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Drinking Water with Uranium below U.S. EPA Water Standard Causes Estrogen Receptor Dependent  
Responses in Female Mice

Stefanie Raymond-Whish<sup>1</sup>, Loretta P. Mayer<sup>1</sup>, Tamara O'Neal<sup>1</sup>, Alisyn Martinez<sup>1</sup>, Marilee A. Sellers<sup>1</sup>,  
Patricia J. Christian<sup>2</sup>, Samuel L. Marion<sup>2</sup>, Carlyle Begay<sup>2</sup>, Catherine R. Propper<sup>1</sup>, Patricia B. Hoyer<sup>2</sup>,  
Cheryl A. Dyer<sup>1</sup>

<sup>1</sup>Northern Arizona University, Department of Biological Sciences, Flagstaff, AZ 86011

<sup>2</sup>University of Arizona, College of Medicine, Department of Physiology, Tucson, AZ 85724

Correspondence to Cheryl A. Dyer, Ph.D., Northern Arizona University,  
Department of Biological Sciences, P.O. Box 5640, Building 21, Room 227, South Beaver Street,  
Flagstaff, AZ 86011  
Phone (928)523-6294, Fax (928)523-7741, e mail [Cheryl.Dyer@nau.edu](mailto:Cheryl.Dyer@nau.edu)

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Abbreviations:

DES	diethylstilbestrol
DU	depleted uranium
E <sub>2</sub>	17 $\beta$ -estradiol
EDC	endocrine disrupting chemical
ER $\alpha/\beta$	estrogen receptor alpha/beta
ICI 182,780	13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-7,8,9,11,12,13,14,15,16
U	uranium
UN	uranyl nitrate hexahydrate
U.S. EPA	United States Environmental Protection Agency
VO	vaginal opening

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Abstract

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## **Abstract**

**Background:** Uranium's deleterious impact on human health has been linked to its radioactive and heavy metal chemical properties. Decades of research has defined the causal relationship between uranium mining/milling and onset of kidney and respiratory diseases 25 years later.

**Objective:** Here we investigated the hypothesis that uranium, similar to other heavy metals such as cadmium, acts like estrogen.

**Methods:** In several experiments, intact, ovariectomized or pregnant mice were exposed to depleted uranium in drinking water ranging from 0.5 µg/L (0.001 µM) to 28 mg/L (120 µM).

**Results:** Mice that drank uranium-containing water exhibited estrogenic responses including selective reduction of primary follicles, increased uterine weight, greater uterine luminal epithelial cell height, accelerated vaginal opening and persistent presence of cornified vaginal cells. Coincident treatment with the antiestrogen ICI 182,780, blocked these responses to uranium or the synthetic estrogen, diethylstilbestrol. In addition, mouse dams that drank uranium-containing water delivered grossly normal pups, but they had significantly fewer primordial follicles than pups whose dams drank control tap water.

**Conclusions:** Decades of uranium mining/milling in the Colorado plateau in the Four Corners region of the American southwest make the uranium concentration and route of exposure used in these studies environmentally relevant. Our data supports the conclusion that uranium is an endocrine disrupting chemical and populations exposed to environmental uranium should be followed for increased risk of fertility problems and reproductive cancers.

## Introduction

Uranium (U) is the heaviest naturally occurring element. It is valued for its radioactive properties. Development of nuclear weapons in the 1940s fueled the U.S. government's desire to become independent of foreign sources of U (Ball 1993; Moure-Eraso 1999; Panikkar and Brugge 2007). The U "boom" in the southwest U.S. lasted from the early 1950s until the market collapsed in 1971 when the U.S. government ceased being the sole purchaser of U ore (Brugge and Goble 2002).

The majority of U mining/milling occurred in the Four Corners region of the U.S. where the Navajo Reservation is located. The Navajo Abandoned Mine Lands (AML) agency reclaims abandoned uranium mines (AUMs) under the authority and with funding from the Surface Mining Control and Reclamation Act of 1977 (Office of Surface Mining, 1977). Navajo AML agency estimates there are approximately 1300 AUMs throughout the 27,000 square miles of the Navajo Nation. About 50% of AUMs have been reclaimed (U.S. EPA 2004). Unremediated AUMs enabled U to disperse into air, soil, water and the food chain (Brugge and Goble 2002). A present day example of unregulated U mining/milling is the Atlas Corporation Moab Uranium Mill Tailing. Nearly 10,000 gallons of U-contaminated water seeps daily into the Colorado River daily (Oak Ridge National Laboratory, 1998) and the adjacent surface water concentration of uranium is greater than 5 mg/l (DOE 2005).

The largest American Indian reservation in the U.S. is the Navajo Nation that is divided into 110 political units called Chapters. Within 33 Chapters the U.S. EPA surveyed 226 water sources. Of these, 90 water sources were contaminated with U above the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM). The U levels found in contaminated water sources ranged from 33.3 – 1131 µg/L, the highest concentration being 38 times the safe drinking water level (U.S. EPA 2004). The surveyed water sources were stock tanks, wells and springs. Chapter officials identified the water sources as providing drinking water for residents without running water (U.S. EPA 2004). According to the 2000 U.S. census, over 175,000 people live on the Navajo Reservation. At least half of these residents haul water from the nearest water source for household use including drinking water,

cooking and clothes laundering, making it a certainty that many Navajo Nation residents are exposed to unsafe levels of U.

U's toxicity is due to its radioactive and chemical properties (Brugge et al. 2005, Taylor and Taylor 1997). U inhalation and/or ingestion leads to malignant and non-malignant respiratory diseases, stomach and kidney cancer, kidney failure and leukemia (Brugge et al. 2005; Roscoe et al. 1995). U's effect on the reproductive system was examined in early studies with rats fed high doses of 2% uranyl nitrate (UN). U exposure caused significant dam weight loss, fewer litters and fewer pups per litter (Maynard and Hodge 1949). When female rats were returned to chow diet without UN they regained the lost body weight. But a reduction in the number of litters and pups/litter persisted, suggesting the ovaries had been permanently damaged (Maynard and Hodge 1949). Female mice treated with uranyl acetate by gavage through gestation, parturition and nursing had an increased number of dead young per litter (Paternain et al. 1989). It is likely the high doses of U in these studies led to reproductive toxicity (Domingo 2001; Hindin et al. 2005).

Heavy metals exhibit estrogenic properties (Dyer 2007). Several heavy metals stimulate proliferation of MCF-7 human breast cancer cells (Brama et al. 2007; Choe et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). Cadmium interacts with estrogen receptor (ER)  $\alpha$  (Brama et al. 2007; Martin et al. 2003) and binds to the ligand binding domain of ER $\alpha$  in cultured cells (Stoica et al. 2000). Cadmium stimulates estrogenic responses *in vivo* (Alonso-Gonzalez et al. 2007; Johnson et al. 2003). Ovariectomized rats injected with cadmium had increased uterine weight, accelerated mammary gland growth/development, and accelerated vaginal opening (Johnson et al. 2003). Cadmium-induced estrogen-like responses were prevented by the antiestrogen ICI 182,780. Cadmium inhibits transcriptional activity of estradiol-activated rainbow trout estrogen receptor in recombinant yeast (Guével et al. 2000). Cadmium treatment stimulates breast cancer cell proliferation by activating ER $\alpha$ -dependent Akt, Erk1/2 and PDGFR $\alpha$  (Brama et al. 2007). Although these studies demonstrate cadmium's estrogen activity, it should be noted that Silva et al. (2006) reported cadmium lacks estrogenic activity in the yeast estrogen screen assay, MCF-7 cell

proliferation or E-screen assay, and also failed to induce Src, Erk1 and Erk2 phosphorylation. Here we tested if depleted U (DU) added to drinking water caused responses in the female mouse reproductive tract like those caused by the potent synthetic estrogen diethylstilbestrol (DES).

## Materials and methods

### Animals

U exposure in intact female mice was done with 28 day old, immature B6C3F<sub>1</sub> mice (Harlan, Indianapolis, IA). *In utero* U exposure experiments were done with 48 day old male and female B6C3F<sub>1</sub> mice (Harlan, Indianapolis, IA). Ovariectomized 28 day old C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used for the prepubertal U and DES exposure experiments. Mice were housed with a 12:12 hour light/dark cycle and received water and food *ad libitum*. Control tap water was tested for U using kinetic phosphorescence analysis (Hedaya et al. 1997) and it was below the limit of detection (< 2 µg/L or < 8 pM). All protocols were approved by the University of Arizona or Northern Arizona University Institutional Animal Care and Use Committees. All mice were treated humanely with regard for alleviation of suffering in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### Treatments

Study 1: Impact of U exposure on ovarian follicle populations.

Experiment 1.1: Impact of U exposure on immature mouse ovarian follicle populations.

Uranyl nitrate hexahydrate (UN) (Sigma Chemical Co., St. Louis, MO) was used for dosing in drinking water and is DU. In our first experiment, mice were exposed to UN in their drinking water at mg/L based on a published study using rats (Gilman et al. 1998). Immature 28 day old B6C3F<sub>1</sub> mice drank water containing UN at 0.5, 2.5, 12.5 and 60.0 mg/L (1, 5, 25, 120 µM respectively), n = 9-10 mice per group. After 30 days, ovaries were analyzed for changes in follicle populations.

Experiment 1.2: Impact of gestational and *in utero* U exposure on dam and female pup ovarian development.

For *in utero* exposure mice drank water containing UN at 0.5, 2.5, 12.5, or 60 µg/L (0.001, 0.005, 0.025 or 0.120 µM U respectively) for 30 days prior to breeding. U dose was reduced a thousand fold to µg/L to correspond to environmentally relevant concentrations. Mice were paired for breeding and males removed when females had vaginal plugs. Females continued to drink U-containing water at

the above doses through gestation. On the day of birth, dams and female pups were euthanized and the ovaries collected for histology. The number of mice used in the experiment were: 5 control/5 each UN dose group, female pups 7 control/7-9 each UN dose group.

Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen.

Experiment 2.1: Impact of U exposure on the uterus of ovariectomized mice

At this juncture, C57Bl/6J mice were used because of strain sensitivity to estrogen in the uterotrophic assay (Ashby et al. 2003). Also anticipated was use of genetically manipulated mice e.g. ER $\alpha$  knockout mice on this genetic background (Lubahn et al. 1993). C57Bl/6J mice were ovariectomized at 28 days of age to remove the endogenous source of estrogen prior to vaginal opening. Seven days post surgery, ovariectomized and intact mice drank tap water or water containing 0.190  $\mu$ M DES or 0.06, 0.12, 1.20 or 12.00  $\mu$ M U for 30 days, n = 5-6 mice per treatment group.

Experiment 2.2: Determination of other estrogen like effects of UN and dependence on estrogen receptor activation

Mice ovariectomized at 28 days of age were exposed to drinking water containing U or DES, at the aforementioned concentrations, beginning at 50 days of age for 10 days. Some mice concurrently received daily i.p. injections of either sesame oil vehicle or 500  $\mu$ g/kg ICI 182,780 (Tocris Coolson Ltd, Avonmouth, UK), n = 6-7 mice per group. Mice were examined daily at the same time for vaginal opening and cytology.

Tissue collection and histology

Following exposure to DES or U, mice were euthanized and organs collected for necropsy. Uteri were removed by dissecting inferior to the Fallopian tubes and superior to the vagina. Wet weights of ovary, uterus, kidney, liver and spleen, were normalized to total body weight. Uterine tissues from 30 day U-exposed mice were fixed in Bouin's solution, embedded in paraffin, serially sectioned every 9  $\mu$ m and every 10<sup>th</sup> section was mounted on slides. Tissue sections were deparaffinized in Citrasolve

(Sigma Chemical Co., St. Louis, MO) and dehydrated in a series of ethanol baths. A Zeiss 435 VP scanning electron microscope and software was used to measure the uterine luminal epithelial cell height. Forty measurements were randomly collected from each individual uterus.

Ovaries were trimmed of adhering tissue and fat then fixed in Bouin's solution. Ovaries were transferred to 70% ethanol, embedded in paraffin, serially sectioned (5  $\mu\text{m}$ ), mounted and stained with hematoxylin and eosin. Nuclei of oogonia and primordial, small primary, large primary, secondary or growing, and healthy antral and atretic follicles were identified and counted in adult ovary every 20<sup>th</sup> section, and in pup ovary every 12<sup>th</sup> section (Mayer et al. 2004).

### Statistical analyses

Oogonia and follicle numbers were determined in ovaries from individual mice and averaged. The means in control versus exposed mice were analyzed for significant differences by one-way analysis of variance (ANOVA) with significance set at  $p < 0.05$ . Tukey-Kramer post-hoc tests were used where appropriate. Organ weights for 10 and 30 day exposed mice were determined for each individual within each experiment and averaged for each exposure group. Uterine luminal epithelial cell height measurements from 30 day exposed mice were collected from individual mice and averaged for each exposure group. Additionally, in the 10 day exposure experiment day, vaginal opening was determined for each individual and averaged for exposure group. The means in control versus exposed mice for organ weights, uterine epithelial cell height and vaginal opening were analyzed for significant differences by one-way ANOVA with significances set at  $p < 0.05$ . Dunnet's post-hoc test was used where appropriate. The means of uterine weights in control, ICI 182,780, U or DES exposed mice were analyzed by two-way ANOVA with significance set at  $p < 0.05$ . Persistent presence of cornified vaginal cells was determined for each individual mouse in the 10 day exposure experiment. Presence and absence of cornified cells was analyzed by Chi-squared test with significance set at  $p < 0.05$ . Statistical significance of persistent presence of cornified cells was analyzed by Fisher's exact test with significance set at  $p < 0.05$ .

## Results

Study 1: Impact of U exposure on ovarian follicle populations.

Experiment 1.1: Impact of U exposure on immature mouse ovarian follicle populations.

### Uranium targets early stage ovarian follicles:

As shown in Table 1, the only effected follicle populations were significantly fewer large primary follicles at 0.5 and 2.5 mg/L UN. There were significantly more secondary or growing follicles at 12.5 mg/L UN. There was no significant increase in the number of atretic follicles or decrease in healthy follicles. Because UN exposure caused a selective change in ovarian follicle populations, and, there were more growing follicles at 12.5 mg/L UN, the changes could not due to heavy metal toxicity.

### Uranium does not lead to overt organ toxicity:

There were no gross anomalies seen in any major organs. Body weight did not significantly change with UN exposure at any concentration. As shown in Table 2, kidney weight was significantly reduced at UN doses of 2.5 and 60.0 mg/L, but was not surprising given the nephrotoxicity of U (Brugge et al. 2005; Taylor and Taylor 1997). These data support the conclusion that there was no systemic UN-mediated toxicity.

There was an interesting, but not statistically significant, trend of increased uterine weight at 12.5 and 60.0 mg/L UN (Table 2). Estrus stage in the mice was not determined at sacrifice, thus uterine weights could not grouped relative to stage.

Experiment 1.2: Impact of gestational and *in utero* U exposure on dam and female pup ovarian development.

### *In utero* uranium exposure reduces pup ovary primordial follicles:

As shown in Figure 1A, compared to control mice, mice exposed to UN for 30 days before mating and through gestation had a significant reduction of small primary follicles at UN concentrations of 0.005, 0.025, and 0.120  $\mu\text{M}$ . All other follicle populations including primordial, secondary/growing, healthy and atretic were unchanged (data not shown). Neonatal mouse ovaries have only oogonia and primordial follicles. There was no difference in the number of pup ovary oogonia among control

and UN exposure groups (data not shown). Primordial follicle numbers were reduced in ovaries of pups whose dams consumed water with UN at 0.001 or 0.120  $\mu\text{M}$ , compared to primordial follicles in pup ovaries from dams drinking control tap water (Figure 1B).

Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen.

Experiment 2.1: Impact of U exposure on the uterus of ovariectomized mice

UN exposure induces estrogen-like changes in uterine morphology and histology:

Mice exposed to drinking water containing UN or DES had significantly increased uterine weight at 0.120  $\mu\text{M}$  U and 0.190  $\mu\text{M}$  DES, 3.6 and 3.8 times greater, respectively, compared to mice drinking control tap water (Figure 2A). Uterine weights were normalized to body weights, that were unchanged across treatment groups. Uterine weights were not increased in ovary intact age-matched mice that drank U-containing water (data not shown).

Experiment 2.2: Determination of other estrogen-like effects of UN and their mediation through estrogen receptor activation.

UN-mediated estrogen-like actions are blocked by concomitant exposure to an estrogen receptor antagonist:

To determine if U-mediated uterotrophic response was dependent on estrogen receptor activation, ovariectomized mice drinking UN-containing water were injected daily with the antiestrogen ICI 182,780. In a pilot experiment it was determined that 10 days of exposure to UN in drinking water caused a significant increase in uterine weight compared to mice drinking tap water (data not shown). Ten days of concomitant ICI 182,780 treatment blocked both UN and DES-mediated increases in uterine weights (Fig. 2B), U at 0.060  $\mu\text{M}$ : 1070  $\pm$  386 mg/kg total body weight compared to 220  $\pm$  28.1 mg/kg total body weight with ICI 182,780 and DES at 0.190  $\mu\text{M}$ : 1530  $\pm$  282 mg/kg total body weight vs. 252  $\pm$  24.7 mg/kg total body weight with ICI 182,780. Uterine weights of mice drinking control tap water were not significantly different from uterine weights of ICI 182,780 treated mice (Figure 2B).

One aspect of the uterotrophic response to estrogen is proliferation of the epithelial cell lining of the uterus (Kang et al. 1975; O'Brien et al. 2006). Uterine epithelial cell height was significantly greater in mice drinking water with U or DES for 30 days (Fig. 3A), U at 0.120  $\mu\text{M}$ :  $31.01 \pm 1.89 \mu\text{m}$  and 1.20  $\mu\text{M}$ :  $23.79 \pm 0.93 \mu\text{m}$ , DES at 0.190  $\mu\text{M}$ :  $40.2 \pm 1.85 \mu\text{m}$ , compared to control tap water  $15.24 \pm 0.77 \mu\text{m}$ . Shown in Figures 3B (control), 3C (0.19  $\mu\text{M}$  DES) and 3D (0.12  $\mu\text{M}$  U), are scanning electron micrographs illustrating changes in uterine luminal epithelial cell height. Seen in Figure 3C and 3D, indicated by arrows, is pseudostratified columnar morphology typical of proliferating epithelial cells due to DES or UN exposure, respectively.

Uranium exposure accelerates vaginal opening and sustains vaginal cell cornification and concomitant exposure to an estrogen antagonist blocks both responses:

Estrogen and EDCs accelerate vaginal opening (VO) in mice (Markey et al. 2001).

Ovariectomized mice drinking water with UN at 0.12  $\mu\text{M}$  or DES at 0.19  $\mu\text{M}$  exhibited significantly accelerated VO, both at 52.5 days, compared to mice drinking control tap water, when VO occurred at 54 days (Figure 4A). UN or DES-mediated acceleration of puberty onset, as indicated by day of VO, was prevented by concomitant treatment with the antiestrogen ICI 182,780 (Figure 4A).

Another indication of estrogenic influence on the female reproductive tract is the persistent presence of cornified cells in vaginal smears (Gordon et al. 1986). As shown in Figure 4B, mice that drank water containing UN at 0.06  $\mu\text{M}$ ; 4 mice, or 0.12  $\mu\text{M}$ ; 5 mice, or DES at 0.19  $\mu\text{M}$ ; 6 mice, had persistent presence of cornified vaginal cells compared to control mice (0 mice). Coincident treatment with the antiestrogen ICI 182,780 prevented the presence of cornified vaginal cells (0.06  $\mu\text{M}$  UN; 0 mice, 0.12  $\mu\text{M}$  UN; 0 mice; and 0.19  $\mu\text{M}$  DES; 1 mouse).

## Discussion

The major contribution of this work is the discovery that U, similar to other heavy metals, has estrogenic activity (Alonso-Gonzalez et al. 2007; Brama et al. 2007; Choe et al. 2003; Dyer 2007; Johnson et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). To our knowledge this has not been demonstrated before. Immature animals exposed to U in drinking water had increased uterine weight and uterine luminal epithelial cell growth, selective reduction of ovarian primary follicles but more growing follicles, accelerated vaginal opening and persistent presence of cornified vaginal cells. U-mediated responses were blocked by co-administration of the antiestrogen ICI 181,720, indicating that an activated estrogen receptor was necessary. In addition, U transplacental exposure caused fewer primordial follicles in developing pup ovaries. These observations support the conclusion that U acts like estrogen in the female mouse reproductive tract.

U caused estrogenic responses at or below the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM) (U.S. EPA 2006). The U.S. EPA safe drinking water level equals the concentration of elemental U and is 47.4 % of UN dissolved in water. Therefore the highest UN concentration of 60 mg/L equals 28 mg/L of elemental U. At first, mg/L amounts UN were used in the drinking water because it was expected U would cause ovarian chemical toxicity as previously reported (Maynard and Hodge 1949). Unexpectedly, at mg/L concentrations, U targeted only large primary follicles causing a reduction in their number but an increase in growing follicles. At the same time there was a trend of increasing uterine weight with increasing U dose. These results directed the research to determine if U could mimic estrogen's effects on the female reproductive system. Subsequently, uterotrophic responses were analyzed in ovariectomized mice using environmentally relevant U concentrations. Significant U effects on the female reproductive system were observed at, or below the U.S. EPA safe levels.

The levels of U used in these experiments are well within the range of U concentrations measured in numerous water sources on the Navajo Reservation, where greater than 1 mg/L have been reported (Brugge and Goble 2002; U.S. EPA 2004). The Navajo Reservation is a vast expanse of

primarily rural and open range land. At least half of the households on the Navajo Reservation rely on water hauled from the nearest source for household use (U.S. Census 2006). Given the frequency of water supplies with unsafe U content, there no doubt that many of the 175,000 residents living on the Navajo Reservation are exposed to hazardous levels of U in their water (Brugge D et al. 2007; Pasternak 2006).

Adult mice exposed to U while immature had fewer primary follicle populations but more secondary follicles. E<sub>2</sub> inhibits mouse oocyte nest breakdown and follicle assembly (Chen et al. 2007). U, mimicking E<sub>2</sub> action, may have reduced follicle assembly leading to fewer primary follicles. Dam ovaries had fewer small primary follicles at a 1000-fold lower U concentration than the adult non-pregnant mice with no significant decrease in primary follicles. The pregnant dam ovaries may have been more sensitive to U due to an up regulation of estrogen receptors that occurs during pregnancy (Spong et al. 2000). Estrogen prevents early follicle assembly (Chen et al. 2007) but stimulates secondary or growing follicles (Drummond 2006). U exposure may have reduced primary follicle populations and stimulated growing follicles via its estrogen like activity.

Developing embryos are exquisitely sensitive to chemical influences. U concentration of 0.001 or 0.120 µM in the dams drinking water led to a significant reduction in pup ovary primordial follicle number. Gestational DES exposure is linked to fewer primordial follicles in pups resulting in fewer ovulated ova (McLachlan et al. 1982). The long term consequence of fewer primordial follicles would lead to accelerated ovarian failure resulting in an earlier menopause onset (Chen et al. 2007). The change in pup ovary primordial follicles with uranium dose was an inverted U-shaped curve. Inverted U-shaped curves are seen in responses that result from *in utero* exposure to E<sub>2</sub> (Welshons et al. 2003).

The rodent uterotrophic assay is used to identify putative EDC. Exposure to chemicals with estrogenic activity are analyzed in immature rodent or ovariectomized mature rodent (Markey et al. 2001; Owens and Ashby 2002; Padilla-Banks et al. 2001). In the first experiment the mice were immature at the outset but became sexually mature during the 30 day exposure to U. These mice

exhibited a trend of increased uterine weight. If these mice had been examined for estrus stage at sacrifice, the uterine weights could have been grouped by estrus stage, possibly enabling the trend to reach statistical significance. Ovariectomized mice were used to avoid the confounding effect of estrus cycling to test if UN caused uterotrophic responses.

The uterotrophic assay measures the consequences of three coordinated responses to estrogen or a chemical that acts like estrogen. These are epithelial cell growth, hyperemia and fluid accumulation or imbibition (O'Brien et al. 2006). DES stimulation of uterine epithelial cell growth in addition to employing classical ER $\alpha$ , may also utilize tethered or non-classical pathways to induce mitogenic uterine responses (O'Brien et al. 2006). This suggests U does not need to directly activate the classical estrogen receptor for uterine epithelial cell growth.

Uranium dose-response in either the uterotrophic assay or increased uterine epithelial cell height was not monotonic. Many EDC elicit low-dose responses resulting in U-shaped or inverted U-shaped dose-response curves (Myers and Hessler 2007; Welshons et al. 2003). Non-monotonic response occurs when a xenoestrogenic compound exerts direct effects by mimicking estradiol or indirect effects by interfering with estrogen receptors or estradiol production and metabolism. Further, xenoestrogenic responses may activate or inhibit different genes at various doses which may result in different outcomes for target endpoints examined at same time points (Coser et al. 2003).

Mice exposed to U for 30 days had a more pronounced uterotrophic response compared to mice exposed for 10 days. This raises questions on how U may be getting into cells/tissues and by which mechanism U interacts with the ER. U enters brain endothelial cells (Dobson et al. 2006) and via specialized transport, enter polarized epithelial LLC-PK<sub>1</sub> cells (Muller et al. 2006). Vidaud et al. (2007) examined the possibility of apotransferrin transporting U into the cell. U binds to transferrin but conformational changes do not enable transferrin receptor recognition of the U-transferrin complex, ruling out this pathway for U to get in to the cell. Other ways in which U may get into the cell, but that have not been investigated, are through divalent metal transporter-1 (DMT-1) or calcium channels. DMT-1 functions to transport iron and other metal ions across the plasma membrane, and

is ubiquitous in plants, insects, microorganisms and vertebrates (Mims and Prchal 2005). U displaces calcium in the bone matrix (Neuman et al. 1949), therefore it is plausible that U may be using calcium channels to enter the cell. The manner and rate by which U gets into the cell may be impeded by U speciation or tissue concentration, which could result in delayed responses as was seen with uterine weight changes after 10 versus 30 day exposure.

Similar to DES, U accelerated VO and stimulated persistent vaginal cornified cells that represents a constant estrus state elicited by estrogen. Uranium stimulated uterine and vaginal responses were blocked by ICI 182,780, indicating estrogen receptor activation was necessary but not sufficient for U to act. We have yet to define the molecular mechanisms of action by which U evokes estrogenic responses. It is possible that U may elicit estrogen-like responses as cadmium is reported to, by binding the ligand binding domain of the ER (Stoica et al. 2000). As mentioned above, U estrogenic stimulation may be the result of U binding some other factor whose responses are “tethered” to the estrogen receptor pathway resulting in crosstalk that induces estrogenic responses. In summary, the stimulatory effects of U on the ovary, uterus and vaginal cells suggests U acts like estrogen in the female reproductive system and is an EDC.

There are few reports relating environmental U exposure to reproductive health outcomes in the Four Corners region. But in one, there was a statistically significant relationship between birth defects and the mother’s proximity to U tailings (Shields et al. 1992). And in another, the incidence of reproductive or gonadal cancer in New Mexico Native American children and teenagers is 8 fold greater than the incidence in age matched non-Native American individuals (Duncan et al. 1986). Environmental estrogens such as DES or bisphenol A may contribute to occurrence of reproductive anomalies and cancer later in life (Maffini et al. 2006; Newbold et al. 2006). Given our results that U is an EDC, health problems may result from inappropriate concentration or timing of exposure to this estrogen mimic.

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Table1: Uranyl nitrate in drinking water effected specific ovarian follicle populations

Follicle Counts per section	Control U < 0.002 mg/L	Uranyl nitrate 0.50 mg/L	Uranyl nitrate 2.50 mg/L	Uranyl nitrate 12.50 mg/L	Uranyl nitrate 60.00 mg/L
Primordial follicles	65.55 ± 7.05	53.80 ± 8.26	37.88 ± 7.01	57.60 ±13.29	61.60 ±12.76
Small primary follicles	26.22 ± 2.50	19.40 ± 3.03	18.56 ± 2.94	32.00 ± 3.51	21.78 ± 2.81
Large primary follicles	12.66 ±0.69	6.50* ± 1.17	7.44* ± 1.27	12.00 ± 1.51	9.11 ± 0.65
Secondary or growing follicles	26.44 ± 1.08	24.20 ± 2.09	21.22 ± 1.85	33.30* ± 1.92	26.78 ± 0.81
Healthy antral follicles	31.22 ± 2.56	31.00 ± 3.49	28.22 ± 3.71	29.00 ± 2.39	23.11 ± 2.78
Atretic antral follicles	17.22 ± 1.37	15.50 ± 2.37	11.44 ± 1.70	16.00 ± 3.26	12.53 ± 1.37

Female B6C3F<sub>1</sub> mice drank control tap water or U containing water for 30 days. Ovaries were collected and prepared for histological analysis to count follicle populations. Values are represented as mean ± SEM (n = 6). Follicle populations significantly different from control are indicated by (\*) where p < 0.05 in the Tukey-Kramer post-hoc tests.

Table 2: The effect of uranyl nitrate exposure on body and tissue weights

Treatment	Body Wt	Ovary	Uterus	Liver	Adrenal	Kidney	Spleen
Control (< 2 µg/L U)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.5 mg/L U	101.2	77.5	97.1	94.2	95.5	96.0	104.0
2.5 mg/L U	100.4	72.5	81.8	94.4	88.4	91.7*	89.9
12.5 mg/L U	104.1	73.9	115.9	99.2	120.8	100.9	103.6
60.0 mg/L U	104.6	62.4	127.8	110.6	108.5	94.2*	109.8

Female B6C3F<sub>1</sub> mice drank control tap water or water containing UN for 30 days.

Tissue weights are expressed as a percent of control values normalized to total body weight.

Organ weights significantly different from control are indicated by (\*) where  $p < 0.05$ .

## Figure legends

Figure 1. Uranyl nitrate in drinking water selectively effects dam follicle populations and *in utero* exposed pup ovary primordial follicles. B6C3F<sub>1</sub> dams were exposed to control tap water (control, black bar), 0.001 – 0.12  $\mu$ M U (clear bar) in drinking water 30 days prior to mating and through gestation. Ovaries from dams (A) and pups (B) were removed on the day of birth. Differences in ovarian follicle populations between U treatments and controls are indicated by (\*)  $p < 0.05$  ANOVA. Values are represented as mean  $\pm$  SEM (n = 7-11).

Figure 2. Uranyl nitrate in drinking water caused uterine weight gain that was blocked by antiestrogen coadministration. Ovariectomized C57Bl/6J mice drank control tap water (control, black bar), or 0.12 – 12.00  $\mu$ M U (clear bar), or 0.19  $\mu$ M DES (gray bar). A. After 30 days uteri were removed, wet weights recorded and normalized to body weight. Differences in wet uterine weight among exposure groups is indicated by (\*) for  $p < 0.001$ . Values are represented as mean  $\pm$  SEM (n = 5-6). B. Ovariectomized C57Bl/6J mice drank control tap water (control, black bar) or 0.06 – 0.12  $\mu$ M U (clear bar) or 0.19  $\mu$ M DES (gray bar) for 10 days and some received daily i.p. injections of either sesame oil vehicle or 500  $\mu$ g/kg ICI 182,780 in sesame oil. Uteri were removed, wet weights recorded and normalized to body weight. Differences in wet uterine weight among exposure groups is indicated by (a, b, c) at  $p < 0.005$ . Values are represented as mean  $\pm$  SEM (n = 6-7).

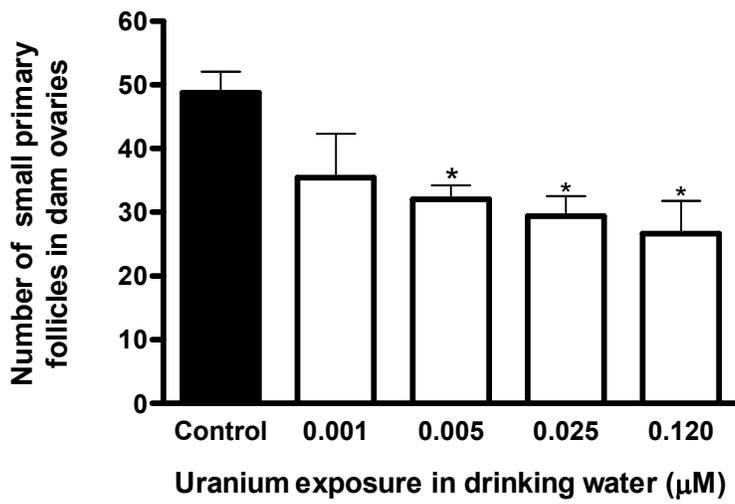
Figure 3. Uranyl nitrate in drinking water stimulated uterine luminal epithelial cell growth. A. Ovariectomized C57Bl/6J mice drank control tap water (control, black bar), or 0.12 – 12.00  $\mu$ M U (clear bar), or 0.19  $\mu$ M DES (gray bar) for 30 days. Uteri were collected and prepared for scanning electron microscopy. Differences in epithelial cell height from control mice are indicated by (\*) with  $p < 0.0001$ . Values are represented as mean  $\pm$  SEM (n = 5 uteri at 40 measurements from each tissue). B. Representative scanning electron microscopy images at the same magnification of

uterine epithelial cell layers from tap water control, C. 0.19  $\mu$ M DES, or D. 0.12  $\mu$ M U. Arrow bars highlight epithelial cell height differences in DES- and U-exposed ovariectomized mice.

Figure 4. Uranyl nitrate in drinking water accelerated vaginal opening and caused persistent presence of cornified vaginal cells. A. For 10 days, ovariectomized C57Bl/6J mice at 50 days old drank control tap water (control, black bar), or 0.19  $\mu$ M DES (gray bar) or 0.06 or 0.12  $\mu$ M U (clear bar) and some mice received daily i.p. injections of either sesame oil vehicle or 500  $\mu$ g/kg ICI 182,780 in sesame oil vehicle. Mice were examined daily for vaginal opening from age day 50 to day of vaginal opening. Differences in day of vaginal opening different from control ovariectomized mice are indicated by (\*) for  $p < 0.001$ . Values are represented as mean day of vaginal opening  $\pm$  SEM (n = 6-7). B. In C57Bl/6J mice exposed to U or DES in drinking water for 10 days, vaginal cell cornification persisted compared to control mice and mice receiving daily i.p. injections of 500  $\mu$ g/kg ICI 182,780. Vaginal smears were collected daily to examine vaginal cytology. Presence and absence of vaginal cornified cells were analyzed by Chi-squared test with  $p < 0.05$ . Statistical significance compared to control are indicated by (\*) for  $p < 0.05$  by Fisher's exact test.

Figure 1

A



B

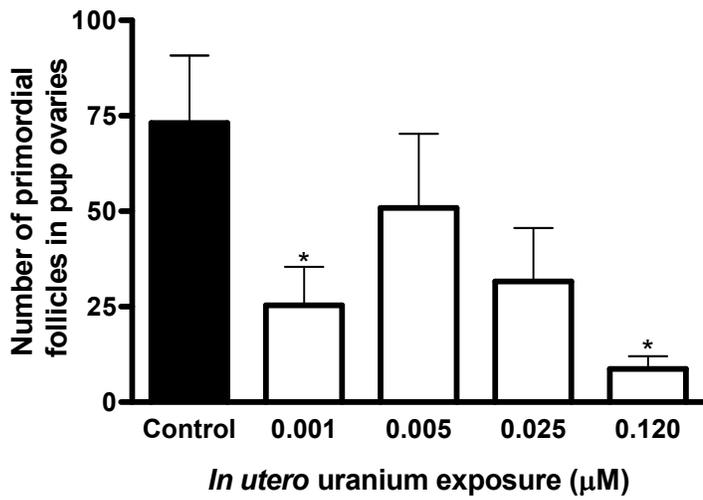
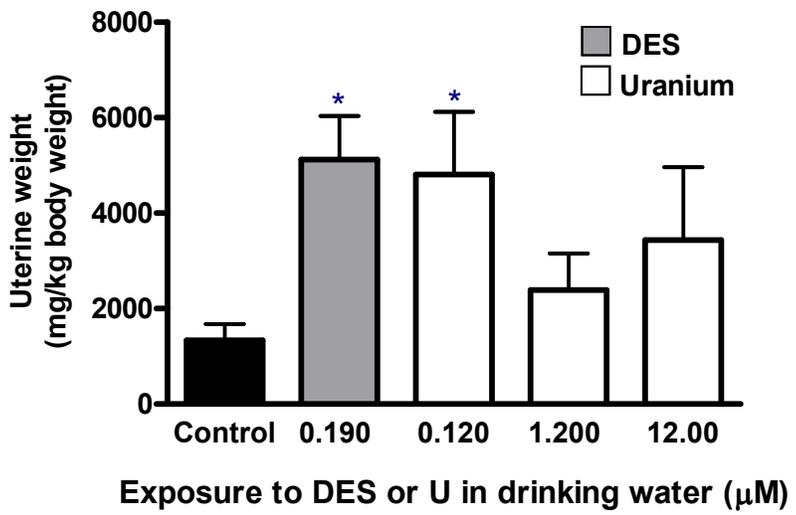


Figure 2

A



B

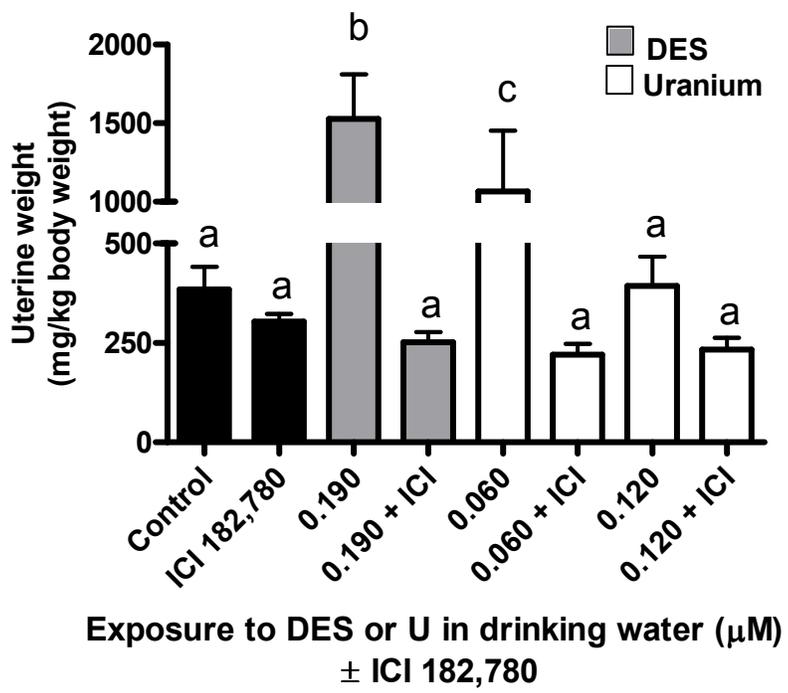
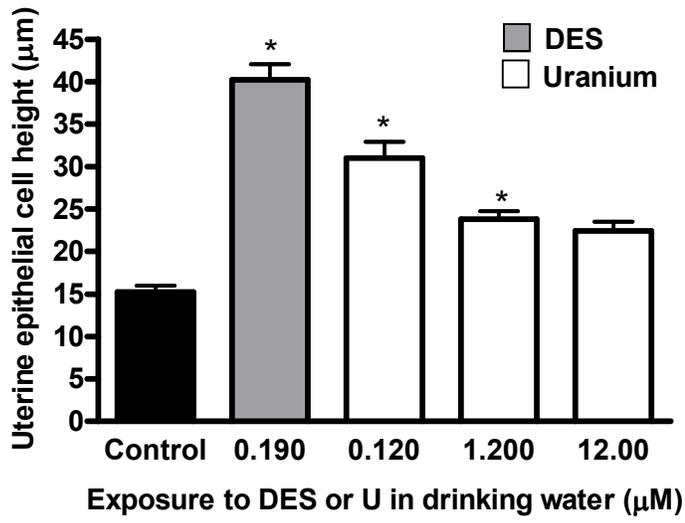
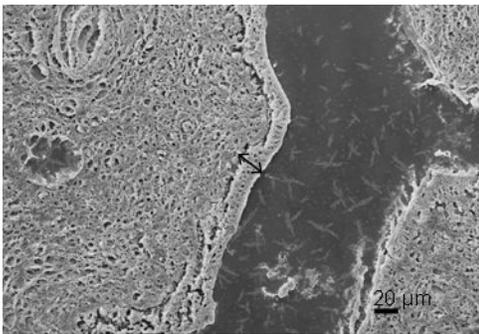


Figure 3

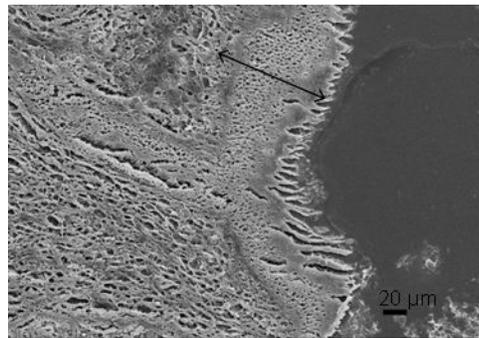
A



B



C



D

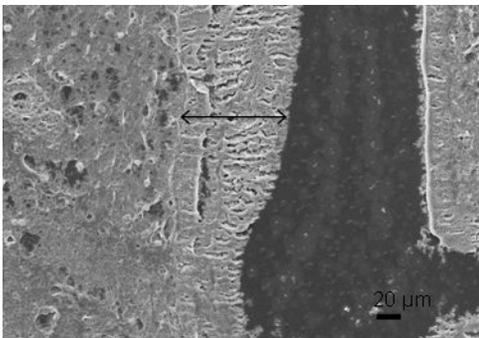
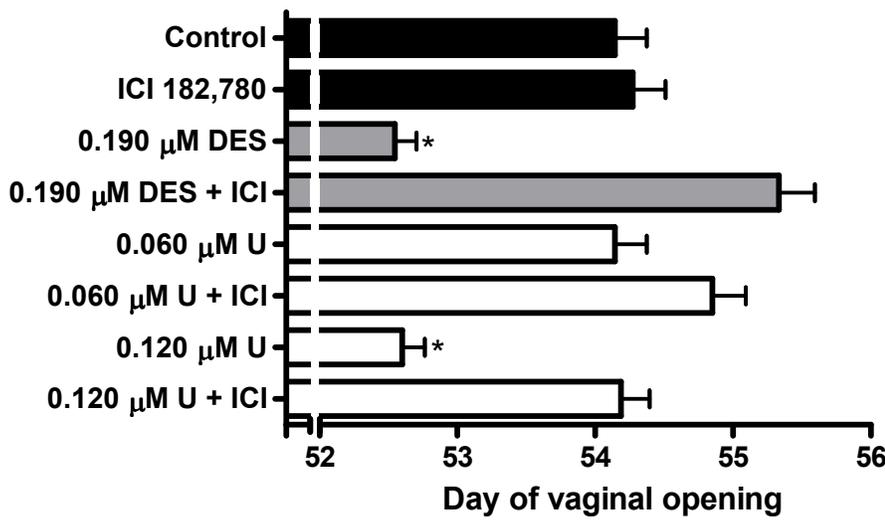


Figure 4

A



B

