Evaluation of Asymmetric Dimethylarginine in Diabetic Rats Treated with Flaxseed Oil

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Abstract: *Objective:* The present study aimed to investigate how far supplementation of dietary omega-3 fatty acids could affect endothelial dysfunction and augment elevation of Asymmetric Dimethylarginine (ADMA) in experimental diabetes.

Methods: To fulfill our objective, forty male albino rats were divided into four experimental groups as follow: Control, flaxseed oil, diabetic, and treated flaxseed oil groups. After blood sample collection from all groups, plasma was used for estimation of different biochemical parameters including blood glucose and plasma insulin. The levels of liver malondialdehyde (MDA), liver superoxide dismutase (SOD) and plasma nitric oxide (NO) were determined by colorimetric methods. ADMA was estimated by HPLC using fluorescence detector.

Results: Streptozotocin (STZ) induced experimental diabetes was shown by the significant elevation in blood glucose levels, insulin resistance and oxidative stress as revealed by a significant increase in the levels of liver MDA accompanied with a significant reduction in SOD activity. In addition, STZ administration significantly elevated ADMA level. In contrast, flaxseed oil supplementation was capable of ameliorating these negative changes in STZ injected rats.

Conclusion: Dietary omega-3 fatty acids present in flaxseed oil can ameliorate endothelial dysfunction and elevation of ADMA in STZ induced diabetes. These results could be assigned to the potential antioxidant, anti-apoptotic, and anti-inflammatory effects of flaxseed oil.

Keywords: Diabetes mellitus, ADMA, Endothelial dysfunction, HPLC, Flaxseed oil.

INTRODUCTION

Diabetes mellitus (DM) is a complicated disease associated with hyperglycemia, impaired insulin production and /or declined insulin action causing the disability of glucose molecules to be transported from blood to peripheral tissues resulting in elevation of blood sugar and urinary glucose excretion [1]. This process is characterized by impaired synthesis and also bioavailability of the vasodilator factor (NO) [2].

Moreover, nitric oxide helps in platelet accumulation inhibition, leucocytes immigration cellular adhesion, and the proliferation of vascular smooth muscle in addition to the providing of vascular homeostasis, the main and the very important action of nitric oxide [3, 4]. Thus, it was observed that endothelial dysfunction starts when nitric oxide decreases [5].

Endothelial cells synthesized ADMA which is released in sufficient amounts and are capable of inhibiting NO production [6, 7], where it contributes as a key molecule to endothelial dysfunction. Also Elevated levels of plasma ADMA are seen in patients with chronic heart failure, in hypertensive and hypercholesterolemic patients, and in other patient groups at high risk of developing cardiovascular disease.

Great interest was concentrated on the relation between diabetic complications and the quantity as well as quality of dietary lipids [8]. It was observed that increased omega-3 fatty acids enhanced insulin sensitivity in adipocytes of sucrose-fed rats [9].

Thus, this study aimed to evaluate how far supplementation of dietary omega-3 fatty acids could affect endothelial dysfunction and diminish elevation of ADMA in experimental diabetes.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ), ADMA (HPLC standard), tetrahydrofuran (THF), O-Phthaldialdehyde (OPA) and mercaptoethanol were purchased from Sigma Aldrich Company St. Louis USA.

Experimental Animals

Male albino rats with average weight between 180-200 g were obtained from the animal house of National Research Centre, Giza, Egypt. They were housed individually in suspended stainless steel cages in a suitable environment (22-25°c) with 12 hour light, 12 hour dark. Animals had sustained access to standard

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rodent chow diet and water. The experimental animals received human care in accordance to guidelines of the Ethical Committee of National Research Centre, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Methods

Induction of Diabetes

Sodium citrate (50 mM and pH 4.5) solution containing 150 mM NaCl was used to dissolve streptozotocin. The solution containing (6.0 mg/100g body weight) was administrated s.c. in rats; fasting blood glucose was measured after 72 hours to confirm diabetes mellitus development [10].

Experimental Design

Forty male albino rats were used in this study and divided into four groups (ten rats each) as follows:

Group I (control): Normal rats received a vehicle.

Group II (flaxseed oil): Normal rats received flaxseed oil (1.2 ml / kg b.w. / day) orally.

Group III (diabetic): Diabetic induced rats received a vehicle.

Group IV (treated): Diabetic induced rats received flaxseed oil (1.2 ml / kg b.w. / day) orally [1].

After the experimental period (4 weeks); blood withdrawn was done using capillary tubes from the orbital vein and collected in a- tubes containing sodium fluoride for blood sugar measurement and b- test tubes contain anticoagulant for other different biochemical analysis.

Liver was quickly removed and placed in iced normal saline, perfused with normal saline solution to remove blood cells, blotted on filter paper and frozen at -80°C. The frozen tissues were cut into small pieces and homogenized in 5 ml buffer (0.7 g of NaH_2PO4 and 0.5 g of Na_2HPO4) in 500 ml deionized water (pH: 7.4) / gram tissue; centrifuged at 4000 rpm for 15 min. at 4°C, the supernatant was kept for estimation of oxidant/antioxidant parameters [11].

Fasting blood glucose was measured by colorimetric method using commercial kit from Vitro Scient, Egypt, based on the previous method [12]. Oxidant/antioxidant parameters were estimated by commercial kits; liver MDA [13] SOD [14] and plasma NO [15] were measured colorimetrically according to the methods described previously. All kits were purchased from Bio. Med. Diagnostics.

Plasma insulin level was estimated by ELISA according to Yalow and Bauman [16] using BioSoure INS-EASIA Kit. Insulin resistance was calculated from the equation:

Insulin resistance = fasting glucose (mg dl^{-1}) X fasting insulin (μIU ml^{-1})/405 [17].

Determination of ADMA by High Performance Liquid Chromatography

ADMA was assayed by modified HPLC method as described previously [18].

Sample Preparation

Twenty-five mg from 5-sulfosalicylic acid (5-SSA) were added to 1 mL plasma, mixed well then left for10 min. in an ice-bath; the precipitated protein was removed by centrifugation at 4000 r.p.m. using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany) for 10 min., the supernatant was filtered through hydrophilic PVDF 0.45 μm filter; then mix 50 μL from the filtered solution with 500 μL of OPA solution and left for 3 min. before injecting into HPLC.

HPLC Condition

Ten μl of (sample-OPA) were injected onto HPLC; separation was achieved on C18 column (150 X 4.6 mm). The mobile phase consisted of sodium acetate buffer / methanol / THF with a ratio (A, 82:17:1and B, 22:77:1, % v, respectively) and eluted in a flow rate 1.0 ml/min. by gradient method as shown in Table **1**. Column temperature was adjusted at 37°C and a fluorescence detector was set at 338 nm excitation and 425 nm emissions. A linear standard curve was constructed by plotting peak areas versus the corresponding concentration in each diluted standard. The concentration in samples was obtained from the standard curve.

Table 1: Mobile Phase A and B Percent

Time (min)	Percent of A	Percent of B
0.00 – 4.90	100	00
5.00- 14.99	90	10
15.00-24.99	60	40
25.00-27.99	25	75
28.00-32.00	10	90

Table 2: Blood Glucose, Insulin and Insulin Resistance Levels in Different Studied Groups

Parameters / Groups	Glucose (mg/dl)	Insulin (µIU/ml)	Insulin Resistance ($mgdl^{-1}$ µIU ml^{-1})
Control	74.60 ± 4.1	11.43 ± 0.9	2. 10 ± 0.1
Flaxseed oil	75.33 ± 2.7[b]	11.65 ± 1.6	2.16 ± 0.1[b]
Diabetic	234.12 ± 9.3[a]	9.31 ± 1.0 [a]	5.38 ± 0.2 [a]
Treated	162.00 ± 5.1 [a,b]	10.94 ± 2.0	4.37 ± 0.1[a, b]

Data represents ± SE
Significant *P* value ≤ 0.05
a: Significant difference compared to control group
b: Significant difference compared to diabetic group
Number of cases = 10

STATISTICAL ANALYSIS

Results were representing as mean ± standard error (SE). Data were analyzed by SPSS (version 15). Differences between groups were considered significant when *P* value <0.05.

RESULTS

Here, the mean values of fasting blood sugar, insulin level and insulin resistance were insignificantly changed in flaxseed oil group when compared to control confirming the safety of flaxseed oil. Whereas, in diabetic rats, fasting blood sugar was significantly increased along with a reduction in insulin level resulting in a significant increase in insulin resistance compared to control one. Contrarily, flaxseed oil administration significantly improved these parameters in treated group compared to diabetic one (Table **2**).

STZ injection significantly increased oxidative stress in diabetic group compared to control which was appeared by the elevation of lipid peroxidation and the reduction of antioxidant enzyme (SOD) activity. However, flaxseed oil significantly decreased MDA and increased the activity of SOD in treated rats compared to diabetic (Table **3**).

In addition, ADMA level was significantly increased in diabetic group along with a significant reduction in NO level compared to control group. Whereas, flaxseed oil supplementation significantly increased NO and significantly decreased ADMA level in treated group compared to diabetic group (Table **4**).

DISCUSSION

Here, the elevation of fasting blood sugar in diabetic group may be resulting from ß-cells destruction following streptozotocin injection [19]. Therefore, in streptozotocin- induced diabetes, ß-cells fail to synthesis insulin resulting in accumulation of blood glucose instead of being utilized or stored. The decline in insulin levels observed in this study was reported by several previous studies [1, 20].

Table 3: Oxidant / Antioxidant Parameters in Different Studied Groups

Parameters / Groups	MDA (nmol/g. Tissue)	SOD (U/g. Tissue)
Control	31.90 ±0.15	453.5±2.1
Flaxseed oil	30.65 ±0.19[b]	467.7±2.5[b]
Diabetic	54.43 ±0.20[a]	246.0±1.8[a]
Treated	40.11 ±0.17[a,b]	382.5±1.9[a,b]

Data represents ± SE
Significant *P* value ≤ 0.05
a: Significant difference compared to control group
b: Significant difference compared to diabetic group
Number of cases = 10

Table 4: Nitric Oxide and ADMA Levels in Different Studied Groups

Parameters / Groups	NO (µmol/L)	ADMA (µmol/L)
Control	131.5 ±1.3	0.39 ± 0.08
Flaxseed oil	133.2 ±1.7[b]	0.36 ± 0.07[b]
Diabetic	82.9 ± 1.2[a]	1.43 ± 0.12[a]
Treated	119.5 ± 1.6[a, b]	0.88 ± 0.09[a, b]

Data represents ± SE
Significant *P* value ≤ 0.05
a :Significant difference compared to control group
b :Significant difference compared to diabetic group
Number of cases = 10

STZ also has the ability to increase oxidative stress as was found in our study by elevation of liver MDA and the reduction of antioxidant enzyme (SOD) activity.

One from the most relevant pathogenic factors of complications in diabetes is increased oxidative stress [21]. Generally oxidative damage is a resultant to the production of highly reactive hydroxyl radical, which causes major oxidative damage to the cell's components like proteins, DNA and lipids [22].

Endothelial dysfunction is one of the important diabetic complications. In the current study, we found an elevation of ADMA level and a reduction in NO level in diabetic group compared to control group.

Several studies indicated that ADMA has been used as an important marker of endothelial dysfunction. There is a proof that elevation of ADMA level may be a cause or a result of rising insulin resistance [23]. Surprisingly, several investigations indicated that insulin resistance plays an indicative role in propagation and proliferation of diabetic complications and retinopathy [24]. However, different studies indicated that increased ADMA level is an important factor in diabetic complications and retinopathy; these evidences reflect a phenomenon of the association of diabetic complication with both ADMA elevation and insulin resistance [25].

In this study flaxseed oil administration significantly decreased blood glucose as well as insulin resistance in treated group compared to diabetic one. Flaxseed oil as a source of omega-3 fatty acids (mainly α-linolenic acid) enhanced omega 3 fatty acids to compete with omega-6 family for incorporation into cell membrane [26, 27] resulting in a reduction of arachidonic acid (omega-6) liberation from the cell membrane.

The reduction of arachidonic acid lead to a reduction of reactive oxygen species (ROS) and increasing antioxidant enzymes as was found in our work. Thus, in this study flaxseed oil administration significantly increased SOD activity and decreased MDA level.

Our previous works indicated the beneficial effect of flaxseed oil supplementation in reducing insulin resistance in treated group compared to diabetic one [1, 28].

In the same line, Kato *et al*. [29] indicated that GLUT-4 in mice treated with α- linolenic acid was significantly elevated compared to control group. In addition, Pifferi *et al*. [30] reported that endothelial Glut-1 significantly reduced in microvessels deficient in n-3 PUFA when compared to control group, while this value was elevated in the microvessels of rats fed the high n-3 polyunsaturated fatty acid diet. The study suggested that n-3 PUFA can improve glucose transport in the brain in endothelial cells of the blood-brain barrier, possibly *via* the protein expression changes in Glut-1.

Supplementation of flaxseed oil in this study slightly increased insulin level in treated group compared to diabetic group (Table **2**); thus it stimulates high secretion of insulin from β-cells; whereas insulin has vasodilatory properties, as well as anti-inflammatory effects [31]. Insulin action in insulin responsive tissues is being mediated by the insulin signaling cascade and stimulates generation of nitric oxide in skeletal muscle and vascular smooth muscle.

In addition, anti-oxidant protection of flaxseed oil elicited decreased cellular production and release of oxygen radicals in the vascular wall, inhibits endothelial activation of oxidation-sensitive genes, and improves the biologic activity of NO through a cell- or tissue-specific antioxidant action. [32]

In conclusion, flaxseed oil serves as a promising agent that effectively attenuates hyperglycemia in experimental diabetes through its antioxidant and anti-inflammatory effects beside its role in attenuation of endothelial dysfunction that appeared through ADMA and NO estimation.

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Received on 11-09-2017 Accepted on 18-10-2017 Published on 27-12-2017