5 Heavy Metals as Endocrine-Disrupting Chemicals

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Contents

1. INTRODUCTION

Heavy metals are present in our environment as they formed during the earth's birth. Their increased dispersal is a function of their usefulness during our growing dependence on industrial modification and manipulation of our environment *(1,2)*. There is no consensus chemical definition of a heavy metal. Within the periodic table, they comprise a block of all the metals in Groups 3–16 that are in periods 4 and greater. These elements acquired the name heavy metals because they all have high densities, >5 g/cm^3 *(2)*. Their role as putative endocrine-disrupting chemicals is due to their chemistry and not their density. Their popular use in our industrial world is due to their physical, chemical, or in the case of uranium, radioactive properties. Because of the reactivity of heavy metals, small or trace amounts of elements such as iron, copper, manganese, and zinc are important in biologic processes, but at higher concentrations they often are toxic.

Previous studies have demonstrated that some organic molecules, predominantly those containing phenolic or ring structures, may exhibit estrogenic mimicry through actions on the estrogen receptor. These xenoestrogens typically are non-steroidal organic chemicals released into the environment through agricultural spraying, industrial activities, urban waste and/or consumer products that include organochlorine pesticides, polychlorinated biphenyls, bisphenol A, phthalates, alkylphenols, and parabens *(1)*. This definition of xenoestrogens needs to be extended, as recent investigations have yielded the paradoxical observation that heavy metals mimic the biologic

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activity of steroid hormones, including androgens, estrogens, and glucocorticoids. Early studies demonstrated that inorganic metals bind the estrogen receptor. Zn(II), Ni(II), and Co(II) bind the estrogen receptor, most likely in the steroid-binding domain, but in this study neither Fe(II) nor Cd(II) bound the receptor *(3)*. Certain metals bind the zinc fingers of the estrogen receptor and could alter the receptor's interaction with DNA *(4)*. Several metals can displace or compete with estradiol binding to its receptor in human Michigan Cancer Foundation MCF-7 breast cancer cells *(5–7)*. Recently, cadmium has been shown to act like estrogen *in vivo* affecting estrogen-responsive tissues such as uterus and mammary glands *(8)*. Metals that mimic estrogen are called metalloestrogens *(9,10)*.

Five heavy metals have been sufficiently investigated to provide insight into the means of their impact on mammalian reproductive systems. Arsenic, a metalloid and borderline heavy metal, is included because it is often found in the earth associated with other heavy metals, such as uranium. Additional heavy metals to be discussed are cadmium, lead, mercury, and uranium—the heaviest naturally occurring element. In this chapter, for each heavy metal, descriptions will be provided for the environmental exposure, history of its use, and thus potential for increased dispersal in our environment, targeted reproductive organs, and specific effects or means of action, usually as a function of low versus high concentration. An important tenet is that earlier (developmental) ages of exposure increase the impact of the endocrine-disrupting chemical or heavy metal on the normal development of reproductive organs, which may be permanently affected. Thus, where known, I will describe the direct action of a heavy metal on a growing embryo, perhaps through epigenetic changes, to set the stage for increased chance of disease later in life when the individual is challenged by another environmental insult. In the case of uranium, I will describe my laboratory's research that supports the conclusion that uranium is a potent estrogen mimic at concentrations at or below the United States Environmental Protection Agency (USEPA) safe drinking water level.

2. ARSENIC

The abundance of arsenic (As) in the Earth's crust is 1.5–3.0 mg/kg, making it the 20th most abundant element in the earth's crust *(11)*. Arsenic has been in use by man for thousands of years. It is infamous as a favored form of intentional poisoning and famous for being developed by Paul Erlich as the first drug to cure syphilis *(12)*. Today, arsenic is used in semiconductor manufacture and pesticides *(13)*. It serves as a wood preservative in chromated copper arsenate (CCA). CCA-treated lumber products are being removed voluntarily from consumer use as of 2002 and were banned as of January 1, 2004. CCA-treated lumber is a potential risk of exposure of children to arsenic in play-structures *(14)*. Another source of environmental arsenic is from glass and copper smelters, coal combustion, and uranium mining. The most extensive environmental exposure is in drinking water. For instance, since the 1980s, the provision of arsenic-contaminated Artesian well water in Bangladesh has exposed an estimated 50–75 million people to very high levels of arsenic *(11)*. Given the latency of 30–50 years for arsenic-related carcinogenesis, epidemiological data on arsenic-induced cancers including skin, lung, urinary bladder, kidney, and liver are only now becoming available *(15)*.

Inorganic arsenic in the forms +3 (arsenite) or +5 charges are the most often encountered forms of arsenic and are most readily absorbed from the gastrointestinal

tract; therefore, these forms cause the greatest number of health problems. A new USEPA limit of arsenic standard for drinking water has recently gone into effect, lowering the limit from 50 to 10 μg/L. Compliance of water systems with this standard became enforceable as of January 23, 2006 *(16)*. However, achieving this limit will be problematic for many smaller water municipalities because of the expense of installing equipment to reduce arsenic to <10 μg/L *(17)*.

2.1. Arsenic as an Endocrine-Disrupting Chemical in Reproductive Systems

Arsenic-mediated endocrine disruption has been reported in research animals and potentially in humans. For instance, adult rats that consume drinking water with arsenite at 5 mg/kg of body weight per day 6 days a week for 4 weeks have reproductive tract abnormalities such as suppression of gonadotrophins and testicular androgen, and germ cell degeneration—all effects similar to those induced by estrogen agonists *(18)*. In this study, it was concluded that arsenite may exhibit estrogenic activity. However, there was no evidence presented to indicate estrogen receptor specificity by demonstrating that an antiestrogen such as ICI 182,780 prevented the arsenic-induced changes. Thus, the degenerative problems could have resulted from arsenic chemical toxicity. Similar to this study are those conducted by Waalkes' research group. In mice that were injected with sodium arsenate at 0.5 mg/kg i.v. once a week for 20 weeks, males had testicular interstitial cell hyperplasia and tubular degeneration that probably resulted from the interstitial cell hyperplasia *(19)*. Arsenate injections in female mice caused cystic hyperplasia of the uterus, which is often related to abnormally high, prolonged estrogenic stimulation. Again, as these changes were unexpected, there was no attempt to determine the dependence on the estrogen receptor by using an antiestrogen to block the changes in the male and female reproductive tissues *(19)*. This same research group went onto to show that *in utero* exposure to arsenic leads to changes in the male and female offspring that indicate they have been exposed to an estrogenic influence *(20)*. In addition, *in utero* arsenic-exposed mice are much more prone to urogenital carcinogenesis, urinary bladder, and liver carcinogenesis when they are exposed postnatally to the potent synthetic estrogen, diethylstilbestrol (DES) or tamoxifen *(21,22)*. The altered estrogen signaling may cause over expression of estrogen receptor-α through promoter region hypomethylation, suggesting an epigenetic change was caused by *in utero* As exposure *(23)*. Together, the *in vivo* data support the hypothesis that arsenic can produce estrogenic-like effects by direct or indirect stimulation of estrogen receptor-α. The levels of As used in the *in vivo* studies are high, similar to the high levels in drinking water in Bangladesh, on average in the 0.1–1 mM range, and thus, these studies are environmentally relevant for people living with one of the worst scenarios of As environmental contamination. Arsenic levels at 0.4 ppm/day, 40 times more than current USEPA safe drinking water level, when given daily in drinking water to rats results in reduced gonadotrophins, plasma estradiol, and decreased activities of these steroidogenic enzymes, 3β hydroxysteroid dehydrogenase (HSD), and 17β HSD *(24)*. At the same time there was no change in body weight, but ovarian, uterine, and vaginal weights were significantly reduced, suggesting that As treatment caused organ toxicity but not general toxicity. For a full description of inorganic arsenic-mediated reproductive toxicity in animals and human see Golub and Macintosh *(25)*.

2.2. Relationship Between Arsenic and Diabetes

In those parts of the world with the most elevated levels of environmental As in drinking water, there is a proposed relationship to type 2 diabetes, as arsenic may cause insulin resistance and impaired pancreatic β-cell functions including insulin synthesis and secretion *(26)*. Blackfoot disease, which is associated with drinking As-contaminated drinking water, is endemic in southwestern Taiwan and also associated with the increased prevalence of type 2 diabetes *(27)*. Type 2 diabetes compromises fertility *(28)*, making As a potential endocrine-disrupting chemical on both the diabetes and reproductive systems. Another mechanism of heavy metals and As is through formation of reactive oxygen and nitrogen species that cause non-specific damage such as oxidative damage to DNA and lipid peroxidation that can contribute to reproductive problems *(29)*. For instance, there are low birth weight infants, more spontaneous abortions, and congenital malformations in female employees and women living close to copper smelters as reported in Sweden and Bulgaria *(30,31)*. However, this mechanism of As action is certainly due to its chemical toxicity rather than its mimicry of endocrine agents such as estrogen.

2.3. Mechanisms of Arsenic Actions on Endocrine Systems

There are limited *in vitro* based studies of the putative estrogenic activity of As. In MCF-7 breast cancer cells, which are often used to assess estrogenic activity of endocrine-disrupting chemicals *(32)*. In these cells, arsenite at low micromolar concentrations stimulated increased proliferation, steady state levels of progesterone receptor, pS2, and decreased estrogen receptor-α mRNA expression *(33)*. The antiestrogen ICI 182,780 or fluvestrant blocked the effects of arsenite indicating the dependence on the estrogen receptor. In addition, by using binding assays and receptor activation assays, it was determined that As interacts with the hormone-binding domain of the estrogen receptor *(33)*. Another group tested the estrogenicity of several heavy metals and arsenite treatment stimulated MCF-7 cell growth but relative to other metals was not very potent *(34)*. In contrast, arsenic trioxide, an approved treatment of acute promyelocytic leukemia, blocks MCF-7 cell proliferation without binding the ligand-binding domain of the estrogen receptor but does interfere with estrogen receptor-signaling pathway indicating that the chemical state of As is key in determining its biologic activity *(35)*.

Arsenite binds to the Zinc (Zn) finger region of the estrogen-binding region of estrogen receptor-α, and the binding affinity is influenced by the amino acid length between two cysteines *(36,37)*. However, these investigations are strictly cell-free assays; so, it is difficult to extrapolate to whole cell responses. Finally, arsenite at 100 μM binds the glucocorticoid receptor in the steroid-binding domain but does not compete for binding to progesterone, androgen, or estrogen receptors in MCF-7 cells at this high concentration *(38)*. On the contrary, arsenite from 0.3 to 3.3 μM, a non-toxic dose, interacts with the glucocorticoid receptor in human breast cancer cells and rat hepatoma cells to inhibit glucocorticoid receptor-mediated gene transcription *(39–41)*. In addition, glucocorticoid receptor binding of its ligand dexamethasone is blocked by low micromolar concentrations of arsenite but not arsenate. Arsenite interacts with the vicinal dithiols of the glucocorticoid receptor as is the case with its interaction with the estrogen receptor *(42,43)*. Arsenite at low micromolar concentrations binds

to the estrogen receptor and glucocorticoid receptor to alter gene expression in rat and human cells. At concentrations >100 μM arsenic may act through chemical toxicity to non-specifically damage DNA or proteins through reactive oxidative species *(29)*. As a whole, these studies suggest influence of As on the stress neuroendocrine system.

In sum, there is suggestive evidence from *in vivo* studies that As may have estrogenic activity. Nevertheless, further proof that antiestrogens may block the responses elicited by As would allow a stronger connection between As and putative estrogenic activity to be drawn. Moreover, there could be indirect endocrine effects of As because of its causing insulin resistance and reducing insulin levels leading to type 2 diabetes that potentially would compromise reproductive tissue responses. The evidence in MCF-7 cell E-Screen bioassays strongly supports the conclusion that As can bind the estrogen receptor in the ligand-binding domain to activate the receptor and exert downstream signaling events that are blocked by the antiestrogen ICI 182,780. In addition, there is strong evidence to support the conclusion that arsenite binds the glucocorticoid receptor to either activate and/or inhibit gene transcription. Thus it appears that at low concentrations (<10 μM) there are observations of specific interaction with steroid receptors whereas at higher concentrations (>100 μM), As reactive chemistry prevails and non-specific interactions with DNA and protein causes toxicity and leads to cell death.

3. CADMIUM

Cadmium (Cd) is dispersed through out the environment primarily from mining, smelting, electroplating, and it is found in consumer products such as nickel/Cd batteries, pigments (Cd yellow) and plastics *(13)*. Tobacco smoke is one of the most common sources of Cd exposure because the tobacco plant concentrates Cd *(13)*. Smoking one pack of cigarettes a day results in a dose of about 1 mg Cd/year *(13)*. Cadmium is very slowly excreted from the body so it accumulates with time. Of all the heavy metals the most data has been collected both regarding Cd's biologic activity as well as in support of its being an endocrine-disrupting chemical *(44)*.

3.1. Cadmium Effects on Pregnancy and the Fetus

The greatest environmental Cd exposure is in the Jinzu River basin in Japan because of an effluent from an upstream mine. Maternal exposure to high levels of Cd has led to a significant increase in premature delivery *(45)*. This has led to investigation of the possible mechanisms for Cd-induced premature delivery, possibly by compromising placental function. There are enhanced concentrations of Cd in follicular fluid and placentae of smokers that are correlated with lower progesterone *(46,47)*. Cd at high concentrations inhibits placental progesterone synthesis and expression of the low-density lipoprotein receptor that is needed to bring cholesterol substrate into the cells *(48,49)*. Detailed analysis of the Cd-mediated reduction in progesterone production by cultured human trophoblast cells indicated that the decrease is not due to cell death or apoptosis. Rather, there is a specific block of P450 side chain cleavage expression and activity. This was shown by blocking P450 side chain cleavage activity with aminoglutethimide and adding pregnenolone, which was converted to progesterone by the unaffected activity of 3β HSD *(50)*.

Placental 11β HSD activity is critical to protect the fetus from maternal cortisol, which suppresses fetal growth, by converting it to inactive cortisone. Mutation or reduced expression of 11β HSD is associated with fetal growth restriction and is a significant risk factor for obesity, type 2 diabetes, and cardiovascular disease later in life *(51,52)*. There is an inverse relationship between birth weight and number of cigarettes smoked per day *(53)*. Cd accumulates in the placenta so significant amounts do not reach the growing fetus *(54)*. Of the thousands of toxic chemicals in cigarette smoke, Cd is one that has been linked to placental deficiencies *(54)*. A recent report describes that Cd at $<1\,\mu M$ reduces 11β HSD type 2 activity and expression in cultured human trophoblast cells *(50)*. Cd's effect was unique because it was not mimicked by other metal divalent cations such as Zn, Mg, or Mn *(51)*. Cadmium may downregulate 11β HSD by mimicking the ability of estrogen to attenuate the expression of this placental enzyme *(55)*. Thus, Cd environmental exposure in cigarette smoke, either first or second hand, could contribute to risk of major diseases later in life, particularly for the low birth weight fetus that was not protected from maternal cortisol.

The detrimental actions of Cd are seen at concentrations $>5\,\mu M$. For instance, in human granulosa cells collected during *in vitro* fertilization (IVF) procedures, $Cd > 16\,\mu M$ inhibited progesterone production *(56)*. However, at concentrations $<5\,\mu M$, Cd stimulates transcription of P450 side chain cleavage in porcine granulosa cells that results in greater progesterone production *(57)*. Cadmium may act to stimulate gene transcription by its high-affinity displacement of calcium from its binding to calmodulin and activation of protein kinase-C and second messenger pathways *(58)*. P450 side chain cleavage is the rate-limiting step for steroidogenesis. Thus, Cd's ability to either stimulate or suppress this enzyme could have a profound impact in all steroidogenic tissues.

In primary ovarian cell cultures from either cycling or pregnant rats, or human placental tissue, Cd at concentrations $>100\,\mu M$ suppressed progesterone and testosterone production *(59,60)*. In addition, *in vivo* Cd-treated rat ovaries exhibited suppressed progesterone, testosterone, and estradiol production in culture *(61)*. All these experiments used Cd concentrations that probably induced toxicity through one or more of numerous mechanisms such as inhibition of DNA repair, decreased antioxidants, activated signal transduction, or cell damage *(62)* rather than acting through a specific receptor or mechanism to inhibit steroidogenesis.

3.2. Cadmium and Testicular Toxicity

There are hundreds of articles describing toxic effects of Cd on the testes, as first reported in 1919 with the finding that testicular necrosis was induced by Cd *(63)*. As in the female, there is a causal relationship between cigarette Cd exposure and impaired male fertility *(64,65)*. In research models, such as rat Leydig cells, Cd is toxic to steroidogenesis but at concentrations $>10\,\mu M$ that coincide with cell death *(66)*. However, in another study, also using rat Leydig cells, $100\,\mu M$ Cd treatment doubled testosterone production with no change in cell viability *(67)*. Consistent with the *in vitro* observation of increased testosterone in the presence of Cd, chronic Cd oral exposure increased plasma testosterone in rats *(68)*. The increase in plasma testosterone was not evident until after more than 1-month exposure to Cd in the drinking water. At the same time, there was an increase in testicular weight *(68)*. In contrast, Cd given by subcutaneous injection to adult rats caused a decrease in plasma testosterone *(69)*.

Discrepancies between these studies suggest that the route of exposure to Cd affects whether it stimulates or inhibits testicular androgen production. Human Cd exposure through ingestion or occupationally also is associated with increased testosterone and estradiol *(70,71)*. Even postmenopausal women demonstrate a correlation between urinary cadmium and significantly elevated serum testosterone *(72)*. The mechanism for Cd-induced increase in human testosterone is unknown.

3.3. Cadmium as a Metalloestrogen

One of the most important studies indicating that Cd is an estrogen mimic, published in 2003 *(8)*, showed that female rats injected with Cd experienced earlier puberty onset, increased uterine weight, and enhanced mammary development. Cadmium treatment induced estrogen-regulated genes such as progesterone receptor and complement component C3. It also promoted mammary gland development with an increase in the formation of side branches and alveolar buds. In utero exposure of female offspring resulted in their reaching puberty earlier and an increase in epithelial area and number of terminal end buds in the mammary glands. Importantly the effect of Cd on uterine weight, mammary gland density, and progesterone receptor expression in uterus and mammary gland was blocked by coadministration of the antiestrogen ICI 182,780 *(8)*. Thus far, this *in vivo* study showing the reversibility of these Cd-induced effects by an antiestrogen is the most robust in supporting the conclusion that Cd is an endocrine-disrupting chemical and a putative metalloestrogen.

Evidence for Cd interaction with the estrogen receptor is the best characterized of all the heavy metals. Cd-treated MCF-7 human breast cancer cells demonstrate many responses to Cd that are the same as those elicited by estrogen. Cadmium treatment stimulates MCF-7 cell growth, downregulates the estrogen receptor, stimulates the expression of the progesterone receptor, and stimulates estrogen response element in transient transfection experiments *(73)*. In these studies, Zn treatment did not elicit these cellular responses demonstrating that Cd's effect was specific and not due to general effects of heavy metals. The specific nature of Cd's interaction with the estrogen receptor was examined in further detail *(74)*. Low concentrations of Cd activate the estrogen receptor-α by interacting non-competitively with the hormone-binding domain to block the binding of estradiol. It is notable that the ability of Cd to block estradiol binding occurs over 8 logs of concentration from 10^{-13} to 10^{-5} M but Zn at 10^{-5} M did not compete. Within the binding domain, the specific amino acids engaged by Cd are cysteines, glutamic acid, and histidine. These residues, particularly the cysteines, react with As through dithiol coordination suggesting that As and Cd share similar chemistry in interacting with the estrogen receptor. The same research group demonstrated that Cd at environmentally relevant concentrations also binds to the androgen receptor in human prostate cancer cells, LNCaP, to activate the receptor and stimulate cell growth *(75)*. As the same heavy metal Cd binds both the estrogen receptor and the androgen receptor, and in many tissues in the reproductive system expresses both types of receptors, it presents the scenario where the same metal exposure could lead to different responses depending on the relative localization and activation of the two steroid receptors in various tissues.

There are additional reports of Cd stimulating MCF-7 breast cancer cell gene transcription and increased cell growth. For instance, Cd-stimulated proliferation of MCF-7 cells is blocked by melatonin, the pineal gland indole hormone *(76)*. Cd

treatment significantly activated both estrogen receptor-α and estrogen receptor-β, with a greater effect on the estrogen receptor-α. Additionally, Cd activated the transcription factor AP-1 through estrogen receptor-α similar to the response caused by estrogens *(77,78)*. To aid identification of estrogen mimetics the cell line, T47D-KBluc, derived from a human breast cancer cell line, has been genetically modified to be a specific, sensitive estrogen-responsive gene expression assay *(79)*. Cd treatment of these cells induced gene expression as indicated by reporter gene luciferase-mediated light generation. At concentrations as low as 0.01×10^{-9} M, Cd induced a significant increase in luciferase gene expression that was completely blocked by the antiestrogen ICI 182,780 *(78)*. Cd induces at least two types of genes: (i) genes for cytoprotective proteins, i.e. metallothioneins, heat-shock proteins and Zn transporter proteins and (ii) early proto-oncogenes related to cell proliferation, i.e. *c-fos (79)*. The first type of genes are induced by Cd at 10–30 μM whereas the stimulation of cell-proliferation related genes occurs at 0.1 μM leading to mitogen-activated protein kinase (MAPK) cascade activation *(80)*. But there is a fly in the Cd ointment. Recently, it was reported that Cd is neither estrogenic, as it does not induce increased MCF-7 cell proliferation, nor does it induce phosphorylation of MAPK *(81)*. Cd was able to interact with the estrogen receptor to prevent estrogen from binding, but these investigators did not observe Cd-mediated increased transcriptional activation as was previously reported by Stoica et al. *(74)*. Therefore, further investigation is needed to clarify the interaction of Cd with the estrogen receptor and downstream consequences.

4. LEAD

Lead (Pb) is a ubiquitous environmental contaminant. In the 1940s, dietary intake of Pb was approximately 500 μg/day in the US population, but now, that intake is <20 μg/day as a result of removing or reducing the primary sources of Pb: leaded-gasoline, lead-based paints, lead-soldered food cans, and lead plumbing pipes *(13)*. Thanks, in particular to the ban on leaded gasoline in 1979, the US population Pb blood level dropped precipitously from 13 μg/dL in the 1980s to <5 μg/dL *(82)*. Lead is similar to calcium in its disposition in the body. Its half-life in the blood is 1–2 months, but depending on exposure, it can accumulate in bone where its half-life is 20–30 years *(13)*. Lead-based paint remains the most common source of Pb exposure for children <6 years old. However, acute lead poisoning as well as chronic low-level Pb exposure can come from handling and/or swallowing metallic charms *(83)*. Pb poisoning is most dangerous to children as it causes mental impairment. There is a 2–4 point IQ deficit for each μg/dL increase in blood Pb within the range of 5–35 μg/dL. Thus, the CDC has set blood lead concentrations of 10 μg/dL or greater to indicate excessive absorption in children and triggers the need for environmental assessment and remediation *(13)*. Recent data suggest that even Pb < 10 μg/dL is associated with impaired intellectual performance in children *(84)*.

4.1. Lead as an Endocrine-Disrupting Chemical in Humans and Animals

Of the five heavy metals discussed here, Pb has the strongest evidence to connect its exposure to endocrine disruption in human populations. Three independent studies indicate that environmental exposure to Pb leads to delay in growth and

pubertal development in girls. The first study found that a blood Pb concentration of 3 μg/dL was associated with delayed puberty after adjustment for body size and other confounders *(85)*. The second study found a similar relationship between blood Pb concentration and delayed attainment of menarche even after adjusting for race/ethnicity, age, family size, residence in metropolitan area, poverty income ratio, and body mass index *(86)*. The most recent study associated blood Pb with later menarche controlling for other toxicants, age, and socioeconomic status in Akwesasne Mohawk girls *(87)*. In all these studies, the relationship between blood Pb and puberty was significant even after adjusting for body size. This indicates that Pb's effect was probably direct through its impact on the hypothalamic–pituitary–ovarian axis rather than secondary to Pb-related decreased body size, which can be associated with the timing of the onset of puberty *(88)*. In the same population, exposure to polychlorinated biphenyls resulted in reduced size at birth but advanced sexual maturation, indicating that different pollutants exert effects through different physiology or endocrinology pathways *(89)*.

How does Pb exposure in children lead to delayed puberty? Research results from experiments with rats show that growth and sex hormones are altered from prenatal, lactational, and prepubertal exposure to Pb. These treatments delayed the age of vaginal opening, first estrus, and disrupted estrous cycling associated with suppressed serum levels of insulin-like growth factor-1 (IGF-1), a liver hormone involved in growth and reproduction *(90–92)*. Moreover, Pb affected hormones and responsiveness of all levels of the hypothalamic–pituitary–ovarian axis *(93,94)*. Dietary Pb may delay the onset of puberty in female mice, as observed in rats *(95)*, although by contrast, very low levels of dietary Pb, 0.02 ppm, were associated with a marked and significant acceleration of puberty in mice *(95,96)*, indicating an effect of dose on the pubertal outcome. In the last decade, puberty onset has advanced in the USA even in children migrating from foreign countries in Western European countries. It has been suggested that environmental endocrine-disrupting chemicals may be contributing to the earlier onset of puberty *(97)*.

4.2. Lead Effects on the Ovary and on Steroidogenesis

There are few studies of the direct effect of Pb on ovarian steroidogenesis. Lead exposure *in vivo* in cynomolgus monkey suppressed circulating luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol without affecting progesterone or causing overt signs of menstrual irregularity *(98)*. Prenatal and neonatal Pb exposure resulted in suppressed rat ovarian homogenate Δ4 androgen production whereas 5α-reduced androgens were increased *(99)*. There are more studies that examine changes in follicle populations after Pb exposure. For instance, mice exposed to Pb *in utero* experienced a significant reduction in the number of ovarian primordial follicles *(100)*. Adult mice given Pb by gavage for 60 days had significant changes in ovarian small, medium, and large follicle populations *(101)*. But, in another study, Pb was given by injection, and there was no change in either antral follicles but decreased primordial follicles and increased growing and atretic follicles *(102)*. Accelerated elimination of ovarian follicles, common to the above-cited reports, will ultimately lead to premature ovarian failure if the reduced follicle pool is the non-regenerating primordial follicles or disrupted cycles if the growing follicles are targeted.

Lead treatment of culture human ovarian granulosa cells retrieved during IVF reduces mRNA and protein levels of both P450 aromatase and estrogen receptor-β *(103)*. However, the mechanism responsible for Pb suppression of these two targets is unknown. Another molecular target of Pb is steroidogenic acute regulatory protein (StAR) that mediates the transfer of cholesterol into mitochondria *(104)*. Female rats exposed to Pb *in utero* have decreased basal ovarian StAR mRNA and protein but this is reversed by stimulating with gonadotrophins before collecting the ovaries. On the basis of these results, it was concluded that Pb acts at the hypothalamic–pituitary level of the reproductive axis.

4.3. Effects of Lead on Testicular Function and Steroidogenesis

Substantial research has been conducted regarding the specific effects of Pb on testicular and male reproductive function *(105)*. Pb accumulates in male reproductive organs. Exposure to Pb causes altered and delayed spermatogenesis accompanied by decreased fertility. For instance, in one IVF clinic, >40 % of the males who were not exposed to Pb occupationally and did not smoke cigarettes had blood and seminal plasma Pb concentrations greater than the permissible limit in men who are exposed to Pb in the work place *(105)*. In fact, there was an inverse relationship between Pb concentration in blood and seminal plasma and rate of fertilization that was due to altered sperm function *(105)*. The progesterone-dependent acrosome reaction was the sperm function most affected by the presence of Pb.

Lead inhibits both Sertoli and Leydig cell steroid production at every step of synthesis. Expression and/or activity of gonadotrophin receptors, StAR, p450 side chain cleavage, 3β HSD, and P450c17, the enzyme that converts progesterone into testosterone, are significantly if not dramatically suppressed by Pb *in vivo, ex vivo*, or *in vitro (106–110)*. Given the high concentrations of Pb used in all of these studies and often more severe inhibition with greater length of exposure, it is likely that the suppression of steroidogenesis at these step is due to toxicity rather than specific action or mimicry of an endocrine hormone.

4.4. Lead and Sex Ratios

Lead has been implicated in shifting the sex ratio to fewer boys born and maybe related to low testosterone at the time of conception *(111)*. Professional drivers exposed to excessive petroleum products father fewer sons *(112)*. Consistent with the occupational exposure to Pb leading to fewer boys born also is observed in filling station workers *(113)*. Both these reports suggest a relationship between occupational exposure to Pb and reduced male sex ratio, although this area is still controversial *(114)*, in part because of separating the consequences of exposure of one or both parents *(112)*. The most recent brief report revealed a dose–response with Pb such that workers with higher blood Pb levels had significantly reduced odds of having male offspring *(115)*. These data are intriguing, but much more investigation is needed to support or reject the hypothesis that Pb exposure impacts paternal-related sex ratio.

Lead can activate estrogen receptor-dependent transcriptional expression assay and stimulate MCF-7 breast cancer cell growth. In both these assays, Pb was not very effective and far less efficient than Cd *(34)*. Specific interaction of Pb with estrogen receptor was not determined in these studies, leaving me to conclude that there

is insufficient evidence to conclude that Pb is an estrogen mimetic. However, the effects of subtoxic doses of Pb on reproductive functions described above support Pb as an endocrine-disrupting chemical whose specific mechanisms need to be determined.

5. MERCURY

Depending on its chemical form mercury (Hg) can be very toxic. Occupational exposure leads to neurodegeneration, behavioral changes, and death. Over 400 years ago, mercuric nitrate used in the felt hat industry gave rise to the phrase "mad as a hatter" depicted by the Mad Hatter in Lewis Carroll's *Alice's Adventures in Wonderland (13)*. Tragically, a recent accidental dimethylmercury poisoning reaffirmed the dangers associated with working with Hg *(116,117)*. Large-scale Hg poisonings have occurred in Minamata, Japan, and Iraq by industrial or inadvertent introduction of Hg into the food chain *(118)*. Industrial uses of Hg range from laboratory, dental, thermometers, paints, electrical equipment, and chloralkali. For the general public, there are three major sources of environmental Hg: fish consumption, dental amalgams, and vaccines *(119)*.

Fish contaminated with Hg poses a serious health risk to pregnant women and their babies. Methyl mercury bioaccumulates and biomagnifies in muscles of predatory fish that are at the top of the food chain, such as albacore *(116)*. Most concern is centered on neurological and behavioral problems that occur following exposure of the fetus or newborn, as Hg easily crossing the placenta and passes through the undeveloped infant blood–brain barrier. Thimerosal is an ethyl mercury-based vaccine preservative used since the 1930s *(116)*. It has received much attention of late because of its possible link to autism, but this is unproven and very controversial.

5.1. Mercury and Reproduction

Women exposed to Hg at work have been reported to experience reproductive dysfunction. Occupational exposure to mercury either in mercury vapor lamp factor or in dentistry is associated with menstrual disorders, subfecundity, and adverse pregnancy outcomes *(120,121)*. Interestingly, at very low Hg exposure, women working in dentistry were more fertile, suggesting a U-shaped dose–response curve *(122)*. Much more research is needed to establish a causal relationship between occupational Hg exposure and compromised fertility *(123)*.

Animal studies provide some insight into the impact of Hg exposure on female reproduction. In hamster, subcutaneous mercuric chloride treatment disrupts estrous cycles, suppresses follicular maturation, reduces plasma and luteal progesterone levels, and may disrupt hypothalamus-pituitary gonadotrophin secretion *(124)*. Similar changes were observed in female rats exposed to Hg vapor. The estrous cycle was lengthened, and morphological changes were evident in the corpora lutea, but ovulation, implantation, or maintenance of first pregnancy was unchanged *(125)*.

In wildlife, it has been proposed that Hg exposure has been responsible for increased cryptorchidism in the Florida panther as a result of exposure through bioaccumulation *(126)*. The authors report no significant difference between serum estradiol levels in male and female panthers, suggesting demasculinization and feminization of males. However, it is important to consider that the reproductive impairment and

cryptorchidism may be genetically rather than environmentally based because there is limited genetic variation in the remaining Florida panther population *(127)*. Nevertheless, based on analysis of panther hair from museum collections, there is no doubt that current Florida panther Hg levels have increased since the 1890s *(128)*.

Investigation of the impact of either intraperitoneal injection of mercury or oral dosing of male rats or mice reveals consistent changes in the reproductive system. For instance, in rats exposed for 90 days to Hg by i.p. injection, testicular steroidogenesis was suppressed at the 3β HSD synthetic step with a significant decrease in serum testosterone and LH *(129–131)*. Oral exposure of rats to mercuric chloride for 45 days resulted in suppressed testosterone and increased testicular cholesterol *(132)*. The authors suggest that the increased cholesterol is due to the block of its biosynthetic conversion to sex steroid hormones such as testosterone. Another possibility is that Hg mimics the effect of estrogen on the testes which is to both inhibit androgen production and cause accumulation of cholesterol probably because of upregulation of the high-density lipoprotein (HDL) receptor, scavenger receptor class B, type I (SR-BI) *(133)*.

5.2. Estrogenic Mechanisms of Mercury

The estrogenicity of Hg was examined in MCF-7 cells *(34)*. Mercuric chloride stimulated both estrogen receptor-dependent transcription and increased proliferation of MCF-7 cells *(34)*. A more detailed study of the methyl mercury impact on MCF-7 cells was performed by Sukocheva et al. *(134)*. In this study, instead of measuring increased number of MCF-7 cells, the number of postconfluent foci that formed with estrogen treatment was counted. Multicellular foci form in response to estrogen agonists and are proportional to hormone dose or concentration *(135)*. A very narrow concentration range, 0.5×10^{-7} to 1×10^{-6} M, of methyl mercury stimulated MCF-7 cell foci formation but did not reach the maximum response elicited by estradiol, indicating that Hg is a weak estrogen mimic. Hg exhibited estrogen receptor agonist–antagonist properties depending on concentration. Its stimulation of foci formation is blocked by the antiestrogen ICI 182,780. However, Hg is poor at competing for ^{3}H estradiol binding to recombinant estrogen receptor as displacement was only observed and 10^{-4} M. As is the case with estradiol, Hg stimulated Erk1/2 activation that was dependent on mobilization of intracellular Ca^{+2}, suggesting similar signaling mechanisms. Methyl mercury reacts with sulfhydryls and could interact with protein thiol groups such as those located in the ligand-binding domain of the estrogen receptor to stimulate MCF-7 cell proliferation. The Erk1/2 activation pathway is involved in cell proliferation and gene expression. A number of extra cellular signals have been shown to induce MAPK Erk1/2 including many other well-known endocrine-disrupting chemicals such as bisphenol A *(136)* (see Chapter 2 by Soto, Rubin, and Sonnenschein).

6. URANIUM

Uranium (U) is the heaviest naturally occurring element, certainly qualifying it as a heavy metal. Similar to Cd, U was used initially for its pigmentation in bright orange-red Fiestaware. Uranium was the first element to be identified as fissile. Uranium supports nuclear chain reactions leading to the development of atomic weapons and later as a fuel for nuclear reactors. Its current use is as depleted uranium (DU) in armed

conflict first in the Balkans and now Iraq for both munitions and armor. Environmental sources and thus risk of contact with U are mining, production of nuclear weapons, nuclear reactor industry and disasters, and US-facilitated armed conflict.

Nuclear accidents at Three Mile Island and Chernobyl quashed hopes of developing nuclear energy to replace dependence on fossil fuels for decades. With today's dwindling petroleum supplies and significant green house gas emissions from coal-produced energy, interest in nuclear energy is renewed. Australia has the world's largest U reserves, but Canada is the largest U exporter. In the USA, the greatest U reserves are in the southwest, specifically the states of the Four Corners, AZ, CO, NM, UT, where the Navajo Reservation Nation is located.

6.1. Uranium, the Navajo Reservation, and Human Exposures

The Navajo Reservation is the largest Native American reservation in the USA. It covers over 27,000 square miles. The Navajo Nation comprises 110 Chapters, the political/social units. According to the 2000 census there are 250,000 members of the Navajo tribe, making it the largest Native American tribe in the USA. This group continues to live its traditional lifestyle and rely on its native language to sustain and nurture the "Navajo way." Over 170,000 people live on Reservation and the majority of the remaining tribal members live in "border towns" such as Farmington, NM, and Flagstaff, AZ. Fifty four percent of households have no indoor plumbing or running water necessitating half of the Reservation households to haul water from the nearest available source. The Navajo Reservation evokes images of a peaceful and healthy environment, but the reality of environmental contamination presents a different picture.

From the 1945 through 1988 there was intensive U mining and milling *(137)*. In 1980, the market price for U crashed and effectively ended the mining. However, the market value of U ore has quintupled in the last four years because of renewed interest in nuclear energy and the ongoing need for depleted U for armed conflict. Thus, U mining is on the upsurge, with 700 claims filed 2005 in the northern part of AZ. At the writing of this chapter, U is poised to climb to a record high of $50/lb in the next 6 months stimulating revived interest in mining on the Navajo Reservation.

Well-documented health problems that arise from U mining and milling are lung cancer and respiratory diseases *(137,138)*. These health problems result from radiation exposure after inhalation of radon-rich dust released during U mining. Hundreds of Navajo and Mormon miners and ore truck drivers have developed these diseases, and if they can document their work experience with pay stubs or other evidence they may qualify for compensation from the Federal Radioactive Exposure Compensation Act (RECA). However, many Navajo miners do not have the documentation to prove their employment history and die before they can be compensated *(137)*.

It is estimated that there are over 1500 abandoned mines on the Navajo Reservation that have not been properly closed, allowing U to be dispersed by the elements over the last 62 years throughout the natural environment. The US Army Corps of Engineers performed a survey of water sources in 30 Chapters on the Navajo Nation, and the results are posted by USEPA on this website http://yosemite.epa.gov/r9/sfund/r9sfdocw.nsf/vwsoalphabetic/Abandoned+Uranium+Mines+On+The+Navajo+Nation?OpenDocument. In every chapter surveyed, there was at

least one water source with U water levels that exceeds the USEPA safe drinking water level of 30 μg/L. Because half of the households on the Reservation haul water, it is certain that many people are exposed to unsafe levels of U in their drinking and household water.

Health problems that result from drinking U-contaminated water include kidney disease, kidney cancer, and possibly stomach cancer *(138)*. Kidney disease results from U's heavy metal poisoning of the proximal tubules and interferes with glucose uptake *(139)*. Cancer in the kidney, stomach and bone marrow results from U-derived alpha radiation causing DNA damage leading to cell transformation and cancer. Owing to its chemical properties, U homes to bone and bioaccumulates, leading to increased risk of leukemia. Other routes of exposure are inhalation of small particles blown from tailings or dust and dirt brought into the home on the miner's clothing often washed with the clothes of other family members *(137)*. Depending on size, the particles can enter the bloodstream leading to the above-mentioned diseases. Allowable exposure limits vary for U. The USEPA safe drinking water limit of 30 μg/L is based on economic feasibility while the World Health Organization standard of 2 μg/L is based on the fractional source of U assuming that an adult drinks 2 L of water a day. However, a recent study suggests that even low U concentrations in drinking water can cause nephrotoxic effects, and after long-term ingestion, elevated concentrations of U in urine can be detected up to 10 months after the exposure has stopped *(140)*.

6.2. Uranium as an Endocrine-Disrupting Chemical on Reproductive Systems

Numerous studies have investigated the reproductive toxicology of both U and DU in experimental mice and rats *(141–143)*. The original study conducted by Maynard and Dodge *(141)* showed that breeder rats consuming chow containing 2 % uranyl nitrate hexahydrate (UN) for 7 months gave birth to half as many litters as the control chow-fed rats. The litter size of the U-consuming rats was significantly less as well. For the next 5 months all rats ate control chow; however, the female rats that had been exposed to UN still only produced less than half as many litters as the rats that were on control chow for the entire 12 months. Rats eating U-containing chow also had irregular estrous cycles compared with the control chow-fed rats. Even though the U-fed rats lost weight, which could have contributed to their reduced fertility, the fact that after the rats were returned to control chow and regained weight, but they still produced half the number of litters indicates that the impact of U was permanent.

Several studies have documented U's toxicity in the male reproductive system. General features reported are degenerative changes in the testes such as aspermia in the testes and epididymis, testicular atrophy, interstitial cell alterations, Leydig cell vacuolization, and reduced successful female mouse impregnation *(142,143)*. DU exposure in human has been a result of warfare. Gulf war 1990–1991 UK veterans may have impaired fertility, and pregnancies took longer to conceive *(144)*. Also, there was a 40 % increased risk of miscarriage among pregnancies fathered by men who served in the first Gulf war *(145)*. Another cohort of US Gulf war veterans struck by friendly fire had DU shrapnel embedded in muscle and soft tissue and were still excreting greater than background levels of U 9–11 years after sustaining their injuries *(146)*.

A follow-up of 30 friendly fire cohort members found evidence of subtle perturbations in the reproductive system indicated by significantly elevated prolactin levels *(147)*. In another DU-exposed friendly fire cohort, there were significantly elevated sperm counts and a higher percentage of progressive spermatozoa among veterans excreting high levels of U as compared with veterans excreting low levels of U in their urine *(148)*. In a different population, Czechoslovakian uranium miners fathered significantly more girls than boys, suggesting a shift in the sex ratio *(149)*. It is important to consider that these reports are preliminary, and there is no evidence to suggest that altered male fertility is caused by exposure to U or DU.

U exposure of pregnant rodents produces maternal toxicity, fetal toxicity, and developmental defects *(150)*. There are more absorptions, dead fetuses, fewer live-born fetuses, and pup body weight and length were significantly reduced. In addition, there is a higher incidence of cleft palate and dorsal and facial hematomas *(150)*. In humans, there is sufficient epidemiological evidence to suggest an increased risk of birth defects in offspring of persons exposed to DU *(151)*. On the Navajo Reservation exposure to environmental U was statistically associated with uranium operations and unfavorable birth outcome, including cleft palate and craniofacial developmental defects, if the mother lived near mine tailings or mine dumps *(152)*. Again, the possible association of environmental U exposure and adverse human health outcomes is correlative, and more research is needed to draw any casual connections.

6.3. Uranium as an Environmental Estrogen

Probably because of U's radioactive nature, it has been studied intensely with regard to harmful effects from its ionizing radiation and chemical toxicity but had not previously been tested for its potential estrogenic activity as a heavy metal. Recently, we have discovered that U is estrogenic in female reproductive tissues *(153,154)*. The source of U in our *in vivo* and *in vitro* studies is UN and is DU. It should be noted that natural U and DU have the same chemistry, and therefore, both will cause the same changes that are dependent on chemical properties.

Although there are many publications describing the effects of U exposure on reproduction in female, none of the reports mentioned the impact of U on ovarian follicle populations *(141–143)*. We wanted to determine whether U exposure would target a specific ovarian follicle population or more likely cause a non-specific general ovarian toxicity. In our original studies, high levels of UN at mg/L in the drinking water were consumed for 30 days by intact immature female mice. UN exposure specifically targeted primary follicles whose number was reduced. Unexpectedly, in these mice, there was a trend, although not statistically significant, of increased uterine weight. In this experiment at these high doses of U, kidney weight was reduced, consistent with U's well-known nephrotoxicity *(139)*. Intrigued by the possible uterotrophic effect of U at mg/L concentrations, we next tested whether μg/L levels would cause uterine weight to increase in ovariectomized mice *(155)*. Ovariectomized mice that drank UN-containing water for 10 days at concentrations starting at the USEPA safe drinking water level of 30 μg/L down to 0.3 μg/L had significantly increased uterine weights *(153)*. If the mice were treated with the antiestrogen ICI 182,780 while drinking the U-contaminated water, the increase in uterine weight did not occur. We concluded from this experiment that U at low, environmentally relevant concentrations, acted like estrogen. Another biological response elicited by estrogen in ovariectomized immature

mice is accelerated vaginal opening. This response was also observed in mice drinking U-contaminated water that was again prevented by the coincident treatment with ICI 182,780 *(153)*. Finally, we observed the persistent presence of vaginal cornified epithelial cells from immature ovariectomized mice drinking U-contaminated water, and as before, the antiestrogen ICI 182,780 ablated this response. Cornified epithelial cells from vaginal smears are an indication that there is an ongoing estrogenic stimulation of the reproductive tract *(156)*. In all these experiments, we compared the responses induced by U to those caused by the potent synthetic estrogen DES. The magnitude of the responses were comparable at equivalent molarity for U and DES, indicating that U is a potent estrogen mimic *in vivo*.

Our preliminary results analyzing the putative estrogenic activity of U *in vitro* using MCF-7 breast cancer cells indicate that UN at nanomolar to low micromolar concentrations stimulates cell proliferation *(154)*. The estrogen receptor was involved because the antiestrogen ICI 182,780 blocked both 17β-estradiol and UN-stimulated MCF-7 cell proliferation. Cell proliferation is a response that takes days to occur through the classic estrogen receptor-dependent genomic pathway. Recent reports indicate that estrogen-mediated responses can occur in just a few minutes *(157)*. To detect whether rapid responses occurred in DES- or UN-treated MCF-7 cells, we used scanning electron microscopy to visualize cell surface morphological changes within minutes of exposure. Both DES and UN treatment caused a significant increase in number and branching of MCF-7 cell surface microvilli, and the cell surface changes were blocked by ICI 182,780 *(154)*. The rapid morphological changes suggest that, similar to estrogen, UN causes cell responses independent of genomic responses *(158)*.

As with the other heavy metals, low concentrations ($<10\,\mu M$) mediate specific responses for instance through the estrogen receptor or other steroid receptors, whereas higher concentrations ($>100\,\mu M$) cause non-specific toxic responses. For instance, in Chinese hamster ovary cells, uranyl nitrate killed the cells and induced chromosome aberrations and sister-chromatid exchanges *(159)*. Uranyl acetate at $200\,\mu M$ killed and mutagenized Chinese hamster ovary cells perhaps by causing DNA strand breaks and forming uranium-DNA adducts *(160)*. Thus, depending on the environmental level of U and exposure or contact, the biologic responses can range from subtle through steroid receptor interaction and activation to striking through cytotoxic and genotoxic mechanisms. The concentrations of U and oral route of exposure at which we see estrogenic responses *in vivo* and *in vitro* are environmentally relevant to exposures on the Navajo Reservation as well as other communities in the USA where U is in drinking water at levels at or below the USEPA safe drinking water limit.

7. CONCLUSIONS

Historically, heavy metal toxicity targeting the kidney or nervous system has been the focus of most research efforts. However, accumulating evidence, particularly when studies are performed with low micromolar concentrations, indicates that heavy metals can act as endocrine-disrupting substances through specific, high-affinity pathways. Thus far, heavy metals primarily have been described to interact with the estrogen receptor giving rise to the term metalloestrogens. This is just the beginning of our understanding of the subtle means by which heavy metals disrupt endocrine function—many more pathways need scrutiny *(161)*.

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