Role of Herbicides & Pesticides on Endocrine Disruption

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INTRODUCTION

Exposure of humans and feral animals to natural and synthetic chemicals having potential endocrine disrupting properties has become a major concern in the field of environmental toxicology (1). This concern is not suddenly new, as public attention was originally drawn to the idea by the publication of Rachel Carson's *Silent Spring* in the 1960's. This book described deleterious reproductive effects of the then-commonly used insecticide DDT on birds and other wildlife. However, the first published evidence for environmental disruptors appeared more than a decade earlier, when it was reported that consumption of a certain type of clover disrupted reproduction in Australian sheep. Subsequent to these studies, a large number of chemicals that act, at least partially, by binding the estrogen receptor, have been identified. However, more recently, various other chemicals have been identified that mimic or inhibit the actions of other hormones, such as androgens, progestins, and thyroid hormone.

While evidence exists for adverse effects of endocrine disruptors on wildlife populations (2, 3), there is as yet only limited evidence that links environmental exposure to these substances and adverse effects on human health. Because wildlife and feral animals appear to provide the first evidence of environmental endocrine effects, it might be argued that wildlife and domesticated animals are sentinel species, which should emphasize the critical nature of this topic, and the necessity to more clearly understand potential effects of endocrine disrupting chemicals on human health. Such concern is further magnified when we consider the potential exposure to just the chemicals produced by industrial agriculture in the United States. For example, it was recently claimed that 70% of the waterway pollution comes from chemicals seeping into farm land and that only about 1% of the pesticides actually reach their intended pests (4). This presentation will focus on endocrine effects in wildlife, recent work with chemicals in river waters of Japan and potential effects of chemicals on development and reproduction in feral animals (2). Finally, we will conclude with new research on the male, which demonstrates that estrogen receptor targets are present in androgen sensitive tissues of the male reproductive tract.

POTENTIAL ENDOCRINE DISRUPTOR-RELATED OUTCOMES IN WILDLIFE

Environmental chemicals known as potential endocrine disruptors often interact with receptors derived from the steroid, thyroid and retinoid gene family. They include ubiquitous and persistent organochlorines, such as PCBs, dioxins and pesticides, as well as plasticizers, pharmaceutics, and natural hormones (1, 5). The best example of a direct link between a commonly used herbicide and endocrine disruption is atrazine. A recent report in the Proceedings of the National Academy of Science clearly demonstrated that atrazine at concentrations found in the environment <50 parts per billion (ppb) induced hermaphroditism of the gonads, decreased concentrations of testosterone and demasculinization of the male larynx in the frog (6). This study is important because atrazine is one of the most abundant herbicides

used in the U.S. (7) and its production and use correlate with the onset and decline in amphibian species (8) and frog populations worldwide (6). It is clear that amphibians cannot tolerate the safety standard of atrazine set for humans in drinking water.

Several other examples of intersex and sex reversal, hypothesized to be induced by environmental chemicals, have been reported in wildlife: female rock shells develop vas deferens and a penis following tributyltin (TBT) exposure; some fish species in the UK have ova in the testis and elevated plasma concentrations of the yolk precursor protein vitellogenin (VTG) in males; 50% of male carps caught in the Tama River, Japan, produce VTG; and a male-like gonopodium develops in female mosquito fish in Florida exposed to pulp mill effluent (see reviews by 2, 9). As with other animals, reptiles are also sensitive to environmental endocrine disruptors. For example, in alligators and turtles, sex determination is influenced by the temperature of the nest during incubation, but also is highly sensitive to exogenous estrogens, including estrogenic chemicals in the environment. Alligators in several contaminated lakes in Florida, USA, show small penis development and depressed plasma T concentrations (10).

In the Japanese aquatic environments, significant levels of chemicals, such as BPA, NP, OP were found in the 109 rivers studied. However, all of these chemicals were degraded by sewage treatment. E_2 was found in the effluent. In sediment samples collected, NP, DEHP, BPA and E_2 were found. Dioxins were found in whales, finless dorphins, kites, cormorants, field mice and raccoon dogs. Dioxin levels were higher in birds living in urban areas than in rural areas.

ESTROGEN RECEPTORS AND DEVELOPMENTAL ESTROGEN EXPOSURE OF THE FEMALE REPRODUCTIVE TRACT

It is now abundantly clear that the most sensitive period for endocrine disruptor chemical exposure is during development (11). Adult effects are less severe and even less likely to occur, simply because the embryo and fetus are experiencing rapid growth and change. Thus, even a small change in the expression of a hormone or receptor during development can be amplified into major endocrine disruption in the adult. After differentiation of the gonads, androgen from Leydig cells stimulates Wolffian ducts and Müllerian inhibiting hormones from Sertoli cells degenerates Müllerian ducts in male mice. Mice exposed antenatally to estrogens provide a model for exploration of the consequences of DES exposure in the human, because mouse genital tract development at birth is similar to that of the human fetus at the end of the first trimester (2, 9, 12-14). In female mice, ovary produces estrogen around 14 days of age. Estrogen receptors (ER) are not expressed in neonatal mouse uterine epithelial cells, but are expressed in stromal cells at the day of birth (day 0). However, early induction of ERα in uterine epithelial cells and vagina was caused by neonatal injection of 17 -estradiol (E_2) or DES in a dose-dependent manner (15, 16). There are species differences in response, with E_2 treatment of ovariectomized adult rats resulting in a reduction of ER mRNA levels, while in mice, DES acts as an ER inducer in the uterus and vagina in both neonatal and ovariectomized adult mice (16). There are also differences between E_2 and DES treatments, but overall the important point is that disruption of ER expression in the female tissue, whether an increase or decrease, is potentially as serious response to estrogenic chemical exposure.

The critical windows of induction of various abnormalities by postnatal estrogen exposure varied from 3 to 30 days, indicating the presence of tissue/organ specificity in mice. In order to understand the molecular basis of estrogenic actions, and effects of hormonally active chemicals on developing organisms, we need to understand the link of exposure levels of chemicals, genes responsive to chemicals and adverse effects of chemicals. In addition, we need

to study effect of chemicals on various species from invertebrates to mammals on the basis of not only toxicology but molecular biology. The utility of the neonatal mouse model in indicating permanent, long-term changes, both overt and cryptic, in a variety of structures, both reproductive and non-reproductive, is emphasized, with particular attention herein to newer information of estrogens on ERs expression, oncogene and *Hox* gene expression, ovarian abnormalities, fertilizability, and skeletal and muscular tissues.

Ontogenic expression and localization of ERα and ERβ in fetal female rat Müllerian duct demonstrated ERα expression on gestational days (GD) 15.5 and an increase of 4.4-fold by GD 21.5. Both ERβ1 and ERβ2 gene expression were at low levels compared to ERα during development (17). ERα nuclear immunolocalization was predominantly seen in the proximal Müllerian epithelium, and middle and caudal Müllerian mesenchyme on GD 15.5-21.5. Staining intensity for ERα increased with development in all regions. However, ERβ immunoreactivity was not detected in any region during prenatal life. These findings provide fundamental information critical for clarifying the species-specific physiological roles of ER subtypes during fetal development and investigating the tissue-specific mechanisms underlying the prenatal response to estrogen and estrogen agonists.

One of the potential mechanisms for DES effect on the female reproductive tract is the primary response of epidermal growth factor (EGF) and its receptor (EGF-R). DES (100 μg/kg BW) injected from GD 15 to 19 in the rat increased Müllerian duct cell proliferation in the epithelium and mesenchyme of the proximal region, but decreased cell division in the caudal epithelium at GD 19.5 (18). No proliferative effect was observed in any cell types in the middle region. Müllerian duct EGF immunoreactivity was suppressed by DES. Expressions of mRNAs for EGF, ER 1 and ER 2, but not ERα and EGF-R were also decreased. These results indicate that EGF could be an important regulatory factor of Müllerian duct cell proliferation, and that DES could alter cell proliferation by disrupting normal EGF, ER 1 and ER 2 expression in the developing female rat reproductive tract.

Effect of bisphenol-A (BPA) at low doses was examined using SD rats to address the questions; whether *in utero* exposure affects the vagina of the offspring and which mechanisms cause the toxic effects (19). Pregnant SD rats were administered either 0.1 (low dose) or 50 mg/kg per day BPA, the no observed effect level, or 0.2 mg/kg per day 17 -ethynyl estradiol (EE2) by gavage. Striking morphological changes were observed in the vagina of postpubertal offspring. Full-length ERα was not expressed during estrus in the vagina of female offspring exposed to either dose of BPA when compared to the control group, whereas ERα expression does not differ from the control group during the diestrus stage. ERα downregulation seems to be responsible for the observed altered vaginal morphology.

ESTROGEN AND ITS DISRUPTION IN THE MALE

Estrogen is produced in sizable quantities in the testis, as well as the brain and is also present in very high concentrations in the semen of several species (see review by 20). Early studies reported that the primary source of estrogen in the immature male was the Sertoli cell. In the adult testis, Leydig cells express aromatase (P450arom) and actively synthesize estradiol at a rate much greater than that seen in the adult Sertoli cell (see reviews by 20, 21), but there is a growing body of evidence indicating that germ cells also synthesize estrogen, and possibly serve as the major source of this steroid in the male reproductive tract of several species (20-24). Others have shown the absence of aromatase in the epididymis (25); thus, the conversion of androgens to estrogens by sperm remains the primary source of estrogen in the lumen of the

tract.

The concentration of estrogens in peripheral blood is typically low in the male, but ranges from 2-180 pg/ml depending upon the species. The horse is an exception, where estrone sulfate is found as high as 2,447 pg/ml (26). Estrogen concentrations are typically higher in the testicular vein and lymph than in the general circulation. Also, in the reproductive tract, estrogen concentrations can reach relatively high concentrations. In one report, estrogen concentration in rete testis fluid of the rat was approximately 250 pg/ml (27), which is higher than the average serum concentration of estradiol in the female (28).

In an effort to elucidate the role of estrogen in the male, the aromatase gene knockout mouse (ARKO) has been used as an alternative model to ERα knockout (29, 30). Lacking the enzyme that converts androgens to estrogens, these mice show an apparent normal development of the testis but with aging there is progressive infertility and impairment of spermatogenesis (30). Infertility was in part due to an inhibition of reproductive mounting behavior. Even though seminiferous tubules dilation or atrophy were not observed in ARKO males, a significant decrease in seminiferous tubules thickness at stage VII was described, suggesting that maybe some rete testis fluid had accumulated in the testis. Rats treated with an aromatase inhibitor partially resembled the Estrogen receptor--α knockout (αERKO) mouse and the ICI-treated rat by showing an increase in testis weight. However, the efferent ductules did not show a consistent response, suggesting that the aromatase inhibitor may not have completely inhibited the enzyme in testicular cells (31). In any case, estrogen receptors are present in ARKO and aromatase inhibitor-treated mice, so the possibility of non-ligand ER transactivation cannot be ignored.

ESTROGEN RECEPTORS AND DEVELOPMENTAL ESTROGEN EXPOSURE IN THE MALE REPRODUCTIVE TRACT

It has been known for at least 25 years that an estrogen receptor-like protein exists in the male reproductive tissue (32). This estrogen receptor was shown to have the highest concentration in the cauda epididymis but a tissue-specific protease in the adult rabbit caput epididymis was capable of decreasing nuclear binding of the ER protein. Further studies showed that the protease was responsible for removing the DNA-binding portion of the ER, thus making estrogen less active in the adult epididymis (see review by 33). Because of this discovery, Danzo concluded that estrogen was more likely to be important during development of the epididymis than in adult function.

Using immunohistochemistry, ER subtypes, α and β, have been localized primarily in the epithelium of efferent ductules but also in some species in the testis and epididymis (20, 34-38). In the mouse at 90 days of age, the efferent ductule epithelium was strongly positive for ERα immunostaining (36). Other epithelia along the epididymis were only slightly positive. The vas deferens has shown no epithelial reactivity in any species, but stromal nuclei have been positive. Iimmunostaining for ER in the mouse provides results similar to autoradiography data previously shown by Schleicher (39). In the rat, ERα localization has been controversial. In one study, ERα positive staining was found only in testicular interstitial cells and epithelial cells of the rete testis and efferent ductules (34). The epididymal tissues were negative. Another study using different antibody showed epididymal staining, too (20). Autoradiography and estradiol binding assays indicate that ER is present in the rat epididymis. RT-PCR data also show that ERα is present in epididymal tissues (40). Therefore, future studies should focus on *in situ* hybridization methods for localizing the mRNA in specific regions and cell types of the epididymis. ER have also been reported in human reproductive tract but not in the testis (41).

Estrogen receptor expression in the male dog and cat provides an excellent comparison in domesticated animal species. In the dog testis, ERα was found in interstitial cells and peritubular myoid cells, but in the cat only interstitial cells were positive (38). In rete testis of the dog, epithelial cells were positive for ERα staining, but in the cat, rete testis epithelium was only weakly positive. In efferent ductules of the dog, both ciliated and non-ciliated cells stained intensely positive, but in the cat, ciliated epithelial cells were only slight positive. Epithelial cells in dog epididymis and vas deferens were completely negative for ERα. However, in the cat, except for the initial region of caput epididymis, ERα staining was positive in the epithelial cells of epididymis and vas deferens. ERβ was positive in multiple cell types of both species. In rete testis and efferent ductules, epithelial cells were weakly positive for ERβ. Most epithelial cells of the epididymis and vas deferens were strongly positive in both species. Co-localization of both ERα and ERβ in efferent ductules was noted in both species. As in all other species, the efferent ductules remained the region of highest concentration of ERα (38).

During development, ERα shows differential expression in the male reproductive tract. Estradiol binding is seen first in epithelia of the efferent ductules on day 16 of gestation in the mouse, while Wolffian ducts and urogenital sinus do not bind estradiol (42). Stromal cells of the efferent ductules, epididymis and other reproductive organs in the male remain ER+ at later times. The epididymis begins expressing epithelial ER soon after its formation on day 19 of gestation. There is a distinct gradient of binding in the developing epididymis, with the efferent ducts and the initial segment of the epididymis containing 3-fold more silver grains per epithelial cell than the more distal regions. Others have shown similar results in the mouse and guinea pig. At birth in the mouse, only the efferent ductule epithelium stains positive for ERα, but stromal cells in the vas deferens are slightly positive (36). At 20 days of age, the efferent ductule epithelium becomes even more positive and select cells of the epididymis begin to express ERα (36). In fetal rats, ERα is immunopositive in cells surrounding the mesonephric tubules, but postnatally the epithelium of the rete testis and efferent ducts at all ages was strongly positive. The epididymal duct was negative at all ages using the 6F11 antibody (34). Overall, ERβ is more widely distributed in the male reproductive tract than ERα. When ERα is negative, ERβ appears to be present. The stromal tissue cells are stain strongly positive for ERβ throughout the male reproductive tract. Thus, we must conclude that the potential for estrogen binding in the epididymis and vas deferens is unequivocal, either through ERα or ERβ.

The effects of diethylstilbestrol (DES) exposure on development and function of the male reproductive tract include cryptorchid testis, testicular atrophy, epididymal cysts, sperm abnormalities, sperm granulomas, adenocarcinoma, prostatic inflammation, and other changes that could affect fertility and male reproductive function (43). Experimental exposure to phthalate esters also induces developmental defects in the male reproductive tract, which are similar to the DES effects (11, 44). It is also of interest that the incidence of cryptorchid testes, testicular cancer and hypospadias are increasing in the human population in certain countries, which has led others to hypothesize that environmental estrogen exposure during development could be an underlying theme that that is common between the human studies and experimental data (11).

One interesting discovery has been the persistence of Müllerian remnants or portions of the female reproductive tract adjacent to the male tract, which apparently interferes with the development of the male tract by decreasing its response to Müllerian inhibiting hormone (45). The induction of adenocarcinoma of the rete testis and formation of epididymal granulomas

following DES exposure during development (46), strongly suggest abnormal growth of the mesonephric tubules. Although, a precise biochemical mechanism to account for this abnormal development is lacking, Sato et al. (35) demonstrated that DES exposure *in utero* induces an early appearance of ER in the male tract and that this appearance is coincident with an increase in cell division. Thus, it appears that DES may be acting directly on the developing ductal tissues in the male, similar to its effects in the female. One hypothesis would be that blind-ending tubules are formed which become filled with stagnant sperm leading to epididymal sperm granulomas. DES stimulation of cell division in the mesonephric tubules may also lead to abnormal growth of the excurrent ducts (43). Similar to DES, the administration of estradiol perinatally also results in abnormalities of the male reproductive system (see review 43) and in the αERKO mouse, the male reproductive tract show developmental anormallies (47) and infertility (48, 49).

The major problem that scientist now face is not whether estrogen can affect development of the male reproductive system, but rather how to answer the question, "do *environmentally relevant* estrogenic chemicals induce effects?" Recent studies suggest that weak estrogens (octylphenol, bisphenol A, or genistein) given during development cause only minor effects that were short-term in duration, compared to the more potent estrogen, DES (50). What may be more important from that study is the discovery that "animals fed a soy-free diet had significantly larger testes than controls fed a soy-containing diet." It is important to consider that in the male there is a critical period of potential effect that exists for the development of testis size, which is influenced by follicle stimulating hormone (FSH) as well as by thyroid hormone (51, 52). Thus, an indirect effect of an endocrine disruptor, such as polychlorinated biphenyls (PCBs), which can induce transient hypothyroidsim, can lead to alteration in adult testis size (51). Developmental exposure to estrogen also affects testis size by altering the release of FSH and decreasing the number of Sertoli cells that divide (53). Thus, we remain concerned that environmental elements may interact with dietary components to effect developmental changes that would not be detected experimentally with single compound exposures, and effects that may not be detected until adulthood.

ESTROGEN FUNCTION IN THE ADULT MALE REPRODUCTIVE TRACT

Estrogen receptors are present in the testis of most species studied to date (38); however, currently there is no known function for estrogen in the adult testis. Spermatogenesis is normal in the ER knockout (αERKO) mice (54) and germ cells from the αERKO testis that are transplanted into a normal testis produce normal sperm capable of fertilization (55). Thus, although Leydig and germ cells of the adult testis synthesize estrogen (20), estrogen's function in the adult is primarily downstream in the reproductive tract, especially the efferent ductules, where the concentration of ERα is abundant (24). Estrogen provides a paracrine role in regulating efferent ductules (56), whose primary function is the reabsorption of nearly 90% of the luminal fluids (57). Several discoveries led to this major conclusion: 1) the estradiol concentration in rat rete testis fluid is relatively high, approximately 250 pg/ml (27), 2) germ cells and sperm contain P450arom and actively convert androgens to estrogen (22, 23), 3) ERα is abundant in efferent ductule epithelium (40) and 4) fluid reabsorption is inhibited when ERα is blocked or deleted (56).

The rete testis in αERKO males is dilated and protrudes into the testis (56). Downstream from the rete, the efferent ductule lumen is also swollen (56). Effects on the rete testis are similar to the developmental effects of DES on the male (58, 59), and thus likely represent

developmental effects in αERKO, too. Effects on the efferent ductules represent both developmental responses but also adult dysfunction, as the antiestrogen ICI 182,780 produces nearly identical effects when treatment is given to the adult mouse or rat (60-62). Histological observations of the αERKO male suggested that luminal fluid was not being reabsorbed by the efferent ductules. As predicted, a transient increase in testis weight in αERKO males was noted between 32-81 days of age and then a continual decrease in weight resulting in long-term atrophy of the testis. Thus, atrophy was not caused by a lack of estrogen function in the testis, but rather was caused by back pressure of the accumulating luminal fluids (47, 56). The αERKO and antiestrogen-treated mice provide strong evidence that estrogen, or more specifically, a functional ERα, is involved in the regulation of fluid transport in the male reproductive tract, and responsible for increasing the concentration of sperm as they enter the head of the epididymis. Thus, disruption of estrogen function in the male reproductive tract in the adult could lead to decreased sperm concentrations and infertility. It remains to be determined whether of not this is a mechanism to explain the reported declines in human sperm counts in some countries (63).

In the αERKO and ICI-treated mice, the efferent ductule epithelium shows a loss of endocytic organelles, a flattening of the nucleus and the loss or shortening of the microvillus border (47, 56, 61). All of these changes are consistent with a decrease in fluid reabsorption (56, 64, 65). Although data from the αERKO mouse appear unequivocal, efferent ductules also contain androgen receptors and the potential for androgen regulation or an androgen/estrogen co-regulation of these ducts remains viable. For example, it has been shown that luminal androgens are required for maintenance of Na^+,K^+-ATPase activity and that estradiol inhibits the activity (64). Previous studies of testosterone propionate, flutamide (an antiandrogen), 17β-estradiol 3-benzoate, or tamoxifen (an antiestrogen), given by injections for 7 days to male rats showed that testosterone caused a small increase in fluid reabsorption and estrogen appeared to inhibit fluid reabsorption (66). The anti-androgen, flutamide, caused a greater reduction in fluid flow than did testosterone and tamoxifen showed the greatest stimulation of fluid reabsorption. Thus, the possibility that both androgens and estrogen or a balance of the steroid hormones is important for efferent ductule function cannot be ignored.

ER presence and function in the efferent ductules is well established. It is less clear if the epididymis and vas deferens epithelia contain ER subtypes (20). However, binding studies suggest that estrogen could have an influence in this region either during development or possibly in the adult. In one of the first experiments to suggest that estrogen could influence epididymal function, Meistrich et al. (67) simply injected intact adult male mice with estradiol benzoate, testosterone proprionate or both hormones and looked for effects on sperm concentrations in the epididymis and the kinetics of sperm transport. What they found was a decrease in transit times with estradiol and estradiol plus testosterone. However, estradiol alone was more effective. More importantly, this rapid transport of sperm through the epididymis resulted in the passage of immature sperm and in total sterility. Unfortunately, this study did not determine the effects on serum hormone concentrations, leaving open the possibility that estrogen was not acting directly, but instead was interfering with gonadotropin secretions and the production of endogenous testosterone. Confirming this possibility, it has now been shown that chloroethylmethanesulphonate, which reduces serum testosterone, and hydroxyflutamide, which blocks the androgen receptor, both decrease sperm transit time through the proximal segment of the epididymis (68). However, there are studies showing that estrogens do influence contractions along the reproductive tract (69). In particular, estradiol blocks the rat vas deferens response *in vitro* to norepinephrine (69). It is also interesting that an environmental antiestrogen compound,

2,3,7,8-tetrachlorodibenzo-p-dioxin (70), when given developmentally, also causes a decrease in sperm transit time through the epididymis (71).

CONCLUSIONS

The male and female reproductive tracts consists of unique gamete producing and tubular organs. Steroid hormone receptors are well recognized for their expression in both the male and female reproductive tracts in the adult, but it is their expression during development that makes it the most sensitive period for exposure to endocrine disrupting chemicals. In light of the chemical load that is present in our environment, significant new efforts are needed to understand endocrine disruptor-induced changes in reproduction and discovery of the critical windows of potential effects following exposure. Finally, although it is difficult to extrapolate experimental animal data to humans, it is not unreasonable to consider feral animals as sentinels for potential human exposure risk.

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