

Impact of Environmental Chemicals (Plasticizers) on Reproduction

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Background

Exposure to synthetic environmental chemicals (e.g., DDT and its metabolites, especially p,p'-DDE, alkylphenol ethoxylates, PCBs and dioxins) produces reproductive problems in a variety of vertebrate species via endocrine mechanisms.

Naturally occurring environmental chemicals (e.g., phytoestrogens and estrogenic mycotoxins) induce infertility in domestic animal species and can alter human reproductive function. Concerns over these findings have been compounded by a series of publications suggesting that *in utero* exposure to environmental chemicals may have contributed to the reported decline in human sperm counts, the increased incidences of urogenital malformations (e.g., hypospadias, testicular cancer and undescended testes) and altered sex ratio over the last 40–50 years.

It is postulated that *in utero* exposure to environmental estrogens could be responsible for the increased incidences of these alterations. This hypothesis is biologically plausible because hormones play critical roles as regulators of development in vertebrates and exposure to hormonally active toxicants during sexual differentiation is known to produce abnormal reproductive phenotypes in humans and other animals.

Physiological Basis

During mammalian sex differentiation, the androgens, Testosterone (T) and the T metabolite Dihydrotestosterone (DHT), produced by the fetal/neonatal male during sexual differentiation, are critical determinants of the male phenotype. Differentiation of the Wolffian structures (e.g., the epididymis, vas deferens and seminal vesicles) is T mediated, while masculinization of the prostate and external genitalia is controlled by the more potent androgen DHT. In the central nervous system of the rat, some sex dimorphisms result from the action of estradiol, locally produced by the conversion of T by aromatase, while others, like play behavior, appear to be dependent upon androgens themselves.

It appears that both T and DHT play important roles in the development of the brain in many mammals, including nonhuman primates and other tissues like the spinal cord and levator ani muscles (T dependent). These developmental events can be altered by exposure to chemicals that bind AR and act either as hormone agonists or antagonists. Androgenic substances, like

Danazol or methyltestosterone, masculinize human fetal females (i.e., female pseudohermaphroditism). Progestins act both as androgen antagonists in male offspring, demasculinizing them such that they display ambiguous genitalia with hypospadias and androgen agonists in females, virilizing the external genitalia. Laboratory studies of chemicals that inhibit or mimic the action of androgens also produce predictable alterations of sex differentiation in rodents.

Impact of *in Utero* Exposure

In the last few years, transgenerational studies on developmental reproductive toxicity of the phthalates have demonstrated that several of these produce malformations in male rat offspring after *in utero* and neonatal treatment. A NOAEL of 50 mg/kg/d has been identified for Dibutyl Phthalate (DBP), but other phthalates have been less thoroughly studied. It has been shown that the plasticizer Bis (2-ethylhexyl) phthalate (DEHP) also alters sexual differentiation in male rats in an antiandrogenic manner much like the effects of DBP. Administration of DEHP to pregnant rats by gavage at 750 mg/kg/day during the period of sexual differentiation (Gestational Day [GD] 14 to Postnatal Day [PND 3]) markedly demasculinizes and feminizes the male offspring. Treated male offspring display female-like Anogenital Distance (AGD) at birth, retained nipples, cleft phallus with hypospadias, undescended testes, blind vaginal pouch, epididymal agenesis and small to absent sex accessory glands.

It has been demonstrated that testicular histological alterations and testis weight reductions when DEHP was administered at dosage levels in the drinking water at 32.5 µl/l or 325 µl/l (estimated as 3 and 30 mg/kg/day) to the dam during gestation and lactation. It is also observed that testicular effects in the male rat at relatively low dosage levels (3.7 mg DEHP/kg/day was identified as a NOAEL and 37 mg/kg/day was the LOAEL) when they administered DEHP in the diet for 90 days to young male rats. DEHP treatment caused mild vacuolation in the Sertoli cells at 36.8 mg/kg/day (500 ppm in the diet considered the LOAEL) in males and mild seminiferous tubular atrophy at 5000 ppm in males. In addition, subtle effects were seen in the testis of males in both the NOAEL dose group and the group below the NOAEL. Four of ten males in each low-dose group (5 ppm or 0.37 mg/kg/day and 50 ppm or 3.7 mg/

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kg/day, which was used as the NOAEL) displayed minimal Sertoli cell vacuolation.

Support from OECD

The Organization for Economic Cooperation and Development (OECD) estimated Margin of Exposure (MOE) of 19 for DEHP for children mouthing toys containing this chemical, whereas for Diisononyl Phthalate (DINP) the value was 75 (EC 11/98 Opinion on Phthalate Migration, found at: http://europa.eu.int/comm/food/fs/sc/sct/out19_en.html)

A survey of phthalates revealed that 32 of 63 toys sampled contained DEHP and 44 of 63 contained DINP. As a consequence of the low MOE, the European commission proposed a ban of several phthalates in childcare articles and toys (found at <http://europa.eu.int/comm/dg03/press/1999/IP99829.htm>), including DINP, DEHP, Di (n-octyl) Phthalate (DNOP), Diisodecyl phthalate (DIDP), Di-n-butyl phthalate (DBP) and Butylbenzyl phthalate (BBP) (Annex 1 to Directive 76/769/EEC and Annex IV of Directive 88/378/EEC).

Although *in utero* DEHP treatment alters androgen-dependent tissues, it does not appear to act as an Androgen Receptor (AR) antagonist like the pesticide vinclozolin. *In vitro* studies found that neither DEHP nor the primary metabolite MEHP compete with androgens for binding to the AR at concentrations up to 10 μ M. In contrast to their lack of ability to bind to the AR, DEHP inhibits fetal Leydig cell testicular testosterone synthesis, reducing fetal male testosterone concentration to a female level from GD 17 to PND 2.

Mechanisms of Antiandrogen Action

The severity of the effects on the T-dependent tissues resulting from exposure to DEHP and BBP (which include a high incidence of epididymal agenesis) differs from the profile of malformations seen with Androgen Receptor (AR) antagonists like flutamide and procymidone. When administered at a dosage level that produces an equivalent degree of hypospadias, the AR antagonists have much less of an effect on T-dependent tissues and the testis than do the PEs.

Histological Basis

Histological evaluation of the fetal and neonatal testis from DEHP- and DBP-treated rats supports the hypothesis that the fetal testis is directly affected by PEs during sexual differentiation. Examination of the testes of fetal and neonatal rats treated with DEHP reveal abnormal fetal Leydig Cell (LC) morphology. When the testes are examined for β 3 HSD staining by immunohistochemistry at GD 20 and at 2–3 days of age, fetal LCs of PE-treated males results in increase in number in the interstitium of the testis. In other words, several PEs act in an antiandrogenic manner distinct from that previously seen with AR antagonists such as vinclozolin and procymidone. The fact that neither the age at puberty, except in malformed males, nor the level of serum T in adulthood are altered in most PE-treated males during adult life indicates that T production by adult LCs during peri-pubertal and adult life is not permanently affected by perinatal PE treatment, in contrast to the marked reductions in T levels and production seen in fetal LCs during gestation.

Hence, the fact that the androgen-dependent tissues are reduced in size by perinatal BBP and DBP treatments is an indication of abnormal organization of these tissues; they have been permanently imprinted and demasculinized and do not have potential to respond normally to the activational effects of androgens after puberty.

Multigenerational Studies

The multigenerational study design is currently the only test protocol that provides an adequate exposure regime and an examination of the reproductive function of the offspring. However, multigenerational

studies that were conducted prior to the new U.S. EPA guidelines occasionally failed to detect malformations produced by chemicals with antiandrogenic activity, as the assessment of the reproductive system of the F1 was not thorough enough.

In this regard, the new U.S. EPA reproductive test guidelines are a considerable improvement, as they have included several end points sensitive to endocrine disruption and require that three F1 animals per sex per litter be examined for gross malformations at weaning. However, it is unclear if the examination of weanling animals for reproductive malformations is entirely adequate, because the reproductive tissues are immature and some of them are quite small at this time. One may be able to detect severe hypospadias at weaning, but it is likely that many effects such as epispadias or agenesis of the sex accessory glands would not be detected at weaning.

Species-Specific Phthalic acid Esters (PE)-Induced Testicular Toxicity?

The reproductive and developmental effects of the PEs as irrelevant due to a species-specific Peroxisome Proliferator-Activated Receptor α (PPAR α) mechanism, with effects occurring only in a few strains of rats and mice. However, a thorough review of the literature indicates otherwise. First, the testicular toxicity of the PEs appears to be unrelated to the species-specific expression of PPAR α , as evidenced by the display of testicular pathology in DEHP-treated PPAR α knockout. Second, a broad range of vertebrate species display testicular toxicity after PE treatment during development.

The PEs have been shown to cause testicular alterations in numerous mammalian species, as long as the exposure included *in utero* or pubertal development. Affected vertebrates include rat, mice, hamsters, ferrets, guinea pigs and rabbits. In addition to mammals, fish and frogs also display adverse reproductive outcomes when exposed to PEs during development. In fact, to date there are no studies of DEHP or effects of Mono-2-ethylhexyl Phthalate (MEHP) that display negative results when the dosing regime includes the perinatal or pubertal periods of life and a sufficient number of animals is adequately evaluated. A study of DEHP in the nonhuman primate, which dosed four adult male marmosets/group with DEHP at 100, 500 and 2500 mg/kg for 13 weeks, did not observe any testicular effects. However, fetal and prepubertal nonhuman primates have not been similarly evaluated. Even in the rat, a species sensitive to PE-induced testicular damage during fetal and pubertal life, the testis is much less sensitive to the effects of PEs during adult life.

SUMMARY

In mammals, exposure to antiandrogenic chemicals during sexual differentiation can produce malformations of the reproductive tract. Perinatal administration of AR antagonists like vinclozolin and procymidone or chemicals like di(2-ethylhexyl) phthalate (DEHP) that inhibit fetal testicular testosterone production demasculinize the males such that they display reduced anogenital distance (AGD), retained nipples, cleft phallus with hypospadias, undescended testes, a vaginal pouch, epididymal agenesis and small to absent sex accessory glands as adults. In addition to DEHP, di-n-butyl (DBP) also has been shown to display antiandrogenic activity and induce malformations in male rats. In this regard, it is likely benzyl butyl (BBP) and diethylhexyl (DEHP) phthalate would alter sexual differentiation, while dioctyl tere- (DOTP or DEHT), diethyl (DEP) and Dimethyl (DMP) phthalate would not.

It is expected that the phthalate mixture diisononyl phthalate (DINP) would be weakly active due to the presence of some phthalates with a 6-7 ester group. While the specific mechanism of action remains to be identified, PE-induced reductions in fetal T levels result in malformations of androgen-dependent tissues. The structure-activity relationship for

these fetal effects resembles that for pubertal testicular toxicity, implying that similar cellular and molecular targets are involved and that this target remains susceptible until fully differentiated after puberty, when reproductive effects can no longer be easily induced.

Given that PE-induced reproductive lesions are displayed by a wide range of vertebrate species and the role of androgens and steroid hormone synthesis are highly conserved throughout the class Mammalia, it is premature to conclude that PE-induced alterations of sexual differentiation would also not be induced in the human if the male was exposed to concentrations of active PE metabolites during critical stages of reproductive development.

Now I wish to answer some hypothetical questions which may be of interest to Pharma staff in the Discovery area:

Question on Aldehyde Impurities in the API: Assuming the drug contains low levels of aldehydes (formaldehyde, acetaldehyde and propionaldehyde), which are all Class 1 or 2 carcinogen (formaldehyde) or bacterial mutagens (acet- and propion-).

Based on the proposed clinical use, a relevant scenario for establishing acceptable intakes of impurities for a drug administered intermittently to treat acute recurring symptoms, a less-than-lifetime exposure approach would seem to be appropriate (ICH M7 Guidance, page 9-12 and Note 7). Assume that the resulting specs are NOT achievable.

The drug is intended for use as a life-saving therapy. There is a reduced life expectancy and limited therapeutic alternatives. The treatment is acute (a single 48-h infusion) for patients in crisis and published literature indicates the average number of times a patient would receive this treatment is less than twice per year. The drug has orphan and fast track status.

Possible Solutions: It seems that a patient might get the drug 2-x/year, but likely might not survive to receive the drug again. Yes? It might be defensible to look at this as a single event. One option would be to submit it to the FDA for an opinion, which may take several months to get a response.

Since the disease claim is life threatening and less than 3 years expected lifespan, the mutagenicity endpoint is usually irrelevant. You can simply qualify a Personal Daily Exposure (PDE) that can be achieved for the aldehydes, provided the available data set is sound.

Question on Ames Cytotoxicity

Assuming the drug under development inhibits bacterial growth but is not an antibiotic.

Possible Solutions: I suggest providing the pharmacology data supporting the bacteriostatic information and state that no Ames assay was performed because of this property and incompatibility with the assay.

By definition, any substance that inhibits cell growth can be considered an antibiotic, regardless of what it is being developed for. There is no reason why it cannot be tested at concentrations that do not inhibit growth (regardless of what OECD 471 may say). The NTP database has a number of antibiotics that were tested at sub-toxic doses. Some antibiotics are used as Ames test positive controls.

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