The Effect of Eicosapentaenoic Acid on the Serum Levels and Enzymatic Activity of Paraoxonase 1 in the Patients With Type 2 Diabetes Mellitus

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Abstract- Paraoxonase 1 is known as one of the most important ant oxidative enzymes associated with HDL-c, and because of its antioxidant and antiinflammatory activities. EPA has the antioxidant, anti inflammatory, antithrombogenic, and antiarteriosclerotic properties. Therefore, we investigated the effect of EPA supplementation on the serum levels and activity of PON1 in type 2 diabetic patients. This study was designed as a randomized, double-blind, and placebo-controlled clinical trial. Thirty-six patients with type 2 diabetes were given written; informed consent randomly was classified into 2 groups. They were supplemented with 2 g/day of the capsules of EPA or placebo for eight weeks. Blood sample was given for measurement of the serum levels of lipids, the activity of PON1, FBS and HbA1c. The patients supplemented with EPA showed a significant increase in the serum levels and activity of PON1 and the serum ratio of PON1/HDL-c. There were no significant differences between the two groups regarding any demographic, clinical or biochemical data, total energy intake, and macronutrient intake at the baseline during the intervention, except for a significant increase of protein intake and the levels of HbA1c in the placebo group, and a significant increase of HDL-c, as well as a slight reduction of total cholesterol, LDL-c, TG and FBS in the supplement group. EPA is atheroprotective via increase in the serum levels and activity of PON1, as well as change in the serum levels of lipids, FBS and HbA1c.

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Keywords: Eicosapentaenoic acid; Paraoxonase 1; Type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus is one of the most common endocrine disorders (1), and in the present century, is recognized as a major public health problem all over the world (2). Over the last two decades, the prevalence of diabetes, in particular, type 2 diabetes, has increased rapidly (3). The prevalence of diabetes in the adults all over the world was estimated 4% in 1995 (4) and is currently estimated to be about 6.4% worldwide (5). In the coming decades, the number of individuals with diabetes in the Middle East region, in particular in India and China, will reach the highest rate all over the world that this is compatible with previous estimates (6). Results of the national survey conducted in Iran indicated that the prevalence of diabetes mellitus in the individuals of 25-65 years age group was 7.7%, which equates to about two million adults (7).

PON1 has been found to play a important role in the various types hydrolysis of substrates, including esters and active metabolites of several organophosphate (OPs) insecticides (such as paraoxon, diazoxon, and chlorpyrifosoxon) and the nerve agents (such as soman and sarin) (8-10), lipid peroxides, and estrogen esters (11), as well as lactones (12). Also, this enzyme is involving in the drug metabolism via its lactonase activity (13), and therapeutic use of it for the drug inactivation has been proposed (14). Furthermore, PON1 inhibits the production of Monocyte Chemoattractant Peptide 1 (MCP-1) in the endothelial cells incubated with oxidized LDL (Ox-LDL) (15), and has a role in innate immunity, as its lactonase activity can hydrolyze the quorum sensing signal molecule produced by Gram-negative bacteria such as *Pseudomonas aeruginosa*, i.e. N-acyl-homoserine-lactone (16), as well as it is known as one of the most important antioxidative enzymes (17) associated with HDL-c in the blood circulation, with the antioxidant and antiinflammatory properties (18).

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Therefore, it can be have the antiatherogenic effects.

Eicosapentaenoic acid (EPA) is one of the ω-3 polyunsaturated fatty acids (ω-3 PUFAs) which are present in the great amounts of fish oil (19). The findings of several studies have shown that EPA has the antioxidant (20), antiinflammatory (21), antithrombogenic (22), and antiarteriosclerotic (23) properties.

Research to find suitable nutritional modulators and/or dietary supplement in order to increase the serum levels and activity of this antiatherogenic enzyme is particularly interesting and as a promising target for the pharmaceutical intervention and therapeutic purposes. The aim of this study is the evaluation of the effect of Eicosapentaenoic acid on the Serum Levels and Enzymatic Activity of Paraoxonase 1 in the Patients with Type 2 Diabetes Mellitus.

Materials and Methods

Patients and study design

Patients

The study subjects were 36 patients with type 2 diabetes mellitus who were selected from Iran Diabetes Association (Tehran, Iran). Only patients with a previous clinical diagnosis of type 2 diabetes mellitus according to the criteria for the diagnosis of diabetes as recommended by American Diabetes Association (24)

Inclusion/Exclusion criteria

Inclusion criteria for the participation in the study were, willingness to collaborate in the study, aged 35-50 years, having a history of at least 1 year of the diagnosis of type 2 diabetes mellitus before the participation in the study based on FBS≥126 mg/dl or 2 hPG≥200 mg/dl (2-hour plasma glucose), 25≤BMI<30 kg/m2, identified and maintaining of the antidiabetic's drug (s) dose from 3 months ago.

Participants were excluded from the study if they had, unwillingness to continue the cooperation in the study, need to take insulin, change in the dose (s) and type of medication to the treatment of diabetes, change in the levels of physical activity, do not use (noncompliance) supplements (<10%), affected by the acute inflammatory diseases; according to the consultant physician endocrinologist.

Study design

The study protocol was designed as a randomized, double-blind, and placebo-controlled clinical trial. At first, the study protocol was approved by the ethics committee of Tehran University of Medical Sciences, and all participants gave written, informed consent before the participation in the study.

The patients were randomly classified into 2 groups to the supplementation with 2 g/day of the capsules of EPA or placebo (supplied as 1-g capsules), the two groups were classified based on sex (or the two groups were randomly allocated to the supplement and placebo groups by balanced permuted block on the sex). The capsules containing Eicosapentaenoic acid ethyl ester (75%) [EPA, Mino Pharmaceutical Co. Iran], or edible paraffin were provided by Mino Pharmaceutical Co., Iran. They were strictly advised to maintain their usual diets and nutritional habits, the level of physical activity, and not to change their medication dose(s) during the study, as well as were asked to record and report any side effect of taking capsules gave to them.

Compliance with the supplementation was assessed by counting the number of capsules had used, and the number of capsules returned to the study center at the time of specified visits. The patients were followed up by telephone each week.

Nutritional assessment

At the beginning and at the end of the intervention, nutrients intakes were estimated using a 24-hour diet recall questionnaire for 3 days.

Questionnaires, anthropometric and biometric measurements

At the start and at the end of the study, each participant was evaluated with the physical examination and a general questionnaire containing questions regarding demographic variables (age, sex), anthropometric data (weight, height, waist and hip circumference, heart rate, and measurements of systolic, diastolic and mean blood pressure (SBP, DBP and MBP), and pulse pressure (PP)), family history of diseases (diabetes, hyperlipidemia and hypertension, cardiovascular, etc), age at the diagnosis of type 2 diabetes, type of the treatment and medication used, and lifestyle habits (including the history of smoking, alcohol consumption). The average of type and duration of all physical activities were measured using the International Physical Activity Questionnaire (IPAQ), at the beginning and at the end of the intervention.

Anthropometric measurements, including weight, height, as well as waist and hip circumference, and blood pressure were measured at the start and at the end of the study according to standard protocols. Weight, changes in the level of physical activity, and any disease

were recorded at the baseline and during weeks 2, 4, 6, and 8 of the intervention.

Subjects were weighed without shoes; in light indoor clothes by a Seca scale with an accuracy of ±100 g. standing height was measured without shoes to the nearest 0.5 cm using a commercial stadiometer. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). According to the recommendation of International Diabetes Federation, hypertension was defined as blood pressure≥130/85 mmHg (25).

Each participant gave a blood sample in the early morning after an overnight fast for 10–12 hours and before taking any oral hypoglycemic agent (s) at the beginning and at the end of intervention (8th week). Samples were drawn from the antecubital vein and were collected into blood tubes containing EDTA or heparin. After at least 30 minutes, plasma and serum were separated by centrifugation at 3000×g for 10 minutes at 4° C. Serum and plasma aliquots of each sample stored at −80° C, for analysis of biochemical parameters [Serum levels and activity of PON1, FBS (fasting blood sugar), HbA1c, the serum total cholesterol (TC), triglyceride (TG), LDL-c and HDL-c]. The blood samples were collected only for this study.

Measurement of the serum levels of PON1 and the serumparaoxonase activity of PON1

The serum activity of PON1 was determined with paraoxon (Sigma-Aldrich Inc., St Louis, MO., USA) as the substrate. The serum activity of PON1 was measured by using procedures previously described (26). The serum levels of PON1was measured using Enzyme-linked immune sorbent assay kits for Human Paraoxonase-1(Biotech, China).

Other laboratory analyses

Serum was used for the determination of lipids and glucose. Glucose and HbA1c were measured by enzymatic methods. Serum lipid (serum total cholesterol, HDL-cholesterol, triglyceride and LDL-cholesterol) analyses were performed by spectrophotometric method (Pars Azmoon, Iran).

Statistical analyses

The data were analyzed using SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA), and the results are expressed as mean±SD. The Independent t-test was used for the comparison of variables between two groups. The Paired t-test and Levene's test were also used for data analysis. 24-hour diet recalls analyzed using Food processor II software (27), and the comparison of means in different intervals of 24-hour diet recalls was performed using Independent t-test. Values of $P<0.05$ were considered statistically significant.

Results

Patient characteristics

The baseline and after characteristics of the two groups of patients are shown in Table 1. There were no significant differences in age, sex, duration of diabetes, weight, height, body mass index (BMI), waist circumference, hip circumference, waist/hip ratio, measurements of systolic, diastolic and mean blood pressure (SBP, DBP and MBP), pulse pressure, heart rate and biochemical data between the two groups at the baseline.

Dietary intake and lifestyle

There were no significant differences in total energy intake, macronutrient intake, and body weight between the two groups of patients at the baseline (Table 1), and no significant changes observed during the intervention, except for a significant increase in protein intake in the placebo group. Medication dose (s), and the levels of physical activity from both groups had no significant difference at the baseline, and remained constant during the intervention period (data not shown).

Compliance and side effect

All patients were fulfilled the intervention program, and were well-tolerated intervention with study capsules for 8 weeks. Also, they have reported no side effects throughout the study.

The serum levels and enzymatic activity of PON1

There were no significant differences in the serumparaoxonase activity of PON1 between the two groups of patients at the baseline (Table 2), whereas as shown in Table 2, the serum paraoxonase activity of PON1 increased significantly ($P=0.001$) in the EPA receiving patients compared with the placebo receiving patients.

As shown in Table 2, no statistically significant differences were observed between the two groups of patients at the baseline with regard to the serum levels of PON1 and the serum ratio of PON1/HDL-c, whereas the serum levels of PON1 and the serum ratio of PON1/HDL-c in the EPA receiving patients compared with the placebo receiving patients increased significantly ($P<0.05$, $P<0.001$, respectively).

The serum levels of lipids

The serum total cholesterol was 226.27±38.73 mmol/L after receiving placebo and 207.16±39.69 mmol/L after the supplementation with EPA. Theserum LDL-cholesterol was 95.73±29.86 mmol/L after receiving placebo and 81.4±32.63 mmol/L after the supplementation with EPA. The serum HDL-cholesterol was 76.50±20.81 mmol/L after receiving placebo and 101.61±16.37 mmol/L after the supplementation with EPA. The serum triglycerides were 162.8±158.81 mmol/L after receiving placebo and 176.48±133.75 mmol/L after the supplementation with EPA (Table 3).

Table 1.Characteristics of the two groups at the baseline and end of study

Variable group	Placebo [n (Female/Male)=18]		*P*	EPA [n (Female/Male)=18]		*P*
	Baseline	After		Baseline	After	
Age (years)	44.72 ± 4.69	--	--	44.44 ± 3.79	--	> 0.05
Duration of DM (years)	6.61 ± 3.68	--	--	6.44 ± 2.83	--	> 0.05
Weight (kg)	78.30 ± 12.34	78.24 ± 13.39	> 0.05	78.03 ± 12.68	77.15 ± 12.68	> 0.05
Height (cm)	165.11 ± 8.85	--	--	165.39 ± 8.12	--	> 0.05
Body mass index (kg/m²)	28.92 ± 5.39	28.87 ± 5.61	> 0.05	28.49 ± 3.95	28.17 ± 3.94	> 0.05
Waist circumference (cm)	97.47 ± 10.93	97.08 ± 11.73	> 0.05	97.55± 9.65	96.44 ± 10.16	> 0.05
Hip circumference (cm)	106.00 ± 11.82	105.61 ± 12.32	> 0.05	105.33 ± 6.70	104.61 ± 7.59	> 0.05
Waist/hip (ratio)	0.92 ± 0.08	0.92 ± 0.07	> 0.05	0.92 ± 0.05	0.92 ± 0.06	> 0.05
Systolic blood pressure (SBP) (mmHg)	124.11 ± 15.32	124.89 ± 18.08	> 0.05	124.00 ± 16.25	123.06 ± 18.78	> 0.05
Diastolic blood pressure (DBP) (mmHg)	80.00 ± 6.69	80.00 ± 7.22	> 0.05	79.78 ± 13.40	79.44 ± 11.83	> 0.05
Mean blood pressure (MBP) (mmHg)	94.70± 7.87	94.96 ± 8.98	> 0.05	94.52 ± 13.69	93.98 ± 13.41	> 0.05
Pulse Pressure (PP) (mmHg)	44.11 ± 14.42	44.89 ± 16.83	> 0.05	44.22 ± 9.59	43.62 ± 11.84	> 0.05
Heart rate (HR) (beat/minute)	89.44 ± 12.49	89.33 ±11.73	> 0.05	89.67 ± 10.50	89.33 ± 10.91	> 0.05
FBS (mg/dL)	138.06 ± 49.13	142.06 ± 52.34	> 0.05	143.72 ± 53.53	137.94 ± 23.566	> 0.05
HbA1C (%)	7.47 ± 1.67	7.77 ± 1.42	0.022	7.89 ± 1.75	7.86 ± 1.58	> 0.05
Total energy intake (kcal)	1953.94±297.12	1961.56±232.21	> 0.05	1955.94 ±279.49	1973.61±274.36	> 0.05
Carbohydrates intake (g/d)	260.32 ± 35.44	265.08±37.22	> 0.05	260.85 ± 41.78	260.82±42.89	> 0.05
Proteins intake (g/d)	63.19 ± 14.78	70.09±11.97	0.041	63.83±14.34	63.92 ±14.06	> 0.05
Lipids intake (g/d)	76.11±22.68	76.39 ±16.56	> 0.05	73.82 ±16.78	86.76 ±13.20	> 0.05
Fibers intake (g/d)	14.75 ± 4.64	14.64 ±2.28	> 0.05	16.66 ± 4.99	16.84 ± 3.82	> 0.05

Data are shown as mean±SD. Statistical analysis was performed using paired t-test and Independent t-test

Table 2. Serum levels and activity of PON1, and serum ratio of PON1/HDL-c at baseline and after of the supplementation with EPA or placebo

Group variable	Placebo		*P*	EPA		*P*
	Baseline	After		Baseline	After	
PON1 activity (U/L)	38.48 ± 8.37	38.91 ± 8.05	0.271	37.89 ± 9.78	45.50 ± 7.69	0.002
PON1 levels (ng/ml)	183.55 ± 169.44	187.33 ±166.57	0.124	176.39 ± 128.59	198.05 ± 144.42	0.026
PON1/HDL-c(Ratio)	2.93 ± 2.94	2.99 ± 3.14	0.940	2.68 ±1.98	3.64 ± 1.79	<0.001

Data are shown as mean ± SD. Statistical analysis was performed using paired t-test

Table 3. Serum levels of lipids (mmol/L) at baseline and after the supplementation with EPA or placebo

Group variable	Placebo		*P*	EPA		*P*
	Baseline	After		Baseline	After	
Total cholesterol (mmol/L)	204.44 ± 43.91	226.27 ± 38.73	> 0.05	211.22 ± 43.57	207.16 ± 39.69	> 0.05
LDL-cholesterol (mmol/L)	92.61 ± 35.92	95.73 ± 29.86	> 0.05	96.33 ± 38.13	81.4 ± 32.63	> 0.05
HDL-cholesterol (mmol/L)	76.22 ± 32.85	76.50 ± 20.81	> 0.05	77.72 ± 14.92	101.61 ± 16.37	< 0.001
Triglycerides (mmol/L)	221.50 ± 121.49	162. 8± 158.81	> 0.05	218.61 ± 94.52	176.48 ± 133.75	> 0.05

Data are shown as mean±SD. Statistical analysis was performed using paired t-test

Discussion

The present study provides evidence for increase in the serum paraoxonase activity of PON1 by EPA in the patients with type 2 diabetes mellitus supplemented with EPA after 8 weeks.

Because of the ability of PON1 to protect against the acute toxicity of certain organophosphate insecticides, and more importantly due to having a significant ant oxidative and lactonase potential against oxidative stress and diseases related to oxidative stress, as well as existing evidence suggesting that a healthy lifestyle, including eating "good" fats, consumption of antioxidants, or exercising may increase the expression and activity PON1, therefore, it seems that strategies aimed at the manipulation of the expression and activity of PON1 by nutritional or pharmacological agents and products can be has an important role in the oxidative stress, and many of diseases related to the inflammation and oxidative stress. Thus, it has major clinical importance and is necessary that more studies be increasingly performed in the future in order to develop specific nutritional and pharmacological agents and products targeting PON1. Several studies have shown that EPA has various effects, including preventing of the insulin resistance (28), increasing the insulin secretion (29), enhancing the size of LDL-c particle(30), reducing the serum levels of TG, lowering the blood viscosity, increasing the production of NO, having the antiinflammatory and antithrombotic properties (21,23,29,31,32), and decreasing the blood pressure (33).

It has been demonstrated that EPA is more effective than docosahexaenoic acid (DHA) in the suppression of inflammatory response (34). EPA plays as a substrate to decreases the production of inflammatory eicosanoids from arachidonic acid, via competing for the cyclooxygenase-2 and lipoxygenase (COX-2/LOX) enzymes. These alternative eicosanoids, which are termed E-series resolvins, have identified as a group of mediators to exert the antiinflammatory functions. Moreover, both DHA and EPA reduce the release of arachidonic acid via the inhibition of Phospholipase A2 (PLA2) (35-37).

Also, EPA has an inhibitory role on the endotoxin-induced expression of adhesion molecules on the endothelial cells (ECs) of humanvein and results in the excessive reduction of monocytes attached to the arterial endothelium (38).

Paraoxonase 1 is a multifunctional antioxidant enzyme, which through its antioxidant and antiinflammatory properties (18,39) can not only prevent of the peroxidation of LDL-c, HDL-c, and macrophages and destroy Ox-LDL but also is involving in the detoxification of HCTL; as a Hcy metabolite; which this active metabolite can pathologically cause damage to the

vessels wall and thereby leads to atherosclerosis (40), and also, since the patients with diabetes mellitus are at high risk of atherosclerosis (41). Therefore, it seems that due to the key role this multifunctional enzyme attached to HDL-c in destroying two main risk factors to the development of atherosclerosis and CVD in the diabetic patients, i.e. Ox-LDL and HCTL, finding an appropriate dietary supplement to increase the serum activity of this antiatherogenic enzyme can be clinically important.

PON1 through the various mechanisms have the protective effects against the development of atherosclerosis, including as an important antioxidant enzyme (17,42) with an antiinflammatory function (17,43), an active role in scavenging free radicals and their metabolic products, protection of the LDL-c, HDL-c, and macrophages against oxidative stress and maintaining the function of HDL-c and macrophages (18,39,44-46), inhibition of the cholesterol influx via the attenuation of Ox-LDL formation and its uptake by macrophages (47), inhibition of the cholesterol biosynthesis in macrophages (48), increase binding HDL-c to macrophages and reduction of the foam cell formation in macrophages via a decrease in the cellular oxidative stress and stimulation of the cholesterol efflux mediated by HDL-c from macrophages (49). PON1 has also been shown to hydrolyze the platelet-activating factor (PAF) (50) and the L-homocysteine thiolactone (L-HCTL) (51). Therefore, this enzyme contributes to the prevention of inflammation in the vessel wall and the development of atherogenesis (52).

It is significant to point out that results of studies previously showed in the diabetic patients than healthy controls, a significant decrease in the serum concentration of PON1 was observed (53-55), although a significant difference was not detected in some studies (56,57). Also, the serum activities (arylesterase and paraoxonase) of PON1 in the patients with diabetes than healthy control individuals were significantly reduced (53,58,59). It was shown that the low serum activity of paraoxonase in type 2 diabetes mellitus was associated with the plasma levels of Ox-LDL and with vascular complications (60), and in some studies the serum paraoxonase or arylesterase activities of PON1 in the diabetic patients did not correlate with the plasma levels of Ox-LDL (61,62), although this low serum activity of PON1 may be influenced by PON1 gene polymorphism .

This is the first time that has been demonstrated EPA can increase the serum activity of PON1 in vivo. Our present study clearly shows that the supplementation of EPA for 8 weeks in the patients with type 2 diabetes mellitus significantly increases the serum activity of PON1.

On the other hand, our present study clearly shows that patients receiving EPA after 8 weeks had the higher serum levels of PON1 and the serum ratio of PON1/HDL-c than patients receiving placebo (Table 2). As yet, the effect of EPA on the serum levels of PON1 and the serum ratio of PON1/HDL-c was not studied, and this is the first time that has been demonstrated EPA can increase the serum levels of PON1 and the serum ratio of PON1/HDL-c in vivo. It is significant to point out that our data are consistent with results of the study was previously established the positive effect of ω-3 PUFA on the plasma levels of paraoxonase in vivo (63).

Meanwhile, several studies have shown that the ω-3 PUFAs have various effects on the lipid profile in type 2 diabetic patients, including enhancing the size of LDL-c particle (64), reducing the serum levels of TG (65), increasing the plasma levels of HDL-c and HDL2-c (65,66), and decreasing the plasma levels of HDL3-c (65). This study demonstrated that EPA could significantly increase the serum levels of HDL-c which is compatible with the results of the other studies with ω-3 PUFAs (65,66) but did not significantly affect the other serum levels of lipids.

Thus, EPA has the beneficial effect for human health in view of its antiinflammatory and antioxidant properties, and as a dietary product and/or supplementation improving the serum activity of PON1. Generally, there are two strategies in order to increase the serum activity of PON1. First, the administration of an exogenous factor(s), such as pharmacological or dietary agents and products that would increase the hepatic expression and serum activity of PON1. However, chronic administration of the drug with the purpose of increasing the serum activity of PON1 is not recommended. Since some of the drugs have potential side effects to individuals health, thereby, is focused on dietary components and/or supplements and it is considered as a promising target for pharmaceutical intervention and therapeutic purposes. Second, it may rely on the direct administration of exogenous PON1. Since it is not determined whether direct administration of PON1 will have potential side effects immune responses. Thus, being applicable this approach to humans is still need to be ascertained, and more research should be performed in order to this issue.

The study limitations

There were several limitations of our study. First, a relatively small sample size of patients, therefore, it

should point out that the results of our study are preliminary and need to be confirmed in a larger sample size of patients. It is better and important that the serum levels of CPR, and inflammatory cytokines, as well as the percentage of EPA in the membrane of RBC measure in the further studies. For these reasons, additional studies will be necessary to determine the general applicability of our study results.

In this study we postulated that Eicosapentaenoic acid induces an antioxidant response via the upregulation of PON-1 activity. Therefore this supplementation can be considered a way to reduce oxidative stress and consequently relapse chronic complication of diabetic patients.

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