Omega-3 Fatty Acid Modifies Serum HSP 70 and hs-CRP in Patients With Cardiovascular Disease: Randomized Double-Blind Placebo-Controlled Trial

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Abstract- All stages of initiation and progression of atherosclerosis are associated with inflammatory responses. Heat shock proteins (HSP-70) can play an important role in the pathogenesis of atherosclerosis. Serum High-sensitivity C-reactive protein (hs-CRP) is significantly associated with the severity of coronary arteriosclerosis. Omega 3 fatty acids contribute to the primary and secondary prevention of cardiovascular disease (CVD). The purpose of the current study was to assess the effect of omega-3 on serum HSP-70 and HsCRP in patients with atherosclerosis. The current study was a randomized, placebo-controlled, double-blind parallel-group clinical trial, involving 42 male patients with coronary artery disease (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 HSP-70 and hsCRP concentrations. The result of the present study revealed that no significant difference was observed between two groups before and after the intervention in terms of serum levels of Cholesterol, Triglyceride, FBS, serum Insulin and homeostasis model of assessment-insulin resistance (HOMA-IR). The difference of HSP-70 between two groups was statistically significant (*P*=0.04). There was no significant difference between two groups for hsCRP. The study showed that taking omega-3 fatty acids can ameliorate serum HSP-70 as inflammatory parameters. The results suggest more investigation to assess the pathway omega-3 leads to lower incidence of CVD.

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Introduction

Atherosclerotic plaque forms in the coronary artery wall and leads to acute coronary syndrome symptoms such as angina and myocardial infarction (1). Inflammation plays an important role in the progression of atherosclerosis. All stages of initiation and progression of atherosclerosis are associated with inflammatory responses (2).

The pathogenesis of cardiovascular disease (CVD) includes variety of factors such as inflammatory and immunologic mechanisms. An autoimmune response against heat shock protein (HSP) is one of the factors causing arteriosclerosis (3,4). The immune response to HSPs is involved in the pathogenesis of atherosclerosis (5). These proteins are divided into seven families based on their molecular size and structure: HSP 10, small HSP (15-30 KDa), HSP-40, HSP-60, HSP-70, HSP-90, and HSP-100 (6). Stressful stimulation, including infection, biomechanical stress, ox-LDL, free radicals, toxins, heat shock, and other types of stress induce HSP production in the vessel wall (7). HSPs are expressed by coronary arterial endothelial cells, smooth muscle cells, and macrophages (8).

Heat shock proteins70 (HSP-70) is secreted into the circulation from the heart muscle, and it causes the innate immune response (9). Serum concentrations indicate HSP-70 expression in tissue (10). The HSP-70 may be involved in the pathogenesis and progression of atherosclerosis or coronary artery disease as damaging agent. High levels of HSP-70 is independently associated with a higher risk of the acute coronary syndrome (11).

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HSP-70 can play an important role in the pathogenesis of atherosclerosis (12). Endothelial cell is the original location of HSP-70 in the heart and is expressed in the large quantities in the human cardiovascular tissues and atherosclerotic plaques (13).

High-sensitivity C-reactive protein (hs-CRP) is an acute phase protein. HsCRP acts as inflammatory mediators that are regulated by Interleukin 6 in the liver. Serum hs-CRP is significantly associated with the severity of coronary arteriosclerosis (14,15).

Epidemiological studies have reported that increased consumption of fish decreases mortality from cardiovascular disease (16). Moreover, it has been reported that a diet rich in Omega-3 fatty acids includes 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) (17). Omega-3 supplementation could decrease the inflammatory response; thus, it is expected that the consumption of omega-3 supplements could reduce stress and inflammation, and serum concentrations of HSP-70 and hsCRP. There is no experience in this field in human specimens with vascular disorders. Therefore, the present trial was aimed to investigate the effects of omega-3 supplementation on serum HSP 70 and hs-CRP in patients with cardiovascular disease.

Materials and Methods

Study design

This was a randomized, double-blind placebo-controlled, 2 parallel groups, trial. Forty-two patients who meet the inclusion criteria were recruited from patient's referred to Tehran Heart Hospital, a referral hospital in Tehran, capital of Iran. The subjects were divided into two 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 received omega-3 supplements (EPA: 720 mg, DHA: 480 mg). The patients were instructed to take two supplements with lunch and two with dinner for 8 weeks. The placebo group received 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.

Patients

Inclusion criteria: Subjects were male patients with CVD proven angiographically, 45 to 65 years of age, a body mass index (BMI) of 18.5 to30, and≥ 50% stenosis demonstrated in at least one coronary angiogram.

Exclusion criteria: Subjects should not have consumed dietary supplements, vitamins, and/or herbal products within a least 3 months of the beginning of the study and throughout the trial. They should not have been diagnosed with the chronic renal disease, GI disease, hepatobiliary disease, a hematological disorder, or hypo- or hyperthyroidism. Potential subjects who are unwilling to participate, who smoke at least 5 cigarettes per day, or who have been smoked within the 6 months prior to the beginning of the study were excluded. Subjects who have undergone cardiac surgery, who were taken Warfarin, who have experienced side effects from the supplementation, who experienced inflammatory disease requiring long-term use of anti-inflammatory drugs (>2 weeks), or who were allergic or sensitive to fish oil were removed.

Outcome measures

Biochemical measurements (primary outcome)

Laboratory tests were carried out on 10 ml venous blood sample taken from the antecubital vein. The serum was separated after being centrifuged at 3000 rpm for 10 minutes. The hsCRP and HSP-70 were measured by ELISA in serum samples drawn at the beginning and end of the study. Serum HSP-70 was measured using ELISA kits (Cat no. E1813Hu Bioassay Technology Laboratory, China). Serum hsCRP high sensitive was measured using ELISA kits (Cat no. DM E-4600 LDN, Germany). Commercial kits (Pars Azmoon, Iran) and auto-analyzer system (Selectra E,Vitalab, Netherland) were used to measure serum levels of glucose, Triglyceride, Total cholesterol. Homeostasis model of assessment-insulin resistance (HOMA-IR) was calculated by following formula: (Glucose×Insulin)/405.

Anthropometric assessment (secondary outcome)

Anthropometric assessment was carried out according to the method proposed by the World Health Organization (WHO) (18). Height was measured in the standing position without shoes using a Seca stadiometer (sensitivity of 0.1 cm). Subjects were asked to wear light clothing and remove shoes before being weighed using a Seca digital scale (accuracy of 0.01 g). BMI was calculated as weight (kg)/height $(m)^2$.

Assessment of diet and physical activity (secondary outcome)

Two 24-hour dietary recall questionnaires and the international physical activity questionnaire (IPAQ) will be completed by the interviewer. We used 24-hour dietary recall at the beginning and end of the study to evaluate usual dietary intake of patients. Nutritionist IV software (version 4.1; First Databank Division, the Hearst Corporation) was used to analyze dietary intake. 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 the metabolic equivalent of task (MET-min/week). Subjects were not asked to change their diets or level of physical activity during the intervention.

Statistical analysis

Data were analyzed using SPSS software version 21 (Chicago, Illinois, USA) and expressed as mean±standard error (SE). Data will be compared between groups using the independent sample t-test before and after the intervention. A value of $P \leq 0.05$ was considered significant for all comparisons. The dietary intake data were analyzed using Nutritionist 4 software. The data was analyzed using the Kolmogorov-Smirnov test for normality of distribution.

Results

Figure 1 summarizes the trial schema. According to table 1, there was no significant difference between the two groups in terms of age, anthropometric indices, physical activity, dietary intake of Linoleic fat, and Linoleic fat before and after the intervention.

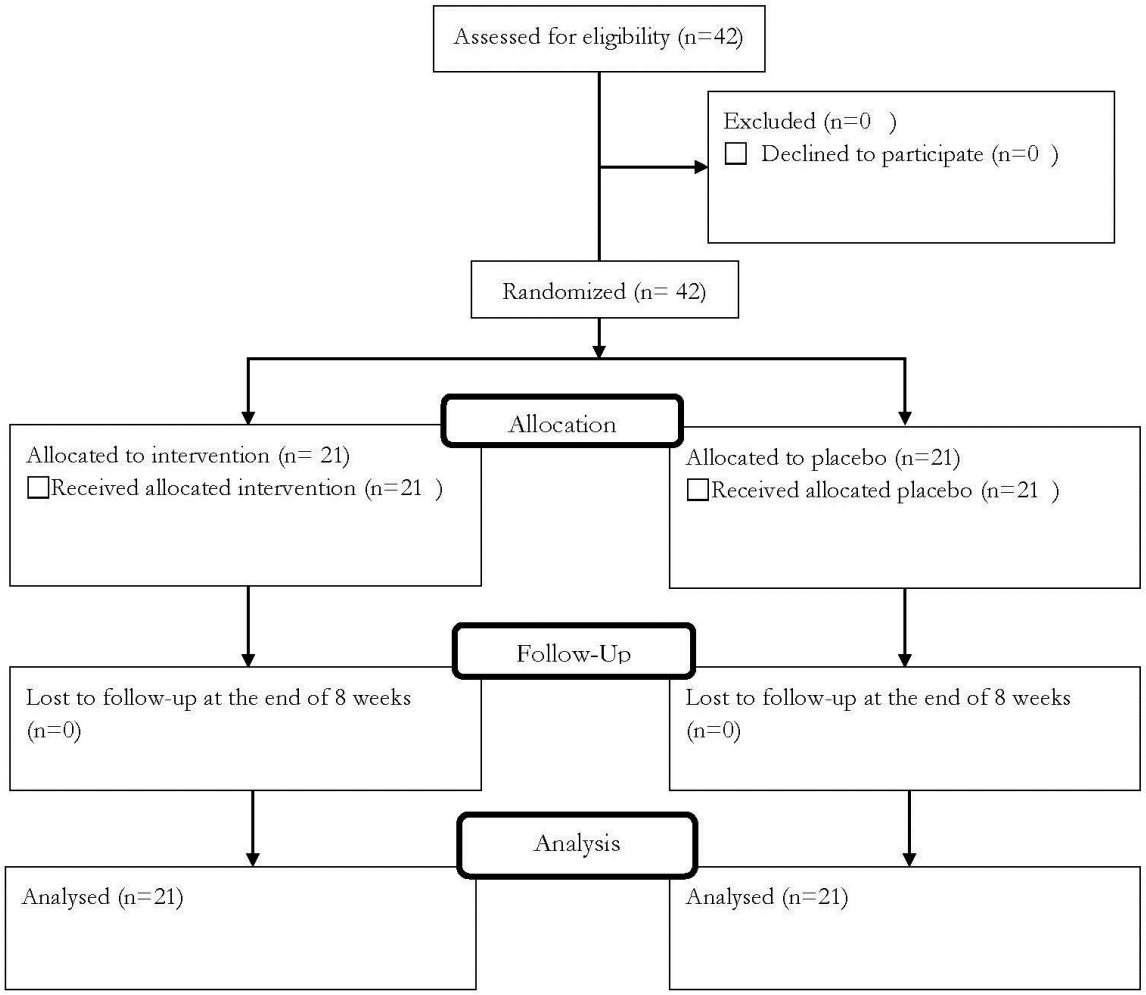

Figure 1. Participant screening, randomization, and follow-up

Table 1. Anthropometric characteristics and fatty acid intake in intervention and placebo groups[#]

		Omega3(n=21)	placebo(n=21)	*P*[*]
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
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
Linoleic fat intake (g/day)	**before**	11.91±1.30	8.61±1.69	0.27
	after	15.08±1.74	12.76±1.68	0.81
Linolenic fat intake (g/day)	**before**	0.15±0.06	0.07±0.01	0.27
	after	0.08±0.03	0.07±0.017	0.81

BMI-Body mass index, [#]Mean±SE,[*]Independent sample t test

As table 2 shows, the serum levels of Cholesterol, Triglyceride, FBS, Insulin serum, and HOMA-IR shows no significant difference between two groups before and after the intervention. Results of this study in Table 3 indicate that the mean serum concentration of HSP70 of the group receiving omega-3 tended to reduce radically (*P*=0.048). The difference of HSP 70 between omega-3 and placebo groups was statistically significant (*P*=0.04). The concentration of hsCRP showed a significant decrease in the group receiving omega-3 fatty acids (*P*=0.02). In addition, the hsCRP difference between omega-3 fatty acid and placebo groups was not statistically significant (*P*=0.07). The mean serum concentration of hsCRP significantly increased after the end of the trial in the placebo group compared with the group receiving omega-3 fatty acids (*P*=0.03).

Table 2. Serum biomarkers before and after omega-3 supplementation in male patients with cardiovascular disease[#]

		Omega-3(n=21)	placebo(n=21)	*P*[*]
FBS(mg/dl)	**before**	87.62±2.90	93.93±4.17	0.22
	after	95.98±3.06	97.67±3.22	0.70
Insulin serum (u/dl)	**before**	14.11±1.68	11.01±1.01	0.12
	after	13.30±1.56	10.90±1.02	0.20
HOMA-IR	**before**	3.09±0.37	2.64±0.32	0.37
	after	3.15±0.39	2.67±0.29	0.33
T.Cholesterol (mg/dl)	**before**	162.12±7.59	173.26±12.18	0.44
	after	153.76±6.71	163.26±8.35	0.38
Triglyceride (mg/dl)	**before**	148.24±10.35	190.07±19.55	0.06
	after	126.07±19.40	167.29±20.15	0.14

FBS-Fasting blood sugar, HOMA-IR- homeostatic model assessment of insulin resistance, T.Cholesterol-total cholesterol. [#]Mean±SE, [*]Independent sample t test

Table 3. HSP-70 and hsCRP serum levels before and after supplementation[#]

		Omega3(n=21)	placebo(n=21)	*P*[a]
HSP-70 (ng/ml)	**before**	33.95±4.92	24.79±3.43	0.13
	after	26.02±2.88	25.19±3.15	0.84
	difference	-7.92±3.75	0.40±0.85	0.04 [a]
	***P*[b]**	0.048 [b]	0.647	
HsCRP (mcg/ml)	**before**	3090±470	2860±360	0.70
	after	1980±160	2960±400	0.03 [a]
	difference	-1100±440	100±480	0.07
	***P*[b]**	0.02 [b]	0.83	

HSP-70- Heat shock protein-70, HsCRP- High-sensitivity C - reactive protein. [a] Independent sample t test, [b] Paired t-test

Discussion

To the best of our knowledge, this was the first investigation on assessing the effect of omega-3 fatty acid on serum levels of HSP 27 and HSP 70 in patients with CVD. The concentration of HSP-70 showed a significant

decrease in the group receiving omega-3 fatty acids. Differences between omega-3 fatty acid and placebo groups were statistically significant. Based on the foregoing, a part of the hypothesis has been accepted. As well, there is no significant difference between two groups in terms of anthropometric characteristics and fatty acid intake. Serum biomarkers did not show significant difference between two groups pre and post intervention.

HSPs are considered as intracellular proteins. In normal condition, there are insignificant numbers of HSP (8). On the other hand, stressful conditions cause increasing amount of them (6). However, under certain conditions, they are released into the extracellular environment (19), where they act as auto antigenic agent (20). The autoimmune response against HSP leads to atherosclerosis pathogenesis (5).

HSPs are released into the blood stream and converted to soluble HSPs (sHSPs). Binding of sHSPs to the complex of Cluster of Differentiation 14 (CD14)/Toll like receptor-4 (TLR-4) leads to the proinflammatory response and autoimmune reaction to the intervention and has an important role in atherosclerosis (8).

The antibodies to HSPs can enter injured cells. It links to the intracellular HSP in macrophages, and foam cells and causes cell lysis. This involves the creation of the necrotic core that exists in more atherosclerotic plaques (19,21).

It was indicated that plasma Hsp70 involves arterial calcification (22). High levels of HSP-70 have been observed in circulation in a variety of diseases such as CVD (23,24), chronic heart failure(25), and acute myocardial infarction (26).

Madden *et al*., have found that patients with peripheral arterial disease (PAD) have lower HSP-70 levels compared with the placebo group (27). This study is not consistent with the result of the present project; it seems that this discrepancy would be originated from study populations, which are much different. Anyway, more studies are needed to clarify the inconsistent results. Low plasma levels of HSP70 are observed in patients with atherosclerosis. Motivated neutrophils act as a source of proteases that lower atheroprotective HSP70 (28).

HSP-70 protects cellular components to reduce oxidation, inflammation, apoptosis, and re-folding of damaged proteins (29). HSP-70 protects the stressed aortic cells in vitro. It prevents cell apoptosis under stress (30). Also, high expression of HSP-70 in cells leads to suppression of apoptosis. The suppression is performed by rapid inactivation of the stress kinases JNK in apoptotic signaling pathway (31).

Although HSP-70 is an intracellular protein, in the presence of stress, it passes through the cell membrane and is found in plasma (9). Ox-LDL leads to HSP70 up regulation in macrophages. Extracellular HSP70 is considered as major paracrine inducer of cytokine expression and secretion in macrophages. Extracellular HSP70 is related to ox-LDL pathway that involves atherosclerosis (32). HSP-70 secretion into the circulation causes the innate immune responses, and it leads to pro-inflammatory cytokines production, including tumor necrosis factor alpha, interleukin 1-6, and adhesion molecule expression in macrophages and endothelial cells (33,34).

The augmented hsCRP serum levels are related to lesion development in CAD patients (35). HsCRP serum level is considered as a marker of carotid atherosclerotic activity during the initial stages of carotid atherosclerosis (36).

HsCRP activates circulating leukocytes. In addition, it reduces nitric oxide activity in endothelial cell and leads to disruption of blood vessels dilation (37). The possible mechanism of CRP is endothelial dysfunction. Human aortic endothelial cells that are cultured with CRP have shown the high expression of Lectin-like oxidized LDL receptor-1 (LOX-1). Finally, LOX-1 leads to endothelial dysfunction. LOX-1 leads to monocyte binding to endothelial cells which are pro-inflammatory and atherogenic (38).

Micallef *et al*., have studied 124 adults into 3 categories based on the levels of hsCRP in the cross sectional study. They showed that plasma concentrations of total omega-3 fatty acids (P=0.05), EPA (P=0.002), and DHA (P=0.01) are inversely associated with hsCRP concentration in healthy people (39). This study confirms our findings.

Niknam *et al*., studied patients more than 45 years with CAD, and have founded Dietary intake of EPA+DHA, and Monounsaturated fatty acids (MUFA) are associated with plasma hsCRP level, but inversely significant (respectively P=0.001 and P=0.002). No significant relationship was observed in the case of alpha-linolenic acid and linoleic acid (40). This study is in line with the present research. However, in line with the present results, C-reactive protein is reduced by fish oil supplement in hemodialysis patients (41). Ordered use of fish oil declines significantly hs-CRP concentrations in adult (42). hsCRP levels decrease by fish oil supplementation (43).

Study limitations

Some limitations must be considered in the interpretation of the findings. The doses of omega-3 fatty acid supplementation were low, and the follow-up period of this trial was relatively short; and some non-significant changes in biomarkers may have become statistically significant with longer follow up. Considering one of the possible mechanisms of Omega-3 fatty acid that is effective on gene expression, so it is recommended to be done in future. In addition, the study with further intervention period and the larger sample size is suggested.

Serum HSP70 decrease in the case of omega 3 fatty acids consumption. The study showed that taking omega-3 fatty acids can ameliorate serum HSP70, reduce inflammatory parameters, and improve the complications of coronary artery disease. Reducing the levels of these markers can be considered as a way of reducing CVD. The present results propose more investigation to assess the pathway in which omega-3 leads to lower incidence of CVD.

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