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The Association of Increasing Dietary Concentrations of Fish Oil with Hepatotoxic Effects and a Higher Degree of Aorta Atherosclerosis in the *ad lib*.-fed Rabbit

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Abstract—The long-term effects of consumption of marine long-chain n-3 polyunsaturated fatty acids (PUFA) on atherosclerosis in the rabbit were examined. Female Dutch rabbits were fed purified diets, containing 40 energy% total fat, for a period of 2.5 years. To study the dose–response relationship between fish oil intake and atherosclerosis, four diets were formulated with fish oil levels being 0, 1, 10 and 20 energy%. A fifth and sixth group were fed an α-linolenic acid-(C18:3, n-3) and linoleic acid-(C18:2, n-6) rich diet, respectively. Every 6 weeks, blood samples were taken for determination of clinical chemical parameters, triacylglycerol and total cholesterol levels. Feeding 10 and 20 energy% fish oil containing diets, resulted in an increase of liver enzymes (AST, ALT and ALP). Histological evaluation of the liver also revealed adverse effects of fish oil containing diets. Triacylglycerol blood levels were similar in all groups, and remained constant throughout the study. Total cholesterol levels in blood was significantly lower in the animals fed a linoleic acid-rich diet, as compared with the other five groups. An n-3 long-chain PUFA concentration dependent increase in aorta plaque surface area was observed in the fish oil groups. A significant positive relationship was found between the group mean score for severity of liver pathology and the aorta plaque surface area. These results indicate that the long-chain n-3 polyunsaturated fatty acids in fish oil may be hepatotoxic to the herbivorous rabbit, which may interfere with the outcome of atherosclerosis studies. This finding necessitates the exclusion of liver pathology in experimental studies on atherosclerosis in animal models.

Abbreviations: ACAT, =acyltransferase; DHA=docosahexaenoic acid; EPA=eicosapentaenoic acid; γ-GT=gamma-glutamyl transferase; PUFA=polyunsaturated fatty acid.

Keywords: rabbit; diet; fish oil; atherosclerosis; liver pathology.

INTRODUCTION

The low incidence of cardiovascular disease in populations consuming high amounts of marine animals may be associated with a high intake of marine n-3 polyunsaturated fatty acids (n-3 PUFA: mainly eicosapentaenoic acid (EPA = C20:5, n-3) and docosahexaenoic acid (DHA = C22:6, n-3). It is suggested that these marine n-3 PUFA have antithrombotic and blood lipid level lowering effects, which may contribute to a lower incidence of atherosclerotic disease (Hornstra *et al*., 1983). Results from rabbit studies from the literature (Adelstein *et al*., 1992; Campos *et al*., 1989; Thiery and Seidel, 1987; Zhu *et al*., 1988) revealed variable results of dietary fish oil on atherosclerosis. Fish oil enhanced atherosclerosis in the cholesterol-fed rabbit (Thiery and Seidel, 1987). In a previous study in our laboratory, dietary fish oil induced more atherosclerotic lesions in the rabbit fed a diet containing no cholesterol (Verschuren *et al*., 1990). The choice for a diet without cholesterol was based on the argument that cholesterol-induced atherosclerosis in

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Table 1. Composition of the experimental fats (energy %)

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en% SF
Fat type						
FO[a]	20	10	1	0	0	0
CB[b]	10.7	15	20.1	21.2	21.8	0
HCN[c]	7	7	7	7	7	0
SF[d]	2.2	1.5	0.9	0.8	0	40
Trisun[e]	0.1	6.5	11	11	4.2	0
LN[f]	0	0	0	0	7	0
Total	40	40	40	40	40	40

[a]FO = fish oil, composed of a refined and cholesterol-free cocktail of fish oils (Unimills BV, Zwijndrecht, The Netherlands); butylated hydroxytoluene was added to the fish oil to ensure stability (300 mg oil/kg).
[b]CB = cocoa butter (Chempri BV, Raamsdonksveer, The Netherlands).
[c]HCN = hydrogenated coconut oil (Chempri BV, Raamsdonksveer, The Netherlands).
[d]SF = sunflowerseed oil (Union NV, Antwerp, Belgium).
[e]Trisun = low linoleic acid/high oleic acid sunflowerseed oil (SVO Enterprises, Eastlake, OH, USA).
[f]LN = linseed oil (Chempri BV, Raamsdonksveer, The Netherlands).

the rabbit may not be the proper model for the human process, because of the poor cholesterol handling by the rabbit. Pathological lesions were observed in the liver of these fish-oil fed rabbits (Verschuren *et al.*, 1990). It was hypothesized that fish-oil feeding may cause liver damage, resulting in hyperlipaemia and subsequent atherosclerosis. In the following study a dose–response curve of dietary fish oil *v.* atherosclerosis in rabbits—fed a diet without cholesterol—was studied. Besides atherosclerosis, the influence on blood lipid levels and haematological parameters and the safety of long-term ingestion of fish oil were evaluated. Those parameters that were considered to be related to cholesterol metabolism, and could indicate possible toxic effects of dietary fish oil, were chosen to be measured. The intake of marine n-3 PUFA was compared with the intake of linoleic acid (C18:2, n-6) and linolenic acid (C18:3, n-3). Long-term intake of a diet high in linoleic acid is expected to prevent atherosclerosis in rabbits, and was therefore used as the negative control group [40 energy(en)% sunflowerseed oil group]. The intake of linolenic acid was studied to evaluate whether the intake of n-3 PUFA from 'vegetable' origin (7 en% linseed oil group) has similar effects compared with those from n-3 marine PUFA. The fish oil diets contained 0 to 20 en% fish oil (approx. 0–4 en% n-3 PUFA). The level of 1 en% fish oil in this study is equivalent to the level recommended for humans by the producer of MaxEPA fish oil capsules (2.5 g maxEPA oil/person/day). The 10 en% fish oil level is a level frequently seen in clinical studies. The 20 en% level was chosen as the possible adverse dose, as it was twice the concentration used in clinical studies.

MATERIALS AND METHODS

Animals and housing conditions

156 Female Dutch rabbits (Ranch Rabbits, Crawley Down, West Sussex, UK), 6–9 wk of age, were housed individually in stainless-steel wire-bottomed cages ($58 \times 44 \times 37$ cm). Animals were housed under conventional conditions and preventive hygienic measures were taken: personnel changed shoes and clothes and washed their hands each time before entering the animal room. Room temperature was $18 \pm 1^{\circ}$C, relative humidity in the room was 40–70% and the lights were on from 7 am–7 pm. Tap water was available *ad lib*.

Diets

The basic composition of the purified diets (for definition see National Research Council, 1995) was similar to that used previously (Verschuren *et al.*, 1990) with 0.04 g/1000 kcal sulfaquinoxalin. All diets contained the same total concentration of *dl*-α-tocopherol (200 mg/kg diet): the vitamin mix delivered 100 mg *dl*-α-tocopheryl acetate and the remaining vitamin E was obtained from the fats and oils and/or were supplemented with *dl*-α-tocopherol (Merck, Amsterdam, Holland). The composition of the experimental fats is given in Table 1 and 2. Diet 2 ("10 en% FO") contained 10 en% fish oil. In order to study possible adverse effects, this amount was doubled in the first group: 20 en% fish oil ("20 en% FO"). The third group ("1 en% FO") contained 1 en% fish oil and the fourth group ("0 en% FO") was similar to group 3 and contained no fish oil. Diet 5 ("7 en% LN") contained linseed oil in order to compare the effects of marine n-3 PUFA with α-linolenic acid (C18:3, n-3), the main 'vegetable' n-3 PUFA. The sixth dietary group ("40 en% SF") served as the negative control group, since this diet was high in linoleic acid and low in saturated fatty acids and is known to inhibit atherosclerosis. Table 3 gives the peroxide values of freshly prepared oil mixtures and diets, as well as of diets that had been kept in the animal room for a period of 3 days. The peroxide values of the diets are higher than those of the oil mixtures. The peroxide values of the diets did not show an increase after keeping them at room temperature.

Table 2. Fatty acid composition of experimental fats (%)

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
Fatty acids						
< 14:0	10.2	10.2	10.5	10.5	10.5	0.0
14:0	7.6	5.6	3.8	3.6	3.6	0.1
16:0	18.2	16.7	16.1	16.3	16.8	7.2
16:1	4.3	2.1	0.2	0.0	0.0	0.0
18:0	12.0	15.0	18.6	19.3	19.7	4.1
18:1	16.0	29.2	39.4	39.9	28.9	16.2
18:2	5.8	5.8	5.8	5.7	5.9	70.9
18:3	0.6	0.3	0.1	0.0	10.0	0.3
20:1	0.7	0.4	0.1	0.1	0.1	0.2
20:5	7.4	3.7	0.4	0.0	0.0	0.0
22:1	0.9	0.5	0.0	0.0	0.0	0.0
22:6	5.3	2.7	0.3	0.0	0.0	0.0
Total[a]	89.0	92.3	95.1	95.4	95.4	99.0
SFA[b]	48.0	47.7	48.9	49.6	50.6	11.4
MUFA[b]	21.9	32.2	39.8	40.0	29.0	16.4
PUFA[b]	18.9	12.5	6.5	5.7	15.9	71.2
n-6/	5.6/	5.8/	5.8/	5.7/	5.9/	70.9/
n-3[b]	13.3	6.7	0.7	0.0	10.0	0.3

[a]The differences between the given percentages and the 100% composition was due to minor fatty acids in the oils used (mainly in the fish oils). They were not incorporated in the table.
[b]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids: n-6/n-3 = ratio of n-6 to n-3 PUFA.

Feeding regimen

The design of the feeding period was as described before by Verschuren *et al.* (1990), in which a gradual replacement of the commercial chow by purified diet was applied. After an acclimatization period of 2 wk, during which the animals received a commercial chow *ad lib.*, the animals were distributed over six dietary groups on the basis of body weight (day 0 of the experimental period), with 24 animals per group. Only the 40 en% SF group contained 36 animals. After a total of 14 wk in the experimental period (as described by Verschuren *et al.*, 1990), the animals received 100% purified diet. The purified diets were pelleted and prepared fresh once a week and stored at −20°C until use. The animals received fresh diets three times a week; however, the fish oil-containing diets were provided fresh every day. Food was given *ad lib.*

Observations

Food intake was measured during a 3-day period when the rabbits consumed 50% commercial chow and 50% purified diet, and again when they were eating 100% purified diet. Body weight was recorded twice a week during the first 5 months of the study and thereafter once a week until termination. Clinical observation was performed once a week. After 17, 53, 90 and 125 wk (non-fasting) blood was collected from the lateral ear vein for the determination of serum lipid levels and enzyme activities of all animals. After 55, 93 and 129 wk (non-fasting) blood was taken from a random subset of 12 animals/group for the determination of serum lipoprotein levels. At 129 wk, collected blood was also used for measurement of haematological parameters. After 129 wk, rabbits were fasted overnight (food was withdrawn at 3 pm). In the morning they were anaesthetized with Hypnorm intramuscularly (0.1 ml/kg; Janssen Pharmaceutica, Beers, Belgium) followed by CO_2/O_2 inhalation and exsanguination via aorta cannulation. The sequence of blood collections and autopsies were always performed at random for groups. All carcasses were subjected to a detailed necropsy. Liver weights were recorded. Samples of liver (median lobe) and adipose tissue (abdominal, near the ovary) were frozen in liquid N_2 for vitamin E analysis. Pieces of liver (median lobe) and heart (five transverse sections from heart base to apex) were fixed in 10% buffered formalin for histological examination. The aorta was split longitudinally from the heart base to the bifurcation and fixed in 10% buffered formalin in a stretched out position. After fixation, the aorta was

Table 3. Peroxide values in the oil mixtures and diets (mmol/2 kg)

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
oil 1[a]	1.9	0.5	0.1	0.1	0.6	0.7
oil 2[a]	2.0	1.6	0.5	0.5	0.9	0.4
oil 3[a]	2.8	1.6	0.7	0.7	1.3	0.8
dietf[b]	22.8	19.6	19.5	18.6	20.5	25.0
dieto[c]	18.5	16.7	13.7	13.2	18.3	23.4

[a]Freshly prepared oil mixtures were analysed for the peroxide value at three different time points (1, 2 and 3; 5–7 month period between sampling).
[b]"dietf(resh)" refers to analysis of freshly prepared diets.
[c]"dieto(ld)": diets were prepared, stored at −20°C for 2 days, thawed and kept at room temperature for a period of 3 days before analyses.

Table 4. Liver weights and liver enzymes

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
Liver weight						
(g)	43.4 ± 1.7^{a}	40.7 ± 1.5^{ac}	33.8 ± 1.2^{b}	36.8 ± 1.4^{bc}	32.6 ± 1.3^{b}	35.4 ± 1.0^{b}
(mg/g BW[1])	19.0 ± 1.1^{a}	17.2 ± 0.4^{a}	14.7 ± 0.4^{b}	15.0 ± 0.3^{b}	14.6 ± 0.6^{b}	14.1 ± 0.3^{b}
AST[2] (mU/litre)						
wk 17	71.0 ± 9.8^{a}	58.0 ± 4.5^{a}	23.0 ± 1.5^{b}	23.0 ± 1.2^{b}	24.0 ± 0.8^{b}	21.0 ± 0.7^{b}
wk 53	72.0 ± 7.7^{a}	58.0 ± 4.7^{a}	23.0 ± 1.2^{b}	23.0 ± 1.8^{b}	25.0 ± 1.5^{b}	23.0 ± 1.2^{b}
wk 90	38.0 ± 3.2^{a}	51.0 ± 5.7^{a}	18.0 ± 1.7^{b}	18.0 ± 1.5^{b}	21.0 ± 1.5^{b}	17.0 ± 0.9^{b}
wk 125	37.0 ± 3.1^{a}	38.0 ± 2.9^{a}	17.0 ± 1.0^{b}	18.0 ± 0.8^{b}	20.0 ± 1.3^{b}	18.0 ± 0.9^{b}
ALT[3] (mU/litre)						
wk 17	137.0 ± 17.0^{a}	102.0 ± 11.0^{a}	24.0 ± 1.5^{b}	24.0 ± 1.2^{b}	27.0 ± 1.9^{b}	20.0 ± 0.9^{b}
wk 53	136.0 ± 21.0^{a}	91.0 ± 5.9^{a}	23.0 ± 1.3^{b}	24.0 ± 1.6^{b}	31.0 ± 2.5^{b}	23.0 ± 1.5^{b}
wk 90	105.0 ± 12.0^{a}	107.0 ± 13.0^{a}	23.0 ± 1.1^{b}	22.0 ± 1.5^{b}	28.0 ± 1.9^{b}	22.0 ± 1.3^{b}
wk 125	45.0 ± 6.9^{a}	43.0 ± 4.4^{a}	10.0 ± 0.8^{b}	9.6 ± 0.5^{b}	15.0 ± 1.1^{b}	11.0 ± 0.8^{b}
γ-GT[4] (mU/litre)						
wk 17	5.8 ± 0.5^{a}	5.4 ± 0.4^{a}	3.0 ± 0.2^{b}	2.8 ± 0.2^{b}	3.2 ± 0.1^{b}	2.8 ± 0.1^{b}
wk 53	7.4 ± 0.9^{a}	5.7 ± 0.4^{a}	2.9 ± 0.3^{b}	3.0 ± 0.2^{b}	3.4 ± 0.2^{b}	2.9 ± 0.2^{b}
wk 90	7.0 ± 0.6^{a}	6.5 ± 0.4^{a}	3.4 ± 0.3^{b}	2.6 ± 0.2^{bc}	2.9 ± 0.3^{bc}	2.3 ± 0.2^{c}
wk 125	7.7 ± 0.7^{a}	6.9 ± 0.5^{a}	4.0 ± 0.3^{b}	3.0 ± 0.2^{b}	3.3 ± 0.3^{b}	3.0 ± 0.1^{b}
Alkaline phosphatase (mU/litre)						
wk 17	134.0 ± 15.0^{a}	145.0 ± 11.0^{a}	62.0 ± 3.1^{bc}	66.0 ± 3.6^{bc}	58.0 ± 3.0^{c}	75.0 ± 3.1^{b}
wk 53	91.0 ± 8.2^{a}	85.0 ± 9.3^{a}	36.0 ± 1.5^{bc}	43.0 ± 2.7^{bc}	35.0 ± 1.2^{c}	48.0 ± 3.9^{b}
wk 90	69.0 ± 7.4^{a}	82.0 ± 8.5^{a}	29.0 ± 1.7^{bc}	33.0 ± 2.1^{bc}	26.0 ± 1.4^{c}	38.0 ± 2.6^{b}
wk 125	73.0 ± 6.1^{a}	85.0 ± 5.6^{a}	33.0 ± 1.5^{bc}	34.0 ± 1.8^{bc}	28.0 ± 1.2^{c}	41.0 ± 3.5^{b}

Values are means $\pm$ SEM; those that do not carry a common superscript are significantly different ($P < 0.05$).
The number of animals per group and time point varied from 15 to 36, depending on the size of the group, the number of animals remaining within the group and whether the blood sample was "valid" (without haemolysis, etc.).
[1]BW = body weight.
[2]AST = aspartate aminotransferase.
[3]ALT = alanine aminotransferase.
[4]γ-GT = γ-glutamyl transferase.

stained with Sudan III/IV. The plaque surface area relative to the total surface area of the aorta was measured morphometrically using an IBAS automatic analyser. After morphometric measurements, histological slides of the aorta were prepared to evaluate the type of aortic lesions. Formalin-fixed tissues were processed to paraplast sections and stained with Harris' Haematoxylin-azophloxine and Masson's Trichrome Elastica stain. Unstained liver sections were studied with a fluorescence microscope. All sections were read under code to avoid reader bias.

In each group a number of animals died or had to be killed before the end of the experiment. All were autopsied: in about 50% of the cases *Pasteurella multocida* abcesses were the cause of death and in the other 50% the cause of death varied.

Analyses

Enzyme activities (alkaline phosphatase, γ-GT, AST, ALT) and total cholesterol, free cholesterol and triacylglycerols, were measured on the programmable analyser PA 800 (Vitatron, Dieren, Holland). The separation of lipoproteins was performed by ultracentrifugation as described by Redgrave *et al.*, (1975). Vitamin E in serum and liver was analysed as described (Verschuren *et al.*, 1990). Haematological parameters measured were: white and red blood cell count, haematocrit, haemoglobin, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, platelets, lymphocyte count and platelet crit (percentage volume of platelets).

Statistics

Data were analysed by means of one-way analysis of variance. Differences between dietary groups were established using Tukey's multiple comparison test. Differences with $P < 0.05$ were considered significant. The relation between dietary fish oil content in groups 1–4 and aorta plaque surface area or serum α-tocopherol was established by linear regression analysis. Histopathological findings were analysed by using the Fisher's exact probability test, comparing each group separately with the negative control group (40 en% SF group). Correlation between liver pathology scores and plaque area was tested with Pearson's correlation coefficient.

RESULTS

Food intake, body and liver weights

The food intake was significantly different during the 3-day period, when the rabbits consumed 50% commercial chow and 50% purified diet. Food intake in the 20 en% fish oil group (61 ± 5 g/day) was significantly lower than that in the 1 en% fish oil group (80 ± 4 g/day) and 40 en% sunflowerseed oil group (81 ± 3 g/day). No significant differences occurred with and among the other groups (10 en% fish oil: 70 ± 4; 0 en% fish oil: 74 ± 4; 7 en% linseed oil: 72 ± 3 g/day). When the rabbits consumed

Table 5. Blood lipids

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
Total cholesterol (mmol/litre)						
wk 17	3.1 ± 0.2^{ab}	3.6 ± 0.2^{b}	2.7 ± 0.1^{ac}	2.5 ± 0.1^{ac}	2.3 ± 0.2^{c}	1.5 ± 0.1^{d}
wk 53	3.4 ± 0.4^{ab}	3.5 ± 0.2^{b}	2.6 ± 0.1^{ac}	2.3 ± 0.1^{c}	2.4 ± 0.1^{c}	1.4 ± 0.1^{d}
wk 90	2.5 ± 0.2^{a}	2.9 ± 0.2^{a}	2.5 ± 0.1^{a}	2.3 ± 0.1^{a}	2.3 ± 0.1^{a}	1.3 ± 0.1^{b}
wk 125	2.3 ± 0.1^{a}	2.7 ± 0.1^{ab}	3.0 ± 0.2^{b}	2.4 ± 0.1^{ab}	2.3 ± 0.1^{ab}	1.6 ± 0.1^{c}
LDL cholesterol (mmol/litre)						
wk 55	1.3 ± 0.3	1.0 ± 0.2*	0.7 ± 0.1*	0.6 ± 0.1*	0.7 ± 0.1	0.5 ± 0.1
wk 93	0.8 ± 0.2	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.6 ± 0.1
wk 129	0.6 ± 0.1	0.7 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
HDL cholesterol (mmol/litre)						
wk 55	1.3 ± 0.1^{a}	$1.3 \pm 0.1^{*a}$	1.2 ± 0.1^{a}	1.0 ± 0.1^{a}	0.9 ± 0.0^{a}	0.5 ± 0.0^{b}
wk 93	$1.3 \pm 0.1^{*abc}$	$1.6 \pm 0.1^{*a}$	1.4 ± 0.1^{ab}	1.2 ± 0.1^{bc}	1.1 ± 0.1^{c}	0.6 ± 0.1^{d}
wk 129	1.3 ± 0.1^{ab}	$1.5 \pm 0.1^{*a}$	1.5 ± 0.1^{a}	1.1 ± 0.1^{b}	1.1 ± 0.1^{b}	0.5 ± 0.1^{c}
VLDL cholesterol (mmol/litre)						
wk 55	0.15 ± 0.05	0.08 ± 0.02*	0.11 ± 0.04	0.15 ± 0.03	0.08 ± 0.01	0.12 ± 0.03
wk 93	0.07 ± 0.02^{a}	0.06 ± 0.01^{a}	0.09 ± 0.01^{ab}	0.15 ± 0.03^{b}	0.11 ± 0.02^{b}	0.09 ± 0.01^{ab}
wk 129	0.09 ± 0.03^{a}	0.05 ± 0.01^{a}	0.11 ± 0.02^{ab}	0.20 ± 0.05^{b}	0.13 ± 0.03^{ab}	0.10 ± 0.02^{ab}
Free cholesterol (mmol/litre)						
wk 17	1.2 ± 0.1^{ab}	1.4 ± 0.1^{b}	1.0 ± 0.0^{ac}	0.9 ± 0.0^{ac}	0.9 ± 0.1^{c}	0.5 ± 0.0^{d}
wk 53	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}	0.4 ± 0.0^{c}
wk 90	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.4 ± 0.0^{b}
wk 125	0.8 ± 0.0^{a}	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.4 ± 0.0^{b}
Triacylglycerols (mmol/litre)						
wk 17	0.8 ± 0.1^{a}	0.7 ± 0.0^{ab}	0.6 ± 0.0^{ab}	0.7 ± 0.0^{ab}	0.7 ± 0.0^{ab}	0.6 ± 0.0^{b}
wk 53	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}
wk 90	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}
wk 125	0.5 ± 0.0^{a}	0.6 ± 0.0^{ab}	0.6 ± 0.0^{ab}	0.8 ± 0.0^{b}	0.7 ± 0.0^{ab}	0.6 ± 0.0^{a}

Values are mean $\pm$ SEM; those that do not carry a common superscript are significantly different ($P < 0.05$). The number of animals per group and time point varied from 19 to 36, depending on the size of the group, the number of animals remaining within the group and whether the blood sample was "valid" (without haemolysis, etc.); the number of animals for lipoprotein determinations was 12 (* or 11, because outliers were excluded) animals per group.

100% purified diet, no significant differences in food intake were measured (20 en% fish oil: 38 ± 3; 10 en% fish oil: 34 ± 3; 1 en% fish oil: 32 ± 2; 0 en% fish oil: 35 ± 2; 7 en% linseed oil: 34 ± 2; 40 en% sunflowerseed oil: 33 ± 2 g/day). Between the groups no significant differences were found in body weight at the end of the experiment (week 129, results not shown). Absolute (g) and relative (mg/g body weight) liver weights of the animals fed the 20 en% fish oil diets were significantly higher than those of all other groups, except for the 10 en% fish oil group. The absolute liver weights (g) of the rabbits fed the 10 en% fish oil diet, were significantly different from those of the 1 en% fish oil, 7 en% linseed oil and 40 en% sunflower seed oil groups. The relative liver weights (mg/g body weight) of rabbits fed the 10 en% fish oil diet, were significantly different from all other groups, except for the 20 en% fish oil group (Table 4).

Blood enzyme activities

A significant elevation of the liver enzymes AST, ALT, γ-GT and AP was found in the 20 and 10 en% fish oil dietary groups during the entire feeding period (Table 4). There was a significant decrease of AST values from wk 53 to wk 90 and for ALT concentrations from wk 90 to wk 125 in the 20 en% fish oil group.

Blood lipids

The rabbits fed the 40 en% SF diet had significantly lower total and free cholesterol blood levels compared with all other dietary groups at all time points measured (Table 5). After 17 and 53 wk, blood total cholesterol levels of the rabbits fed the 10 en% fish oil diets were significantly higher than those of all other dietary groups, except for the 20 en% fish oil diet. The animals fed the 10 and 20 en% fish oil diets had significantly higher serum free cholesterol levels after 53 wk.

After 17 wk, serum triacylglycerol levels were significantly higher in the animals fed the 20 en% fish oil diet as compared with those fed the 40 en% SF diet (Table 5). At the time points of 53 and 90 wk these differences had disappeared. Low-density lipoprotein (LDL)-cholesterol concentrations did not differ between the groups at any time point (Table 5). The rabbits that had been fed the 40 en% SF diet had significantly lower high-density lipoprotein (HDL)-cholesterol levels than all other groups at all time points measured (Table 5). At wk 55 no significant differences in very-low-density lipoprotein (VLDL) levels between the groups were observed (Table 5). VLDL levels in the 20 and 10 en% fish oil groups were significantly lower than in the 0 en% fish oil and 7 en% linseed oil group after 93 wk. After 129 wk, VLDL levels in the 20 and 10 en% fish oil groups were significantly lower than those in the 0 en% fish oil group.

Table 6. Haematological parameters at the end of the experiment

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
WBC (g/litre)	5.2 ± 0.3	6.2 ± 0.5	5.7 ± 0.4	4.9 ± 0.3	5.6 ± 0.5	5.9 ± 0.3
RBC (T/litre)	5.5 ± 0.1[a]	5.8 ± 0.1[ab]	5.8 ± 0.1[ab]	5.8 ± 0.1[ab]	5.8 ± 0.1[ab]	5.9 ± 0.1[b]
Hb (mmol/litre)	7.7 ± 0.1	8.2 ± 0.1	8.2 ± 0.2	8.2 ± 0.2	8.1 ± 0.2	8.2 ± 0.1
Ht (litre/litre)	0.39 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	0.41 ± 0.01
MCV (fl)	71.1 ± 0.62	69.6 ± 0.61	70.4 ± 0.70	69.5 ± 0.45	68.8 ± 0.69	69.4 ± 0.51
MCH (fmol)	1.42 ± 0.02	1.40 ± 0.02	1.41 ± 0.021	1.40 ± 0.02	1.39 ± 0.02	1.39 ± 0.01
MCHC (mmol/litre)	19.8 ± 0.3	20.1 ± 0.2	20.1 ± 0.2	20.2 ± 0.2	20.2 ± 0.2	20.1 ± 0.2
LC (g/litre)	2.0 ± 0.2	2.4 ± 0.2	2.5 ± 0.3	2.2 ± 0.3	2.7 ± 0.5	2.5 ± 0.2
PCT (ml/litre)	0.97 ± 0.05[a]	0.87 ± 0.05[a]	1.36 ± 0.07[b]	1.59 ± 0.09[b]	1.57 ± 0.10[b]	1.51 ± 0.05[b]
Plt (g/litre)	245 ± 14[a]	240 ± 14[a]	339 ± 14[b]	366 ± 20[b]	386 ± 21[b]	339 ± 11[b]

Hb = haemoglobin concentration Ht = haematocrit LC = lymphocyte count MCH = mean cell haemoglobin MCHC = mean cell haemoglobin concentration MCV = mean cell volume PCT = platelet crit Plt = platelets RBC = red blood cells WBC = white blood cells

Values are mean ± SEM; those that do not carry a common superscript are significantly different ($P < 0.05$). The number of animals per group and time point varied from 13 to 31, depending on the size of the group, the number of animals remaining within the group and whether the blood sample was "valid" (without haemolysis, etc.).

Haematology

At the termination of the experiment, several haematological parameters were measured. No effects were found on white blood cell count, haematocrit, haemoglobin, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration and leucocyte count (Table 6). The erythrocyte cell count in the 20 en% fish oil diet fed animals was significantly lower than in the 40 en% SF diet fed rabbits. Platelet count and platelet crit were significantly lower in the groups fed the 20 and 10 en% fish oil diets as compared with the other groups.

Vitamin E levels

α-Tocopherol levels in the livers of the rabbits that had been fed the 20 en% fish oil diets, were significantly lower than that in all other groups (Table 7). Serum α-tocopherol levels were lowest in the animals fed the 20 en% fish oil diet and intermediate in the animals fed the 10 en% fish oil and 40 en% SF diets. As vitamin E is transported in the serum by cholesterol-containing lipoproteins, a correction was made for total cholesterol levels, expressed as a ratio: (α-tocopherol concentration/total cholesterol level). This ratio was the lowest in the animals fed the 20 en% fish oil diet and intermediate in the rabbits fed the 10 en% fish oil diet. There was a negative linear relation ($P < 0.01$) between dietary fish oil concentration and serum (α-tocopherol concentration and between dietary fish oil level and the ratio of (serum α-tocopherol/total cholesterol level).

Aorta plaque surface

Absolute total aorta plaque surface was significantly higher in the rabbits fed the 20 and 10 en% fish oil diets as compared with those fed the 40 en% SF diet (Table 7). Relative total aorta plaque surface was significantly higher in the 20 en% fish oil diet only (Table 7). The amount of plaques in the aorta was significantly higher in the 20 en% fish oil dietary group as compared to the 40 en% SF group (Table 7). There was a positive linear relation ($P < 0.05$) between the amount of fish oil in the diet (groups 1–4: 0, 1, 10 and 20 en%) and the absolute and relative aorta plaque surface and the total number of plaques. on comparison of three dietary fish oil concentrations (1, 10 and 20 en%) and the group mean values of relative aorta plaque surface, a significant positive linear relation ($r = 0.97$, $P < 0.05$) was observed. on inclusion of the 0 en% fish oil group, this linear relation was no longer significant ($r = 0.98$, $P = 0.07$).

Liver histology

The animals fed 20 and 10 en% fish oil diets clearly showed pathological liver changes (Table 8): hepatocellular vacuolation ("lipidosis"), hepatocellular hypertrophy, ductular cell hyperplasia, and the

Table 7. α-Tocopherol concentrations in liver and serum and atherosclerosis in aorta

Group	20 en%FO	10 en%FO	1 en%FO	0 en%FO	7 en%LN	40 en%SF
Liver α-tocopherol concentration (μg/g)						
	45.0 ± 2.7^{a}	85.0 ± 7.5^{b}	78.0 ± 3.7^{b}	80.0 ± 4.5^{b}	77.0 ± 2.7^{b}	82.0 ± 2.7^{b}
Serum α-tocopherol concentration (μg/ml)						
	5.9 ± 0.3^{a}	8.9 ± 0.3^{b}	14.9 ± 0.9^{c}	13.5 ± 0.8^{c}	12.9 ± 0.5^{c}	8.1 ± 0.4^{b}
Ratio (serum α-tocopherol concentration/total cholesterol concentration)						
	2.7 ± 0.1^{a}	3.4 ± 0.1^{b}	5.3 ± 0.3^{c}	5.7 ± 0.2^{c}	5.7 ± 0.2^{c}	5.5 ± 0.3^{c}
Total number of plaques in aorta						
	42.1 ± 7.8^{a}	29.5 ± 4.2^{ab}	19.9 ± 4.1^{ab}	22.8 ± 4.2^{ab}	30.4 ± 8.1^{ab}	16.7 ± 3.2^{b}
Total plaque surface in aorta (mm^2)						
	150.0 ± 34.1^{a}	131.0 ± 40.5^{a}	73.0 ± 21.2^{ab}	73.0 ± 17.6^{ab}	95.0 ± 26.8^{ab}	40.0 ± 8.7^{b}
Relative plaque surface in aorta (%)						
	11.5 ± 2.7^{a}	9.6 ± 2.8^{ab}	5.6 ± 1.6^{ab}	5.7 ± 1.4^{ab}	7.2 ± 2.0^{ab}	$3.1. \pm 0.7^{b}$

Values are mean ± SEM; those that do not carry a common superscript are significantly different ($P < 0.05$). The number of animals per group and time point varied from 18 to 31, depending on the size of the group and the number of animals remaining within the group.

presence of multinuclear giant macrophages with brown pigment accumulation (lipogranulomas). The incidence of hepatocellular vacuolation and autofluorescence, inflammatory cell infiltrates and autofluorescence in macrophages and/or giant cells was significantly increased in the rabbits that had been fed the 20 and 10 en% fish oil diets as compared with those fed the 40 en% SF diet.

Aorta histology

The histopathological changes of the aorta of the rabbits fed the 20, 10 and 1 en% fish oil diets did not show statistically significant differences compared with those fed the 40 en% SF diet (Table 8). The incidence and severity of aortic plaques was about equal in the various feeding groups and the type of lesion found was mainly the fibrolipid plaque.

Heart histology

Incidence, type and severity of coronary arterial plaques was similar in the various groups (Table 8).

Relation liver histology and plaque surface area

A total score was given to each liver slide (on a scale from 0–5), to indicate the severity of the pathological changes. The group means of this histopathological score were positively correlated with the means of the relative aorta plaque surface (N = 6, correlation coefficient = 0.96, $P = 0.003$; see Fig. 1). Between individual values no significant correlations were found.

DISCUSSION

The long-term consumption of high levels of dietary fish oil (20 and 10 en%) led to more severe aortic atherosclerosis in our experiment. In several studies using the rabbit as a model, supplemental fish oil had variable effects on the level of atherosclerosis: Thiery and Seidel (1987) found that daily supplements with MaxEPA led to an increase, whereas Adelstein *et al.* (1992) and Campos *et al.* (1989) showed no effect of daily fish oil supplements on the extent of atherosclerosis. Zhu *et al.* (1988) gave three dose levels of fish oil supplements: a protective effect on atherosclerosis of the second dose compared with the first was followed by an aggravating effect of the third (highest) dose. These differences between rabbit studies can result from a different experimental setup, for example, species used, sex and age of the animals, mode of induction of atherosclerosis (e.g. dietary cholesterol), the period and dose of experimental treatment, etc. We used the Dutch rabbit, whereas others used New Zealand White rabbits (Adelstein *et al.*, 1992;

Table 8. Histopathological findings in liver, aorta and heart

Group	20 en%FO	10 en%FO	1 en %FO	0 en%FO	7 en%LN	40 en%SF
Liver: incidence of hepatocellular vacuolation (no. of animals/total no.)						
	15/20*	22/22*	0/21	0/21	0/21	3/31
Liver: incidence of hepatocellular enlargement (no. of animals/total no.)						
	7/20*	3/22	0/21	0/21	0/21	0/31
Liver: incidence of hepatocellular auto-fluorescence (no. of animals/total no.)						
	19/20*	12/22*	2/21	1/21	0/21	0/31
Liver: inflammatory cell infiltrate (no. of animals/total no.)						
	19/20*	19/22*	11/21	9/21	10/21	9/31
Liver: brown pigment accumulation in macrophages and giant cells (no. of animals/total no.)						
	19/20*	18/22*	3/21	6/21*	8/21*	1/31
Liver: increased autofluorescence in macrophages and giant cells (no. of animals/total no.)						
	19/20*	14/22*	1/21	5/21	2/21	4/31
Aorta: incidence of fibrolipid plaques (no. of rabbits with plaques/total no.)						
	13/20	15/22	12/21	9/21	10/21	16/31
Heart: incidence of coronary artery fibro (lipid) plaques (no. of positive rabbits/total no.)						
	16/20	15/22	9/21	15/21	16/21	25/31

*Statistically significant different from the negative control group (40 en% SF) ($P < 0.05$) in Fisher's exact probability test.

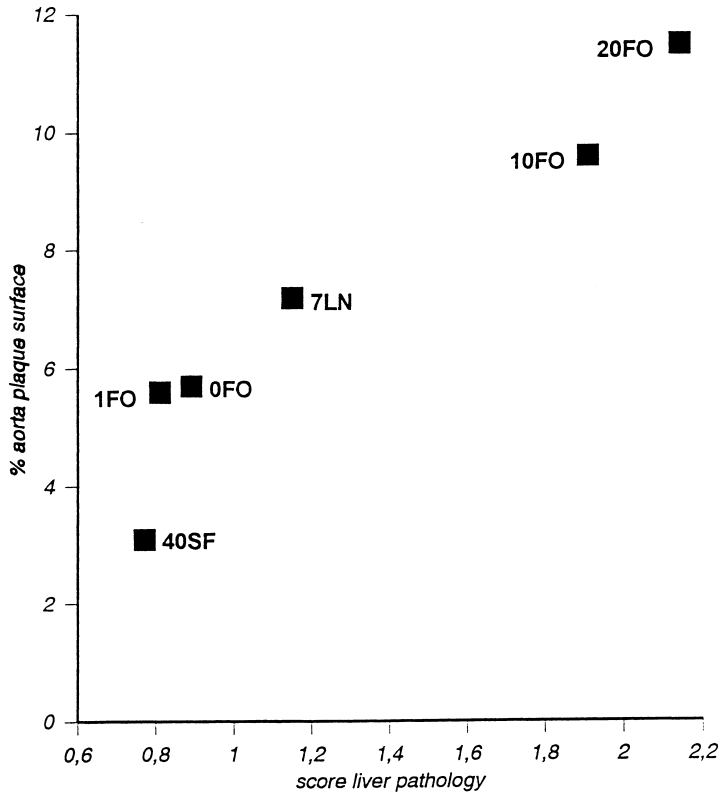

Fig. 1. Correlation between group mean scores for liver pathology (X-axis) and relative aorta plaque area (Y-axis). N = 6, correlation coefficient = 0.96, $P = 0.003$. 20FO = 20 en% fish oil diet; 10FO = 10 en% fish oil diet; 1FO = 1 en% fish oil diet; 0FO = 0 en% fish oil diet; 7LN = 7 en% linseed oil diet; 40SF = 40 en% sunflowerseed oil diet.

Campos *et al.*, 1989; Thiery and Seidel, 1987; Zhu *et al.*, 1988). In our study, diets were cholesterol free. In other studies, variable concentrations of cholesterol were added to the diets to induce atherosclerosis, concentrations used were 1.5% (Thiery and Seidel, 1987), 0.3% (Zhu *et al.*, 1988) and 0.25% (Campos *et al.*, 1989). In these three last-mentioned studies fish oil supplements were given without caloric exchange in the control groups, whereas in our experiment all diets were isocaloric. None of the studies reported in the literature lasted as long as our study of 2.5 yr.

As expected, the rabbits fed the 40 en% SF diet had the lowest serum total cholesterol levels and mean aorta plaque surface. Increased dietary fish oil levels caused higher degrees of aortic atherosclerosis. This might result from a direct effect of dietary fish oil components on the aortic wall, or an indirect influence. The significant positive correlation between the group mean scores for liver pathology and relative plaque surface area in the aorta supports the latter. Possibly, long-chain marine n-3 polyunsaturated fatty acids cannot adequately be metabolized by the liver of the herbivorous rabbit, leading to hepatotoxic effects and more aortic atherosclerosis. The observed pathological liver changes in rabbits fed the 20 and 10 en% fish oil diets, indicate an altered hepatic fat metabolism. Hepatocellular vacuoles often contained slightly birefringent inclusions of varying size, resembling fat vacuoles (lipoid inclusions). It is suggested that these inclusions contain either non-metabolized/less metabolizable fat, or fat that did not disappear after tissue processing, which may have resulted from polymerization of fat that had occurred *in vivo*. Hepatocellular hypertrophy was typically associated with fish oil feeding and may have resulted from hypertrophy or hyperplasia of SER, mitochondria or peroxisomes. The incidence of autofluorescence in macrophages and/or giant cells had significantly increased in the 20 and 10 en% FO groups, probably indicative of increased peroxidation of polyunsaturated fatty acids from fish oil. These results indicate that fish oil is less readily metabolized in the rabbit liver, resulting in fat accumulation in hepatocytes. This was accompanied by fatty degeneration of individual or clustered hepatocytes with inflammatory (granulomatous) reactions. An illustration of differences in the handling of fatty acids by rabbit liver tissue was given by Benner *et al.* (1990). When rabbit hepatocytes *in vitro* were incorporated with eico-

sapenaenoic acid (EPA) and oleic acid, EPA was preferentially incorporated into phospholipids, whereas oleic acid became predominantly esterified in triglyceride. In addition, EPA, unlike oleic acid, failed to stimulate rabbit hepatocyte VLDL secretion (Benner *et al.*, 1990). Feeding a diet containing 10% menhaden oil to rabbits for 14 days clearly influenced the activity of the two microsomal liver enzymes ACAT (acyl-coenzyme A:cholesterol acyltransferase) and HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (Field *et al.*, 1987). These enzymes control the rates of intracellular cholesterol esterification (ACAT) and synthesis (HMG-CoA reductase), respectively (Field *et al.*, 1987). Adding menhaden oil to the diet resulted in increased liver ACAT and decreased HMG-CoA reductase activity and elevated plasma cholesterol levels. Also in our study, mean serum total cholesterol levels in the 20 and 10 en% FO groups were elevated at wk 17 and 53. A temporary rise in serum total cholesterol levels may have contributed to an increased aortic plaque surface.

Our results do not demonstrate a beneficial effect of fish oil consumption on aortic atherosclerosis in the rabbit. From epidemiological studies, a positive association is found between the intake of fish and the prevention of coronary heart disease (Duthie and Barlow, 1991; Kromhout *et al.*, 1985). The long-chain polyunsaturated fatty acids could be one of the aetiological factors involved. Feeding fish oil to rabbits appeared to be hepatotoxic, as concluded from the increased serum levels of liver enzymes and histopathological examination. Long-chain polyunsaturated fatty acids *per se* might lead to hepatotoxicity due to (per)oxidation. However, the diet containing long-chain polyunsaturated fatty acids from vegetable origin did not lead to adverse effects in the liver. In a previous rabbit study it was found that raising dietary vitamin E levels only partly prevented liver damage caused by dietary fish oil (Verschuren *et al.*, 1990). In the present study a high dietary level of vitamin E (200 mg/kg) was chosen, which exceeds the concentrations that are advised to protect againts oxidation of highly unsaturated fatty acids (0.6 TE linoleic acid/g, 0.9 TE α-linolenic acid/g, 1.6–1.8 TE EPA + DHA/g). A maximum protective effect of this high vitamin E level was expected, as based on the results of the previous study (Verschuren *et al.*, 1990). It is clear that liver pathology was not prevented by this level of vitamin E. Significantly lower serum and liver vitamin E levels in the 20 en% FO group may indicate increased use of vitamin E and/or lack of protection of oxidation of marine long-chain polyunsaturated fatty acids. This may have contributed to increased liver damage and atherosclerosis. A higher content of n-3 long-chain PUFA in serum lipoproteins is expected to render them more susceptible for oxidation (Witzum and Steinberg, 1991). This may have contributed to a higher level of atherosclerosis.

The significantly lowered erythrocyte cell count in the 20 en% FO group as compared with the 40 en% SF group, might have resulted from increased erythrocyte breakdown by the liver. However, although the mean liver pathology score in the 10 en% FO group was only slightly lower and serum liver enzyme levels similarly high as the 20 en% FO group, erythrocyte cell count was not influenced.

Measuring aorta plaque surface area was more discriminatory towards the severity of atherosclerosis than the histological evaluation of the atherosclerotic lesions in aorta and heart. In our study, increasing dietary fish oil levels led to more liver damage and an increased aorta atherosclerotic plaque surface in the rabbit. These results question the usefulness of the herbivorous rabbit as a model to examine the influence of dietary fish oil on atherosclerosis. Because of the positive correlation between group mean liver pathology score and aortic atherosclerosis, it is advisable to monitor liver functioning in atherosclerosis studies. Whenever possible, liver pathology should be excluded in animals used as models in atherosclerosis research.

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