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MATERNAL FLAXSEED DIET DURING PREGNANCY OR LACTATION INCREASES FEMALE RAT OFFSPRING'S SUSCEPTIBILITY TO CARCINOGEN-INDUCED MAMMARY TUMORIGENESIS

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Abstract

Flaxseed contains several dietary components that have been linked to low breast cancer risk; i.e., n-3 polyunsaturated fatty acids (PUFAs), lignans and fiber, but it also contains detectable levels of cadmium, a heavy metal that activates the estrogen receptor (ER). Since estrogenic exposures early in life modify susceptibility to develop breast cancer, we wondered whether maternal dietary intake of 5% or 10% flaxseed during pregnancy or lactation (between postpartum days 5 and 21) might affect 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis in the rat offspring. Our data indicated that both *in utero* and postnatal 5% and 10% flaxseed exposures shortened mammary tumor latency, and 10% flaxseed exposure increased tumor multiplicity, compared to the controls. Further, when assessed in 8-week-old rats, *in utero* 10% flaxseed exposure increased lobular ER- α protein levels, and both *in utero* and postnatal flaxseed exposures dose-dependently reduced ER- β protein levels in the lobules and terminal end buds (TEBs). Exposures to flaxseed did not alter the number of TEBs or affect cell proliferation within the TEBs, lobules or ducts. In a separate group of immature rats that were fed 5% defatted flaxseed diet (flaxseed source different than in the diets fed to pregnant or lactating rats) for 7 days, cadmium exposure through the diet was 7-fold higher than allowed for humans by World Health Organization, and cadmium significantly accumulated in the liver and kidneys of the rats. It remains to be determined whether the increased mammary cancer in rats exposed to flaxseed through a maternal diet *in utero* or lactation was caused by cadmium present in flaxseed, and whether the reduced mammary ER- β content was causally linked to increased mammary cancer risk among the offspring.

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Keywords

Flaxseed; breast cancer; estrogen receptor

Introduction

The human diet contains several components that are proposed to promote health. For example, n-3 polyunsaturated fatty acids (PUFAs) and phytoestrogens reduce the risk of cardiovascular diseases, allergies and other inflammatory conditions (arthritis, psoriasis, eczema), diabetes (1–3) and cancer (4–6). n-3 PUFAs are obtained mostly from fish, while the main sources of phytoestrogens are different beans and seeds. Flaxseed is an oilseed that is high both in n-3 PUFA, containing about 57% of this fatty acid, and phytoestrogens (lignans). The main lignan in flaxseed is secoisolariciresinol diglucoside, which is metabolized by gut microbes via its aglycone secoisolariciresinol (SECO) into mammalian lignans enterodiols (END) and enterolactone (ENL) after ingestion. Flaxseed is gaining popularity as an ideal dietary component to prevent various diseases and promote health. Of some concern, however, are findings indicating that flaxseed influences endocrine functions, in particular if exposed early in life (7). It is possible that these endocrine effects are due to a heavy metal cadmium that flaxseed effectively accumulates (8;9), since cadmium has estrogen-like effects both *in vitro* and *in vivo* (10).

Maternal nutrition during pregnancy can have long lasting effects on offspring's health (11), perhaps including the breast. In humans, breast cancer risk is elevated in women who had a high birth weight (12–14). Animal studies have shown that maternal dietary exposures to a high fat diet (15) or genistein (16) increase, while whole wheat flour (17) or n-3 PUFA (18) reduce female offspring's later mammary tumorigenesis. Maternal dietary exposures during lactation (19) also modify offspring's breast cancer risk.

The effect of early life dietary exposure to flaxseed on mammary gland development and tumorigenesis has been investigated previously. These studies show that rats exposed to a flaxseed diet from conception until weaning; i.e., *in utero* and during lactation, exhibit changes in the mammary gland (20;21) predicting a reduced risk of developing mammary tumors. Specifically, early life exposure to flaxseed reduces the number of targets for malignant transformation (terminal end buds, TEBs) and promotes their differentiation to lobulo-alveolar structures that do not give rise to malignant breast tumors. Consistent with this observation, a dietary exposure to 10% flaxseed during lactation reduced carcinogen-induced mammary tumorigenesis (22). However, we have found that maternal dietary exposure to 15% defatted flaxseed during pregnancy increased offspring's mammary cancer risk (17). Studies performed in adult animals indicate that flaxseed diet reduces carcinogen-induced mammary tumor promotion (23) and inhibits the growth of human breast cancer cells in athymic mice (24;25).

In the present study, we determined the level of cadmium in 5% defatted flaxseed diet and investigated whether it had estrogenic effects in the uterotrophic assay. We also compared the effects of either *in utero* or early postnatal exposure to 5% or 10% flaxseed diet through a pregnant or lactating dam on mammary tumorigenesis and possible biomarkers of increased risk of developing breast cancer; i.e. mammary gland morphology, cell proliferation, and expression of the estrogen receptor (ER)- α and ER- β .

Materials and Methods

In utero dietary flaxseed exposure

Female Sprague-Dawley rats were obtained from Charles River Laboratories on day 7 of gestation, and housed singly. The rat dams either received the standard semipurified AIN93 (American Institute of Nutrition) diet (n=21) or were divided into three groups of 7 dams each on the day of arrival; i.e., those that were fed either 0, 5 or 10% flaxseed diet. The flaxseed source was brown flax obtained from BulkFoods (<http://www.bulkfoods.com>). The detailed composition of the diets is shown in Table 1. The diets were designed by Dr. Carolyn Good and they were based on those reported previously (20) and were prepared by Harlan Teklad (Madison, WI, USA). To avoid oxidation of the volatile n-3 PUFAs in the flax, the diets were vacuum packed in foil bags that contained enough food for one day, and stored in +4 °C. The animals, thus, obtained fresh food each day.

Pregnant rats were kept on the flaxseed diets until they gave birth, and at that point switched to AIN93 lab chow. Two days after the offspring were born, all female pups were pooled and randomly assigned to be housed with a dam that was fed the same diet during pregnancy as the pups. Each dam had a total of 10 female pups. After weaning on postnatal day 25, the pups were housed in groups of 3–5 animals. Studies were approved and performed in accordance with the appropriate institutional and federal regulations.

Postnatal dietary flaxseed exposure

To expose some animals to flaxseed diets during postnatal period, those dams that were fed AIN93 diet throughout pregnancy were divided to three groups of 3 lactating dams each on post-delivery day 5, and fed either 0, 5 or 10% flaxseed diets. The pups housed with these dams were all females (see above). Pups were exposed to the flaxseed diets during the first 10 days through milk and during the next 10 days both through milk and by consuming the food pellets. Dams and pups were kept on these diets until post-delivery day 25. Pups were then weaned and housed in groups of 3–5 animals.

Mammary gland morphology

Wholemounts of the right 4th abdominal glands, obtained at 8 weeks of age from rats exposed to flaxseed diets either *in utero* or during early postnatal period, were used to assess changes in mammary gland morphology. Five rats per group were used. The wholemounts were processed as previously described (15), and the total number of TEBs was counted double-blinded under an Olympus dissecting microscope.

Other assays

We determined cell proliferation and ER- α and ER- β expression in the mammary glands of 8-week-old rats exposed to flaxseed *in utero* or early postnatal period. In each assay, 4–7 animals per group were used and their left 4th abdominal mammary glands were fixed with 10% phosphate-buffered formalin overnight at +4°C, dehydrated with graded ethanol, and then embedded in paraffin. The paraffin-embedded tissues were sliced into 5 μ m thick sections and mounted on silane-coated glass slides.

PCNA assay (proliferating cell nuclear antigen)—To determine changes in cell proliferation, sections were heated overnight at 57°C, rehydrated in graded alcohol, and reheated in microwave in high power for 10–15 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. After cooling the slides in room temperature and washing with PBS, the sections were incubated for 10 min in 3% H₂O₂ to denature any endogenous peroxidase. Sections were washed again with PBS, exposed to PCNA antibody (Goat polyclonal IgG,

SC-9857, Santa Cruz), and incubated with 20% rabbit serum for over night at 4°C. Sections were then washed and treated with biotinylated rabbit antiserum to goat IgG (Vector laboratories) for 30 min followed by a 30 min incubation with streptavidin – peroxidase conjugate (Vectastin ABC Kit, Vector Laboratories). Antigen antibody complex was visualized by incubation with 3,3'diaminobenzidine (Vector laboratories). Finally, sections were treated with Gills haematoxyline, stained, dehydrated through graded alcohol, and mounted. To determine the level of cell proliferation, the number of cells showing PCNA staining was assessed separately in TEBs, lobules and ducts per 1,000 cells per structure and per gland.

Tunel assay—(Terminal deoxynucleotidyl transfersase dUTP nick end labeling). In the mammary gland sections, the nuclei containing degraded DNA were stained by using the TUNEL assay, an in situ apoptosis ApopTag Peroxidase detection kit (Serological corporation, S –7100), as recommended by the manufacturer. Briefly, after rehydrated by graded alcohol and pre-treated with protein digesting enzyme (Proteinase –K), each slide was quenched by hydrogen peroxide. The Equilibrium Buffer was then administered and slides were incubated with TdT enzyme at 37°C for 1 hour. After keeping the slides in buffer for 10 min, tissues were incubated with Anti -Digoxigenin Peroxidase conjugate for 30 minute. Then, washing with PBS, the slides were covered by Peroxidase substrate until the optimal staining had taken place. The reaction was stopped by water rinse. The sections were counter stained with methyl green, dehydrated by xylene, and mounted on the slides.

The proportion of cells undergoing apoptosis was determined by counting the number of stained cells divided by the total number of cells (1,000) counted. In the rats exposed to flaxseed *in utero*, the cells were counted separately in lobules and ducts (the number of TEBs per slide was too low to count apoptotic cells), while an equal number of lobules and ducts per gland were included to the analysis among the postnatally flax exposed rats. Only those stained cells that appeared apoptotic based on morphological evaluation were counted.

Estrogen receptors—The antibody for ER- α was MC-20 (rabbit polyclonal IgG, Santa Cruz Biotechnology, CA), used at 1:100 dilution, and for ER- β H-150: SC-8974 (rabbit polyclonal IgG, Santa Cruz Biotechnology, CA), used at 1:300 dilution with 20% goat serum over PBS.

To quantitate ER- α protein levels, the percentile of cells showing nuclear staining was assessed in three ductal and three lobulo-alveolar units (LAUs) per gland with a scale of 0–5, and the staining intensity was assessed with the scale of 0–3. The sections used for these assays did not contain enough TEBs to include these structures for this study. The scores for staining density and intensity were combined to determine the level of ER- α expression.

To determine the protein levels of ER- β , we cut a new set of sections from the tissue blocks that all contained TEBs, lobules and ducts. Further, instead of using the scoring method utilized for ER- α assays, the number of cells showing ER- β staining was assessed separately in TEBs, lobules and ducts by counting 1,000 cells per structure and per gland.

Effects on offspring's mammary tumorigenesis—Mammary tumors were induced by an administration of 10 mg 7,12-dimethylbenz[a]anthracene (DMBA) to 50-day-old female rats exposed to control or 5%, or 10% flaxseed *in utero* or during lactation. Each group contained 22–25 rats. Importantly, the histopathology, estrogen-dependence, estrogen and progesterone receptor expression, and antiestrogen responsiveness of the DMBA-induced tumors closely reflect human breast cancer (26).

The animals were examined for mammary tumors by palpation once per week. The end-points for data analysis were (i) week of tumor appearance (latency), (ii) number of animals with tumors (tumor incidence), and (iii) number of tumors per animal (tumor multiplicity). Histopathology of the tumors was also assessed. The animals were sacrificed when detectable tumor burden approximated 10% of total body weight, as required by our institution. All surviving animals, including those that did not appear to develop mammary tumors, were sacrificed 18 weeks after carcinogen administration.

In vivo estrogenicity of flaxseed—The estrogenic effects of dietary flaxseed diet in immature Sprague-Dawley rats were studied in an uterotrophic test. The uterotrophic test was performed at the University of Turku animal department, and was approved by the local authorities. The time-mated rat dams were housed in 12h–12h light-dark cycle, constant temperature and humidity, and had free access to tap water and feed (semisynthetic estrogen-poor C1000 basal diet, Altromin, Lage, Germany) throughout the experiment. The pups were weaned on postnatal day 19, divided into three groups (n=8 in each) and allocated to the flaxseed or control diet.

The flaxseed diet consisted of 5 % defatted flaxseed flour of brown variety (MTT Agrifood Research Finland, Jokioinen, Finland) in the C1000 basal diet, while the control rats received C1000 diet corrected for the main nutrients to correspond the composition of the flaxseed diet (prepared at Altromin, Lage, Germany). Some of the control rats were injected s.c. with 1 mg/kg estradiol (E₂) (Sigma, St Louis, MO, USA) in rape seed oil daily. These exposures were started on postnatal day 19 and they continued for 7 days.

During the treatment period, weight gain and food consumption were measured daily. On postnatal day 26, rats were sacrificed by carbon dioxide suffocation and neck dislocation. Serum was collected and stored in –20 °C for later lignan analysis. The uteri were dissected, carefully blotted to remove extra fluids, weighted, and collected in formalin. Livers and kidneys were dissected, weighted, and stored in –20 °C until cadmium analysis. Abdominal mammary glands were dissected and routinely processed into whole mounts.

Cadmium analysis—Concentration of cadmium in the flaxseed and control diets, and dissected kidneys and livers was analyzed by GFAAS (Solaar M6 Dual Zeeman AAS Spectrometer, Thermo Electron Spectroscopy Ltd., Cambridge, England) after microwave-assisted digestion with ultrapure HNO₃ (Fluka Chemie GmbH, Buchs, Switzerland) and H₂O₂ (J.T. Baker, Deventer, Holland) (5/2 vol/vol). Palladium (0.5 g/l) was used as matrix modifier. Commercial reference material (lyophilized bovine liver, CRM-185R, IRMM, Geel, Belgium) handled in similar manner was used as quality control sample.

Lignan analysis—Serum lignans were analyzed as described before for rat urine (27;28) and human serum (29). Shortly, 100 µl of serum samples were enzymatically hydrolyzed with filtered *Helix pomatia* over night at +37 °C, after which internal standards, d₆-ENL and d₆-MR, were added. Quality controls and standard samples containing enterolactone (ENL), enterodiol (END) and secoisolariciresinol (SECO) were prepared from pure reference compounds. Serum collected from rats on a lignan poor diet was used as blank serum in the quality control samples and standard samples. After solid phase extraction at pH 4.0 with Oasis HLB Extraction Cartridges (Waters, Milford, U.S.A.) samples were evaporated to dryness under N₂-flow, reconstituted in MeOH/0.1% HAc 20/80 (v/v) and analyzed with HPLC-MS/MS as described (27;29).

Histology of uteri and mammary glands—Uteri were processed into 5 µm thick tissue sections and stained with hematoxylin and eosin (H&E). Histology of the uteri was assessed

under Olympus BX51 Microscope and estrogenic responses; i.e., uterine size, thickness and proliferation of epithelium, and infiltration of immune cells, were determined.

Mammary gland whole mounts were evaluated for the amount and size of TEBs on the 4th abdominal mammary gland. Total number of TEBs was calculated under Olympus SZX9 Stereo Microscope. The methods to identify and count TEBs in these wholemounts were similar as described above. In addition, the size of 10 TEBs per mammary gland was measured with ImageJ program (<http://rsb.info.nih.gov/ij/>) from photographs taken with an Olympus DP70 Digital Camera System.

Statistical analysis—Results obtained for (i) total number of TEBs; (ii) percentile of proliferating cells or cells expressing ER- β protein, separately in the TEBs, lobules and ducts, (iii) scores for the level of ER- α expression separately in the lobules and ducts, and (iv) mammary tumor multiplicity were analyzed using one-way analysis of variance (ANOVA). Where appropriate, between-group comparisons were done using Fisher's Least Significant Difference (LSD) test. Mammary tumor latency was assessed using non-parametric Kruskal-Wallis One way ANOVA on Ranks, followed by Dunn's method to identify which groups differed from each other. Possible differences in the tumor incidence were determined by estimating tumor presentation by the methods developed by Kaplan and Meier (30). Differences among the dietary exposure arms were tested using an extension of the log rank test. The differences were considered significant if the p-value was less than 0.05. All probabilities are two-tailed.

In the experiment focusing on cadmium content and the estrogenicity of flaxseed, cadmium exposure, cadmium concentration in organs, the organ weights, body weights, and the number and area of TEBs were analyzed with one-way ANOVA followed by Fisher's LSD test. The difference between flaxseed-fed and control rats in serum lignan concentrations was analyzed with Mann Whitney U-test.

Results

Mammary gland

The number of TEBs was not significantly altered in the 8-week-old rats exposed to 5% or 10% flaxseed diet *in utero* or during postnatal period (data not shown).

PCNA

The epithelial structures (TEBs, lobules and ducts) of rats fed 10% flaxseed diet *in utero* contained approximately 34%-41% more proliferating cells than those of the controls, but the difference did not reach statistical significance (Fig. 1a). No statistical differences in the proportion of proliferating cells were noted among the TEBs, lobules or ducts.

Postnatal exposure to 5% or 10% flaxseed exposure did not have any significant effects on the mammary gland cell proliferation either, when assessed in the TEBs, lobules or ducts (Fig. 1b). The mammary glands of rats fed 10% flaxseed diet, in fact, contained 4–19% fewer proliferating cells than the glands of control rats.

Apoptosis

The rate of apoptosis was not altered in the TEBs, lobules or ducts of rats exposed to flaxseed diets *in utero* or during postnatal period (data not shown).

ER- α and ER- β

In utero exposure to 10% flaxseed diet altered the expression of ER- α and ER- β in the mammary glands. Specifically, this *in utero* exposure increased ER- α protein levels both in the lobules [F(2,12)=33.98, $p<0.001$] and ducts [F(2,12)=5.78, $p<0.017$] (Fig. 2). In contrast, it caused a significant reduction in ER- β levels in the TEBs [F(2,10)=5.06, $p<0.030$], but not in the lobules or ducts (Fig. 3). No significant changes in the expression of these two receptors were seen between the control rats and the rats exposed to 5% flaxseed diet *in utero*.

Postnatal 10% flaxseed exposure did not have any significant effects on mammary ER- α expression. However, an exposure to 5% flaxseed diet during postnatal period caused a significant reduction in the ER- α expression in the lobular structures [F(2,11)=4.46, $p<0.038$] (Fig. 2). Further, mammary ER- β expression was dose-dependently reduced in the TEBs [F(2,11)=12.77, $p<0.001$] and lobules [F(2,11)=4.10, $p<0.047$] (Fig. 3).

Mammary tumorigenesis

Three end-points were used to assess the effect of early life exposure to flaxseed in affecting mammary tumorigenesis: latency – the week the first tumor was recorded in a rat; incidence – proportion of animals with mammary tumors at the end of the 18-week follow-up period; and multiplicity – number of tumors per animal.

Histopathological assessment indicated that all the tumors were adenocarcinomas. The median latency to the appearance of the first tumor per rat (among those rats that developed at least one tumor) was significantly shorter in the rats that were exposed to 10% flaxseed diet *in utero* [H=8.20, df=2, $p<0.017$] or postnatally [H=9.29, df=2, $p<0.010$], when compared to the control animals (Table 2). Rats exposed to 5% flaxseed diet *in utero* also exhibited reduced tumor latency.

Neither *in utero* nor postnatal exposure to 5% or 10% flaxseed diet affected final mammary tumor incidence (Fig. 4). Tumor multiplicity among all animals per group was significantly elevated in the rats exposed to 10% flaxseed diet *in utero* [F(2,70)=3.56, $p<0.034$] and during postnatal period [F(2,63)=4.36, $p<0.017$] (Table 2, Fig. 4). No changes in the multiplicity were seen in the rats exposed to 5% flaxseed diet during early life. When tumor multiplicity was assessed only among the rats that developed tumors, the results were slightly stronger (data not shown), but essentially similar than when all rats were included to the analysis.

Estrogenicity of flaxseed

Exposure to 5% defatted flaxseed diet for seven days did not affect the weight of the uterus, liver or kidney (Table 3) or the body weight (data not shown). As expected, E₂ exposure significantly increased the wet weight of the uterus ($p<0.0001$) (Table 3). Histological examination of the H&E-stained uteri showed expected histological changes in the E₂-exposed animals, i.e. hyperplastic epithelial lining and infiltration of immune cells (Fig. 5). No signs of estrogen exposure were observed in the flax group (Fig. 5). After adjustment against body weight, the weight of the kidneys was increased in the E₂-group compared to the controls ($p<0.05$) (data not shown). Food consumption was similar in all treatment groups throughout the 7-day exposure to flaxseed diet (data not shown).

Mammary whole mount assays indicated that an exposure to E₂ reduced the number of TEBs ($p<0.05$), when determined at the end of the 7-day exposure period, and increased their size ($p<0.001$) (Table 3). Flaxseed diet did not impact the TEBs.

The amount of cadmium was 7-fold higher in the flaxseed diet compared to the basal diet (Table 3). Based on the food consumption and animal weight gain during the 7-day treatment period, the rats in the flaxseed-group were exposed to over 40 µg/kg of cadmium (Table 3), which clearly exceeds the PTWI value set by WHO for this heavy metal (7 µg/kg/week). Despite reported poor absorption of dietary cadmium in the rat (31), the amount of cadmium in the liver and kidneys was significantly higher in the flaxseed fed group compared to both control and E₂-exposed group ($p < 0.001$ for both organs) (Table 3). Serum ENL, END, and SECO concentrations were also significantly higher in the flaxseed-group than in the controls ($p < 0.01$ for all) (Table 3).

Discussion

Our results showed that an exposure to 10% flaxseed diet *in utero* or postnatally through a pregnant or nursing dam increased offspring's susceptibility to mammary tumorigenesis. These findings are in accordance with a recent study showing that maternal dietary exposure to 15% defatted flaxseed diet during pregnancy increased DMBA-induced mammary tumorigenesis among female offspring (17). However, another study (22) reports that postnatal 10% flaxseed exposure or exposure to SECO, the lignan present in flax, reduced DMBA-initiated mammary tumorigenesis. This and our postnatal exposure study are identical in regard of the flaxseed exposure, animals, and carcinogen used. The source of flaxseed was different, as our flaxseed was from the US and Finland, whilst the previous study used flaxseed obtained from Canada. The location of flaxseed could be important in determining how it affects breast cancer risk; for example, cadmium content of flaxseed is known to vary depending on the location (8;9).

The increase in mammary tumorigenesis in animals exposed to 10% flaxseed diet *in utero* or during postnatal period reflected increased tumor multiplicity and shortened tumor latency. It is puzzling as to why the higher flaxseed diet that is high in lignans and n-3 fatty acids, both of which have been linked to a reduced breast cancer risk, led to an increase in mammary tumorigenesis. One possible explanation is the effect of flaxseed on circulating estrogen levels. Rats fed 10% flaxseed diet have been reported to have elevated serum estradiol levels (32). Since an exposure to estradiol *in utero* increases DMBA-induced mammary tumorigenesis in rats (15), estradiol levels might have been elevated in the pregnant rats fed 10% flaxseed diet, increasing later susceptibility to mammary cancer. However, high prepubertal estrogen levels have been linked to a reduced susceptibility to develop mammary tumors (33), and therefore a possible increase in estrogen levels in rats exposed to flaxseed during postnatal period cannot explain why this dietary component increased their later breast cancer risk.

Another explanation for increased mammary tumorigenesis may relate to the fact that flaxseed accumulates high levels of cadmium, resulting rats in this study to be exposed to levels that exceeded the maximum dietary guideline levels set by the World Health Organization (Provisional Tolerable Weekly Intake of 7 µg/kg body weight cadmium per week). We have previously shown that cadmium doses lower than 7 µg/kg body weight activate the ER- α , induce early puberty onset, and alter mammary gland development in a manner predictive of increased breast cancer risk (10;34). In addition, low doses of cadmium (5 µg/kg) administered by injection, induce strong estrogenic effects in the rat uterus (10). Thus, it is possible that the increased mammary tumorigenesis in rats exposed to flaxseed *in utero* at least partly is caused by the presence of this heavy metal in flaxseed.

Lignans in flaxseed are often suggested to possess estrogenic effects as well, mainly due to their structural resemblance with estrogens. The mammalian lignin enterolactone increases proliferation of estrogen dependent MCF-7 and T-47D cells (35–37) at concentrations

observed in the flaxseed fed rats' serum in this study. Enterolactone weakly activates transcription of estrogen dependent reporter genes and endogenous genes *in vitro* (35;37;38). No evidence, however, indicating that lignans would have estrogenic properties *in vivo* has materialized.

Despite being exposed to a mixture of compounds with endocrine activities (lignans and cadmium), the uteri of rats fed flaxseed diet showed no signs of estrogen exposure; i.e., uterine wet weight or morphology were not altered. Further, although 7-day exposure of immature rats to estradiol reduced the number of TEBs, in accordance with previous studies (33), flaxseed diet had no effects on TEBs (Table 3). We also noted that estradiol (but not cadmium) exposure increased the size of TEBs; this is a novel observation that we are planning to pursue further.

We wondered whether some biomarkers proposed to be predictive of increased breast cancer risk were altered in the animals exposed to 10% flaxseed diet during early life. The biomarkers studied were mammary gland morphology, cell proliferation, and the levels of expression of ER- α and ER- β . No significant changes in mammary gland morphology were noted. This is in contrast to the findings reported by Thompson et al. who showed that rats fed 10% flaxseed diet either from conception throughout nursing or during nursing period only exhibit a reduced number of TEBs and increases LAUs in the mammary epithelium (20;21). In our study, the number of proliferating cells was not altered in the mammary glands of rats exposed to flaxseed diets *in utero* or during postnatal period.

Although uterine assay did not indicate any estrogenic effects in immature rats fed 5% defatted cadmium diet for a 7-day period, we found that *in utero* exposure to 10% flaxseed was associated with increased expression of ER- α , and reduced expression of ER- β . Postnatal exposure to 10% flaxseed did not affect mammary ER- α content; this is consistent with an earlier study showing no changes in the expression of this receptor in the mammary glands of rats exposed to 10% flaxseed during lactation (22). Similarly to *in utero* flaxseed exposure, postnatal exposure reduced the expression of ER- β . ER- α is believed to mediate the proliferative actions of estrogens (39). The specific functions of ER- β in the breast are not known, but there is strong evidence that this receptor inhibits the ER- α -mediated transcription (40–42) and functions as a tumor suppressor (43). Our finding obtained in rats fed 10% flaxseed diet *in utero* are consistent with the idea that high ER- α /low ER- β ratio is associated with increased breast cancer risk.

In summary, maternal dietary exposures during pregnancy or lactation to 10% flaxseed diet increased susceptibility to develop mammary tumors in their female rat offspring. The mechanisms that led to an increased carcinogen-induced mammary tumorigenesis remain to be established, but might relate to high cadmium content of flaxseed. We found that a 7-day exposure of immature rats to a flaxseed diet containing high levels of cadmium and lignans was devoid of any estrogenic effects in the classic uterine assay. However, since *in utero* and early postnatal exposure to flaxseed diet down-regulated ER- β expression in an adult mammary gland, this dietary compound might have estrogenic properties after all.

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Abbreviations

DMBA	dimethylbenz[a]anthracene
E2	17 β -estradiol
ER	estrogen receptor
LAU	lobuloalveolar unit
PUFA	polyunsaturated fatty acid
TEB	terminal end bud

Reference List

1. Bucher HC, Hengstler P, Schindler C, Meier G. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am J Med.* 2002; 112:298–304. [PubMed: 11893369]
2. Prasad K. Secoisolariciresinol diglucoside from flaxseed delays the development of type 2 diabetes in Zucker rat. *J Lab Clin Med.* 2001; 138:32–39. [PubMed: 11433226]
3. James MJ, Cleland LG. Dietary n-3 fatty acids and therapy for rheumatoid arthritis. *Semin Arthritis Rheum.* 1997; 27:85–97. [PubMed: 9355207]
4. Klein V, Chajes V, Germain E, et al. Low alpha-linolenic acid content of adipose breast tissue is associated with an increased risk of breast cancer. *Eur J Cancer.* 2001; 36:335–340. [PubMed: 10708934]
5. Rose DP. Dietary fatty acids and cancer. *Am J Clin Nutr.* 1997; 66:998S–1003S. [PubMed: 9322580]
6. Trock BJ, Hilakivi-Clarke L, Clarke R. Meta-analysis of soy intake and breast cancer risk. *J Natl Cancer Inst.* 2006; 98:459–471. [PubMed: 16595782]
7. Tou JC, Chen J, Thompson LU. Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. *J Nutr.* 1998; 128:1861–1868. [PubMed: 9808635]
8. Angelova V, Ivanova R, Delibaltova V, Ivanov K. Bio-accumulation and distribution of heavy metals in fibre crops (flax, cotton, hemp). *Industrial Crops and Products.* 2004; 19:197–205.
9. Hocking PJ, McLaughlin MJ. Genotypic variation in cadmium accumulation by seed of linseed, and comparison with seed of some other crop species. *Australian Journal of Agricultural Research.* 2006; 52:427–433.
10. Johnson MD, Kenney N, Stoica A, et al. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. *Nat Med.* 2003; 9:1081–1084. [PubMed: 12858169]
11. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. *Science.* 2004; 305:1733–1736. [PubMed: 15375258]
12. Michels KB, Trichopoulos D, Robins JM, et al. Birthweight as a risk factor for breast cancer. *Lancet.* 1996; 348:1542–1546. [PubMed: 8950880]
13. Sanderson M, Williams M, Malone KE, et al. Perinatal factors and risk of breast cancer. *Epidemiology.* 1996; 7:34–37.
14. McCormack VA, dos SS I, De Stavola BL, Mohsen R, Leon DA, Lithell HO. Fetal growth and subsequent risk of breast cancer: results from long term follow up of Swedish cohort. *BMJ.* 2003; 326:248. [PubMed: 12560272]
15. Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E, Lippman ME. A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proc Natl Acad Sci USA.* 1997; 94:9372–9377. [PubMed: 9256489]
16. Hilakivi-Clarke L, Cho E, Onojafe I, Raygada M, Clarke R. Maternal exposure to genistein during pregnancy increases carcinogen-induced mammary tumorigenesis in female rat offspring. *Oncol Rep.* 1999; 6:1089–1095. [PubMed: 10425307]

17. Yu B, Khan G, Foxworth A, Huang K, Hilakivi-Clarke L. Maternal dietary exposure to fiber during pregnancy and mammary tumorigenesis among rat offspring. *Int J Cancer*. 2006
18. Hilakivi-Clarke L, Cho E, Cabanes A, et al. Modulation of pregnancy estrogen levels by maternal dietary exposure to soy isolate or n-3 polyunsaturated fatty acid and breast cancer risk among female rat offspring. *Clin Cancer Res*. 2002 In press.
19. Hilakivi-Clarke L, Cho E, DeAssis S, et al. Maternal and prepubertal diet, mammary development and breast cancer risk. *J Nutr*. 2001; 131:154S–157S. [PubMed: 11208953]
20. Ward WE, Jiang FO, Thompson LU. Exposure to flaxseed or purified lignan during lactation influences rat mammary gland structures. *Nutr Cancer*. 2000; 37:187–192. [PubMed: 11142092]
21. Tou JC, Thompson LU. Exposure to flaxseed or its lignan component during different developmental stages influences rat mammary gland structures. *Carcinogenesis*. 1999; 20:1831–1835. [PubMed: 10469631]
22. Chen J, Tan KP, Ward WE, Thompson LU. Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. *Exp Biol Med (Maywood)*. 2003; 228:951–958. [PubMed: 12968067]
23. Rickard SE, Yuan YV, Chen J, Thompson LU. Dose effects of flaxseed and its lignan on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. *Nutr Cancer*. 1999; 35:50–57. [PubMed: 10624706]
24. Chen J, Stavro PM, Thompson LU. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. *Nutr Cancer*. 2002; 43:187–192. [PubMed: 12588699]
25. Dabrosin C, Chen J, Wang L, Thompson LU. Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. *Cancer Lett*. 2002; 185:31–37. [PubMed: 12142076]
26. Russo, J.; Russo, IH. Toward a unified concept of mammary carcinogenesis. In: Aldaz, MC.; Gould, MN.; McLachlan, J.; Slaga, TJ., editors. *Progress in Clinical and Biological Research*. New York: Wiley-Liss; 1997. p. 1-16.
27. Saarinen NM, Smeds A, Makela SI, et al. Structural determinants of plant lignans for the formation of enterolactone in vivo. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002; 777:311–319.
28. Smeds AI, Saarinen NM, Hurmerinta TT, Penttinen PE, Sjöholm RE, Makela SI. Urinary excretion of lignans after administration of isolated plant lignans to rats: the effect of single dose and ten-day exposures. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004; 813:303–312.
29. Smeds AI, Hakala K, Hurmerinta TT, Kortela L, Saarinen NM, Makela SI. Determination of plant and enterolignans in human serum by high-performance liquid chromatography with tandem mass spectrometric detection. *J Pharm Biomed Anal*. 2006; 41:898–905. [PubMed: 16460900]
30. Kaplan EL, Meijer P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc*. 1958; 53:457–481.
31. House WA, Hart JJ, Norvell WA, Welch RM. Cadmium absorption and retention by rats fed durum wheat (*Triticum turgidum* L. var. durum) grain. *Br J Nutr*. 2003; 89:499–508. [PubMed: 12654168]
32. Tou JC, Chen J, Thompson LU. Dose, timing, and duration of flaxseed exposure affect reproductive indices and sex hormone levels in rats. *J Toxicol Environ Health A*. 1999; 56:555–570. [PubMed: 10321385]
33. Cabanes A, Wang M, Olivo S, et al. Prepubertal estradiol and genistein exposures up-regulate BRCA1 mRNA and reduce mammary tumorigenesis. *Carcinogenesis*. 2004; 25:741–748. [PubMed: 14729590]
34. Stoica A, Katzenellenbogen BS, Martin MB. Activation of estrogen receptor-alpha by the heavy metal cadmium. *Mol Endocrinol*. 2000; 14:545–553. [PubMed: 10770491]
35. Sathyamoorthy N, Wang TTY, Phang JM. Stimulation of pS2 expression by diet593 derived compounds. *Cancer Res*. 1994; 54:957–961. [PubMed: 8313386]
36. Mousavi Y, Adlercreutz H. Enterolactone and estradiol inhibit each other's proliferative effect on MCF-7 breast cancer cells in culture. *J Steroid Biochem Mol Biol*. 1992; 41:615–619. [PubMed: 1562532]

37. Welshons WV, Murphy CS, Koch R, Calaf G, Jordan VC. Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast Cancer Res Treat.* 1987; 10:169–175. [PubMed: 3427225]
38. Mueller SO, Simon S, Chae K, Metzler M, Korach KS. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. *Toxicol Sci.* 2004; 80:14–25. [PubMed: 15084758]
39. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endo Rev.* 1987; 8:29–39.
40. Hall JM, Chang CY, McDonnell DP. Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. *Mol Endocrinol.* 2000; 14:2010–2023. [PubMed: 11117531]
41. Pettersson K, Delaunay F, Gustafsson JA. Estrogen receptor beta acts as a dominant regulator of estrogen signaling. *Oncogene.* 2000; 19:4970–4978. [PubMed: 11042684]
42. Matthews J, Wihlen B, Tujague M, Wan J, Strom A, Gustafsson JA. Estrogen receptor (ER) beta modulates ERalpha-mediated transcriptional activation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters. *Mol Endocrinol.* 2006; 20:534–543. [PubMed: 16293641]
43. Zhao C, Lam EW, Sunter A, et al. Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene.* 2003; 22:7600–7606. [PubMed: 14576822]

Figure 1.

The number of proliferating cells, identified using PCNA stain, per 1,000 epithelial cells counted, in the mammary glands of 8-week-old rats fed 0%, 5% or 10% flaxseed diet *in utero* or during postnatal period (n=4–5 rats per group). The counting was done separately in the TEBs, lobules and ducts. No significant differences were seen.

Figure 2.

ER- α protein expression, determined by immunohistochemistry, in the mammary glands of 8-week-old rats exposed to a 0%, 5% or 10% flaxseed diet *in utero* or during postnatal period (n=4–5 rats per group). ER- α protein levels were quantified using a subjective scale in which the proportion of stained cells was scored using a scale from 0–5, and the intensity of staining using a scale of 0–3: the two scores were then combined. Rats exposed to 10% flaxseed *in utero* exhibited elevated, and rats exposed to 5% flaxseed during postnatal period reduced expression of this receptor. * (p<0.05).

Figure 3.

(a) Representative section obtained from mammary glands of rats exposed to 0%, 5% or 10% flaxseed diet *in utero* or during postnatal period showing cells staining for ER-β antibody; and (b) ER-β protein expression, determined by immunohistochemistry, in the mammary glands of 8-week-old rats exposed to a 0%, 5% or 10% flaxseed diet *in utero* or during postnatal period (n=4–5 rats per group). The level of expression was quantified by counting the number of stained cells per 1,000 cells, per structure (TEB, lobule or duct) and per section. ER-β expression was significantly reduced in the TEBs of rats exposed to 10% flaxseed diet *in utero* and dose-dependently reduced in the TEBs and lobules in the rats exposed to flaxseed diet during postnatal period: * (p<0.05)

Figure 4.
Cumulative tumor multiplicity and incidence in the DMBA-treated rats fed 0%, 5% or 10% flaxseed diet *in utero* or during postnatal period.

Figure 5. Representative examples of H&E-stained uteri in the rats exposed to 5% defatted flax in diet for seven days. Control (Ctrl) rats received basal diet only, and E₂-group 1 mg/kg estradiol as a daily subcutaneous injection. Scale bars are 200 μ m.

Table 1

Dietary composition (g)	Control	5% flax (50g/kg feed)	10% flax (100 g/kg feed)
Protein			
• Casein	200	189	174
• Flax	0	10	20
Carbohydrate			
• corn starch	397.5	389	385
• maltodextrin	132	132	132
• sucrose	100	100	100
• flax	0	8.5	17
Fat			
• corn oil	70	51	31.5
• flax	0	20	40
Fiber			
• cellulose	50	38	27
• flax	0	12	23
Mineral mix (TD 94046)	35	35	35
Vitamin mic (TD 94047)	10	10	10
L-cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
TBHQ	0.014	0.014	0.014
Total	1000 g	1000 g	1000 g
Energy calculations			
Energy content/1000 g	3758	3941	3835
Protein %	19	18	18
Carbohydrate %	64	65	65
Fat %	17	17	17

Table 2

Tumor multiplicity (number of tumors per rat at the end of tumor monitoring period $\pm$ SEM) and median tumor latency in weeks, and 25% and 75% range, among rats exposed to 5% or 10% flaxseed diet or control diet, either *in utero* or during postnatal period. Only those animals that developed at least one tumor (n) were included to the analysis. Both *in utero* and postnatal exposures to 10% flaxseed diet increased tumor multiplicity and reduced mammary tumor latency.

	N (rats with tumors/ rats per group)	Tumor multiplicity (mean $\pm$ SEM number of tumors per rat)	Median (number of weeks after DMBA exposure)	25% – 75% range
<i>In utero</i> exposure				
Control	25/25	2.56 $\pm$ 0.42	10	7.75 – 13
5% flax	20/24	2.75 $\pm$ 0.45	7 ^a	6 – 9.5
10% flax	23/24	4.08 $\pm$ 0.45 ^a	8 ^a	6 – 9
Postnatal exposure				
Control	18/23	1.81 $\pm$ 0.36	10	8 – 11
5% flax	16/22	1.86 $\pm$ 0.40	8	8 – 10.5
10% flax	18/22	3.48 $\pm$ 0.57 ^a	7 ^a	6 – 8

^a significantly different from the controls

Table 3

Results of the uterotrophic test. Immature rats were exposed to 5% defatted flax in diet for seven days. Control (Ctrl) rats received basal diet only, and estradiol (E₂)-group 1 mg/kg estradiol as a daily subcutaneous injection. Flaxseed diet contained more cadmium than that basal diet, and cadmium accumulated to the kidneys and livers of the rats consuming the flaxseed diet. No estrogenic effects were observed in the uterus or mammary glands of the rats exposed to flaxseed diet. Results are presented as mean $\pm$ SD unless stated otherwise.

	Cd in diet ($\mu\text{g/kg}$)	Exposure ($\mu\text{g/kg/}$ week)	Cd in organs ($\mu\text{g/kg}$)		Lignans (nM) Median (Range)			Uterine weight (g)	Terminal end buds (TEBs)	
			Liver	Kidneys	ENL	END	SECO		Number	Size
Ctrl	6.7	6.0 $\pm$ 0.4	0.7 $\pm$ 0.1	0.6 $\pm$ 0.4	7.9 (4.1–104)	22.6 (14.6–518)	10.6 (3.7–64.6)	0.04 $\pm$ 0.02	52 $\pm$ 11	336 $\pm$ 53
Flax	45.6	42.4 $\pm$ 2.8 ^{**}	2.3 $\pm$ 0.4 ^{**}	1.9 $\pm$ 0.1 ^{**}	3200 ^{**} (816–8351)	3758 ^{**} (452–5927)	850 ^{**} (350–1517)	0.04 $\pm$ 0.01	47 $\pm$ 9	380 $\pm$ 42
E₂	6.7	5.3 $\pm$ 0.5	0.9 $\pm$ 0.3	0.8 $\pm$ 0.1				0.17 $\pm$ 0.02 ^{**}	38 $\pm$ 4 [*]	583 $\pm$ 125 ^{**}

Significantly different from control

* (p<0.05);

** (p<0.01)