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14. ABSTRACT Exposures to estrogen-mimickers correlate strongly with biomarkers of breast cancer risk in epidemiological and animal studies. The heavy metal cadmium activates ERa 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 (ER α) 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 (Serenio, 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 *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.

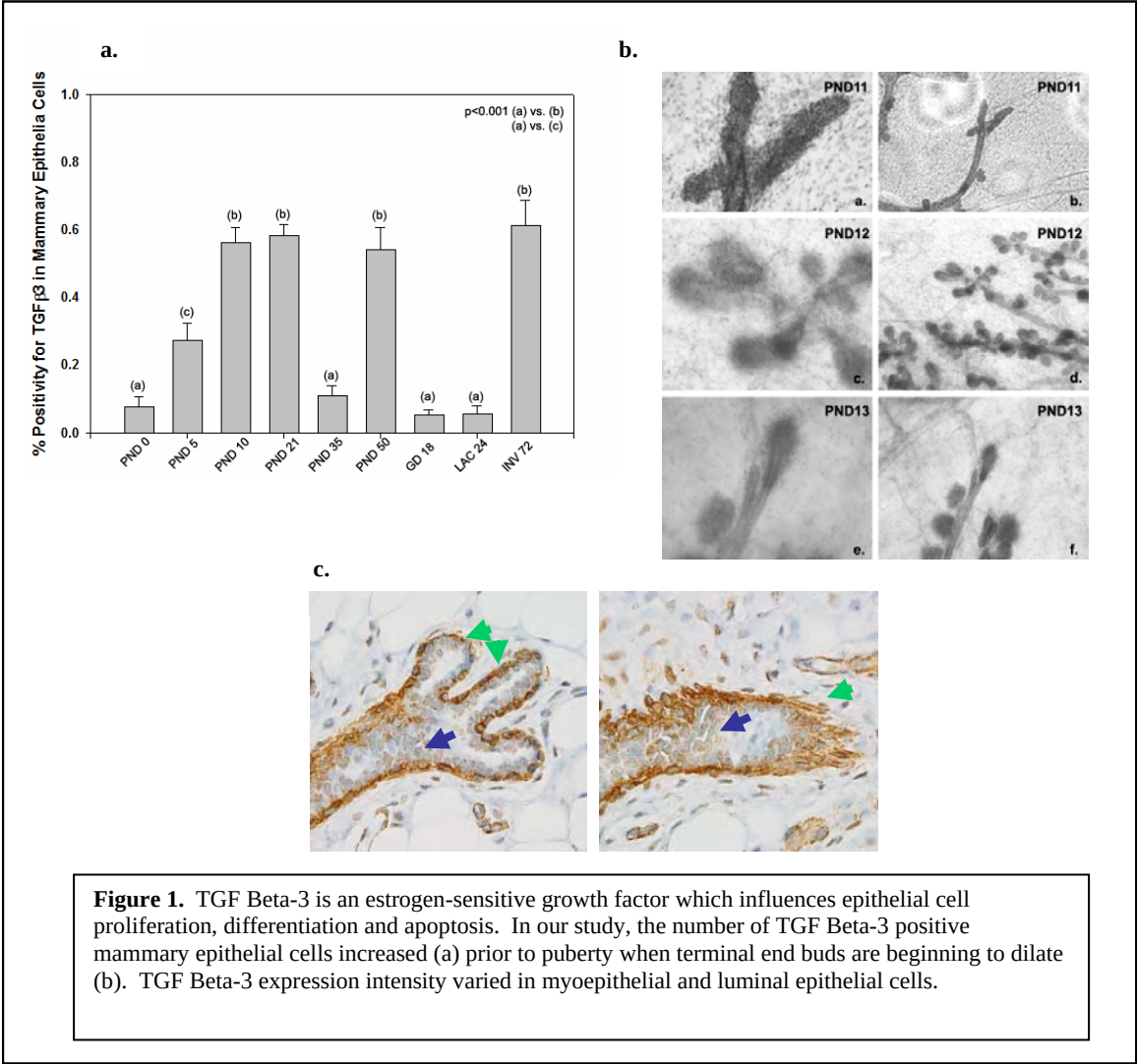
In the moderate-dose cadmium exposed group, testosterone levels were higher pre-pubertally. Cadmium binds to the androgen receptor, but when compared to the natural ligand (testosterone), cadmium is only a weak competitor. Nevertheless it is possible that moderate doses of cadmium accelerate puberty 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%).

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APPENDICES



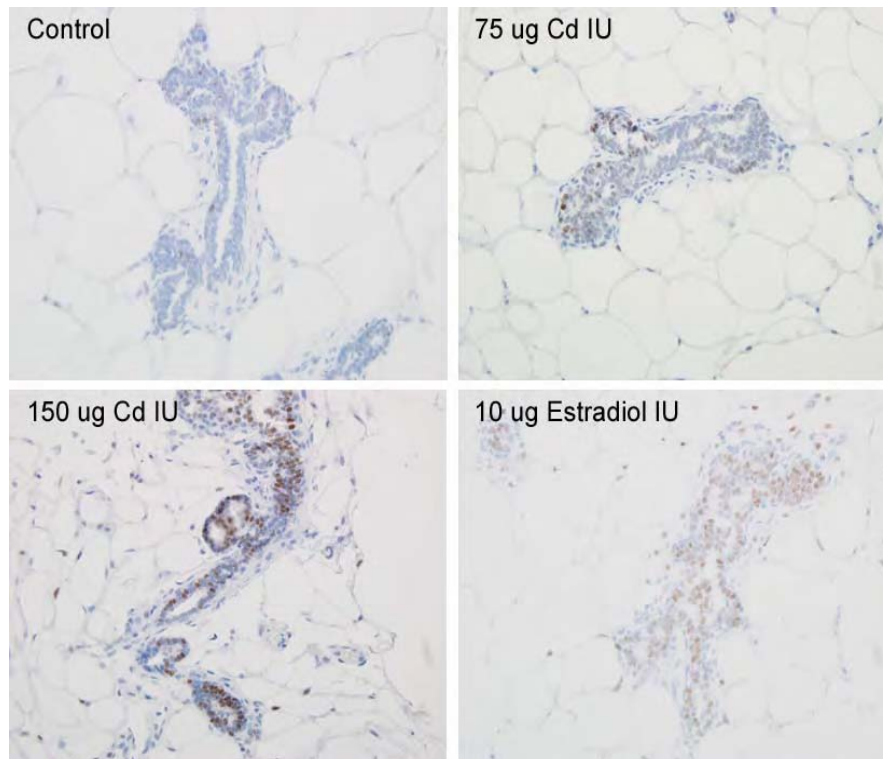


Figure 2. Post-natal day 28 qualitative changes in epithelial expression of ER- α . Receptor expression was significantly increased in high dose and estrogen treated groups compared to the lower-dose cadmium and vehicle exposed groups.

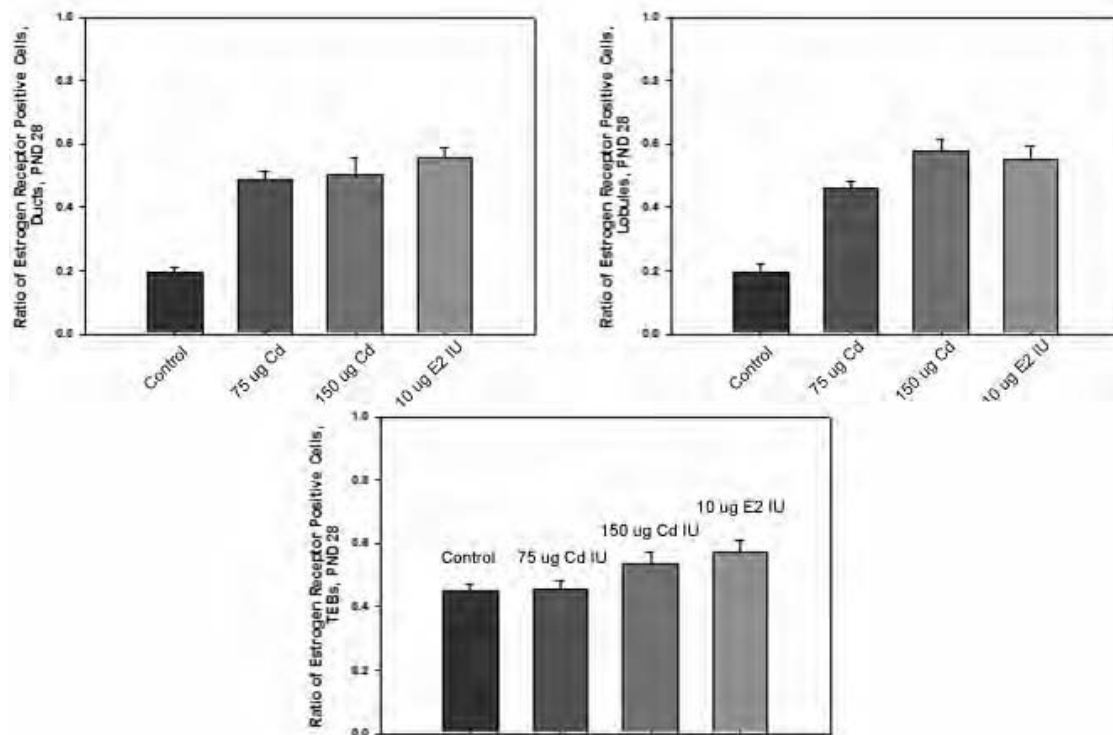


Figure 3. At post-natal day 28 ER- α levels were significantly increased in offspring exposed to estrogen or the higher dose cadmium group compared to vehicle-exposed group. This was most significant in ducts and lobules.

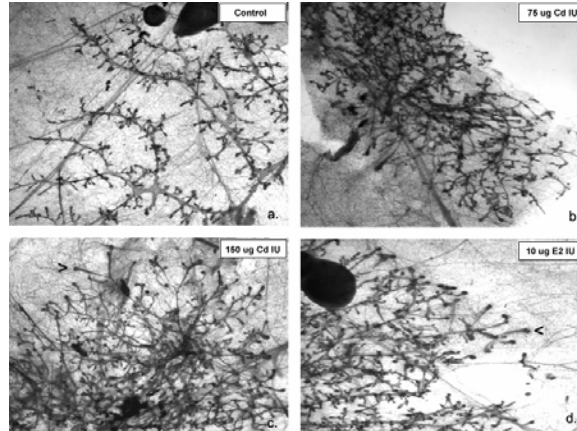
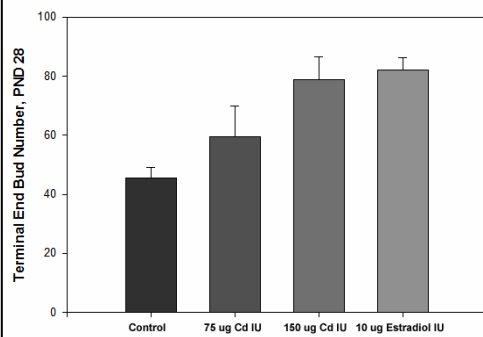


Figure 4. Post-natal day 28 terminal end bud number was significantly increased in both the moderate-dose cadmium and estrogen in utero-exposed groups compared to the vehicle exposed group ($p < 0.05$). Qualitative changes in epithelial density and branching were also apparent.

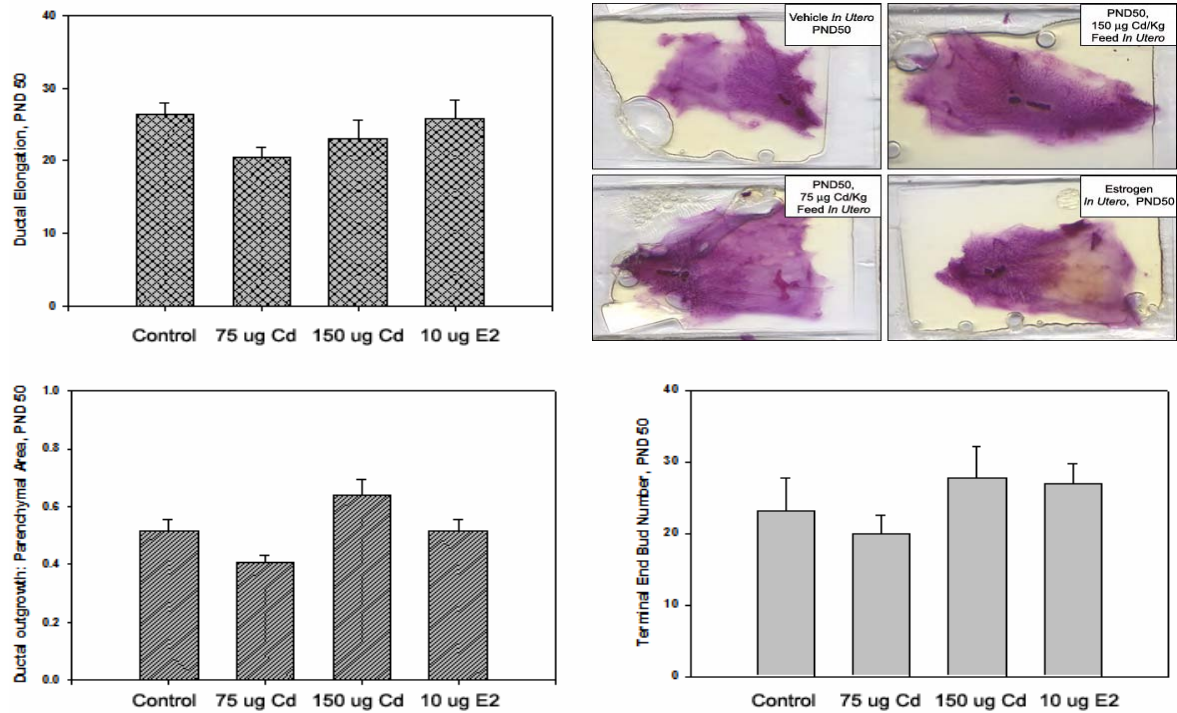


Figure 5. Post-natal day 50 mammary morphometric analysis. Terminal end bud number was increased in both the moderate-dose cadmium and estrogen in utero-exposed groups. Ductal outgrowth and ductal elongation were also increased in the moderate dose versus lower dose cadmium groups.

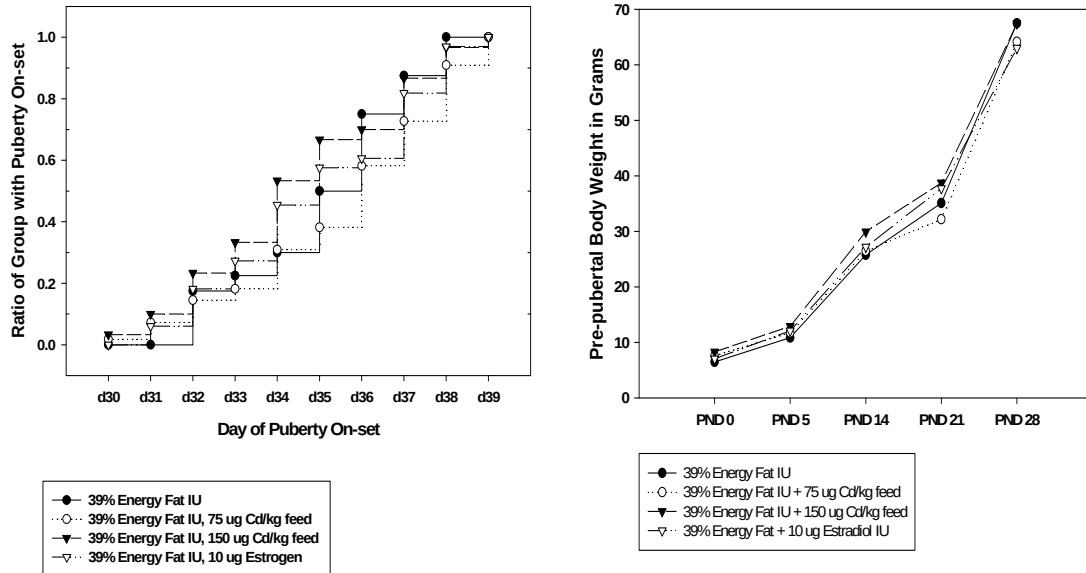


Figure 6: Puberty on-set was significantly accelerated in the higher-dose in utero cadmium-exposed group (150 ug Cd/kg feed), Log rank 8.4, $p < 0.004$ compared to all other groups. This was associated with accelerated body weight again post-natally in the higher dose group, ($p < 0.04$).

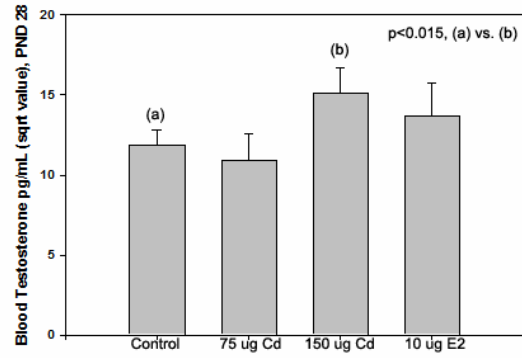
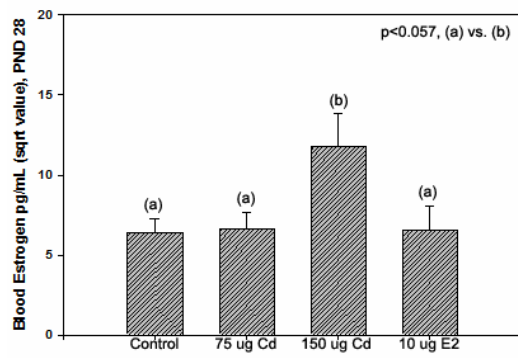


Figure 7: Pre-pubertal estrogen and testosterone levels were increased in the higher-dose *in utero* cadmium-exposed group. This correlated to accelerated puberty on-set.

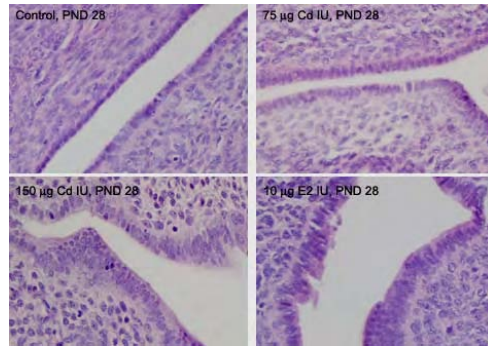
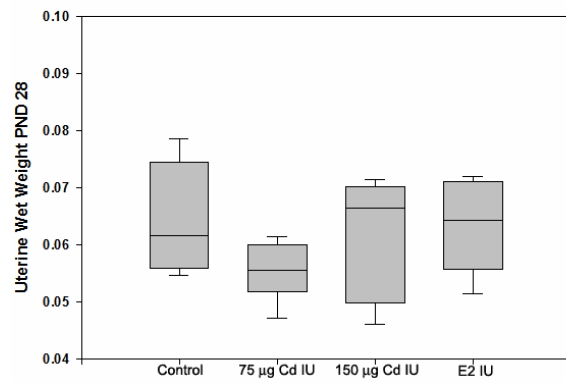
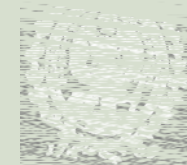


Figure 8: a) The uterine wet weight at PND 28 did not display statistically significant changes, but (b) there were qualitative changes in uterine epithelial thickness. Higher cadmium and estrogen in utero-exposures increased the uterine thickness (qualitative assessment).

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

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ABSTRACT

In utero exposure to high levels of estrogen (or estrogen mimickers) alters mammary development in rats and mice and increases their susceptibility to mammary cancer. Cadmium, a heavy metal with a half-life of over 20 years in the mammalian body, potentially binds to and activates the estrogen receptor. Previous studies have shown that in utero exposure to cadmium—at levels present in some human environments—accelerated puberty onset and altered mammary development in rats. In this study we sought to determine whether in utero exposure to low doses of dietary cadmium altered body weight, mammary development, and ultimately mammary cancer risk. To test whether in utero exposure to low doses of dietary cadmium have significant effects on mammary development and breast cancer risk, we exposed pregnant rat dams to a diet consisting of 39% fat (similar to a typical human diet), and very low cadmium doses (75 or 150 µg/kg feed), throughout pregnancy. Control groups received daily injections of vehicle (corn oil) or estrogen (10 µg dissolved in corn oil) from days 14 through 20 of pregnancy. After parturition, all rats were switched to AIN93-G laboratory chow and the offspring were cross-fostered. The effects on (i) birth weight, (ii) postnatal weight development, (iii) puberty onset, (iv) mammary development, and (v) DMBA-induced mammary tumorigenesis were investigated. The higher cadmium dose induced a long-lasting increase in postnatal body weight that was first detected on post-natal day (PND50) ($p < 0.04$) and accelerated puberty on-set as measured by vaginal opening ($p < 0.03$) compared to controls and lower-dose groups. Mammary development was also significantly altered by in utero exposure to cadmium. At PND50 offspring of lower-dose cadmium-exposed rats had a significantly decreased epithelial density ($p < 0.05$) compared to the estrogen-treated group. Differences in alometric growth were measured by comparing fat pad to mammary epithelial tree length. An average of 71% of the length of the fat pad was filled with the mammary epithelial tree in the higher-dose cadmium group compared to an average of 53% in the lower-dose group ($p < 0.005$), while the vehicle group had an average of 65% and the estrogen group 67%. Proliferation-to-apoptosis ratio—an indication of cellular turnover—was significantly increased in the higher-dose cadmium group ($p < 0.05$ compared to higher-dose and estrogen treated groups) at PND50. These differences in mammary development were strongly correlated to later mammary cancer incidence. Final mammary tumor incidence was lowest in the lower-dose cadmium group (56% of rats developed tumors) and highest in the higher-dose cadmium group (80% tumor incidence) ($p < 0.001$). 73% of the vehicle-treated rats developed mammary tumors. This study presents initial findings on the effects of in utero exposure to low doses of dietary cadmium on post-natal body weight, mammary development, and breast cancer risk. Follow-up studies based on this work will aid in breast cancer prevention efforts that include dietary guidelines for pregnant women.

INTRODUCTION

Exposures to estrogens or estrogen-mimics correlate strongly with indicators of increased breast cancer risk in epidemiological and animal studies. The heavy metal cadmium binds to and activates oestrogen receptor alpha (ER α) and to a lesser degree the androgen receptor (AR). Previous studies demonstrate that cadmium levels present in human environments mimic the effects of estrogens in the mammary glands and uterus of adult rats. Humans are exposed to cadmium through several food sources including wheat, soy, rice, sunflower and flax seeds. These food products accumulate cadmium from pesticide residues, contaminated ground water and cadmium-based fertilizers. 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 because of its long half-life in the mammalian body, in utero exposure to low doses of dietary cadmium, would be associated with post-natal changes in puberty on-set, mammary development and mammary tumour incidence. To test these hypotheses we exposed pregnant rat dams to a diet consisting of 39% of energy from fat and low cadmium levels (75 or 150 µg/kg feed) throughout pregnancy. Based upon typical food consumption in pregnant rats, this resulted in a weekly intake of either 2.6-5 µg/kg of maternal body weight of cadmium or 3.5-7 µg/kg of maternal body weight of cadmium. Both doses are below or at the WHO weekly tolerable intake standards for humans (7 µg cadmium/kg of body weight weekly). Control diets consisted of 39% of energy from fat and vehicle or estradiol injection daily between days 14 through 20 of gestation. After parturition, all groups were switched to AIN93-G diet. We chose two time-points to examine mammary development; post-natal day 28 (pre-pubertal) and post-natal day 50 (pubertal).

MATERIALS AND METHODS

Treatment groups: Pregnant Sprague-Dawley dams were exposed to corn oil vehicle, 75 µg or 150 µg/kg feed cadmium throughout pregnancy. For the estrogen treatment group, daily injections of 10 µg of estradiol in 0.05 mL volume of corn oil gestational days 14 through 20. These treatments were in the context of 39% energy from dietary fat, similar to that of a human diet. At birth all offspring were maintained on AIN93-G chow (16% energy from dietary fat). Measurement of developmental endpoints: Body weight and monitoring for vaginal opening (an indication of puberty on-set) were performed during development. Mammary tissue collection: At pre-pubertal day 28 and, post pubertal day 50, animals were sacrificed (n=5 per group). Mammary tissue was collected for whole mount (4th inguinal gland), immunohistochemistry (5th inguinal gland), protein and mRNA expression (2nd gland). Tissue collected for immunohistochemistry was fixed in 10% formalin, washed in PBS, dehydrated in 70% ethanol, and paraffin embedded. Slide sections were 4 µm thick. Collection of Uteri: Upon sacrifice offspring uteri were collected, weighed, graded by thickness and fixed in 10% formalin for later embedding, sectioning and hematoxylin/eosin staining. Proliferation analysis: 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 BX100, ImagePro software. Positive and negative nuclei were counted using ImageJ (NIH). Apoptosis Analysis: Chemicon Apoptag TUNEL Assay Kit. Carmine Stain of Whole Mount Mammary Gland: NIH protocol at mammary.nih.gov Day 50 determination of estrus stage: Estrus status was graded for each animal using morphological criteria of Schedin et al. Tumor multiplicity and latency: Per group, 19-24 rats were monitored for tumors for 20 weeks post DMBA challenge. Data Analysis: Pre-pubertal One-Way ANOVA Test and Post-natal Day 50, Two-Way ANOVA Test (taking into account estrus status) using SigmaStat.

RESULTS

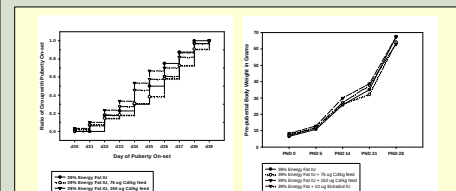


Figure 1: Puberty on-set was significantly accelerated in the higher-dose in utero cadmium-exposed group (150 µg Cd/kg feed). Log rank 8.4, $p < 0.004$ compared to all other groups. This was associated with accelerated body weight gain post-natally in the higher dose group, ($p < 0.04$).

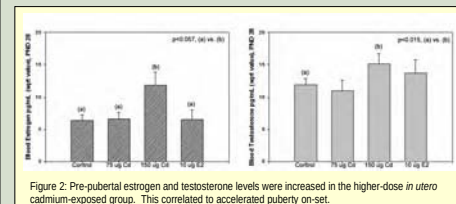


Figure 2: Pre-pubertal estrogen and testosterone levels were increased in the higher-dose in utero cadmium-exposed group. This correlated to accelerated puberty on-set.

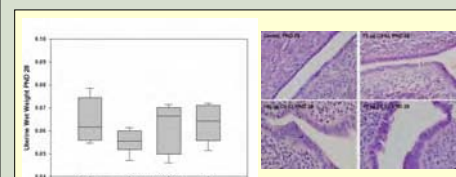


Figure 3: a) The uterine wet weight at PND 28 did not display statistically significant changes, but (b) there were qualitative changes in uterine epithelial thickness. Higher cadmium and estrogen in utero-exposures increased the uterine thickness (qualitative assessment).

RESULTS CONTINUED

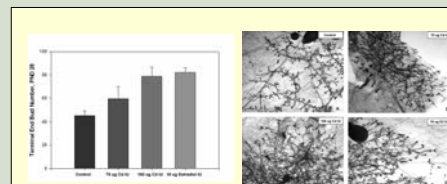


Figure 4: Post-natal day 28 terminal end bud number was significantly increased in both the higher-dose cadmium and estrogen in utero-exposed groups compared to the lower-dose cadmium exposed group ($p < 0.05$). Qualitative changes in epithelial density and branching were also apparent.

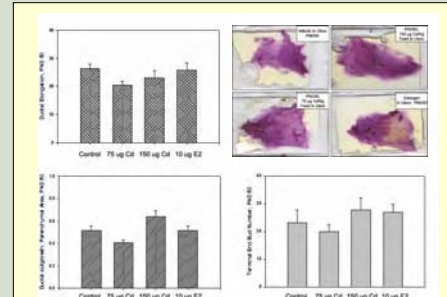


Figure 5: Post-natal day 50 mammary morphometric analysis. Terminal end bud number was increased in both the higher-dose cadmium and estrogen in utero-exposed groups. Ductal outgrowth and ductal elongation were also increased in the higher dose versus lower dose cadmium groups.

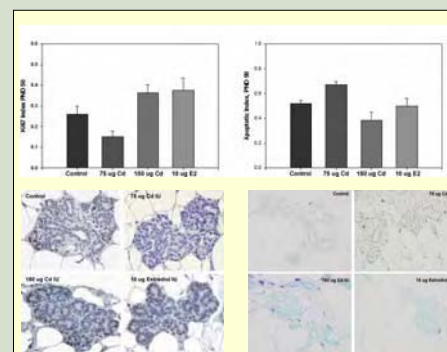


Figure 6: Post-natal day 50 proliferation and apoptosis. Proliferation as measured by Ki67 was decreased at PND50 in the lower-dose cadmium group. Apoptosis was also increased in the lower-dose cadmium group.

CONCLUSIONS

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 higher-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 higher 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 higher 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 higher doses of cadmium were associated with an elevated tumor incidence (80%) compared to control (73%).

SUMMARY

We found that the in utero effects of exposure to dietary cadmium were long-lasting and not directly comparable to estrogenic stimulation. Instead some of the effects were similar to those reported in rodents when given androgen receptor agonists. The higher-dose cadmium-exposed group appeared to have biomarkers (delayed puberty on-set and high number of terminal end buds) which are indicative of enhanced breast cancer risk and increased tumour incidence (80%). However, the lower dose cadmium-exposed group had a reduced tumour incidence (56%) and biomarkers which are associated with protective effects (reduced terminal end bud number, reduced epithelial area). Our results highlight the fact that endocrine disruptors may stimulate more than one type of steroid receptor, and that this may be dependent upon dose. Further studies on the combined effects of estrogenic and androgenic stimulation by endocrine disruptors are necessary and will increase our understanding of their long-term effects upon breast cancer risk.

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