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

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BACKGROUND: The deleterious impact of uranium 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: We investigated the hypothesis that uranium, similar to other heavy metals such as cadmium, acts like estrogen.

METHODS: In several experiments, we exposed intact, ovariectomized, or pregnant mice 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 with the antitestosterone ICI 182,780 blocked these responses to uranium or the synthetic estrogen diethylstilbestrol. In addition, mouse pups 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: Because of the decades of uranium mining/milling in the Colorado plateau in the Four Corners region of the American Southwest, the uranium concentration and the route of exposure used in these studies are environmentally relevant. Our data support 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.

KEY WORDS: depleted uranium, endocrine disruption, estrogen, estrogen receptor, female reproduction, heavy metal, Navajo reservation.

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weight, accelerated mammary gland growth/development, and accelerated vaginal opening (VO) (Johnson et al. 2003). Cd-induced estrogen-like responses were prevented by the antiestrogen ICI 182,780. Cd inhibits transcriptional activity of estradiol-activated rainbow trout ER in recombinant yeast (Guével et al. 2000). Cd treatment stimulates breast cancer cell proliferation by activating ER-α–dependent Akt (protein kinase B), Erk1/2 (extracellular signal-regulated kinase), and platelet-derived growth factor receptor-α (Brama et al. 2007). Although these studies demonstrate the estrogen activity of Cd, it should be noted that Silva et al. (2006) reported that Cd lacks estrogenic activity in the yeast estrogen screen assay, MCF-7 cell proliferation, or the E-SCREEN assay, and also failed to induce Src, Erk1, and Erk2 phosphorylation. In the present study we tested whether depleted U 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.** We performed U exposure in intact female mice using 28-day-old immature C57Bl/6J mice (Harlan, Indianapolis, IN). For in utero U exposure experiments, we used 48-day-old male and female B6C3F1 mice (Harlan). We used ovariectomized 28-day-old C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) for the prepubertal U and DES exposure experiments. Mice were housed with ad libitum access to food and water for 30 days. Mice were paired for breeding, and the day of birth, dams (n = 5 mice per treatment group) and female pups (n = 7–9 pups per treatment group) were euthanized and the ovaries collected for histology.

**Tissue collection and histology.** After exposure to DES or U, mice were euthanized and organs were 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 were fixed in Bouin’s solution, embedded in paraffin, and serially sectioned every 9 µm; every 10th section was mounted on slides. Tissue sections were deparaffinized in Citrasolve (Sigma Chemical Co.) and dehydrated in a series of ethanol baths. We used a Zeiss 435 VP scanning electron microscope and LE032 V02.01 software (Carl Zeiss SMT Inc., Peabody, MA) to measure the height of uterine luminal epithelial cells. Forty measurements were randomly collected from each individual uterus.

Ovaries were trimmed of adhering tissue and fat and then fixed in Bouin’s solution. They were transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 µm), 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 20th section, and in pup ovary every 12th 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 a posteriori Tukey-Kramer test.

### Table 1. Effects of UN exposure on specific ovarian follicle populations (follicle counts per ovary; mean ± SE) in B6C3F1 mice exposed to UN in drinking water for 30 days.

| Follicle type          | Control (U < 0.002 mg/L) | 0.5 | 2.5 | 12.5 | 60.0 |
|-----------------------|--------------------------|-----|-----|------|------|
| Primordial            | 65.55 ± 7.05             | 53.80 ± 8.28 | 37.88 ± 7.01 | 57.60 ± 13.29 | 61.60 ± 12.76 |
| Small primary         | 26.22 ± 2.50             | 19.40 ± 3.03 | 18.56 ± 2.94 | 32.00 ± 3.51 | 21.79 ± 2.81 |
| Large primary         | 12.66 ± 0.69             | 6.50 ± 1.17* | 7.44 ± 1.27* | 12.00 ± 1.51 | 9.11 ± 0.65  |
| Secondary or growing  | 26.44 ± 1.08             | 24.20 ± 2.09 | 21.22 ± 1.85 | 33.30 ± 1.92* | 26.79 ± 0.81 |
| Healthy antral        | 31.22 ± 2.96             | 31.00 ± 3.48 | 28.22 ± 3.71 | 29.00 ± 2.39 | 23.11 ± 2.78 |
| Atretic antral        | 17.22 ± 1.37             | 15.50 ± 2.37 | 11.44 ± 1.70 | 16.00 ± 2.56 | 12.53 ± 1.37 |

*n = 6 per group.

*Significantly different from control (p < 0.05, Tukey-Kramer post hoc test).

### Table 2. Effects of UN exposure on body weight and tissue weight in B6C3F1 mice exposed to UN in drinking water for 30 days.

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

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

*Significantly different from control (p < 0.05).
variance (ANOVA) with significance set at \( p < 0.05 \). We used Tukey-Kramer post hoc tests where appropriate. For mice exposed for 10 and 30 days, organ weights were determined for each individual within each experiment and averaged for each exposure group. In the 30-day–exposure group, uterine luminal epithelial cell height measurements were collected from individual mice and averaged for each exposure group. Additionally, in the 10-day–exposure group, VO was determined for each individual and averaged for the exposure group. The means for control versus exposed mice for organ weights, uterine epithelial cell height, and VO were analyzed for significant differences by one-way ANOVA with significance set at \( p < 0.05 \). We used Dunnett’s post hoc test where appropriate. The means of uterine weights in controls or in mice exposed to ICI 182,780, U, or DES 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 group. Presence and absence of cornified cells was analyzed by chi-square 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: U exposure in immature mice. Experiment 1.1 showed that U targets early stage ovarian follicles. As shown in Table 1, there were significantly fewer large primary follicles at 0.5 and 2.5 mg/L UN and significantly more secondary or growing follicles at 12.5 mg/L UN. However, we found 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 because there were more growing follicles at 12.5 mg/L UN, the changes could not be caused by heavy metal toxicity.

This experiment also showed that U does not lead to overt organ toxicity. We found no gross anomalies in any major organs, and body weight did not significantly change with UN exposure at any concentration. As shown in Table 2, kidney weight was significantly reduced at doses of 2.5 and 60.0 mg/L UN, but this 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.

We found an interesting, but not statistically significant, trend of increased uterine weight at 12.5 and 60.0 mg/L UN (Table 2). We did not determine estrous cycle stage in mice at sacrifice, thus uterine weights could not be grouped relative to stage.

Experiment 1.2: Gestational and in utero U exposure in dams and female pups. Experiment 1.2 showed that in utero uranium exposure reduces pup ovary primordial follicles. As shown in Figure 1A, 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 \( \mu M \) compared with control mice. 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. We found 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 0.001- or 0.120-\( \mu M \) UN, compared with 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: U exposure in ovariectomized mice. Experiment 2.1 showed that UN exposure induces estrogen-like changes in uterine morphology and histology. Mice exposed to UN or DES had significantly increased uterine weight at 0.120 \( \mu M \) U and 0.19 \( \mu M \) DES, 3.6 and 3.8 times greater, respectively, compared with mice drinking control tap water (Figure 2A). We normalized uterine weights to body weights, which 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: Other estrogen-like effects of UN and their mediation through ER activation. Experiment 2.2 showed that UN-mediated estrogen-like actions are blocked by concomitant exposure to an ER antagonist. To determine if the U-mediated uterotrophic response was dependent on ER activation, ovariectomized mice drinking UN-containing water were injected daily with the antiestrogen ICI 182,780. In a pilot experiment, we determined that 10 days of exposure to UN in drinking water caused a significant increase in uterine weight compared with 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 (Figure 2B): 0.06 \( \mu M \) U alone, 1.070 \( \pm 386 \) mg/kg total bw; 0.06 \( \mu M \) U plus ICI 182,780, 220 \( \pm 28.1 \) mg/kg total bw; 0.19 \( \mu M \) DES alone, 1.530 \( \pm 282 \) mg/kg total bw; 0.19 \( \mu M \) DES plus ICI 182,780, 252 \( \pm 24.7 \) mg/kg total bw. Uterine weights of control mice were \( < 0.05 \). We used Tukey-Kramer post hoc test with significance set at \( p < 0.05 \). Persistent presence of cornified vaginal cells was analyzed by Fisher’s exact test with significance set at \( p < 0.05 \).

\*Significantly different compared with controls (\( p < 0.05 \), ANOVA).
not significantly different from controls treated with ICI 182,780 (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 containing U or DES for 30 days (Figure 3A); 0.120 µM U, 31.01 ± 1.89 µm; 1.20 µM U: 23.79 ± 0.93 µm; 0.19 µM DES, 40.2 ± 1.85 µm; controls, 15.24 ± 0.77 µm. Figures 3B (control), 3C (0.19 µM DES), and 3D (0.12 µM U) show scanning electron micrographs illustrating changes in uterine luminal epithelial cell height. Arrows in Figure 3C and 3D indicate pseudostratiﬁed columnar morphology typical of proliferating epithelial cells due to DES or UN exposure, respectively.

**Effects of U on VO and vaginal cell corniﬁcation.** Estrogen and endocrine-disrupting chemicals (EDCs) accelerate VO in mice (Markey et al. 2001). Ovariectomized mice exposed to 0.12 µM UN or 0.19 µM DES exhibited signiﬁcantly accelerated VO (both at 52.5 days), compared with control mice (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 exposed to 0.06 µM UN (4 mice) or 0.12 µM UN (5 mice), or 0.19 µM DES (6 mice) had persistent presence of corniﬁed vaginal cells compared with control mice (5 mice). Coincident treatment with ICI 182,780 prevented the presence of corniﬁed vaginal cells (0.06 µM UN, 0 mice; 0.12 µM UN, 0 mice; 0.19 µM DES, 1 mouse).

**Discussion**

The major contribution of the present study 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 VO, and persistent presence of corniﬁed vaginal cells. U-mediated responses were blocked by coadministration of the antiestrogen ICI 181,720, indicating that an activated ER was necessary. In addition, transplacental exposure to U 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, we used milligram per liter amounts of UN in the drinking water because we expected U to cause ovarian chemical toxicity as previously reported (Maynard and Hodge 1949). Unexpectedly, at milligram per liter 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 led us to determine whether U could mimic estrogen’s effects on the female reproductive system. Subsequently, we analyzed uterotropic responses in ovariectomized mice using environmentally relevant U concentrations. We observed signiﬁcant effects of U on the female reproductive system at or below the U.S. EPA safe levels.

The U levels used in these experiments are well within the range of U concentrations measured in numerous water sources on the Navajo Reservation, where concentrations > 1 mg/L have been reported (Brugg 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 is 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 et al. 2007; Pasternak 2006).

Adult mice exposed to U while immature had fewer primary follicle populations but more secondary follicles. 17β-Estradiol (E2) inhibits mouse oocyte nest breakdown and follicle assembly (Chen et al. 2007). U, mimicking E2 action, may have reduced follicle assembly leading to fewer primary follicles. Dam ovaries had fewer small primary follicles at a 1,000-fold lower U concentration than did the adult nonpregnant mice, which had no signiﬁcant decrease in primary follicles. The pregnant dam ovaries may have been more sensitive to U because of an up-regulation of ERs 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 concentrations of 0.001 or 0.120 µM in the dams’ drinking water led to a significant reduction in the number of primordial follicles in pup ovaries. 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 resulting from in utero exposure to E2 (Welshons et al. 2003).

The rodent uterotrophic assay is used to identify putative EDCs. Exposure to chemicals
with estrogentic activity are analyzed in immature rodents or ovariectomized mature rodents (Markey et al. 2001; Owens and Ashby 2002; Padilla-Banks et al. 2001). In our 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 estrous stage at sacrifice, the uterine weights could have been grouped by stage, possibly enabling the trend to reach statistical significance. We used ovariectomized mice to avoid the confounding effect of estrous cycling to test whether UN caused uterotrophic responses.

The uterotrophic assay measures the consequences of three coordinated responses to estrogen or a chemical that acts like estrogen: 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-α, may also use tethered or nonclassical pathways to induce mitogenic uterine responses (O’Brien et al. 2006). This suggests that U does not need to directly activate the classical ER for uterine epithelial cell growth.

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

Mice exposed to U for 30 days had a more pronounced uterotrophic response than mice exposed for 10 days. This raises questions about 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 it enters polarized epithelial LLC-PK1 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 enter the cell. Other ways that U may enter the cell have not been investigated: 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 use 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 we observed with uterine weight changes after 10-day exposure compared with 30-day exposure.

Similar to DES, U accelerated VO and stimulated persistent vaginal cornified cells, which represents a constant estrus state elicited by estrogen. U-stimulated uterine and vaginal responses were blocked by ICI 182,780, indicating that ER 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 Cd is reported to, by binding the ligand binding domain of the ER (Stoica et al. 2000). As mentioned above, U estrogen stimulation may be the result of U binding some other factor whose responses are “tethered” to the ER pathway, resulting in cross-talk that induces estrogenic responses. In summary, the stimulatory effects of U on cells of the ovary, uterus, and vagina suggest that U acts like estrogen in the female reproductive system and is an EDC.

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