FLAXSEED HAVE BENEFICIAL EFFECTS ON ENDOTHELIAL DYSFUNCTION AND OXIDATIVE STRESS IN EXPERIMENTAL ATHEROSCLEROSIS

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Summary

The present study examined the effects of flaxseed, a rich source of polyunsaturated fatty acids n3 (PUFA n3) and lignans, on endothelial dysfunction and oxidative stress biomarkers in an animal model of atherosclerosis induced by ovariectomy associated with high-fat diet. Forty-two female Wistar rats were either sham-operated (Sham) or ovariectomized (Ovx) and each of the two groups were randomly assigned to three different diets: low-fat diet (8 % energy as fat); high-fat diet (40% energy as fat, lard-based); high-fat diet enriched with ground flaxseed 15 g/100 g of food, for 36 weeks. The circulating soluble adhesion molecules of endothelial origin and von Willebrand factor (sVCAM-1, vWF), and oxidative stress markers were measured to assess the endothelial dysfunction and atherosclerosis risk biomarkers. Serum VCAM-1 increased in Lard groups compared to low-fat diet groups and the addition of flaxseeds significantly decreased this endothelial marker in Sham and Ovx groups. The ovariecetomized rats fed with lard+flaxseeds had significantly lower serum concentrations of vWF as compared to Ovx+Lard group. Ovariectomy significantly increased serum and liver TBARS. High-fat diet resulted in significant increasing of serum TBARS in Sham group and liver TBARS in Ovx group as compared to low-fat diet. The flaxseed addition to the high-fat diet led to reductions of serum and liver TBARS in Sham and Ovx groups. The supplementation of diet with flaxseed significantly increased liver GSH in Sham and Ovx animals, as compared to animals fed with standard and high-fat diet. Supplementation of the diet with integral flaxseeds had beneficial effects on the vascular wall protection in experimental menopause associated with high-fat diet, through improvement of endothelial dysfunction and oxidative stress biomarkers.

Keywords: endothelial dysfunction, oxidative stress, flaxseed, atherosclerosis.

Introduction

Atherosclerosis has ceased to be considered a simple “lipid disease” in recent years, and is now understood to be a dynamic and progressive process stemming from endothelial dysfunction and inflammation of the vessel wall that can lead to an acute event due to plaque rupture or thrombosis (Ribeiro et al, 2009). Cardiovascular diseases (CVD) are the main causes of death in Europe, accounting for over 4 million deaths each year (52% of deaths in women and 42% of deaths in men). The risk for CVD increases drastically when women reach the age of menopause (Lucas et al, 2004). The fact that hormone replacement therapy (HRT) is associated with increased risk of endometrial, breast and ovarian cancer, systemic lupus erythematosus and thromboembolism (Judd et al, 1983) have conducted postmenopausal women and their practitioners for non-pharmacological alternative regimens of reducing CVD risks. Previous studies reported animal ovariectomy as a suitable model of
postmenopausal hypercholesterolemia and atherosclerotic lesions formation (Lucas et al., 2004). In the present study, we followed the effects of ovarian hormones deficiency in surgical menopause (experimental ovariectomy), cumulative with a diet rich in saturated (SFA) and monounsaturated fatty acids (MUFA), as risk factors for progression of endothelial dysfunction. The absence of endogenous estrogens disturbs the lipid metabolism, increases oxidative stress and decreases the antioxidant capacity, while the excess of SFA and MUFA determines hyperlipidemia and hypercholesterolemia, with increased atherogenic potential. Plasma concentrations of von Willebrand factor (vWF) are a standard indicator of endothelial injury, while vWF is playing a crucial role in platelet adhesion to damaged arterial wall (Vlot et al., 1998; Paulinska et al., 2009). Several studies have also investigated the association between serum cell adhesion molecules levels and progression of CVD and have found elevated levels of intercellular adhesion molecules (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in individuals with atherosclerotic disease (Tousoulis et al., 2001).

Flaxseeds (Linum usitatissimum) are known to contain 35-40% fat, of which 55% is represented by alpha-linolenic acid – ALA (n3 polyunsaturated fatty acids - PUFA) and 15 - 18% linoleic acid (n6 PUFA). In addition of being one of the richest plant source of ALA and lignans (mainly, secoisolariciresinoldiglucoside, SDG), which have phytoestrogen properties, flaxseed is an essential source of dietary fiber (Babu et al., 2000). All of these components could positively influence women's CVD risk profile (Mocanu et al., 2011; Martinchik et al., 2012). In our current diet there are excessive amounts of SFA and MUFA compared with the intake of PUFA n3 and n6, representing an increased atherogenic risk.

The aims of this study were to investigate the effects of flaxseed supplementation, a rich source of ALA, lignans and fibers, on endothelial inflammation and dysfunction markers (sVCAM-1, vWF) and parameters of oxidative stress in an animal model of ovariectomy associated with high-fat diet, rich in SFA and MUFA.

**Materials and methods**

**Animals**

Fourty-two female Wistar rats (14 weeks old, weight 200±20 g) were used in the experiment. The rats were anaesthetized with an intraperitoneal injection of a mixture of Ketamine, dose of 100 mg/kg bodyweight and Xylazine, dose of 10 mg/kg bodyweight. Half of the rats (n = 21) were subjected to bilateral ovariectomy (Ovx), using the dorsolateral approach. The remaining animals (n = 21) were subjected to sham surgery, during which the ovaries were exteriorized, but replaced intact (Sham). This study was approved by the Laboratory Animal Care Committee of “Gr. T. Popa” University of Medicine and Pharmacy and the rats were maintained in accordance with the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities (Council of European Communities, 1986).

**Conditions and diets**

The rats were kept in standard laboratory conditions, with a controlled temperature (20±2°C) and a 12 h light/12h dark cycle. The animals were provided with laboratory chow 20 g food/rat/day and tap water ad libitum. Each of the two groups (Ovx and Sham) were randomly assigned for 36 weeks to three different diets: 1) low-fat diet (8 % energy as fat, deficient in ALA, Control); 2) high-fat diet (40% energy as fat, lard-based, Lard group); 3) high-fat diet enriched with ground flaxseed 15 g/100 g of food, rich in alpha-linolenic acid, ALA (Lard+Flaxseed group).
Diets had similar carbohydrate, total fiber, protein, and fat content (*Table 1*).
Table 1. The composition of experimental diets

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>8% Fat</th>
<th>40% Fat</th>
<th>40% Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lard</td>
<td>Lard + Flaxseed</td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>62.00</td>
<td>32.00</td>
<td>32.00</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>L-cystein</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>Choline</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Fat</td>
<td>8.00</td>
<td>40.00</td>
<td>40.00</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>8.00</td>
<td>15.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Lard</td>
<td>-</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Flaxseeds**</td>
<td>-</td>
<td>-</td>
<td>8.00</td>
</tr>
</tbody>
</table>

** Flaxseeds (*Linum usitatissimum*) belonged to the Olin variety and were provided by the Department of Phytotechny, Faculty of Agronomy Iasi. The composition of flax seeds was: 40.2 % oil (55.6 % linolenic acid) and 19.5 % proteins.

Animal necropsy and processing of samples

After 36 weeks, the animals were sacrificed, by thiopental anesthesia (1ml/100 g body weight from 0.01% solution), followed by opening the chest and collecting the blood by cardiac puncture. Blood samples were collected using sodium citrate as anticoagulant buffer, blood/citrate ratio of 9:1, or without anticoagulant. Aliquots of serum were frozen and kept at -80°C for later analysis. The liver was immediately removed, rinsed with icecold saline, homogenized and stored at -20°C until analyzed.

Parameters of endothelial dysfunction

Serum VCAM-1 was measured by ELISA method for quantitative evaluation of human sVCAM-1 (Bender Medical System) (Hession et al., 1992). Serum vWF was measured by an immuno-enzymatic "sandwich" method for vWF antigen (Life Therapeutics) (Bartlett et al., 1976).

Parameters of oxidative stress

Serum and liver thiobarbituric acid reactive substances (TBARS) are an index of lipid peroxidation and oxidative stress. TBARS were determined by an adapted method from Phelps (Phelps S. and Harris W.S., 1993). The quantity of the TBARS was measured using a TECAN microplate reader at a wavelength of 540 nm.

Liver reduced glutathione (GSH) was determined by an enzymatic reaction, based on the oxidation of reduced glutathione (GSH) by 5.5'- diithiobis (acid 2-nitrobenzoic) (DTNB), in the presence of glutathione reductase and NADPH2, monitored at a wavelength of 405 nm (Tietze F., 1969).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Univariate statistical analysis was performed using the Student’s t-test and Bonferroni’s Multiple Comparison Test (Statistical Software Package SPSS®, version 13, SPSS Incorporation, Chicago, IL, USA).

Results and discussion

In our research, the anti-atherogenic mechanism of flaxseed-enriched diet was investigated in ovariectomized female rats, a model of experimental atherosclerosis (Lucas et al., 2004). The absence of endogenous estrogens disturbs the lipid metabolism, increases oxidative stress and decreases the antioxidant capacity, and alters the expression of adhesion molecules and platelet adhesion to endothelial cells (Cossette et al., 2012; Cutini et al., 2012). Moreover, the excess of saturated FA determines hypercholesterolemia and could increase the atherogenic potential (Cutini et al., 2012).
Table 2. Mean ± SD values for endothelial markers in studied groups

<table>
<thead>
<tr>
<th>MEASURES</th>
<th>Sham</th>
<th>Sham +Lard</th>
<th>Sham +Lard +Flaxseed</th>
<th>Ovx</th>
<th>Ovx +Lard</th>
<th>Ovx +Lard +Flaxseed</th>
</tr>
</thead>
<tbody>
<tr>
<td>sVCAM (ng/ml)</td>
<td>175±64</td>
<td>286±26b</td>
<td>294±11b</td>
<td>252±53a</td>
<td>539±162ab</td>
<td>404±10bc</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>111±6</td>
<td>118±6</td>
<td>104±10</td>
<td>178±13a</td>
<td>193±16ab</td>
<td>155±10bc</td>
</tr>
</tbody>
</table>

Values are means±S.D., n = 7 in each group. *p< 0.05 as compared to corresponding Sham groups. 

b p< 0.05 as compared to groups fed with standard diet. 

c p< 0.05 between Lard and Lard+flaxseed fed groups.

Table 2 shows the changes in endothelial markers by addition of lard or lard+flaxseed in Sham and Ovx groups. In our study, serum sVCAM-1 increased in Lard groups as compared to low-fat diet groups and the addition of flaxseeds significantly decreased this endothelial marker in Sham and Ovx groups. Serum vWF increased in Ovx groups as compared to Sham groups. The ovariectomized rats fed with lard+flaxseeds had significantly lower serum concentrations of vWF as compared to Ovx+Lard group.

Table 3. Mean ± SD values for oxidative stress parameters in studied groups

<table>
<thead>
<tr>
<th>MEASURES</th>
<th>Sham</th>
<th>Sham +Lard</th>
<th>Sham +Lard +Flaxseed</th>
<th>Ovx</th>
<th>Ovx +Lard</th>
<th>Ovx +Lard +Flaxseed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum TBARS (nmol/ml)</td>
<td>4.0±0.6</td>
<td>5.0±0.7b</td>
<td>4.5±0.4</td>
<td>4.8±0.4a</td>
<td>5.1±0.4</td>
<td>4.7±0.7</td>
</tr>
<tr>
<td>Liver TBARS (nmol/mg protein)</td>
<td>7.3±1.1</td>
<td>8.2±1.0</td>
<td>7.3±1.6</td>
<td>8.6±5.3a</td>
<td>12.2±3.8ab</td>
<td>9.4±2.2</td>
</tr>
<tr>
<td>Liver GSH (µmol/mg protein)</td>
<td>5.3±0.7</td>
<td>6.2±0.7</td>
<td>8.3±0.8bc</td>
<td>6.6±1.9</td>
<td>7.1±1.5</td>
<td>9.1±2.8bc</td>
</tr>
</tbody>
</table>

Values are means±S.D., n = 10 in each group. *p< 0.05 as compared to corresponding Sham groups. 

b p< 0.05 as compared to groups fed with standard diet. 

c p< 0.05 between lard and lard+flaxseed fed groups.

Table 3 shows the changes in serum and liver TBARS levels, as markers of oxidative stress and liver GSH, as marker of antioxidant defence, by addition of lard or lard+flaxseed in Sham and Ovx groups. Ovariectomy significantly increased serum and liver TBARS. High-fat diet resulted in significantly increasing of serum TBARS in Sham group and liver TBARS in Ovx group as compared to low-fat diet. The flaxseed addition to the high-fat diet led to reductions of serum and liver TBARS in Sham and Ovx groups. The supplementation of diet with flaxseed significantly increased liver GSH in Sham and Ovx animals, as compared to animals fed with standard and high-fat diet.

In the present study, ovariectomy and lard-based diet increased serum concentrations of endothelial dysfunction markers (sVCAM-1 and vWF) and oxidative stress parameters (serum and liver TBARS), factors with high contribution to the initiation and development of atherosclerotic lesions in female rats fed on high-fat diet. The addition of ground flaxseed (15 g Linum usitatissimum/100 g food) to lard-based diet significantly reduced serum concentrations of endothelial integrity markers (sVCAM, vWF), reduced serum and liver TBARS and significantly increased antioxidant defence, and these effects could prevent the progression of atherosclerotic lesions in estrogen deficiency states. Our results clearly demonstrated that the flaxseed-enriched diet may protect against atherosclerotic lesions by decreasing endothelial dysfunction.
markers, decreasing oxidative stress and increasing antioxidative defence.

The results of our study suggested that, in a condition associated with two cardiovascular risk factors, estrogen deficiency and increased saturated fatty acids intake, the endothelial markers and oxidative stress are significantly changed and the diet supplementation with flaxseed had a beneficial effect.

Our results are consistent with literature studies that have found the relation between endothelial dysfunction and plasma levels of adhesion molecules (Jenkins et al., 1999). Thus, flaxseeds (exogenous antioxidants) and estrogens (endogenous antioxidants) in physiological concentrations, may have synergistic effects of endothelial dysfunction prevention and thus prevention of atherosclerosis, as found by Lucas et al., 2004. A recent metaanalysis revealed that supplementation of the diet with n-3 PUFA significantly improves the endothelial function, without affecting endothelium-independent dilation (Wang et al., 2012). Since increased sVCAM-1 and vWF means endothelium activation, we believe that our results confirm the presence of endothelial dysfunction caused by high-fat diet rich in SFA and MUFA, both in Sham group and especially in those with endogenous estrogen deficiency (Ovx). Dietary supplements with PUFA n3 and lignans reduced sVCAM-1 and vWF levels, with statistical significance, improving endothelial function.

Studies investigating the effects of n3 PUFA dietary supplements on oxidative stress in normal or pathological conditions have reported mixed results, and sometimes contradictory. Cardiovascular risk factors can be significantly reduced with PUFA n3 from the plants/seeds, while PUFA n3 from marine plants have a lower impact (Mozaffarian et al., 2005). Some studies have noted the ineffectiveness of flaxseed oil in the prevention of MDA increase in dietary hypercholesterolemia in rabbits, which confirms that n3 PUFA-rich flaxseed oil has contradictory effects on the reduction of oxidative stress (Lee, Prasad, 2003). Recent studies showed that seicosolariiresinol diglucosid (SDG), the lignan from flaxseed, and its metabolites, have antioxidant activity (Prasad, 2009). The effects of SDG on lipids, oxidative stress parameters and development of atherosclerotic lesions in the aorta in rabbits fed a high cholesterol diet have been studied, the results suggesting that SDG had beneficial effects by reducing hypercholesterolemia-induced atherosclerotic changes; these effects were associated with reduced lipid peroxidation products, total and LDL cholesterol, and increased antioxidant defense (Prasad et al., 1998; Prasad, 2005).

Conclusions

In our study, high dose of ground flaxseed incorporated to lard-based diet prevented the progression of atherosclerotic lesions in estrogen deficiency female rats by decreasing endothelium activation and oxidative stress. Comparing with the dates from the speciality studies, it appears that n3 PUFA from flaxseeds improved the endothelial function assessed by sVCAM and vWF, while SDG lignan exerted antioxidant effects. Assessment of endothelial dysfunction by serum soluble adhesion molecule sVCAM and endothelium integrity molecule vWF and by measuring oxidative stress parameters could be useful to detect the risk for atherosclerotic lesions in estrogen deficiency states and to estimate the effects of flaxseed supplementation. Supplementing the diet with high doses of ground flaxseed may lower the atherosclerotic risk in postmenopausal women by increasing the vascular wall protection and decreasing oxidative stress.

References

Babu, U.S.; Mitchell, G.V.; Wiesenfeld, P.; Jenkins, M.Y.; Gowda, H.: Nutritional and hematological impact of dietary flaxseed and


