Exposure to flaxseed or its lignan component during different developmental stages influences rat mammary gland structures

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Reduction of the highly proliferative terminal end bud (TEB) structures in the developing mammary gland by differentiation to alveolar buds (ABs) and lobules has been suggested to be protective against mammary cancer. Flaxseed is high in \( \alpha \)-linolenic acid (ALA) and secoisolariciresinol diglycoside (SDG). SDG is the precursor of mammalian lignans, which can affect mammary gland structures. Thus, the objective of this study was to determine the effect of lifetime, gestation and lactation or after-weaning exposure to 5 or 10% flaxseed or SDG and flaxseed oil components on the mammary gland structures of virgin female rat offspring at post-natal day 50. Lifetime or gestation and lactation exposure to flaxseed altered mammary gland structure development, whereas exposure to flaxseed after weaning had no effect. Lifetime or gestation and lactation exposure to 5% flaxseed caused endocrine changes, as suggested by delayed puberty onset and reduced number of estrous cycles. These changes reduced exposure to endogenous estrogens, leading to atrophy of mammary TEB structures. SDG, but not flaxseed oil, at the level found in 5% flaxseed produced similar effects as 5% flaxseed. This suggested that the lignans were the component in flaxseed responsible for the observed effects. Lifetime or gestation and lactation exposure to 10% flaxseed also caused endocrine changes, as suggested by early puberty onset and lengthened cycles due to prolonged estrus. This increased exposure to endogenous estrogens and stimulated mammary gland differentiation, as indicated by fewer TEBs and more ABs. Thus, lifetime or gestation and lactation exposure to 5 or 10% flaxseed induced structural changes in the mammary gland that may potentially reduce mammary cancer risk.

Introduction

Flaxseed has components that may alter mammary gland structures. It is the richest source of the mammalian lignan precursor secoisolariciresinol diglycoside (SDG) (1). Mammalian lignans have been shown to exert weak estrogenic or anti-estrogenic activity in vitro (2,3). This has important implications because the development and differentiation of mammary gland structures are hormone dependent. During early development, rising endogenous estrogen promotes mammary duct branching which ends in highly proliferative structures termed terminal end buds (TEBs) (4). At post-natal day (PND) 21 the number of TEBs is maximal. After PND 21, puberty and the onset of estrous cycles result in changing progesterone and estrogen levels that promote differentiation of TEBs to the less proliferative alveolar buds (ABs) and lobules (5,6). Studies have shown that administering the phytoestrogen genistein to neonatal and prepubertal rats reduced TEBs by promoting TEB differentiation (7,8). Flaxseed is also rich in \( \alpha \)-linolenic acid (ALA) (C18:3n-3) (9). High dietary ALA inhibits metabolism of linoleic acid (LA) (C18:2n-6) (10). This has important implications because exposure to high dietary LA during the gestation and early neonatal stages has been reported to reduce differentiation of TEBs to ABs and lobules (11,12).

Differentiation of the mammary gland is important because the highest number of tumors per animal was observed when carcinogen exposure occurred in rats at PND 40–46, a time when the mammary gland exhibits a high density of the highly proliferative TEBs (5). The incidence of carcinomas is positively correlated with the number of TEBs in the mammary gland of the young virgin rat at the time of carcinogen exposure (13). Female rats administered genistein during the prepubertal or neonatal stage had reduced TEBs and treatment of these rats with carcinogen at PND 50 resulted in fewer tumors and increased latency to tumor development (7,8). Thus, promoting the differentiation of highly proliferative TEBs to the less proliferative ABs and lobules may reduce the risk of breast cancer (14,15).

The objective of this study was to determine the effect of exposing female virgin rats to 10% flaxseed (10F), 5% flaxseed (5F), flaxseed oil (FO) or SDG at the level found in 5% flaxseed during (i) gestation and lactation, (ii) after weaning (PND 21–50) or (iii) continuous from gestation to PND 50 (i.e. lifetime) on mammary gland structures and its potential to reduce cancer risk. Indicators of hormonal changes were also measured.

Materials and methods

Diet

The basal diet (BD) composition was based on the semi-purified AIN-93 G diet (16). BD supplemented with 5 (5F) or 10% (10F) ground flaxseed (Linott variety; Omega Products, Melfort, Canada) or 1.82% flaxseed oil (FO; Omega Nutrition; Vancouver, Canada) was corrected for protein, fat and fiber contributed by flaxseed or oil so that the energy values of the diets were the same as previously described (17,18). The SDG in flaxseed was isolated and purified using a modification of the Klosterman method (19,20) as described by Rickard et al. (21). All ingredients were from Dyets Inc. (Bethlehem, PA). Diets were stored at 4°C and fresh diets were provided to the rats every 2 days.

Experimental design

Twenty-eight pregnant Sprague-Dawley rats (77 days old; Charles River Canada, Montreal, Canada) were randomly assigned to either BD or BD supplemented with 5F, 10F, FO or SDG. Dams were given free access to water and their assigned diet throughout pregnancy (22 days) until the end of lactation (21 days). At weaning (i.e. PND 21), female offspring were separated from their dams and individually housed in polycarbonate cages in 22–24°C rooms with 50% humidity and a 12 h light:dark cycle. For no exposure to flaxseed, at PND 21 female offspring were continued on their dam’s BD (BD-BD). For lifetime exposure to flaxseed, at age 21 days the female offspring

Abbreviations: AB, alveolar bud; ALA, \( \alpha \)-linolenic acid; BD, basal diet; 5F, 5% flaxseed; 10F, 10% flaxseed; FO, flaxseed oil; LA, linoleic acid; PND, post-natal day; SDG, secoisolariciresinol diglycoside; TEB, terminal end bud.
of dams fed flaxseed diet were continued on their dam’s diet of 5 (5F-5F) or 10% flaxseed (10F-10F). To limit exposure to flaxseed after weaning, at age 21 days the female offspring of dams given BD were fed 5 (BD-5F) or 10% flaxseed (BD-10F). To limit exposure to flaxseed to gestation and lactation, at age 21 days the female offspring of dams given 5 (5F-BD) or 10% flaxseed (10F-BD) were fed BD. To determine the effect of exposure to flaxseed components, dams were fed either BD supplemented with FO (FO-BD) or BD plus a daily gavage of 1.5 mg SDG (SDG-BD) in 1 ml distilled water throughout gestation and lactation. The SDG level is estimated to be approximately equivalent to that given to rats in the 5F diets on the basis of a SDG concentration of 2.93 µmol/g flaxseed according to HPLC analysis (23) and a diet intake of 15 g/day. The dams not gavaged with SDG were given a daily gavage of distilled water throughout gestation and lactation. Animals were given free access to water and their assigned diets throughout the study period. Animal care and use conformed with the Guide to the Care and Use of Experimental Animals (23). The experimental protocol was approved by the University of Toronto Animal Care Committee.

Mammary gland whole mounts

At puberty (i.e. PND 50), virgin female rats (*n* = 6, consisting of 1–2 offspring per litter from each dam) were killed by CO₂ inhalation. The entire skin pelt was removed from the rats, stretched by pinning on corkboard and then fixed in 10% neutral buffered formalin (24). The abdominal gland (gland number 4) was dissected from the skin pelt and processed for whole mounts. The remaining steps were conducted under continuous agitation. Dissected mammary glands were defatted in acetone for 10 days, after which the glands were hydrated in decreasing concentrations of 100, 95 and 70% ethanol for 1 h each then stored overnight in distilled water. Glands were stained in 0.025% toluidine blue solution for 2 h, washed in distilled water and destained in methanol followed by 70% ethanol for 30 min each. After washing in distilled water, destained glands were fixed in 4% ammonium molybdate for 30 min and stored in distilled water overnight. The following day the glands were dehydrated in 70, 95 and 100% ethanol for 1 h each then transferred to xylene. The mammary glands that stained too darkly were destained. This was done by hydrating through a decreasing series of graded ethanol (70–100%) then dehydrating through an increasing series of graded alcohol (70–100%). The whole mount mammary glands were preserved in heat-sealed polyethylene packages containing methyl salicylate.

Counting of mammary structures

Counting of mammary structures was based on the method described by Russo and Russo (6) with some modifications. The whole mount mammary glands were coded so that the investigator was blind to the identity of the glands. The distal portions of the mammary glands were examined under a stereomicroscope at 3x magnification. Whole mounts were evaluated for TEB, AB and lobule structures. The number of TEB, AB or lobule structures was determined in 10 randomly chosen 1 mm² areas in the distal portion of the mammary gland, the area which normally contains most of the terminal duct structures in the gland.

Puberty onset and estrous cyclicity

In female rats, the age and weight at puberty onset as indicated by visible opening of the vaginal aperture were determined. Vaginal smears were taken and examined microscopically at vaginal opening until PND 50 to determine the length of the first full estrous cycle. The estrous phase of animals was classified as (i) proestrus (mainly epithelial cells present), (ii) estrus (mainly cornified cells present), (iii) metestrus (cornified cells and leukocytes present in large numbers) or (iv) diestrus (mainly leukocytes present). Rat estrous cycle length was determined as the number of days required for completion of the four phases of proestrus, estrus, metestrus and diestrus. A normal cycle was defined as being 4–5 days and having 1–2 days of estrus (25). Cycles were considered prolonged if rats remained in one phase for >3 days and acyclic if they remained in one phase for >10 days.

Relative ovarian weight and serum estradiol

At PND 50, female offspring were killed and the ovaries removed, blotted dry and weighed. Ovarian weight was expressed as relative ovarian weight, i.e. wet organ wt/body wt. Trunk blood was also collected in non-heparinized tubes (Becton-Dickson, Mississauga, Canada), allowed to clot and the serum for estradiol analysis was separated by centrifugation at 1500 g (model 670-1234; Beckman, Palo Alto, CA) for 10 min. Extracted serum was used for estradiol radioimmunoassay. Anhydrous diethyl ether (4 ml) was added to serum (100 µl) and then vortexed and centrifuged for 30 min. The phases were separated by freezing in dry ice and the liquid phase was decanted (to be evaporated to dryness under a gentle stream of nitrogen and analyzed for estradiol using a commercially available radioimmunoassay kit with 125I-labeled 17β-estradiol antibody-coated tubes (ICN, Montreal, Canada). Counting was done using a Packard COBRA II auto-gamma counter (Canberra Packard, Malton, Canada). Serum samples were analyzed for estradiol in one batch and the intra-assay coefficient of variation was 4.5%.

**Results**

Mammary gland structures

Gestation and lactation (10F-BD) or lifetime (10F-10F) exposure to 10F reduced (*P* < 0.05) the TEB density and enhanced AB or lobule density compared with the control (BD-BD) group (Figure 1). Exposure to 10F after weaning (BD-10F) reduced the TEB density to levels that did not differ significantly from the control but also did not differ significantly from those exposed during gestation and lactation or lifetime.

Exposure to 5F after weaning (BD-5F) had no effect on the mammary gland structures whereas gestation and lactation (5F-BD) or lifetime (5F-5F) exposure to 5F reduced (*P* < 0.05) the TEB density but had no significant effect on AB or...
or estrous cyclicity. However, lifetime or gestation and lactation exposure to SDG delayed puberty onset and reduced the number of estrous cycles from vaginal opening to PND 50 compared with the control group but had no significant effect on the length of estrous cycles (Table II). Gestation and lactation exposure to flaxseed components at the level found in 5F, showed that SDG delayed puberty onset and reduced the number of estrous cycles whereas FO had no effect (Table II).

A two-way ANOVA showed that dose ($P < 0.001$) and timing of flaxseed treatment ($P < 0.013$) significantly affected age of puberty onset but there was no dose and timing of treatment interaction. On the other hand, although timing of treatment had no effect, the dose ($P < 0.001$) and timing and dose interaction ($P < 0.001$) significantly affected the number of estrous cycles.

### Relative ovarian weight and serum estradiol

Exposure to 10F after weaning had no significant effect on relative ovarian weight or serum estradiol whereas lifetime or gestation and lactation exposure to 10F resulted in higher relative ovarian weight and serum estradiol levels compared with the control group (Table III). In contrast, exposure to 5F during the different developmental stages had no significant effect on relative ovarian weight or serum estradiol levels (Table IV).

### Relative ovarian weight and serum estradiol

A two-way ANOVA indicated significant dose ($P < 0.001$), treatment timing ($P < 0.001$) and timing and dose interaction ($P = 0.001$) effects on serum estradiol levels and significant dose ($P < 0.002$) and dose and treatment interaction ($P < 0.033$) effects on ovarian weight but no effect of treatment timing. At

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### Table I. The effect of timing of exposure to 10F on puberty onset and estrous cyclicity in female rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Puberty onset (day)</th>
<th>Estrous cycle length (days)</th>
<th>Number of estrous cycles ($n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-BD</td>
<td>31.83 ± 1.28</td>
<td>5.00 ± 0.37</td>
<td>3.77 ± 0.43</td>
</tr>
<tr>
<td>BD-10F</td>
<td>29.50 ± 1.41</td>
<td>6.00 ± 0.56</td>
<td>4.09 ± 0.49</td>
</tr>
<tr>
<td>10F-10F</td>
<td>25.67 ± 0.33</td>
<td>7.00 ± 0.58</td>
<td>3.58 ± 0.26</td>
</tr>
<tr>
<td>10F-BD</td>
<td>24.83 ± 0.40</td>
<td>7.33 ± 0.67</td>
<td>3.60 ± 0.38</td>
</tr>
</tbody>
</table>

For definition of abbreviations see Figures 1 and 2.

$^a$ Determined at vaginal opening until PND 50.

$^b$-d Values are means ± SEM, $n = 6$. Values with different letters within the same column are significantly different ($P < 0.05$) by one-way ANOVA followed by Tukey’s test.

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### Table II. The effect of timing of exposure to 5F or its components on puberty onset and estrous cyclicity in female rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Puberty onset (day)</th>
<th>Estrous cycle length (days)</th>
<th>Number of estrous cycles ($n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-BD</td>
<td>31.83 ± 1.28</td>
<td>5.00 ± 0.37</td>
<td>3.77 ± 0.43</td>
</tr>
<tr>
<td>BD-5F</td>
<td>31.67 ± 1.41</td>
<td>5.67 ± 0.33</td>
<td>3.32 ± 0.38$^d$</td>
</tr>
<tr>
<td>5F-5F</td>
<td>36.67 ± 0.56$^c$</td>
<td>6.17 ± 0.17</td>
<td>1.86 ± 0.42$^c$</td>
</tr>
<tr>
<td>5F-BD</td>
<td>36.17 ± 0.79$^b$</td>
<td>6.50 ± 0.43</td>
<td>2.18 ± 0.19$^c$</td>
</tr>
<tr>
<td>SDG-BD</td>
<td>35.83 ± 0.48$^a$</td>
<td>6.75 ± 0.70</td>
<td>2.58 ± 0.20$^{ab}$</td>
</tr>
<tr>
<td>FO-BD</td>
<td>31.50 ± 0.50$^a$</td>
<td>5.33 ± 0.21</td>
<td>3.83 ± 0.17$^d$</td>
</tr>
</tbody>
</table>

For definition of abbreviations see Figures 1 and 2.

$^a$-d Values are means ± SEM, $n = 6$. Values with different letters within the same column are significantly different ($P < 0.05$) by one-way ANOVA followed by Tukey’s test.

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### Fig. 2. The effect of exposure to 5F or flaxseed components at various developmental stages on the density of mammary gland TEBs, ABs and lobules. Values are the means ± SEM of $n = 6$. Control is the group fed BD (BD-BD); BD-5F is after-weaning exposure to BD supplemented with 5F (w/w); 5F-5F is lifetime exposure to BD supplemented with 5F; 5F-BD is BD and a daily gavage of 1.5 mg SDG in 1 ml distilled water during gestation and lactation; FO-BD is gestation and lactation exposure to BD supplemented with 1.82% FO. The rat dams not gavaged with SDG were given a daily gavage of distilled water throughout gestation and lactation.

lobule density compared with the control group (Figure 2). Gestation and lactation exposure to SDG (SDG-BD) at the level found in 5F reduced ($P < 0.05$) TEB and AB density but FO (FO-BD) had no effect (Figure 2).

A two-way ANOVA indicated that timing of flaxseed treatment significantly affected TEB density ($P = 0.002$) and lobule density ($P = 0.019$) but dose and treatment timing interaction had no effect. However, AB density was affected by dose ($P < 0.001$), treatment timing ($P = 0.012$) and their interaction ($P < 0.001$).

### Puberty onset and estrous cyclicity

Exposure to 10F after weaning had no effect on puberty onset or estrous cyclicity. However, lifetime or gestation and lactation exposure to 10F delayed age ($P < 0.05$) of puberty onset and reduced the number of estrous cycles from vaginal opening to PND 50 compared with the control group but had no significant effect on the length of estrous cycles (Table II). Gestation and lactation exposure to flaxseed components at the level found in 5F, showed that SDG delayed puberty onset and reduced the number of estrous cycles whereas FO had no effect (Table II).

A two-way ANOVA showed that dose ($P < 0.001$) and timing of flaxseed treatment ($P < 0.013$) significantly affected age of puberty onset but there was no dose and timing of treatment interaction. On the other hand, although timing of treatment had no effect, the dose ($P < 0.001$) and timing and dose interaction ($P < 0.001$) significantly affected the number of estrous cycles.

### Relative ovarian weight and serum estradiol

Exposure to 10F after weaning had no significant effect on relative ovarian weight or serum estradiol whereas lifetime or gestation and lactation exposure to 10F resulted in higher relative ovarian weight and serum estradiol levels compared with the control group (Table III). In contrast, exposure to 5F during the different developmental stages had no significant effect on relative ovarian weight or serum estradiol levels (Table IV). Gestation and lactation exposure to SDG or oil components at the level found in 5F during gestation and lactation also had no effect on relative ovarian weight or serum estradiol (Table IV).

A two-way ANOVA indicated significant dose ($P < 0.001$), treatment timing ($P < 0.001$) and timing and dose interaction ($P = 0.001$) effects on serum estradiol levels and significant dose ($P < 0.002$) and dose and treatment interaction ($P < 0.033$) effects on ovarian weight but no effect of treatment timing. At
Table III. The effect of timing of exposure to 10F on relative ovarian weight and serum estradiol levels in female rats on PND 50

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative ovarian weight* (mg/100g)</th>
<th>Serum estradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-BD</td>
<td>58.31 ± 3.40b</td>
<td>33.83 ± 1.51b</td>
</tr>
<tr>
<td>BD-10F</td>
<td>69.80 ± 5.37bc</td>
<td>34.83 ± 2.26b</td>
</tr>
<tr>
<td>10F-10F</td>
<td>95.04 ± 9.53bc</td>
<td>54.33 ± 1.38c</td>
</tr>
<tr>
<td>10F-BD</td>
<td>90.14 ± 5.97c</td>
<td>47.33 ± 3.69c</td>
</tr>
</tbody>
</table>

For definition of abbreviations see Figures 1 and 2.

Values are means ± SEM, n = 6. Values with different letters within the same column are significantly different (*P < 0.05) by one-way ANOVA followed by Tukey’s test.

Table IV. The effect of timing of exposure to 5F or flaxseed components on relative ovarian weight and serum estradiol levels in female rats on PND 50

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative ovarian weight* (mg/100g)</th>
<th>Serum estradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-BD</td>
<td>58.31 ± 3.40</td>
<td>33.83 ± 1.51</td>
</tr>
<tr>
<td>BD-5F</td>
<td>72.14 ± 5.11</td>
<td>34.00 ± 1.57</td>
</tr>
<tr>
<td>5F-5F</td>
<td>70.53 ± 4.51</td>
<td>35.50 ± 2.68</td>
</tr>
<tr>
<td>5F-BD</td>
<td>61.06 ± 4.81</td>
<td>30.50 ± 1.62</td>
</tr>
<tr>
<td>SDG-BD</td>
<td>55.33 ± 2.97</td>
<td>30.33 ± 1.52</td>
</tr>
<tr>
<td>FO-BD</td>
<td>59.17 ± 2.97</td>
<td>33.67 ± 1.86</td>
</tr>
</tbody>
</table>

For definition of abbreviations see Figures 1 and 2.

Values are means ± SEM, n = 6.

*Relative ovarian weight calculated as wet wt/body wt.

PND 50, the female offspring exposed to 10F during gestation and lactation or throughout their lifetime had higher ovarian weights and serum estradiol levels compared with the female offspring given 5F during the different developmental stages.

Discussion

This study shows that flaxseed can influence mammary gland structures depending on the dose and timing of exposure. Gestation and lactation exposure to 10F reduced TEB density and increased AB density in the mammary gland of 50-day-old virgin female rats compared with the control (BD-BD) group. A similar effect was produced by lifetime 10F treatment that includes the gestation and lactation period. Exposure to 10F starting at weaning (PND 21) produced a small reduction in TEB density, although this did not differ significantly from the control or those exposed during gestation and lactation or throughout their lifetime. Similarly, exposure to 5F after weaning had no effect on mammary gland structures whereas gestation and lactation or lifetime exposure to 5F reduced mammary gland TEB density compared with the control group. This suggests that gestation and lactation is the critical period for inducing structural changes in the mammary gland.

Flaxseed has components that may alter mammary gland structures. It is the richest source of the mammalian lignan precursor SDG (1). Mammalian lignans have been shown to exert weak estrogenic or anti-estrogenic activity in vitro (2,3). This has important implications because the development and differentiation of mammary gland structures are hormone dependent. Flaxseed is also rich in ALA (9). High dietary ALA inhibits metabolism of LA (10). This has important implications because exposure to high dietary LA during the gestation and early neonatal stages has been reported to reduce differentiation of TEBs to ABs and lobules (11,12). To determine which component was responsible for the observed effects, female offspring were exposed to FO or SDG at the level found in 5F during gestation and lactation. SDG but not FO reduced TEBs and ABs. This suggested that the lignans were responsible for the observed effects on mammary gland structures. In agreement, treatment of rats during the neonatal period with the phytoestrogen genistein, found in soybean, resulted in reduced TEBs (7). Unfortunately, the effect of SDG and FO at the level found in 10F on mammary gland structures was not determined. This would have been of interest because the mechanism whereby 10F and 5F reduce mammary TEB density apparently differ.

Reduced TEB density in female rats given 10F during gestation and lactation or throughout the lifetime appeared to be the result of enhanced mammary gland differentiation, as indicated by reduced TEB density and higher AB density. On the other hand, reduced TEB density with lifetime or gestation and lactation exposure to 5F appeared not to be the result of differentiation, since the reduction in TEB density was not accompanied by increased AB and lobule densities. In studies with the phytoestrogen genistein, TEBs were also reduced, with or without increases in the number of differentiated gland structures, at PND 50 (7).

The different endocrine effects produced by low (5%) compared with high (10%) flaxseed dose may be responsible for the different effects exerted on the mammary gland. Our previous study has shown that at PND 21, free serum estradiol was reduced in female offspring exposed to 5F or SDG but not in those exposed to 10F compared with the control group (18). During prenatal and early post-natal life in rats, estradiol is required for normal mammary gland growth and branching. Branches end in highly proliferative TEB structures. The number of TEBs is maximal at PND 21 (4). 5F or SDG acting as an estrogen antagonist prior to PND 21 may have reduced estrogen-dependent mammary branching resulting in the development of fewer TEBs. Gestation and lactation exposure to SDG also reduced mammary AB density. This may have resulted because each estrous cycle promotes differentiation of TEBs to ABs and lobules (26). SDG delayed puberty and reduced the number of estrous cycles. Treatment with 5F also delayed puberty onset and reduced the number of estrous cycles. This resulted in a small reduction in AB density that was not significantly different from the control group. The oil or other components in flaxseed may have counteracted the inhibitory effects of SDG on mammary gland differentiation.

In contrast, gestation and lactation or lifetime exposure to 10F resulted in female rats with earlier age of puberty onset. This would be expected to increase the number of estrous cycles over a period of time. The progressive differentiation of TEBs into ABs is accentuated by each estrous cycle (26), suggesting that the lignans were responsible for the observed differ-
Flaxseed and rat mammary gland development


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References


