

DIETARY OMEGA-3 FATTY ACIDS PREVENT ERYTHROCYTE MEMBRANE ATPASE REDUCTION IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Objective: This study aimed to compare the effect of flaxseed oil and fish oil administration on erythrocyte membrane phospholipids fractions and their role on the activities of membrane bound enzymes in experimental diabetic rats.

Methods: sixty male albino rats were used in this study and classified into six groups including control, flaxseed oil, fish oil, diabetic, treated flaxseed oil and treated fish oil groups. Fasting blood sugar, urinary isoprostane, erythrocyte membrane ATPase were determined. Fractionation of erythrocyte membrane phospholipids was carried out by HPLC using stainless steel phenomenx bond with 250x 4.60 mm, 5µl silica. Photodiode array UV-visible detector was used and set at 203nm.

Results: Fasting blood glucose was elevated in diabetic group concomitant with the elevation of urinary isoprostane and phospholipids fractions, in addition to a reduction of erythrocyte membrane ATPase. However, supplemented oils improved these complications.

Conclusion: flaxseed and fish oils have an important effects in improving cell membrane phospholipids and decreasing oxidative stress in addition to the prevention of ATPase reduction during diabetes. However, fish oil is more potent than flaxseed oil in attenuating diabetic complications.

Keywords: Diabetes, ATPase, Isoprostane, Erythrocyte membrane, HPLC

INTRODUCTION

Diabetes is a major health problem affecting major populations worldwide. Epidemiological studies and clinical trials strongly support the notion that hyperglycemia is the principal cause of complications [1]

Diabetes mellitus induces changes in rheological properties i.e. specific changes in mechanical properties eg. increase in erythrocyte microviscosity, aggregation and adhesiveness which causes the changes in lipid composition, dysfunctioning of membrane structure and functions [2, 3], involved in the action of insulin receptor binding enzyme and transporter activities [4].

Rodrigo *et al.* [5] indicated that lipid peroxidation alters the cellular structure of membrane bound enzymes by changing phospholipids and fatty acid composition. In addition, the membrane bound proteins are also glycosylated decreasing the activity of proteins [6]

Most methods available to assess oxidant stress *in vivo* lack specificity and sensitivity. However, a substantial body of evidence has been obtained to indicate that the measurement of isoprostanes, in urine or plasma, provides a reliable approach to assess lipid peroxidation *in vivo* [7].

F2 isoprostanes, a class of prostanoids produced by non enzymatic free radical-catalyzed peroxidation of arachidonic acid, esterified to phospholipids and subsequently released, possibly by a phospholipase [8]. They circulate in the blood stream and are finally excreted in urine [9].

The activities of erythrocyte membrane bound enzymes such as ATPase were significantly inhibited in STZ diabetic rats. The fair degree of ATPases activities in erythrocyte membrane could be serving as simple, safe and useful marker of intracellular damage [1].

Food derived antioxidants have a strong potential for long term use as chemo-preventive agents in disease states involving oxidative stress such as diabetes [10].

Omega-3 fatty acids (n-3) comprise a family of unsaturated fatty acids that consists of α -linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential nutrients that must be obtained from food because of the inability of mammals to synthesize these fatty acids *de novo* [11].

Hussein *et al.* [12] indicated that flaxseed oil (a rich source of ALA) administration has a beneficial effect on decreasing insulin resistance in diabetic rats through the scavenging of free radicals and increasing antioxidant enzymes. Also, De Felice *et al.* [13] reported that, oral supplementation with fish oil (which is a good source of EPA and DHA) significantly decreased oxidative stress parameters such as isoprostane.

Thus, the present investigation was carried out to compare the effect of flaxseed oil and fish oil administration on erythrocyte membrane phospholipids fractions and their role in the activities of membrane bound enzyme in experimental diabetic rats.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ) was purchased from Sigma Chemicals Co. (Munich, Germany).

Phospholipids standards (PC, PE, PS and SM) from bovine sources were purchased from Sigma Chemical Company, St. Louis, USA.

Acetonitrile, methanol, ethanol, N-hexane, 2-propanol and phosphoric acid (HPLC grade) were purchased from ALDRICH, Germany.

Flaxseed and fish oils were purchased from a local market (Cairo, Egypt). Tris buffer was purchased from (laboratory Rasayan) and ATP was purchased from (Oxford laboratory, India).

Experimental Animals

Sixty male albino rats weighing (180-200 g) were used in this study and obtained from the animal house of the National Research Center (Cairo, Egypt). They were housed in stainless steel cages under environmentally controlled conditions. The ambient temperature was 25 ± 2 °C and the light/dark cycle was 12/12 hours. The animals had free access to water and standard rodent chow diet. All animals received human care in compliance with guidelines of the Ethical Committee of National Research Center, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Methods

Induction of diabetes mellitus

Streptozotocin (STZ) was dissolved in 50 mM sodium citrate (PH 4.5) solution containing 150 mM NaCl. The solution (6.0 mg/0.5 ml/100g body weight) was subcutaneously injected into rats; fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus [14].

Experimental design

Sixty male albino rats were used in this study and divided into six groups (ten rats in each group) as follows:

Group I (control group): healthy rats received 1.2 ml corn oil / kg b.w. / day orally.

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

Group III (fish oil group): healthy rats received 1.2 ml fish oil / kg b.w. / day orally.

Group IV (diabetic group): diabetic rats received 1.2 ml corn oil / kg b.w. / day orally.

Group V (treated flaxseed oil group): diabetic rats received 1.2 ml flaxseed oil / kg b.w. / day orally.

Group VI (treated fish oil group): diabetic rats received 1.2 ml fish oil / kg b.w. / day orally [15].

After the experimental period (8 weeks), animals were kept individually in metabolic cages for 24 hours for urine collection then kept fasting for 12 hours before blood sampling. Blood was withdrawn from the retro-orbital venous plexus of the eye using a capillary tube and the blood of each rat was collected into two tubes; one contains sodium fluoride for blood glucose estimation and the other contains heparin for other biochemical parameters and erythrocyte membrane component.

Heparinized blood was centrifuged at 2000 rpm for 10 minutes using cooling centrifuge, plasma was separated and immediately frozen, packed RBCs were divided into two parts; the 1st part for erythrocyte membrane lipids extraction while the 2nd part for erythrocyte membrane proteins extraction.

Fasting blood sugar was determined using enzymatic colorimetric method. Centronic, Germany[16].

Urinary F2 isoprostane was estimated by enzyme immunoassay (ELISA) by a kit derived from Cayman Ann Arbor, USA [17].

Extraction of erythrocyte membrane lipids

Total lipids in red blood cells membrane were extracted by chloroform: methanol method [18], modified from the method described by Bligh and Dyer [19].

Determination of erythrocyte membrane phospholipids fractions

Fractionation of phospholipids was carried out by HPLC[20]. The separation was achieved on a stainless steel phenomenx bond with 250 x 4.60 mm, 5µl silica.

Standards preparation

All standards were dissolved in N-hexane/2-propanol (3/1) v/v, then serial concentrations were prepared from each standard. 20 µl from each concentration were injected in HPLC.

Sample preparation

2 ml of lipid solution (chloroform containing lipids) were dried under nitrogen gas stream, the residue (dry sample) was redissolved in N hexane / 2 propanol (3/1) v/v to be ready for injection in HPLC. 20 µl of each sample were injected.

HPLC condition

The mobile phase was acetonitrile-methanol -85% phosphoric acid (1000:40:0.4) v/v. It was delivered to the column at flow rate of 1.5

ml/min and a pressure of 75 bar at room temperature (25°C). Photodiode array UV-visible detector was used and set at 203nm.

A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentrations in samples were obtained from the curve.

Extraction of erythrocyte membrane proteins

The method used for erythrocyte ghost preparation was based on washing of backed RBCs by isotonic phosphate buffer and hemolysis of RBCs for removal of hemoglobin by hypotonic phosphate buffer (pH was adjusted at 7.4) [21, 22], centrifuged at 4000 rpm, the supernatant was aspirated very carefully, and the ghost button was resuspended by swirling while adding sufficient fresh buffer of the same strength, the ghost was washed three times, and used for determination of the following:

- Erythrocyte membrane total protein that was estimated according to Passing and Bablok [23], using BICON diagnostic Kit, Germany.
- Erythrocyte membrane ATPase activity that was assayed according to the method described previously [24], Enzyme activity is measured as the amount of inorganic phosphate (Pi) released from ATP and ATPase activity expressed as µmol of phosphorus liberated/hr/mg protein at 37°C.

Statistical analysis

Results were expressed as mean ± standard error. Data were analyzed by independent sample t test (SPSS) version 15 followed by (LSD) test to compare significance between groups. Difference was considered significant when P value <0.05.

RESULTS

In this study, the mean value of fasting blood glucose was significantly increased in diabetic group compared to control, while this value decreased by flaxseed and fish oils administration. This value was significantly decreased in treated fish oil compared to treated flaxseed oil group (Fig.1).

In this study, the mean value of urinary isoprostane was significantly increased in diabetic group compared to control while supplemented oils significantly decreased these values in treated groups compared to diabetic one (fig.2).

As shown in figure (3), erythrocyte membrane ATPase was significantly decreased in diabetic group compared to control while it significantly increased by supplemented oils in treated groups. In addition, phospholipids fractions (PE, PC and SM) were significantly decreased in diabetic group compared to control; these values were decreased again by treatment, however the mean value level of PS was not changed during the experimental period (fig.4-7).

DISCUSSION

In the present study, STZ significantly increased blood glucose level in diabetic group compared to control group, this result was in agreement with Yang *et al.* [25] who indicated that, STZ- induced diabetes produced a significant increase in fasting glucose levels that was associated with a decrease in serum insulin levels.

Streptozotocin-induced diabetes is a well documented model of experimental diabetes [26]. It can begin an autoimmune process that results in the destruction of the Langerhans islets beta cells and results in the toxicity of beta cells [27].

This study appeared elevations in urinary isoprostane, PE, PC and SM along with a reduction of membrane ATPase in diabetic group compared to control. Although PS was not changed during the experimental period.

The elevation of isoprostane in diabetic group was due to the generation of free radicals from the arachidonic acid (omega-6) in cell membrane phospholipids [8].

Increased oxidative stress could be one of the common pathogenic factors of diabetic complications [28]. Oxidative damage is generally

attributed to the formation of highly reactive OH· [29]. The major targets of these damaging species are the long chain polyunsaturated fatty acids (LC-PUFAs) of cellular phospholipids, which are particularly prone to attack because of the arrangement of double and single bonds. The resultant lipid peroxide frequently

decomposes to radicals [30], which react with most biological molecules, including proteins and lipids. In addition, oxygen free radicals exert their cytoplasmic effect by peroxidation of membrane phospholipids, which leads to changes in the permeability and loss of membrane integrity [31].

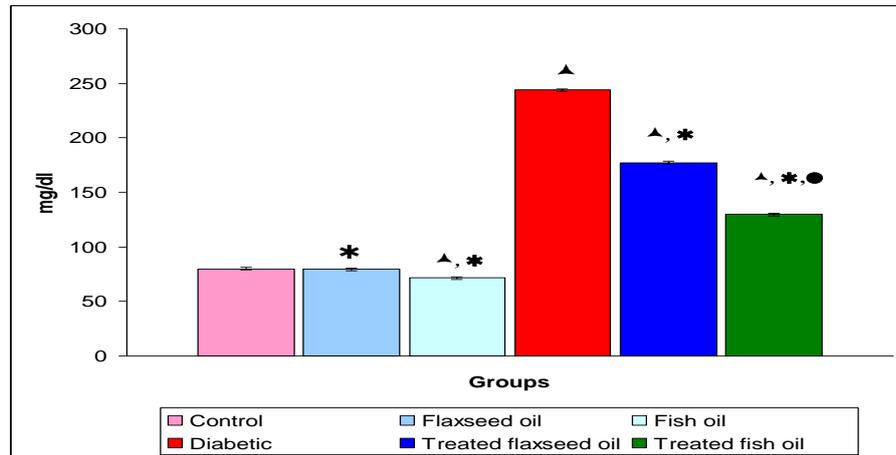


Fig. 1: Fasting blood sugar in different studied groups. [▲] Significant difference compared to control group. * Significant difference compared to diabetic group. • Significant difference between treated fish oil and treated flaxseed oil group.

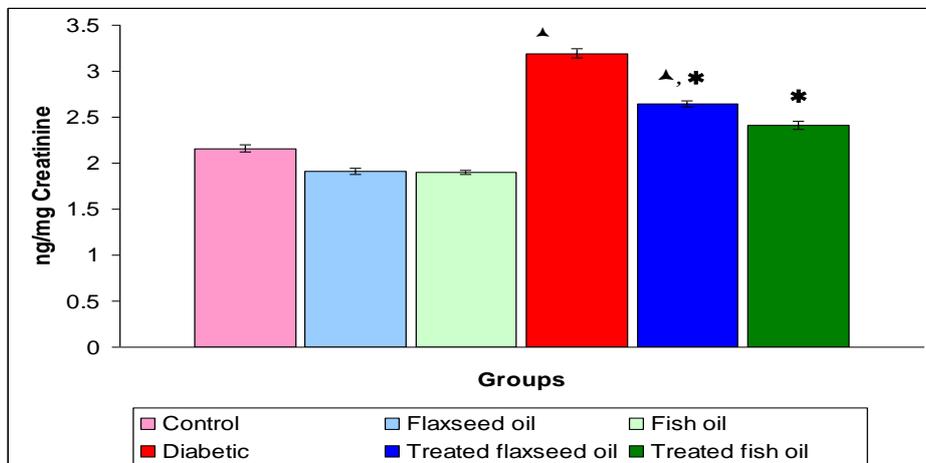


Fig. 2: Urinary isoprostane in different studied groups. [▲] Significant difference compared to control group. * Significant difference compared to diabetic group.

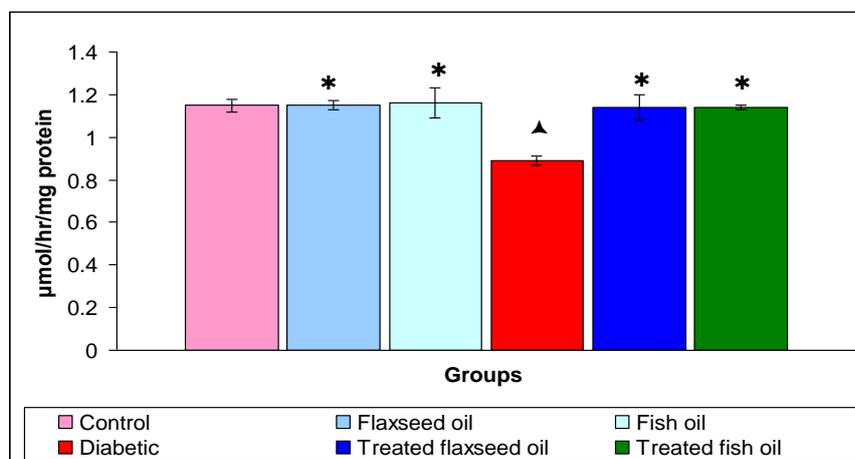


Fig. 3: Erythrocyte membrane total ATPase in the different studied groups. [▲] Significant difference compared to control group. * Significant difference compared to diabetic group.

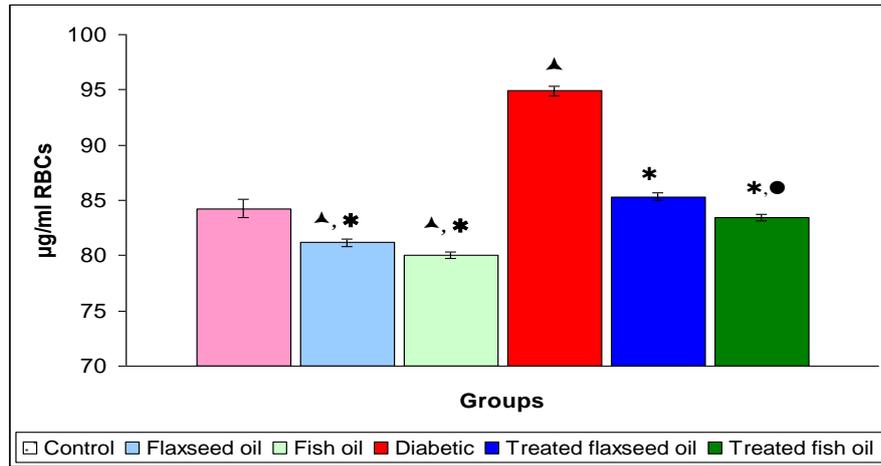


Fig. 4: Erythrocyte membrane phosphatidylethanolamine in different studied groups. ▲Significant difference compared to control group. * Significant difference compared to diabetic group. ● Significant difference between treated fish oil and treated flaxseed oil group.

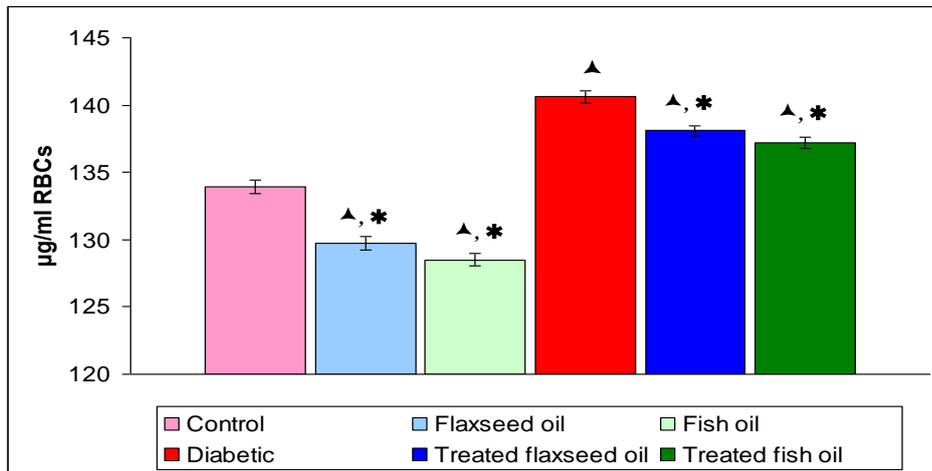


Fig. 5: Erythrocyte membrane phosphatidylcholine in different studied groups. ▲Significant difference compared to control group.* Significant difference compared to diabetic group.

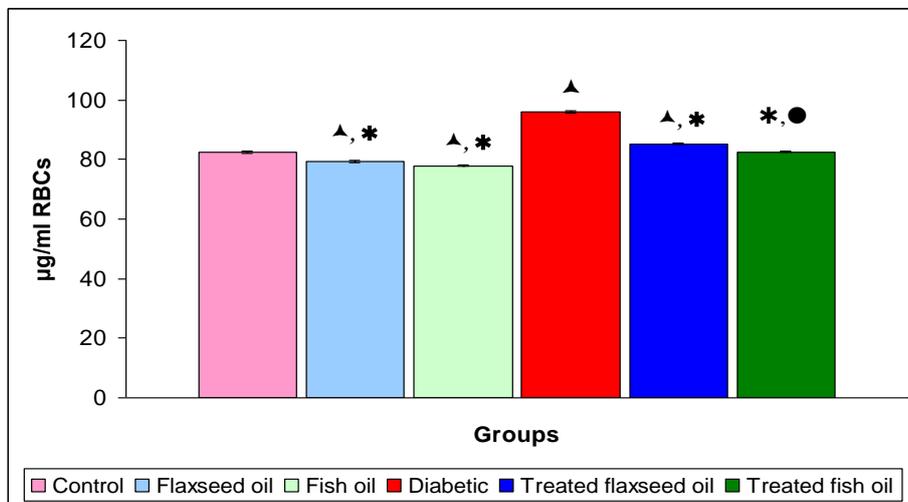


Fig. 6: Erythrocyte membrane sphingomyelin in different studied groups. ▲Significant difference compared to control group. * Significant difference compared to diabetic group. ● Significant difference between treated fish oil and treated flaxseed oil group

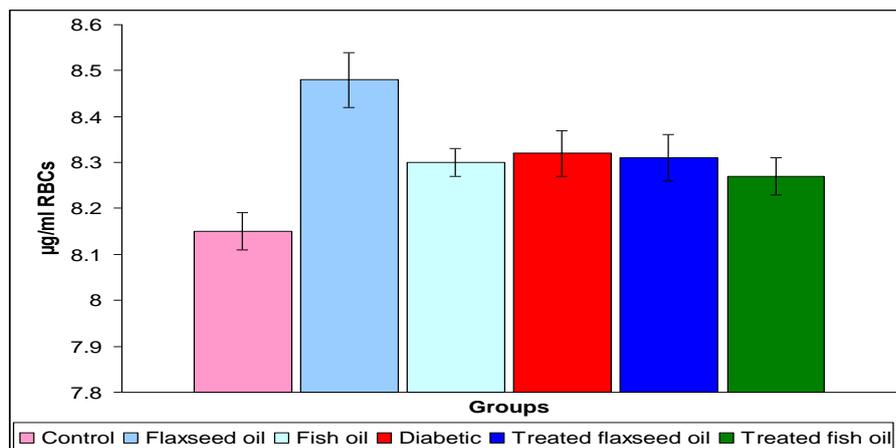


Fig. 7: Erythrocyte membrane phosphatidylserine in different studied groups.

The elevation of phospholipids fractions (PC, PE, SM) may be attributed to the increase of free radicals that attach the polyunsaturated fatty acids, so the concentration of saturated fatty acids in the cell membrane increased and elevated the PC, PE and SM that are consisting mainly of saturated fatty acids [32].

Impairment of ATPase activity could be due to the loss of its optimal interaction with the membrane components, as a consequence of increased lipid peroxidation [33].

Fatty acids composition of membrane phospholipids such as SM, PE and PC are tissue specific [34], but are affected by the composition of the dietary fat [35]. Changes in the fatty acids composition of erythrocyte membrane, which are easily accessible cells, reflects changes in that of membrane phospholipids of less accessible tissues [36].

In the present study, phospholipids fractions SM, PE and PC significantly decrease in treated flaxseed and fish oils groups compared to diabetic group, this result was in agreement with another study which indicated that the amount of dietary fat as well as the nature of fatty acids regulate various steps in the biosynthesis of membrane phospholipids, thus, total PC, PE and SM in rats fed a diet high in saturated fat were 1.5, 2 and 5 fold respectively higher than in rats fed on an unsaturated high fat diet [32].

A reduction in lipid peroxidation in both treated groups as was found in this study can prevent diminution in the activities of ATPase which is beneficial because any reduction in ATPase activity can affect the intracellular concentrations, alter the signal transduction pathway, and affect contractility, which in turn leads to cellular dysfunction. Administration of flaxseed oil and fish oil to diabetic rats showed significant elevation in the activities of total ATPase in erythrocyte membrane could be due to increase in antioxidant defense as flaxseed oil and fish oil offered protection to cells against oxidative stress by scavenging free radicals generated during diabetes [24].

In this study it was observed that fish oil attenuated the diabetic complications such as altering cell membrane structure, function and oxidative stress more than flaxseed oil. This observation may be due to the fact that fish oil is an animal source that rich of omega-3 fatty acids in the form of EPA and DHA which are very beneficial fatty acids that have antioxidant and anti-inflammatory effects in addition to their incorporation in the cell membrane.

On the other hand, flaxseed oil is a plant source of ω -3 FAs in the form of α -linolenic acids, this fatty acid is beneficial fatty acid as well, but it must be converted to EPA and DHA to do its effect, this conversion must be done by desaturase enzyme which is decreased by aging and in some diseases including diabetes so, not all fatty acids in flaxseed oil could be converted to EPA and DHA but a limited value only. We concluded that flaxseed oil and fish oil effectively attenuated the elevation of oxidative stress and phospholipids fractions during diabetes. As well as the prevention of ATPase

reduction. In addition, this result observed that, fish oil is more potent than flaxseed oil in attenuating diabetic complications.

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