

The Effect of Dietary Oil Capsules on Reducing Serum Concentrations of Oxidized Low Density Lipoprotein- β 2-Glycoprotein-I Complex

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Abstract

Circulating levels of oxidized low density lipoprotein β 2-glycoprotein I complex (oxLDL-GP) have been previously correlated with adverse cardio-vascular events. This study measured the effect of consuming various dietary oil capsules on oxLDL-GP serum concentrations in healthy human subjects. The dietary oil capsules tested were: enzymatically liberated salmon oil, as a proxy for the entire lipid soluble fraction of whole salmon, high DHA algae oil and standard 18/12 fish oil. Our results showed that only the enzymatically liberated salmon oil had a significant lowering of circulating serum concentration of oxLDL-GP, after 3 weeks of treatment. This result lends support to the developing theory, that eating whole functional foods for their nutritive and health benefiting effects may not be reproduced by highly-refined supplements derived from these foods.

Keywords: Oxidized-LDL; β 2-glycoprotein; Salmon; Fish; Algal; Oil

Introduction

Atherosclerosis is a multifactor chronic pathologic process of the arterial system associated with significant cardiovascular morbidity and mortality. Arterial wall inflammation leads to atherosclerotic lesions that lead to plaque build-up resulting in intraluminal narrowing.

Numerous serologic markers of inflammation have been associated with coronary artery disease (CAD) progression [1]. Recent clinical evaluations have shown that oxidative modification of low-density lipoprotein plays a central role in the initiation and progression of atherosclerosis [2]. Oxidized low-density lipoprotein is highly inflammatory with proatherogenic properties that promote endothelial dysfunction [3].

Unlike native LDL, oxLDL binds *in vitro* to β 2-glycoprotein I, a phospholipid-binding plasma protein, in a time- and temperature-dependent manner to form covalently bound stable oxidized low density lipoprotein- β 2-glycoprotein-1 (oxLDL-GP) complexes [4]. The association of oxLDL-GP complexes with CAD severity and adverse outcomes in patients with ACS has been clearly recorded [5].

The primary aim of this study is to examine the potential for nutraceutical marine oil capsules to lower circulatory oxLDL-GP in healthy human subjects. The secondary end-point is to examine whether there is a difference between a fish oil which is minimally treated during extraction (enzymes only) and thus contains the total un-damaged lipid fraction of a fish versus a highly refined fish oil in which most of the sensitive minor constituents have been destroyed and the polyunsaturated fatty acid balance has been artificially changed to have more eicosapentaenoic acid and docosahexaenoic acid. Non-fish based algae oil and significantly lower Omega-3 concentration sunflower oil were used for further comparisons.

In our previous animal studies, we have observed a lowering of serum levels of oxLDL-GP upon oral administration of different marine and algae oils incorporated into feed. Salmon oil extracted with enzyme digestion showed the maximum lowering of oxLDL-GP levels while algae oil from fermentation and fish oil which was steam extracted and fractionally distilled were much less active at lowering oxLDL-GP levels.

Sunflower oil used as a control oil since it does not contain natural anti-oxidants and has a completely different mix of fatty acid ratios did not show any lowering of oxLDL-GP concentrations.

In this new healthy human study, we investigated the effect of daily capsules of these same nutraceutical oils as soft gel capsules on lowering circulatory oxLDL-GP concentrations. The 500 mgs soft gel capsules were provided by:

- Salmon oil (EVSO)-Green Earth Industries Inc., USA.
- Algal oil capsules (AO)-Martek Inc., USA.
- 18/12 Fish oil capsules (FO)-Ranbaxy Ltd., India
- Sunflower oil (SU)-Flora, USA

Fish oils have been the subject of numerous cholesterol and triglyceride lowering studies [6] and the presence of anti-oxidants present in crustacean and seaweeds has been reviewed for chronic disease treatments [7] but neither the consumption of fish nor fish oil supplements have been studied for their impact on lipid downstream oxidative complexes such as oxLDL-GP.

Materials and Methods

Study design and participants

This study was conducted according to GCP guidelines issued by the ICH, CDSCO and ICMR ethical guidelines in accordance with the laws and regulations of India where the trial was performed. The final approved protocol, informed consent documents and all the study related documents were reviewed and approved by an Institutional

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Ethics Committee before the start of the study. The procedures and possible hazards to which the subjects would be exposed were explained and an informed consent statement was read and signed by all participants.

28 subjects of mixed gender and age between 20-60 years completed this 3 week randomized open label study. All subjects underwent a standard diagnostic procedure as shown below at the beginning of the trial and only patients within normal healthy ranges were enrolled in the trial.

1. History and complete physical examination
2. Complete blood counts with platelet
3. Standard 12 lead electrocardiogram (ECG)
4. 12 hours fasting lipid profile
5. Fasting Blood Glucose (FBG)
6. Complete thyroid profile
7. Liver function test
8. Renal function test
9. oxLDL-GP test

Subjects with known Coronary Artery Disease and concomitant use of Statins/Fibrates or other lipid lowering drugs in the preceding 10 days or who were enrolled in any other clinical trials in the preceding 3 months and pregnant or lactating women were excluded from the study.

The enrolled subjects were assigned to one of four groups (Group A-Group D) such that the average starting concentration of oxLDL-GP levels in each group were statistically equivalent. At the beginning of the trial, 15 ml of blood was collected on Day 0 from each subject and the serum separated and oxLDL-GP levels measured to facilitate this distribution.

Each subject was given a bottle containing 60x500 mg soft gel capsules (Group A-EVSO Capsules; Group B - AO Capsules; Group C - FO; Group D - SU). They were instructed to take two capsules per day together with any meal. Subjects were also informed to maintain their normal diet during this period but were asked to note down any extraordinary increase or decrease if applicable.

At the end of the trial period (Day 28) 15 ml of blood was again collected and serum separated for oxLDL-GP analysis. A truncated end-of-trial diagnostic procedure was carried out - physical examination and blood count only. A palatability and acceptability assessment was also carried out using a questionnaire at the end of the trial.

The fatty acid profile of all four oils was carried out using a gas chromatograph equipped with a flame ionization detector and recorder using an AB-35 capillary column. (Injector temp=260°C; oven temp=180°C; Detector=270°C; nitrogen 3 ml/min; Injected volume 1 μ l). All four oils were converted into methyl esters using the AOCs method Ce 1-b-89.

ELISA assay for oxLDL/ β 2GPI complexes

To a pre-prepped 96 microwell plate (Cayman Chemicals Inc, USA) was added 100 μ L of patient serum samples diluted 1:100 in sample diluents (100 mmol/L tris(hydroxymethyl) amino methane hydrochloride saline containing 2% BSA and 5 mmol/L magnesium chloride, pH 8.5) and allowed to incubate at room temperature for

1 hour. The microwells were washed four times with phosphate-buffered saline containing 0.05% polysorbate-20 between each step. Biotinylated 2E10 anti-body (IgG murine monoclonal anti-human ApoB-100) diluted in 20 mmol/L HEPES-saline containing 2% BSA at pH 7.4 was added to the microwells and incubated for 30 minutes at room temperature, followed by horseradish peroxidase-streptavidin for 30 minutes. Color was developed with tetramethyl benzidine/hydrogen peroxide for 30 minutes and the reaction stopped with 0.3N sulfuric acid. Optical density was read at a wavelength of 450 nm. The functional sensitivity was 0.05 U/mL. Serum oxLDL/ β 2GPI complex concentration (expressed in U/mL) was calculated against a reference curve generated from 3-fold serial dilutions of the reference serum. The specific interaction between oxidized LDL and β 2GPI and the specificity of the assay for oxLDL/ β 2GPI complexes has been previously reported [8].

The average (of duplicate) serum concentrations measured for each subject at the start and end of the trial period was recorded. We used the student t-test within Microsoft Excel 2007 to carry out statistical significance evaluation of the data. We used 2-tail distribution since we did not know if the oxLDL-GP value would stay the same or increase or decrease for each of the groups and described as paired samples since the oxLDL-GP level was compared in the same subject at the start and end of treatment, reducing some of the effects of confounding factors given the small sample sizes.

Results

The fatty acid distribution and astaxanthin concentrations (as representative of the presence or absence of anti-oxidants) for each of the four oils and the results are shown below in Table 1.

There was no significant change in BMI, hip circumference and waist circumference at the end of the trial for all four groups. All four test substance capsules were also found equally acceptable and palatable based on the response from the questionnaire at the end of the trial as shown below in Table 2.

The plasma collected was analysed for oxLDL-GP concentration in

	EVSO (mg/g)	AO (mg/g)	FO (mg/g)	SU (mg/gm)
Saturated Fatty acids	200	340	150	300
Monounsaturated fatty acids	490	110	400	580
Omega-3 fatty acids	200	400	320	30
Omega-6 fatty acids	100	130	110	80
Eicosapentaenoic acid	55	30	110	15
Docosapentaenoic acid	20	< 5	< 5	< 5
Docosahexaenoic acid	105	380	185	10
Astaxanthin (mcg/gm)	9	< 1	< 1	< 1

Table 1: Fatty acid analysis of the Test Oils.

	Group A	Group B	Group C	Group D
Test Oil Capsules	EVSO	AO	FO	SU
Study ID	09R1	09R2	09R3	09R4
# Subjects	8	7	7	6
Mean Age (years)	48	45	51	47
Mean BMI	26.8	27.2	26.6	27.0
Hip Circumference (cm)	103.1	102.6	101.7	102.2
Waist Circumference (cm)	90.7	93.0	92.4	92.8
Palatable & Acceptable	Yes	Yes	Yes	Yes

Table 2: Summary of Test Parameters and Results.

duplicate using the ELISA assay described above. Using a calibration formula generated from the standard curve, the corrected concentration values were calculated for start and final serum circulatory levels of oxLDL-GP. The average reduction across all four groups for the serum concentration levels of oxLDL-GP at the start and end of the trial are shown below as Figure 1.

Statistical analysis of the results of each Group was carried out using the paired, two-tailed student t-test. The results summarized in Table 3 below, show that only the EVSO treated Group A had a statistically significant reduction ($p < 0.01$) in mean oxLDL-GP serum concentration from the start to end of the trial. None of the other Groups B-D showed a statistically significant decrease ($p < 0.01$). Due to the small sample size, no comparison was able to be made between the groups.

Discussion

Chronic inflammatory diseases including atherosclerosis are a major cause of morbidity and mortality worldwide. However, the factors that trigger the processes that determine the outcome of an inflammatory response are still poorly understood. Accumulating evidence suggests that certain oxidized low-density lipoprotein complexes, such as oxLDL- β 2-glycoprotein-I assayed here, represent endogenously formed factors that are capable of triggering vascular inflammation and plaque build-up.

Several studies have shown the value of including oily fish in the diet for improved cardio-vascular and cerebro-vascular health [9] while similar studies on fish oil supplements have shown mixed results with many limitations placed on the effect of omega-3 supplements [10]. Recent Meta analysis of published studies [11] has actually reported no correlation between omega-3 supplements and cardio-vascular health.

One aspect of our results examines the effect of nutraceutical oil capsules with omega-3 concentrations ranging from 30mg/1000 mg to 400mg/1000 mg to reduce serum concentration levels of the new cardio-vascular biomarker, oxLDL-GP through their anti-oxidation effectiveness. Although sunflower oil, which contained the lowest omega-3 concentration (30 mg/1000 mg), showed no oxLDL-GP

lowering effect, the only statistically significant and highest lowering effect was actually observed by the next lowest omega-3 product (EVSO at 200 mg/1000 mg).

Another aspect of our results, examines the effect of the presence or absence of naturally bound astaxanthin (anti-oxidant) ranging from 9 mcg/gm in EVSO to < 1 mcg/gm in the other oils. Our results show a direct correlation between increased astaxanthin levels and a lowering of oxLDL-GP.

It may be concluded from our results that some minimum quantity of omega-3 acids may be required for this beneficial cardio-vascular effect but that the omega-3's alone are not sufficient. Further, the presence of the maximum lipid soluble factors in minimally treated salmon oils (EVSO) seems to play an important role in potentiating the effect of lowering oxLDL-GP serum concentrations when given as a supplement.

We are planning further trials to investigate the role of bio-active factors such as astaxanthin in lowering oxLDL-GP serum concentrations by selectively destroying components in EVSO. We also have some unpublished *in-vitro* data that is pointing to a myeloperoxidase (MPO) inhibiting pathway for this observed effect and we are planning further trials with this enzyme marker as well.

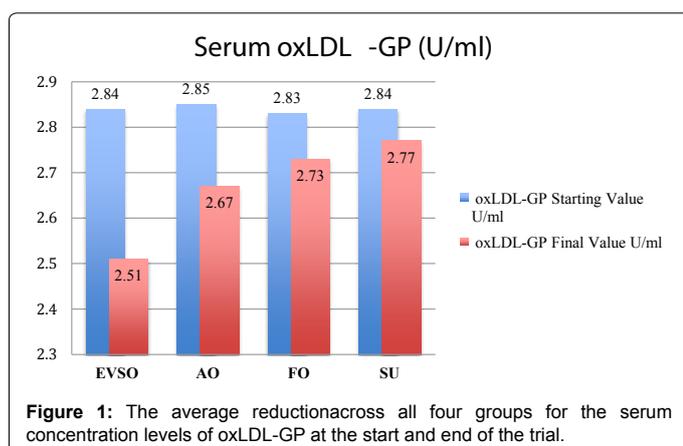
Our result does still reinforce other studies [12] that show consuming a fractionated extract of a functional food may not result in the same quantitative biological effect as consuming the natural whole food and adds further evidence to the theory that eating whole foods for their nutritive and health benefiting effects cannot always be substituted by highly-refined supplements derived from these foods but may be substituted with a non-fractionated whole-food extract.

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Marine Oil	n	Starting Mean	Mean SD	Final Mean	Mean SD	Deg of freedom	t value	p
EVSO	8	2.8387	0.2115	2.5062	0.1889	14.000	3.3159	0.00509
AO	7	2.8457	0.1765	2.6702	0.1775	12.000	1.8567	0.08806
FO	7	2.8257	0.1841	2.7317	0.1840	12.000	0.9728	0.34984
SU	6	2.8417	0.1875	2.7733	0.1852	10.000	0.6349	0.53969

Table 3: Statistical Summary – Serum oxLDL-GP concentration (U/ml).

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