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Marine n-3 fatty acids attenuate pro-inflammatory mediators in ovariectomized rats as a model of postmenopausal

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ABSTRACT

Postmenopause represents a major health apprehension for aging populations that might be prevented by nutritional strategies. In this study we examined whether the supplemented n-3 fatty acids could contribute with arachidonic acid (n-6) in the cell membrane to augment inflammation in ovariectomized rats. Forty female Sprague-Dawley rats were used in this study and divided into four groups: sham control, fish oil, ovariectomized (OVX) and treated groups. Erythrocyte membrane was used in this study to examine how far fish oil could augment any disturbances in its components, so that arachidonic acid (AA), linolenic acid (LA) and α-linolenic acid (ALA) were estimated by RP- HPLC using UV detector that set at 200 nm. The results appeared that fish oil supplementation in a dose of 1.2 ml /kg body weight /day orally for 8 weeks effectively attenuated inflammation in OVX rats. We concluded that supplementation of marine omega-3 fatty acids reduced the inflammation that induced by ovarictomy in female rats, we suggested that omega-3 fatty acids compete with omega-6 in incorporation in cell membrane resulting in a reduction in arachidonic acid and then a reduction in inflammatory mediators.

Keywords: Arachidonic acid, HPLC, OVX, 5-lipoxygenase, Cell membrane

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INTRODUCTION

Inflammation is a congregation process that involves prevalent changes in molecular components of physiology. Controlled inflammation is very necessary for a series of protective process including tissue repair, wound healing and defense against pathogens. Whereas, prolonged chronic inflammation is mostly linked to a number of diseases, including diabetes, cardiovascular and cancer [1, 2].

It is well documented that inflammation is associated with osteoporosis, and the reduction of bone mineral density that may be actually quickened during menopause [3]. Elevation of cytokines mainly interleukins 1 and 6 (IL-1& IL-6) and TNF-α, the tumor necrosis factor-α is well documented in postmenopausal women [4]. Indeed, estrogen deficiency accelerates many processes involved in vascular aging, including cells proliferation [5] and endothelial dysfunction [6].

Due to side effects of medical treatment, post-menopausal women have a complicated problem about the inflammation symptoms. Thence, we need to introduce another treatment that may be reduce inflammation with minimize side effects.

Hussein indicated that both polyunsaturated fatty acids (omega -3 and omega-6) have an important role in inflammatory path-ways. Although omega-6 fatty acids accelerate pro-inflammatory mediators and produce inflammatory responses, omega-3 has anti-inflammatory effects [7].

From this light, we investigated in this study whether administration of fish oil as a rich source of omega-3 might contribute with omega -6 (mainly arachidonic acid) in the cell membrane to augment inflammation in ovariectomized rats, the animal model of experimental menopause. Erythrocyte membrane fatty acids and inflammatory cytokines were measured to determine the relationship between omega-3 contents and inflammation pathway.

MATERIALS AND METHODS

Chemicals:

- Refined fish oil was purchased from local pharmacy.
- Fatty acids standards (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals:

Forty female Sprague-Dawley rats (weighing 180–200 g) at the beginning of the experiment) were obtained from the animal house of the National Research Centre (NRC) and used in this study. The rats were individually housed in clean polypropylene cages and maintained in a controlled temperature room (22 ±2ºC) with a 12 h light and a 12 h dark cycle with free access to water and standard rat food pellets. Animals were allowed 14 days to acclimatize to the laboratory conditions prior to the experiment. Experiments were conducted between 8:00 a.m. and 3:00 p.m. All procedures were conducted in accordance with ethical guidelines and with approval from the ethical committee.

Methods

The ovariectomy and sham operations were done according to the method described by [8].

Experimental Design:

Forty female albino rats were used in this study and classified into four groups (10 rats each) as follow:
Group I: Control group, sham-operated rats received a vehicle.
Group II: Fish oil group, sham group received fish oil in a dose of 1.2 ml /kg body weight /day orally for 8 weeks [9].
Group III: Ovariectomized group, ovariectomized rats received a vehicle.
Group IV: Treated group, ovarictomized rats received fish oil (1.2 ml /kg body weight /day) orally for 8 weeks.

After the experimental period, 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- Tubes contain sodium fluoride for blood glucose estimation, b- Heparinized tubes for other biochemical parameters.

Blood was centrifuged at 3000 rpm for 10 minutes using cooling centrifuge. Plasma was separated and immediately frozen. Packed RBCs were used for isolation and extraction of erythrocyte membrane lipids. The following parameters were estimated:

Fasting blood sugar was determined using enzymatic colorimetric method Centronic, Germany, according to [10]; Plasma insulin level was estimated by ELISA according to [11] using BioSoure INS-EASIA Kit.

Plasma total cholesterol and triglycerides were estimated according to [12] and [13] respectively using enzymatic colorimetric method Centronic, Germany kit. Plasma high-density lipoprotein (HDL) cholesterol was measured according to [14].

The concentration of plasma estrogen will be measured using a commercial immunosorbent assay kit (Estradiol sensitive ELISA, DRG Instruments, Marburg, Germany) according to the method of [15].

The levels of 5-lipoxygenase (5-LOX) was determined according to [16] using Enzyme-linked immunosorbent assay (ELISA) for rat according to the manufacturers protocols (R&D systems).

Tumor necrosis factor alpha (TNF - α) was determined by an enzyme amplified sensitivity immunoassay (EASIA) according to [17] the kits were purchased from Biosource, Belgium.

Analysis of cell membrane fatty acids by HPLC

Cell membrane was homogenized in 2 % acetic acid- ethyl ether mixture (2:1 volume ratio). The solution was then filtered and centrifuged at 500 xg, the organic phase was evaporated to dryness. The extract was dissolved in 200 ul acetonitrile [18].

Fish oil samples (3 different samples from different places) were treated as cell membrane samples by 2 % acetic acid- ethyl ether mixture.

HPLC Condition

This method was carried out after modification of the method described previously [19]. HPLC column C 18 (260 X 4.6, particle size 5 µl), mobile phase was acetonitrile / water mixture (70/30) v/v by isocratic elution with flow rate 1 ml / min and 200 nm wave length. Serial dilutions of standards were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

RESULTS AND DISCUSSION

Postmenopaus represents a major health apprehension for aging populations that might be prevented by nutritional strategies [20]. In our study, we investigated the effect of marine omega-3 on ovariectomized rats, the experimental model of postmenopausal women. Taken together, our data demonstrated that fish oil was able to abolish inflammation.

Table (1): Changes in plasma lipid profile in studied groups

	SH	SH + FO	OVX	OVX+ FO
Cholesterol (mg/dl)	86.8 ± 4.6 [a]	78.8 ± 3.1 [b]	125.8 ± 4.6 [c]	96.75 ± 5.6 [d]
Triglyceride (mg/dl)	49.7 ± 1.4 [a]	39.2 ± 1.4 [b]	89.2 ± 10.6 [c]	70.7 ± 4.6 [d]
HDL-Cholesterol (mg/dl)	53.7 ± 2.5 [a]	60.1 ± 1.7 [b]	33.6 ± 2.7[c]	42.3± 5.6 [d]

Values sharing the same superscript means not significant. $P > 0.05$. Values are means ± standard error of mean (SEM). Different superscript letters within a row indicate significant differences by Duncan s multiple range test $P< 0.05$. SH= sham rat, FO= fish oil; OVX= ovarectomized rat. Number of animals = 40

We hypothesized that OVX rats would exhibit lower erythrocyte omega-3 compared to sham (the control group); this reduction would be augmented by supplementation of fish oil, the marine omega-3 fatty acids .This hypothesis was confirmed by the current data; thus, our results indicated a significant increase in lipid profile (cholesterol and triglycerides) along with a significant reduction in HDL in overictomized rats compared to sham group. Although, fish oil supplementation significantly controlled these values (Table.1).

Table (2): Membrane polyunsaturated fatty acids (mg/ml RBC) in different studied groups

	SH	SH + FO	OVX	OVX+ FO
Arachidonic acid(AA, ω-6)	0.05 ± 0.0047[a]	0.08 ± 0.006 [b]	0.18 ± 0.001[c]	0.1 ± 0.001 [d]
linoleic acid (LA, ω-6)	0.73 ± 0.03[a]	0.54 ± 0.04 [b]	0.83 ± 0.04 [c]	0.55 ± 0.05 [b]
alpha- linolenic acid (ALA, ω-3)	0.69 ± 0.03 [a]	0.73 ± 0.05 [b]	0.48 ± 0.008 [c]	0.55 ± 0.05 [d]

Values sharing the same superscript means not significant. $P> 0.05$. Values are means ± standard error of mean (SEM). Different superscript letters within a row indicate significant differences by Duncan s multiple range test $P< 0.05$. SH= sham rat, FO= fish oil; OVX= ovarectomized rat. Number of animals = 40

In addition, erythrocyte membrane omega-3 (α- linolenic acid) was reduced in overictomized rats with an elevation of omega-6 fatty acids (arachidonic and linoleic acids). In contrast, fish oil supplementation significantly increased ($P< 0.05$) α- linolenic acid and decreased ($P< 0.05$) both arachidonic and linoleic acids in treated group compared to overictomized group (Table 2).

There are two metabolic pathways of AA, the first one is lipoxygenase (LOX) pathway and the second is cyclooxygenase (COX) pathways. AA is converted to thromboxanes , prostaglandins and prostacyclins by COX and converted to leukotrienes (LTs) or hydroxyeicosatetraenioc acids (HETEs) by LOX [21]. This study investigated the relationship between AA metabolic process and the degree of inflammation during postmenopausal experimental model and how far replacement of this fatty acid by omega-3 fatty acids can augment inflammation.

Table (3): Plasma levels of TNF-α , 5-lipoxygenase and estrogen in different studied groups

	SH	SH + FO	OVX	OVX+ FO
TNF-α (ng/L)	40.2 ± 2.5 [a]	42.6 ± 3.4 [a]	170.0 ± 11.0[b]	140.0 ± 6.1[c]
5-Lipoxygenase (5-LOX) (U/ml)	6.1 ± 1.36 [a]	7.5 ± 1.7[b]	9.4 ± 1.3 [c]	12.9 ± 1.13 [d]
Estrogen (Pg/ml)	43.3 ± 1.87 [a]	41.3 ± 4.3 [a]	12.5 ± 2.3[b]	20.4 ± 3.4 [c]

Values sharing the same superscript means not significant. $P > 0.05$. Values are means ± standard error of mean (SEM). Different superscript letters within a row indicate significant differences by Duncan s multiple range test $P< 0.05$. SH= sham rat, FO= fish oil; OVX= ovarectomized rat. Number of animals = 40

The anti-inflammatory and pro- inflammatory cytokines are considered as signaling molecules that are responsible for immune response' regulation. Thus, anti-inflammatory cytokines (IL-4, 10 and 13) reduce inflammatory pathway, whereas, pro-inflammatory cytokines including TNF- α and IL-1 & 6 are elevated in inflammatory processes [22]. In the same line, several studies indicated the elevation of pro-inflammatory cytokines including TNF- **α** and IL-1 in postmenopausal women [23, 24].

It was reported that, there is a connection between estrogen deficiency and pro-inflammatory markers. Pro-inflammatory cytokines, which are released due to inflammatory process, may be involved in the pathophysiology of menopause. Thus, there is a tight connection among inflammation and estrogen deficiency. From this scenery, ovariectomy is a well-documented model for postmenopausal that comparable estrogen deficiency in females [25] that explained the reduction of estrogen along with elevation of pro- inflammatory marker (TNF-α) in ovariectomized group in this study.

Diet-induced changes in the composition of cell membrane including polyunsaturated fatty acids has an important role in the functions of all cells; that may be related to the role of different fatty acids in communication between cells; particularly, n-3 family competes with n-6 family (mainly arachidonic acid) to incorporate in the cell membrane [26].

When the living cells are exposed to external stimuli, the main member of omega-6 (arachidonic acid) is liberated from cell membrane phospholipids and converted to potent cellular mediators including leukotrienes ,prostaglandins [27] and lipoxins [28].

These compounds are very important in pharmacological treatments, thus pharmaceutical products now are targeting their biosynthesis and actions [29]. Indeed, Riccioni [30] indicated that the metabolism of arachidonic acid is considered as a target of cyclooxygenase-2 (COX-2) inhibitors, leukotriene antagonists and anti- inflammatory drugs.

Supplementation of n-3 fatty acids regulate arachidonic acid liberation and metabolism directly because n-3 fatty acids supplant n-6 (arachidonic acid) from the membrane phospholipids and contend with it for the enzymes that affect and catalyze the biosynthesis of prostaglandins and leukotrienes [31].

So that, elevation of α linolenic acid in the membrane phospholipids lowers the production of AA's eicosanoids. The current data confirmed this finding by the reduction of 5-lipoxygenase (5-LOX) level significantly ($P< 0.05$) by fish oil supplementation in treated group compared to OVX group (Table 3).
Thus, supplementation of food rich in omega-3 fatty acids like fish oil and flaxseed oil attenuates the synthesis and production of inflammatory mediators resulting from arachidonic acid metabolism [32].

Our results confirmed the supposition of Yano et al. [33] who reported that n-3 family decreases the production , releasing and activity of the pro-inflammatory cytokines including IL-1&6 and TNF-α . Contrarily, n-6 family has the obverse effect. The powerful effect of n-3 PUFA to reduce pro-inflammatory cytokines leads to the idea of using fish oil to mitigate pain [34]. The profitable effects of n-3 must be investigated not only in context of their absolute level in diet, or serum and tissue, but also in context of their ratio to n-6 fatty acids (n-3/n6) [7].

CONCLUSION

Our experiment represents encouraging results for using n-3 PUFA in postmenopausal women and favors the need to test our hypothesis in a clinical tuning.

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