Endocrine Disruptors
MARCOM V

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Teaching Objectives

• Provide an overview of agents affecting male reproductive tract development.
• Describe the endocrine disruptor hypothesis as a cause of testicular dysgenesis syndrome (TDS).
• Evaluate human risk from exposure to endocrine disrupting chemicals using phthalates as an example.

Endocrine Disruptor Hypothesis

• There are evident trends in declining male reproductive health over the past 20 years in developed countries.
• Trends consist of increased incidence of testicular cancer, declining semen quality, increased prevalence of undescended testes (UDT) and hypospadias at birth, and a growing demand for assisted reproduction.
• These are considered symptoms of an underlying entity called the testicular dysgenesis syndrome (TDS)
• Environmental and/or life-style factors, rather than accumulation of genetic defects, are considered the most likely cause.

(Skakkebaek et al, 2001)
Endocrine Disruptor Hypothesis

• Exposure to endocrine disruptors (environmental estrogenic and anti-androgenic agents) during prenatal life is considered the primary cause.
• There is general support for the view that development and programming of the hypothalamic-pituitary-gonadal axis during fetal life could be the most sensitive window for permanent alteration in the homeostatic mechanisms of the endocrine system.

Environmental Estrogens

• Chlorinated hydrocarbon pesticides (o,p’-DDT, kepone, toxaphene, dieldrin); DDT banned in 1972.
• Polychlorinated biphenyls (PCBs); major risk for bioaccumulation; banned in 1977.
• Methoxychlor: pesticide developed as a substitute for DDT; metabolites are estrogenic.
• Phenolic derivatives: p-phenylphenol (rubber additive), o-phenylphenol (disinfectant); alkylphenols (detergents, paints, herbicides). Degradation products have estrogenic activity.
• Non-oxyinol: surfactant in pesticides, cosmetics, spermicides.
• Atrazine: herbicide.
• Phytoestrogens: plant estrogens.
• Bisphenol A: polycarbonate plastics and epoxy resins.

Environmental Anti-Androgens

• AR antagonists: vinclozolin (fungicide), procymidone, limuron (herbicide), p,p’ DDT (herbicide), methoxychlor (herbicide), DES, fenitrothion.
• T synthesis inhibitors: phthalate esters (plastics), atrazine (herbicide).
• AhR receptor antagonists: dioxin.
Phthalates

- Phthalates are industrial chemicals added to many consumer products such as food packaging, plastics, adhesives, detergents and personal care products.
- When incorporated into a polymer matrix (as a plasticizer), phthalates are not covalently bound to the polymer and are easily released from the product to air, water, saliva, blood, food and other extracting materials.
- Within this large class of chemical agents, only those phthalates with esters in the ortho position and with side chains of length C4-C6 are associated with reproductive toxicity in animals (DBP, BzBP, DEHP).
- According to NHANES surveys, over 75% of urine samples from the general population had measurable levels of phthalates

Phthalate Metabolites Examined

<table>
<thead>
<tr>
<th>Parent Diester Compounds</th>
<th>Monoester Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl phthalate (DMP)</td>
<td>Monomethyl phthalate (MMP)</td>
</tr>
<tr>
<td>Diethyl phthalate (DEP)</td>
<td>Monoethyl phthalate (MEP)</td>
</tr>
<tr>
<td>Dibutyl phthalate (DBP) *</td>
<td>Monobutyl phthalate (MBP)</td>
</tr>
<tr>
<td>Benzylbutyl phthalate (BzBP) *</td>
<td>Monobenzyl phthalate (MBzP)</td>
</tr>
<tr>
<td>Di (2-ethylhexyl) phthalate (DEHP) *</td>
<td>Monoo(2-ethylhexyl)phthalate (MEHP)</td>
</tr>
<tr>
<td></td>
<td>Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)</td>
</tr>
<tr>
<td></td>
<td>Mono (2-ethyl-5-oxohexyl) phthalate (MEOHP)</td>
</tr>
</tbody>
</table>

* Produce male reproductive tract anomalies in laboratory animals
Outline

• Summary of Laboratory Animal Studies with the Phthalate Esters
• Summary of Human Studies
• Future Directions in Translational Research Using Amniocyte Banks

Design of Rat Studies to Assess Effects on Male Reproductive Tract Development

- Evaluate AGD; gonadal identification of sex
- Evaluate male and female offspring for nipple development
- Evaluate external genitalia; necropsy and measure accessory organ weight

Summary of Animal Studies with DBP

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Hypospadias/UDT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Accessory gland agenesis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Testes histopathology</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Male nipples</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Testicular T/INSL3</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Gene expression changes</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Doses in mg/kg/day on GD 12-12 (Mylchreest et al, 2000; Lehmann et al, 2004)
Review of Human Studies

- Decrease in Anogenital Distance in Male Infants with Prenatal Phthalate Exposure (Swan et al., 2005)
- Human Breast Milk Contamination with Phthalates and Reproductive Hormones in Infants at 3 Months of Age (Main et al., 2006)
- Molecular Epidemiology Study of Hypospadias (Manson et al., 2007)

Swan et al., 2005

- Phthalate monoester metabolites measured in maternal urine (mean 28 weeks gestation) were correlated with the anogenital index in male offspring (12 ± 7 months) in 85 maternal-infant pairs
- Maternal urinary concentrations of 4 metabolites (MEP, MBP, MBzP and MiBP) and a global phthalate score were inversely correlated with AGI.
- The median concentration of phthalate metabolites associated with decreased AGI are below those found in one-quarter of the female population in the USA.
- These data support the hypothesis that prenatal phthalate exposure at environmentally relevant exposure levels can adversely affect male reproductive tract development.

Estimated Phthalate Exposure in Mothers of Male Infants with Reduced AGI

<table>
<thead>
<tr>
<th>Phthalate</th>
<th>RfD</th>
<th>Mean Exposure US Populationa</th>
<th>Median Exposure Infants &lt; AGIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEP</td>
<td>800 μg/kg/day</td>
<td>2.6 μg/kg/day</td>
<td>6.6 μg/kg/day</td>
</tr>
<tr>
<td>DEHP</td>
<td>20 μg/kg/day</td>
<td>5.8 – 8.2 μg/kg/day</td>
<td>1.3 μg/kg/day</td>
</tr>
<tr>
<td>DBP</td>
<td>100 μg/kg/day</td>
<td>1.7 μg/kg/day</td>
<td>1.0 μg/kg/day</td>
</tr>
<tr>
<td>BBzP</td>
<td>200 μg/kg/day</td>
<td>0.84 μg/kg/day</td>
<td>0.5 μg/kg/day</td>
</tr>
</tbody>
</table>

(*Calafat and McKee, 2006; †Marsee et al, 2006)
Swan et al, 2005

- **Strengths:** Novel study design, accurate analytical measurements of urinary phthalate metabolites, break-through study for human health effects consistent with findings from animal models.
- **Weaknesses:**
  - MEP is not associated with reproductive toxicity in animal models.
  - A single urine sample was obtained late in pregnancy.
  - The reliability of a single AGI measurement has not been established; results should be replicated.

Main et al, 2006

- Breast milk samples collected at 1-3 months postpartum were analyzed for phthalate levels; serum samples from male infants collected at 3 months of age and analyzed for reproductive hormone levels (62 cases UDT/68 healthy controls).
- There was no difference between cases and controls in phthalate metabolite concentrations in breast milk.
- SHBG levels were positively correlated with MEP and MBP levels.
- LH:free T ratio was positively correlated with MMP, MEP and MBP levels.
- Free T was negatively correlated with MBP levels.

Main et al, 2006

- **Strengths:** Relevant sampling period for male infants; results suggestive of an effect on Leydig cell function in human infants consistent with animal models.
- **Weaknesses:** Inconsistent association between phthalate metabolites known to be reproductive toxicants and infant hormone levels; inconsistent pattern in changes of reproductive hormones with individual phthalate levels; incomplete data analysis; need to control for infant BMI.
- Long-branched phthalates such as DBP, DEHP and DiNP are excreted unmetabolized or as primary monoester metabolites at relatively high levels in human breast milk.
Molecular Epidemiology of Hypospadias

- Examine relationship between occupational/home exposures, clinical risk factors and genotype in a case-control study of hypospadias.
- Families recruited in a Pediatric Urology Clinic at 6 months of age and questionnaire data and DNA samples obtained.
- Maternal history of subfertility, maternal family history of hypospadias and low birth weight are clinical risk factors.
- Paternal exposures at home to pesticides, and maternal exposures at home to paints and pesticides are environmental risk factors.
- SRD5A2 is a susceptibility gene and the V89L SNP is significantly associated with the risk for hypospadias; the wild-type allele is the risk factor and it is a site of DNA methylation.
- The clinical, environmental and genetic risk factors are independent and there is no significant gene-environment interaction.

Final Genetic and Environmental Multivariable Logistic Regression Model

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 8 lbs</td>
<td>1.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>&lt; 5 lbs 8 oz</td>
<td>5.5</td>
<td>1.8 - 17</td>
<td>.002</td>
</tr>
<tr>
<td>Maternal relative with RTA</td>
<td>3.7</td>
<td>1.4 – 9.7</td>
<td>.008</td>
</tr>
<tr>
<td>Difficulty conceiving 1st pregnancy</td>
<td>2.5</td>
<td>1.3 - 5.1</td>
<td>.008</td>
</tr>
<tr>
<td>Paternal pesticide exposure home</td>
<td>2.1</td>
<td>1.2 – 3.6</td>
<td>.005</td>
</tr>
<tr>
<td>Maternal paint + pesticide exposure home</td>
<td>1.1</td>
<td>.64 - 1.8</td>
<td>.80</td>
</tr>
<tr>
<td>Infant V89L genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VV</td>
<td>1.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VL</td>
<td>1.3</td>
<td>.33 – 2.0</td>
<td>.24</td>
</tr>
<tr>
<td>LL</td>
<td>.29</td>
<td>.10 – .84</td>
<td>.02</td>
</tr>
</tbody>
</table>

* Case-control status the dependent variable; controlled for infant race, maternal smoking during pregnancy, education grade, prematurity and parity.

Manson et al, 2003

- Strengths: Large study with good control of phenotype, clinical risk factors and recall bias.
- Weaknesses: Environmental exposures ascertained through self report. Analytical methods would provide a much stronger level of ascertainment.
Summary of Human Studies

- Alterations in AGI and reproductive hormones in offspring have been associated to date with phthalate exposure.
- Reproductive tract malformations (hypospadias, UDT) have not been associated to date but the possibility of a gene-environment interaction or a small effect size cannot be ruled out.
- The most difficult aspect of human studies is quantifying prenatal exposure to the phthalates. The most appropriate matrix (urine, breast milk, amniotic fluid, etc), metabolic intermediates and meaningful human reproductive endpoints need to be identified.

Future Directions in Translational Research Using Amniotic Fluid

- Findings from laboratory animal studies.
- Phthalate monoester metabolites in amniotic fluid as biomarkers of exposure to phthalates
- Fetal androgenic hormone levels in amniotic fluid as biomarkers of effect

Measurement of Phthalates in Amniotic Fluid of Rats (Calafat et al, 2005)

- DEHP was administered to rats at 0, 11, 33, 100 or 300 mg/kg on GD 7-18; maternal urine and amniotic fluid were collected.
- Concentrations of MEHP in amniotic fluid were strongly correlated with corresponding maternal urinary levels of MEHP and with the maternal DEHP dose.
- Maternal urinary levels of MEHP may be useful as surrogate markers for fetal exposure to MEHP.
- Reproductive tract malformations observed in male offspring of rats given 300 mg/kg/day of DEHP would be expected to yield an amniotic fluid MEHP level of 3000 ng/mL.
Phthalate Metabolites in Human Amniotic Fluid Samples (Silva et al, 2004)

- Ten phthalate metabolites were measured in human amniotic fluid samples obtained from 54 anonymous donors.
- Three monoester metabolites were quantifiable; MBP (93% of samples); MEP (39%) and MEHP (39%). MMP, MBzP, MCHP, MNP, MOP, MEHP, MEHHP and MEOHP were not detectable.
- Concentrations of MBP, MEP and MEHP in amniotic fluid samples were 4 to 7-fold lower than urinary levels in children and adults from NHANES.

Use of Amniocyte Banks to Assess the Effects of Human In Utero Exposure to Phthalates

- Human exposure assessment has consistently identified a high prevalence of exposure to phthalates in the general population.
- Measurement of human fetal exposure is difficult; maternal blood, urine or breast milk levels may not accurately reflect fetal exposure during the critical period; questionnaire assessment for phthalate exposure is not likely to be useful.
- There is a need to develop sensitive and specific biomarkers of exposure and effect to determine whether there is a relationship between human in utero exposure to phthalates during the critical period and alterations in steroidogenic pathways involved in male reproductive tract development.

Amniocyte Banks

- Amniocyte banks (amniotic fluid and cells) are available and are a potential resource for assessing the relationship between exposure during the critical period and early endocrine and biological perturbations in the fetus indicative of altered reproductive tract development (14-18 weeks gestation).
- Studies using amniocyte banks can provide crucial proof of concept linking in utero exposure and mechanistically relevant early fetal effects.
- Such proof of concept studies are necessary to justify costly efforts in launching large-scale postnatal follow-up studies.
Database Elements of Amniocyte Banks

- Amniocytes and amniotic fluid obtained from women undergoing amniocentesis at ~14-18 weeks gestation
- Patient enrollment (race, ethnicity, age, LMP date personal identifiers)
- Reproductive genetics intake (reproductive history, indications for the procedure, pedigree)
- Reproductive genetics chart review (1st, 2nd trimester screening tests, ultrasound findings level I, II, any genetic findings)
- Pregnancy outcome (complications during pregnancy and delivery, live birth, fetal death, miscarriage, elective termination, and any abnormality in these outcomes)

Biomarkers of Effect: INSL3

- Insl3 expression in the fetal Leydig cell is associated with the transabdominal phase of testes descent; KO have UDT.
- Insl3 expression is a reflection of the number and differentiation status of Leydig cells; the proximate intracellular pathways activated by Insl3 have not been identified.
- Both estrogenic (DES) and antiandrogenic (phthalate) exposure to the fetal rat testes reduce Insl3 expression and intratesticular T levels.
- INSL3 and testosterone levels have been recently examined in human amniotic fluid.

INSL3 Levels in Amniotic Fluid from Male Fetuses

Gestational age determined from a Level II ultrasound
Testosterone Levels in Amniotic Fluid from Male and Female Fetuses

Future Plans

- If elevated phthalate levels are associated with a decrease in levels of fetal androgenic hormones:
  - Collect maternal urine samples at the time of amniocentesis and determine the correlation between maternal and amniotic fluid levels of the phthalate metabolites.
  - Design prospective follow-up studies to identify the postnatal sequelae of the reduction in androgen levels.
  - Focus should be on evaluation of sexually dimorphic markers such as morphological indices (otoacoustic emissions, finger ratio and dermatoglyphics) behavioral indices (eye contact, vocabulary size, social relationships and gender-typed play).
  - These measurements can be made in the first 2 years of age and are reasonably predictive of prenatal androgenic levels in healthy infants (Cohen-Bendeham et al, 2005).
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References

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