Effects of DHA Supplementation on Vascular Function, Telomerase Activity in PBMC, Expression of Inflammatory Cytokines, and PPARγ-LXRα-ABCA1 Pathway in Patients With Type 2 Diabetes Mellitus: Study Protocol for Randomized Controlled Clinical Trial

Omid Toupchian[1], Gity Sotoudeh[2], Anahita Mansoori[1,3], Mahmoud Djalali[1], Seyyed Ali Keshavarz[4], Ensieh Nasli-Esfahani[5], Ehsan Alvandi[1], and Fariba Koohdani[1,5]

[1] Department of Cellular and Molecular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Community Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

[3] Nutrition and Metabolic Diseases Research Center, Jundishapur University of Medical Sciences, Ahvaz, Iran

[4] Department of Clinical Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

[5] Diabetes Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

Abstract- Docosahexaenoic acid (DHA), as an omega-3 fatty acid, in a natural ligand of peroxisome proliferator-activated receptors (PPARs). Regarding the combinative effects of Nutrigenomics and Nutrigenetics and due to the lack of in vivo studies conducted using natural ligands of PPARs, we aimed to evaluate the effects of DHA supplementation on vascular function, telomerase activity, and PPARγ-LXRα-ABCA1 pathway, in patients with type 2 diabetes mellitus (T2DM), based on the Pro12Ala polymorphism in PPARγ encoding gene. 72 T2DM patients (36 dominant and 36 recessive allele carriers), aged 30-70, with body mass index of 18.5 to 35 kg/m^2, will be participated in this double blind randomized controlled trial. In each group, stratification will be performed based on sex and age and participants will be randomly assigned to receive 2.4 g/day DHA or placebo (paraffin) for 8 weeks. PPARγ genotyping will be carried out using PCR-RFLP method; Telomerase activity will be estimated by PCR-ELISA TRAP assay; mRNA expression levels of target genes will be assessed using real time PCR. Serum levels of ADMA, sCD163 and adiponectin, will be measured using ELISA commercial kits. The present study is designed in order to help T2DM patients to modify their health conditions based on their genetic backgrounds, and to recommend the proper food ingredients as the natural agonists for PPARs in order to prevent and treat metabolic abnormalities of the disease.

Introduction

Type 2 diabetes mellitus (T2DM) is one of the prevalent causes of mortality and morbidity worldwide (1-3) which is characterized by macro- and micro-vascular complications leading to cardiovascular diseases (CVD) (4,5). Changes in intima–media thickness (IMT) have a major role in increasing CVD risk (6). IMT increases with aging and is associated with metabolic disorders such as T2DM and metabolic syndrome (7).

One of the IMT related pathways is the role of nuclear receptors in the proliferation of smooth muscle cells in the vascular wall. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors, which function as transcription factors and regulate the expression of a wide variety of genes. One of the members of this family, PPARα, is shown to have

Corresponding Authors: G. Sotoudeh* and F. Koohdani**

*Department of Community Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

**Department of Cellular and Molecular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

Tel: +98 21 88955975, Fax: +98 21 88955975, E-mail addresses: koohdanif@gmail.com, gsotodeh@tums.ac.ir

a key role in up-regulation of several tissue-specific genes involved in fatty acid uptake and β-oxidation (8,9), modulation of local cellular inflammation (10), plaque stabilization (11), prevention of plaque rupture, and controlling central obesity (12,13). Besides, recent studies have proposed that PPARα has a key role in the proliferation of intima-media smooth muscle cells (14). In the vascular wall, PPARα inhibits cell-cycle progression at G1/S transition through the induction of p16INK4a (14). P16 is a tumor suppressor and can inhibit the activity of cyclin-CDK complexes. Moreover, telomerase stabilizes telomeres and prevents cellular senescence, and it is shown that in humans, p16 could inhibit the telomerase activity through the inhibition of reverse transcriptase subunit of the telomerase (15,16).

In addition, PPARα activation by synthetic ligands such as fibrates could inhibit the expression of cytokine genes involved in acute-phase and inflammatory responses, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). This suggests an anti-inflammatory role for PPARα (17). Other studies also showed that PPAR activation might have an impact on serum level of asymmetric dimethylarginine (ADMA). This metabolic by-product impairs normal endothelial function by inhibiting endogenous nitric oxide synthetase enzyme and nitric oxide production (18,19). Amounts of this mediator are up-regulated along with sCD163 in a series of inflammatory diseases such as type 2 diabetes and CAD.

Reverse cholesterol transport is a multi-step process, initiated by ABCA1 (ATP-binding cassette transporter), to bring cholesterol to Apo A-I in HDL structure. Since LXR response elements are located in the promoter region of ABCA1 gene, LXR can alter ABCA1 expression level. Also, LXR agonists can stimulate ABCA1 gene expression and upregulate reverse cholesterol transport (20).

The idea of the current study was originated from three topics: a) the major role of the aforementioned molecules in vascular function, IMT, cholesterol reverse transport, and CVD risk; b) strong relationship between PPARγ2 Pro12Ala polymorphism with obesity and diabetes; c) lack of in vivo studies on natural PPARα and PPARγ ligands. Therefore, we decided to design an in vivo study to evaluate the effects of docosahexaenoic acid on the relevant factors affecting vascular function, intima-media smooth muscle proliferation, and PPARγ-LXRα-ABCA1 pathway in patients with T2DM.

Main objectives

The present trial is one of the first investigations to study the combinative effect of nutrigenetics and nutrigenomics. This study will run in two steps. First, in the nutrigenetics part of the study, PPARγ2 Pro12Ala polymorphism will be examined in about 600 T2DM patients in order to find the carriers of dominant and recessive alleles as the preliminary data for the second step. The main objective of the second step (intervention) is to investigate the effects of DHA supplementation on vascular function, telomerase activity in PBMC, expression of inflammatory cytokines, and PPARγ- LXRα- ABCA1 pathway.

Specific objectives

I) Determining the frequency of PPARγ2 genetic variants in the study population.

II) Comparing the within- and between-group changes regarding the telomerase activity; serum levels of ADMA, sCD163, and adiponectin; P16, IL-6, TNF-α, ABCA1, and LXRα gene expression between carriers of dominant and recessive alleles and between DHA and placebo groups.

III) Comparing within- and between-group changes in serum lipid levels, including serum triglycerides, total cholesterol, HDL and LDL cholesterol; fasting blood sugar, fasting insulin, and insulin resistance between carriers of dominant and recessive alleles and between DHA and placebo groups.

IV) Comparing within- and between-group changes in anthropometric; obesity and central obesity indices (WHR and WHtR), dietary intakes, and physical activity levels between carriers of dominant or recessive alleles and between DHA and placebo groups.

Questions and hypotheses

I) what is the frequency of subjects with dominant and recessive alleles of PPARγ2 polymorphism?

II) DHA supplement could alter telomerase activity, serum levels of ADMA, sCD163, and adiponectin, and P16, IL-6, TNF-α, ABCA1, and LXRα gene expression.

III) DHA supplement could ameliorate lipid profile, fasting blood sugar, fasting Insulin, and insulin resistance.

IV) DHA supplementation could improve anthropometric parameters.

Materials and Methods

Study design

We will conduct an 8-week, double blind, randomized, controlled trial (RCT) in parallel groups to determine the effects of DHA supplementation on

factors affecting vascular function, telomerase activity, and PPARγ-LXRα-ABCA1 pathway in T2DM patients. Before initiating the intervention, about 800 T2DM patients have been contacted and screened for eligibility and consent. Previously, PPARγ2 Pro12Ala (rs1801282) polymorphism (CCA>GCA) has been genotyped by PCR-RFLP method on 600 T2DM subjects. Among them, 36 recessive allele carriers (recessive group) and 36 CC genotype (dominant group) were included. In each group, stratified randomization (based on age and sex) will be performed to randomly allocate the patients to receive DHA or placebo. Finally, the following groups, with 18 patients in each, will be studied as follow: I) subjects with GC or GG genotypes to receive DHA supplement; II) subjects with GC or GG genotypes to receive placebo; III) CC genotype group to receive DHA supplement and IV) CC genotype group who will receive placebo. Patients in each group will receive 2.4 g/d of DHA or placebo (4 soft gels per day) for 8 weeks. All the assessments (nutritional, anthropometric, metabolic, and genetic assessments) will be conducted twice (baseline and endpoint) during the intervention.

Initially, demographic and physical activity questionnaires will be provided, and food record forms will be delivered to the patients to be completed at home (three days in the first week of intervention and three days in the final week of intervention). Anthropometric measurements, body composition, metabolic profile, telomerase activity, mRNA expression of Il-6, TNF-α, LXR, and ABCA1 genes, and serum levels of ADMA, sCD163, and adiponectin will be measured once at the beginning and the other at the end of the intervention.

In order to reduce the confounding effects of changes in physical activity and dietary intakes on the studied parameters, all the patients will be requested to maintain their common diets, physical activity levels, and lifestyle factors throughout the intervention.

Ethics and trial registration

The patients who meet the inclusion criteria will be fully informed about the study's protocol. This protocol, approved by Medical Ethics Committee of Tehran University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: 92.01.16.20454.89521). Each subject will sign an informed consent form. This investigation was registered on Iranian Registry of Clinical Trials on 15 September 2013 (Irct registration number: IRCT2013071213964N1).

Participants
Inclusion criteria

The inclusion criteria are as follows for both male or female subjects: Type 2 diabetes mellitus (lasted at least 3 months after the diagnosis), BMI range of 18.5 to 35 kg/m2, age 30-70 years old.

Exclusion criteria

The participants with any of the following criteria will be excluded: insulin therapy, pregnancy or lactation, thiazolidinedione therapy, the history of liver, kidney, coagulation and thyroid disorders, the consumption of anticoagulant, non-steroidal anti-inflammatory and lipid lowering agents, the history of allergic reaction to fish or fish oil, daily consumption of fish or omega-3 supplements and vitamins A, D or B12.

Recruitment

All 800 T2DM patients were previously recruited from Iranian Diabetes Society and Gabric Diabetes Society, and PPARγ2 Pro12Ala genotyping was performed on approximately 600 patients. Then, considering the inclusion and exclusion criteria, the patients will be invited on schedule (Figure 1).
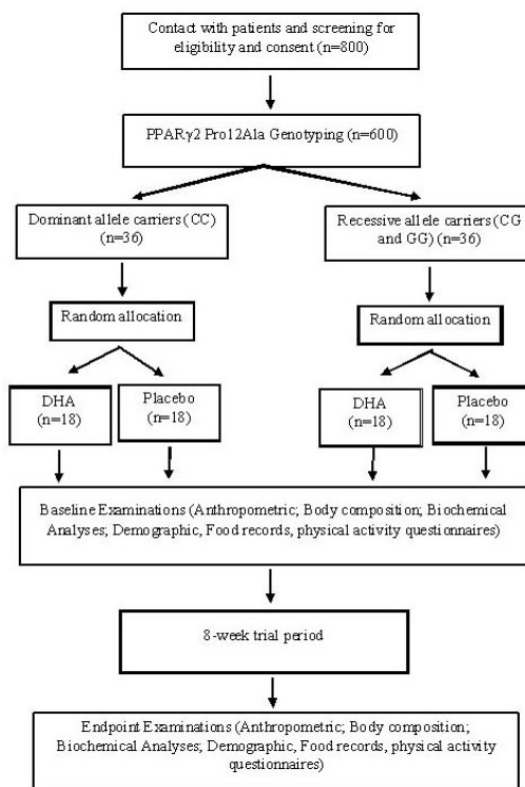

Figure 1. Overview of the Study

Sample size calculation

The sample size was calculated according to the changes in ADMA level, in response to EPA supplementation, based on the study conducted by Hagiwara *et al.*, (21). It was calculated considering 95% confidence interval, 80% power ($\alpha=0.05$ and $\beta=0.2$), and the related mean and SD of ADMA levels in the mentioned study ($\mu1=0.48$; $\mu2=0.44$; $SD1=0.06$; $SD2=0.05$). It was then increased by 20% because of the possible dropout in the recruited subjects. Four groups were defined, each including 18 subjects, with the total sample size of 72 participants. Since the recessive allele frequency of Pro12Ala SNP in T2DM patients of the Iranian population was approximately 6%, the primary sample size for genotyping, i.e. before the intervention, was calculated as 600 subjects (22).

Randomization

After identification of the genotypes in PPARγ Pro12Ala polymorphism, the carriers of Ala allele will be matched with non-Ala carriers according to age (<50 or ≥50), sex (male or female), and body mass index (18.5-24.9, 25-29.9, and 30-34.9 kg/m2). Next, the eligible participants will be randomly allocated to receive DHA or placebo. Randomization in each genotype group will be undertaken using permuted blocks. Stratification factors are age (<50 or ≥50) and sex (male or female).

Blinding

In this double blind clinical trial, the supplements (DHA or placebo soft gels) will be packaged into containers in order not to be detectable by the patients or administrators. Each bottle contains 120 soft gels which are the required amount for one-month usage. All the patients will be requested to give back the first container (empty or remained capsules) at the end of the first month and receive the second bottle. A person, who is not involved in this project will label the containers as A or B and also will enumerate and record the remained capsules of each container that will be returned by the patients. At the end of the first week of intervention, all the patients will be contacted separately and will be questioned about the bottles' content (DHA or placebo) and their opinion about the procedure to check the blinding nature of the intervention.

Intervention

All the patients will take four soft gels (orally) per day for 8 weeks. Two soft gels should be taken in the morning (after breakfast), and two others after the dinner. Each DHA capsule contains 600 mg of omega-3 fatty acids, more specifically, 363 mg of DHA plus 100 mg of EPA, according to the manufacturer (Pure Encapsulations, Boston, USA). Other ingredients are gelatin capsule; fish (tilapia), natural mixed tocopherols, and rosemary extract (leaf). The placebo group will receive paraffin soft gels that are identical in appearance to DHA capsules and contains 600 mg paraffin (Zahravi, Tabriz, Iran). The participants will continue their usual medical treatment throughout the supplementation period.

Compliance

All the participants will receive a notepad with the written date of admission. During the two-month intervention, all the subjects will be requested to record the number of consumed soft gels daily. This notepad also contains some other notifications, such as changes in drugs consumed by patients and changes in physical activity during the intervention period. After two months of intervention, the patients will be requested to bring their notepads and the second bottle containing the remained soft gels. Each subject should consume 240 soft gels during the study. The remained soft gels will be counted and, according to the check marks in the notepads, the number of capsules used by each patient will be calculated. At the end of the intervention, if the remained capsules exceed 10% of total administered capsules (12 capsules), that subject will be excluded from the study.

Forms and questionnaires

A questionnaire about patients' demographic situations, diseases, and medications, diabetes history and probable supplementations will be recorded at the beginning of the study. METs physical activity questionnaire will be recorded for estimating the physical activity at the beginning and end of the intervention.

Assessment of dietary intake

The participants will be requested to complete a 3-day diet record (2 weekdays and 1 weekend day) in the first week of intervention and another 3-day diet record in the last week of intervention, according to the instructions on food recording by a dietician. Dietary data will be analyzed using Nutritionist IV (The Hearst Corporation, San Bruno, CA).

Anthropometric parameters and body composition

Anthropometric parameters will be measured by the same dietician, after overnight fasting, with minimal clothing, and without shoes. Body weight will be

measured with the accuracy of 100 g using Seca scale. Stature will be measured in a relaxed position and freely hanged arms using Seca stadiometer with the accuracy of 0.5 cm. BMI will be calculated as body weight (kg) divided by the square of the height (m). Waist and hip circumference will be assessed at above the iliac crest, just below the lowest rib margin at normal minimal respiration, and as the maximum circumference over the buttocks to the nearest 0.5 cm, respectively. Afterward, waist to hip ratio (WHR) will be estimated as the index of body fat distribution. Waist to stature ratio (WSR) will be calculated by dividing waist circumference to stature.

TANITA BC-418 (Tanita Corporation of America, Inc.) segmental body composition analyzer will be employed to calculate total and segmental body composition, including total body fat and fat percent, total body water, fat-free mass, fat-free mass percent, and abdominal fat.

General biochemical variables

After 12-hour overnight fast, 20 ml of venous blood samples (10 ml in EDTA-coated sterile tubes and 10 ml in regular tubes) will be collected. The blood in EDTA-coated tubes will be used for collecting WBC and subsequent molecular analysis. Second tubes will be used for biochemical analyses such as the determination of adiponectin, glucose, insulin, HBA1C, total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol in serum. The plasma glucose, total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol levels will be measured by the automated enzymatic method. HbA1c and circulating insulin will be measured by HPLC and Immunoassay, respectively. Commercially available ELISA kits will be used to measure serum adiponectin (Mediagnost, Germany), ADMA (Biovendor, Czech), and sCD163 (My Biosource, San Diego, CA). HOMA-IR score, as an index for insulin resistance, quantitative insulin sensitivity check index (QUICKI) as an insulin sensitivity (IS) index, and homeostasis model assessment of beta-cell function (HOMA-B) as beta cell function index will be calculated using the following formulas (23).

HOMA-IR: (Fasting plasma glucose mmol/l×fasting serum insulin mU/l)/22.5

QUICKI: 1/(log (fasting insulin µU/mL)+log (fasting glucose mg/dL)

HOMA-B: (20×fasting insulin µU/ml)/(fasting glucose mmol/l−3.5)

Genotyping of PPARy2 Pro12Ala polymorphism

Genomic DNA was extracted from the whole blood samples of 600 T2DM patients using salting-out method (24). PCR was performed in 25 µL mixture, containing 2X Taq polymerase mix (Amplicon co.), 75 ng DNA, and 0.6 mM of each primer (Forward: 5'-CTGATGTCTTGACTCATGGGTGTATTCAC-3'; Reverse: 5'-ACAGTGTATCAGTGAAGGAATCGCTTTCCG-3') in 35 cycles of 94°C for 30s, 58°C for 40s, and 72°C for 40s on PeQSTAR thermocycler system (PeQLab, Germany). Genotyping was carried out by RFLP method using BstU-I at 37°C overnight. The digested PCR products (CC: one 196bp band; CG: 196, 166, and 30bp bands; GG: two 166 and 30 bp bands) were run on 8% polyacrylamide gel electrophoresis with subsequent staining by fluorescent red dyes (Biotium Inc.).

RNA extraction

Anticoagulated blood will be diluted with an equal volume of PBS. Then, PBMCs will be isolated by density gradient centrifugation with Ficoll for 40 min at 800 g at 4°C. The mononuclear cell layer will be collected, washed with PBS and centrifuged for 10 min at 275 g at 4°C. Total mRNA will be extracted from PBMCs using Hybrid-R blood RNA isolation kits (GeneAll, Korea). RNA samples will be converted into cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Real-time polymerase chain reaction

The expression levels of P16, TNF-α, IL-6, ABCA1, and LXRα genes will be analyzed by quantitative Real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.) on Step-One real-time PCR system (Applied Biosystems). All the primer sequences are listed in table 1. The relative changes in mRNA expression will be determined by Pfaffl method.

Table1. Real-time PCR primers

	Forward	Reverse
TNF-α	CCAGGGACCTCTCTCTAATCAG	TGAGGTACAGGCCCTCTGATG
IL-6	GACAGCCACTCACCTCTTCAG	GTGCCTCTTTGCTGCTTTCAC
ABCA1	ATGGCACTGAGGAAGATGCTG	CTCAGCCATGACCTGCCTTG
LXR-α	CGCACTACATCTGCCACAG	TCAGGCGGATCTGTTCTTCT
P$_{16}^{INK}$	CTTCCTGGACACGCTGGTG	GCATGGTTACTGCCTCTGGTG
GAPDH	TGGTATCGTGGAAGGACTCATG	GCTTCACCACCTTCTTGATGTC

Measurement of telomerase activity

Telomerase activity will be assayed by telomerase PCR ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) based on the telomeric repeat amplification protocol (TRAP). The process has two steps: First, telomerase adds telomeric TTAGGG sequences to the 3′ end of the biotinylated synthetic P1-TS-primer and then the products will be amplified by PCR (elongation/amplification step). Second, PCR product will be hybridized to a probe, immobilized to a streptavidin-coated microplate, and is detected by an anti-digoxigenin antibody conjugated to peroxidase (25).

Statistical analyses

One-sample Kolmogorov-Smirnov test will be used to establish the normal distribution of data, and non-parametric values will be transformed logarithmically for statistical analyses and then back-transformed to natural units for exhibition in tables or figures. All data will be expressed as mean±SD and P.value<0.05 will be defined as statistically significant for all comparisons. All Data will be analyzed using SPSS software (ver. 17.0).

Independent sample t-test or Analysis of Variance (ANOVA) followed by post-hoc analysis will be used for comparing parametric continuous data among the groups. Mann-Whitney U test will be used to test the differences in asymmetric variables between the groups. Pearson's correlation coefficient will be applied to show the correlation between biochemical and anthropometric indices. General linear models will be used to assess the effects of DHA relative to paraffin after adjustment for baseline values.

Discussion

Since diabetes affects many vital organs in the body and long-term complications of diabetes, such as cardiovascular involvement, neuropathy, retinopathy and nephropathy develop gradually; an accurate metabolic profile control can help prevent these complications. Administered drugs, such as lipid and blood pressure reducing medications, have beneficial effects for controlling the metabolic profile and preventing hazardous conditions. However, there are some reported adverse side effects which in some cases were dangerous for patients. For example, statins are associated with deteriorating glucose homeostasis, increased risk of diabetes mellitus, and renal failure in a dose-dependent manner. Other rare conditions attributed to statin therapy include myalgia, myopathy, changes in hepatic enzymes, and rhabdomyolysis (26,27). Some other side effects also reported by fibrates are myopathy, increased risk for gallstones, acute renal injuries (28), increased concentration of plasma homocysteine (29), and increased the concentration of plasma creatinine (30). Furthermore, multi-drug therapy, which is prevalent in diabetic patients, can lead to drug-drug interactions and reduced drug efficacy.

In this study, our challenge is to investigate the effects of DHA supplementation, as a natural ligand for PPARs in metabolic pathways, on the prevention and reduction of CVD risk and controlling the complications caused by diabetes. This approach can lead to a reduction in the number or dose of synthetic drugs and the improvement in the quality of life for patients with T2DM.

Trial status

About 600 patients were genotyped for Pro12Ala polymorphism in PPARγ2 gene and trial step started with the inclusion of patients. While submitting the manuscript, 58 patients were included after signing the informed consent.

Acknowledgement

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