Effect of Eicosapentaenoic acid Supplementation on the Serum Profile of Apolipoprotein (apo) A-I, apo B-100, and apo B-100/apo A-I Ratio in the Patients with Non Insulin-Dependent Diabetes

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Abstract: Background: There is sufficient evidence that the apo B-100 and apo A-I are as accurate indicators in the prediction of atherosclerotic vascular disease in the diabetic patients. EPA has the antioxidant, antiinflammatory, antithrombogenic, and antiarteriosclerotic properties. Therefore, we investigated the effect of EPA supplementation on the serum levels of apo B-100, apo A-I and apo B-100/apo A-I ratio in the diabetic patients. **Methods:** 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 were classified into 2 groups. They were supplemented with 2 g/day of the capsules of EPA or placebo. Blood sample for measurement of the serum levels of apolipoproteins and lipids, as well as FBS and HbA1c were given. **Results:** 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, and during the intervention, except for a significant increase of HDL-c and apo A-I, and a significant decrease in the serum levels of apo B-100 and apo B-100/apo A-I ratio, as well as a slight reduce of TC, LDL-c, TG and FBS in the supplement group. **Conclusions:** EPA is atheroprotective via increase in the serum levels of apo A-I, and decrease in the serum levels of apo B-100 and apo B-100/apo A-I ratio, as well as change in the serum levels of lipids, and FBS.

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Introduction

In the present century, type 2 diabetes mellitus is recognized as a major public health problem all over the world [1], and its prevalence has reached the epidemic proportions worldwide [2]. International Diabetes Federation (IDF) has predicted that the number of people with diabetes will increase from 240 million in 2007 to 380 million for the year 2025 [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 [3].

Apolipoproteins are the principal components of lipoprotein particles, and each class of lipoprotein particles is associated with the specific polipoproteins to play an essential role in the stabilization of lipoprotein structure and in the regulation of metabolism. Some of the apolipoproteins are as ligands to tissue receptors and other some act in the activation or inhibition of enzymes involved in metabolic processes in the blood circulation or tissues [6]. 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 cardiovascular disease (CVD) than total

cholesterol (TC) or LDL-c [7], and as markers of lipid lowering therapy [6].

There is only one molecule of apo B in each atherogenic particle. Therefore, apo B displays the total number of potentially atherogenic particles, and is a more important factor which contributes to the development of atherosclerosis, thus the measurement of this protein could be better and more reliable in the prediction of risk [8].

The apo B/apo A-I ratio indicates the balance between two atherogenic and antiatherogenic processes i.e. transport of cholesterol to the peripheral tissues along with its subsequent influx inside artery, and reverse transport of cholesterol to the liver [9]. There is sufficient evidence to demonstrates the apo B/apo A-I ratio is a more adequate and powerful indicator than the TC/HDL-c and LDL-c/HDL-c ratios in the prediction of atherosclerotic vascular disease risk [10], so that a linear relationship was found between the apo B/apo A-I ratio and risk for CVD [11].

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 [12], and the endothelial dysfunction is a main initiating step in the development of atherosclerosis in the patients with type 2 diabetes mellitus [13], thereby, these patients are at increased risk of atherosclerotic vascular disease [14]. Therefore, the interventions aimed in order to decrease the hepatic secretion of lipoproteins containing apo B could be have great clinical importance.

However, there is evidence that indicate dietary fatty acids can influence the plasma levels of apo B-100 [13]. Eicosapentaenoic acid (EPA) is one of ω-3 PUFAs which are present at the great amounts in the fish oil [15]. The findings of several studies have shown that EPA has the antioxidant [16], antiinflammatory [17], antithrombogenic [18], and antiarteriosclerotic [19] properties. The present study provides the first description of the effects of Eicosapentaenoic acid on the serum profile of apolipoprotein (apo) A-I, apo B-100, and apo B-100/apo A-I ratio in the patients with type 2 diabetes mellitus.

Material and Methods

1. Patients and Study Design:

1. 1. 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 [20] were recruited.

1.1.1. 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 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 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 to the acute inflammatory diseases; according to the consultant physician endocrinologist.

1.2. Study Design:

The study protocol was designed as a randomized, double-blind, and placebo-controlled clinical trial. At the 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 softgels of EPA or placebo (supplied as 1-g softgels), the two groups were randomly 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 were provided by Mino Pharmaceutical Co., Iran. They were strictly 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 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 softgels had used and the number of softgels returned to the study center at the time of specified visits. The patients were followed up by telephone each week.

1.2.1. 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.

1.2.2. 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. 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/height² (kg/m²). According to the recommendation of International Diabetes Federation, hypertension was defined as blood pressure ≥130/85 mmHg [21].

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 of apo A-I, apo B, apo B/apo A-I ratio, FBS (fasting blood sugar), HbA1c, the serum TC, TG, LDL-c and HDL-c]. The blood samples were collected only for this study.

1.2.3. Measurement of the Serum Levels of Apolipoprotein (apo) A-I and apo B:

The serum levels of apolipoprotein A-1 and apo B were measured by immunoturbidimetry (Hitachi autoanalyzer, model 705, Daiichi). Standardization was performed with commercially available material (Boehringer, Mannheim, Germany). Measurement ranges were 104 to 202 (for male) and 108 to 225 mg/L (for female) for apo A-1, as well as 66 to 133 (for male) and 60 to 117 mg/L (for female) for apo B, respectively.

1.2.4. 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).

1.2.5. Statistical Analyses:

The data were analysed 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. 24-hour diet recalls analysed using Food processor II software [22], 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

1. Patient Characteristics:

The baseline 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.

2. 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 (data not shown). 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).

3. Compliance and Side Effect:

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

4. The Serum Levels of Apolipoprotein (apo) A-I and apo B-100 and apo B-100/apo A-I Ratio:

There were no significant differences in the serum levels of apo A-I between the two groups of patients at the baseline (Table 2), whereas as shown in Table 2, the serum levels of apo A-I 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 apo B-100, whereas the serum levels of apo B-100

in the EPA receiving patients compared with the placebo receiving patients decreased significantly ($p < 0.\ 0.001$)

There were no significant differences in the serum ratio of apo B-100/apo A-I between the two groups of patients at the baseline (Table 2), whereas as shown in Table 2, the serum ratio of apo B-100/apo A-I decreased significantly ($p < 0.005$) in the EPA receiving patients compared with the placebo receiving patients.

5. 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. The serum 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 31.38 ± 4.76 mmol/L after receiving placebo and 37.11 ± 5.97 mmol/L after the supplementation with EPA. The serum triglycerides was 162.8 ± 158.81 mmol/L after receiving placebo and 176.48 ± 133.75 mmol/L after the supplementation with EPA (Table 3).

Discussion:

1. Apolipoprotein B, Apolipoprotein A-I and apo B/apo A-I Ratio in the diabetes mellitus:

It is important to point out that several studies have shown that apo B and non-HDL-c are as better and more reliable predictors of CVD than LDL-c in the diabetic patients [8, 23]. Also, the apo A-I is previously reported as a new marker predicting risk in the diabetes mellitus to be independent of the apo B categories and apo E genotype [24]. Furthermore, the more accurate and close relationship apo B/apo A-I ratio with carotid intima-media thickness (IMT) than the LDL-c/HDL-c ratio has been demonstrated in the patient with diabetes and non-diabetic individuals [25].

2. Functions and Molecular Mechanisms of Action of EPA:

Several studies have shown that EPA has various effects, including preventing of the insulin resistance [26], increasing the insulin secretion [27], enhancing the size of LDL-c particle [28], reducing the serum levels of TG, lowering the blood viscosity, increasing the production of nitric oxide (NO), having the antiinflammatory and antithrombotic properties [29-31], and decreasing the blood pressure [32].

It has been demonstrated that EPA is more effective than docosahexaenoic acid (DHA) in the suppression of inflammatory response [33]. EPA plays as a substrate to 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-2 (PLA2) [34-36].

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 [37].

The findings of an epidemiological study of Greenland Eskimos suggested that EPA could be has the antithrombogenic and antiarteriosclerotic properties [19]. It has been postulated that the mechanisms of these actions are including the suppression of platelet aggregation and the improvement of blood rheologic properties [38].

It has also been reported that EPA has the 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 [18]. These results suggest that the administration of EPA to the patients with type 2 diabetes may prevent 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 the antiinflammatory, antiatherosclerotic, and antithrombotic effects caused by EPA.

3. Effects of ω-3 PUFAs on Apolipoprotein (apo) A-I, apo B-100 and apo B/apo A-I Ratio:

There are only a small number of human interventional studies regarding the effects of ω-3 PUFA on apo A-I, but the findings of them are contradictory [39-41]. These inconsistencies can be attributed to several factors, such as discrepancies in the population studied, the duration of study, the content of ω-3 PUFA in the supplement or the history of diet.

Our findings clearly show that the supplementation of EPA for 8 weeks in the patients with type 2 diabetes mellitus significantly increases the serum levels of apo A-I (Table 2). As yet, the effect of EPA on the serum levels of apo A-I in vitro and in vivo was not studied, and this is the first time that has been demonstrated EPA can enhance the serum levels of apo A-I in vivo. Meanwhile, this finding is in accordance with that of the interventional study performed in this regard with the fish oil supplementation on obese subjects with the metabolic syndrome [42].

On the other hand, several studies have shown different results regarding the effects of ω-3 PUFA on apo B [13, 42]. These inconsistencies could be related to the same reasons explained to apo A-I. However, as

yet, the effect of EPA on the serum levels of apo B-100 in vitro and in vivo was not studied, and this is the first time that has been demonstrated EPA can decrease the serum levels of apo B-100 in vivo. So that our present study clearly shows that the supplementation of EPA for 8 weeks in the patients with type 2 diabetes mellitus leads to a significant reduction in the serum levels of apo B-100 than the placebo group (Table 2).

Meanwhile, there is yet no study in vitro and in vivo regarding the effect of ω-3 PUFAs among EPA on the serum ratio of apo B-100/apo A-I, and this is the first time to has been shown EPA can reduce the serum ratio of apo B-100/apo A-I in vivo. So that the results of present study show that the supplementation of EPA for 8 weeks in the patients with type 2 diabetes mellitus leads to a significant reduction in the serum ratio of apo B-100/apo A-I than the placebo group (Table 2).

Thus, it is significant to point out that our data provide evidence compatible with the hypothesis that EPA influences the serum levels of apo B-100, apo A-I and apo B-100/apo A-I ratio in the patients with type 2 diabetes mellitus.

4. ω-3 PUFAs and the lipid profile

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 [43], reducing the serum levels of TG [44], increasing the plasma levels of HDL-c and HDL2-c [44, 45], and decreasing the plasma levels of HDL3-c [44]. This study demonstrated that EPA can significantly increase the serum levels of HDL-c which is compatible with the results in the other studies with ω-3 PUFAs [44, 45], but did not significantly affect the other serum levels of lipids.

5. The study limitations:

There were several limitations for 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. Second, the exact mechanisms by which EPA decrease the serum level of apo B-100 and increase the serum level of apo A-I have not been clarified, and further work is necessary to delineate the molecular mechanism of action of EPA on the regulation of serum levels of apo B-100 and apo A-I. Third, 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, the additional studies will be necessary to determine the general applicability of our study results.

Conclusions:

From findings it can be concluded that the supplementation of EPA is very effective in the reduction of oxidative stress and endothelial dysfunction as a main initiating step in the development of atherosclerosis, through an improvement in the serum levels of apo B-100, apo A-I and apo B-100/apo A-I ratio, which may contribute in the prevention of vascular complications in the patients with type 2 diabetes mellitus.

In general, measurement of the serum levels of apo A-I, apo B and apo B/apo A-I ratio provides the additional information to that obtained by measuring the serum levels of LDL-c and HDL-c. The serum levels of these proteins are associated with particle numbers in these major atherogenic and antiatherogenic lipoproteins, and if be especially used along with the plasma levels of TG are a reflection of the metabolic status. The assays for apo A-I and apo B are accurate, reliable, standardized and inexpensive. Inclusion of these indicators in the standard lipid profile would be as useful parameters in strategies to the prevention of atherosclerotic vascular disease. Since they are beneficial first to help in the prediction of vascular risk, and then in the assessment of lipid lowering treatment.

Table 1. The baseline and after characteristics of the two groups of patients

Variable / Group	Placebo [n (Female/Male)=18]		P-value	EPA [n (Female/Male)=18]		P-value
	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	274.36 ± 1973.61	> 0.05
Carbohydrates intake (g/d)	260.32 ± 35.44	37.22 ± 265.08	> 0.05	260.85 ± 41.78	42.89 ± 260.82	> 0.05
Proteins intake (g/d)	63.19 ± 14.78	11.97 ± 70.09	0.041	14.34 ± 63.83	63.92 ± 14.06	> 0.05
Lipids intake (g/d)	22.68 ± 76.11	76.39± 16.56	> 0.05	16.78 ± 73.82	20.13 ± 76.86	> 0.05
Fibers intake (g/d)	14.75 ± 4.64	2.28 ± 14.64	> 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 of apolipoprotein (apo) A-I, B-100 and apo B-100/apo A-I ratio at baseline and after of the supplementation with EPA or placebo

Group / Variable	Placebo		P-value	EPA		P-value
	Baseline	After		Baseline	After	
apo A-I (ng/ml)	**147.94 ± 22.65**	**147.22 ± 20.85**	**0.837**	**128.50 ± 11.70**	**137.72 ± 10.67**	**< 0.001**
apo B-100 (ng/ml)	**96.89 ± 22.89**	**97.72 ± 18.21**	**0.883**	**95.00 ± 21.13**	**87.94 ± 20.49**	**< 0.001**
apo B-100/apo A-I (Ratio)	**0.6689 ± 0.19**	**0.6672 ± 0.10**	**0.971**	**0.7461 ± 0.19**	**0.6683 ± 0.16**	**0.003**

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-value	EPA		P-value
	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)	**31.11 ± 4.24**	**31.38 ± 4.76**	**> 0.05**	**29.72 ± 5.31**	**37.11 ± 5.97**	**< 0.05**
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.

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