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The effect of omega-3 supplementation on serum levels of YKL-40, BMP-4, and ox-LDL in men with coronary artery disease: a randomized double-blind placebo-controlled trial

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Summary. *Objective:*Bone morphogenic protein-4 (BMP-4), oxidized low density lipoprotein (ox-LDL), and YKL-40 are the potential biomarkers which may causes inflammation and cardiovascular disease. Omega-3 fatty acid-rich diets may have anti-inflammatory effects. This study was designed to determine the effects of omega-3 fatty acid supplementation on serum YKL-40, BMP-4, and ox-LDL levels in men with coronary artery disease (CAD). *Methods:* The current study was a randomized, placebo-controlled, double-blind parallel-group clinical trial that involved 42 male patients with CAD. The volunteers were randomly allocated into two groups to receive 4 g omega-3 (containing 720 mg EPA plus 480 mg DHA) supplements (n = 21) or placebo (n = 21) per day for 8 weeks. Fasting blood samples were taken at the beginning and end of the trial to quantify serum levels of ox-LDL, YKL-40 and BMP-4 concentrations. *Result:* The results identified that omega-3 fatty acid supplementation for 8 weeks had a noticeable effect on serum ox-LDL (P = 0.03), YKL-40 (P = 0.01), and BMP-4 (P = 0.01) concentrations between groups. A significant difference was found between placebo and omega-3 supplementation groups only for BMP-4 (P = 0.046) after removing counfounding effects of vitamin C and carbohydrates. *Conclusions:* The results of this study indicate that omega-3 supplementation decreased serum BMP-4 significantly. Serum YKL-40 correlated with ox-LDL before intervention. Further study is recommended to elucidate the exact mechanisms for this process.

Key words: Coronary Artery Disease, BMP-4, omega-3 fatty acid, YKL-40, ox-LDL

Abbreviations

BIA: Bioelectrical Impedance Analysis, BMI: Body mass index, BMP-4: Bone Morphogenic Protein-4, cFLIP:cellular FLICE (Fas-associating protein with death domain-like interleukin-1–converting enzyme)-inhibitory protein,CHD: Coronary Heart Disease, CVD: Cardiovascular disease, DBP: Diastolic Blood pressure, DHA: Docosahexaenoic Acid, EPA: Eicosapentaenoic Acid, ELISA: Enzyme-Linked Immunosorbent Assay, ICAM: Intercellular adhesion molecule, LS: Laminar shear,MGP: Matrix GLA protein,MUFA:Monounsaturated Fatty Acids, NO: Nitric oxide, OS: Oscillatory shear stress, Ox-LDL: Oxidized Low Density Lipoprotein,RPM: Revolutions per minute,PUFA:Polyunsaturated Fatty Acids,SBP: Systolic Blood Pressure,SFA:Saturated Fatty Acid, SRs: scavenger receptors,TLRs: Toll like receptors, TNF α: Tumor necrosis factor alpha, VCAM: Vascular cell adhesion molecule,VSMC's: Vascular smooth muscle cells, WHO: World Health Organization.

Introduction

Cardiovascular disease is a chronic inflammatory disease caused by endothelial dysfunction (1, 2). Studies have reported that some risk factors lead to endothelial dysfunction resulting in adhesion of monocytes to arterial walls (2, 3). The monocytes differentiate into macrophages, which result in the accumulation of lipids and formation of foam cells. Inflammatory cytokines from foam cells accelerate the process of atherosclerosis (3).

YKL-40 is also called human cartilage 39 glycoprotein, and chitin or heparin-binding protein (4). Its name stems from the three N-terminal amino acids tyrosine (Y), lysine (K), and leucine (L) and its molecular weight of 40 KD (5). YKL-40 is synthesized by the Vascular smooth muscle cells (VSMCs) (6), macrophages (7), and CD 68^{+} macrophages *in vivo* (6). YKL-40 is associated with increased severity of atherosclerosis (8). Rathcke et al. reported that YKL-40 is a biomarker of inflammation, endothelial dysfunction, and cardiovascular disease (9).

Bone morphogenic protein (BMP) are members of transforming growth factor, and play an important role in bone formation, embryonic growth, and differentiation (10, 11). Oscillatory shear (OS) stress, oxidation, and pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) up regulate BMP expression (12-14). BMP-4 is an inflammatory factor that plays an important role in atherogenesis (12). Yin reported that BMP-4 also effects endothelial inflammation. BMP promotes vascular inflammation in endothelium-dependent behavior (15), and exists in calcified atherosclerotic plaque (16).

Low density lipoprotein (LDL) penetration beneath endothelial cells and its oxidative modification are involved in the early stages of atherosclerosis and activation of inflammatory cells (17). Augmented levels of oxidized-low density lipoprotein (ox-LDL) autoantibodies may cause chest pain in patients with Coronary heart disease (CHD) who are not diabetic (18).

Epidemiological studies have reported that increasing consumption of fish decreases mortality from cardiovascular disease (19). Moreover it has been reported that a diet rich in Omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may reduce inflammatory cytokines (IL-6, IL-1ra, TNFα, and C-reactive protein), and increase anti-inflammatory cytokines (soluble IL-6r, IL-10) (20). Mechanisms through which EPA and DHA affect cardiovascular disease include anti-inflammatory effects, anti-thrombotic effects, and retardation of development of atherosclerotic plaque. Omega-3 fatty acids feature vascular effects, such as amelioration of endothelial function, reduction of vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (21).

Considering the anti-inflammatory effects of omega-3 fatty acids, the present trial was aimed to investigate the effects of omega-3 supplementation on serum YKL-40, BMP-4, and ox-LDL in male patients with coronary artery disease (CAD).

Material and Methods

Patients

The subjects were 42 male patients aged 45 to 65 years that were selected based on defined inclusion and exclusion criteria between July 2012 and June 2013. The subjects were recruited from Tehran Heart Hospital, a referral hospital in Tehran, capital of Iran. The inclusion criteria were willingness to participate, being 45 to 65 years of age, being of the male gender, and having been diagnosed with at least 50% stenosis in one coronary artery as confirmed by angiography during the previous 3 months. Additional exclusion criteria were BMI>30kg/m2, diabetes mellitus, thyroid, liver, kidney and digestive diseases, supplementation with omega-3 for at least 3 months prior to the onset of the study, and coronary artery surgery. Patients who smoked at least 5 cigarettes per day for a period of 6 months were also excluded from the study.

Study design

This was a randomized double-blind placebo-controlled, 2 parallel groups, trial conducted for 8 weeks. Originally, 44 male patients suffering from cardiovascular disease with more than 50% stenosis proven by at least 1 coronary artery were recruited.

The subjects were divided into two groups of 21 subjects (placebo and omega-3 groups) using a permuted block randomization (block size 4) to the active or control groups. The randomization sequence was computer-generated by a blinded statistician not involved in data collection or analysis. All investigators, study staff, and participants were blinded to group allocations, and the randomization code was not broken until statistical modeling of outcomes was complete.

The intervention group underwent the supplementation with 4 g capsules containing 720 mg EPA plus 480 mg DHA daily for 8 weeks. The control group was administered a placebo supplement (4 g edible paraffin) in the same manner as for the intervention group. The supplements and placebo were of the same size, color, and taste. The participants were followed weekly to assess their compliance.

At the begining of the study, subjects signed written informed consent forms. The patients were informed of the aim and possible risks of the clinical trial, and were free to leave the study at any time. The study protocol was approved by the Ethics Committee at Tehran University of Medical Sciences. The present study was registered in the appropriate clinical trial registration system as IDNCT02117960.

Outcome measures

Biochemical measurements (Primary outcome)

Subjects were referred to a laboratory after fasting overnight. Venous blood (10 ml) was drawn from the antecubital vein of all subjects at the beginning and end of intervention. It was kept in a test tube without anti-coagulant at room temperature for 30 min. Coagulated blood samples were centrifuged at 3000 RPM for 10 min. The separated serum was transferred to a clean micro-tube and placed in a -80° refrigerator until testing. Serum YKL-40 was measured using enzyme-linked immunosorbent assay (ELISA) kits (E2063Hu; Bioassay Technology Laboratory, China). Serum ox-LDL was measured by ELISA kits (E1542Hu; Bioassay Technology Laboratory, China). Serum BMP-4 was also measured using ELISA kits (E1990Hu; Bioassay Technology Laboratory, China).

Anthropometric assessment (Secondary outcome)

Anthropometric indices were determined before and after intervention according to World Health Organization standards (22). Height was measured in the upright position without shoes using a stadiometer (Seca; Germany). Weight was measured with a digital scale (Seca; Germany) while the subject were wearing minimal clothing and no shoes. The body mass index (BMI) was calculated as weight (kg)/height (m)2. Body composition was measured using a bioelectrical impedance analysis device (BC-418; Takara; Japan) before and after intervention. Subjects were told to consume an adequate amount of fluid and empty their bladder 2 hours before measurement.

Dietary and physical activity assessment (Secondary outcome)

Dietary intake was determined by 2-day 24-hour dietary recall by a trained nutritionist to assess the food intake of subjects at baseline and after supplementation. The dietary recalls were analyzed using Nutritionist IV software (version 4.1; First Databank Division; Hearst). Data were based on USDA database with minor modification for the special national foods like breads. An international physical activity questionnaire (IPAQ) was used to evaluate the physical activity level at baseline and after supplementation. Physical activity was measured based on the average of metabolic equivalent of task (MET-min/week). Subjects were not asked to change their diets or level of physical activity during intervention. Blood pressure was measured using a digital manometer after 5-10 min in a resting position.

Statistical analysis

The data was analyzed using SPSS software, version 21 (Chicago, Illinois, USA). The mean ± standard error (SE) and percentage was used to express the data. All variables were assessed as 2-tailed tests. The data was analyzed using the Kolmogorov-Smirnov test for normality of distribution. ANCOVA was used to remove the effect of confounders. Results were considered significant at $P \leq 0.05$. A comparison between groups was performed using an independent sample t-test for quantitative data. To compare variables before and after intervention, the paired t-test was used for the quantitative records.

Results

Figure 1 summarizes the trial schema. Among 44 patient enrolled in the study before the randomization, two patients were excluded for personal reasons. Table 1 indicates that there was no significant difference between groups for age, height, weight, BMI, systolic and diastolic blood pressure, physical activity, fat mass, and fat free mass before and after supplementation.

Table 2 reports the macronutrient intake for energy, carbohydrates, protein, fat, MUFA, SFA, PUFA, omega-3 fatty acids, omega-6 fatty acids, EPA-omega-3, DHA-omega-3, oleic fat intake, and vitamins C and E. There was no significant difference observed between groups before and after intervention except for carbohydrates (P=0.03) and vitamin C (P=0.038).

Table 3 indicates that the YKL-40, BMP-4, and ox-LDL concentrations revealed a significant decrease of mean scores after supplementation with omega-3 fatty acids (P= 0.021, P= 0.009, P= 0.016, respectively). A significant difference in mean scores of YKL-40, BMP-4 and ox-LDL levels also were observed before and after placebo and supplementation for omega-3 (P= 0.01, P= 0.01, P= 0.03, respectively). A significant difference

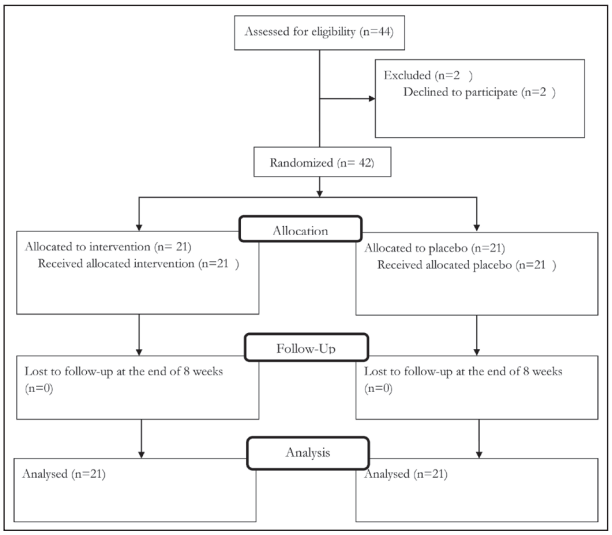

Figure1. Effect of obesity on plasma paraoxonase level and activity

Table1. Anthropometric indices, blood pressure, body composition, and Physical activity score of the study participants

		Omega3(n=21)	placebo(n=21)	P-value*
Age(y)	before	56.19±1.35	57.86±1.45	0.40
Height(cm)	before	169.11±1.20	167.36±1.47	0.36
Weight(kg)	before	80.61±1.81	74.70±2.45	0.06
	after	80.42±1.74	75.40±2.41	0.10
BMI (kg/m^2)	before	28.22±0.64	26.68±0.86	0.16
	after	28.15±0.60	26.91±0.83	0.23
SBP(mmHg)	before	124.57±3.38	125.43±3.76	0.86
	after	117.43±3.53	124.24±4.34	0.23
DBP(mmHg)	before	81.00±2.77	79.38±2.84	0.68
	after	76.14±2.36	77.95±2.51	0.60
Physical activity score (met- minutes/week)	before	1.57±0.13	1.48±0.13	0.61
	after	1.62± 0.14	1.43± 0.13	0.33
Fat mass(kg)	before	19.19±1.37	16.80±1.46	0.12
	after	19.72±1.21	17.51±1.26	0.21
Fat free mass(kg)	before	60.01±1.32	57.86±1.45	0.28
	after	60.55±1.23	58.00±1.48	0.19

Values are expressed as mean ± SE; *Independent sample t teat; BMI = Body mass index, DBP = Diastolic Blood pressure, SBP = Systolic Blood Pressure

Table2. Dietary intakes of the study participants

		Omega-3(n=21)	Placebo(n=21)	P-value*
Energy intake **(kcal)**	before	1616.36±141.83	1454.51±127.13	0.40
	after	1838.11±145.50	1533.62±81.67	0.07
Carbohydrate intake **(g)**	before	248.22±24.58	235.77±22.53	0.71
	after	297.35±24.16	237.34±13.42	0.03*
Protein intake **(g)**	before	61.60±5.60	59.34±7.55	0.81
	after	59.16±4.71	61.09±5.38	0.78
Fat intake **(g)**	before	45.45±6.84	33.33±4.17	0.13
	after	50.16±5.64	39.90±3.16	0.12
MUFA intake **(g)**	before	10.61±2.31	7.92±1.10	0.26
	after	10.62±0.96	9.53±0.81	0.39
SFA intake **(g)**	before	11.33±2.50	8.84±1.15	0.37
	after	10.49±0.88	10.53±1.04	0.97
PUFA intake **(g)**	before	14.87±2.01	10.91±2.03	0.75
	after	17.89±1.75	14.69±1.97	0.23
Omega3 fatty acids intake **(g)**	before	0.18±0.071	0.08±0.01	0.17
	after	0.15±0.06	0.12±0.04	0.80
Omega6 fatty acids intake **(g)**	before	14.68±1.98	9.59±1.70	0.06
	after	17.74±1.77	14.56±1.97	0.23
Vitamin C intake **(mg)**	before	96.48±16.36	90.62±16.99	0.80
	after	108.85±18.10	61.74±12.33	0.038*
Vitamin E intake **(mg)**	before	3.30±0.80	2.33±0.42	0.29
	after	4.34±0.96	3.16±0.49	0.28
EPA- Omega3 intake **(g/day)**	before	0.008±0.007	0.001±0.0005	0.32
	after	0.02±0.01	0.01±0.01	0.82
DHA-Omega3 intake **(g/day)**	before	0.02±0.01	0.008±0.003	0.31
	after	0.05±0.03	0.04±0.02	0.86
Oleic fat intake **(g/day)**	before	7.85±1.50	6.90±1.69	0.67
	after	7.76±1.27	7.59±0.97	0.91

*Values are expressed as mean ± SE; *Independent sample t teat; DHA=Docosahexaenoic Acid, EPA=Eicosapentaenoic Acid,MUFA =Monounsaturated Fatty Acids, PUFA = Polyunsaturated Fatty Acids, SFA = Saturated Fatty Acid.*

was found between placebo and omega-3 supplementation groups only for BMP-4 (P = 0.046) after removing counfounding effects of vitamin C and carbohydrates.

Discussion

This is the first investigation to assess omega-3 fatty acid supplementation on serum YKL-40, ox-LDL, and BMP-4 in patients with cardiovascular disease. Anthropometric indices, blood pressure, body composition, and physical activity scores of the study participants suggested no significant change between groups before and after intervention, and were not considered to be confounders of the results. The BMI in both groups was in the overweight range, but there was no significant difference between group by the variable; thus, it was also not considered to be a confounder. No signifi-

Table3. YKL-40,BMP-4and ox-LDL concentrationbefore and aftersupplementationwith omega-3 fatty acids in patients withcardiovascular disease

		Omega-3(n=21)	placebo(n=21)	P-value[a]	P-value[-]
YKL-40 (ng/ml)	before	117.47±20.65	90.76±12.92	0.28	
	after	71.06±3.77	95.76±11.25	0.04[a]	
	difference	-46.41±18.48	5.00±8.44	0.01[a]	0.10[-]
	P-value[b]	0.021[b]	0.560		
BMP-4(ng/ml)	before	172.28±26.77	129.85±21.57	0.22	
	after	103.63±6.03	130.14±17.91	0.17	
	difference	-68.64±23.87	0.28±5.70	0.01[a]	0.046[-]
	P-value[b]	0.009[b]	0.96		
Ox-LDL(ng/L)	before	4798.76±865.33	3511.66±507.47	0.20	
	after	3018.52±234.33	3296±488.77	0.61	
	difference	-1780.23±675.12	-215.66±140.26	0.03[a]	0.16-
	P-value[b]	0.016[b]	0.140		

Values are expressed as mean ± SE or percentage; [a]Independent sample t teaT; [b]Pair t test; [c]Ancova BMP4= Bone Morphogenic Protein-4, Ox-LDL= Oxidized Low Density Lipoprotein.

cant differences were recorded for dietary intake of the subjects between groups before and after intervention except for vitamin C and carbohydrates. These were assessed as confounders and the results were reported in Table 3 after removing for their effect.

The main findings from this randomized double-blind placebo-controlled trial was that omega-3 supplementation for 8 weeks significantly decreased serum YKL-40, ox-LDL, and BMP-4 levels. The results demonstrated a significant decrease in BMP-4 levels at post-intervention.

Zheng et al. reported that YKL-40 serum concentration increased in subjects with lesion progression over the control group, and was not related to the severity of atherosclerosis (23). Michelsen et al. reported that carotid plaque in subjects with ischemic symptoms during the previous 2 month up-regulates YKL-40 (24).

YKL40 expression by macrophages increased in the presence of the inflammatory cytokine TNFα (25). Conversion of CD14 monocytes to CD16 macrophages increased YKL-40 expression in CD16 macrophages (7). YKL-40 induces lymphocyte adherence, abnormal angiogenesis (9).

This glycoprotein leads to chemotaxis, adhesion cells, and ascular smooth muscle cells (VSMC) immigration and plays a role in convection of monocytes to active macrophages ininflammatory tissue (26-28). Omega-3 fatty acids decrease TNF α, IL1, and IL6 activation (21), likely decreasing the production of YKL-40.

Toll-like receptor 2 (TLR2) and TLR4 agonists induce up-regulation of YKL-40 in THP-1 monocytes *in vitro* (24). Arachidonic acid **(**AA), EPA, and DHA supplementation decreases TLR-4 (29). Schaebel et al. reported no reduction in inflammatory markers such as YKL-40 in subjects who consumed marine mammal-based diets. They considered YKL-40 to be a marker of disease instead of a disease inducer (30). Their results are not in line with the results of the present study. One possible reason could be the presence of organic pollutants in marine mammals of Greenland (31) that contribute to inflammatory disease (32).

The present study has identified that BMP signaling is overcome in pro-atherogenic conditions. BMP signaling causes inflammation and monocyte recruitment (33). This investigation identified that BMP signaling is involved in atherosclerosis and calcification, and its prevention could be beneficial to medical treatment of atherosclerosis and vascular calcification (34). Son et al. reported that, after adjustment for car-

diovascular risk factors in patients with type-2 diabetes, decreased serum BMP-4 is an independent predictor of the cardio-ankle vascular index and increased arterial intima-media thickness (35). It could be the result of measurement of BMP-4 in patients with diabetes.

Ox-LDL is associated with thickening of the covers of the middle intimal vessels (36), endothelial dysfunction (37), and acute myocardial infarction (38). Ox-LDL induces expression of cell adhesion molecules from endothelial cells that cause cell leukocyte adhesion (monocytes and T- cells) (17). Iraz et al. demonstrated that omega-3 fatty acid supplementation could preserve tissues from ROS injury when the immune system does not function well (39). Moreover, a styudy by Chen et al. identified that EPA and DHA diminish ox-LDL-induced expression of adhesion molecules (40).

Some limitations must be considered in the interpretation of the findings. The doses of omega-3 fatty acid supplementation was low; and the follow-up period of this trial was relatively short; and some nonsignificant changes in biomarkers may have become statistically significant with longer follow up.We recommended that future large-scale studies with larger sample sizes and longer duration would be needed to better investigate the subject. The findings of the current study can be considered a basis for further study to elucidate the exact mechanism for the decrease in CVD in response to omega-3 fatty acid.

Conclusions

The current study demonstrated that serum BMP-4 decreased dramatically after supplementation with omega-3 fatty acids for a period of 8 weeks. It appears that omega-3 fatty acids reversed the steps of BMP-4 involvement in atherosclerosis pathogenesis. The current clinical trial identified that it is a possible mechanism for reducing the incidence of coronary heart disease in populations consuming a rich source of omega-3 or omega-3 supplements.

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