Impaired nitric oxide production, brachial artery reactivity and fish oil in offspring of ischaemic heart disease patients

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ABSTRACT

The offspring of coronary heart disease (CHD) patients are at particularly high risk for developing CHD. Endothelial dysfunction is present in the majority of CHD and atherosclerosis patients. Fish oil, rich in n-3 fatty acids has been shown to augment endothelium-dependent vasodilatation in human peripheral and coronary arteries. The aims of this study are to investigate presence of endothelial dysfunction determined by the brachial flow-mediated diameter, nitric oxide, plasma lipids and fibrinogen, and the effect of high doses of fish oil on these parameters. Twenty-four healthy offspring of CHD patients (study group) were supplemented with 9 g/day Alsepa fish oil (each gram containing 180mg EPA and 120mg DHA), for a period of two weeks. Plasma nitric oxide, urine nitric oxide, fibrinogens and flow-mediated vasodilatation (FMD) were determined prior to fish oil therapy, two weeks into therapy and four weeks after the end of therapy with fish oil. Twelve healthy subjects (control group) with no family history of heart disease were studied as controls (day one only). The offspring had a lower increase in FMD and lower nitric oxide production, compared with the control group. No other parameters varied between the two groups. The administration of fish oil did not result in any changes in the studied parameters. In healthy offspring of CHD patients, early endothelial dysfunction was documented before evidence of atherosclerosis. Ingestion of fish oil over a 13-day period did not improve endothelial dysfunction.

KEY WORDS: Endothelium, vascular. Fish oils. Nitric oxide. Offspring.

Introduction

Endothelial dysfunction has been documented in many independent cardiovascular risk factors, including diabetes mellitus, smoking, ageing, obesity, essential hypertension and hyperlipidaemia.[1-6] Impairment of nitric oxide synthesis or chronic inactivation of nitric oxide by oxygen free radicals may account for the increased vascular resistance, vascular hypertrophy, increased platelet and monocyte adhesion to the endothelium, atherosclerosis, myocardial infarction and stroke.[7] Fibrinogen, a known coronary risk factor, seems also to contribute to endothelial cell anomalies.[8-11]

There is increasing supporting evidence that a functional impairment of the endothelium, resulting in a reduction in the availability of nitric oxide and enhanced release of vasoconstricting factors, may predispose patients to monocyte and platelet adhesion, the proliferation of smooth muscle cells, and increased accumulation of macrophages and lipoproteins in the arterial wall. These effects promote atherosclerotic structural lesions at an early age.[12]

n-3 polyunsaturated fatty acid (PUFA) is derived almost exclusively from cold water fish and marine animals, and in areas where consumption of n-3 PUFA is high, the incidence of cardiovascular disease is reported to be low.[13] Fish oil therapy can influence vascular tone and reactivity and alter the functional properties of the systemic circulation.[14,15] The mechanisms by which n-3 fatty acids influence the function of endothelium are still under investigation but it is known that n-3 fatty acids must be incorporated into the cellular phospholipids to exert their effect.[16] This incorporation results in a concomitant reduction of n-6 fatty acids in the phospholipids,[17] suggesting that a specific ratio of n-3 to n-6 fatty acids is important in reducing endothelial activation.

It also appears that the reduction in cell surface expression of the transcriptional n-3 fatty acids may be due to modulation at the transcriptional level, given the finding of a reduced level of cell adhesion molecule messenger RNA after incubation with n-3 fatty acids.[18]

As fish oils favourably influence many of the mechanisms involved in atherogenesis, they are attractive candidates for therapy in atherosclerotic high-risk subjects,[19] and we have previously described[20,21] a protocol for the enhanced exchange of n-3 for n-6 PUFA in serum phospholipids when fish oil is administered (together with intermittent one-day fasting), promoting the depletion of n-6 PUFA and subsequent increase of n-3 PUFA concentration during fish oil supplementation. This resulted in an increased level of 20:5 n-3 eicosapentaenoic acid (EPA) and 22:6 n-3 docosahexaenoic acid (DHA) and a decrease in serum arachidonic acid (AA) in just 13 days, compared to at least 12 weeks in other studies.[22-24]

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The use of this procedure (using 9 g/day fish oil) in hypertensive patients causes a significant reduction in systolic/diastolic blood pressure (BP), serum triglycerides, and platelet aggregation on the extracellular matrix. Positive family history increases an offspring's cardiovascular risk.[25]

The aim of this study is to investigate whether or not healthy offspring of CHD patients do have reduced endothelial-dependent vasodilatation, what the effect of our protocol using high doses of fish oil will be, bearing in mind that it has already proved beneficial for enhanced exchange of n-3 for n-6 PUFA on endothelial-dependent vasodilatation. Here, the nitric oxide system, brachial flow-mediated vasodilatation measurement, plasma lipids and fibrinogen are determined as markers of endothelial function and atherosclerosis.

Materials and methods

Study population

Twenty-four healthy offspring (12 males, 12 females; aged 37.6±5.1 years [range 26–47]) of CHD patients participated in the study. All had a positive family history of CHD; specifically, a first-degree relative (mother, father or brother/sister) diagnosed with CHD before the age of 45 (males) and before the age of 55 (females).

After a complete physical examination, including BP measurement, 24-h urine collection, laboratory determination and brachial flow-mediated vasodilatation measurement, all subjects were treated with nine capsules (3x1g TID) of Alsepa fish oil concentrate for two weeks.[20,21] Each capsule contained 180 mg EPA and 120 mg DHA (Arko, NY, USA). Total daily n-3 supplement was 1620 mg EPA and 1080 mg DHA.

All subjects were advised to adhere to a low cholesterol diet (cholesterol intake <300 mg/day, 50% carbohydrates, 30% fat and 20% protein). All examinations were performed after a 20-h fast, on day zero (prior to fish oil therapy), on day 14 (end of fish oil therapy) and four weeks after the last dose of fish oil.

Twelve healthy subjects (6 males, 6 females; aged 36.2±6.3 years [range 18–45]), match for age and sex, with no family history of any heart disease, served as the control group (day zero only). The local ethics committee approved the study and written informed consent was obtained from all subjects.

Laboratory measurements

Laboratory tests were performed at one particular clinical laboratory, using identical techniques.

Blood pressure

A constant set of observers took three BP measurements from each patient, at 3-min intervals, in the seated position, between 08.00—10.00 hours, and average results were recorded.

Plasma lipid analysis

Triglycerides (TG) were assayed by an enzymatic method (bioMerieux, France) following TG hydrolysis by lipase. Total cholesterol was assayed by an enzymatic method (bioMerieux) using cholesterol-oxidase, phenol reaction to quinoneimine. High-density lipoprotein (HDL)-cholesterol was assayed by precipitation of plasma HDL with heparin, and $MnCI_2$ LDL was calculated using the formula of Friedewald *et al*.[26]

Brachial flow-mediated vasodilatation measurements

This method has been described elsewhere.[5] Briefly, the diameter of the brachial target artery was measured from B-mode ultrasound images, using a 10-MHz linear array transducer and a standard action ATL HDI300 (Advanced Technology Laboratory, Bothell, USA). In all trials, scans were taken at rest and during reactive hyperaemia, after 90 sec and 5 min. The subjects remained in a supine position for 10 min prior to the initial scan.

The brachial artery was scanned in a longitudinal section, 2–15 cm above the elbow, wherever the clearest ultrasound image was obtained. The centre of the artery was identified when the clearest picture of the anterior and posterior intimal layers was obtained. The transmit (focus) zone was set to the depth of the near vessel wall, in view of the greater difficulty of evaluating the near compared with the far wall 'm' line (the interface between media and adventitia), and gain settings were set to optimise images of the lumen/arterial wall interface.

Images were magnified using a resolution box function (leading to a video line width of approximately 0.065 mm), and machine operating parameters were not changed during any part of the study.

When a satisfactory transducer position was found, the skin was marked and the arm was held in an identical position throughout the study. A resting scan was recorded and arterial flow velocity was measured using a pulsed Doppler signal at a 60° angle to the vessel, with the range gate (1.5 mm) in the centre of the artery.

Increased flow was then induced by inflation of a pneumatic tourniquet around the forearm (to a pressure of 240 mmHg for 4.5 min), followed by release. A second scan was taken, continuously and 90 seconds after cuff deflation, including a repeat flow velocity recording for the first 5 min after the cuff was released.

NO measurements

Blood samples were obtained following overnight fasting. Both urine and serum samples were stored at –70°C until laboratory determinations for levels of nitrite (NO_2), nitrate (NO_3) and stable metabolites of NO (NO_x) were measured in all samples.[27] After enzymatic reduction of NO_3 (prepared in our laboratory from *Escherichia coli* and NADPH), NO_2 was determined spectrophotometrically using the Griess reaction. The results are given as mmol/L in serum and nmol/mg creatinine in urine. Based on the 24-h urea excretion, a similar daily intake of nitates was assumed.

Statistical analysis

Descriptive data are expressed as means (±SD). SPSS for Windows analysis was carried out to test significance between the different parameters studied. $P<0.05$ was considered statistically significant.

Results

All 24 subjects (study group) treated with fish oil completed the protocol. The clinical and laboratory data of both fish-oil treated and control subjects are presented in Table 1. Both

Table 1. Haemodynamic and biochemical variables at baseline and after fish-oil supplementation (P=NS)

	Control Group	Study Group		
	Day 1 only	Day 1	Day 13	Day 43
Weight (kg)	71.08±13	73.08±10	73.3±11	73.5±10
BMI (kg/m^2)	25.1±4.2	24.9±3.6	25.1±3.8	25.1±3.9
SBP (mmHg)	112±6	116±9	114±7	112±7
DBP (mmHg)	71±3.6	73±5.4	73±4.5	73±4.7
CHO (mg%)	191.3±16	188.4±18	191.4±19	190.6±17
TG (mg%)	148.2±17	152.7±20	142.3±18	141.8±19
HDL (mg%)	38±6.2	44.1±7.1	45.1±7.4	43.8±7.2
LDL (mg%)	124.9±11.3	114±97	118.8±8.9	116.1±9.2
GLU (mg%)	92.4±4.2	91±3.6	91.6±3.7	92±4.4
Fib (mg%)	298±26	318±21	311±21	321 ±26
UA (mg%)	4.7± 1.1	4.5±0.6	4.5±0.7	4.5±0.6

Table 2. Brachial artery flow-mediated vasodilatation (FMD) at baseline and after fish-oil supplement (P=NS)

	Control Group	Study Group		
		Day 1	Day 13	Day 43
FMD(%)	7.5±3	4.5±2	4.4 ±2	5.2±2
	P<0.001	P=0.81	P=0.72	P=0.71
	(Control *vs* Study)	(13 *vs* 0)	(43 *vs* 0)	(43 *vs* 13)

Table 3. NO production and excretion at baseline and after fish-oil supplement

	Control Group	Study Group		
		Day 1	Day 13	Day 43
Serum NO (μmol/L)	33.7±11.7	26.8±13.4	28.1±12.7	28.4±12.1
Urine NO (nmol/mg CR)	872.7±157.3	393.4±25.6	441.2±269.5	387.1±290
	***P* value**	***P* value**	***P* value**	***P* value**
	(control *vs* day 1)	(day 43 *vs* 1)	(day13 *vs* 1)	(day 43 *vs* 13)
Serum NO	0.08	0.51	0.51	0.85
Urine NO	<0.0001	0.13	0.91	0.13

NO = (NO_2+NO_3)

groups had normal BP and laboratory values of total cholesterol, TG, HDL, low-density lipoprotein (LDL), creatinine, urea, glucose, uric acid and plasma fibrinogen.

FMD changes in the two groups are presented in Table 2. The baseline change in FMD for the study group was 4.5±2%, which was significantly lower (P<0.001) than for the control group (7.5±3%). The fish-oil treatment had no effect on FMD throughout the whole study period.

Table 3 shows the serum and urine NO_x (NO_2+NO_3). Mean plasma NO_x was lower in the study group (26.8±13.4 μmol/L) than in the controls (33.7±11.7 μmol/L), but did not attain statistical significance (P=0.08). Urine NO_x was significantly lower in the study group (393.4±25.6 nmol/mg creatinine) than in the controls (872.7±157.3 nmol/mg creatinine) (P<0.00001). These results suggest significantly lower NO production in the study group than in the control group. Throughout the study, the fish oil did not affect serum NO_x or urine NO_x.

Discussion

The major finding of the present work was a significantly different delta FMD in the offspring of CHD patients, compared with a control group. The lower delta FMD in these subjects seems to be associated with lower NO production. Although plasma fibrinogen and lipid levels were normal, the results suggest early endothelial dysfunction in patients with a family history of CHD. In the presented protocol, fish oil given for two weeks had no

effect on any of the studied parameters.

There is increasing evidence of the importance of endothelial dysfunction as an early indicator of atherosclerosis and an important cause of ischaemia in patients with atherosclerosis.[28] Using invasive and non-invasive approaches to study endothelial function, a close correlation was shown between the coronary vasculature and the brachial artery.[29,30] Endothelial dysfunction is associated with various cardiovascular risk factors, including: diabetes mellitus, hypertension, dyslipidaemia (especially low LDL), smoking and obesity.[1-6] A positive family history of CHD in patients is also a risk factor for their offspring.[8,25]

Reduced FMV of the brachial artery reflects endothelial dysfunction of subcutaneous resistance arteries and, to a lesser degree, structural changes of these vessels in the offspring of CHD patients. This supports the hypothesis that impaired endothelial function in these subjects is systemic, affecting conduct and resistance size vessels.[6] Although NTG was not used in the present study (guidelines[31] published after the study was completed), the results show obvious differences between the study and control groups. Endothelial dysfunction may result from decreased production of NO, inactivation of NO by oxygen-derived free radicals and/or increased production of endothelium-derived contracting factors, which oppose the vascular effect of NO.[30]

Recently, Lee *at al*.[8] found that genetic factors associated with endothelial dysfunction affect the onset of coronary artery disease in Korean males, and these were related to eNOS polymorphism and NADH/NADPH oxidase p22 phox gene C242T polymorphism. However, the distribution of the fibrinogen H1/H2 genotypes was not associated with the development of CHD. Impaired basal nitric oxide production was found in offspring of patients with hypertension,[30] and, to a lesser degree, in offspring of diabetic patients.[32] Recently, offspring of premature myocardial infarction patients were also found to have endothelial dysfunction (measured by brachial artery reactivity) and impaired NO production,[33] a finding that reinforces the results of the present study. It is also interesting to note that the administration of L-arginine in CHD patients with endothelial dysfunction did not affect the FMD.

Epidemiological and clinical studies show an inverse correlation between the consumption of fish oil and cardiovascular disease mortality rates.[34,35] Fish oil rich in n-3 PUFA EPA and DHA is known to reduce BP and serum TG levels and to normalise the hypercoagulability state.[20,21] n-3 PUFA have been shown to modify several key risk factors for cardiovascular disease, with benefits that include increased HDL cholesterol concentration, reduced triacylglycerol-rich lipoprotein concentrations, reduced post-prandial lipaemia and reduced remnant concentration. In contrast, LDL-cholesterol concentration has sometimes been found to rise. Additional benefits of fish oils include improved endothelial function and better arterial compliance (elasticity).

Future trials will be needed to determine minimum effective dosages of EPA and DHA over long periods and to show cardiovascular disease reduction through intervention. In previous studies,[20,21] we showed that an n-6 for n-3 PUFA exchange in the platelets membrane caused a reduction in platelet aggregation, BP and serum TG levels within two weeks, and that the higher the initial levels of such parameters were, the higher the fish-oil reduction effect.

In the present study, initial baseline parameters were normal (BP, TG, platelet count), by definition of the inclusion criteria used, and, as expected, no changes were found in these using the protocol (Table 1). Perhaps it was because of these unchanged parameters (even the change in baseline TG was minimal and not significant) that we failed to show any effect on NO production or excretion, or on FMD, during the 13 days of the study protocol.

In summary, in healthy offspring of CHD patients, FMD is decreased in parallel with decreased NO production, reflecting endothelial dysfunction and resulting in increased cardiovascular risk. Short-term supplementation with fish-oil treatment does not affect either FMD or NO production, thus failing to improve endothelial function. In the present study, endothelial dysfunction was apparent in the offspring of CHD patients prior to the development of atherosclerosis, and may be related to a possible genetic polymorphism of endothelial nitric oxide synthesis. We were unable to show that the administration of fish oil for 13 days improves the impaired vessels' endothelial function; however, a longer period of treatment might affect the parameters studied.

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References

1 Caridllo C, Kilcoyne CM, Quyyumi AA *et al*. Selective defect in nitric oxide synthesis may explain the impaired endothelium-dependent vasodilation in patients with essential hypertension. *Circulation* 1998; **97**: 851–6.

2 Stroes E, de Bruin T, de Valk H *et al*. NO activity in familial combined hyperlipidemia: potential role of cholesterol remnants. *Cardiovasc Res* 1997; **36**: 445–52.

3 Cosentino F, Luscher TF. Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* 1998; **32**: S54–S61.

4 Celermajer DS, Adams MR, Clarkson P *et al*. Passive smoking and impaired endothelium dependent arterial dilatation in healthy young adults. *N Engl J Med* 1996; **334**: 150–4.

5 Celermajer DS, Sorensen KE, Georgakopoulos D *et al*. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* 1993; **88**[5 pt 1]: 2149–55.

6 Park JB, Charbonneau F, Schiffrin EL. Correlation of endothelial function in large and small arteries in human essential hypertension. *J Hypertens* 2001; **19**: 415–20.

7 Massaeli H, Pierce GN. Involvement of lipoproteins, free radicals, and calcium in cardiovascular disease processes. *Cardiovasc Res* 1995; **29**: 597–603.

8 Lee WH, Hwang TH, Oh GT *et al*. Genetic factors associated with endothelial dysfunction affect the early onset of coronary artery disease in Korean males. *Vasc Med* 2001; **6**: 103–8.

9 Koenig W. Fibrinogen and coronary risk. *Curr Cardiol Rep* 1999;**1**: 112–8.

10 Ernst E. The role fibrinogen as a cardiovascular risk factor. *Atherosclerosis* 1993; **100**: 1–12.

11 Mirshahi F,Vasse M, Vincent L *et al*. Fibrinogen: a vascular risk factor, why? Contributing effect of oncostatin M on both fibrinogen biosynthesis by hepatocytes and participation in atherothrombotic risk related to modifications of endothelial cells. *Ann N Y Acad Sci* 2001; **936**: 621–4.

12 Cooke JP, Tsao PS. Is NO an endogenous antiatherogenic molecule? *Arterioscler Thromb* 1994; **14**: 653–5.
13 Shrapnel WS, Calvert GD, Nestel PJ *et al*. Diet and coronary heart disease. The National Heart Foundation of Australia. *Med J Aust* 1992; **156**: S9–S16.
14 Shimokawa H, Vanhoutte PM. Dietary cod-liver oil improves endothelium-dependent response in hypercholesterolemic and atherosclerotic porcine coronary arteries. *Circulation* 1988; **78**: 1421–30.
15 Chin JP, Gust AP, Nestel PJ *et al*. Marine oils dose dependently inhibit vasoconstriction of forearm resistance vessels in humans. *Hypertension* 1993; **21**: 22–8.
16 De Caterina R, Cybulsky MI, Clinton SK *et al*. The omega–3 fatty acid docoshexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. *Arterioscler Thromb* 1994; **14**: 1829–36.
17 De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation. *Am J Clin Nutr* 2000; **71**(1 suppl): 213S–23S.
18 De Caterina R, Bernini W, Carluccio MA *et al*. Structural requirements for inhibition of cytokine-induced endothelial activation by unstaturated fatty acids. *J Lipid Res* 1998; **39**: 1062–70.
19 McVeigh GE, Brennan GM, Cohn JN *et al*. Fish oil improves arterial compliance in non-insulin dependent diabetes mellitus. *Arterioscler Thromb* 1994; **14**: 1425–9.
20 Yosefy C, Viskoper JR, Laszt A *et al*. The effect of fish oil on hypertension, plasma lipids and hemostasis in hypertensive, obese, dyslipidemic patients with and without diabetes mellitus. *Prostaglandins Leukot Essent Fatty Acids* 1999; **61**: 83–7.
21 Yosefy C, Viskoper JR, Varon D *et al*. Repeated fasting and refeeding with 20:5, n-3 eicosapentaenoic acid (EPA): a novel approach for rapid fatty acid exchange and its effect on blood pressure, plasma lipids and hemostasis. *J Hum Hypertens* 1996; **10**(Suppl 3): S135–S139.
22 Blonk CM, Bilo HJ, Nauta JJ *et al*. Dose-response effects of fish oil supplementation in healthy volunteers. *Am J Clin Nutr* 1990; **52**: 120–7.
23 Marangoni F, Angeli MT, Colli S *et al*. Changes of n-3 and n-6 fatty acids in plasma and circulating cells of normal subjects, after prolonged administration of 20:5 (EPA) and 22;6 (DHA) ethyl esters and prolonged washout. *Biochim Biophys Acta* 1993; **1210**: 55–62.
24 Reis GJ, Silverman DI, Boucher TM *et al*. Effects of two types of fish oil supplements on serum lipids and plasma phospholipids fatty acids coronary artery disease. *Am J Cardiol* 1990; **66**: 1171–5.
25 Leander K, Hallqvist J, Reuterwall C *et al*. Family history of coronary heart disease, a strong risk factor for myocardial infarction interacting with other cardiovascular risk factors: results from the Stockholm Heart Epidemiology Program (SHEEP). *Epidemiology* 2001; **12**: 215–21.
26 Friedewald WT, Levi RI, Fredrickson DS. Estimation of the concentration low-density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. *Clin Chem* 1972; **18**: 499–502.
27 Blum M, Yachnin T, Wollman Y *et al*. Low nitric oxide production in patients with chronic renal failure. *Nephron* 1998; **79**: 265–8.
28 Suwaidi JA, Hamasaki S, Higano ST *et al*. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000; **101**: 948–54.
29 Anderson TJ, Uehata A, Gerhard MD *et al*. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 1995; **26**: 1235–41.
30 McAllister AS, Atkinson AB, Johnston GD *et al*. Basal nitric oxide production is impaired in offspring of patients with essential hypertension. *Clin Sci* (Lond) 1999; **97**: 141–7.
31 Corretti MC, Anderson TJ, Benjamin EJ *et al*. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; **39**: 257–65.
32 Lee BC, Shore AC, Humphreys JM *et al*. Skin microvascular vasodilatory capacity in offspring of two parents with type 2 diabetes. *Diabet Med* 2001; **18**: 541–5.
33 Gaeta G, De Michele M, Cuomo S *et al*. Arterial abnormalities in the offspring of patients with premature myocardial infarction. *N Engl J Med* 2000; **343**: 840–6.
34 Drevon CA. Marine oils and their effects. *Nutr Rev* 1992; **50**: 38–45.
35 Nestel PJ. Fish oil and cardiovascular disease: lipids and arterial function. *Am J Clin Nutr* 2000; **71**(1 Suppl): 228S–31S.