Relationship between cell membrane fatty acids and inflammatory markers in ovariectomized diabetic rats treated with fish oil

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Dietary omega-3 fatty acids decrease pro-inflammatory markers by displacing arachidonic acid (AA) from cell membranes and competing with it for the enzymes that catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes. This study aimed to investigate the beneficial role of fish oil (FO) as a source of omega-3 fatty acids on pro-inflammatory markers in diabetic ovariectomized rats in context of AA releasing status from the cell membrane. Forty female rats were divided into four groups: sham operated rats (control group), sham operated rats received orally fish oil, ovariectomized diabetic rats (OVX-D) and OVX-D rats received fish oil (treated group). Interleukin-1β (IL-1β), 15-lipoxygenase (15-LOX) and Lipoxin-A4 (LXA4) were measured. The concentration of arachidonic acid (AA) and alpha-linolenic acid (ALA) were estimated by HPLC. Results indicated that hyperglycemia in OVX diabetic rats significantly increased pro-inflammatory markers and erythrocyte membrane arachidonic acid, concomitant with a reduction in ALA, fish oil supplementation attenuated this parameters, indicating a significant effect in managing several complications of streptozotocin (STZ) induced diabetes in rats which may be related to the efficiency of omega 3 fatty acids in suppressing inflammation of pancreatic beta cell, enhancement of insulin secretion and uptake of glucose in adipose tissue.

Keywords: Insulin resistance; HPLC; OVX; ω-3 fatty acids; Lipoxin; cell membrane

INTRODUCTION

Menopause is accompanied with atherosclerosis and endothelial dysfunction (Taddei et al., 1983). During menopause; inflammation is associated with osteoporosis whereas, the reduction of bone mineral density may be accelerated (Lencel and Magne, 2011). Deficiency of estrogen increases the risk of cardiovascular diseases, as a result of modifications of plasma lipid atherogenic profile, excess production of reactive oxygen species (ROS) which scavenge nitric oxide (NO) and activation of the renin-angiotensin system (Yung et al., 2011). In addition, inflammatory cytokines mainly tumor necrosis factor-α (TNF-α) and interleukins (IL-1& IL-6) are elevated in postmenopausal women (Weitzmann and Pacifici, 2006).

Estrogen deficiency also accelerates most of the processes involved in vascular aging, including cells proliferation (Morey et al., 1997) and endothelial dysfunction which is determined by a reduction in nitric oxide bioavailability that facilitates the progression of atherosclerosis and its complications. Impaired function of vascular system because of reactive oxygen species overproduction which happens throughout
deficiency of estrogen is attenuated by hormonal replacement therapy in experimental and human menopause (Majmudar et al., 2000; Wassmann et al., 2001)

In diabetes mellitus (DM), increased pro-inflammatory cytokines synthesis such as TNF-α and IL-1β enhances apoptosis of beta cells and increases resistance of peripheral insulin (Hanifi-Moghadam et al., 2003). There has been a great deal of interest in the role of alternative and complementary medicine for the treatment of various acute and chronic diseases such as diabetes mellitus (Hussein et al., 2012), liver disorders (Hussein et al., 2010, Hussein et al., 2016a) and brain injury (Hussein et al., 2016b).

Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are important fatty acids that have both antioxidant and anti-inflammatory effects in addition to their high incorporation in the cell membrane phospholipids; when these fatty acids incorporate into the cell membrane, the incorporation of omega-6 fatty acids such as arachidonic acid in cell membrane decreased (Hussein et al., 2014). Thus, dietary omega-3 fatty acids directly affect AA metabolism because they displace AA in the cell membranes and compete with it for the enzymes that catalyze the biosynthesis of leukotrienes, thromboxanes and prostaglandins (Wassmann et al., 2001). So, consumption of food enriched in omega-3 fatty acids (such as fish oil) is diminished potential for cells like monocytes, neutrophils and eosinophils to synthesize these powerful arachidonic acid-derived mediators of inflammation and a diminished potential for platelets to produce the prothrombotic agent thromboxane A2 (Hussein et al., 2014).

Arachidonic acid is metabolized by lipoxygenases (LOX), cytochrome P450 (CYP) and cyclooxygenase (COX) enzymes into eicosanoids, which participate in different diseases, such as type1 & 2 diabetes. Eicosanoids are derived either from omega-6 (ω-6) or omega-3 (ω-3) fatty acids. The ω-6 eicosanoids are mainly pro-inflammatory; whereas, ω-3 eicosanoids are anti-inflammatory. Unlike omega 3 fatty acids, AA metabolites arising from omega 6 fatty acids are significant factors to beta cell destruction and dysfunction through COX, 12-lipoxygenase (12-LOX) and 5-lipoxygenase (5-LOX) pathways and consequently participating in the pathogenesis of diabetes and its complications (Adkins and Kelley, 2010).

We hypothesized that OVX rats with diabetes having high dietary ratio of ω-3/ω-6 fatty acids have a lower risk of complications. The board of our study was to investigate the relationship between cell membrane fatty acids and inflammatory markers in OVX-diabetic rats treated with fish oil.

MATERIALS AND METHODS

ALA and AA (HPLC standards) and STZ were purchased from Sigma Aldrich Chemicals Company (St Louis, Missouri, USA).

- Fish oil was purchased from Western Pharmaceutical Industries.

Experimental Animals

Forty female albino rats weighing 180-200 g were obtained from the animal house of National Research Centre, Giza, Egypt, and fed a standard commercial diet (control diet) purchased from the Egyptian Company of Oils and Soaps. Water was available ad libitum for acclimatization before starting the experiment; rats were kept under constant environmental conditions at room temperature. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre (ethical clearance number 10/219).

Ovariectomy

Bilateral ovariectomy and sham surgeries were performed via a mid-abdominal route after rats be sedated and anesthetized using xylazine and ketamine intra-peritoneal (i.p.) injection (Goseki et al., 1996). Anesthesia was evaluated by complete absence of limb retraction upon painful stimulation. Body weight and food consumption were recorded regularly.

Induction of diabetes

After one week of ovariectomy and overnight fasting, diabetes was induced by subcutaneous injection of freshly prepared streptozotocin (6 mg/100 g body weight (b.w.)). STZ was dissolved in 50 mM sodium citrate solution (pH 4.5) containing 150 mM sodium chloride. Fasting blood sugar was estimated after 3 days to confirm induction of diabetes mellitus (Hardya et al., 2005), animals with glucose levels > 200 mg/dl were considered diabetic.

Experimental design

Forty female albino rats were classified into four groups (10 rats in each group) as follows:

Group I: Sham operated rats, received a vehicle.
Group II: Sham operated rats, received 1.2 ml fish oil /kg body weight / day orally (Hussein et al., 2014).
Group III: Diabetic bilaterally ovariecotimized rats, received a vehicle.
Group IV: Diabetic bilaterally ovariecotimized rats, received 1.2 ml fish oil/kg body weight / day orally.
After the experimental period (8 weeks), animals were kept fasting for 12 hours before blood sampling, blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and collected in; a- Clean dry tubes for serum separation. b- heparinized tubes for cell membrane fatty acids estimation. Blood was centrifuged at 4000 rpm for 15 min. using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany).
Serum and plasma were separated immediately frozen. Packed RBC's were used for isolation and extraction of erythrocyte membrane fatty acids.

**Ghost preparation**
The method used for erythrocyte ghost preparation was based on washing packed RBC's by isotonic phosphate buffer and hemolysis of RBC's for removal of hemoglobin by hypotonic phosphate buffer (pH was adjusted at 7.4) (Hussein et al., 2014).

**Determination of fasting blood sugar**
Glucose was measured in blood by standard glucose oxidase method as described by Trinder, (1969) by UV-Visible recording spectrophotometer model (Spectro UV Double Beam UVD-3500, USA).

**Determination of lipid profile**
Serum cholesterol and triglycerides levels were determined according to (Meiattini et al., 1978 and Buccolo et al., 1973) respectively using colorimetric enzymatic assay kits (BioMed Diagnostics). HDL-cholesterol was measured using commercial colorimetric kit (BioMed Diagnostics) according to Friedwald et al. (1972), however LDL-cholesterol was calculated from the documented equation using cholesterol, triglyceride and HDL levels
\[
LDL-\text{cholesterol} (\text{mg/dl}) = \text{Total cholesterol} - (\text{HDL-c+} \times \text{TG/5})
\]
according to Friedwald et al. (1972).

**Determination of insulin and estrogen levels**
Serum insulin levels were determined using ELISA according to the method described previously (Yallow and Bawman, 1983), following the protocol given by the manufacturer (Crystal Chem Inc.). The concentration of plasma estrogen was measured using a commercial immunosorbert assay kit (Estradiol sensitive ELISA, DRG Instruments, Marburg, Germany) according to the method described by Taddei et al. (1983).

**Determination of IL-1β levels**
Levels of the pro-inflammatory mediator IL-1β, in serum was determined using ELISA according to the method described previously (Reira et al., 2010) following the manufacturer’s protocol (R&D systems).

**Determination of lipoxygenases (15- LOXs) and lipoxin-A4 (LXA4)**
15- lipoxygenase (15-LOX) level was determined according to Hardya et al. (2005) and lipoxin A4 (LXA4) in the sample was determined according to the method of Lieb et al. (2011) using Enzyme-Linked Immunosorbert Assay (ELISA) for rat according to the manufacturers protocols (R&D systems).

**Estimation of cell membrane fatty acids by HPLC**
Erythrocyte membrane fatty acids were estimated by HPLC, Agilent technologies 1100 series, equipped with a quaternary pump (Quat. pump, G131A model).

**Sample preparation**
Cell membrane was homogenized in 2% acetic acid: ethyl ether mixture (2:1 volume ratio). The solution was then filtered and centrifuged at 5000 rpm, the organic phase was evaporated to dryness. The residue was dissolved in 500 µl acetonitrile and filtered by PVDF 0.45µm filter before injection onto HPLC (De Vriese et al., 2003).

**HPLC condition**
This method was carried out according to the method described previously and modified by El-Khayat et al. (2013). HPLC column C18 (length: 250 mm X ID: 4.6 mm, particle size 5µm), mobile phase was acetonitrile / ultrapure water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min and 200nm wave length. Serial dilutions of each standard were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak
areas versus the corresponding concentrations. The concentrations in samples were obtained from the curve.

**Statistical analysis**

All data from the current comparative cross sectional study were expressed as mean ± standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16). Duncan's new multiple-range test was used to assess differences between means. A significant difference was considered at the level of P < 0.05.

**RESULTS**

In this study, the mean value levels of fasting blood sugar was significantly increased in OVX-D group compared to sham group which was related to a significant reduction in insulin level compared to sham control group. However, fish oil supplementation significantly attenuated these parameters in treated group. In addition, plasma estrogen level was significantly decreased in OVX-D compared to sham group, whereas fish oil administration slightly increased this level in treated group but still significantly decreased compared to sham group (table 1).

Serum cholesterol and triglycerides levels were significantly increased in OVX-D group compared to control group along with a significant reduction in HDL-cholesterol level. Fish oil supplementation significantly attenuated these parameters in treated group although it still far away from the control group (table 2).

As shown in table (3), IL-1β and LA-4 levels were significantly increased in OVX-D group compared to control group, however fish oil supplementation significantly decreased IL-1β in treated group compared to OVX-D group but it still significantly increased compared to control. In addition, fish oil increased LA-4 again in treated group to become significantly increased compared to both OVX-D and sham groups. 15-LOX in this study was elevated in SH+FO group compared to sham, also in OVX-D+FO compared to OVX-D indicating the role of fish oil in elevation of this parameter (table 3).

<table>
<thead>
<tr>
<th>Table (1): Blood glucose, insulin and estrogen levels in different studied groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Groups</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
</tr>
<tr>
<td>Estrogen (Pg/ml)</td>
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</tbody>
</table>

Values are means ± S.E. Number of animals in each group = 10. Values sharing the same superscript means not significant. Superscript different letters in the row mean significant differences p<0.05. SH: sham rat, FO: fish oil, OVX: ovariectomized rat, D: diabetic.

<table>
<thead>
<tr>
<th>Table (2): Serum lipid profile in different studied groups</th>
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<tbody>
<tr>
<td><strong>Groups</strong></td>
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<tr>
<td>---------------</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
</tr>
</tbody>
</table>

Values are means ± S.E. Number of animals in each group = 10. Values sharing the same superscript means not significant. Superscript different letters in the row mean significant differences p<0.05. SH: sham rat, FO: fish oil, OVX: ovariectomized rat, D: diabetic.
Table (3): Inflammatory markers in different studied groups

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>SH</th>
<th>SH + FO</th>
<th>OVX-D</th>
<th>OVX-D+ FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (Pg/ml)</td>
<td>28.89 ± 1.20 a</td>
<td>25.62 ± 1.70 b</td>
<td>48.16 ± 1.17 c</td>
<td>36.84 ± 1.29 d</td>
</tr>
<tr>
<td>15-LOX (U/L)</td>
<td>11.4 ± 0.86 a</td>
<td>12.0 ± 1.8 b</td>
<td>11.1 ± 0.59 a</td>
<td>13.38 ± 1.15 b</td>
</tr>
<tr>
<td>LA-4 (Pg/L)</td>
<td>78.6 ± 12.5 a</td>
<td>129.0 ± 24.9 b</td>
<td>153.0 ± 13.1c</td>
<td>172.0 ± 8.4d</td>
</tr>
</tbody>
</table>

Values are means ± S.E. Number of animals in each group= 10 Values sharing the same superscript means not significant.Superscript different letters in the row mean significant differences p<0.05.


Table (4): Erythrocyte membrane fatty acids in different studied groups

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>SH</th>
<th>SH + FO</th>
<th>OVX-D</th>
<th>OVX-D+ FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid (mg/ml RBC's)</td>
<td>0.05 ± 0.0047 a</td>
<td>0.08 ± 0.006 b</td>
<td>0.16 ± 0.03 c</td>
<td>0.09 ± 0.01 d</td>
</tr>
<tr>
<td>alpha- linolenic acid (mg/ml RBC's)</td>
<td>0.60 ± 0.03 a</td>
<td>0.73 ± 0.05 b</td>
<td>0.35 ± 0.04 c</td>
<td>0.6 ± 0.02 d</td>
</tr>
<tr>
<td>ω-6/ ω-3 ratio</td>
<td>0.08 a</td>
<td>0.11 b</td>
<td>0.45 c</td>
<td>0.15 d</td>
</tr>
</tbody>
</table>

Values are means ± S.E. Number of animals in each group= 10 ,Values sharing the same superscript means not significant.Superscript different letters in the row mean significant differences p<0.05.


Of ω-6/ ω-3 ratio was calculated in this study by determination of both AA (ω-6) and ALA (ω-3) levels in the cell membrane that gave us a complete picture about the ability of fish oil to change cell membrane component. Thus, in this study fish oil supplementation significantly decreased AA and increased ALA in treated group compared to OVX-D group, furthermore, ω-6/ω-3 was significantly increased in OVX-D compared to sham group and significantly decreased by fish oil supplementation in treated group compared to OVX-D group (table 4).

DISCUSSION

In the current study fasting blood sugar was significantly increased in diabetic group compared to sham; whereas, fish oil supplementation significantly attenuated this elevation. The hypoglycemic effect of fish oil may be due to the enhancement of insulin production from the existing β cells of islets of langerhans and it may increase the peripheral uptake of glucose (Arquilla et al., 1978). Our results agreed with Shariati et al. (2011) who stated that diet containing either fish oil or corn oil had a restrained effect on elevated blood glucose in experimental diabetes. Djousse et al. (2006) reported that high exhaustion of omega 3 (present in FO) is usually accompanied with elevated plasma insulin and maintained glucose use and efficacy.

In this study insulin level in diabetic OVX group was significantly decreased compared to sham control group. The same results were approved by Mahmud et al. (2004) who stated that insulin levels are lowered in OVX-D rats. Meanwhile, fish oil supplementation increased insulin levels (table1) in OVX-D rats, which may be due to the synthesis of insulin by β cells and its ulceror transport to the extra-cellular medium up regulation (Soltan, 2012). This valuation is related to the fact that components of FO such as EPA and DHA significantly enhance fluidity of plasma membrane (Hashimoto et al., 1999). Moreover, Punithavathi et al. (2011) reported that insulin levels are increased as a result of the stimulatory action of omega-3, and increasing the activity of the existing β cells in diabetic rats. Besides, previous studies documented that ω -3 fatty acids may improve insulin sensitivity in experimental diabetes (El-Khayat et al., 2013).

In our study there was a significant increase in HDL cholesterol levels and a significant decrease in cholesterol level in OVX-D rats that received fish oil when compared to OVX-D group. These results are explained by the beneficial action of dietary FO on clearance of serum cholesterol, which may also be related to the high content of
omega-3 long chain polyunsaturated fatty acids (Tzang et al., 2009).

The reduction of triglycerides in treated group compared to OVX-D group was in agreement with Strolien et al. (2007) who indicated that plasma triglycerides are reduced by n-3 fatty acids. Thus omega-3 FA inhibited hepatic lipogenesis and lowered levels of circulating triglycerides. Also Soltan, reported that, the net action of n-3 fatty acids in diet lowered plasma TG levels on lipoproteins and plasma lipids (Soltan, 2012).

Triglycerides might influence the binding of insulin to its receptor or interfere with early post binding steps (Norris et al., 2007), higher serum triglycerides leads to a resistance to the antilipolytic effect of insulin, therefore, a reduction in serum TG levels might improve insulin sensitivity (Arquilla et al., 1978; El-Khayat et al., 2013), and attenuates hyperglycemia as was observed in our study.

In this study, serum levels of LDL were lowered by administration of fish oil in OVX-D group resulting in improved levels of plasma lipids. It was documented that FO reduced LDL-cholesterol and significantly increased HDL-C in rats fed on omega-3 enriched diet (Nestel, 2000).

Fish oil was shown to decrease Apo lipoprotein B; the major protein in LDL, representing almost 90% of total mass of protein (Dorfman et al., 2005). Therefore, FO was shown to favorably modify the balance of Apo lipoproteins as was observed in our results.

In both types of diabetes mellitus, there is an increased production of reactive oxygen species (ROS) and pro-inflammatory cytokines such as (tumor necrosis factor-α and interleukins) that induce β cells apoptosis and cause peripheral resistance of insulin (Das, 2013).

In our work plasma levels of Interleukin 1ß (IL-1ß) in ovariectomized diabetic rats showed a significant increase compared to sham group while in ovariectomized diabetic rats that received fish oil IL-1 ß levels showed a significant decrease compared with ovariectomized diabetic rats. This result was approved by Stephens et al. (2004) who reported that IL-1ß levels in circulation are increased in states with insulin resistant such as impaired glucose tolerance (IGT), obesity, and type 2 diabetes mellitus. There is a growing evidence suggesting that IL-1ß produced by fat cells is capable of induction of insulin resistance in these cells. Common functional variant has been accompanied with high plasma IL-1ß including insulin resistant and increased risk of cardiovascular diseases.

One of the important results of our study is the elevation of LA-4 concomitant with the elevation of AA and a reduction of ALA in OVX-diabetic group compared to sham group, however, 15-LOX was insignificantly changed. Fish oil supplementation significantly increased 15-LOX, LA- 4 and ALA in treated group compared to OVX-D group.

Lipoxins are endogenous anti-inflammatory lipid-based autacoids, resulting from the biosynthesis of prostaglandins and leukotrienes. They are produced endogenously in most murine species during inflammation and disease pathogenesis (Xu et al., 2012; Karra et al., 2015).

Lipoxygenase (LOX) pathway involves the conversion of arachidonic acid (AA) to 5-, 12-, or 15- hydro-peroxyeicosatetraenoic acids (HPETE) by 5-, 12-, or 15-LOX, respectively; HPETEs being rapidly metabolized to 5-, 12, or 15-hydroxyeicosatetraenoic acids (HETE). 5- HPETE could be dehydrated into leukotriene A4 (LTA4), which was enzymatically hydrolyzed to LTB4 (Vincent et al., 2008).

The ability of LTB4, 12-HETE and 15-HETE to regulate important functions of the immune system has been widely explored. These compounds activate various blood cell types and stimulate their pro-inflammatory cytokine productions, indicating an ability of LTB4, 12-HETE and 15-HETE to augment and prolong tissue inflammation (Vincent et al., 2008).

In treated group, DHA and EPA from fish oil effectively replace AA in the cell membrane resulting in a reduction of insulin resistance by increasing the residency time of glucose transporter-4 (GLUT-4) in the plasma membrane and extending of intracellular pool of glucose-6-phosphate (Hussein, 2013) in addition to inhibition of inflammatory markers and pro-inflammatory mediators.

**CONCLUSION**

From our findings we concluded that fish oil can significantly manage several complications in diabetic ovariectomized rats. This indicative effect may be related to the high contents of omega-3 fatty acids in fish oil in suppressing hyperglycemia, hyperlipidemia and pro-inflammatory mediators as well as enhancement of insulin production and action through the improvement of cell membrane fatty acids contents (reducing ω-6/ ω-3 ratio).

**CONFLICT OF INTEREST**

The authors declared that present study was performed in absence of any conflict of interest.
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AUTHOR CONTRIBUTIONS
All authors equally contributed in the paper. All authors read and approved the final version.

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