Testicular Toxicology *In vitro* Models Workshop:

Overview of Phthalates as a Model for Assessing Testicular Toxicity

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Goal: To recapitulate *in vivo* spermatogenesis *in vitro*
Testis Developmental Time Frame

- **Fetal testis**
  - **Testosterone**
  - Sertoli cell proliferation (testis size)

- **Masculinisation programming window**
- **Masculinisation of the reproductive tract**

- **Determination of penile length**
- **Determination of AGD**

- **Elongation/growth of penis**
- **Expansion of AGD**

- **Gestational weeks**
  - 10
  - 20
  - 30
  - 40
  - Birth
  - Postnatal weeks
  - 10
  - 20
Toxicity can occur at all Life Stages

• *In utero*

• Postnatal

• Puberty

• Adult
Cellular Targets of Toxicity

- Sertoli
- Leydig
- Germ cells
- Peritubular Myoid Cells
- Vasculature
Histopathology as the tool for assessing Male Reproductive Toxicity
Methods to assess toxicity

Direct damage to the germ cells

- Testis Size and Body Size
- Seminiferous Tubule Diameter
- Spermatid Head Counts
- Accessory Sex Organ weights
- Fertility
- Arrays
- Hormonal Measurements

Apoptosis (TUNEL)

Rasoulpour et al., Endocrinology 147(9):4213–4221 (2006)

Immunofluorescence and Confocal Microscopy as a Powerful Tool to assess male repro toxicity \textit{in vitro}?

Chromatin structure plays an important role in the regulation of gene expression in the testis. To gain further insight into the regulation of gene expression during postnatal development of the testis, we are beginning to determine the localization patterns of various markers associated with chromatin remodeling.
Can we utilize similar techniques for *in vitro* toxicity models?
Male Reproductive Health Trends

Cryptorchidism

Hypospadias

Sperm quality/quantity

Testicular Cancer

Toppari et al. Env Health Persp 1996; (104) 4
Male Reproductive Health Trends

Environmental Exposures
Genetic Defects

- altered Leydig Cell function
- lowered Testosterone/InsL3 production
- altered Sertoli Cell function
- impaired germ cell development

Cryptorchidism
Hypospadias
Sperm quality/quantity
Testicular Cancer

Testicular Dysgenesis Syndrome*
- Spectrum of disorders
- Interrelated in nature
- May share common origin

*Skakkebaek et al. Hum Reprod 2001; 16(5) 972-978
What are Phthalates Esters?

- Phthalate esters are a large group of chemical compounds used frequently as:
  - Non-covalently linked to polymer
  - Metabolized by gut esterases

- Loff et al (2000) report that critically ill infants may receive 10-20 mg of phthalates per day from PVC-based medical devices
- NTP reports critically ill infants may receive doses as high as 6000μg/kg bw/day
Rodent Models

• *In utero* exposures
• Postnatal exposures
## Phthalate-Induced Effects on Fetal Testis

### Rat
- **Seminiferous Cord Effects:** YES
  - Multinucleated germ cells
  - ↑ Cord diameter
- **Leydig Cell Effects:** YES
  - ↓ Steroidogenic genes
  - ↓ Testicular testosterone
    - ↓ Anogenital distance
    - Hypospadias
    - Cryptorchidism
    - Leydig cell hyperplasia
    - Nipple/areola retention

### Mouse
- **Seminiferous Cord Effects:** YES
  - Multinucleated germ cells
  - ↑ Cord diameter
- **Leydig Cell Effects:** NO*
  - No decrease in gene expression
  - No decrease in testosterone
  - No change in anogenital distance
  - No hypospadias
  - No cryptorchidism
  - No Leydig cell hyperplasia

### Human
- **Seminiferous Cord Effects:** ?
- **Leydig Cell Effects:** ?

How to Assess the Human Response?

Epidemiology?  

In Vitro cultures?  

Xenografting

Isolate testes  

+ phthalate  + corn oil  

Are responses intrinsic to the testis, or a species-dependent effect?

What is the human response?

Working Hypothesis: A fetal testicular transplant bioassay determines the human response to developmental di (n-butyl) phthalate exposure

<table>
<thead>
<tr>
<th>Testis</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Rat</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mouse</td>
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</tbody>
</table>

Boekelheide Lab
How to Assess the Human Response?

Isolate gd16 rat testes

Xenograft into adult male Rat host

Di-(n-butyl) phthalate (DBP); oral gavage

gd16 ...... | ...... gd17 ...... | ...... gd18 ...... | ...... gd19 ...... | ......

peak Testosterone production

Collect testis implants 6hr after dosing

- Histopathology
- Gene expression
Bar: 100um (top); 50um (bottom)  H&E staining

Heger, N. unpublished
MNG induction

Steroidogenic gene suppression

Suppressed Testosterone production

Consistent with observed in utero effects

*\(p<0.05\)  **\(p<0.01\)  ***\(p<0.005\)  
\(t\)-test

Values in parentheses

Heger, N. unpublished
Heger, N. unpublished
Consistent with observed *in utero* effects
Cross-species Transplants

- Xenograft model recapitulates *in utero* effects of phthalate exposure
  - Optimized parameters: timing, graft location, host hormones
- Host species does not determine response
  - *Seminiferous cord effects*
  - *Leydig Cell effects*
- Intrinsic to the testis
- Proof of Principle

Consistent with observed *in utero* effects

Consistent with observed *in utero* effects
Human Fetal Testis Xenografts

MOUSE

RAT

DBP

Control

BrdU

CD31

Bar: 50um; H&E staining

Heger, N. unpublished

RAT

MOUSE

HUMAN testis

RAT
Summary of Phthalate-Induced Effects on Fetal Testis

Seminiferous Cord Effects: **YES**
- Multinucleated germ cells
  \[\uparrow\text{Cord diameter}\]

Leydig Cell Effects: **YES**
- Steroidogenic genes
- Testicular testosterone
  \[\downarrow\text{Anogenital distance}\]
  - Hypospadias
  - Cryptorchidism
  - Leydig cell hyperplasia
  - Nipple/areola retention

Leydig Cell Effects: **NO**
- No decrease in gene expression
- No decrease in testosterone
- No change in anogenital distance
- No hypospadias
- No cryptorchidism
- No Leydig cell hyperplasia

Host does not determine response
- Intrinsic to the testis
- Species-specific

Developmental phthalate exposure is an unlikely cause of anti-androgenic related effects of Testicular Dysgenesis Syndrome, namely hypospadias and cryptorchidism

Heger, N. unpublished
If you can do it in the female....

Whole organ culture

Phthalates,

Moyer, B. unpublished
And if we can’t do whole TESTIS organ culture, what about Seminiferous Tubule Floating Rafts ???

24hour control

24hour post bleomycin

Next TUNEL
γH2AX

Moyer, B. unpublished
Figure 1: In vitro culture of primary (S23Y) and immortalized (S23Y/hTERT) adult human Sertoli cells.

(A) Primary or immortalized human Sertoli cells were stained with the neutral lipid stain Oil Red O or processed for transmission electron microscopy. Arrowheads indicate cytoplasmic lipid droplets. (B) For growth kinetic studies, S23Y cells were plated at 50,000 cells per 25cm² flask and passaged before cells reached 80% confluence. Cell counts were determined at each passage and population doublings were calculated as $\log_2(\text{cell number}_{\text{final}}/\text{cell number}_{\text{initial}})$. 

Pietruska, J. unpublished
Figure 4: MEHP exposure reduces proliferation and viability of adult human Sertoli cells

S23Y cells exposed to MEHP for 48hr were harvested and stained with Trypan Blue for determination of cell viability (A) and total cell number (B). Means ± SD are shown

Pietruska, J. unpublished
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