA also led to significant increases of EPA proportions in LDL phospholipids by 42, 43 and 65%, respectively. Proportion of EPA returned to baseline at the end of the 8-week WO period. In addition, there was a decrease by 35% in docosapentaenoic acid (22:5 n-3) proportion following supplementation with the highest dose of DHA. Concerning n-6 PUFAs, proportions of linoleic acid (18:2 n-6) significantly decreased after 800 and 1600 mg DHA and proportions of arachidonic acid (20:4 n-6) decreased after ingestion of DHA ranging from 200 to 1600 mg DHA. Linoleic and arachidonic acids proportions returned to baseline values after the WO period.

Fatty acid composition of LDL cholesteryl esters

As shown in Table 2, the ingestion of increasing doses of DHA resulted in a stepwise increase in its proportion in cholesteryl esters : 20%, 57%, 88% and 148%, after 200, 400, 800 and 1600 mg DHA, respectively. Proportion of EPA increased significantly after each DHA supplement until two-fold after the highest dose. DHA and EPA proportions did not completely return to baseline levels after the WO period. No differences were observed for oleic (18:1 n-9) and linoleic acids between each step of supplementation and pre-

supplementation period and the proportion of arachidonic acid decreased by 11 % after 1600 mg DHA.

Tocopherol concentrations in LDL

As shown in Figure 1A, supplementation with 200, 400 and 800 mg DHA resulted in an increase by 27, 20 and 17% of α-tocopherol concentrations, respectively, when compared with baseline values. There was no significant change of α-tocopherol concentrations after 1600 mg DHA and the WO period compared with baseline concentration. γ-tocopherol concentrations also increased after 200 mg/day DHA for 2 weeks compared with baseline concentrations (1.50 ± 0.22 versus 1.16 ± 0.14 nmol/mg protein respectively, $P < 0.05$, n=12) but did not change significantly after 400 mg (1.25 ± 0.14 nmol/mg protein), 800 mg (1.31 ± 0.16 nmol/mg protein), 1600 mg DHA (1.17 ± 0.11 nmol/mg protein) and the WO period (1.18 ± 0.13 nmol/mg protein).

Malondialdehyde concentrations in LDL

When compared to baseline values, MDA concentrations significantly decreased by 37%, 45% and 25% after 200, 400 and 800 mg DHA supplements, respectively (Figure 1B). No change in MDA level was observed after the highest dose (1600 mg DHA). MDA concentration returned to baseline value after the WO period.

Susceptibility of LDL to copper-induced oxidation

The effect of DHA on LDL susceptibility to oxidation *in vitro* was derived from the formation of conjugated dienes upon addition of cupric ions to LDL and the corresponding lag time to oxidation (Figure 2). Supplementation with DHA resulted in significant increases in lag times after doses ranging from 200 to 800 mg DHA, compared to the pre-

supplementation lag time. The maximum increase (34%) was observed after 400 mg DHA. There were no differences in the lag time after the highest dose of DHA and the WO period, compared with baseline.

Concentrations of 4-hydroxy-nonenal and 4-hydroxy-hexenal in plasma

4-HNE is one of the major aldehydes derived from the peroxidation of n-6 fatty acids, essentially linoleic and arachidonic acids, while 4-HHE is the major aldehyde derived from the peroxidation of n-3 fatty acids. As shown in Table 3, plasma 4-HNE concentrations did not vary significantly prior to and post supplementation periods with DHA. Compared to its baseline concentration in plasma, no significant changes of plasma 4-HHE levels were observed after supplementation with 200 and 400 mg DHA, although there was a trend. Supplementation with 800 mg DHA resulted in a 7.2-fold increase of plasma 4-HHE concentration, reaching 10.5-fold after the highest dose of supplementation (1600 mg DHA). Hydroxy-alkenal concentrations returned to baseline ones after the WO period.

Discussion

The salient findings of the present study are that the supplementation of healthy men volunteers with 200, 400 and 800 mg/day DHA during two-week consecutive periods can improve LDL redox status and protect LDL against *in vitro* oxidation.

Concerning LDL redox status, our results show a significant and noticeable increase of α-tocopherol concentration in LDL following supplementation with 200 to 800 mg/day DHA. It is very unlikely that the increased α-tocopherol LDL concentration following DHA supplementation was due to the tiny amount of α-tocopherol contained in each capsule (around 0.5% of the normal daily intake). In addition, supplementation with 1600 mg/day DHA had no effect on tocopherol concentrations. No other study has ever reported that DHA or n-3 PUFAs may lead to increased concentrations of vitamin E in LDL. In plasma, most studies either reported no effect [14] or decreases of plasma vitamin E [15,16] following the supplementation with n-3 PUFAs. Only one study reported increases of plasma α-tocopherol and β-carotene concentrations [17]. We also show for the first time that supplementation with 200 to 800 mg DHA resulted in lower concentrations of MDA in LDL. The concentration of MDA, one of the end-products of lipid peroxidation, was assessed in LDL by the separation and quantification of the TBA-MDA adducts by HPLC. Some previous studies showed an increase of thiobarbituric acid reactive substances (TBARS) levels in plasma after high doses of n-3 PUFAs (from 4 to 15g/day fish oils) [16,18,19] whereas others reported no effect on TBARS formation in plasma and LDL after lower doses of n-3 PUFAs (from 0.3 to 2.4g fish oil/day) [17,20].

Despite numerous studies, there is no consensus on the effects of n-3 PUFAs on LDL oxidation and very few have been performed with DHA alone and none with increasing doses of DHA. Most intervention studies reported an increased *in vitro* oxidation of LDL

following supplementation with fish oils [21-23]. However, some studies showed that fish oil [24], n-3 PUFAs [25] or DHA [26] did not increase the susceptibility of LDL to oxidation and even decreased it [27]. Such discrepancies may be related to differences in the populations studied, in the composition and dosage of the n-3 PUFAs supplement, in the duration of studies and in methodologies. We show that supplementation with 200 to 800 mg/day DHA increased the lag time for copper-induced LDL oxidation, indicating a decreased susceptibility of LDL to oxidation. Since the lag phase is inversely corelated with severity of clinical atherosclerosis [28], the prolongation of the lag phase with DHA may prove beneficial. The length of the lag period is known to be determined by the concentrations of antioxidants, the concentrations of substrates available for oxidation and the amount of endogenous peroxides [11]. In our study, the candidate molecules responsible for an increased lag time could be the lesser amount of lipid peroxides attained after DHA supplementation because the lag time was inversely correlated with MDA concentration ($P < 0.05$, data not shown). Supplementation with 1600 mg DHA had no effect on the lag phase compared to baseline, and corroborates the absence of effect of this dose on LDL redox status. These results strengthen *in vitro* results [29] showing differential effects of DHA on platelet redox status with antioxidant and prooxidant effects at low and high DHA concentrations and no effect at moderate DHA concentrations.

Our results confirm that supplementation with DHA dose-dependently increased DHA proportion in LDL lipids [20]. Noteworthy, daily supplementation with as little as 200 mg DHA for 2 weeks led to significant increases of DHA in phospholipids and cholesteryl esters. EPA proportion significantly increased in both LDL phospholipids and cholesteryl esters after supplementation with 400 to 1600 mg DHA, indicating that some retroconversion of DHA to EPA may have occurred. Based on LDL phospholipid data, the retroconversion of DHA to EPA was estimated to 12% at the end of the 8-week

supplementation period. This estimated value is close to the one estimated in serum phospholipids after 6-week supplementation with an EPA-free preparation of DHA (11.3%) [30]. Supplementation with 1600 mg DHA also resulted in a decreased proportion of docosapentaenoic acid (22:5 n-3) in LDL phospholipids, corroborating other additional results of the present study in plasma and platelets [5]. Such a decrease could reflect the competition of 22:5 n-3 with DHA for esterification at the sn-2 position into phospholipids. In addition, plasma concentrations of triglycerides, total cholesterol, LDL-cholesterol remained unchanged throughout the study [5]. The only significant change was a slight increase of HDL-cholesterol after 1600mg DHA compared with baseline (1.60 ± 0.12 versus 1.43 ± 0.09 nmol/L, n=12, $P < 0.05$). Besides LDL parameters, 4-HNE and 4-HHE were measured in some of plasma samples which allowed us to differentiate between specific oxidation of n-6 PUFAs and that of n-3 PUFAs, respectively. While free 4-HNE concentrations did not vary at all throughout the supplementation periods, the intake of 800 and 1600 mg DHA supplements resulted in significant increased concentrations of 4-HHE but represented as low as 0.01% of plasma n-3 PUFAs amount. Our results indicate that DHA was specifically peroxidized into 4-HHE [31] but to a very small extent. Although further studies are required, we may speculate that the formation of 4-HHE from DHA might be a way to explain the antioxidant effect of DHA which might serve as a primary target for oxidative stress, sparing endogenous antioxidants at an early stage.

The present study has some limitations such as the sample size and therefore our results need to be confirmed on a larger scale.

In conclusion, our results show that supplementation of healthy men with 200-800 mg DHA/day may have protective and antioxidant effects on LDL and are supportive of the global recommendations for long-chain n-3 PUFAs for primary prevention of coronary diseases [32]. It gives new information on the most adequate doses of DHA individually to

confer benefits for the prevention against LDL oxidation and possibly for cardiovascular health.

Acknowledgements

We are grateful to the volunteers for their reliable participation and to the staff from CRNH for their expert blood drawing and some lipid analyses. This work was supported by INSERM, Groupe Lipides et Nutrition (GLN) and Agence Nationale de la Recherche (ANR) 2005 "Cardiovasculaire, Obésité et Diabète". Catherine Calzada is supported by CNRS.

Abbreviations

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 4-HNE, 4-hydroxy-nonenal; 4-HHE, 4-hydroxy-hexenal; LDL, low-density lipoproteins; MDA, malondialdehyde; PUFAs, polyunsaturated fatty acids; WO, wash out.

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Figure legends

Figure 1. α-Tocopherol (A) and malondialdehyde (B) concentrations in LDL.

Results are means ± S.E.M. for the 12 volunteers (except for the WO, n=11) at baseline (0), after each 2-week period of supplementation (200, 400, 800 and 1600 mg/day DHA) and at the end of the WO period. $P < 0.05$ compared with baseline.

Figure 2. Lag time for conjugated diene formation in copper-oxidized LDL.

LDL (50 µg protein /mL) were oxidized in the presence of 1 µM $CuSO_4$ and the lag time was calculated from the time courses of conjugated diene formation. Results are means ± S.E.M. for the 12 volunteers (except for the WO, n=11). $P < 0.05$ compared with baseline.

Table 1. Fatty acid composition of LDL phospholipids.

	DHA (mg/day)					
Fatty acid (mol %)	0	200	400	800	1600	0 (WO)
Palmitic (16:0)	32.4 ± 0.4	32.4 ± 0.5	32.3 ± 0.7	33.4 ± 0.7	33.5 ± 0.5	33.6 ± 0.7
Stearic (18:0)	15.5 ± 0.6	14.9 ± 0.3	15.1 ± 0.3	14.9 ± 0.3	15.0 ± 0.4	14.8 ± 0.5
Oleic (18:1 n-9)	10.0 ± 0.5	10.0 ± 0.5	9.3 ± 0.5	9.8 ± 0.5	9.6 ± 0.6	10.4 ± 0.6
Linoleic (18:2 n-6)	21.8 ± 0.7	21.7 ± 0.6	21.4 ± 0.8	20.2 ± 0.7*	19.8 ± 0.9*	20.5 ± 0.7
Dihomo-γ-linolenic (20:3 n-6)	2.8 ± 0.3	2.5 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	1.9 ± 0.1	2.5 ± 0.1
Arachidonic (20:4 n-6)	7.8 ± 0.4	7.3 ± 0.4*	7.3 ± 0.5*	6.7 ± 0.4*	6.1 ± 0.3*	7.2 ± 0.4
Eicosapentaenoic (20:5 n-3)	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.2*	1.0 ± 0.1*	1.2 ± 0.2*	1.0 ± 0.2
Docosanoic (22:0)	1.3 ± 0.3	1.6 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.1	1.6 ± 0.2
Docosapentaenoic (22:5 n-3)	0.7 ± 0.1	0.7 ± 0.04	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.02*	0.8 ± 0.1
Docosahexaenoic (22:6 n-3)	3.0 ± 0.2	3.5 ± 0.2*	4.4 ± 0.3*	5.4 ± 0.3*	6.3 ± 0.3*	3.2 ± 0.3
Nervonic (24:1 n-9)	2.2 ± 0.3	2.4 ± 0.2	2.1 ± 0.3	2.0 ± 0.2	2.2 ± 0.1	2.2 ± 0.2

Results, expressed as mol % of main fatty acids, are means ± S.E.M. for the 12 volunteers (except for the WO, n=11) before (0), after each 2-week period of supplementation (200, 400, 800 and 1600 mg/day DHA) and at the end of the WO period.

* : $P < 0.05$ compared with baseline (0).

Table 2. Fatty acid composition of LDL cholesteryl esters.

	DHA (mg/day)					
Fatty acid (mol %)	0	200	400	800	1600	0 (WO)
Palmitic (16:0)	12.6 ± 0.3	12.4 ± 0.3	12.3 ± 0.5	12.9 ± 0.3	12.5 ± 0.2	12.8 ± 0.6
Palmitoleic (16:1 n-7)	3.6 ± 0.4	3.4 ± 0.5	2.7 ± 0.2	3.6 ± 0.5	3.3 ± 0.6	4.1 ± 0.7
Stearic (18:0)	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.03	1.0 ± 0.1
Oleic (18:1 n-9)	20.5 ± 0.6	20.6 ± 0.7	19.8 ± 0.5	20.4 ± 0.6	20.4 ± 0.9	21.3 ± 1.0
Linoleic (18:2 n-6)	50.8 ± 1.3	51.4 ± 1. 5	52. 8 ± 1.4	50.7 ± 1.3	51.5 ± 1.7	49.2 ± 2.1
Gamma-linolenic (18:3 n-6)	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.2
Arachidonic (20:4 n-6)	6.0 ± 0.4	5.9 ± 0.3	5.9 ± 0.5	5.6 ± 0.4	5.3 ± 0.3*	5.9 ± 0.3
Eicosapentaenoic (20:5 n-3)	0.7 ± 0.1	0.8 ± 0.1*	1.1 ± 0.2*	1.1 ± 0.1*	1.4 ± 0.2*	1.1 ± 0.1*
Docosahexaenoic (22:6 n-3)	0.5 ± 0.1	0.6 ± 0.1*	0.8 ± 0.1*	1.0 ± 0.1*	1.3 ± 0.1*	0.6 ± 0.1*

Results, expressed as mol % of main fatty acids, are means ± S.E.M. for the 12 volunteers (except for the WO, n=11) before (0), after each 2-week period of supplementation (200, 400, 800 and 1600 mg/day DHA) and at the end of the WO period.

* : $P < 0.05$ compared with baseline (0).

Table 3. Plasma 4-hydroxy-nonenal (4-HNE) and 4-hydroxy-hexenal (4-HHE) concentrations.

DHA (mg/day)	4–HNE (ng/ml)	4–HHE (ng/ml)
0	1.11 ± 0.34	1.01 ± 0.44
200	1.00 ± 0.31	1.73 ± 0.60
400	1.04 ± 0.32	3.25 ± 1.69
800	1.24 ± 0.48	7.25 ± 2.41 *
1600	0.91 ± 0.36	10.57 ± 3.68 *
0 (WO)	0.73 ± 0.27	0.56 ± 0.12

Results, expressed in ng/ml plasma, are means ± S.E.M. for 7 volunteers (except for the WO, n=6) before (0), after each 2-week period of supplementation (200, 400, 800 and 1600 mg/day DHA) and at the end of the WO period.

* : $P < 0.05$ versus baseline (0).