Original Article

Effect of Eicosapentaenoic acid (EPA) supplementation on cardiovascular markers in patients with type 2 diabetes mellitus: A randomized, double-blind, placebo-controlled trial

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\begin{abstract}
Aims: Cardiovascular complications are one of main cause of increased mortality and morbidity among Diabetes Mellitus (DM) patients. Altered metabolism of sulphur amino acids in diabetes reflected as increases in concentration of methionine and cysteine/cystine in the blood which known as a markers of Cardiovascular Diseases (CVD). The aim of present study was to determine the effect of Eicosapentaenoic acid (EPA) supplementation on sulphhydryl amino acids and Atherogenic Index of Plasma (AIP) in patients with type 2 DM (T2DM).

Method: A randomized, double-blind, placebo-controlled clinical trial was performed in 36 control and patients with DM. The subjects were randomly assigned to obtain 2 g/d EPA (n = 18) or placebo (n = 18) for 8 weeks. Fasting serum level of Cystein and Methionine were measured using HPLC method and atherogenic index of plasma (AIP) as a proxy measure of atherosclerosis was computed.

Results: Eight weeks supplementation with EPA led to significant reductions in Met (p < 0.002) and Cys (p < 0.001) compared with the placebo (p < 0.06). In addition, compared to placebo a significant reduction in AIP were seen after taking EPA (p < 0.04).

Conclusion: EPA supplementation in patients with T2DM for eight weeks had beneficial effects on Met, Cys and AIP, which may attribute to the prevention of vascular complications in the T2DM patients.© 2018 Diabetes India. Published by Elsevier Ltd. All rights reserved.
\end{abstract}

1. Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder with hyperglycemia in which insulin becomes disabled to carry out main role because of impaired insulin secretion, insulin resistance or both [1]. The prevalence of diabetes is rising all over the world and it is anticipated that the prevalence of type 2 DM (T2DM) increase to more than 366 million people worldwide in 2030 [2]. T2DM is more prevalent than T1DM, and is responsible for 90% of diabetes cases [3]. Chronic hyperglycemia is contributed to many chronic microvascular and macrovascular complications such ascardiovascular diseases, neuropathy and nephropathy [4,5].

Recently, amino acids have been suggested as new biomarkers indicating metabolicmarks of insulin action [6]. Furthermore, a positive relationship between branched-chain and aromatic amino acids and risk for future insulin resistance and cardiometabolic disorders has been implicated [7–9].

Literature review revealed that inappropriate insulin action is associated with alteration in sulphur amino acid metabolism reflected as subtle increases in concentrations of methionine and cysteine/cystine in the blood [10]. DM thorough coronary artery disease, dyslipidemia and hypertension may lead to cardiovascular complication [11]. Diabetes dyslipidemia is described as a mainroot for the development of atherosclerosis and cardiovascular complication [12]. Particularly, atherogenic dyslipidemia defines as high triglyceride (TG) and low high-density lipoprotein cholesterol (HDL-C) levels directly related with adverse clinical outcomes [13]. The TG/HDL-C ratio consider as a marker of insulin resistance [14]. Furthermore, a greater TG/HDL-C ratio was implicated with cardiovascular disease [15] and type 2 diabetes patients [16]. The Atherogenicindex of Plasma (AIP), calculated as log(TG/HDL-C), is useful formula in the estimation of atherosclerosis and coronary-heart disease than other lipid profile measurements [17]. HDL are normally associated with reduced CVD risk because of their

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antioxidant and reverse cholesterol transport capabilities and their ability to stimulate glucose uptake and fatty acid oxidation contrasting insulin resistance [18]. However function of HDL may be flawed in T2DM due to increased lipid peroxidation [19].

We hereby present data of a double-blind, placebo-controlled, randomized; studyin T2DM patients receiving daily 2 g purified EPA or placebo for 8 weeks. Fasting levels of circulating Methionine and Cystein and AIP were measured.

2. Subjects, materials and methods

2.1. Participants

All participants aged 30–65 years with type 2 diabetes diagnosis based on available guidelines (American Diabetes Association [20]) were recruited during February 2014 to June 2015 from Iran Diabetes Association, Tehran University of Medical Sciences (TUMS), Tehran, Iran. The cases were enrolled in the study if they met these inclusion criteria: [1] Affliction history of at least 1 year before the study [2] BMI < 35 [3]; taking the anti diabetic's drug(s) dose at least for 3 months. All the subjects excluded if they [1] had history of Hypertension, Renal, Arrhythmia, Gastrointestinal, Hepatic, Endocrinological or hematological disease, [2] need to take insulin, do not use (noncompliance) supplements (<10%). The study was based on the Declaration of Helsinki. All the subjects filled up written informed consent after verification the study protocol by the TUMS ethical committee. The trial was registered in the Clinical Trials (www.clinicaltrials.gov) for registration of clinical trials (NCT03258840).

2.2. Study design

This was a randomized, placebo-controlled, double-blind parallel-group clinical trial. At study baseline and after stratification for gender and age, Subjects were randomly allocated to receive 2 g/day of the softgels of EPA (n = 18) ([softgels containing Eicosapentaenoic acid ethyl ester (75%), Mino Pharmaceutical Co, Iran]) (supplied as 1 g softgels) or placebo (Edible paraffin by Mino Pharmaceutical Co., Iran) (n = 18) for 8 weeks. EPA and its placebo were in the same appearance such as colour, shape, size and packaging, which coded by the manufacturer to ensure blinding. Randomized allocation sequence and assigned participants to the groups was done by study technician allocate patients to EPA group and placebo group. Randomized allocation was not disclosed to the researchers and patients until the main analysis.

The participants were strictly advised to not change their usual diets and nutritional habits, dosage and type of medication during study to avoid their possible effect on the study findings. Compliance to the EPA supplementation was controlled through phone interviews, weekly and asking participants to return the medication containers, monthly.

2.3. Sample size calculation

Sample size calculated according to type one (α) and type two errors (β) as 0.05 and 0.20 (power = 80%), respectively and according to the previous study. Standard deviation (SD) and difference in mean or effect size (d) of serum Paraoxonase considered as 11.61 U/ml and 11.4 U/ml respectively as the key variables [21]. Although we needed 16 subjects in each group but because of possible dropouts 36 participants were participated in the study.

2.4. Anthropometry and physical activity assessment

Body weight was measured without shoes and in a minimum clothes condition by the use of a digital scale (Seca, Hamburg, Germany) to the nearest 0.1 kg. Height was calculated to the nearest 0.1 cm by using aninelastic tape measure (Seca, Hamburg, Germany). BMI was calculated as weight in kg divided by height in meters squared. Waist circumference was assessed by tape measure (Seca; Germany) at the midpoint between the costal margin and the iliac crest.

2.5. Nutritional and medical history assessment

All patients filled up a general questionnaire regarding demographic variables (age, sex) and lifestyle habits (including the history of smoking, alcohol consumption) medical and drug history (heart rate, and measurements of systolic, diastolic blood pressure (SBP and DBP)), family history of diseases (diabetes, hyperlipidemia and hypertension, cardiovascular, etc) at the beginning and end of study.

Dietary intake was measured using a 24-h recall method for 3 days (including 2 working days and 1 weekend day) a week before and at the end of supplementation. The dietary recalls were analyzed using Nutritionist IV software (First Databank, San Bruno, CA, USA) adjusted for Iranian foods.

2.6. Biochemical assessment

Fasting blood samples (10 ml) (overnight fast for 10–12 h) were obtained from the antecubital vein at the beginning and the end of study (8th week) then centrifuged (3000 rpm for 10 min at 4 °C) for serum acquisition and stored at −80 °C until analysis.

2.7. Chemicals

Amino acid standard (Cys and Met), tri-n-buthylphoshine, dimethylformamide, sodium acetate, 7-Fluoro-2,13-Benzoxadiazole-4-Sulfonamide (ABD-F), were from Sigma (St. Louis, MO, USA). Acetonitrile, methanol and tetrahydrofuran was from Merck (Merck, Germany).

2.8. Sample preparation and HPLC analysis

The HPLC method includes reduction of the plasma samples with tri-n-buthylphoshine, in dimethylformamide in order to reduce thiols and to decouple them from proteins. Then sample were mixed with trichloroacetic acid solution containing Na2EDTA under vigorous vortexing, followed by centrifugation. At last borate buffer, NaOH and ABD-F were added to the cleared supernatant.

The sample injected in reverse phase (RP) high-performance liquid chromatographer (Agilent, 1260). The the chromatographic column (Intersil ODS–3 V, 5 μm-4.6”250 mm, C/N 5020-01802) was equilibrated with acetonitrile in acetate and methanol buffer then amino acids concentration fluorometrically measured (Excitation: 340, Emission: 450, 37C, pH = 7). Quantization was performed by a standard curve equation in a range of 0.05–10 μM. Tests with standard fresh solutions were frequently injected during analysis.

2.9. Atherogenic index of plasma equation

Based on previous studies atherogenic index of plasma were calculated according to following formula:

Atherogenic Index of Plasma: [Log(Triglycerides/HDL-Cholesterol)]

2.10. Statistical analysis

Normal distribution of all variables was tested by the Kolmogorov-Smirnov test. All variables were reported as mean ± standard deviation (SD). Within group comparisons were
completed by paired-sample t-test. The Independent t-test was carried out to identify differences between the two groups independently. Analysis of covariance (ANCOVA) was performed to identify any differences between the two groups at the end of the study, adjusting for baseline value. P < 0.05 was considered as statistically significant. All statistical analyses were performed by the Statistical Package for Social Science version 17 (SPSS Inc., Chicago, Illinois, USA).

3. Results

As presented in Table 1, at baseline, the demographic characteristics, were not significantly different between two groups. There were no significant differences between two groups in duration of disease (p = 0.73). There were no significant differences between two groups in dietary intake of total energy, carbohydrates, proteins, fats, and fibers at the baseline and end of the trial.

Table 2 represents the effect of EPA supplementation on Met, Cys and AIP. Baseline Met and Cys were not significantly different between two groups. Compared with the placebo, after adjusting for baseline value EPA intake led to significant reductions in Met and Cys (p < 0.05). In addition, significant reductions in AIP were seen after taking EPA supplementation compared with placebo (p = 0.04) (Table 2).

4. Discussion

Present study is one of few studies which evaluating EPA supplementation on serum fasting level of amino acids in a double-blind, placebo-controlled, randomized study. Our results showed that EPA supplementation cause significant reduction in Met and Cys in comparison with control group. However AIP were decreased significantly in EPA group in comparison with control group. These results obtained while dietary intake also showed any statistical difference between EPA and control group.

Recent reports like Framingham offspring study have been indicated that plasma-free amino acid profiles altered before development of T2DM and CVD [22]. Comprehensive metabolite profiling studies have showed that differences in plasma concentrations of cysteine [23] or methionine [24] follow insulin resistance. Significantly higher Cysteine concentration reported in T2DM patients compared to nondiabetics [25]. Obese nondiabetic patients also showed modest increases of methionine of 9% to 22%, cysteine/cysteine were 10–14% concentration compared with non-obese controls. The aggregate of results revealed that impaired insulin action is associated with increases in blood level of methionine and cysteine/cysteine [10].

Adams explain impaired amino acid metabolism by impaired insulin action, increased FAA oxidation, and co-occurring changes in mitochondrial redox status that attenuate specific catabolic pathways that sequentially increase tissue and blood concentrations of BCAA and sulphur amino acids [26]. Some studies introduce methionine restriction as an effective dietary manipulation to improve insulin sensitivity relative to animals on a control diet [27].

Previous reports indicated increased total Cys levels in patients with vascular diseases including peripheral and cerebral vascular diseases and coronary heart disease [28], El-Khair et al. reported that serum total Cys levels are associated with several CVD risk factors such as BMI and DBP [29]. Low HDL-c levels, high triglyceride levels and high blood pressure, components of Metabolic Syndrome, also were reported in subjects with high serum Cys levels [26,30]. Inflammatory markers like TNF-α (Tumor Necrosis Factor-alpha) and C-reactive protein (CRP) closely related with serum Cys level [26]. Cys could be suitable candidates for early biomarkers in asymptomatic subjects at increased risk of developing MetS. Recent study reported that Stage 2 Metabolic Syndrome is related with higher Cystein concentration compared with stage 0 MetS. In that study in MetS1 group, Cys were 10-fold higher than in group MetS0, while in group MetS2 were 19.3 times higher than in the group MetS0 [26]. Cys also may involve in

Table 1

<table>
<thead>
<tr>
<th>Variables</th>
<th>Placebo (n = 18)</th>
<th>EPA (n = 18)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (Female/Male)</td>
<td>9/9</td>
<td>9/9</td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>44.72 ± 4.69</td>
<td>44.44 ± 3.79</td>
<td>0.846</td>
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<tr>
<td>Duration of DM (years)</td>
<td>6.61 ± 6.85</td>
<td>6.44 ± 2.83</td>
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<td>Weight (kg)</td>
<td>78.30 ± 12.34</td>
<td>78.03 ± 12.68</td>
<td>0.947</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.11 ± 8.85</td>
<td>165.39 ± 8.12</td>
<td>0.922</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>28.92 ± 5.39</td>
<td>28.49 ± 3.95</td>
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</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>97.47 ± 10.93</td>
<td>97.53 ± 9.65</td>
<td>0.981</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124.11 ± 15.32</td>
<td>124.00 ± 16.25</td>
<td>0.983</td>
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<tr>
<td>DBP (mmHg)</td>
<td>80.00 ± 6.69</td>
<td>79.78 ± 13.40</td>
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</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>138.06 ± 49.13</td>
<td>143.72 ± 53.53</td>
<td>0.743</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.47 ± 1.67</td>
<td>7.89 ± 1.75</td>
<td>0.459</td>
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<tr>
<td>Total energy intake (kcal)</td>
<td>1953.94 ± 297.12</td>
<td>2070.67 ± 307.27</td>
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<tr>
<td>Carbohydrates intake (%)</td>
<td>260.32 ± 35.44</td>
<td>270.63 ± 50.37</td>
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<tr>
<td>Protein (g)</td>
<td>63.19 ± 14.78</td>
<td>68.95 ± 20.26</td>
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<tr>
<td>Total fat (g)</td>
<td>86.11 ± 22.68</td>
<td>92.71 ± 19.85</td>
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<tr>
<td>Fibers intake (g/1000 kcal)</td>
<td>14.75 ± 4.64</td>
<td>16.66 ± 4.99</td>
<td>0.243</td>
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</table>


* Independent Sample t-test.

Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (n = 18)</th>
<th>EPA (n = 18)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
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<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>27.60 ± 0.961</td>
<td>27.50 ± 1.39</td>
<td>0.94</td>
</tr>
<tr>
<td>End of trial</td>
<td>22.80 ± 0.899</td>
<td>28.92 ± 1.54</td>
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</tr>
<tr>
<td>P</td>
<td>0.001</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-4.71 ± 1.11</td>
<td>-2.43 ± 1.93</td>
<td>0.003</td>
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<tr>
<td>End of trial</td>
<td>20.15 ± 0.64</td>
<td>20.09 ± 0.94</td>
<td>0.95</td>
</tr>
<tr>
<td>P</td>
<td>0.001</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Atherogenic Index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.48 ± 0.64</td>
<td>-0.96 ± 0.71</td>
<td>0.001</td>
</tr>
<tr>
<td>End of trial</td>
<td>0.97 ± 0.11</td>
<td>1.02 ± 0.11</td>
<td>0.76</td>
</tr>
<tr>
<td>P</td>
<td>0.009</td>
<td>0.72</td>
<td></td>
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<tr>
<td>Change</td>
<td>-0.33 ± 0.11</td>
<td>-0.05 ± 0.14</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Obtained from Independent sample t-test.
* Obtained from paired T test.
* Obtained from ANCOVA, adjusted for baseline values.

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atherosclerosis and is a strong marker of endothelial dysfunction, in terms of abnormal flow-mediated dilatation as well [31]. Aminothiols may be particularly atherogenic in patients with dyslipidemia because it can support superoxide-mediated modification of LDL, production of free radicals species and forming foam cell [32]. Met metabolism is tightly regulated. Its metabolism, homocysteine, can be either remethylated to methionine or undergo transsulfuration to form cysteine [33]. Transsulfuration is elevated in high Met levels and by peroxides while being decreased by antioxidants [34]. Hyperhomocysteinemia and Cys-Hcy mixed-disulfide level associated with CVD maybe thorough increases in oxidative stress and impaired endothelial function [35]. Not only autooxidation of Cys occurs more readily than Hcy, but also in the presence of Cys autooxidation of Hcy and generation of reactive oxygen species increased dramatically [36]. We found that EPA supplementation decrease Met and Cys serum level significantly so it could be effective supplementation for prevention of cardiovascular complications of diabetes.

Dyslipidemia is a well-documented risk factor for CVD especially TG and HDL-C which are also key factors of Met5 and insulin resistance development [37]. AIP, a new biomarker of CVD and plasma atherogenicity, is associated with lipoprotein particle size and thus is more promising in the estimation of CVD than other lipid profile measurements [38]. In a study of 1433 subjects from 35 cohorts with various risks of atherosclerosis a strong positive correlation between AIP and indirect measure of LDL-C particle size were reported [39]. AIP is a strong marker for prediction of atherosclerosis and coronary heart disease the general population [13] and various populations with high cardiovascular risk [40,41]. Possible mechanisms by which higher AIP influence increased risk of CVD could be LDL-C particle size [39], insulin resistance [42], and metabolic syndrome [43] due to TG and HDL serum level. We found that after eight week supplementation with EPA, AIP decreased significantly in comparison with control group.

The present study has some limitations that must be mentioned. First, a small sample size which prevents any solid conclusion so it must be conducted in larger trials. Next, the duration of trial and the more long term should be considered. Third, molecular trials examining the exact mechanism that EPA decrease the serum levels of Met and Cys and reduce AIP is necessary. Maybe serum levels of inflammatory markers, as well as the percentage of EPA in the membrane of RBC measure in the future studies could be helpful. For these reasons, additional studies will be essential to conclude the common effect of our study results.

5. Conclusion

The aim of this study was to determine the effect of EPA supplementation on some CVD risk factors that have not been studied yet. We found that supplementation of EPA is very efficient in the reduction of Met and Cys which may attribute to the prevention of vascular complications in the T2DM patients.

Conflicts of interest

All the authors declared that they have no conflicts of interest.

Ethical considerations and Funding/Support

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors. Vice-chancellor of Tehran University of Medical Services supported this work financially.

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References


