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TITLE: In Utero Exposure to Cadmium, Mammary Gland Development, and Breast Cancer Risk

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**In Utero Exposure to Cadmium, Mammary Gland Development, and Breast Cancer Risk**

Exposures to estrogen-mimickers correlate strongly with biomarkers of breast cancer risk in epidemiological and animal studies. The heavy metal cadmium activates ERα and the androgen receptor. Food sources of Cd include wheat, soy, rice, sunflower and flax seeds. Because the half-life of cadmium in the mammalian body is >20 years its endocrine-disrupting effects are long-lasting. We hypothesized that in utero exposure to low doses of dietary cadmium, would be associated with post-natal changes in puberty on-set, mammary development and tumour incidence. To test these hypotheses we exposed pregnant rats to a diet consisting of low cadmium levels throughout pregnancy. After parturition, all groups were switched to standard rodent chow. We chose two time-points to examine mammary development; post-natal day 28 (pre-pubertal) and post-natal day 50 (pubertal).
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INTRODUCTION

Exposures to estrogens or estrogen-mimics correlate strongly with indicators of breast cancer risk in epidemiological and animal studies. The heavy metal cadmium binds to and activates estrogen receptor alpha (ERα) and to a lesser degree the androgen receptor (AR) (Stoica, Katzenellenbogen et al. 2000; Martin, Voeller et al. 2002). Previous studies demonstrate that cadmium levels present in human environments mimic the effects of estrogens in the mammary glands and uterus of adult rats (Johnson, Kenney et al. 2003). Humans are exposed to cadmium through several food sources. Wheat, soy, rice, sunflower and flax seeds contain detectable levels of cadmium (Chaudri, Allain et al. 2001; Khan, Penttinen et al. 2007). These food products accumulate cadmium from pesticide residues, contaminated ground water and cadmium-based fertilizers (Knight, Chaudri et al. 1998; Chaudri, McGrath et al. 2007). In rice, for example, cadmium levels may be as high as 600 ug/kg of food material (Reeves and Chaney 2004) and sugar cane also absorbs cadmium so easily that it has been suggested as a phyto-remediation source to remove cadmium from soil (Sereno, Almeida et al. 2007). Regular consumption of cadmium-containing foods could result in exposure levels above the world health organization’s weekly tolerable intake of 7 ug/kg of body weight. Because the half-life of cadmium in the mammalian body is over 20 years, its endocrine-disrupting effects may be amplified by repeated life-time exposures.

We hypothesized that in utero exposure to low doses of dietary cadmium, would alter post-natal puberty on-set, body weight gain and biomarkers of breast cancer risk. To test this hypothesis we exposed pregnant rat dams to a diet consisting of 39% of energy from fat and low to moderate cadmium levels (75 or 150 ug/kg feed, both resulting in levels below World Health Organization tolerable weekly intake) throughout pregnancy. The energy content of the rat maternal diet during pregnancy in our study was within the range of typical fat content in the diets of pregnant American women (Swensen, Harnack et al. 2001; Siega-Riz, Bodnar et al. 2002), although the current recommendation is for pregnant women to consume 30% of daily energy from fat. Our control dietary exposures consisted of a cadmium-free diet plus estradiol or vehicle injections from days 14 through 20 of gestation. After parturition, all groups were switched to AIN93-G diet. Our goal in this study was to determine whether changes in post-natal body development (puberty-onset, post-natal body weight gain), mammary development and ultimately tumor incidence were linked to \textit{in utero} exposure to dietary cadmium.

BODY

1. Determine whether in utero exposure to cadmium alters the proliferation and differentiation of the epithelial cells of the mammary gland.

   a. Epithelial cell expression of TGF Beta-3 (a moderator of proliferation, differentiation and apoptosis) during normal mammary gland development (Figure 1).

   Transforming growth Factor Beta-3 (TGF Beta-3) is an estrogen-sensitive cytokine and growth factor. It is hypothesized that TGF Beta-3 is a protective factor which is induced by mammary gland differentiation which has been induced by completion of pregnancy (D’Cruz, Moody et al. 2002). To determine the normal patterns of expression of TGF Beta-3 during rat mammary development, mammary tissue was collected, along a developmental time continuum beginning with neonate through post-natal day 50 (post-pubertal) and times of mammary gland cellular differentiation (pregnancy and lactation) and apoptosis (involution upon cessation of weaning). Of interest we found that TGF Beta-3 epithelial cell expression is highest on PND 10, and 21, which is also a time in which terminal end bud numbers are highest in rat. Further investigation of whole-mounted carmine stained mammary gland from untreated rats at PND 11, 12 and 13 indicate that at this time end buds appear to undergo dilation. These findings are interesting in light of the fact that terminal end buds are considered malignant target sites for breast cancer initiation. In our future studies we intend
to examine the expression patterns of TGF Beta-3 in offspring exposed and unexposed to cadmium or estradiol.

b. Epithelial cell expression of estrogen receptor alpha (ER-α) in mammary of offspring exposed in utero to low doses of dietary cadmium, or control diet plus vehicle or estrogen. (Figures 2 and 3).

ER-α expression was increased within the mammary epithelial cells of offspring exposed to lower and moderate dose of cadmium and estrogen in utero (p<0.001). It remains to be determined whether this is linked to altered TGF Beta-3 expression.

c. Changes in mammary morphology due to exposure to low doses of dietary cadmium, vehicle or estradiol. Time-points examined are post-natal (PND) day 28 (pre-pubertal), and PND 50 (post-pubertal) (Figure 4 and 5).

Terminal end bud number, ductal elongation and ductal outgrowth were significantly altered in moderate and low dose cadmium groups compared to either estradiol or vehicle exposed groups at PND 28 and 50. At PND 28 terminal end bud number was significantly increased in the moderate dose cadmium and estrogen in utero exposed groups (p<0.05). At PND 50 ductal elongation, terminal end bud number and ductal outgrowth were significantly reduced in the lower dose cadmium exposed group compared to control (p<0.05).

d. Acceleration of puberty onset, pre-pubertal body weight gain (Figure 6). Puberty on-set and pre-pubertal body weight gain were increased in the moderate dose cadmium group compared to all other treatment groups.

e. Estrogen and testosterone levels at pre-pubertal day 28, (Figure 7) and changes in uterine wet weight per 100 grams of offspring body weight and uterine epithelial thickness at PND 28 (Figure 8).

Testosterone and estrogen levels were significantly increased (p<0.057 and 0.015) in the moderate dose cadmium exposed offspring at pre-pubertal day 28. Testosterone is produced in the ovaries and is aromatized both in ovarian tissues and in peripheral body tissues to make estrogens. Both testosterone and estrogen levels are known to be increased prior to puberty, and because puberty on-set was accelerated in the higher dose cadmium group compared to all other groups, this result may have been due to accelerated puberty on-set. Another possibility is that this effect was due to a global disruption of the estrogen/testosterone endocrine system. To test this, blood samples from PND 50 offspring exposed in utero to cadmium are under analysis currently.

Uterine wet weights at PND 28 were altered in offspring exposed in utero to low doses of dietary cadmium, suggesting that pre-natal exposure to cadmium had long-lasting effects. The uterine wet weight in mg per 100 grams of body weight was decreased in the lower dose cadmium exposed offspring compared to all other groups, (control 75.163±5.7 SEM; lower dose cadmium 65.2±3.0; moderate dose cadmium 79.9±4.8; and estrogen groups 79.9±3.9) but the difference did not reach statistical significance when compared by One-Way ANOVA. This could be due to the increased fat content of the in utero diet (39% of energy from fat). However in spite of the higher fat diet in utero, when the lower dose cadmium group was compared to the moderate dose cadmium group the reduction in uterine wet weight per 100 grams of body weight was significant (p<0.043). In a study by K. Yamasaki
et. al. a uterotrophic assay was used to test the effects of low doses of testosterone enanthate upon uterine wet-weight in female PND 21 (pre-pubertal) Crj:CD Sprague-Dawley Rats. The results indicated that low doses of testosterone enanthate (2 mg/kg of body weight per day for 3 days) significantly reduced uterine wet weight (control group 61.3±4.2 and testosterone group 50.5±6.3). This effect was biphasic and at higher doses of testosterone enanthate (40 mg/kg of body weight for 3 days) significantly increased uterine wet weight (147.3±10) and noted. This was even more pronounced when rats were treated with estrogen in addition to testosterone treatment (Yamasaki, Takeyoshi et al. 2003). Our study found that in utero exposure to cadmium at 75 ug/kg of feed or 150 ug/kg of feed had opposing effects upon uterine wet weight. This may represent a biphasic effect caused by in utero exposure to cadmium. It is interesting to note that this effect was found although the cadmium containing diet was removed at birth. Future studies could repeat this work using a lower fat diet to determine whether in utero exposure to low doses of cadmium can induce significantly altered uterine wet weights. In our study we will analyze both uterine endometrial and myometrial thickness as a further indication of hormonally influenced changes in uterus of offspring exposed to low doses of dietary cadmium.

2. Determine whether in utero exposure to cadmium alters the arcuate nucleus, the center of hypothalamic control of both puberty on-set and appetite regulation, and the mechanism by which this occurs.
   
   a. We ask for permission to change the statement of work for this task based upon current results. A separate letter outlining a proposed new statement of work is forthcoming.

3. Data analysis, writing and submission of manuscripts.
   
   a. An initial meeting for review of data with a biostatistician has been accomplished. Most data has been examined for normality and equal variance and where necessary transformed to pass these tests. A follow-up meeting with biostatistician will be arranged prior to manuscript submissions.

KEY RESEARCH ACCOMPLISHMENTS

- In this study we determined that in utero exposure to cadmium alters pre-pubertal body weight development, timing of puberty on-set and mammary gland development.

- Some of these changes are significant and represent altered biomarkers of breast cancer risk (such as changed terminal end bud number).

- ER-α content in mammary tissue is increased in offspring exposed to low doses of dietary cadmium in utero.

- TGF Beta-3, an estrogen sensitive growth factor, is expressed at high levels in pre-pubertal mammary epithelial cells of unexposed offspring. Other times of high expression include involution.

- Uterine weight in mg per 100 grams of body weight was lower in the low dose cadmium exposed group compared to all other groups.
Testosterone levels and estrogen levels were increased in offspring exposed to moderate doses of cadmium in utero at PND 28. It remains to be determined whether this is a long term effect or due to accelerated puberty on-set.

**REPORTABLE OUTCOMES**

Post Presentations:

2008  
*In utero exposure to cadmium and mammary gland cancer risk in rats.* Jennifer D. Davis, Galam Khan, Mary Beth Martin, and Leena Hilakivi-Clarke. DOD Era of Hope Meeting, Baltimore, MD.

2008  
*Transforming Growth Factor Beta-3 (TGF Beta-3) expression, during normal mammary development and upon exposure to high levels of estrogen in utero in female Sprague Dawley Rat.* Jennifer D. Davis, Leena Hilakivi-Clarke. Mammary Gland Biology Gordon Conference, Il Ciocco, Italy

**CONCLUSION**

The mammary gland morphometry in this study indicated that some endpoints were altered depending on dietary cadmium dosage. Other studies in our laboratory have demonstrated that later breast cancer risk is increased when rats are exposed to cadmium-containing flaxseed either in utero or neonatally. In the present study we demonstrate that puberty on-set, post-natal body weight gain, and biomarkers of breast cancer risk are changed in opposing manners depending upon dietary cadmium dose.

In the moderate-dose cadmium exposed group, testosterone levels were higher pre-pubertally. Cadmium binds to the androgen receptor, but when compared to the natural ligand (testosterone), cadmium is only a weak competitor. Nevertheless it is possible that moderate doses of cadmium accelerate puberty on-set by mimicking testosterone. The dissociation of cadmium for the estrogen receptor ER-α is lower than that of the androgen receptor and so preferential binding of cadmium is almost 3-fold higher for the estrogen receptor as compared to the androgen receptor. A dual stimulation of ER-α and androgen receptors could contribute to the opposing effects of low dietary cadmium versus moderate dietary cadmium dosage. Further work in this area could examine the synergistic effects of androgen and estrogen stimulation *in vivo* on the mammary gland. These effects are not well defined in relation to in utero exposures and breast cancer risk. In our study we found that lower doses of cadmium were associated with a decrease in final tumor incidence (56%) and moderate doses of cadmium were associated with an elevated tumor incidence (80%) compared to control (73%).

**REFERENCES**


Figure 1. TGF Beta-3 is an estrogen-sensitive growth factor which influences epithelial cell proliferation, differentiation and apoptosis. In our study, the number of TGF Beta-3 positive mammary epithelial cells increased (a) prior to puberty when terminal end buds are beginning to dilate (b). TGF Beta-3 expression intensity varied in myoepithelial and luminal epithelial cells.
Figure 2. Post-natal day 28 qualitative changes in epithelial expression of ER-α. Receptor expression was significantly increased in high dose and estrogen treated groups compared to the lower-dose cadmium and vehicle exposed groups.
Figure 3. At post-natal day 28 ER-α levels were significantly increased in offspring exposed to estrogen or the higher dose cadmium group compared to vehicle-exposed group. This was most significant in ducts and lobules.
Figure 4. Post-natal day 28 terminal end bud number was significantly increased in both the moderate-dose cadmium and estrogen in utero-exposed groups compared to the vehicle exposed group (p<0.05). Qualitative changes in epithelial density and branching were also apparent.
Figure 5. Post-natal day 50 mammary morphometric analysis. Terminal end bud number was increased in both the moderate-dose cadmium and estrogen in utero-exposed groups. Ductal outgrowth and ductal elongation were also increased in the moderate dose versus lower dose cadmium groups.
Figure 6: Puberty on-set was significantly accelerated in the higher-dose in utero cadmium-exposed group (150 ug Cd/kg feed), Log rank 8.4, p<0.004 compared to all other groups. This was associated with accelerated body weight again post-natally in the higher dose group, (p<0.04).
Figure 7: Pre-pubertal estrogen and testosterone levels were increased in the higher-dose in utero cadmium-exposed group. This correlated to accelerated puberty on-set.
Figure 8: a) The uterine wet weight at PND 28 did not display statistically significant changes, but (b) there were qualitative changes in uterine epithelial thickness. Higher cadmium and estrogen in utero-exposures increased the uterine thickness (qualitative assessment).
The mammary gland morphometry in this study indicated that some endpoints were influenced by exposure to high levels of estrogen (or estrogen mimickers) altering mammary development. In utero exposure to cadmium led to lower doses of dietary cadmium altered body weight, mammary development, and ultimately mammary cancer risk. To assess the impact of in utero exposure to cadmium on mammary development and breast cancer risk, we exposed pregnant rat dams to a diet consisting of 39% of energy from fat and very low cadmium doses (75 or 250 µg/kg feed), throughout pregnancy. After parturition, all rats were switched to AIN-93-G chow and the offspring were cross-fostered. At pre-pubertal day 28 and post-pubertal day 50, animals were sacrificed (n = 15 per group). Mammary tissue was collected for whole slide staining and analysis.

**RESULTS CONTINUED**

Slides were stained using a rabbit monoclonal antibody to Ki67 (Abcam ab16667-500), utilizing DAKO Envision kit for Rabbit (DAKO, K4002). Imaging was performed using Olympus Scanning microscope IX50, images were analyzed. Positive and negative nuclei were counted using imageJ (NIH).

**Apoptosis Analysis:**

Slides were stained with a rabbit monoclonal antibody to TUNEL (AHCAB 1179-500), utilizing DAKO Envision kit for Rabbit (DAKO, K4002). Imaging was performed using Olympus Scanning microscope IX50, images were analyzed. Positive and negative nuclei were counted using imageJ (NIH).

**Estrous Cycle:**

Day 50 determination of estrus stage: NIH protocol at mammary.nih.gov

**REFERENCES**