The Effects of Eicosapentaenoic acid Supplementation in The Patients with Type II Diabetes Mellitus: Study protocol for a randomized controlled trial

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Abstract: Background: Diabetes mellitus is one of the most common endocrine disorders in the world, and as a public health challenge due to the substantial financial and economic burden related to this disease and its chronic complication on the individuals, families, society, and healthcare system of the country. Cardiovascular disease is the most important cause of death among these patients, and ω-3 fatty acids have the antioxidant properties and improving the antioxidant status and the serum levels of HDL-c. Meanwhile, the antioxidants result in an increase in the gene expression and serum activity of PON1. **Methods:** The present study is a randomized, double-blind, and placebo-controlled clinical trial. Thirty six patients with type 2 diabetes mellitus will be selected. They will give written; informed consent, will randomly be allocated to the supplement and placebo groups. Blood sample for measurement of the biochemical parameters and gene expression of PON2 will be given. **Discussion:** EPA could be has the antioxidant, antiinflammatory, antithrombotic and antiarteriosclerotic effects, and PON family has antioxidant and antiinflammatory roles in the context of atherosclerosis. We expect that EPA supplementation has been desirable effects on the serum levels of sVCAM-1, sE-selectin, HDL-c and its subfractions, apoB-100, apoA-I, Malondialdehyde, Hcy, and FBS, and increases the antioxidant activities of serum PON1 and HCTLase, as well as up-regulate the gene expression of PON2.
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Background

Type 2 diabetes is as a serious chronic metabolic disease, and one of the most common endocrine disorders resulting from defect in the insulin secretion, the insulin resistance, or both[1]. In the twenty-first century, type 2 diabetes is recognized as a major public health problem all over the world[2], and its prevalence has reached epidemic proportions worldwide[3]. One of the most costly diseases to manage is diabetes[4]. This disease and its chronic complications impose a substantial economic burden on individuals, families, society, and the healthcare system of the country and make it as a public health challenge[5]. It is anticipated that in the year 2025, the healthcare expenditures of diabetes will be between 7% and 13% of the healthcare budget of worldwide[6]. Enhanced oxidative stress is a characteristic of patients with diabetes, and it plays an important role in the development of diabetic complications such as atherosclerosis and particularly vascular disease among cardiovascular disease (CVD)[7], so that the risk of developing CVD is two to fourfold higher in people with diabetes than in those without diabetes and the general population, and includes approximately 80% of all mortality in the diabetic patients[8].

The paraoxonase (PON) multigene family is consisting of three different isozymes; PON1, PON2, PON3; with genes adjacent to each other on chromosome 7 q21-q22[9]. PON1, PON2, and PON3 share homocysteine thiolactonase (HCTLase) activity to metabolize aromatic and long-chain aliphatic lactones[10].

PON1 is a calcium dependent ester hydrolase or glycoprotein which is tightly associated with the high density lipoprotein cholesterol (HDL-c) particles[11]. PON1 has arylesterase, paraoxonase, and HCTLase activities[12, 13]. 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 chlorpyrifos oxon) and the nerve agents (such as soman and sarin)[14], lipid peroxides, and estrogen esters[15], as well as lactones[9, 16]. Furthermore, PON1 inhibits the production of Monocyte Chemoattractant Peptide 1 (MCP-1) in the endothelial cells incubated with oxidized LDL (Ox-LDL)[17], and it is known as one of the most important antioxidative enzymes[18] associated with HDL-c in the blood circulation, with the antioxidant and antiinflammatory properties[19]. Therefore, it can be have the antiatherogenic effects.

PON2 is nearly expressed in all human tissues, including the liver, heart, kidney, small intestine, stomach, lung, spleen, testis, and placenta[20]. Moreover, PON2 mRNA in humans (hPON2 mRNA) is also found in the cells of the artery wall; including smooth muscle cells, endothelial cells, and macrophages[21, 22]; white blood cells, and skeletal muscle[22]. Although there is still little information about its specific functions, characteristics and regulation of PON2, Paraoxonase 2 acts as an antioxidant at the cellular and not humoral level. PON1, PON2 and PON3 have a protective role against the development of atherosclerosis (antiatherogenic)[11, 19, 23], and has also demonstrated that this role of PON2 can be related to its antioxidative properties[22]. In addition, PON2 indicates the lower antioxidant properties than PON1, but has the uttermost lactonase activity[24]. Thus, the PON2 preventing role of the development of atherosclerosis can also be dependent on its lactonase activity. PON2 is able to reduce the intracellular oxidative stress, prevent the cell-mediated oxidation of LDL, alter the properties of LDL and HDL via the interactions of these lipoproteins with a number of cell types including macrophages[22], and protect from triglyceride (TG) accumulation in the macrophages[25].

In fact, homocysteine (Hcy) is a nonessential amino acid containing sulfur and a byproduct generated from the amino acid methionine (Met); which serves as a precursor; via the biologic transmethylation reactions, and removes from the body[26]. In the normal conditions, total homocysteine (tHcy) is used to the production of RNA and DNA for making and maintenance of tissue[27], while findings of several studies among epidemiological studies and clinical trials have shown that elevated the plasma or serum levels of Hcy is well-known as an modifiable and independent risk factor for atherosclerosis in peripheral, coronary, and cerebral arterial diseases such as CVD, and in venous thrombosis[28-30]. Diabetic patients with the high serum levels of Hcy have a higher intima-media thickness, and are more susceptible to the harmful effects of the high serum levels of Hcy than nondiabetic individuals[31]. Therefore, research for this modifiable risk factor in the patients with type 2 diabetes mellitus is important.

Malondialdehyde (MDA) is one of highly toxic products of the peroxidation of polyunsaturated fatty acids (PUFAs) of membrane phospholipids due to exposure to reactive oxygen species (ROS) and free radicals, and it may be used as an index of the oxidative damage of membrane[32, 33], which is usually measured as thiobarbituric acid reactive substances (TBARS) reaction[32].

Apolipoprotein metabolism is strongly associated with the development of atherosclerosis. So that in clinical studies, the high levels of apolipoprotein B (apo B) and/or low levels of apolipoprotein A-I (apo A-I) have been associated with an increase risk of cardiovascular events. These two apolipoproteins are as important, better and more accurate predictors of the risk of CVD than total cholesterol (TC) or LDL-c[34], and as markers of lipid lowering therapy[35]. An increased in the hepatic synthesis of lipoproteins containing apo B is one of characteristics associated with obesity, non insulin dependent diabetes mellitus and the metabolic syndrome[36], and the endothelial dysfunction is a main initiating step in the development of atherosclerosis in patients with type 2 diabetes mellitus[37], thereby, these patients are at increased risk of atherosclerotic vascular disease[38]. Therefore, interventions aimed in order to decrease the hepatic secretion of lipoproteins containing apo B could be have great clinical importance.

It seems that the expression of serum selectins molecules (sP-selectin and sE-selectin) and soluble cell adhesion molecules (intracellular cell adhesion molecule, sICAM-1; vascular cell adhesion molecule, sVCAM-1) regulate at the transcription level[39]. Although adhesion molecules are quite requirement and play a key role in the normal development and function of cardiovascular system[40], but plasma measurement of adhesion molecules are considered as markers of endothelial dysfunction, and predictors of early atherothrombotic and atherosclerosis processes and vascular disease[41].

HDL-c particles are a highly heterogenous class of particles to differ in apolipoprotein (apo) and lipid composition, density, size, and charge[42]. They are with a density >1.063g/mL[43, 44] and can differentiate based on the density in HDL2-c larger

particles, and HDL3-c less dense subpopulation[43]. HDL-c and each of the subfractions are regarded as one of the most important independent protective and modifiable factors against arteriosclerosis, which current therapies for their improvement are inadequate[45]. Larger subfractions of HDL-c may protect against atherosclerosis, whereas the smaller subfractions are more atherogenic[46].

It has been demonstrated that the ω-3 fatty acids have the antioxidant properties and improving the antioxidant status in the diabetic patients[47]. Also, a lot of epidemiological studies have demonstrated that there is a strong relationship between the content of ω-3 PUFAs in the diet and a decrease in cardiovascular diseases (CVDs) and total mortality[48, 49]. Eicosapentaenoic acid (EPA) is one of ω-3 PUFAs which are present at the great amounts in the fish oil[50]. The findings of several studies have shown that EPA has the antioxidant[47], anti-inflammatory[51], antithrombogenic[52], and antiarteriosclerotic[50] properties.

In spite of the promising beneficial effects of ω-3 PUFAs in the CVDs, there is yet a concern about increment intake of ω-3 PUFAs may cause increased in the lipid peroxidation. Furthermore, in recent years, a lot of studies have been done on ω-3 PUFAs, but results from clinical trials are conflicting. Therefore, the beneficial metabolic effect of this type of fatty acids in the patients with type 2 diabetes mellitus is still under debate.

Primary Objective of the Study:

The aim of this study is to determine the efficacy of the supplementation of Eicosapentaenoic acid on the serum activities of Paraoxonase 1 and Homocystein thiolactonase, and the serum levels of some of indicators of vascular inflammation, and the gene expression of PON2 in PBMC in the patients with type 2 diabetes mellitus.

Methods

1. Patients and Study Design:

1. 1. Patients:

Sample size and Recruitment:

The study subjects are 36 patients with type 2 diabetes mellitus who are 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 (ADA)[53] are recruited.

1.1.1. Inclusion/Exclusion Criteria:

Inclusion criteria for the participation in the study are, 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 2hPG ≥200 mg/dl (2-hour plasma glucose), 25≤BMI<30 kg/m^2, identified and maintaining of the antidiabetic's drug (s) dose from 3 months ago.

Participants will be excluded from the study if they have, 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 to the acute inflammatory diseases; according to the consultant physician endocrinologist.

1.2. Study Design:

The study protocol has been designed as a randomized, double-blind, and placebo-controlled clinical trial. Flow chart of study protocol is shown in Figure 1.

Ethics

At the first, the study protocol has been approved by the ethics committee of Tehran University of Medical Sciences (ID: 84153). The patients are informed about the aim of the study and are free to leave the study at any time. All participants will give written, informed consent before the participation in the study. The study protocol is registered in the Clinical Trial. gov-register (……..).

Randomization:

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

Compliance:

Compliance with the supplementation will be assessed by counting the number of softgels used and the number of softgels returned to the study center at the time of specified visits. The patients will be followed up by telephone each week.

1.2.1. Outcome Measurements:

1.2.1. 1. Questionnaires, Anthropometric and Biometric Measurements:

At the start and at the end of the study, each participant will be 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 will be 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 will be measured at the start and at the end of the study. Weight, changes in the level of physical activity, and any disease will be recorded at the baseline and during weeks 2, 4, 6, and 8 of the intervention.

Subjects will be weighed without shoes, in light indoor clothes by a Seca scale with an accuracy of ±100 g. Standing height will be measured without shoes to the nearest 0.5 cm using a commercial stadiometer. Body mass index (BMI) calculates as weight/height² (kg/m²). According to the recommendation of International Diabetes Federation, hypertension is defined as blood pressure ≥130/85 mmHg[54].

1.2.1. 2. Nutritional Assessment:

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

1.2.1.3. Laboratory Assessment:

Each participant will give 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 will be drawn from the antecubital vein, and collected into blood tubes containing EDTA or heparin. After at least 30 minutes, plasma and serum will be separated by centrifugation at 3000 ×g for 10 minutes at 4 °C. Serum and plasma aliquots of each sample will be stored at −80 °C, for analysis of biochemical parameters. For the evaluation of gene expression, at the first, PBMCs (peripheral blood mononuclear cells) will be isolated. Then RNA isolation and cDNA synthesis will be done. Analysis and measurement of the selected genes expression will be performed by real-time PCR. The blood samples will be collected only for this study.

1.2.1.3.1. Molecular Analysis:

Isolation of PBMCs*:* At the first, published guidelines will be followed to guard against bacterial and nucleic acid contamination [55]. Subsequently, PBMCs (peripheral blood mononuclear cells) will be isolated. Briefly, the remaining blood will be returned to its original volume with adding PBS, and then blood gently will be added to tubes containing Ficoll, followed by the centrifugation at 2500 rpm (1100 ×g) for 20 minutes at room temperature (25 °C), PBMC-containing band (buffy coat) will be aspirated, washed with adding PBS and centrifuge at 1600 rpm (450 ×g) for 15 minutes at room temperature (25 °C). Finally, cells will be analysed microscopically.

RNA isolation and cDNA synthesis: After PBMCs separated, total RNA will be isolated from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA synthesis will be done by Quanti Tect Reverse Transcription (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers design: Using information contained public database in Gene Bank of the National Center for Biotechnology Information (NCBI), the sequences of primers are designed by Primer 3 online software (http://www.basic.nwu.edu/biotools/Primer3.html or http://www.cgivo.2 [56].), and theirs specificity are verified in BLAST domain (http://www.ncbi.nlm.nih.gov/BLAST). Primers for the PON2 and β-actin genes are synthesized by Bioneer Co. Ltd. The sequences of primers to will be used for real time PCR reactions are shown in Table 1.

Real-time PCR: Analysis and measurement of the selected genes expression will be performed by real-time PCR using ABI Step One (Applied Biosystems, Foster City, CA, USA). PCR amplification will be performed in 40 cycles of 15 seconds at 95 °C, followed by 40 seconds at 60 °C.

The β-actin gene will be used as a housekeeping and endogenous control. Thus, the mRNA expression of PON2 gene will be normalized by β-actin. The real time PCR results will be imported into Microsoft Excel, and the ratios of the expression levels of PON2 and β-actin genes will be calculated (using the Pfaffl method [57]) by subtracting the threshold cycle number (Ct) of target gene (PON2) from the Ct of β-actin and raising 2 to the power of this difference. The Ct values are defined as the number of PCR cycles at which the fluorescent signal while the PCR reaches a constant threshold. Target gene expressions will be expressed relative to β-actin expression, and will be shown as mean ± SD.

1.2.1.4. Data Analyses:

The data will be analysed using SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA), and the results express as mean ± SD. The Independent t-test will be used for the comparison of variables between two groups. The Paired t-test and Levene's test will also be used for data analysis. 24-hour diet recalls analyse using Food processor II software[58], and the comparison of means in different intervals of 24-hour diet recalls will be performed using Independent t-test. Values of $p < 0.05$ consider statistically significant.

Table 1. The sequences of primers to will be used for real time PCR reactions.

Primer		Sequence (5′→ 3′)
	Forward	TGTAGACCTTCCACACTGCCACCT
PON2	Reverse	TGGTGCAAAGCTGTGGAGTCCTG
β -actin	Forward	CCTGGCACCCAGCACAATGAAG
	Reverse	CTAAGTCATAGTCCGCCTAGAAGC

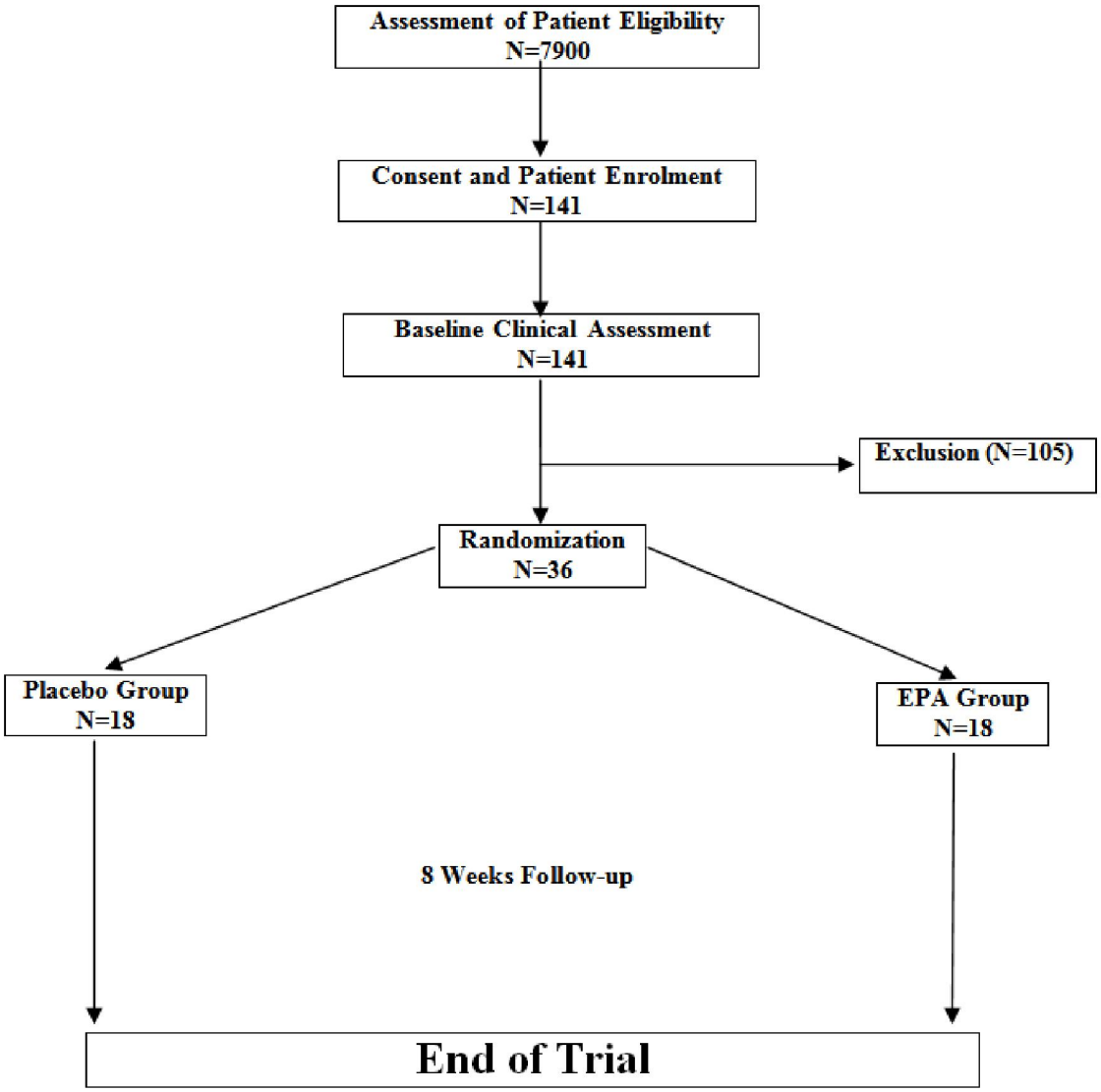

Figure 1 Flow chart of study protocol.

Discussion

Several studies have shown that EPA has various effects, including preventing of the insulin resistance[59], increasing the insulin secretion[60], enhancing the size of LDL-c particle[61], reducing the serum levels of triglyceride, lowering the blood viscosity, increasing the production of nitric oxide (NO), having the antiinflammatory and antithrombotic properties[62-64], and decreasing the blood pressure[65].

It has been demonstrated that EPA is more effective than docosahexaenoic acid (DHA) in the suppression of inflammatory response[66]. EPA plays as a substrate that decreases the production of inflammatory eicosanoids from arachidonic acid, via competing for the cyclooxygenase-2 and lipooxygenase (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)[67, 68].

Also, EPA has an inhibitory role on the endotoxin-induced expression of adhesion molecules upon the endothelial cells (ECs) of human vein, and results in the excessive reduction of monocytes attached to the arterial endothelium[68, 69].

The findings of an epidemiological study of Greenland Eskimos suggested that EPA could be has the antithrombogenic and antiarteriosclerotic properties[70]. It has been postulated that the mechanisms of these actions are including the suppression of platelet aggregation and the improvement of blood rheologic properties[71].

It has also been reported that EPA has beneficial effects on the serum levels of lipids to is suggesting that EPA may be useful as a supplement for the prevention and treatment of arteriosclerotic disease[52]. These results suggest that the administration of EPA to the patients with type 2 diabetes may prevent of the development of cardiovascular complications caused by some different risk factors. It seems that a combination of these actions and mechanisms explained above are responsible for antiinflammatory, antiatherosclerotic, and antithrombotic effects caused by EPA.

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 [72], reducing the serum levels of TG [73], increasing the plasma levels of HDL-c and HDL2-c [73, 74], and decreasing the plasma levels of HDL3-c [73].

We hypothesise that the Eicosapentaenoic acid supplementation has desirable effects on the serum levels of some of markers of vascular inflammation, and HDL-c anti-atherogenic lipoprotein and its subfractions, B-100 atherogenic apolipoprotein, A-I anti-atherogenic apolipoprotein, and transformed amino acid containing sulfur i.e. Hcy, and leads also to the improvement of FBS. Despite the worries about oxidative stress following intake of ω-3 fatty acids, this study is aimed that EPA not only does not exacerbate oxidative stress, it but also results in the reduction of lipid peroxidation index**)** Malondialdehyde (MDA) **(** and increases the antioxidant activities of serum PON1 and HCTLase, as well as up-regulate the gene expression of PON2.

If this trial is successful, its findings may be applicable to people diagnosed with type 2 diabetes mellitus with minimal costs. Thus appropriate use of EPA in the diet of the patients with type 2 diabetes mellitus can be as a controller factor and preventive from the development of CVDs in these patients.

Trial status

This trial is performing now.

Abbreviations

ADA: American Diabetes Association, apo A-I: Apolipoprotein A-I, apo B: Apolipoprotein B, BMI: Body mass index, COX-2/LOX: Cyclooxygenase-2 and lipooxygenase, CVD: Cardiovascular disease, DBP: Diastolic blood pressure, DHA: Docosahexaenoic acid, FBS: Fasting blood sugar, ECs: Endothelial cells, EPA: Eicosapentaenoic acid, HCTLase: Homocysteine thiolactonase, Hcy: Homocysteine, HDL-c: High density lipoprotein cholesterol, 2hPG: 2-hour plasma glucose, hPON2 mRNA: PON2 mRNA in humans, IPAQ: International Physical Activity Questionnaire, MBP: Mean blood pressure, MCP-1: Monocyte Chemoattractant Peptide 1, MDA: Malondialdehyde, Met: Methionine, NO: Nitric oxide, Ops: Organophosphate, Ox-LDL: Oxidized LDL, PBMCs: Peripheral blood mononuclear cells, PLA2: Phospholipase A2, PON: Paraoxonase, PP: Pulse pressure, PUFAs: Polyunsaturated fatty acids, ROS: Reactive oxygen species, sAAs: Sulfydryl amino acids, SBP: Systolic blood pressure, TBARS: Thiobarbituric acid reactive substances, TC: Total cholesterol, TG: Triglyceride, tHcy: Total homocysteine.

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Competing interests

The authors declare that they have no competing interests.

Trial Registration: Clinical Trials.gov-Identifier.

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