Effects of maternal exposure to cow’s milk high or low in isoflavones on carcinogen-induced mammary tumorigenesis among rat offspring

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Abbreviations: DMBA = 7,12-dimethylbenz[a]anthracene; E2 = 17β-estradiol; IGF-1 = insulin like growth factor; GD = gestational day; PND = postnatal day; VO = vaginal opening

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Abstract

We investigated whether maternal exposure during pregnancy to cow’s milk containing endogenous estrogens and insulin like growth factor 1 (IGF-1) and either high or low levels of isoflavones from dietary legumes (HIM and LIM, respectively) affected carcinogen-induced mammary carcinogenesis in female rat offspring. Pregnant Sprague-Dawley rats were given HIM, LIM or tap water (control) from gestational day (GD) 11 until birth; hereafter all rats received tap water. Mammary tumorigenesis was induced by administrating 7,12-dimethylbenz[a]anthracene (DMBA) on postnatal day (PND) 50. No differences in maternal serum estradiol ($P = 0.19$) and IGF-1 levels ($P = 0.15$) at GD 19 or birth weight among the milk and water groups were seen, but estradiol, and IGF-1 levels and birth weight were numerically higher in the LIM than in the HIM group. Puberty onset occurred earlier in the LIM offspring than in controls ($P = 0.03$). Although the high isoflavone content seemed to prevent the effect on circulating estradiol and IGF-1 levels and advanced puberty onset seen in the LIM group, HIM increased DMBA-DNA adducts in the mammary gland and tended to increase mammary tumorigenesis. In contrast, offspring exposed to LIM in utero, did not exhibit increased breast cancer risk, despite having higher estradiol and IGF-1 environment and consequently earlier puberty onset. These results indicate that the phytochemical content in the cow’s milk, consumed by a pregnant dam, determines how milk affects the offspring.
Introduction

Dietary phytoestrogens, particularly isoflavones and lignans, have received a great deal of attention due to their potential to affect hormone dependent cancers, especially breast cancer. Soy products contain high levels of isoflavones (0.2-6.5 mg/ml) (1), but isoflavones are also present in cow’s milk (2, 3), although not in levels comparable to soy products. Equol is usually the far most abundant isoflavone in cow’s milk, and the levels vary from 0.045 to 1 μg/ml, depending on the amounts and type of legumes fed to cows. The content of equol is on average 5-7 times higher in organic milk than in conventionally produced milk (2), due to the more intensive use of legumes in the feed. Equol is produced from the precursors formononetin and daidzein by ruminal and intestinal bacteria (4, 5). All cows produce equol, but only about one third of the Western population is estimated to be able to produce this isoflavone, most likely due to inter-individual variability in the intestinal bacterial flora (5). Cow’s milk is therefore a potential source of equol for non-equol producers. It is not clear whether equol exposure affects breast cancer risk. In humans, urinary equol excretion correlates with a high intake of soy products and a reduced risk of breast cancer (6), but equol also shows proliferative effects on human breast cancer cells (7), and in rodent studies dietary equol exhibits estrogenic effects (8).

Results from animal studies suggest that maternal dietary or hormonal exposures during pregnancy may have long lasting effects on mammary tumorigenesis among the female offspring (9-12). For example, maternal exposure to a high fat diet during pregnancy increases a female offspring’s susceptibility to develop carcinogen-induced mammary tumors in rats (9, 13), whereas whole wheat flour, containing lignans, and fish oil reduces the risk (10, 12). The effects of maternal exposure to the isoflavone genistein during pregnancy on offspring’s mammary tumorigenesis have also been intensively studied but results are conflicting (14).
Neonatal and prepubertal exposure to equol alters the morphology of the mammary gland epithelial tree in a manner that can be considered to be protective, but it has no effect on mammary tumorigenesis (15).

Some animal (16, 17) and human studies (18) suggest that milk intake increases breast cancer risk. However, most studies have reported either a reduced risk or no change in risk being associated with milk consumption (19, 20). Findings in humans suggest that childhood milk intake reduces breast cancer risk (21, 22). No animal studies have addressed the effect of maternal milk intake during pregnancy on mammary tumorigenesis among the offspring. Human epidemiological data suggest that high milk consumption during pregnancy is associated with increased birth weight (23), and a high birth weight has been linked to elevated maternal estrogen levels (24) and increased risk of later development of breast cancer (25). Consistent with the human data, mammary cancer risk is increased in rats which had a high birth weight (13).

The present study was designed to investigate whether in utero exposure to cow’s milk high or low in isoflavones affects carcinogen-induced mammary tumorigenesis among the female offspring, compared to a tap water control group.

Material and methods

Animals

Twenty-seven female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) on day 7 at gestation and weighed at arrival. The animals were housed individually in standard rat plexiglas cages at a constant temperature and humidity under a 12-h light-dark cycles. Animals were fed the American Institute of Nutrition (AIN-93G)
phytoestrogens-free, semi-purified diet ad libitum throughout the study period. The study was performed in accordance with the appropriate institutional and federal regulations.

Maternal liquid exposures

Pregnant dams were randomly assigned to the following four treatments (n = 9 per group) from gestational day (GD) 11 until they gave birth: Low isoflavone milk (LIM), high isoflavone milk (HIM) or tap water (control). Since we have previously shown that maternal exposure to estradiol (E2), either at a very low concentration (0.1 μg/g) or at a higher concentration (50 μg/g), significantly increases offspring’s mammary tumorigenesis, an E2 control group was not included to this study. Fresh milk was provided daily, and the drinking bottles were weighed before and after milk was added to calculate the daily intake. The average daily feed intake was calculated based on the difference in weight of the food provided and the remaining feed. The daily calorie intake (kcal/day) for dams during pregnancy was calculated from the energy content of the AIN93G diet provided in the information leaflet and the kcal content in skim milk (0.35 kcal/g milk).

When pups were born the litter was weighed and all dams were switched to tap water and continued on the AIN93G diet. To avoid litter effect, female pups were cross-fostered 1-2 days after the dams gave birth; female pups from 3-4 dams were pooled and housed with a nursing dam which had received the same treatment as the pup’s mother during pregnancy. Male pups were sacrificed. All female pups were weaned on postnatal day (PND) 23.

Milk and milk analyses

The two milk types were obtained directly from two privately owned commercial Danish dairy farms, that were selected based on a screening of bulk milk phytoestrogen content on 20
Danish farms (organic and conventional) representing various different feed ration compositions especially regarding varying types and amounts of legumes. Cows producing HIM were fed a ration consisting of 24% whole crop barley-pea silage, 71% clover grass silage including red clover and 5% grains (triticale, oats, barley, peas). LIM was obtained from a herd where cows were fed 24% horsebeans, 66% clover grass silage (white clover) and 10% grains (oats). Unprocessed bulk milk from one days milking was obtained from both herds and stored at -20°C until analyzed or fed to rats as skim milk in the experiment. Milk was centrifuged 10 min at 4000 rpm, 4°C and fat discarded to obtain skim milk. All subsequent analysis were carried out on skim milk. The IGF-1 concentration was determined as described previously (26), and the content of phyt oestrogens was measured according to the method described elsewhere (27). Unfortunately this method was not able to detect genistein in milk although it is known typically to be present in the amounts of 0.9-3 ng/ml (28, 29) and might have been higher in HIM than LIM.

Pregnancy E2, IGF-1 and isoflavone levels

Blood from all pregnant dams was collected by tail puncture on GD 19. Serum was separated and kept at -80°C until use. A commercial EIA kit from Cayman Chemicals (Ann Arbor, MI) was used to measure serum E2. Serum was purified by diethyl-ether extraction prior to E2 analysis according to the manufacturer’s instructions. IGF-1 was measured by an EIA kit from R&D Systems, Inc. (Minneapolis, MN), according to manufacturer’s instructions. The contents of genistein, daidzein and equol were measured in serum using a validated isotope dilution liquid chromatography electrospray mass spectrometry (LC-ES/MS) method (30).

Puberty onset - vaginal opening
Starting on PND 25, female offspring were clinically examined daily to evaluate vaginal opening (VO). Rats were recorded positive for VO when the vagina exhibited complete canalization and patency. VO typically occurs in the Sprague-Dawley rat around 32 days of age under the influence of ovarian and adipose-derived estrogens and other hormones, and represents the initial stages of attaining sexual maturity.

**Offspring mammary tumorigenesis**

Mammary tumors were induced by oral administration of 10 mg 7,12-dimethylbenz[a]anthracene (DMBA) to 50-day-old female rats exposed to the different liquids *in utero*. The histopathology, estrogen-dependence, estrogen and progesterone receptor expression and antiestrogen responsiveness of DMBA induced tumors show similarities to estrogen-sensitive human breast cancer (9, 10, 13) The animals were examined for mammary tumors by palpation once per week starting from week 8 post-DMBA treatment. The end-points for data analysis were (i) number of animals with tumors (tumor incidence), (ii) number of tumors per animal (tumor multiplicity) and (iii) the total tumor volume per animal (volume). The animals were sacrificed when the detectable tumor burden approximated 5% of the total body weight, as required by our institution. All surviving animals, including those that did not develop mammary tumors, were sacrificed 18 weeks after carcinogen administration.

**DMBA-DNA adduct measurement**

DNA from mammary tissue at 18 weeks after DMBA administration was isolated by standard phenol extraction procedures and DMBA-DNA adducts were subsequently measured by $^{32}$P-postlabelling (31). In short, 10 µg DNA was digested using micrococcal endonuclease (0.06
U/μl, Sigma) and spleen phosphodiesterase (0.5 μg/μl, Sigma) for 4 hours at 37°C. Subsequently, samples were treated with nuclease P1 (0.26 μg/μl, MP Biomedicals) for 30 minutes at 37°C in a total volume of 12.5 μl. The largest fraction of the DNA digest (10.5 μl) was labeled with [γ-32P]-ATP (50 μCi/sample, MP Biomedicals) by incubation with T4-polynucleotide kinase (0.68 U/μl, Fermentas) for 30 minutes at 37°C. Radiolabeled adduct nucleotide biphosphates were separated by thin-layer chromatography on polyethyleneimine (PEI) cellulose sheets (Macherey-Nagel, Germany). Finally, DMBA-DNA adduct spots were quantified using Phosphor-Imaging technology (Fujifilm FLA-3000, Rotterdam, The Netherlands). A fraction of the digest (2 μl) was used to determine the exact amount of DNA in the assay by HPLC-UV.

Statistical analysis

Data obtained on pregnancy-related parameters (birth weight, numbers of pups per litter and pregnancy hormone levels), tumor volume/animal and tumor multiplicity at sacrifice, together with DNA-adduct formation, were analyzed using one-way analysis of variance (ANOVA) followed by between group comparisons using Tukey’s test. Kaplan-Meier curves were used to compare differences in vaginal opening and tumor incidence, followed by the Log-Rank test. All tests were performed using the SPSS SigmaStat software and differences were considered significant if the p-value was less than 0.05.

Results

Milk composition

There were no macronutrient differences between the two types of milk (data not shown). The total content of phytoestrogens was approximately 4 times higher in high isoflavone milk.
(HIM) (429 ng/ml) compared with low isoflavone milk (LIM) (101 ng/ml) \( P < 0.001 \) (Table 1), with equol being the most abundant phytoestrogen in HIM (366 ng/ml). However, the levels of enterodiol and enterolactone were significantly higher in LIM (0.21 and 42 ng/ml, respectively) than HIM (0.09 and 19.8 ng/ml, respectively) \( P = 0.04 \) and \( P = 0.01 \), respectively) (Table 1).

Pregnancy outcome and serum estradiol, IGF-1 and isoflavones

Pregnant dams were started on cow’s milk on gestation day (GD) 11 and continued until they gave birth. During pregnancy, the intake of liquid was higher in the two milk groups \( P < 0.001 \), but milk treated dams also consumed significantly less feed than the tap water group \( P < 0.001 \), and therefore intake of energy (kcal/day) was not significantly different among the three groups \( P = 0.07 \) (Table 2). The total daily phytoestrogen exposure was determined between GD 19 and birth, and it was 16.9±1.1 and 75.1±2.2 μg/kg body weight for LIM and HIM dams, respectively \( P < 0.001 \) (Table 2). Neither milk group differed from tap water control offspring in female \( P = 0.14 \) and male \( P = 0.09 \) birth weight and the female/male offspring ratio was similar among the three groups (Table 2). However, both female and male offspring of HIM dams tended to be lighter than the offspring of LIM dams, although maternal energy intake was highest in the HIM group.

There was no difference in serum levels of E2 at GD 19 \( P = 0.19 \), but when HIM and LIM were compared to each other, the E2 concentration was significantly lower in the HIM than LIM group (419 pg/ml vs 482 pg/ml, \( P = 0.03 \); control dams had E2 concentrations in between the two values (468 pg/ml) (Table 3). Similarly, HIM exposed dams exhibited significantly lower IGF-1 level at GD 19 (782 ng/ml) compared with LIM (904 ng/ml) \( P = 0.03 \) and control dams (824 pg/ml), but the difference among the three groups did not reach
Statistically significance \((P = 0.15)\) (Table 3). Serum levels of isoflavones were low in all dams and neither LIM nor HIM altered the circulating levels of genistein or daidzein at GD 19, compared with water controls (Table 3). Equol levels were undetectable in most dams (in 23 of 27 dams). Since isoflavones cross the placenta and the aglycone (biologically active) form accumulates in the fetus (32), it is possible that had we been able to measure isoflavones in the fetus, differences would have been seen.

Puberty onset

Maternal exposure to LIM during pregnancy caused an earlier puberty onset in the female offspring, determined by assessing the age of vaginal opening, compared to the control group (Log Rank = 7.169, \(P = 0.03\)) (Fig. 1). No difference was seen between the HIM and control offspring. The age at which 50% of the rats showed vaginal opening was 33.6 ± 0.3, 34.3 ± 0.3 and 35.1 ± 0.4 for the LIM, HIM and the control groups, respectively.

DMBA-DNA adducts

DMBA-DNA adducts were determined at the end of the tumor monitoring period (18 weeks) in non-tumorous mammary tissue. Although DMBA-DNA adduct levels that were initially formed after the exposure will gradually decline in time due to DNA repair and cell turn-over, DMBA-DNA adducts were still detectable in all samples. DMBA-induced DNA adduct levels were 2-fold higher in the HIM than in the control rat mammary glands (23.4±3.1 vs 11.2±3.4 adducts per \(10^8\) nucleotides, \(P < 0.001\)) (Fig. 2). DNA adduct levels in mammary glands of rats that received LIM in utero did not differ from the levels observed in control rats (16.3±3.0 adducts per \(10^8\) nucleotides) (Fig. 2).
Tumorigenesis

There was no difference between LIM and HIM offspring relative to water offspring with respect to mammary tumor incidence (Log Rank = 2.544, *P* = 0.28) (Fig. 3a). Compared with water and HIM of which 74% and 75% developed mammary tumour, respectively, maternal LIM intake during pregnancy resulted in the lowest incidence (60% mammary tumors). The number of mammary tumors per animal at sacrifice (tumor multiplicity) (Fig. 3b) was non-significantly higher in the HIM offspring (2.08 ± 0.37), than in the tap water control and LIM offspring (1.42 ± 0.34 and 1.32 ± 0.27, respectively) (*P* = 0.19). The final tumor burden at sacrifice (total tumor volume) was 34% higher in the HIM group (6241 ± 1161 vs. 4815 ± 1301 and 4797 ± 990 mm³ the control and LIM offspring, respectively) (Fig. 3c).

Discussion

Maternal dietary intake of excess fat or other dietary factors during pregnancy have been shown to affect female offspring’s later susceptibility to develop mammary cancer in rats (10-12). This increase in risk is thought to occur due to an increase in fetal estrogenic environment caused by the maternal diet (33, 34). Our study is the first to investigate the effect of maternal milk consumption during pregnancy on the offspring’s risk of mammary cancer. Milk contains endogenous estrogens and IGF-1 (26, 35) and various dietary phytoestrogens (2, 3, 27, 29), particularly isoflavones and lignans, with concentrations reflecting the composition of the cow’s diet. These phytoestrogens have potential to exert estrogenic effects. Different feeding practices have been reported to result in up to 10-fold difference in milk’s phytoestrogen content (118 vs 1215 ng/ml) (3), but the two milk types in our experiment only reached a four-fold difference in total phytoestrogen content. Further, milk phytoestrogens occur almost entirely as biologically inactive glucuronide and sulfate conjugates (28, 36) that
are hydrolysed by intestinal glucuronidases and sulfatases upon ingestion before free
biologically active forms can be absorbed.

We fed pregnant rats skim milk which contained either high or low levels of
isoflavones; control dams received tap water. Skim milk was chosen for this study to avoid
any confounding effects caused by possible differences in fatty acid composition between the
LIM and HIM, and to minimize the effect of unconjugated biologically active endogenous
estrogens that are suggested to increase with fat content (37). Phytoestrogens in milk are
reported to be independent of milk fat content (28). HIM contained significantly more
daidzein and its metabolite equol than LIM. The latter, in turn, contained significantly higher
levels of enterodiol and enterolactone, two key lignan metabolites. These findings probably
reflect the fact that cow’s producing HIM consumed a feed ration which included red clover
silage containing high levels of isoflavones (29) typically used in organic milk production,
whereas LIM cows consumed a feed ration typical for conventional milk production which
includes white clover having a relatively high content of lignan precursors (38).

The different content of isoflavones in the two milk types could not be detected
as a difference in circulating levels of genistein, daidzein and equol at GD 19, perhaps
reflecting the overall relatively low content of phytoestrogens in these two types of cow’s
milk compared to for example soy milk. Lignans were not determined. However, since the
serum levels of E2 and IGF-1 at GD 19 were lower in the HIM than LIM dams, the higher
phytoestrogen content of HIM may have affected the dams. Commercial milk contains
relatively high levels of both IGF-1 and estrogens (26, 37), and this could explain why these
two tended to be higher in the LIM group than in the controls. The fact that they were lower
in the HIM dams is consistent with previous findings in women showing that red clover-
derived isoflavones non-significantly reduce circulating IGF-1 levels (39) and that intake of
soy-derived isoflavones is associated in some, but not all studies, with lower serum estrogen levels (40). The tendency of higher circulating levels of IGF-1 and E2 levels in the dams consuming LIM than in the control dams is consistent with milk containing relatively high levels of both (26, 35), and perhaps thereby increasing them also in individuals consuming milk (41), although milk protein consumption is believed to stimulate IGF-1 synthesis over IGF-1 absorption (42).

Offspring of dams consuming HIM during pregnancy exhibited significantly higher levels of DMBA-induced DNA adducts in their mammary glands than the controls; no difference was seen between the control and LIM offspring. Consistent with this, mammary tumor multiplicity and total tumor burden tended to be higher in the HIM group than in the other two groups. Animal studies have shown an increase in mammary cancer risk among offspring of dams which were exposed to high levels of isoflavones during pregnancy (10, 14, 43). Maternal exposure during pregnancy in these earlier studies was purified genistein, while milk phytoestrogens are a mixture of several different phytoestrogens with isoflavones daidzein and equol being the most abundant in HIM. Equol exists in two enantiometric forms: $S$-(-) and $R$-(+); $S$-equol is the naturally occurring metabolite of formononetin and daidzein in the rumen of cows and in the human intestine (4, 5, 44) and most likely the sole form in milk. In a recent study of carcinogen-induced mammary tumorigenesis in rats, $S$-equol administration from day 35 onwards showed no chemopreventive action, nor was it stimulatory, while $R$-equol reduced mammary tumorigenesis significantly (45). In another study, rats were exposed to $S$- or $R$-equol during neonatal life or pre-pubertally (day 21-35) (15). The exposures did not significantly alter later mammary tumorigenesis. The results obtained in our study suggest that in utero exposure to equol in milk increases, rather than protects against breast cancer. This is consistent with earlier studies which indicate that in
uterine and prepubertal isoflavone exposures are reported to have opposing effects on later breast cancer risk (14). However, the possibility that reduced lignan levels in the HIM, compared to the LIM, led to increased tumorigenesis, rather than high isoflavone levels, cannot be ruled out. Lignans have been linked to reduced mammary cancer risk in animal studies (46).

Offspring of dams consuming LIM milk during pregnancy exhibited several changes which are predictive of increased breast cancer risk. Specifically, their dams had the highest pregnancy IGF-1 and E2 levels, and they tended to have higher birth weight and they exhibited the earliest puberty onset. High pregnancy E2 levels increase offspring’s mammary tumorigenesis (9), and it has been proposed that high IGF-1 also increases the risk (47). Human epidemiological studies have reported that a high milk intake during pregnancy is associated with increased birth weight (23). Further, high birth weight is a risk factor for breast cancer in both humans and rats (25, 48). Early puberty onset is known to be related to increased breast cancer risk (49), although the effect has become less significant in recent years (50). Thus, regardless of the increased presence of breast cancer biomarkers in the LIM group suggesting that these rats should develop most mammary tumors, they were not at an increased risk, illustrating that the chosen biomarkers are not always predictive of mammary tumorigenesis risk.

In summary, maternal HIM intake during pregnancy, relative to tap water intake, led to increased carcinogen-induced DNA adduct formation in the mammary gland and non-significantly increased some key parameters of tumorigenesis among the offspring. In contrast, maternal intake of LIM which contained more lignans than HIM, had no effect on offspring’s mammary cancer risk, although it increased dam’s pregnancy estradiol and IGF-1 levels, induced higher birth weight and accelerated puberty onset. These finding suggest that
the content of isoflavones and lignans in cow’s milk determines whether maternal exposure to
this milk during pregnancy contributes to accumulation of DNA adducts following carcinogen
exposure in the offspring’s mammary gland.

Acknowledgements

We thank Dr. Daniel R. Doerge at the National Center for Toxicological Research, Jefferson,
AR, USA for performing the isoflavone analysis on rat serum.
Figure 1. Effects of maternal exposure to tap water (control), low or high isoflavone milk (LIM or HIM, respectively) from GD 11 until birth on vaginal opening among female offspring (n = 28, 47 and 36 in the control, LIM and HIM groups, respectively). Vaginal opening occurred significantly earlier in the LIM group (p = 0.03) compared with the control group.

Figure 2. Effects of maternal exposure during pregnancy to tap water (control), or low or high isoflavone milk (LIM or HIM, respectively) on DMBA-DNA adduct formation measured in non-tumorous mammary tissue at the end of the tumor monitoring period (18 weeks) among the offspring. Values represents means ± SEM. Bars marked with different letters are significantly different from each other (p<0.05).

Figure 3. Effects of maternal exposure during pregnancy to tap water (control), or low or high isoflavone milk (LIM or HIM, respectively) on offspring (a) tumor incidence (% rats with tumors), (b) tumor multiplicity measured as the number of tumors per animal at the time of sacrifice, (c) tumor volume measured as the volume of tumors per animal at the time of sacrifice (n = 19, 25 and 24 in the control, LIM and HIM groups, respectively). Values are expressed as mean ± SEM.
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30. Twaddle NC, Churchwell MI, Doerge DR. High-throughput quantification of soy isoflavones in human and rodent blood using liquid chromatography with electrospray


Key TJ, Appleby PN, Reeves GK, Roddam AW. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: pooled individual data analysis of 17 prospective studies. Lancet Oncol 2010;11:530-42.


Figure 1.
Figure 2
Table 1. Content of IGF-1 and phytoestrogens in low and high isoflavone milk (LIM and HIM, respectively). Means ± SEM

<table>
<thead>
<tr>
<th></th>
<th>LIM</th>
<th>HIM</th>
<th>p-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGF-1 (ng/ml)</strong></td>
<td>2.9 ± 0.06</td>
<td>1.2 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Phytoestrogens (ng/ml)&lt;sup&gt;2&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Formononetin</td>
<td>6.0 ± 0.6</td>
<td>7.9 ± 0.3</td>
<td>0.054</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>5.2 ± 0.6</td>
<td>12.0 ± 5.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Daidzein</td>
<td>1.7 ± 0.6</td>
<td>5.8 ± 0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Prunetin</td>
<td>-</td>
<td>7.5 ± 2.4</td>
<td>-</td>
</tr>
<tr>
<td>Equol</td>
<td>37 ± 2.0</td>
<td>366 ± 4.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Matairesinol</td>
<td>-</td>
<td>0.63 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>Secoisolariciresinol</td>
<td>9.0 ± 0.05</td>
<td>9.1 ± 0.4</td>
<td>0.43</td>
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<tr>
<td>Enterodiol</td>
<td>0.21 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Enterolactone</td>
<td>42.4 ± 3.4</td>
<td>19.8 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total phytoestrogen</strong></td>
<td>101 ± 3.3</td>
<td>429 ± 11.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup> Probability for no difference between the two milk types based on an unpaired t-test

<sup>2</sup> Milk samples from two different days of the study period from GD 11 until birth were obtained and analyzed.
Table 2. Feed and liquid intake in dams during pregnancy (n=7-9 per group) exposed to low or high isoflavone milk (LIM or HIM, respectively) or tap water (control) from GD 11 until they gave birth. Birth weight of pups and female-male ratio in the litters. Values are means ± SEM

<table>
<thead>
<tr>
<th>Maternal treatment</th>
<th>LIM</th>
<th>HIM</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake, g/day</td>
<td>15.2 ± 0.3a</td>
<td>16.9 ± 0.8a</td>
<td>19.2 ± 0.8b</td>
<td>&lt;0.001</td>
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<td>Liquid intake, g/day</td>
<td>48.4 ± 3.0a</td>
<td>52.4 ± 2.0a</td>
<td>30.2 ± 2.3b</td>
<td>&lt;0.001</td>
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<td>Energy intake, kcal/day</td>
<td>75.9 ±1.8</td>
<td>84.1 ± 3.3</td>
<td>74.9 ± 3.5</td>
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<tr>
<td>Daily phytoestrogen exposure (μg/kg body weight)</td>
<td>16.9 ± 1.1</td>
<td>75.1 ± 2.2</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female birth weight, g/pup</td>
<td>7.4 ± 0.3</td>
<td>6.8 ± 0.2</td>
<td>7.0 ± 0.1</td>
<td>0.14</td>
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<tr>
<td>Male birth weight, g/pup</td>
<td>7.9 ± 0.4</td>
<td>7.1 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>0.09</td>
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<tr>
<td>Female/male ratio</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Different superscripts indicate statistical difference at the p<0.05 level
Table 3. Serum 17β-estradiol (E2), IGF-1 and isoflavones in dams at GD 19 (n=5-9 per group) exposed to low or high isoflavone milk (LIM or HIM, respectively) or tap water (control) from GD 11 until they gave birth. Means ± SEM.

<table>
<thead>
<tr>
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<th>Maternal treatment</th>
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<th>p-value</th>
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<td></td>
<td>LIM</td>
<td>HIM</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>482 ± 10.2</td>
<td>419 ± 17.8*</td>
<td>468 ± 26.1</td>
<td>0.19</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>904 ± 32.7</td>
<td>782 ± 37.4*</td>
<td>824 ± 52.1</td>
<td>0.15</td>
</tr>
<tr>
<td>Equol (μM)(^1)</td>
<td>Below detection</td>
<td>Below detection</td>
<td>Below detection</td>
<td></td>
</tr>
<tr>
<td>Genistein (μM)(^1)</td>
<td>0.23 ± 0.07</td>
<td>0.23 ± 0.08</td>
<td>0.29 ± 0.03</td>
<td>0.76</td>
</tr>
<tr>
<td>Daidzein (μM)(^2)</td>
<td>0.33 ± 0.10</td>
<td>0.26 ± 0.10</td>
<td>0.37 ± 0.02</td>
<td>0.63</td>
</tr>
</tbody>
</table>

\(^1\) LOD; limit of detection: 0.05 μM and LOQ; limit of quantification: 0.15 μM. Equol could be detected in 4 out of 27 samples and therefore considered below detection. Genistein could be detected in 22 out of 27 samples and a surrogate value 0.05 μM was inserted for samples below the LOD

\(^2\) LOD; 0.03 μM and LOQ; 0.08 μM. Daidzein could be detected in 24 out of 27 samples and a surrogate value 0.03 μM was inserted for samples below the LOD

* p<0.05, compared with the LIM group