Daily Consumption for Six Weeks of a Lignan Complex Isolated from Flaxseed Does Not Affect Endothelial Function in Healthy Postmenopausal Women

Jesper Hallund, 3 Æ Inge Tetens, 4 Susanne Bügel, 1 Tine Tholstrup, 3 Marika Ferrari, 5 Tom Teerlink, 6 Andreas Kjaer, 2 and Niels Wiinberg 8

1Department of Human Nutrition, Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark; 2Department of Nutrition, the Danish Institute for Food and Veterinary Research, Søborg, Denmark; 3Human Nutrition Unit, National Institute for Research on Food and Nutrition, Rome, Italy; 4Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands; 5Department of Clinical Physiology, Nuclear Medicine and PET, Rigshospitalet, Denmark; and 6Department of Clinical Physiology and Nuclear Medicine, Frederiksberg University Hospital, Frederiksberg, Denmark

Abstract

The occurrence of menopause is associated with an increased risk of cardiovascular events, and this has partly been attributed to the decline in circulating levels of estrogen. A lignan complex rich in the plant lignan secoisolariciresinol diglucoside (SDG) was isolated from flaxseed. SDG is metabolized by the colonic microflora to the mammalian lignans enterodiol and enterolactone and is hypothesized to be cardioprotective due to their structural similarity to estrogen. The aim of this study was to investigate the effect of a lignan complex, providing 500 mg/d of SDG, on markers of endothelial function. Healthy postmenopausal women (n = 22) completed a randomized, double-blind, placebo-controlled, crossover study. Women consumed daily a low-fat muffin, with or without a lignan complex, for 6 wk, separated by a 6-wk washout period. Flow-mediated, endothelium-dependent vasodilatation (FMD) and nitroglycerine-mediated, endothelium-independent vasodilatation were measured at the end of each intervention period. The sum of Plasma nitrite and nitrate (NOx), endothelin-1 (ET-1), and asymmetric dimethylarginine (ADMA) were measured at the beginning and end of each intervention period. FMD was 3.6 ± 0.9% (mean ± SEM) after the lignan complex intervention period compared with 3.9 ± 0.7% after the placebo period (P = 0.72). Plasma concentrations of NOx, ET-1, and ADMA were not affected. We conclude that daily consumption for 6 wk of a low-fat muffin enriched with a lignan complex had no effect on endothelial function in healthy postmenopausal women. J. Nutr. 136: 2314–2318, 2006.

Introduction

Women have an increased risk of cardiovascular disease (CVD) 9 after the occurrence of menopause, and this has partly been attributed to the decline in circulating levels of estrogen (1). The occurrence of menopause is associated with a more atherogenic lipid profile (2) and altered vascular reactivity. Endothelial function, which plays a central role in the development, progression, and clinical manifestations of atherosclerosis, has been shown to decline around the time of menopause (3), and a similar decline has been reported for the distensibility of the aorta (4).

Flaxseed is the richest dietary source of the plant lignan secoisolariciresinol diglucoside (SDG), which is metabolized to the mammalian lignans enterodiol and enterolactone (ENL) by colonic bacteria (5). Plant lignans belong to the group of phytoestrogens that are structurally similar to endogenous estrogen and have binding affinity to the sex steroid-binding globulin (6). It has been hypothesized that isoflavones may be cardioprotective due to their structural similarity to estradiol (7). Recently, a number of observational studies have shown that mammalian lignans may have an effect on the vascular system and, thus, a protective effect against the risk of CVD. A higher habitual plant lignan intake has been associated with improved vascular function through increased systemic arterial compliance (8). In addition, it has been associated with a small reduction in CVD risk among past and current smokers, but not with overall risk among postmenopausal women (7). High serum ENL

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2 No reprints available.
3 Abbreviations used: ADMA, asymmetric dimethylarginine; BP, blood pressure; CVD, cardiovascular disease; ENL, enterolactone; eNOS, endothelial NO synthase; ET-1, endothelin-1; FMD, flow-mediated, endothelium-dependent vasodilatation; LDL-C, LDL cholesterol; MMD, nitroglycerine-mediated, endothelium-independent vasodilatation; NO, nitric oxide; NOx, sum of Plasma nitrite and nitrate; SDMA, symmetric dimethylarginine; SDG, secoisolariciresinol diglucoside; TC, total cholesterol.
4 To whom correspondence should be addressed. E-mail: jeha@kvl.dk.

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concentrations have prospectively been associated with a reduced risk of acute cardiovascular events and lower coronary heart disease and CVD-related mortality in healthy men (9,10).

The mechanisms underlying these potentially beneficial effects of mammalian lignans are not fully understood. A number of randomized controlled studies have shown that flaxseed and defatted flaxseed may reduce serum cholesterol in normal and hyperlipidemic subjects (11–13). In addition, animal studies have shown that isolated flaxseed lignans reduce total cholesterol (TC) and LDL cholesterol (LDL-C) concentrations and the extent of hypercholesterolemic atherosclerosis (14,15).

Another plant lignan, sesamin, has been shown to improve endothelial dysfunction in hypertensive rats (16) and increase nitric oxide (NO) production and reduce endothelin-1 (ET-1) production in human umbilical vein endothelial cells (17). To our knowledge, no other human intervention studies have been published in relation to the possible role of the plant lignan SDG on cardiovascular risk markers. To test the hypothesis that a lignan complex isolated from flaxseed would affect endothelial function in healthy postmenopausal women, we measured endothelial function by flow-mediated, endothelium-dependent vasodilatation (FMD) and nitroglycerine-mediated, endothelium-independent vasodilatation (NMD). In addition, changes in plasma concentrations of the sum of nitrate and nitrite (NOx), ET-1, and the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) were measured. The effects on arginine and symmetric dimethylarginine (SDMA) were also investigated.

Subjects and Methods
The primary objective of the present study was to determine whether a lignan complex isolated from flaxseed would affect endothelial function. Secondly, its effect on plasma lipids, plasma antioxidant capacity, and inflammation was examined. Results on plasma lipids and antioxidant capacity have been published previously (18).

Subjects. Healthy postmenopausal women (defined as no menstrual period for >24 mo), aged 61 ± 7 y (mean ± SD), were recruited from Copenhagen, Denmark and the surrounding areas by advertisement in the local media. Before the study, none of the women had used hormone-replacement therapy for at least 6 mo; fatty acid-, isoflavone-, vitamin-, or mineral-containing supplements for 2 mo; or antibiotics for 3 mo. None of the women had a history of diabetes, inflammatory diseases, or CVD, and they did not use antihypertensive, antiinflammatory, or lipid-lowering drugs on a regular basis. All women were nonsmokers and had a blood pressure (BP) <160/90 mm Hg. Screening blood samples were taken before entry, and all subjects had TC <5 mmol/L, triacylglycerol <1.7 mmol/L, Hg >7.0 mmol/L, and fasting glucose <6.5 mmol/L. A total of 23 women were included in the study. One woman withdrew from the study due to the use of antibiotics during the study period. Twenty-two women completed the study according to the protocol.

Ethical approval. Ethical approval was obtained from the Local Research Ethical Committee of Copenhagen and Frederiksberg (KF 11–0477/03). All women received oral and written information about the study before they gave written informed consent.

Study design. We performed a randomized, double-blind, placebo-controlled, crossover study. The women consumed a low-fat muffin, with or without a lignan complex, for 6 wk, separated by a 6-wk washout period. The women were instructed to consume the muffin at least 1 h after dinner and to keep daily records of muffin consumption and well-being in a study diary. The muffins were identical in all respects other than enrichment with a lignan complex providing 500 mg/d of SDG. The average nutrient content of each muffin (58 g) was as follows: energy, 642 kJ; protein, 2.6 g; carbohydrate, 34.1 g; fat, 0.5 g; and dietary fiber, 0.7 g. The muffins were produced in a single batch and were frozen at −20°C until use. The lignan complex was added to the dough just before baking. The major components of the lignan complex were as follows: 32.9% SDG, 13.9% cinnamic acids, 11.8% protein, 10.0% 3-hydroxy-3-methyl glutaric acid, 3.5% fat, 3.3% moisture, and 1.0% ash. The women were instructed to avoid any flaxseed consumption during the study period. Compliance was assessed using study diaries, as well as ENL concentrations in serum and urine.

Frozen muffins were handed out to the women every 2nd wk from the department for consumption at home. In addition, the women visited the department for 5 examinations during the study: 1 wk before the beginning of the study (wk −1), at the beginning of the study (wk 0), after the first intervention period (wk 6), at the beginning of the second intervention period after the 6-wk washout period (wk 12), and after the second intervention period (wk 18). FMD and NMD were measured at wks 6 and 18. Height was measured at wk 0. Body weight, BP, 24-h urinary ENL excretion, serum ENL concentrations, and plasma NOx, ET-1, ADMA, arginine, and SDMA concentrations were measured at wks 0, 6, 12, and 18. A 3-d weighed food record was performed 1 wk before the study and during the last week of each intervention period to estimate habitual dietary intake. Energy and nutrient intake were calculated using the Dankost 2000 dietary assessment software (National Food Agency, Herlev, Denmark).

Blood samples were collected from subjects during the morning after they had fasted for 12 h and had rested for 15 min in a supine position. A total of 180 mL of blood was collected during the entire study. Women were instructed to consume a standardized low-fat meal providing a maximum of 15 g of fat the evening prior to blood collection. Vascular measurements of FMD and NMD were performed during the afternoon. The time of the measurements was standardized, and each woman was measured at the same time during the afternoon of each visit. A standardized breakfast and lunch were provided on the day of the measurements, which together contained a total of <7 g fat, <25 mg vitamin C, and <2 mg vitamin E. All the women were instructed to finish the meals at least 3 h before the measurements and to abstain from coffee, caffeine-containing beverages, and tea during the day.

Vascular measurements. Endothelial function was measured by ultrasound using the method described by Corretti et al. (19). FMD and NMD were calculated as the change in the diameter of the right superficial brachial artery following ischemia and sublingual nitroglycerine administration, respectively, as a percentage of the baseline diameter. The baseline diameter of the right superficial brachial artery was measured at rest and 1 min after reactive hyperemia induced by an arterial occlusion cuff around the proximal part of the forearm to a pressure of >200 mm Hg for 4.5 min, causing FMD. After 10 min the baseline diameter was measured again and compared with arterial response 4 min after 400 μg of nitroglycerine spray was administered sublingually as glyceryl trinitrate, causing NMD. The diameter was measured by Q LAB Quantification software (Philips) using stored images of the brachial artery. Each diameter was measured at 2 cardiac cycles at the end of diastole, defined by the R wave. Images were obtained by a B-mode scan on a standard Acuson 128 XP/10k system using a 7- to 10-MHz linear array transducer while an ECG trace was simultaneously recorded. Images were magnified using the resolution box function. All measurements were performed in a supine position after 15 min of rest at room temperature in a calm environment.

Laboratory measurements. For the analysis of serum ENL, a fasting blood sample was drawn into a 5-mL tube with no additives (Becton Dickinson 366454) and centrifuged at 3000 × g for 15 min at 20°C and stored at −20°C until further analysis. Twenty-four-hour urinary excretion was collected into 2.5-L containers with 2 g of added boric acid and stored at −20°C until further analysis. Serum and urine ENL concentrations were determined using time-resolved fluoroimmunoassay (Lambmaster Diagnostics) as described previously (20,21). The intraassay and interassay CVs were 15.6 and 14.0% and 10.2 and 8.3% for serum and urinary ENL, respectively. A fasting blood sample was drawn into a 10-mL Na heparin tube for the NOx analysis and a 10-mL EDTA tube...
for the ET-1 analysis. Tubes were centrifuged at 1600 × g for 10 min at 4°C and stored at −80°C until further analysis. The concentration of NOx was measured by reduced NADPH-dependent nitrate reductase assay (22). Intraassay and interassay CVs were 3.4 and 3.7%, respectively. ET-1 concentrations were determined using the Parameter human ET-1 immunoassay kit (R&D Systems Europe). Intraassay and interassay CVs were 6 and 9%, respectively. A fasting blood sample was drawn into a 5-mL Na heparin tube for the arginine, ADMA, and SDMA analyses. Tubes were centrifuged at 3000 × g for 15 min at room temperature and stored at −80°C until further analysis. The concentrations of ADMA, arginine, and SDMA were determined simultaneously by HPLC as described previously (23). Intraassay and interassay CVs were better than 1.2 and 3.0%, respectively (23).

**Power calculations.** The number of women needed in this study was calculated using the method of least standardized difference (24). The study was primarily designed to demonstrate an improvement in FMD of at least 2 percentage points. The inclusion of 23 women gave the study enough power (80%) to detect a significant difference \( (P < 0.05) \) of 0.65 × SD of the study outcome.

**Statistical analysis.** Data describing the characteristics of the volunteers are summarized as the means ± SD. Data on the outcome of the study are expressed as means ± SEM. Data on NOx, ET-1, ADMA, SDMA, and arginine were analyzed in SAS 8.02 (SAS Institute) using a mixed-model analysis of covariance with treatment (lignan complex muffin or placebo muffin) and period (first or second period) as fixed factors, subjects as random factor, and the baseline measurements as a covariate. Further fixed terms corresponding to treatment-period interactions were included to test for any carryover effect between periods and the treatment-covariate interaction were included to test if the treatment effect of the lignan complex varied according to the baseline values of the covariate. For the analysis of FMD and NMD, data were analyzed using a mixed-model analysis without covariates. Differences were considered significant when \( P < 0.05 \).

**Results**

**Baseline characteristics and compliance.** The baseline characteristics of the women were age 61 ± 7 y, BMI 24.1 ± 3.4 kg/m², TC 5.97 ± 1.02 mmol/L, LDL-C 3.77 ± 1.01 mmol/L, HDL cholesterol 1.75 ± 0.45 mmol/L, triacylglycerol 0.98 ± 0.26 mmol/L, systolic BP 124 ± 13 mm Hg, and diastolic BP 75 ± 8 mm Hg.

The volunteers perceived the lignan complex and placebo muffins identical in appearance and taste. Compliance assessed using study diaries showed that 98% of all muffins were consumed during the 2 intervention periods.

**Mammalian lignans.** ENL concentrations in serum and urinary ENL excretion increased from 46 ± 8 nmol/L and 17 ± 2 μmol/d, respectively, at baseline to 385 ± 67 nmol/L \( (P < 0.001) \) and 94 ± 11 μmol/d \( (P < 0.001) \), respectively, following the lignan complex intervention period. We observed no changes in serum ENL concentrations or urinary ENL excretion during the placebo intervention period. As previously reported, serum ENL concentrations and urinary ENL excretion did not change during the placebo intervention period (18).

**Endothelial function.** Endothelium-dependent and endothelium-independent vasodilatation did not differ between treatments. FMD was 3.6 ± 0.9% after the lignan complex treatment compared with 3.9 ± 0.7% after placebo treatment. NMD was 15.9 ± 3.1% after the lignan complex treatment compared with 12.9 ± 1.1% after placebo treatment.

**Endothelium-derived relaxing and constricting factors and ADMA.** We found no significant differences in plasma concentration of NOx, ET-1, the ratio of NOx to ET-1, ADMA, arginine, and SDMA after the lignan complex treatment period and placebo periods (Table 1).

**Body weight and BP.** We observed no significant changes in body weight \( (P = 0.482) \), BMI \( (P = 0.438) \), systolic BP \( (P = 0.799) \), and diastolic BP \( (P = 0.642) \) following the intervention study.

**Dietary intake.** We observed no significant differences in energy \( (P = 0.558) \), fat \( (P = 0.855) \), SFA \( (P = 0.751) \), monounsaturated fatty acid \( (P = 0.701) \), polyunsaturated fatty acid (PUFA) \( (P = 0.881) \), protein \( (P = 0.402) \), carbohydrate \( (P = 0.350) \), total fiber \( (P = 0.956) \), dietary cholesterol \( (P = 0.612) \), and alcohol \( (P = 0.596) \) intake calculated at baseline and wk 6 of the intervention and placebo periods.

**Discussion**

In this double-blind, placebo-controlled, and randomized crossover study, we have shown that 6 wk of consumption of a muffin enriched with a lignan complex isolated from flaxseed, providing 500 mg/d SDG, had no effect on FMD and plasma concentrations of NOx, ET-1, and ADMA. To our knowledge, no other human intervention studies have investigated the effect of plant lignans on cardiovascular risk factors related to endothelial function. We have previously reported additional results from the same study where we have shown that the lignan complex intervention has no effect on plasma lipid concentrations and antioxidant capacity (18).

In the present study, a lignan complex isolated from flaxseed was chosen to focus on the isolated effect of the plant lignans.

**Table 1** Plasma concentration of NOx, ET-1, the ratio of NOx to ET-1, ADMA, arginine, and SDMA in healthy postmenopausal women at baseline and wk 6 of the lignan complex intervention and placebo periods

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lignan complex</th>
<th>Placebo</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 0</td>
<td>Wk 6</td>
<td></td>
</tr>
<tr>
<td>NOx, μmol/L</td>
<td>16.69 ± 1.92</td>
<td>20.55 ± 2.64</td>
<td>0.88</td>
</tr>
<tr>
<td>ET-1, ng/L</td>
<td>12.70 ± 0.79</td>
<td>12.66 ± 0.70</td>
<td>0.43</td>
</tr>
<tr>
<td>NOx/ET-1, μmol/ng</td>
<td>1.47 ± 0.20</td>
<td>1.74 ± 0.29</td>
<td>0.75</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>0.47 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>Arg, μmol/L</td>
<td>89.65 ± 3.09</td>
<td>92.92 ± 3.36</td>
<td>0.61</td>
</tr>
<tr>
<td>SDMA, μmol/L</td>
<td>0.52 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.26</td>
</tr>
</tbody>
</table>

1 All values are mean ± SEM, n = 22.
2 P-values are shown for the treatment effect analyzed using a mixed-model analysis of covariance.
without the physiological effects of fiber, PUFA, and protein. The lignan complex was baked into a low-fat muffin and provided to the women in 1 daily dose, which they were instructed to consume at least 1 h after dinner. This was chosen to reduce any potential interaction with the dinner-related fat intake as it has been shown that a high intake of fat is associated with lower concentrations of ENL (25). In addition, earlier studies have shown that serum ENL concentrations can be maintained with 1 daily dose of lignans after a period with constantly high lignan intake (26). SDG has been shown to be stable to the baking process and almost all SDG is recovered (27). The dose of 500 mg/d of SDG used in our study corresponds to ~21–42 g defatted flaxseed or 38–82 g whole flaxseed according to recent analysis of SDG content in flaxseed (28). The dose was chosen to allow for comparison between results in the present study and earlier studies using 38–50 g of ground and defatted flaxseed (11–13).

Some issues about the methodology used in the present study need to be addressed before the results are discussed. The number of women included in this study was calculated using the method of least standardized difference (24) and the study was designed to demonstrate an improvement in FMD of at least 2 percentage points. It has earlier been suggested that a mean improvement in FMD of at least 2 percentage points would be required to detect a treatment benefit (29). Post-hoc power calculations based on the results from this study have shown that inclusion of 23 women was sufficient to detect an improvement in FMD of at least 2 percentage points. In the present study, the protocol included a high level of standardization to minimize intraindividual variation. In addition, compliance was high according to the study diaries, and this was further confirmed by the significant rise in serum and urine ENL concentration. Therefore, if there had been a specific effect of the lignan complex on FMD, it would likely have been apparent in the present study.

Endothelial function is modulated by the release of endothelium-derived constricting factors such as ET-1 and relaxing factors such as NO. NO is synthesized from the substrate L-arginine by the enzyme endothelial NO synthase (eNOS) and is rapidly oxidized into nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Due to the rapid conversion and distribution of NO, endogenous NO production is commonly assessed by measuring NOx in plasma. However, the use of NOx to assess eNOS activity has been challenged because it was shown that changes in NOx did not necessarily reflect altered eNOS activity (30). In the present study, no differences in plasma NOx and ET-1 concentrations were found between the lignan complex intervention and placebo period. Earlier studies have shown that the plant lignan sesamin induces NO production and decreases ET-1 production in human umbilical vein endothelial cells (17). Sesamin has been shown to be a major precursor of ENL in humans, but only moderate concentrations of free sesamin are found in plasma after sesame seed intake (31). Possibly, ENL does not have the same effect on NO and ET-1 production as those proposed for sesamin based on in vitro studies.

ADMA is an endogenous competitive inhibitor of eNOS, and elevated plasma concentrations of ADMA have been associated with endothelial dysfunction (32). Recently, it has been shown that estrogen-replacement therapy reduces plasma concentrations of ADMA in healthy postmenopausal women (33). However, we found no changes in plasma ADMA concentrations after the lignan complex intervention period.

The results from the present study do not suggest that plant lignans isolated from flaxseed may affect endothelial function in healthy postmenopausal women. In addition, previously published results from the same study showed no effect on plasma lipid concentrations and antioxidant capacity (18). Taken together, these results are not in agreement with earlier published observational studies finding an association between a higher habitual intake of plant lignans and improved vascular function (8), as well as an association between high serum ENL concentrations and reduced risk of acute cardiovascular events and lower coronary heart disease and CVD-related mortality (9,10). Several factors should be considered. First, women in the present intervention study consumed daily a low-fat muffin enriched with a lignan complex for 6 wk. Although 6 wk is an acceptable time period for a clinical intervention study, it may be an unrealistically short period to observe the benefits from plant lignans that were observed after a lifetime exposure in observational studies. Second, it is possible that plant lignans are not responsible for the cardioprotective effects found in observational studies and that the results from observational studies may be an effect of the fiber fraction alone or in combination with SDG. Plant lignans are associated to the fiber fraction and flaxseed contains ~8% viscous polysaccharides, which have been shown to reduce TC and LDL-C concentration (34). Serum ENL may be a biomarker of a healthy diet rich in fiber. Third, the women in the present study were healthy and it may be possible that a different treatment effect of the lignan complex would have occurred in women with a different CVD risk profile, e.g., obesity and diabetes.

In conclusion, daily consumption for 6 wk of a low-fat muffin enriched with a lignan complex, providing 500 mg/d of SDG, had no effect on FMD, NMD, or plasma concentrations of NOx, ET-1, and ADMA. These results do not suggest that plant lignans isolated from flaxseed may affect endothelial function in healthy postmenopausal women.

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