Omega-3 fatty acid supplements improve the cardiovascular risk profile of subjects with metabolic syndrome, including markers of inflammation and auto-immunity

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Objective — Fish-oil contains high concentrations of omega-3 fatty acids that have been shown to have anti-inflammatory properties. We have evaluated the effects of purified omega-3 fatty acid supplements on several anthropometric and biochemical parameters, including heat shock protein (Hsp) 27 antibody titres in subjects with metabolic syndrome.

Methods — Subjects (n = 120) with metabolic syndrome (mean age of 52.9 ± 11.9 years) were randomly allocated to one of two groups: sixty subjects were given 1 gram of fish oil as a single capsule, containing 180 mg eicosapentaenoic acid and 120 mg docosahexaenoic acid daily for 6 months. Control subjects did not receive any supplementation over the same period.

Results — The study was completed by 47 subjects in the intervention group and 42 subjects in the control group. Treatment with omega 3 supplements was associated with a significant fall in body weight ($P < 0.05$), systolic blood pressures ($P < 0.05$), serum low density lipoprotein cholesterol ($P < 0.05$), and total cholesterol ($P < 0.05$), triglycerides ($P < 0.05$), high-sensitivity C-reactive protein (hs-CRP) ($P < 0.01$), and Hsp27 antibody titres ($P < 0.05$). No significant changes were observed in the control group.

Conclusion — It appears that omega 3 improves the cardiovascular risk profile of subjects with metabolic syndrome, having effects on weight, systolic blood pressure, lipid profile and markers of inflammation and autoimmunity.

Keywords: *antibody titres – dietary supplementation – fish oil – heat shock proteins – Hsp27 – metabolic syndrome.*

Introduction

Metabolic syndrome comprises a clustering of cardio-metabolic risk factors that include abdominal obesity, impaired glucose tolerance, dyslipidaemia, elevated blood pressure, and insulin resistance[1]. The importance of identifying patients with metabolic syndrome is that it is associated with an increased risk of both cardiovascular disease and type 2 diabetes, and therefore provides an opportunity for implementing early lifestyle change.

C-reactive protein (CRP) is an acute phase protein synthesized by the liver in response to circulating inflammatory cytokines such as IL-6[2]. Experimental studies *in vitro* suggest that CRP may have a direct role in the pathogenesis of atherosclerosis[2;3]. Furthermore, elevated levels of serum CRP are associated with an increased risk of cardiovascular disease, including

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acute myocardial infarction and coronary death among individuals with angina pectoris[4]. We and others have shown that metabolic syndrome is associated with high serum CRP concentrations[5].

The heat shock proteins (Hsps) are a family of 20-25 molecules expressed by cells in response to environmental stressors, including high temperature, free radicals, sheer stress, and toxins, including oxidized-LDL-C[6]. Hsps are involved in the renaturation of damaged proteins, allowing them to refold into their native conformation. High antibody titres to Hsp-60, -65 and -70 have been reported to be associated with coronary risk factors[7;8], increased risk of cardiovascular disease[9], the severity of cardiovascular, and vascular endpoints in patients with established disease[10;11].

Several studies have shown that Hsp27 is over expressed in cardiac myocytes following ischaemia-reperfusion, and may potentially have a cardioprotective effect[12].

An increased intake of ω-3 polyunsaturated fatty acids (PUFA) has been proposed to be beneficial for cardiovascular disease[13]. Intervention studies using ω-3 PUFA have shown that they have anti-inflammatory effects in patients with chronic inflammatory conditions[14]. In vivo and in vitro studies have shown that ω-3 PUFA can modulate the immune system[15,16], probably by decreasing production of prostaglandins such as PGE_2 and leukotrienes such as LTB_4. It has also been demonstrated that ω-3 PUFA can reduce platelet aggregation, clotting, smooth muscle contraction, and leukocyte chemotaxis, and can modulate inflammatory cytokine production and immune function.

Romano et al. have reported that a diet rich in saturated fatty acids induces the expression of Hsps including Hsp-25, -60, and -70 in mice[27], and we have previously reported that Hsp 65 antibody titres rise in rabbits fed a high-cholesterol diet[17]. To our knowledge, there have been no previous reports on the effect of dietary of ω-3 fatty acids on Hsp-27 antibody titres. In this current study, we have investigated the effect of dietary ω-3 fatty acid supplements on several established cardiovascular risk factors and also serum anti-Hsp27 titres in patients with metabolic syndrome.

Methods

Subjects

This study was conducted in Mashhad, Iran. A total of 120 subjects with metabolic syndrome (defined using the International Diabetic Federation criteria, 2005), aged 40-70 years, were recruited from one area of Mashhad. The volunteers had not previously taken ω-3 fatty acid capsules or other nutritional supplements. However, 14.9% of intervention group subjects were taking anti-hypertensive medication and 17.0% of them were being treated with anti-diabetic therapy. In the control group, 14.3% and 16.7 were taking anti-hypertensive and anti-diabetic drugs respectively. In both groups, the treatment did not change during the course of the study.

Each subject gave informed written consent to participate in the study, which was approved by the Mashhad University of Medical Science Ethics Committee. Subjects were randomly allocated to either a group given ω-3 fatty acid capsules (intervention group, n = 60), or a control group who were reviewed in the same way but not given supplements (n = 60). Subjects who were < 40 or > 70 years old were excluded from the study, as were subjects who entered the trial but changed their area of residence. Compliance was monitored by weekly visiting, as assessed by counting tablets, and subjects who did not take their ω-3 fatty acid tablets regularly, or, who were intolerant, were excluded from the study.

Omega-3 fatty acid treatment

Each ω-3 fatty acid capsule contained 180 mg eicosapentaenoic acid (EPA) and 120 mg docosahexaenoic acid (DHA), (Seven Seas, Ltd Marfleet, Hull, United Kingdom) and were provided in weekly batches to the intervention group of subjects to take daily for 6 months. Control subjects did not take any supplements for the duration of the study period. If any subjects were taking other medication, its dose was kept constant for the duration of the study. Subjects from both groups were given general dietary advice. Forty-seven individuals from the intervention group (7 men and 40 women) and 43 control subjects (4 men and 39 women) completed the study. Subjects, who failed to take the ω-3 fatty acid capsules regularly, or became intolerant during the study, were excluded from the study. Anthropometric and laboratory test data were collected in both groups of subjects at the end of the trial.

Anthropometric measurements

Anthropometric parameters including weight, height, and waist circumference were determined using a standard protocol. Subjects were asked to breathe normally, and to breathe out gently at the time of the measurement. Height and body weight were measured with the subjects dressed in light clothing after an overnight fast. The body weight of each subject was measured with a standard scale to an accuracy of ± 0.1 kg, and height was measured to an accuracy of ± 0.1 cm. Body mass index (BMI) was also calculated. Blood pressure was measured twice while the patients were seated and rested for 15 minutes, using a

standard mercury sphygmomanometer calibrated by the Iranian Institute of Standards and Industrial Research. The interval between the two blood pressure measurements was at least 30 minutes, and the average of the two measurements was taken as the blood pressure. The systolic blood pressure was defined as the appearance of the first sound (Korotkoff phase 1) and the diastolic blood pressure was defined as the disappearance of the sound (Korotkoff phase 5) during deflating of the cuff. The BMI was calculated as weight (kg) divided by height squared (m^2).

Collection of serum samples

Blood samples were collected in the morning after an overnight fast from each subject. After being allowed to clot, the tubes were centrifuged at 2500 rpm for 15 minutes at room temperature to obtain serum. Haemolysed samples were excluded from analysis. Serum was stored at -80°C prior to analysis.

Routine biochemical analysis

Laboratory data including full fasting lipid profile comprising total cholesterol, HDL-C, LDL-C, triglycerides and fasting blood sugar (FBS) were determined for each patient. All of the above were measured by routine methods. CRP was measured by a high sensitivity PEG-enhanced immunoturbidimetry method with an Alycon analyzer (ABBOTT, Chicago, IL, USA).

Serum Hsp 27 antibody titres

Serum Hsp27 antibody titres were measured using an in-house ELISA. Microtitre plates (Nunc Maxisorp, Nunc) were coated with 100 ng per well recombinant human Hsp-27 dissolved in 50 μl carbonate buffer (pH = 9.6) incubated for 18 hours at 4°C under humidified conditions**.** The wells were washed 3 times in wash buffer (PBS containing 0.05% Tween-20). Non-specific binding was reduced by blocking each well with 2% goat serum in PBS and 250 μl added to each well and incubated for 30 minutes in 37°C and 30 minutes at room temperature. Wells were washed 3 times with PBS. Serum was diluted 1:100 with 2% goat serum in PBS and 100 μl added to each well in duplicate and incubated for 30 min at room temperature. After washing (4 times in wash buffer and 2 times in PBS), 100 μl peroxidase conjugated-goat anti-human IgG (Sigma Sigma-Aldrich, Inc., USA) diluted 1:500 with 2% goat serum in PBS, was added to each well, and incubated for 30 min at room temperature. After washing (4 times in wash buffer and 2 times in PBS), 100 μl of TMB substrate (100 μl of 6 mg/ml TMB in DMSO was added to 10 ml of 50 mM acetate buffer pH 4.5 containing 3 μl H_2O_2) was added per well and plate incubated for 15 min in the dark at room temperature.

The reaction was terminated by adding 50 μl 2M HCl per well. Optical density at 450 nm was measured using a Labsystems iEMS Reader MF microtitre plate reader with a reference wavelength of 620 or 570 nm. The within assay and between assay precision was 3.5% and 5.2%, respectively. After correction for the non-specific background absorbance (subtracting the absorbance of uncoated wells from the antigen-coated wells for each sample) the results were expressed in optical density units.

Statistical analysis

Statistical analysis was undertaken with the use of MINITAB software (release 13; Minitab Inc, State College, PA), with determination of descriptive statistics (i.e, means, medians, SEMs, and interquartile ranges) for all variables. Data were assessed for normality by using the Kolmogorov-Smirnov test. Between-group comparisons of biochemical variables were assessed by analysis of variance. Categorical data were compared by using Fisher's exact test or chi-square test. Values were expressed as means ± SDs or medians and interquartile ranges (for non-normally distributed data). Analysis of covariance was used to assess differences after adjustment for important confounding factors, such as age and physical activity. The serum titres of Hsp27 antibody were non-normally distributed and were therefore logarithmically transformed before parametric analysis. For comparison pre- and post-intervention, a paired *t*-test was used for normally distributed data and Mann-Whitney U test for non-normally distributed data.

Results

Comparison of the baseline characteristics of intervention group and control subjects

As would be expected, for subjects with metabolic syndrome, there was a high mean BMI and prevalence of lipid abnormalities in both groups at baseline as summarized in table 1. The intervention and control groups did not differ significantly for age, gender, weight, height, BMI, prevalence of diabetes (FBS > 7 mmol/L) and hypertension (systolic blood pressure (SBP) > 140 mmHg or diastolic blood pressure (DBP) > 90 mmHg) or current smoking habit. Nor did the groups differ with respect to SBP or DBP or any of the measured biochemical parameters (table 1).

Table 1. – *Clinical characteristics of the group treated with omega-3 fatty acid supplements and control subjects*

Variables	Values			
	Cases		Control subjects	
	Pre	Post	Pre	Post
N	47	47	42	42
Age (years)	53.5 ± 12.7	53.5 ± 12.7	52.3 ± 11.1	52.3 ± 11.1
Weight (kg)	68.3 ± 11.7	65.7 ± 11.6*	69.5 ± 14.6	68.5 ± 14.1
Height (m)	150.7 ± 7.5	150.7 ± 7.5	151.3 ± 7.3	151.3 ± 7.3
BMI (kg/m^2)	30.3 ± 5.2	29.1 ± 5.1*	30.4 ± 6.1	29.9 ± 5.8
DM (%)	27.7	27.7	30.2	30.2
HTN (%)	31.9	31.9	32.6	32.6
Smokers (%)	4.3	4.3	2.3	2.3
SBP (mmHg)	130.7 ± 14.7	123.6 ± 23.7*	129.6 ± 19.8	127.8 ± 16.8
DBP (mmHg)	81.7 ± 9.7	81.2 ± 9	78.3 ± 13.4	82.3 ± 9.1
Anti-DM medication (%)	14.9	14.9	14.3	14.3
Anti-hypertensive medication (%)	17.0	17.0	16.7	16.7

Values are expressed as mean ± SD, or percentages. Comparison between pre- and post-treatment was assessed by paired *t* test for normally distributed data, or by Mann-Whitney U test for nonparametric data ($P^* < 0.05$). T test was used for comparison between pretreatment case and control subjects and no significant differences were observed. BMI = body mass index, DM = diabetes mellitus, HTN = hypertension, SBP = systolic blood pressure, DBP = diastolic blood pressure.

Table 2. – *Biochemical parameters in the case group treated with omega-3 fatty acid supplements and control subjects*

Variables	Values					
	Cases (n = 47)			Control subjects (n = 42)		
	Pre	Post	% changes	Pre	Post	% changes
FBS (mmol/l)	6.10 ± 2.07	6.34 ± 3.16	+ 3.93	5.91 ± 1.73	6.11 ± 2.72	+ 3.38
TC (mmol/l)	5.99 ± 1.07	5.24 ± 1.15*	-12.52	5.75 ± 1.04	5.04 ± 1.28	-12.35
LDL-C (mmol/l)	3.77 ± 0.89	2.98 ± 0.79*	-20.96	3.71 ± 0.72	2.78 ± 0.74	-25.07
HDL-C (mmol/l)	1.18 ± 0.15	1.23 ± 0.16	+ 4.24	1.12 ± 0.19	1.18 ± 0.11	+ 5.36
TG (mmol/l)	1.76 (1.16-2.24)	1.45 (1.04-2.06)*	-17.61	1.64 (1.06-2.20)	1.41 (0.96-2.29)	-14.02
Hsp 27 antibody titre (absorbance unit)	0.26 (0.03-0.34)	0.11 (0.03-0.16)*	-57.69	0.21 (0.04-0.28)	0.19 (0.03-0.25)	-9.52
Hs-CRP (mg/l)	9.37 (5.40-19.39)	3.12 (2.43-9.01)**	-66.70	7.73 (5.02-16.32)	6.80 (3.78-9.36)	-12.03

Values are expressed as mean ± SD, or percentages. Comparison between pre- and post-treatment was assessed by paired t test for normally distributed data, or by Mann-Whitney U test for nonparametric data ($P^* < 0.05$). T-test was used for comparison between pretreatment case and control subjects and no significant differences were observed. FBS = fasting blood sugar, TC = total cholesterol, LDL-C = low-density lipoprotein cholesterol, HDL-C = high-density lipoprotein cholesterol, TG = triglycerides, Hsp 27 antibody = antibody against heat shock protein 27.

Effects on clinical parameters in the group treated with omega-3 fatty acid supplements compared with the control subjects

On recruitment, each subject was given general dietary advice. No significant changes were observed in the frequency of diabetes mellitus (DM), hypertension (HTN) and smoking habit in either group (table 1, ($P > 0.05$). However, the ω-3 fatty acid-treated group lost significantly more weight ($P < 0.05$), than the control group at 6 months [2.63 kg (3.8%) weight loss ($P < 0.05$) vs. 1 kg (1.4%, $P > 0.05$)]. SBP was also significantly lower at the end of the study in the group receiving omega 3 fatty acid with a mean fall of 7.1 mmHg (5.4%, $P < 0.05$). The fall in SBP in the control group (1.8 mm Hg, 1.4%) did not reach statistical significance ($P > 0.05$). DBP did not differ significantly pre and post treatment in both intervention group and control groups ($P > 0.05$, table 1).

Comparison of biochemical parameters in intervention group and control groups

As seen in table 2, there was a statistically significant reduction in serum fasting triglycerides (17.53%, $P < 0.05$), total cholesterol (12.36%, $P < 0.05$) and LDL-C (20.95%, $P < 0.05$) in the subjects who received ω-3 fatty acids.

There was also a significant reduction in hs-CRP (66.70%, $P < 0.01$) and Hsp27 antibody titres (57.69%, $P < 0.05$) in subjects receiving ω-3 fatty acids, with no significant change in the control group. Whilst FBS

and HDL-C did not change significantly in either group ($P > 0.05$) (table 2).

There was a non-significant ($P > 0.05$) reduction in some of the clinical and biochemical parameters in the control group; these included weight (1.43%), BMI (1.64%), total cholesterol (12.37%), LDL-C (25.20%), triglycerides (14.55%), hs-CRP (12.03%) and HSP 27 antibody titre (-9.52%), and may relate to the general dietary advice given to the subjects at the start of the study (table 2).

Discussion

This is the first reported investigation of the effect of dietary supplementation of ω-3 on Hsp27 antibody titres and coronary risk factors in Iranian subjects with metabolic syndrome. Our results indicated that dietary supplementation of ω-3 for six months at relatively low doses can significantly reduce Hsp27 antibody titres and some features of metabolic syndrome.

Effect of dietary supplementation of omega-3 on lipid profile

In the present study, beneficial effects of ω-3 PUFA were noted on lipid profile, specifically TG, and HDL-C. Consistent with our finding, Harris and Muzio reported a 32% decrease in plasma triglycerides levels after taking 64 mg omega-3 fatty acids/kg/body weight compared to an olive oil placebo daily for 4 weeks in a double-blind, crossover design. A 17.5% decrease in plasma triglycerides levels was observed after 6 months supplementation with omega-3. In another study, there was a significant decrease in triglycerides and very low density lipoproteins but a significant increase in total cholesterol from 5.9 to 6.7 mmol/l after fish oil[18]. According to Varga, fish oil therapy causes a marked decrease in serum triacylglycerol and very low density lipoprotein levels and moderately increases high density lipoprotein levels without any adverse effects[35]. In our study the percentage changes in total and LDL cholesterol were similar for the groups and is probably related to other dietary changes associated with the advice given to each subjects.

Effect of dietary supplementation of omega-3 on blood pressure

Our results suggest that ω-3 supplementation may have a beneficial effect on blood pressure. This observation is in agreement with the results of population-based intervention studies that have demonstrated that PUFAs in fish oil lowers blood pressure in subjects with hypertension[19].

In their meta-analysis of 31 trials, Morris et al.[20] concluded that the hypotensive effect of fish oil when used at high doses might be greatest in subjects with hypertension and in those with established atherosclerotic disease, or hypercholesterolaemia.

In our study the antihypertensive effect of ω-3 fatty acids may be dependent on their direct vascular effects, with an associated improvement in endothelial function. Bao et al. found that the incorporation of fish into a weight-reducing diet had additive effects in reducing ambulatory blood pressure, as well as beneficial effects on heart rate, in overweight hypertensive subjects. These antihypertensive effects were also associated with improvements in platelet function, plasma triglycerides, endothelial function, and inflammatory cell cytokines[21].

Effect of dietary supplementation of omega-3 on plasma glucose

The ω-3 PUFA supplements did not have a significant effect on plasma glucose. Other reports have shown a significant increase in fasting glucose after consumption of fish[22] or fish oils[23]. According to Puhakainen et al., differences in the dosage of ω-3 PUFA may provide a potential explanation for the differences in effects of fish oil on glycaemia[24].

Effect of dietary supplementation of omega-3 fatty acids on body weight

We found that dietary ω-3 supplementation may enhance the reduction in body weight when combined with lifestyle advice for weight reduction. Dietary ω-3 PUFAs have been reported to reduce body fat deposition by inducing the expression of genes involved in lipid metabolism and thermogenesis, increasing total body heat generation and calorie utilisation[25]. Because of their constituent fatty acids, some dietary oils may have beneficial effects on lipid metabolism and on obesity. Another study has shown that a moderate calorie-restricted, cod-based diet promoted weight loss, and was accompanied by a specific improvement in indices of oxidative stress. The low saturated fat content and the high fish protein content of this diet may be important factors in causing these effects[26].

Heat shock protein antibody titres, coronary risk factors and dietary fatty acids

Several studies suggest that ω-3 fatty acid supplementation may reduce the inflammatory response[27]. It is proposed that they do so by several mechanisms including: decreasing lymphocyte proliferation, cytokine

production, natural killer (NK) cytotoxicity and antibody production[28].

We have previously reported that serum hs-CRP concentrations are strongly related to serum LDL-C concentration in serum[29]. CRP has been proposed to be actively involved in the initiation of atheromatous lesion formation[30] and the development of the mature plaque[31].

Park et al. found that the plasma levels of Hsp27 were significantly increased in patients with acute coronary syndrome compared with the healthy reference group, and it has therefore been suggested that circulating levels of Hsp27 antibody titres may be a coronary risk marker[16].

Romano et al.[28] showed that a diet rich in saturated fatty acids induces the expression of Hsp-25, -60, and -70 in mice splenic lymphocytes, and we have previously shown that antibody titres to Hsp-60, -65 and -70 rise following cholesterol feeding in rabbits[32]. These associations may be due to a combination of increased expression of the Hsps and an enhanced immune response, both of which are associated with a high-saturated-fat diet. Ghayour-Mobarhan et al. reported that higher antibody titres to Hsp-60, -65, and -70 are found in the dyslipidaemic patients, and that other features of the metabolic syndrome may be related to a heightened state of immunoactivation associated with atherosclerosis in this group[36].

The fact that we have not used a double-blind placebo-controlled trial design for this study does limit the interpretation of our data. Subjects in the intervention group may have had a more substantial reduction in weight compared to the control group due to the so-called 'Hawthorne effect'[37] and a randomized double-blind placebo-controlled trial would be necessary to confirm our finding.

Conclusion

It appears that a low dose of fish oil supplementation (1 g/day) for a period of six months improves the cardiovascular risk profile of subjects with metabolic syndrome; and has effects on BMI, systolic blood pressure, lipid profile and markers of inflammation and autoimmunity. This is the first report that fish oil decreases antibody titres to Hsp27, which is mainly related to general anti-inflammatory effects of ω-3 supplements. However, these findings will need to be confirmed by a formal double-blind placebo-controlled trial.

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Conflict of interest: none.

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