SOT 59th Annual Meeting & ToxExpo
March 15–19, 2020 • Anaheim, California

# Continuing Education Course

Sunday, March 15 | 1:15 PM to 5:00 PM

# PM14: The Male Reproductive Tract: Development, Toxicology, and Pathology

## Chair(s)

Vicki Sutherland, NIEHS/NTP

Nicole Principato, Bristol-Myers Squibb Company

## Primary Endorser

Reproductive and Developmental Toxicology Specialty Section

## Other Endorser(s)

Comparative Toxicology, Pathology, and Veterinary Specialty Section

Regulatory and Safety Evaluation Specialty Section

## Presenters

Justin Vidal, Charles River

Kim Boekelheide, Brown University

Catherine Picut, Charles River

Cynthia Willson, Integrated Laboratory Systems Inc.

SOT Anaheim, California 2020

# Course Participant Agreement



## Continuing Education Committee



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# The Male Reproductive Tract: Development, Toxicology, and Pathology

| | | |
|---|---|---|
| 1:30 PM–2:15 PM | **An Overview of the Male Reproductive System: Applied Anatomy and Physiology from a Pathologist's Perspective**<br>Justin Vidal, Charles River, Mattawan, MI | 4 |
| 2:15 PM–3:00 PM | **Prenatal Development of the Male Reproductive Tract in the Rat, Dog, and Human: Critical Developmental Windows and Later-Life Consequences of Exposure**<br>Kim Boekelheide, Brown University, Providence, RI | 34 |
| 3:00 PM–3:30 PM | **Break** | |
| 3:30 PM–4:15 PM | **Postnatal Development of the Juvenile Male Reproductive Tract in Rats: Microscopic Evaluation, Interpretation, and Time Points of Toxicologic Significance**<br>Catherine Picut, Charles River, Durham, NC | 61 |
| 4:15 PM–5:00 PM | **Pathology in Reproductive Toxicology Assessments and the Role of Stage-Awareness for Testis Evaluation**<br>Cynthia Willson, Integrated Laboratory Systems Inc., Research Triangle Park, NC | 90 |

# An Overview of the Male Reproductive System: Applied Anatomy and Physiology from a Pathologist's Perspective

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## Conflict of Interest Statement

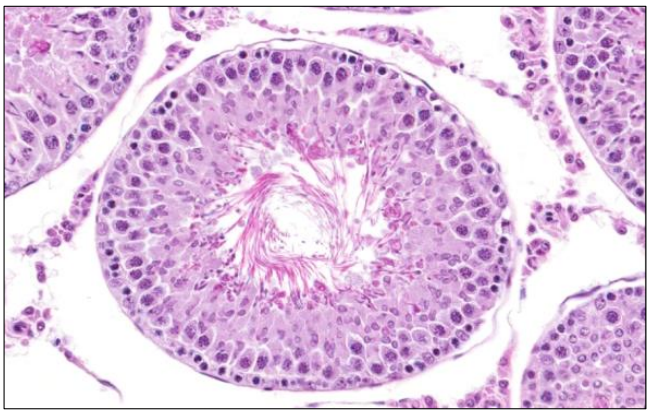
The author declares no conflict of interest.

# Abbreviations

- DHT: dihydrotestosterone
- E2: estradiol
- FSH: follicle stimulating hormone
- GnRH: gonadotropin releasing hormone
- ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
- IHC: immunohistochemistry
- LH: luteinizing hormone
- OAT: organic anion transporters
- OATP: organic anion transporting polypeptides
- OCT: organic cation transporters
- OECD: Organisation for Economic Co-operation and Development
- T: testosterone
- US FDA: US Food and Drug Administration

# Introduction

Before administering the first dose in healthy male volunteers, the sum total of the assessment on the male reproductive system falls on the study (and peer-review) pathologist(s)!



# ICH Guidance

- ICH: *Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility* S5(R2)
  - Note 12 (4.1.1)
    - Information on potential effects on spermatogenesis can be derived from repeated-dose toxicity studies
    - Histopathology of the testis has been shown to be the most sensitive method for the detection of effects on spermatogenesis

# ICH Guidance: Continued

- ICH: *Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* M3(R2)
- 11.1 Men
  - Men can be included in Phase I and II trials before the conduct of the male fertility study since an evaluation of the male reproductive organs is performed in the repeated-dose toxicity studies (Note 2)
  - A male fertility study should be completed before the initiation of large scale or long duration clinical trials (e.g., Phase III trials)
  - Note 2: An assessment of male and female fertility by thorough standard histopathological examination on the testis and ovary in a repeated-dose toxicity study (generally rodent) of at least 2-week duration is considered to be as sensitive as fertility studies in detecting toxic effects on male and female reproductive organs

## ICH Guidance: Continued

- ICH: *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals* S6(R1)
- 5.2 Fertility
  - It is recognized that mating studies are not practical for nonhuman primates (NHP). **However, when the NHP is the only relevant species, the potential for effects on male and female fertility can be assessed by evaluation of the reproductive tract (organ weights and histopathology evaluation) in repeat-dose toxicity studies of at least 3-months duration using sexually mature NHPs.**

## OECD

- **OECD Test No. 421** (Repro./Dev. Tox. Screening Test) and **422** (Combo. Repeated Dose Tox. Study with the Repro./Dev. Tox. Screening Test):
  - "Detailed histological examination should be performed on the ovaries, testes, and epididymides (with special emphasis on stages of spermatogenesis and histopathology of interstitial testicular cell structure)"
- **OECD Test No. 416** (Two-Generation Reproduction Toxicity):
  - "Detailed testicular histopathological examination . . . should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen"
- **OECD Guidance 106**: Guidance Document for Histologic Evaluation and Reproductive Tests in Rodents

# US EPA

- Health Effects Test Guidelines OPPTS 870.3800 Reproduction and Fertility Effects
  - ". . . testicular histopathological examination should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen"

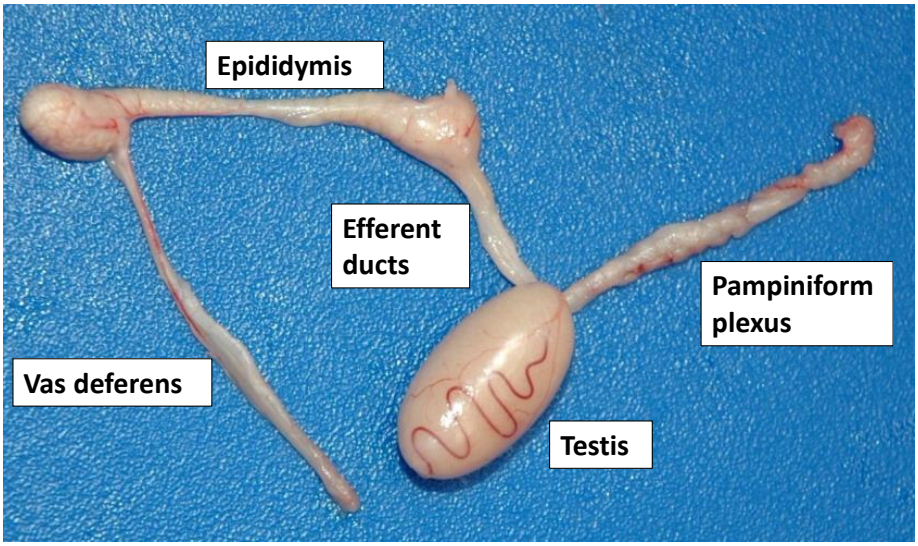
# Background

- Age
  - Puberty
  - **AGE AT NECROPSY**
- Species differences
  - Also consider strain and supplier differences
- Pathology is often linked to fertility/infertility and not necessarily the overall health of the animal
  - Definition of adversity can be challenging

# Male Reproductive System—Endpoints in a Repeat-Dose Tox Study

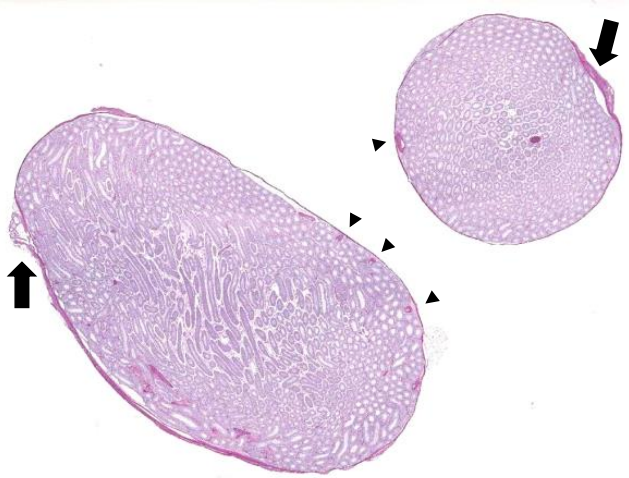
| Organ | Weight | Histopathology |
|---|---|---|
| Testis | + | + |
| Epididymis | +/- | + |
| Prostate | + | + |
| Seminal vesicles | +/- | + |
| Pituitary | +/- | + |
| Mammary gland (rats only) | NA | +/- |

# Basic Anatomy



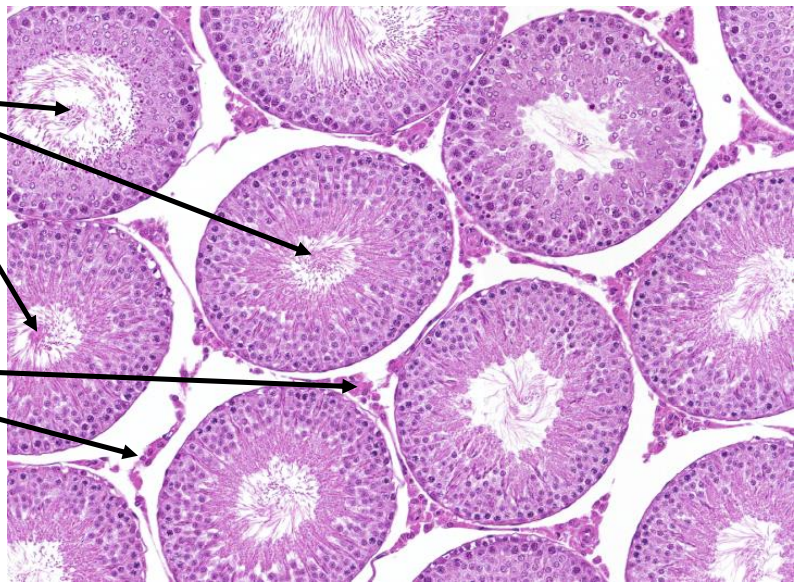
# Rat Testis

- Rete is peripherally located at the proximal pole (arrows)
- Opposite the large vessels (arrowheads)
- No lobular pattern with minimal connective tissue

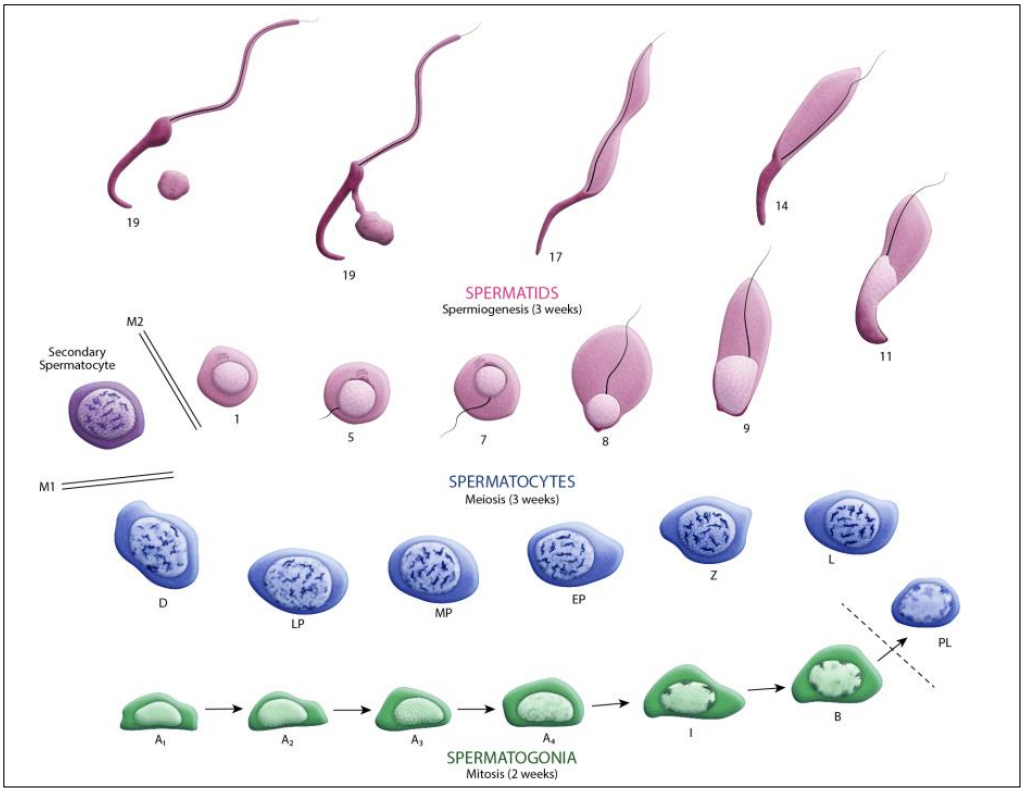


# Rat Testis: Continued

- **Seminiferous tubules**
  - Germ cells
  - Sertoli cells
  - Basement membrane
  - Peritubular myoid cells
- **Interstitium**
  - Leydig cells
  - Macrophages
  - Vasculature

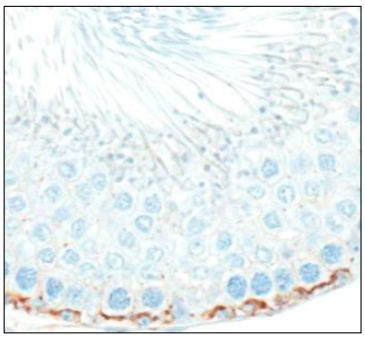


## Spermatogenesis

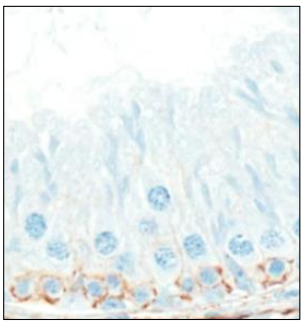



## Blood-Testis Barrier

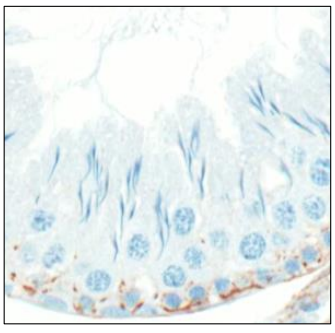
Stage VII–VIII



Stage X–XI



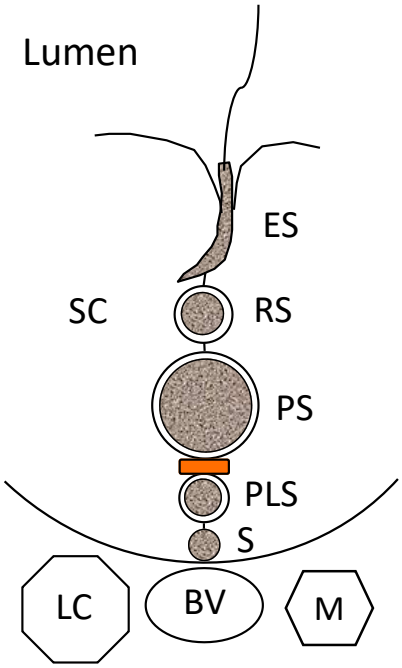
Stage XII



B-catenin IHC: Demonstrates the location and dynamic nature of the blood-testis barrier. Note that the barrier is within the tubule!

# Blood-Testis Barrier: Continued

SC: Sertoli Cells
ES: Elongated Spermatids
RS: Round Spermatids
PS: Pachytene Spermatocytes
PLS: Preleptotene Spermatocytes
S: Spermatogonia
LC: Leydig Cells
BV: Blood Vessels
M: Macrophages




Luminal Surface
Integrin
Laminin
Efflux pumps (P-glycoprotein)
Influx pumps (e.g., OATP, OAT, and OCT)

Blood-Testis Barrier
Tight Junction Proteins
Ectoplasmic specializations
Efflux pumps (P-glycoprotein)
Influx pumps (e.g., OATP, OAT, and OCT)

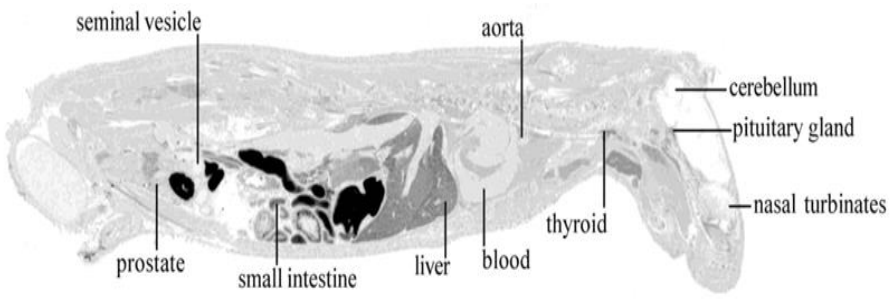
# Case Example: Drug Accumulation in Testis

| Dose (mg/kg/day) | Male | | | |
|---|---|---|---|---|
| | Mean $C_{max}$ (µg/mL) | | Mean $AUC_{(0-t)}$ (µg.h/mL) | |
| | Day 1 | Day 7 | Day 1 | Day 7 |
| 100 | 4.6 | 2.4 | 22.5 | 11.9 |
| 300 | 16.1 | 5.5 | 96.9 | 35.7 |
| 1000 | 25.7 | 9.70 | 260 | 118 |

Toxicokinetics: systemic exposure of compound “X” on Days 1 and 7 in a one-week repeated-dose toxicity study

# Case Example: Drug Accumulation in Testis

1 hour

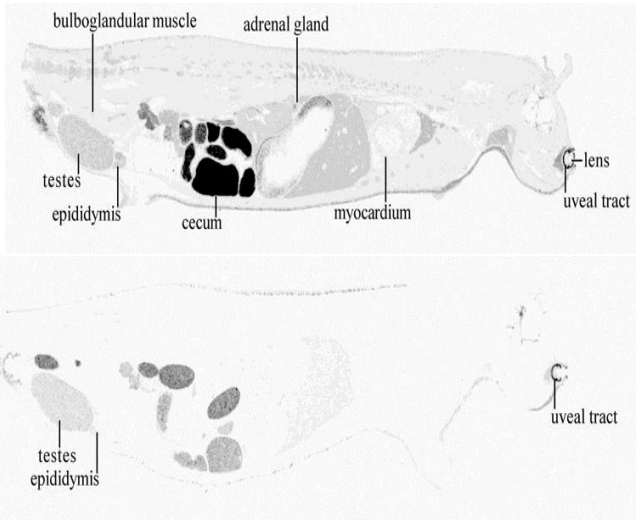



Whole-body autoradiogram: Single Oral Administration of [14C] Labeled Compound "X" at 300 mg/kg

# Case Example: Drug Accumulation in Testis

8 hours

24 hours

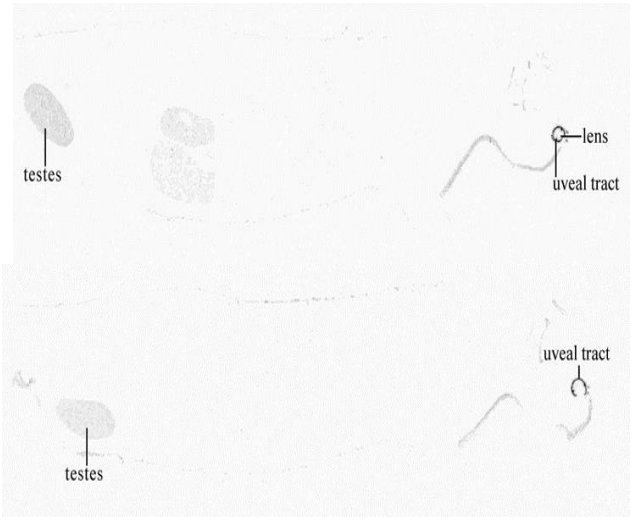



Whole-body Autoradiogram: Single Oral Administration of [14C] Labeled Compound "X" at 300 mg/kg

# Case Example: Drug Accumulation in Testis

3 Days

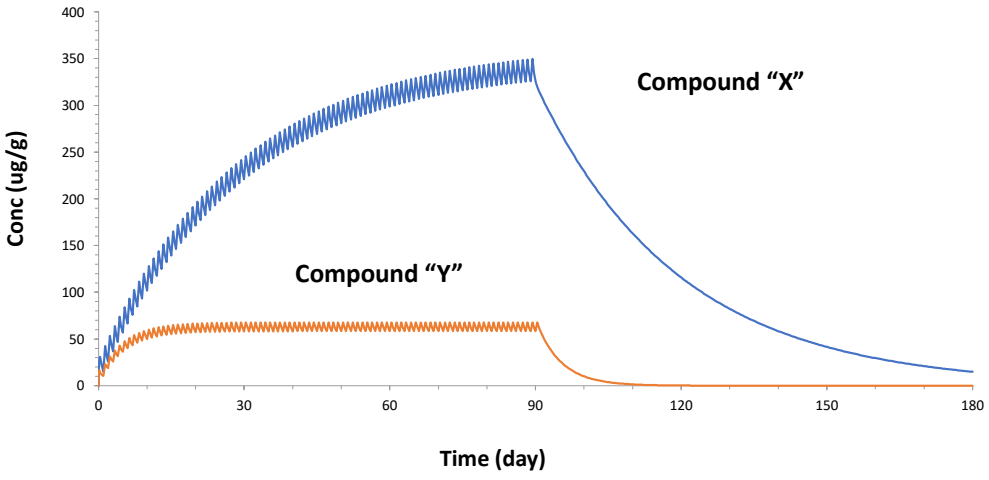
7 Days




Whole-body Autoradiogram: Single Oral Administration of [14C] Labeled Compound "X" at 300 mg/kg

# Case Example: Drug Accumulation in Testis

**Predicted Testis Concentration following 90-Day Repeat-Dose Oral Administration**



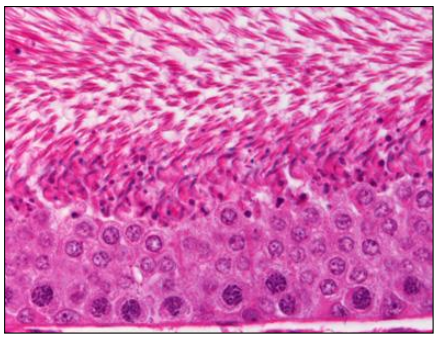

Modeling the predicted testis concentration during repeat dosing based on tissue distribution studies for compound "X" and compound "Y." Compound "Y" has the same target engagement but different chemical class. Note the difference in time to tissue steady state and the difference in tissue clearance.

# Species Comparisons

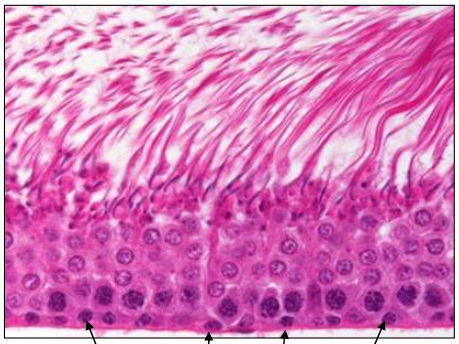
| Species | Number of Stages | Cycle Length (Days) | Total Duration of Spermatogenesis (Days) | Epididymal Transit Time (Days) |
|---|---|---|---|---|
| Rat | 14 | 12.9 | 51.6 | 8 |
| Mouse | 12 | 8.62 | 34.5 | 5 |
| Dog | 8 | 13.6 | 54.4 | 10 |
| Pig | 8 | 8.6 | 40 | 10 |
| NHP | 12 | 10.5 | 46.4 | 11 |
| Human | 6 | 16 | 64 | 6 |
| Marmoset | 9 | 10 | 37 | ? |

# Early Mitotic Inhibition: 1–2 Weeks

**Depletion, preleptotene spermatocytes: Stage VII–VIII**



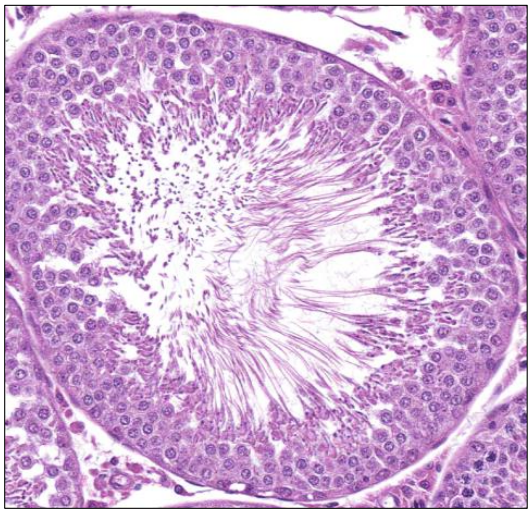
**Control**



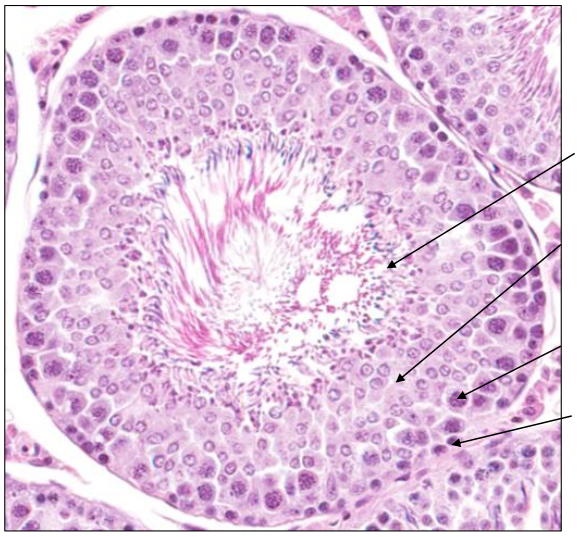

Note: Inhibition of spermatogonia mitotic activity leads to loss of spermatogonia and ultimately loss of more mature cell types. Detecting the initial loss of spermatogonia can be difficult and loss of early spermatocytes is often the first readily identifiable finding.

# Continued Mitotic Inhibition: 4 Weeks

**Depletion, preleptotene spermatocytes, and pachytene spermatocytes: Stage VII**

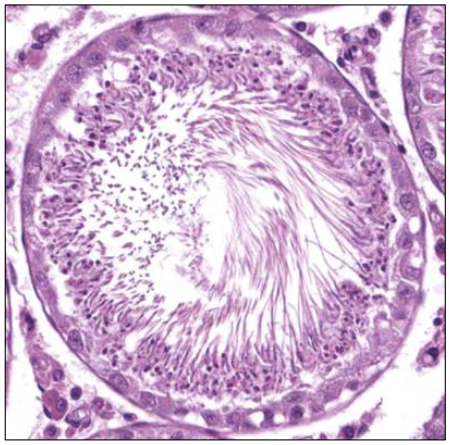


**Control**

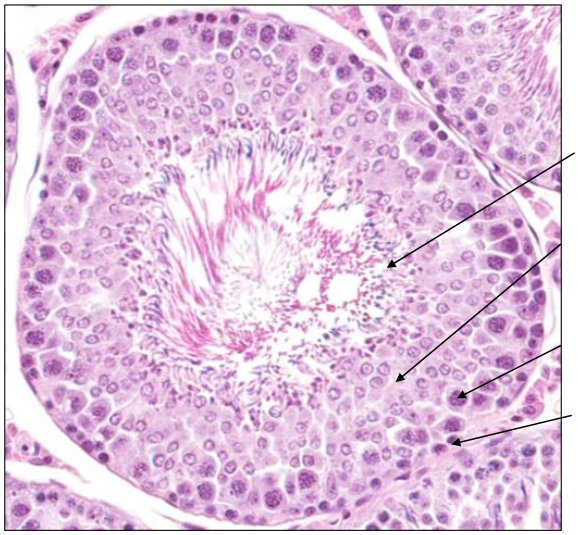



# Continued Mitotic Inhibition: 6 Weeks

**Depletion, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids: Stage VII–VIII**

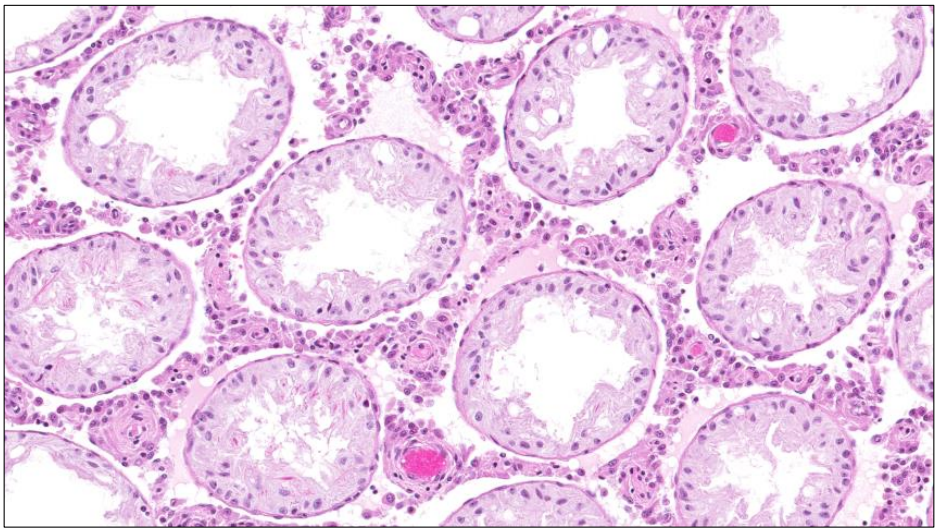


**Control**

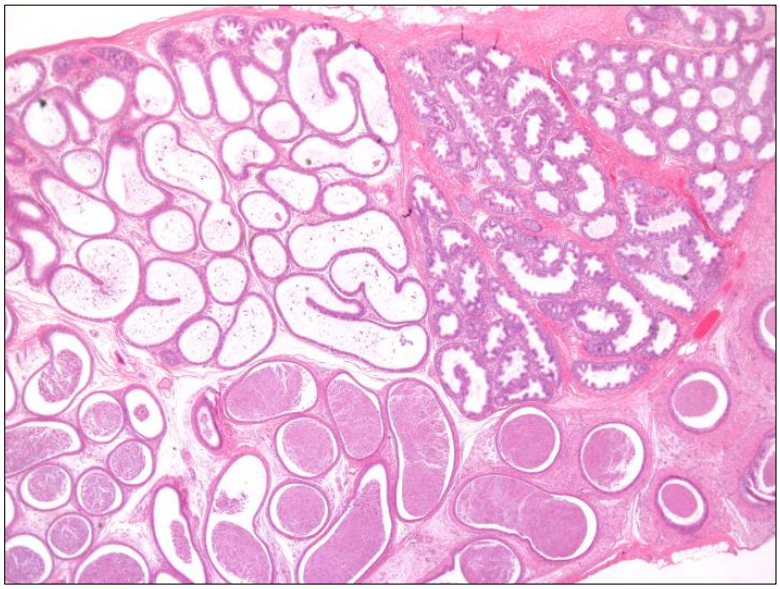


# Continued Mitotic Inhibition: 8 Weeks

**Tubular atrophy**

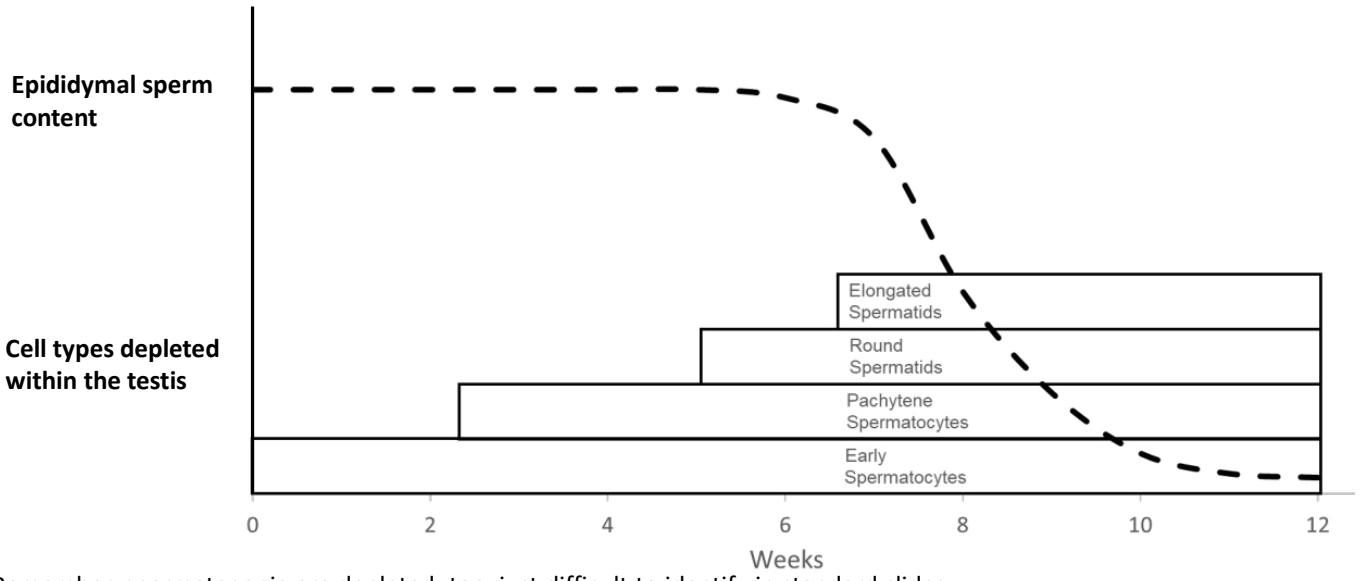


# Continued Mitotic Inhibition



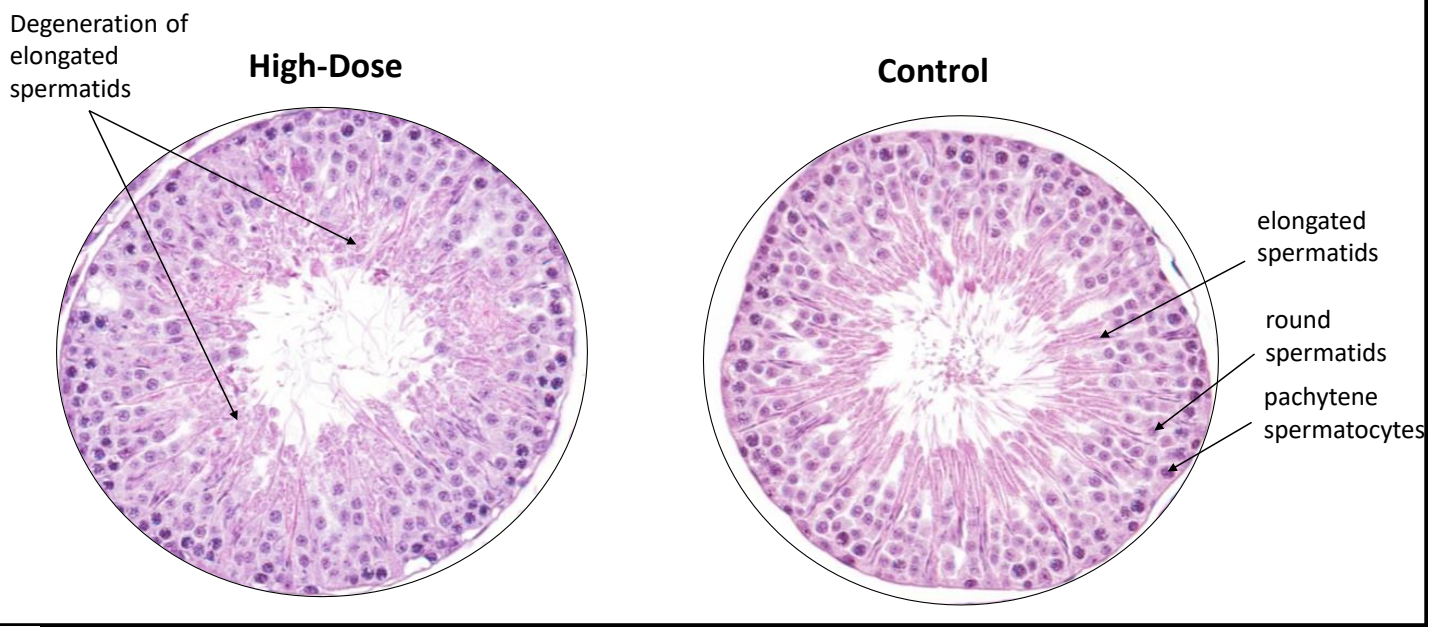
Changes can be observed within the epididymis in approximately 8 weeks

# Time Course: Mitotic Inhibition

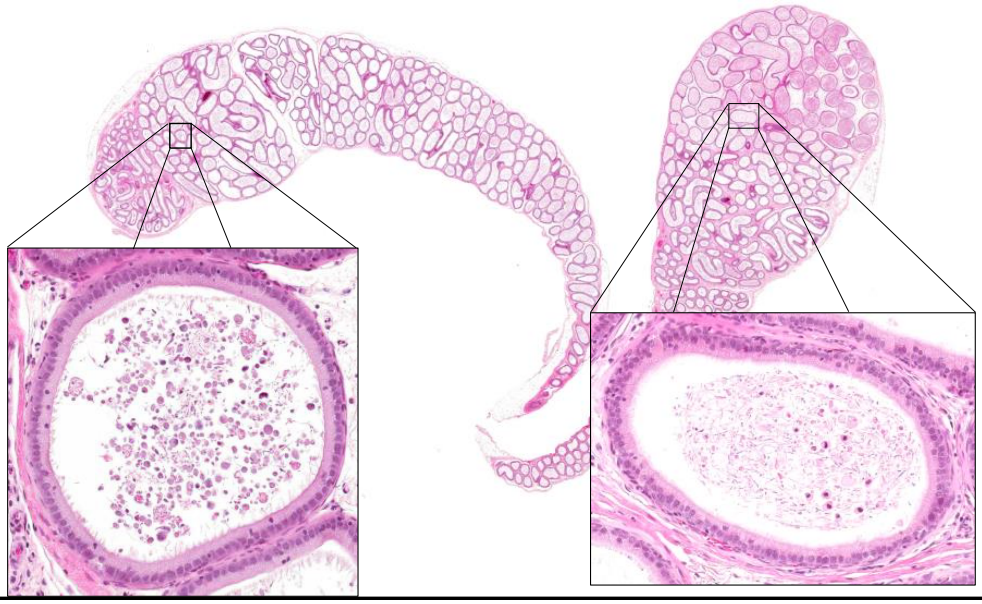



Remember, spermatogonia are depleted, too, just difficult to identify in standard slides

# Rat: Germ Cell Degeneration and Spermatids



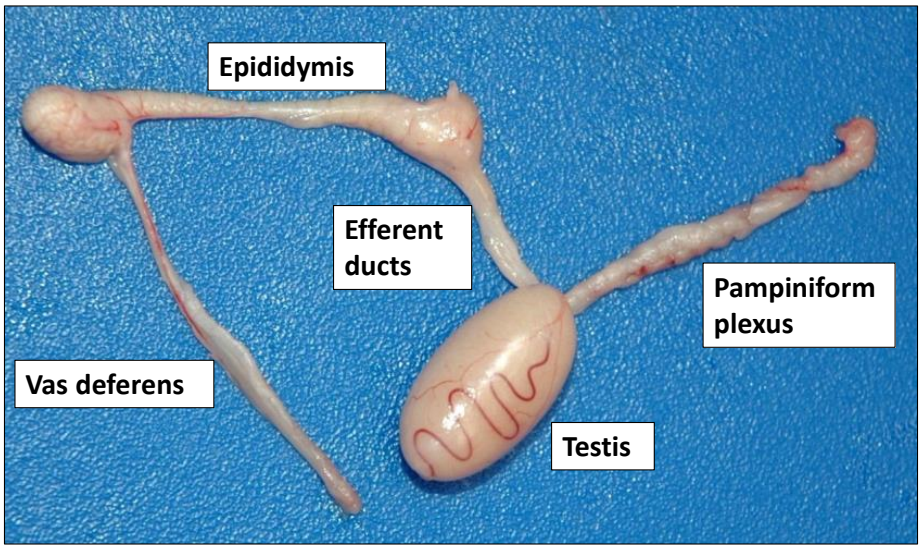
## Changes Can Be Observed within the Epididymis Quickly: < 2 Weeks



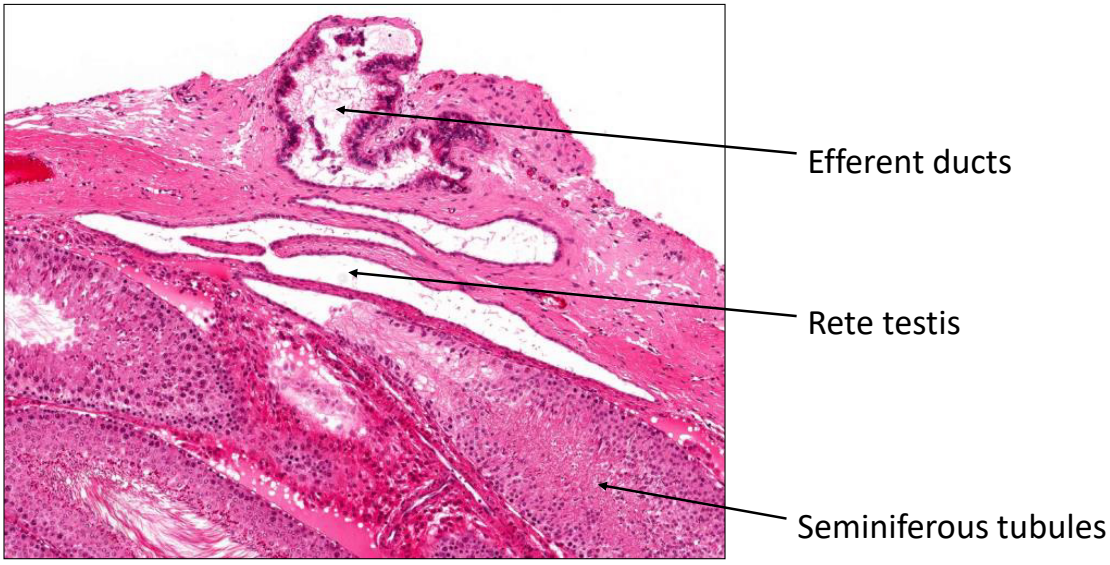
## Testicular Effects

- Understanding the cell type(s) affected within the testis is critical to design of follow-up studies and is needed before discussing length of dosing, length of recovery, endpoints, etc.
- This is key component of the hazard characterization and risk assessment!!

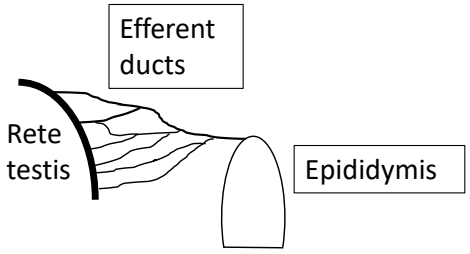
# Basic Anatomy



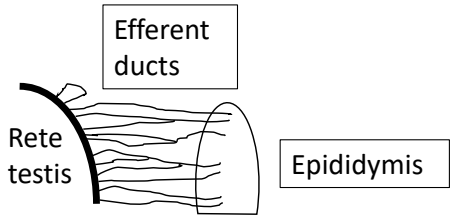

# Rat: Rete Testis and Efferent Ducts



# Species Differences in Efferent Duct Anatomy

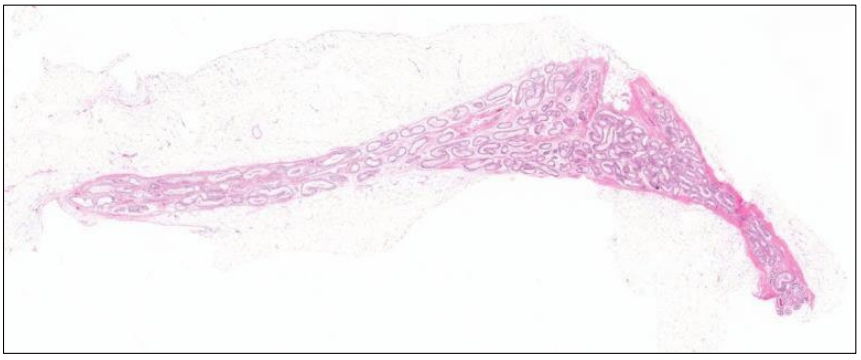



**Funnel Pattern**
Rats, mice



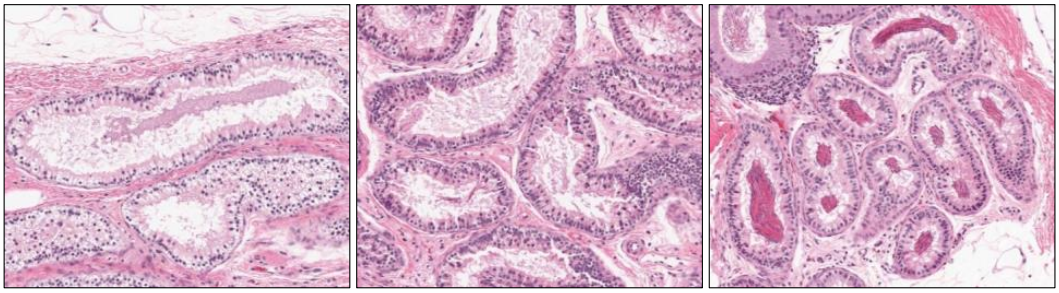

**Parallel Pattern**
Most mammals
although number of tubules and exact points of insertion vary

# Rat: Efferent Ducts



In rats, the efferent ducts are embedded in a fat pad, which is discarded in most toxicity studies.
If a potential target organ, must request prospectively!

# Rat: Efferent Ducts

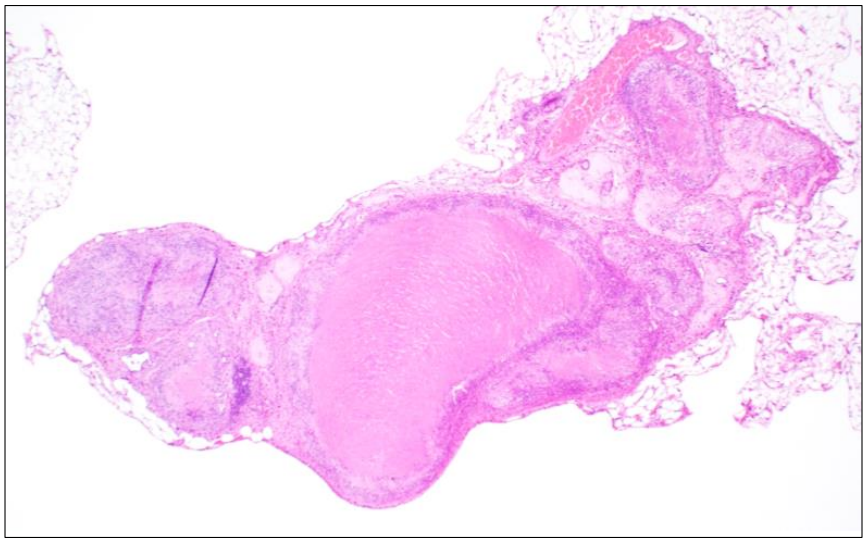


**Proximal Zone (P)** | **Conus region (C)** | **Terminus (T)**

Testis → Epididymis

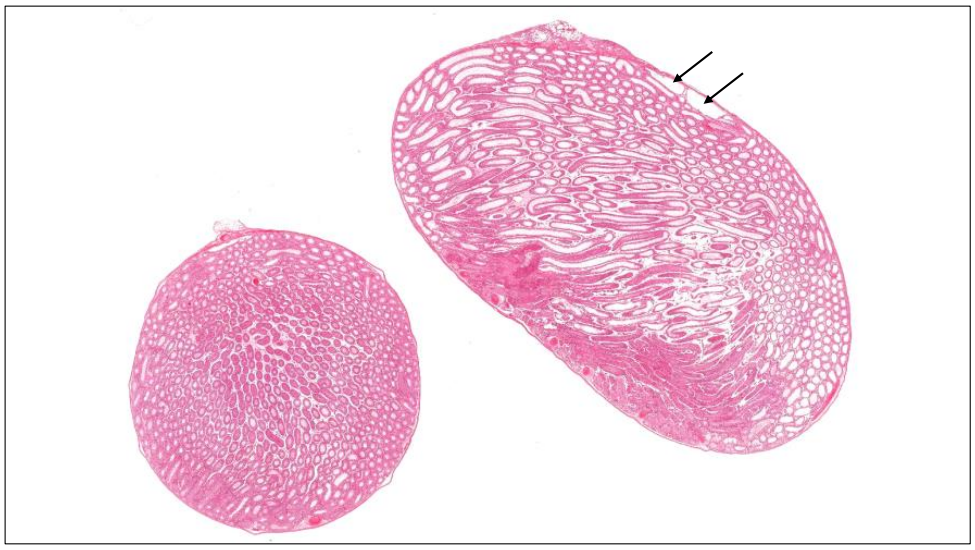
Sperm are concentrated in efferent ducts as fluid is resorbed

# Rat: Efferent Ducts



Sperm Granuloma

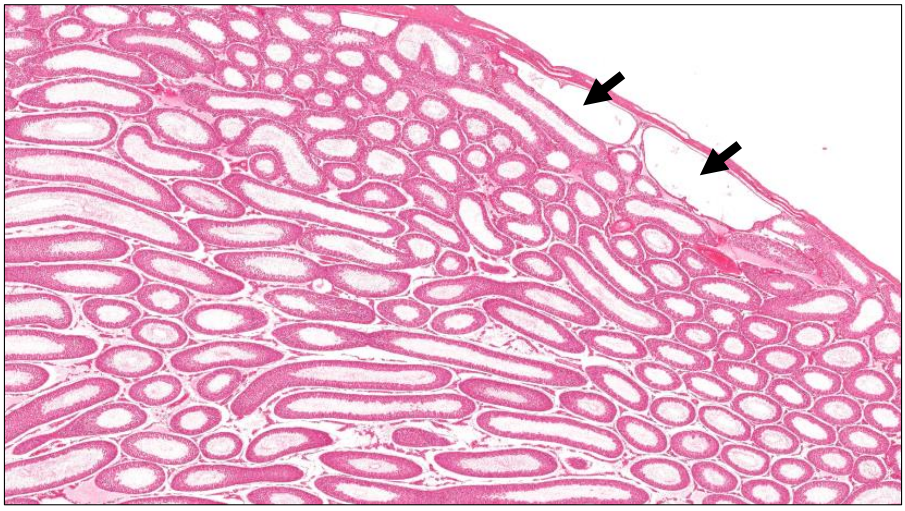
# Rat: Testis



Right: Normal

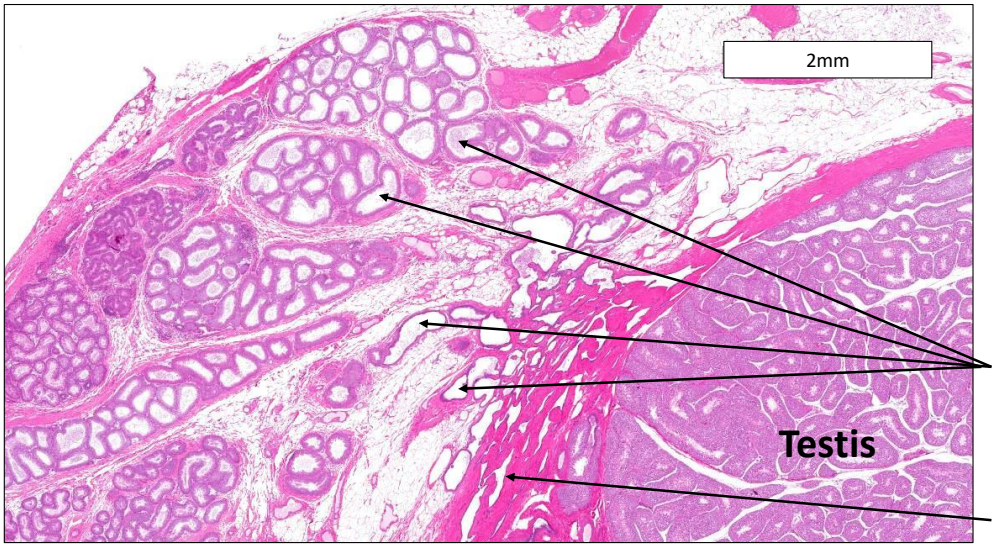
Left: Unilateral dilation of the seminiferous tubules with dilation of the rete testis (arrows)

# Rat: Testis

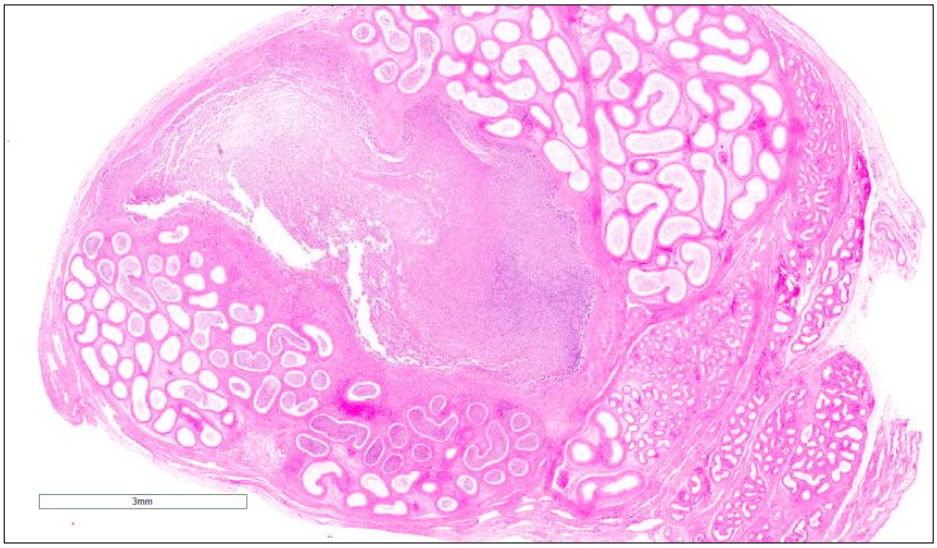


Higher magnification: Unilateral dilation of the seminiferous tubules with dilation of the rete testis (arrows)

# NHP: Rete Testis and Efferent Ducts

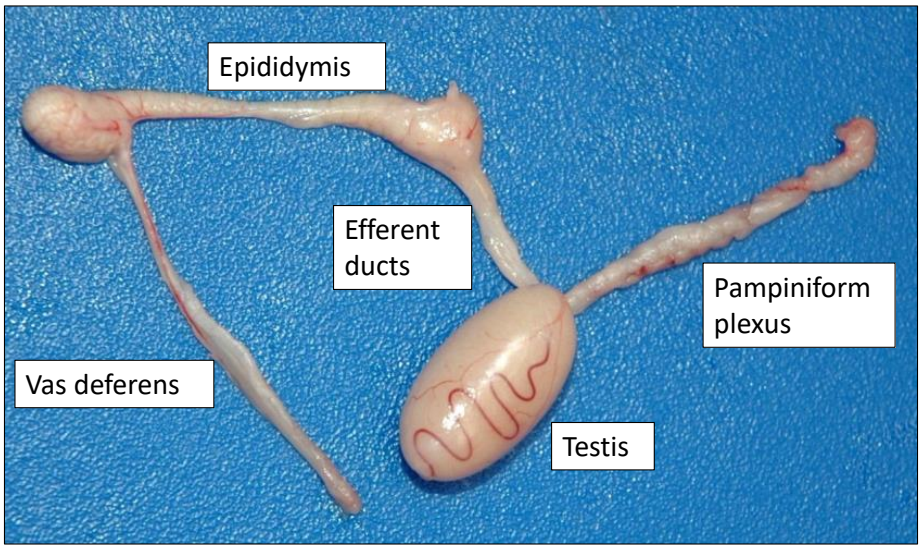



# Dog: Efferent Ducts/Head of Epididymis

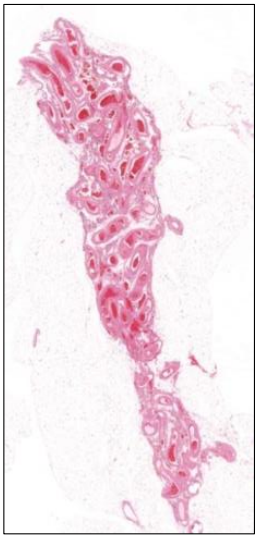


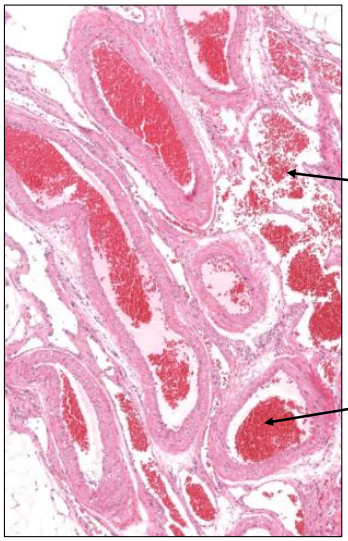

Sperm Granuloma

# Pampiniform Plexus

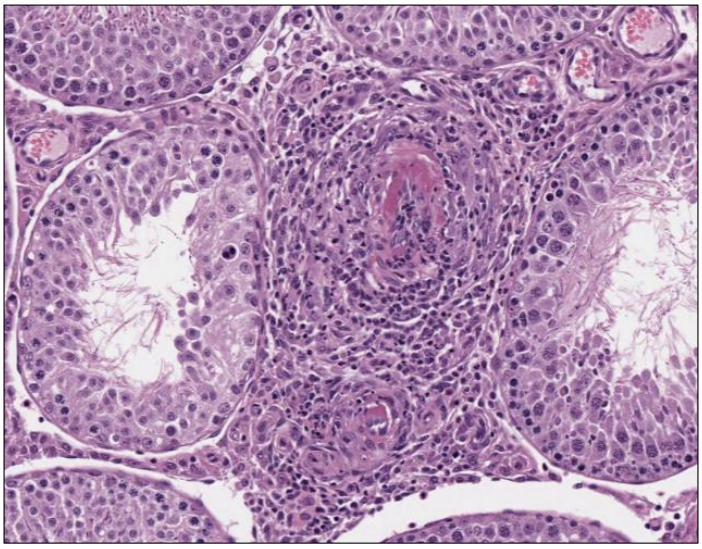



# Pampiniform Plexus



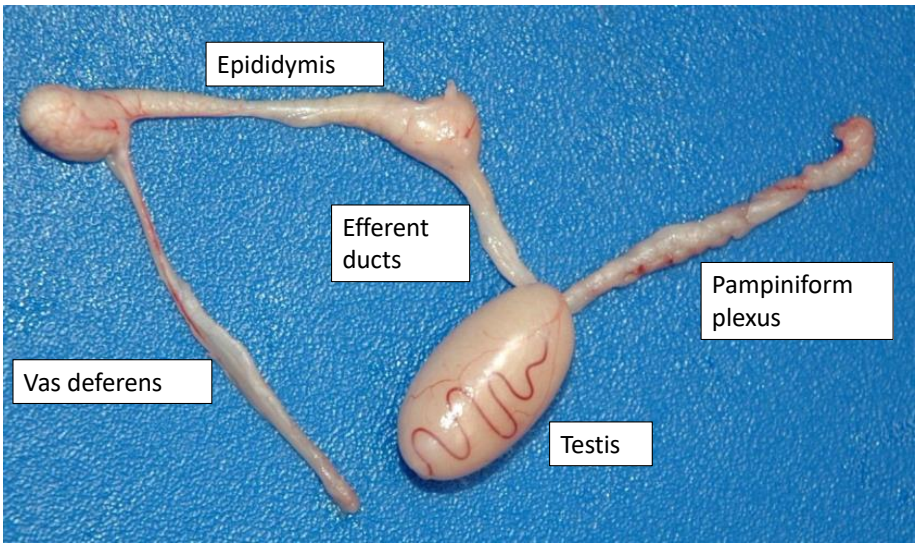


# Rat: Testis

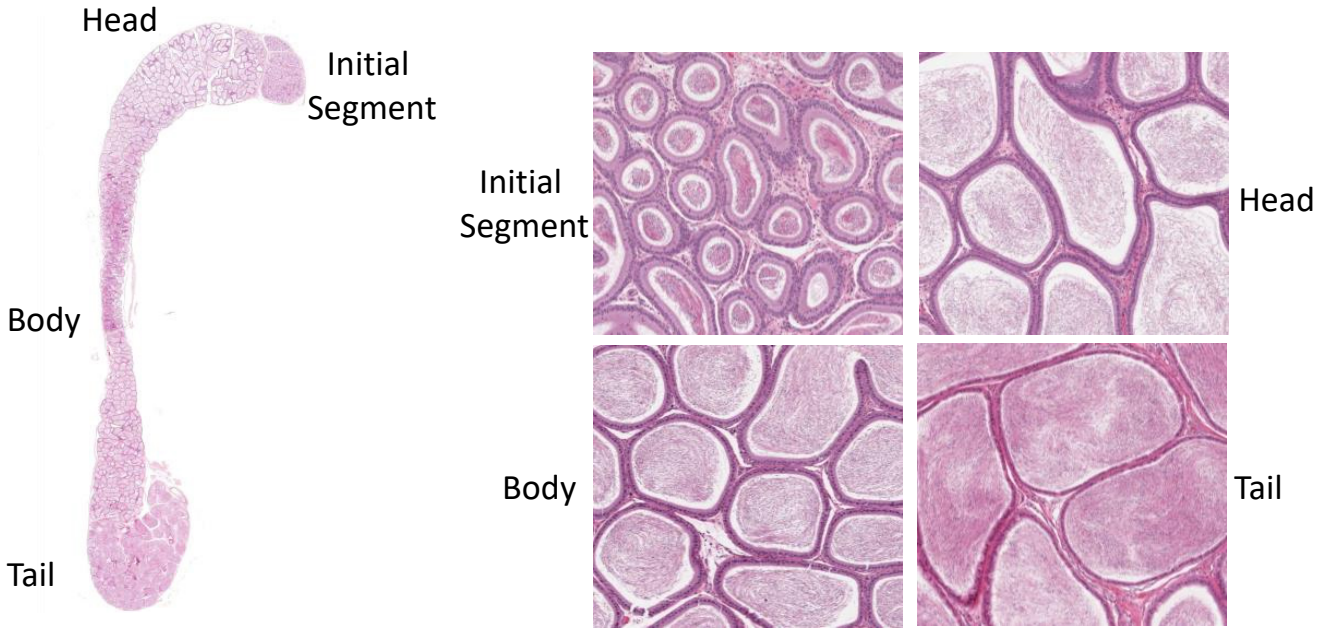


Vascular necrosis and inflammation with tubular degeneration

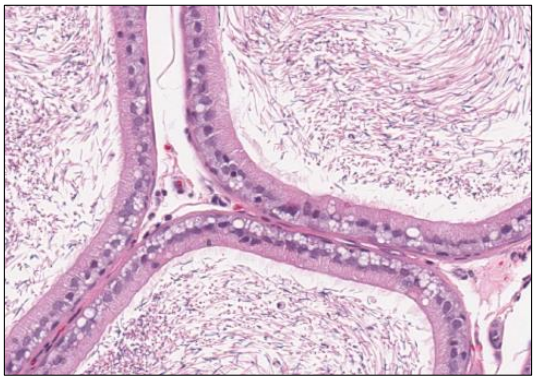
# Epididymis



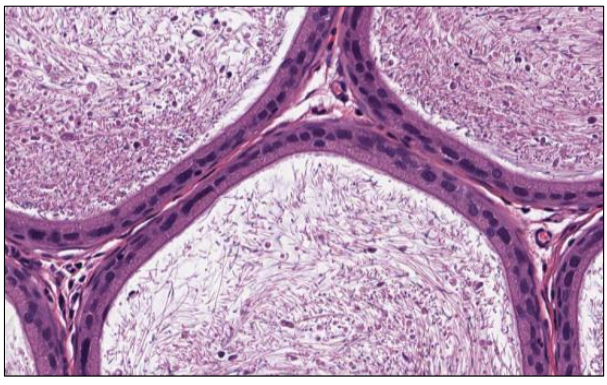
# Rat Epididymis




# Rat Epididymis



Epithelial vacuolation



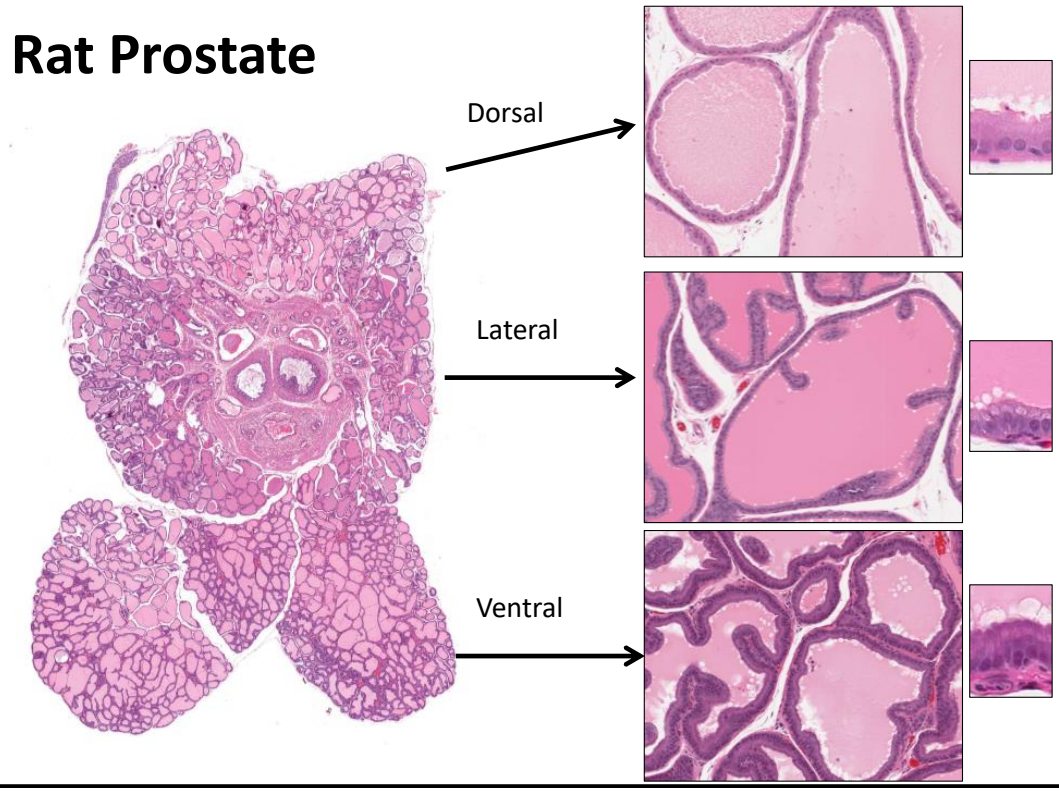
Increased luminal cell debris

# Accessory Sex Organs

| Accessory Sex Organ | Rodent | Dog | NHP | Minipig |
|---|---|---|---|---|
| Prostate | + | + | + | + |
| Coagulating Gland | + | - | +/-* | - |
| Seminal Vesicles | + | - | + | + |
| Bulbourethral Gland | + | - | + | + |
| Ampullary Gland | + | + | - | - |
| Preputial Gland | + | - | - | - |
| Preputial Diverticulum | - | - | - | + |

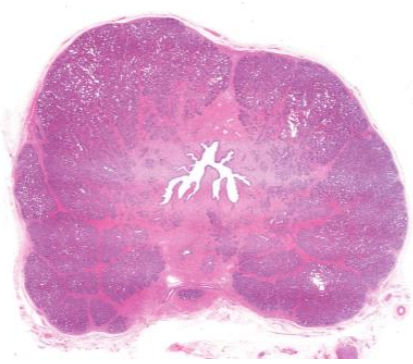
*Macaques have cranial and caudal lobes of the prostate and the cranial lobe serves a coagulating gland-like function

# Rat Prostate



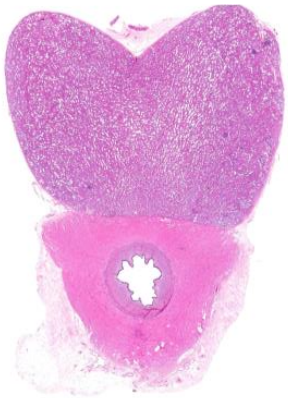
# Nonrodent Accessory Sex Organs

Dog Prostate



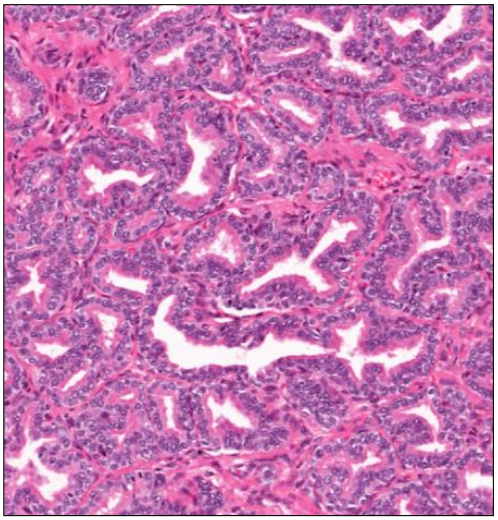
-Prostate encircles the urethra with right and left lobes
-No seminal vesicles

NHP Caudal Prostate



-Prostate does not encircle the urethra and have cranial and caudal lobes
-Large seminal vesicles

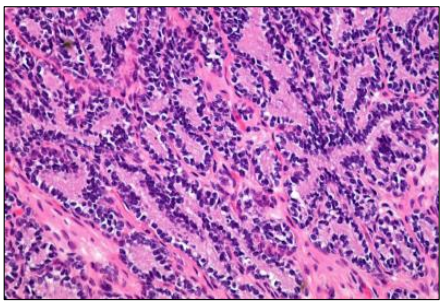
# Dog Prostate



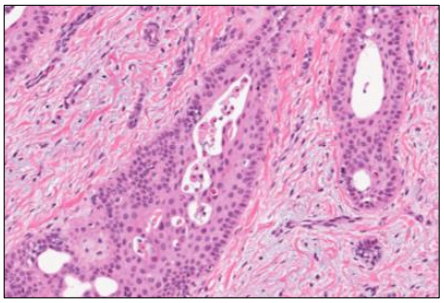
Control

Decreased androgen production

Atrophy



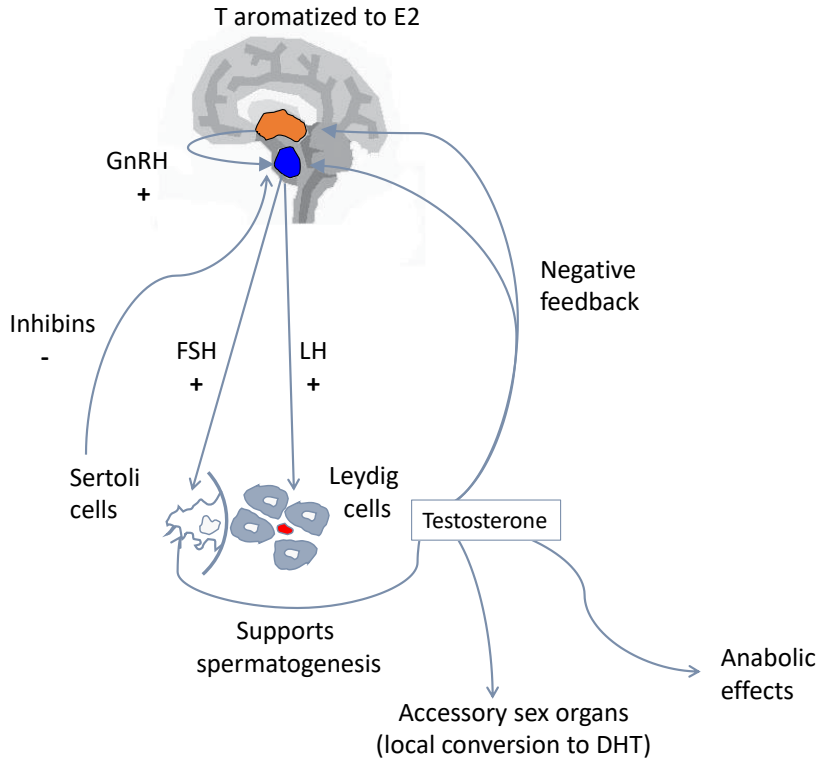
Estrogenic stimulation



Squamous metaplasia and stromal edema

# Endocrinology

- Hypothalamus
  - Gonadotropin releasing hormone (GnRH)
- Pituitary
  - Follicle stimulating hormone (FSH)
  - Luteinizing hormone (LH)
  - Prolactin
- Testis
  - Testosterone (T)
  - Inhibins
  - Estradiol (E2) species dependent
- Peripheral conversion
  - Dihydrotestosterone (DHT)
  - Estradiol (E2)



## Variation in Testosterone Secretion

- Rats
  - No clear diurnal rhythm
  - Highly variable over the day
  - T peaks do not always follow LH peaks
- Dogs
  - No clear diurnal rhythm
  - T peaks follow LH peaks every hour to 1.5 hours
- Pigs
  - No clear diurnal rhythm
  - T peaks follow LH peaks every 2–3 hrs (age dependent)
- NHP
  - Peaks at night
  - T peaks follow LH peaks

## Summary/Conclusion

- Pathology assessment plays a major role in understanding male reproductive effects
- Remember species-specific differences in anatomy and physiology
- Must have clear understanding of the cell types affected in the testis
- Carefully consider timing of sample collection and assessment

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- OPPTS (Office of Prevention, Pesticides and Toxic Substances) (US EPA) Health Effects Draft Guidelines: OPPTS 870.3800 Reproduction and Fertility Effects [EPA 712-C-96-208] Posted: 06/23/2011.

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# Prenatal Development of the Male Reproductive Tract in the Rat, Dog, and Human: Critical Developmental Windows and Later-Life Consequences of Exposure

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Brown University
Providence, RI
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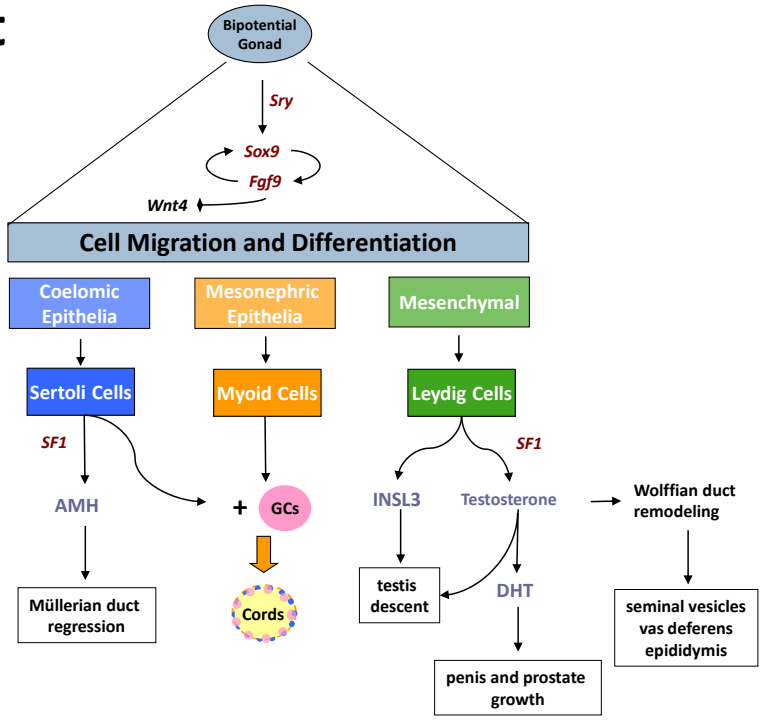
## Disclosure

Current funding from NIEHS. Occasional expert consultant for chemical and pharmaceutical companies, including Tb Alliance and Lantmännen Medical.

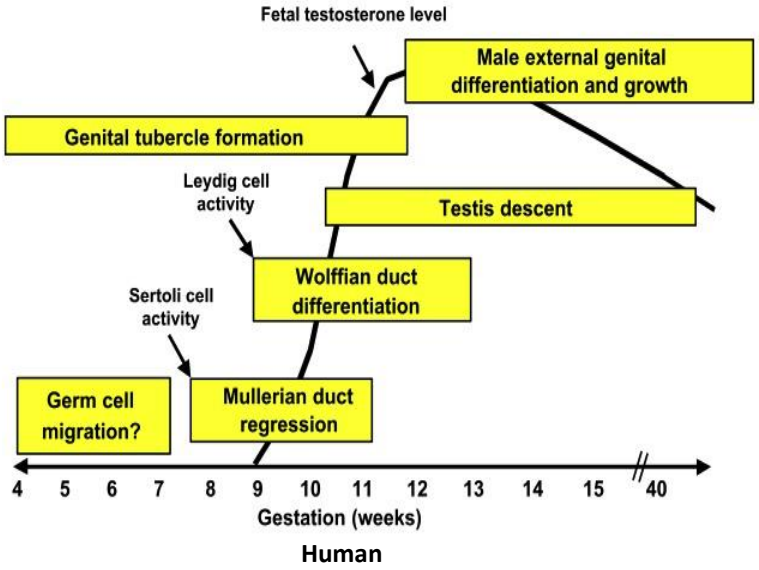
# Fetal Testis Development

- A sequence of molecular and hormonal signaling events drive the bipotential gonad down the path of male differentiation
- Disruption of these signals results in dysgenesis, including intersex conditions, testicular dysgenesis, and abnormalities in the external genitalia

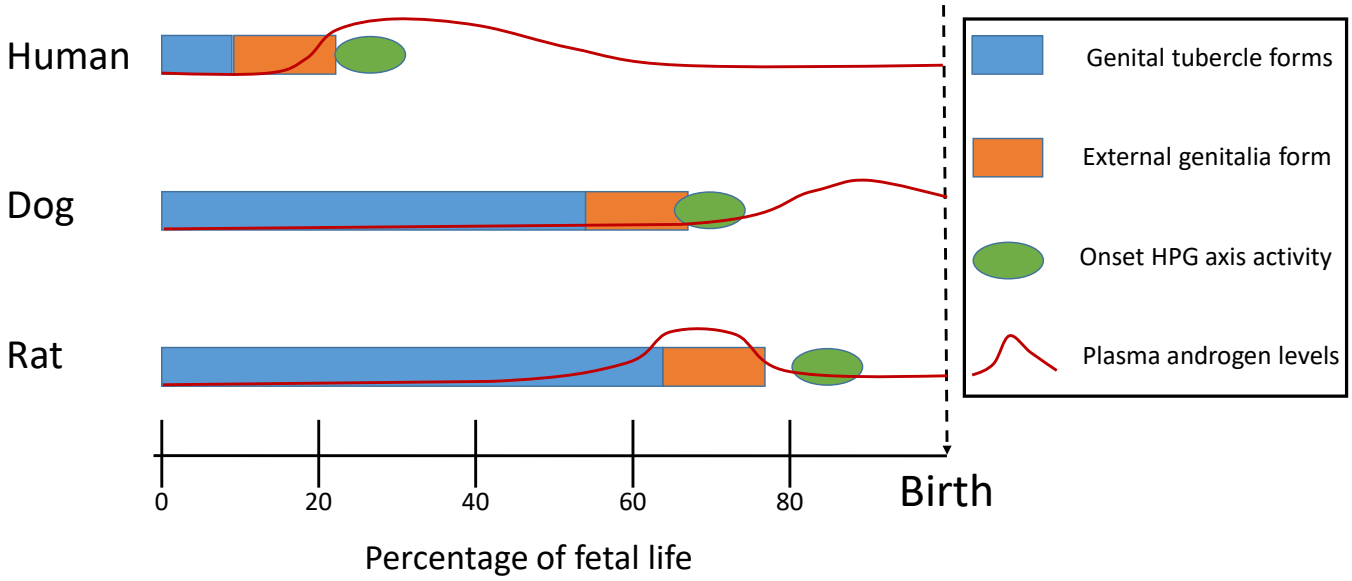



# Fetal Testis Development

- The order of developmental events is similar across mammals
- But the timing, relative to parturition, differs significantly

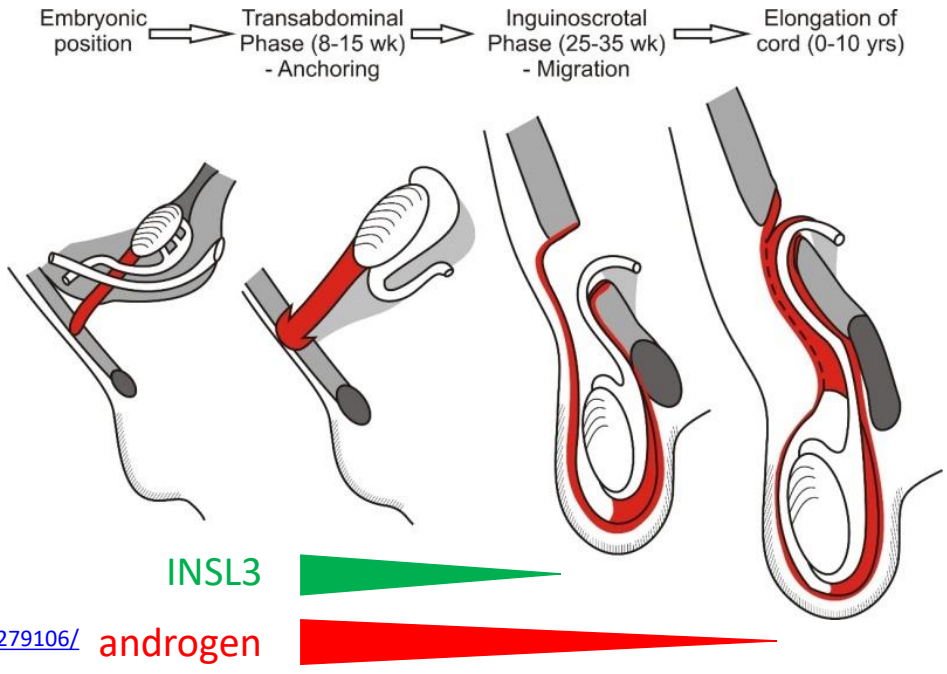


# Fetal Male Reproductive Developmental Milestones




# Normal Human Testicular Descent

- Insulin-like 3 protein (Insl-3) is made by Leydig cells and regulates growth and differentiation of gubernaculum, mediating intra-abdominal testicular descent
- Androgens produced by the Leydig cell mediate all phases of testicular descent




ENDOTEXT: Hutson et al., Cryptorchidism and Hypospadias
https://www.ncbi.nlm.nih.gov/books/NBK279106/

Descended testis

Cryptorchid testis

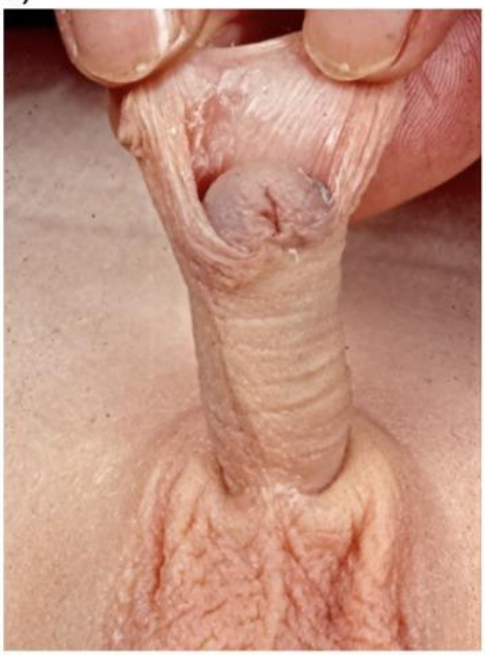
## Human Hypospadias

ENDOTEXT: Hutson et al., Cryptorchidism and Hypospadias https://www.ncbi.nlm.nih.gov/books/NBK279106/

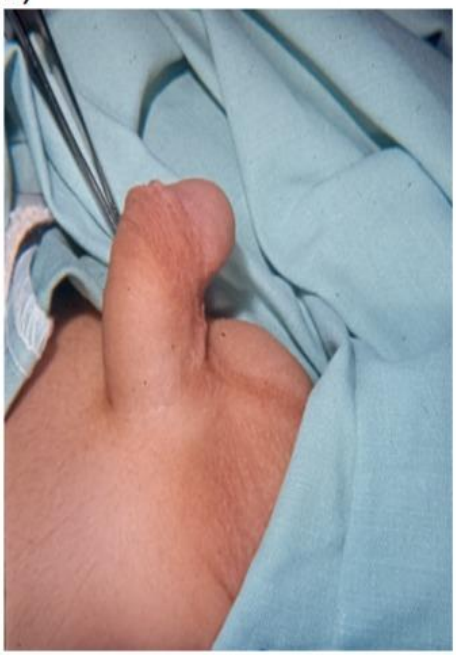
Hypospadias is associated with:

- Failure of the urethral meatus to be located on the tip of the glans
- Failed ventral fusion of the prepuce, causing a “dorsal hood”
- Inadequate growth of the ventral shaft around the urethra, leading to bend

a)

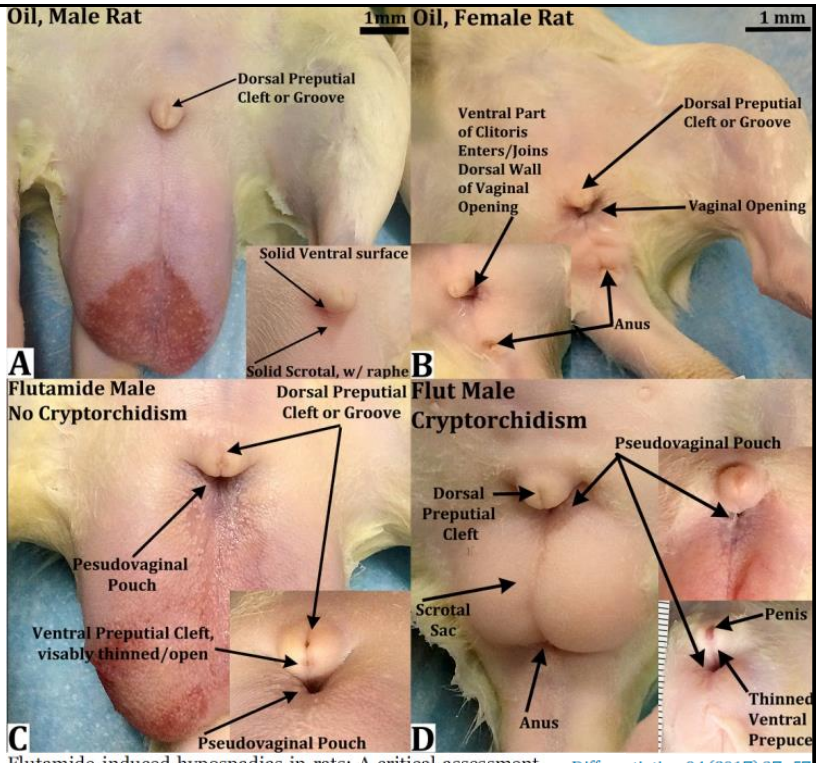


b)



# Flutamide-Induced Hypospadias in Rat

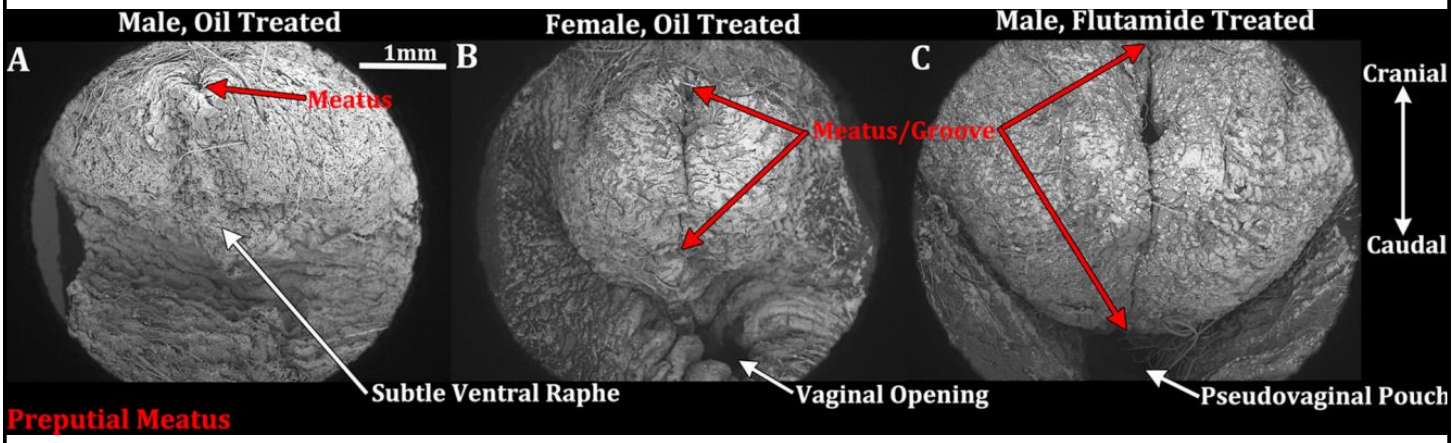
- Flutamide is a potent androgen receptor antagonist that produces hypospadias
- A ventral preputial cleft is present in all flutamide-treated males as well as a pseudovaginal pouch
- The inset in (D) shows the tip of the penis, which normally would not be visible




Flutamide-induced hypospadias in rats: A critical assessment Differentiation 94 (2017) 37–57

Adriane Watkins Sinclair[a], Mei Cao[a], Andrew Pask[b], Laurence Baskin[a], Gerald R. Cunha[a,*]

# Flutamide-Induced Hypospadias in Rat



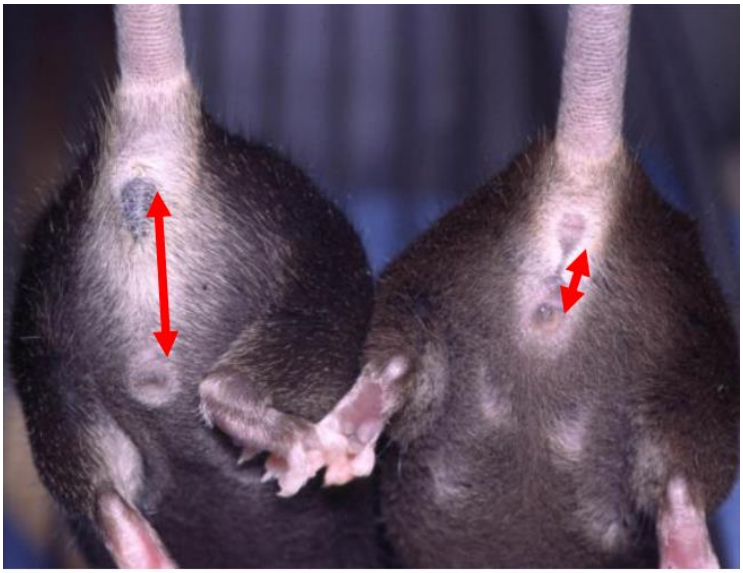

- The size of the preputial meatus is defined in red, with the flutamide-treated male having the longest preputial meatus/groove for males

Flutamide-induced hypospadias in rats: A critical assessment Differentiation 94 (2017) 37–57

Adriane Watkins Sinclair[a], Mei Cao[a], Andrew Pask[b], Laurence Baskin[a], Gerald R. Cunha[a,*]

# Anogenital Distance

Anogenital distance (AGD) is a sexually dimorphic endpoint useful in both animal and human studies of *in utero* male reproductive tract development



MALE FEMALE

The Jackson Laboratory, Reproductive Biology of Mice Webinar, August 9, 2018

## Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract?

THE LANCET VOL 341: MAY 29, 1993

RICHARD M. SHARPE NIELS E. SKAKKEBAEK

"We argue that the increasing incidence of reproductive abnormalities in the human male may be related to increased oestrogen exposure *in utero*, and identify mechanisms by which this exposure could occur."

Development of the male reproductive tract

Estrogen suppresses Leydig cell testosterone:
- Inhibits testis descent
- Inhibits masculinization

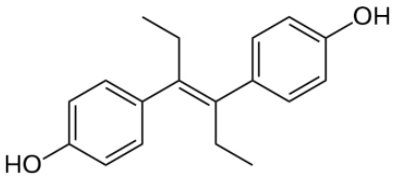
→ Testis "descends" into scrotum

Hormonal control of the fetal testis

Estrogen suppresses HPG axis:
- Inhibits Sertoli cell function
- Reduces sperm production

→ Sertoli cell proliferation and germ cell support

# Diethylstilbestrol (DES)




- Powerful synthetic estrogen
- Prescribed to pregnant women 1941–1971
- Strong evidence of effects on male fetuses in animal studies
- Epidemiologic evidence of reproductive effects in males exposed *in utero* (relatively low risk)
- Human fetal testis xenotransplants

# DES—Animal Studies

**Lesions in the reproductive tract of male mice exposed prenatally to DES. Males were the 9- to 10-month-old offspring of CD-1 mice treated with DES (100 µg/kg, subcutaneously) on days 9 to 16 of gestation.**

| Prenatal treatment | Incidence of lesions* | Location of lesions | | |
|---|---|---|---|---|
| | | Testis † | Epididymal cysts | Accessory sex gland § |
| Corn oil | 0/14 | 0 | 0 | 0 |
| DES | 18/24 | 15 | 10 | 6 |

† cryptorchidism and/or fibrosis/calcification; § nodular masses with squamous metaplasia

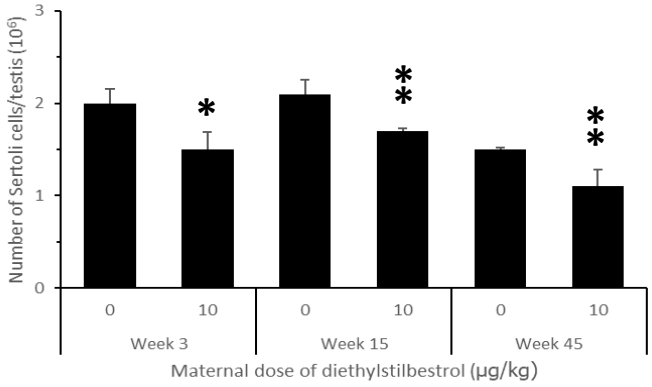
**Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol**

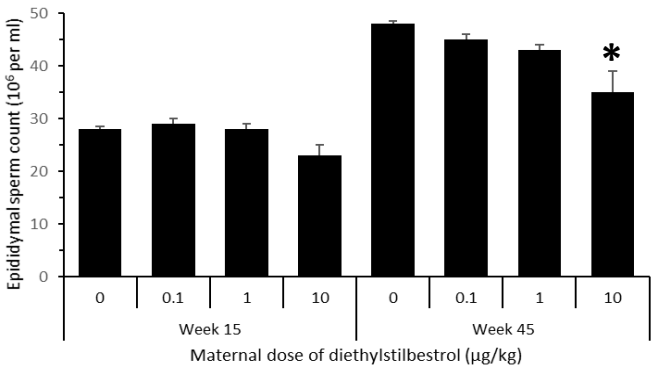
JA McLachlan, RR Newbold and B Bullock 5 DECEMBER 1975 *Science* **190** (4218), 991-992.

**Gestational and Lactational Exposure of Male Mice to Diethylstilbestrol Causes Long-Term Effects on the Testis, Sperm Fertilizing Ability *in Vitro*, and Testicular Gene Expression**

MARK R. FIELDEN, ROBERT G. HALGREN, CORA J. FONG, CHRISTOPHE STAUB, LARRY JOHNSON, KAREN CHOU, AND TIM R. ZACHAREWSKI

- Sertoli cells proliferate until PND~15
- DES exposure during gestation and lactation inhibits Sertoli cell proliferation
- Germ cell numbers are proportional to the number of Sertoli cells
- Therefore, ↓Sertoli cells means ↓germ cells and ↓epididymal sperm count




# DES—Epidemiology

## DES exposure in relation to urogenital abnormalities

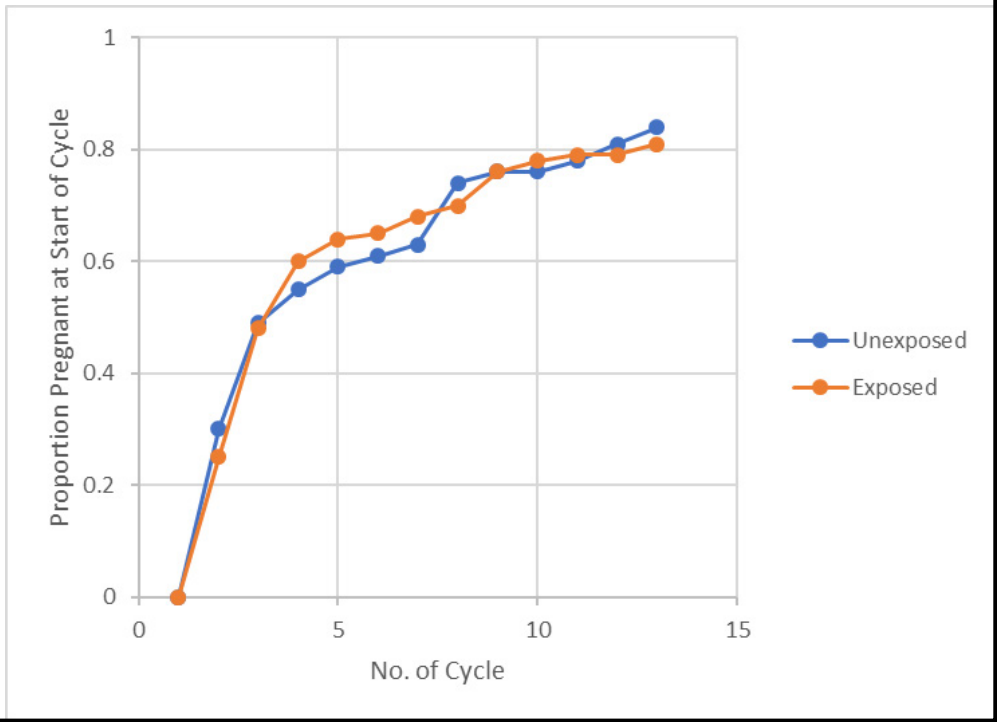
| Urogenital abnormalities | DES-Exposed (N = 1197) | Unexposed (N = 1038) | Risk ratio | 95% Confidence interval |
|---|---|---|---|---|
| Cryptorchidism | 38 | 17 | 1.9 | 1.1–3.4 |
| Epididymal cyst | 55 | 19 | 2.5 | 1.5–4.3 |
| Varicocele | 63 | 61 | 0.9 | 0.6–1.3 |
| Penis abnormality | 9 | 7 | 1.1 | 0.4–3.0 |
| Urethral stenosis | 14 | 9 | 1.3 | 0.6–3.1 |

**Urogenital abnormalities in men exposed to diethylstilbestrol *in utero*: a cohort study** Julie R Palmer *Environmental Health* 2009, 8:37

**FERTILITY IN MEN EXPOSED PRENATALLY TO DIETHYLSTILBESTROL**

ALLEN J. WILCOX J Med 1995;332:1411-6.

No difference in fertility in men exposed to DES prenatally compared to those unexposed




**Testicular Dysgenesis Syndrome and the Estrogen Hypothesis: A Quantitative Meta-Analysis** *Health Perspect* 116:149–157 (2008).

*Olwenn V. Martin,[1,2] Tassos Shialis,[1] John N. Lester,[3] Mark D. Scrimshaw,[4] Alan R. Boobis,[2] and Nikolaos Voulvoulis[1]*

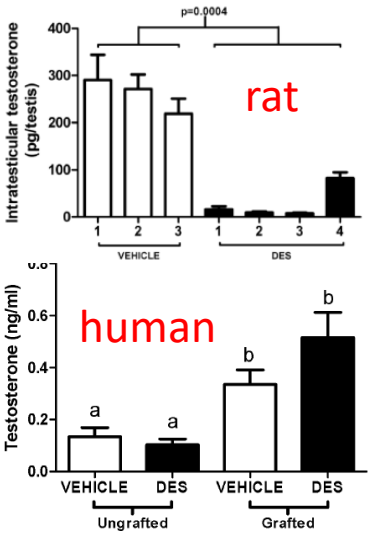
"While it is clear that hypospadias, cryptorchidism, and testicular cancer are all positively associated with prenatal exposure to DES, this meta-analysis was unable to produce evidence that such effects were associated with environmental estrogens or even accidental use of oral contraceptives during pregnancy."

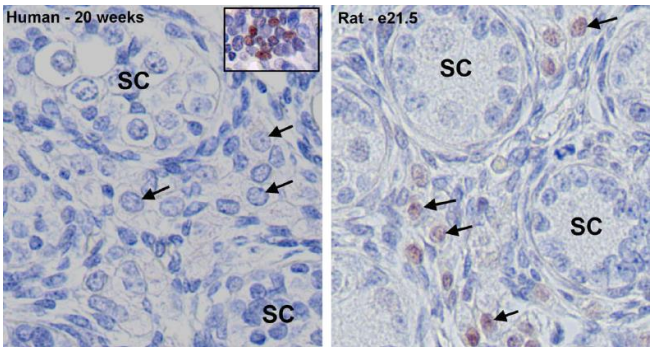
# DES—Testis Xenotransplants

## Diethylstilboestrol Exposure Does Not Reduce Testosterone Production in Human Fetal Testis Xenografts

**Rod T. Mitchell** et al., PLoS ONE 8(4): e61726.

Note the abundant estrogen receptor alpha (ER-α) staining in the rat Leydig cell compared with the human, and the corresponding difference in diethylstilbesterol (DES) exposure on testis xenotransplant production of testosterone

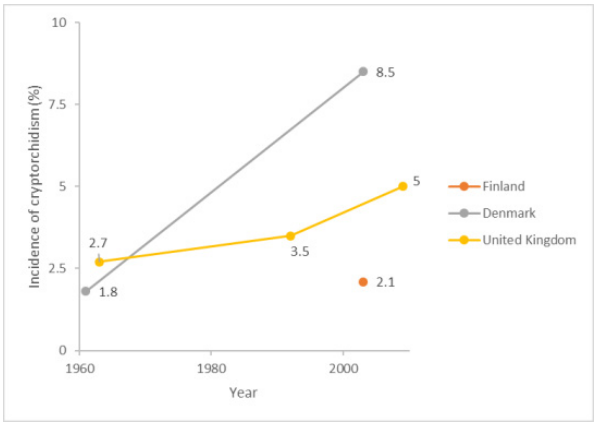



Immunohistochemistry for ER-α
human<< rat

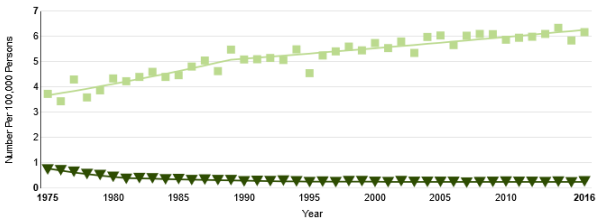
# Human Testicular Dysgenesis Syndrome (TDS)

Epidemiological research suggests that male reproductive disorders—including cryptorchidism, hypospadias, infertility, and testicular cancer—have been increasing in incidence




DOI: (10.1152/physrev.00017.2015)

Testis germ cell cancer incidence




NIH NATIONAL CANCER INSTITUTE
Surveillance, Epidemiology, and End Results Program

# Male Reproductive Health Trends

*Altered Leydig cell function*

↓

Lowered testosterone/Insl3 production

↙ ↘

*Cryptorchidism* *Hypospadias*

*Altered Sertoli cell function*

↓

Impaired germ cell development

↙ ↘

*Sperm quality/quantity* *Testicular cancer*

# Male Reproductive Health Trends

*Altered Leydig cell function*

↓

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↙ ↘

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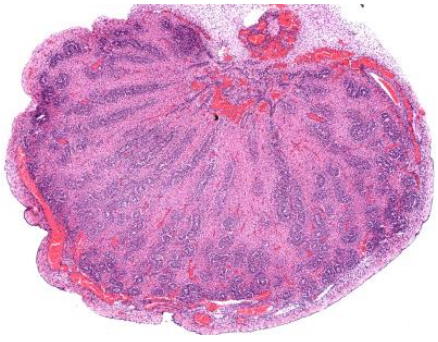
*Altered Sertoli cell function*

↓

Impaired germ cell development

↙ ↘

*Sperm quality/quantity* *Testicular cancer*



# Male Reproductive Health Trends

*Altered Leydig cell function*

↓

Lowered testosterone/Insl3 production

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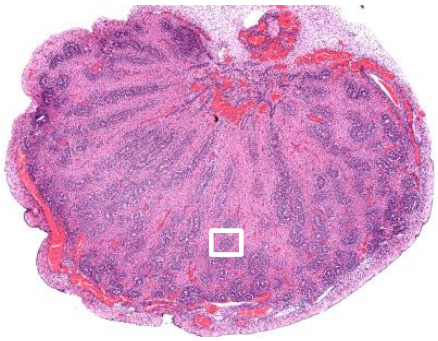
*Altered Sertoli cell function*

↓

Impaired germ cell development

*Sperm quality/quantity* *Testicular cancer*



# Male Reproductive Health Trends

*Altered Leydig cell function*

↓

Lowered testosterone/Insl3 production

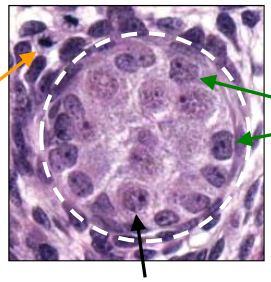
*Cryptorchidism* *Hypospadias*

*Altered Sertoli cell function*

↓

Impaired germ cell development

*Sperm quality/quantity* *Testicular cancer*



# Male Reproductive Health Trends

Environmental Exposures or Genetic Defects

*Altered Leydig cell function* ⟷ *Altered Sertoli cell function*

Lowered testosterone/Insl3 production

Impaired germ cell development

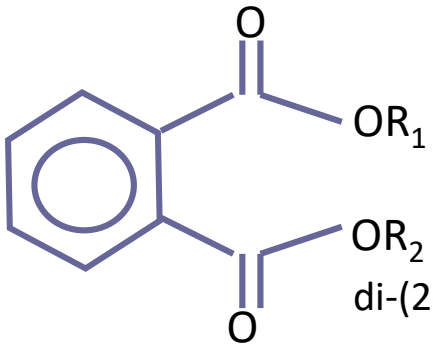
*Cryptorchidism* *Hypospadias* *Sperm quality/quantity* *Testicular cancer*

**Testicular Dysgenesis Syndrome***

- Spectrum of disorders
- Interrelated in nature
- May share common origin in disrupted development

*Skakkebaek et al., *Hum Reprod* 2001; 16(5) 972-8

# The Phthalate Syndrome




$R_1$, $R_2$ =

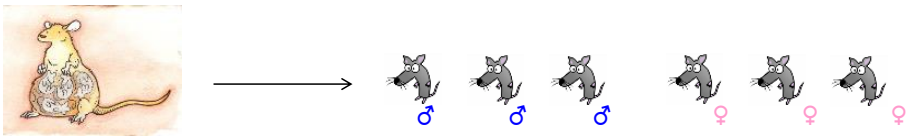
di-(2-ethylhexyl) phthalate (DEHP)

- Testis atrophy after exposure first reported in the 1930s
- Focus was on adult animal model effects until fetal sensitivity observed
  - Rat fetal testis effects on testosterone synthesis and seminiferous cords
  - Rat adult testis effects limited to seminiferous tubules
- "Drug-like" mode of action

# Phthalates and Exposure in Development

- Maternal exposure → fetal exposure
  - Cross placental barrier
  - Metabolites detectible in urine and amniotic fluid
- Critically ill neonates
  - Intensive medical interventions
  - 10–20 mg/day (Loff et al., 2000)
  - "*Serious Concern*" (NTP CERHR/OHAT 2006)
- Children aren't small adults . . .
  - Higher body burden
  - Active development
  - Immature detoxification functions

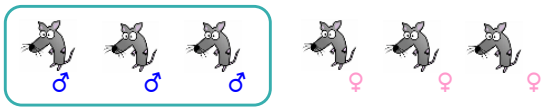
# Phthalate-Induced Effects on Fetal Testis




RAT

# Phthalate-Induced Effects on Fetal Testis

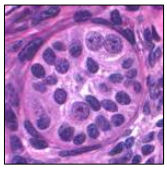
+ phthalate



Seminiferous Cord Effects: ***YES***

**Multinucleated germ cells**

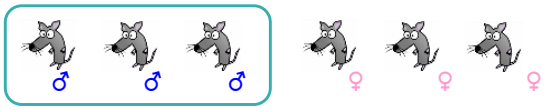
↑ Cord diameter



**RAT**

# Phthalate-Induced Effects on Fetal Testis

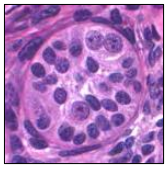
+ phthalate



Seminiferous Cord Effects: ***YES***

**Multinucleated germ cells**

↑ Cord diameter



Leydig Cell Effects: ***YES***

↓ **Steroidogenic genes**

↓ **Testicular testosterone**

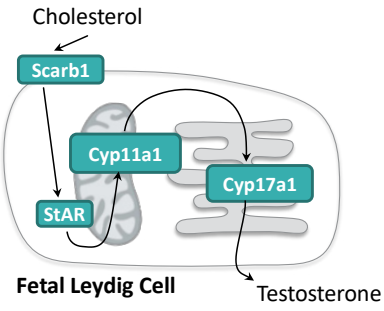
Hypospadias

Cryptorchidism

↓ Anogenital distance

Leydig cell hyperplasia

Nipple/areola retention




**RAT**

# Phthalate-Induced Effects on Fetal Testis

Seminiferous Cord Effects: ***YES***
**Multinucleated germ cells**
↑ Cord diameter

Leydig Cell Effects: ***YES***
↓ **Steroidogenic genes**
↓ **Testicular testosterone**

Hypospadias
Cryptorchidism
↓ Anogenital distance
Leydig cell hyperplasia
Nipple/areola retention

**RAT**

# Phthalate-Induced Effects on Fetal Testis

Seminiferous Cord Effects: ***YES***
**Multinucleated germ cells**
↑ Cord diameter

Leydig Cell Effects: ***YES***
↓ **Steroidogenic genes**
↓ **Testicular testosterone**

Hypospadias
Cryptorchidism
↓ Anogenital distance
Leydig cell hyperplasia
Nipple/areola retention

**RAT**

Seminiferous Cord Effects: ***YES***
**Multinucleated germ cells**
↑ Cord diameter

**MOUSE**

# Phthalate-Induced Effects on Fetal Testis

| RAT | MOUSE |
|---|---|
| Seminiferous Cord Effects: ***YES***<br>**Multinucleated germ cells**<br>↑ Cord diameter | Seminiferous Cord Effects: ***YES***<br>**Multinucleated germ cells**<br>↑ Cord diameter |
| Leydig Cell Effects: ***YES***<br>↓ **Steroidogenic genes**<br>↓ **Testicular testosterone**<br>Hypospadias<br>Cryptorchidism<br>↓ Anogenital distance<br>Leydig cell hyperplasia<br>Nipple/areola retention | Leydig Cell Effects: ***NO***<br>**No decrease in gene expression**<br>**No decrease in testosterone**<br>No hypospadias<br>No cryptorchidism<br>No change anogenital distance<br>No Leydig cell hyperplasia |
|  |  |

# Phthalate-Induced Effects on Fetal Testis

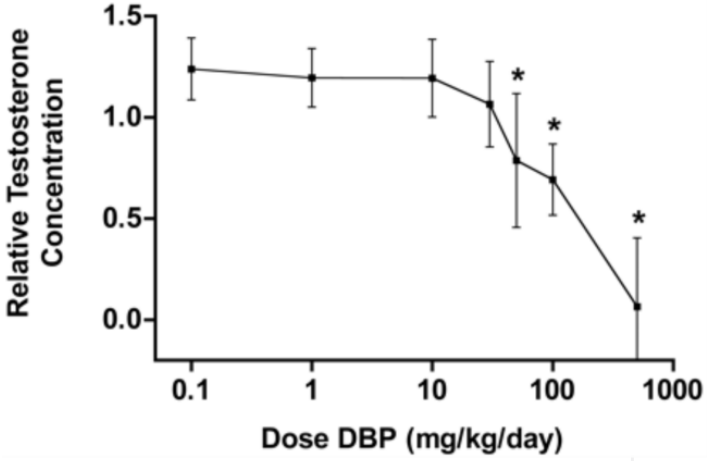
| RAT | MOUSE | HUMAN |
|---|---|---|
| Seminiferous Cord Effects: ***YES***<br>**Multinucleated germ cells**<br>↑ Cord diameter | Seminiferous Cord Effects: ***YES***<br>**Multinucleated germ cells**<br>↑ Cord diameter | Seminiferous Cord Effects:<br>? |
| Leydig Cell Effects: ***YES***<br>↓ **Steroidogenic genes**<br>↓ **Testicular testosterone**<br>Hypospadias<br>Cryptorchidism<br>↓ Anogenital distance<br>Leydig cell hyperplasia<br>Nipple/areola retention | Leydig Cell Effects: ***NO***<br>**No decrease in gene expression**<br>**No decrease in testosterone**<br>No hypospadias<br>No cryptorchidism<br>No change anogenital distance<br>No Leydig cell hyperplasia | |
|  |  |  |

# Phthalate-Induced Effects on Fetal Testis

| RAT | MOUSE | HUMAN |
|---|---|---|
| Seminiferous Cord Effects: ***YES*** | Seminiferous Cord Effects: ***YES*** | Seminiferous Cord Effects: |
| **Multinucleated germ cells** | **Multinucleated germ cells** | ? |
| ↑ Cord diameter | ↑ Cord diameter | |
| Leydig Cell Effects: ***YES*** | Leydig Cell Effects: ***NO*** | Leydig Cell Effects: |
| **↓ Steroidogenic genes** | **No decrease in gene expression** | ? |
| **↓ Testicular testosterone** | **No decrease in testosterone** | |
| Hypospadias | No hypospadias | |
| Cryptorchidism | No cryptorchidism | |
| ↓ Anogenital distance | No change anogenital distance | |
| Leydig cell hyperplasia | No Leydig cell hyperplasia | |
| Nipple/areola retention | | |

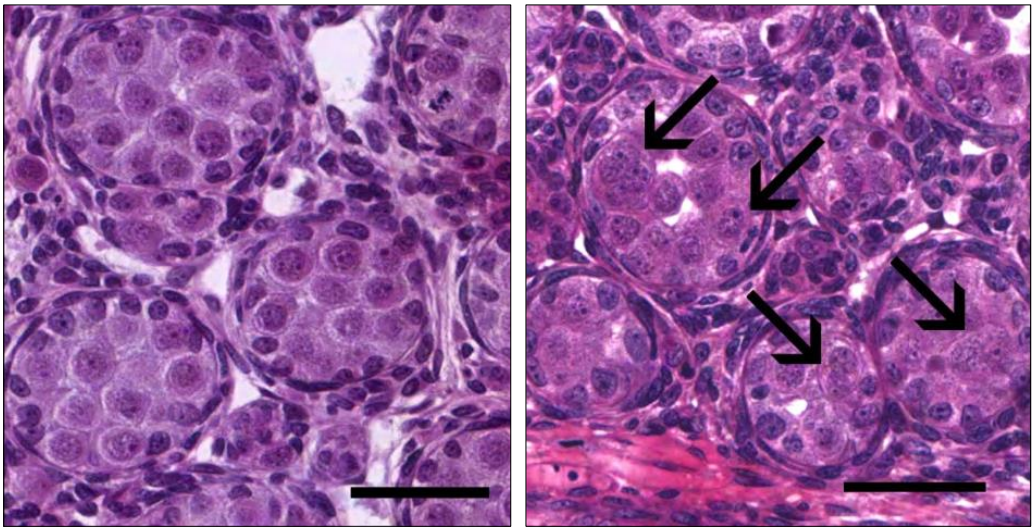
# Di-(n-butyl) Phthalate and Rat Fetal Testosterone

Exposure of rat dams to di-(n-butyl) phthalate significantly inhibits fetal testicular Leydig cell synthesis of testosterone with a LOAEL of ~30 mg/kg/d



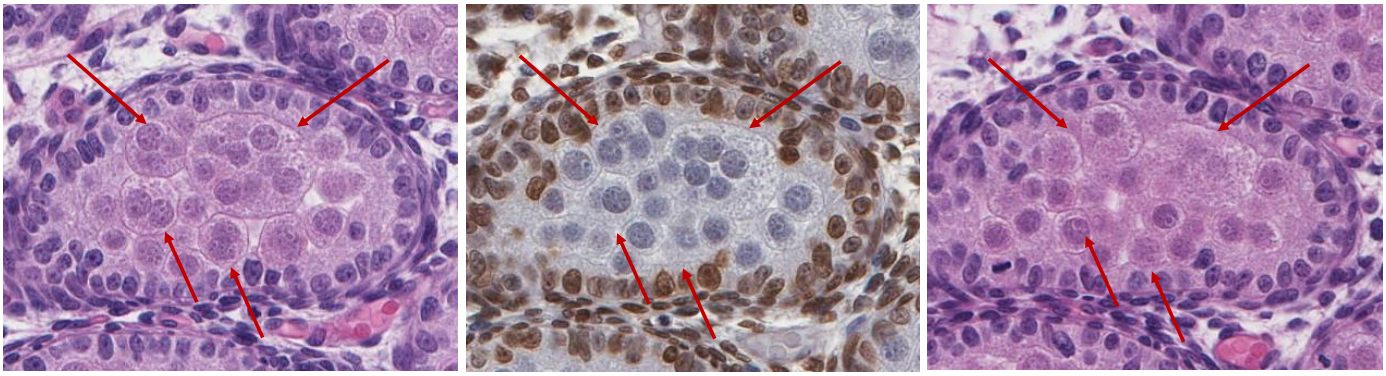

From: Dose-Dependent Alterations in Gene Expression and Testosterone Synthesis in the Fetal Testes of Male Rats Exposed to Di (n-butyl) phthalate
*Toxicol Sci*. 2004;81(1):60–68. doi:10.1093/toxsci/kfh169

## Multinucleated Germ Cells (MNGs) Are a Biomarker of Phthalate-Induced Seminiferous Cord Effects in Rat



Heger et al., *Environ Health Perspect*. 2012;120:1137–43

## MNGs Do Not Form by a Proliferative Mechanism

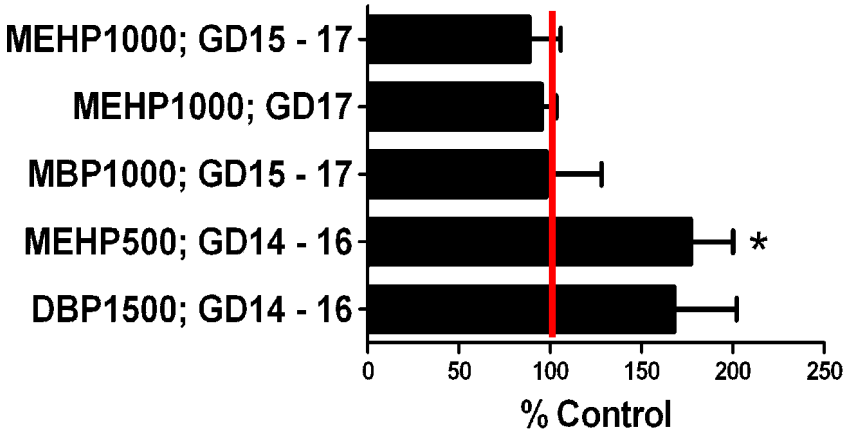


- Late gestational exposure of rat dams to di-(n-butyl) phthalate induces MNGs
- Serial sections of fetal testis labeled with BrdU (middle panel) show unlabeled MNGs

*Biology of Reproduction* 2015;93(5):1–10. https://doi.org/10.1095/biolreprod.115.131615

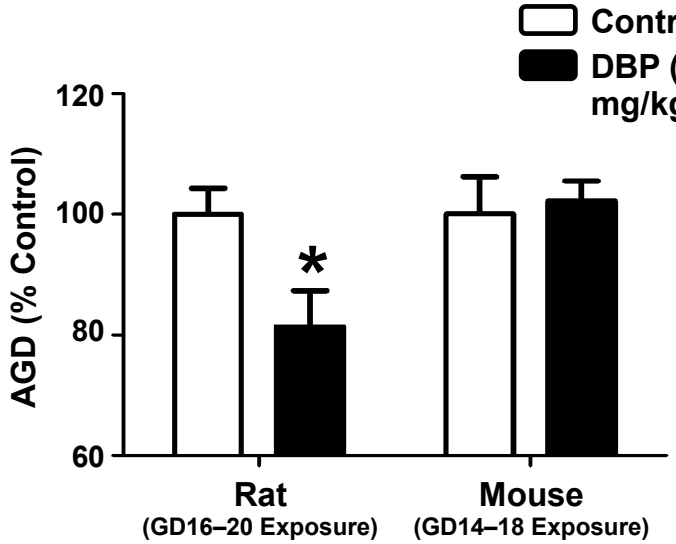
# The Mouse Is Resistant to Phthalate Endocrine Disruption

**Fetal Testis Testosterone**




Gaido et al., *Toxicol Sci* 97: 491 (2007)

# The Mouse Is Resistant to Phthalate Endocrine Disruption




Model: daily fetal DBP gavage during the male programming window

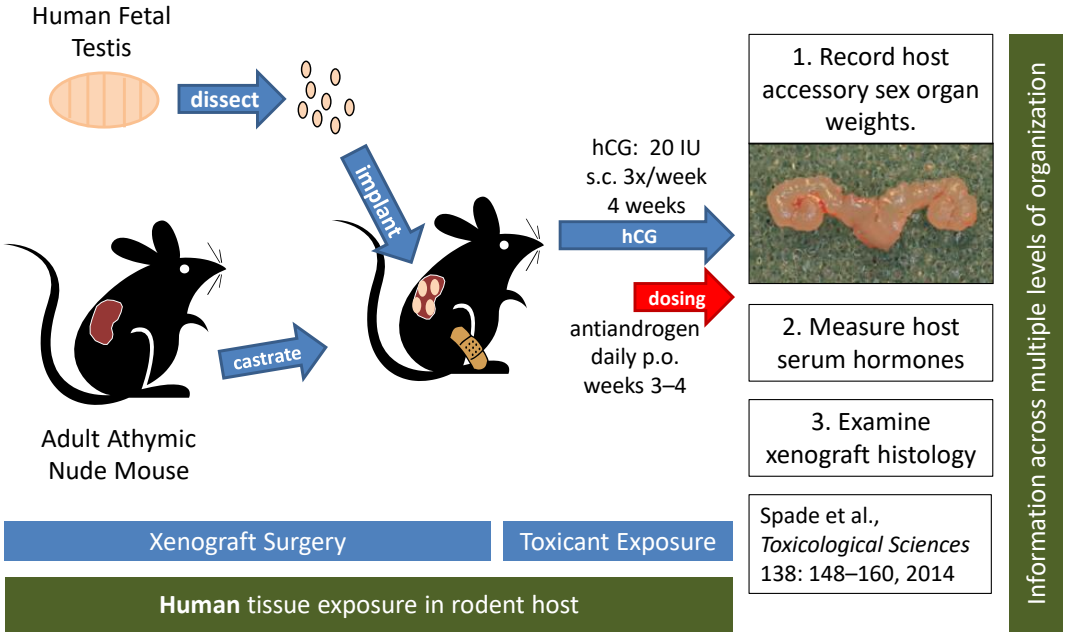
Endpoint: anogenital distance (PND5 in SD rat and PND3 in CD1 mouse)

# Conclusion: Rats Are Sensitive but Mice Are Resistant to Phthalate-Induced Endocrine Disruption

| Endpoint | Rat | Mouse |
|---|---|---|
| Multinucleated Germ Cells | Yes | Yes |
| Global Gene Expression Changes in Fetal Testis | Yes | Yes |
| Steroidogenic Gene Expression | ↓ | ↑ or ↔ |
| Testosterone Production | ↓ | ↑ or ↔ |
| Anogenital Distance Decreases | Yes | No |
| Phthalate Syndrome | Yes | ? |

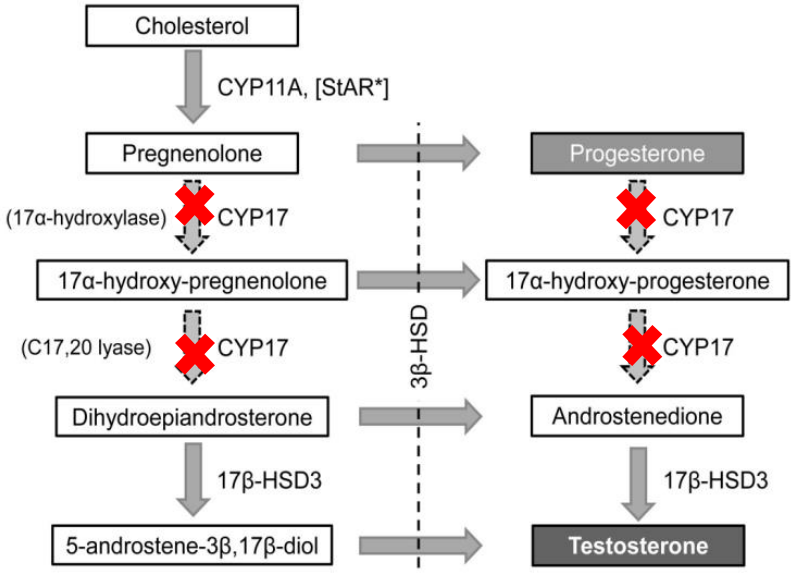
Johnson et al., *Toxicological Sciences* 129:235–248 (2012)

# A Hershberger-Like Xenotransplant Model: An hCG-Stimulated Castrate Mouse Host

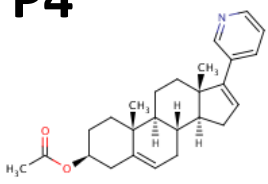


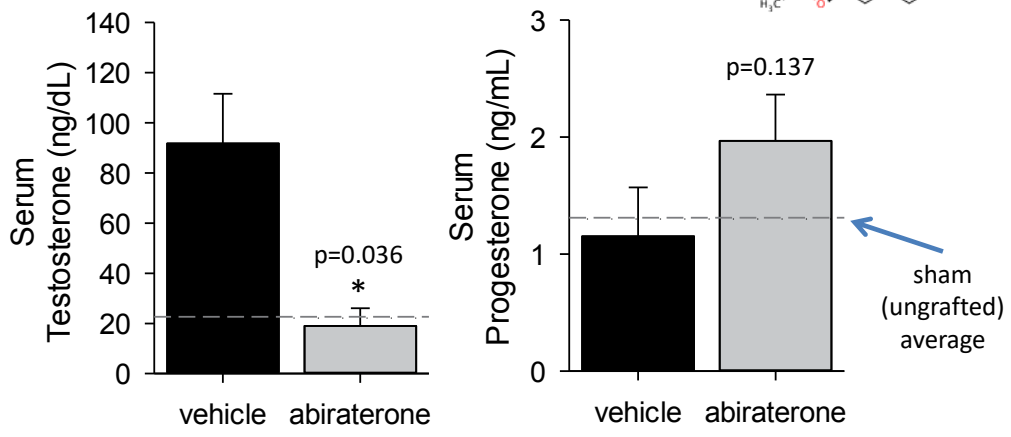
# Abiraterone Acetate Is a Positive Control for Determining Xenograft Response to Steroidogenic Inhibition

Exposure to abiraterone acetate, an irreversible CYP17 inhibitor, leads to reduced testosterone synthesis and shunting of steroidogenesis to the production of progesterone




# Human Fetal Testis Exposure to Abiraterone Acetate Reduces T and Increases P4

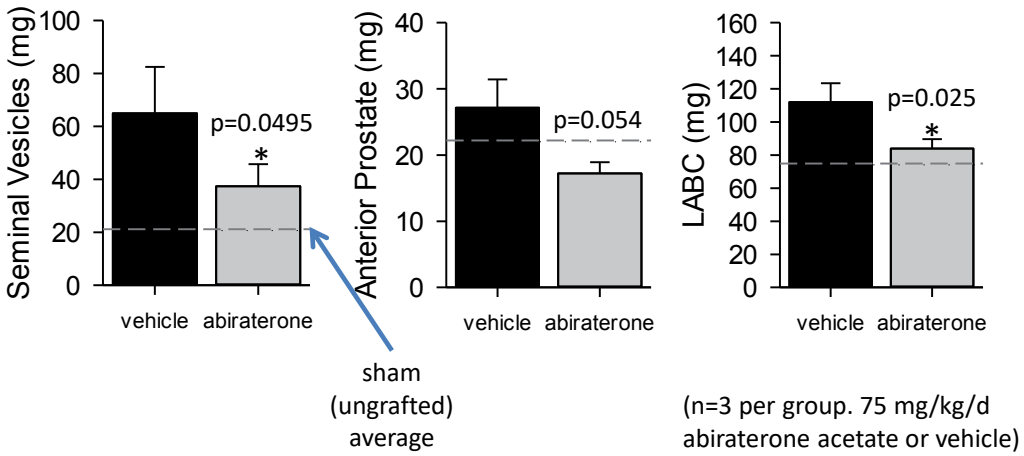





Spade et al., *Toxicological Sciences* 138: 148–160, 2014

(n=3 per group. 75 mg/kg/d abiraterone acetate or vehicle)

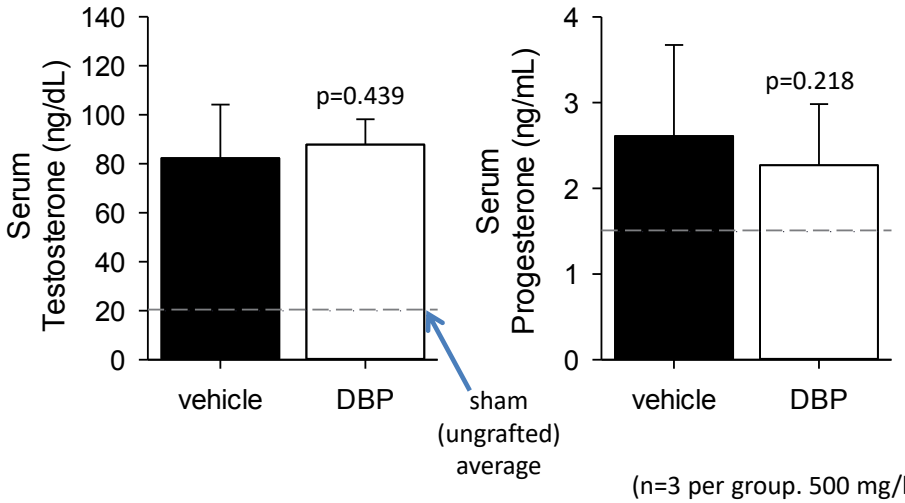
# Abiraterone Acetate Reduces Host Accessory Sex Organ Weights




(n=3 per group. 75 mg/kg/d abiraterone acetate or vehicle)

Spade et al., *Toxicological Sciences* 138: 148–160, 2014

# DBP Has No Antiandrogenic Effect on Human Fetal Testis Xenografts

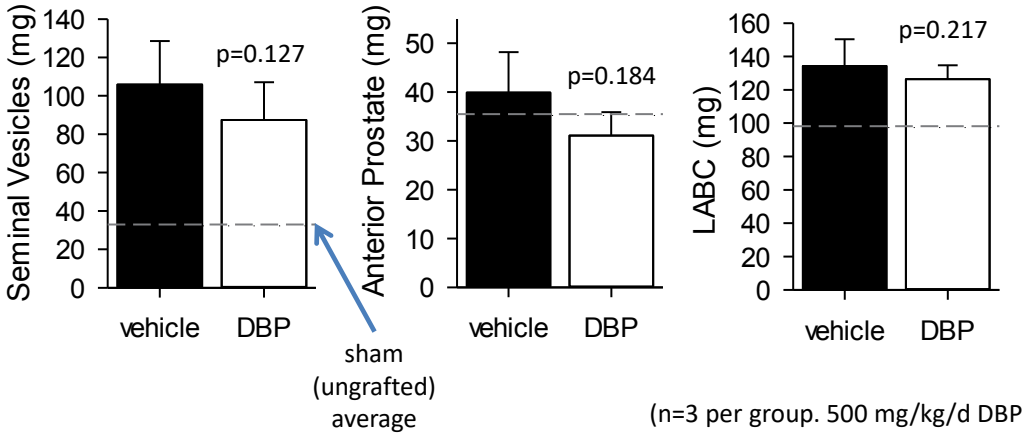



(n=3 per group. 500 mg/kg/d DBP or vehicle)

- No indication that DBP reduces testosterone

Spade et al., *Toxicological Sciences* 138: 148–160, 2014

# DBP Has No Antiandrogenic Effect on Human Fetal Testis Xenografts

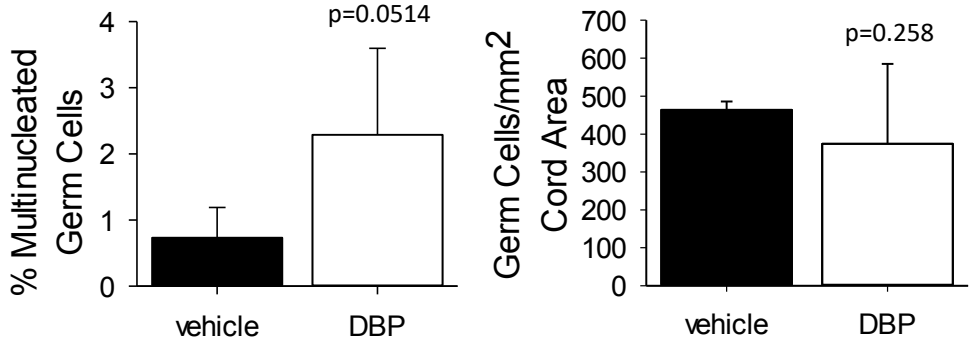



(n=3 per group. 500 mg/kg/d DBP or vehicle)

- No decrease in accessory sex organ weights

Spade et al., *Toxicological Sciences* 138: 148–160, 2014

# Xenograft Morphology




- DBP treatment does not significantly alter germ cell or MNG counts (n=3)
- MNGs trend higher after DBP treatment

(n=3 per group. 500 mg/kg/d DBP or vehicle)

Spade et al., *Toxicological Sciences* 138: 148–160, 2014

## Phthalate Exposure and Male Reproductive Outcomes: A Systematic Review of the Human Epidemiological Evidence

| Timing of exposure | Outcome | DEHP | DINP | DBP | DIBP | BBP | DEP |
|---|---|---|---|---|---|---|---|
| In utero | Anogenital distance | M | S | M | S | S | S |
| | Hypospadias/cryptorchidism | I | S | S | S | S | I |
| In utero or childhood | Pubertal development | I | I | I | I | I | I |
| Adult | Semen parameters | M | M | R | S | M | I |
| | Time to pregnancy | S | I | M | S | M | I |
| | Testosterone | M | M | S | M | I | I |
| | Male repro overall | R | M | R | M | M | S |

Robust (R) | Moderate (M) | Slight (S) | Indeterminate (I)

Level of confidence in association

Radke et al., *Env Int'l* 121 (2018) 764–793

"Overall, despite some inconsistencies across phthalates in the specific outcomes associated with exposure, these results support that phthalate exposure at levels seen in human populations may have male reproductive effects, particularly DEHP and DBP. The relative strength of the evidence reflects differing levels of toxicity as well as differences in the range of exposures studied and the number of available studies."

## Summary

- While the male reproductive tract development sequence is similar across mammals, the timing varies
- Male reproductive tract development is hormonally sensitive
  - Both testis descent and formation of the urethra are androgen dependent
- Estrogenic exposures, such as to diethylstilbesterol (DES), alter male reproductive tract development
  - Rodents may be more sensitive than humans to estrogenic exposures
- Testicular Dysgenesis Syndrome (TDS) is a hypothesis to explain the increased incidence in multiple male reproductive tract developmental abnormalities
- Developmental phthalate exposure in rodents recapitulates several TDS abnormalities
  - The Phthalate Syndrome
- The developing male reproductive tract sensitivity to phthalate varies across species
  - Rat>>Mouse~Human

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# Postnatal Development of the Juvenile Male Reproductive Tract in Rats: Microscopic Evaluation, Interpretation, and Time Points of Toxicologic Significance

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## Conflict of Interest Statement

The author declares no conflict of interest.

# Abbreviations

- AMH: Anti Mullerian hormone
- DHT: dihydrotestosterone
- Esp: elongating spermatid
- GATA: transcription sequence 5'-AGATAG-3
- GD: gestation day
- HSD: hydroxysteroid dehydrogenase
- IHC: immunohistochemistry
- LC: Leydig cell
- MAGE-A4: melanoma associated antigen
- MPW: masculinization programming window
- OCT: Octamer binding protein
- PCNA: proliferating cell nuclear antigen
- PLAP: placenta-like alkaline phosphatase
- PND: postnatal day
- Rsp: round spermatid
- SC: Sertoli cell
- SG: spermatogonia
- SP: spermatocyte
- T: testosterone
- TDS: Testicular Dysgenesis Syndrome
- TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
- VASA: protein encoded by *vasa* gene

# Introduction: Postnatal Stages of Development

| Stage | Rat | Human |
|---|---|---|
| **Neonatal** | Days 0–7 | Days 0–1 Month |
| **Infantile** | Days 8–21 | 1 Month–2 Years |
| **Juvenile/Childhood** | Days 22–32 | Years 2–11 |
| **Peripubertal** | Days 33–55 | Years 11–14 |
| **Sexual Maturity** | Days 56–70 | Years 14–16 |

Stages are approximate, as development occurs on a continuum

# Introduction

## We DOSE "Juvenile" Animals Often, but NO REGULATORY REQUIREMENT to Examine Immature Testes Microscopically

- **Pubertal and Thyroid Function Assay** in Intact Juvenile/Peripubertal Male Rats (OCSPP Guideline 890.1500). US EPA, 2001 (dose PND 23–PND 53). **No requirement.**
- **Juvenile Toxicity Studies** (US FDA 2006). Guidance for Industry. Nonclinical safety evaluation of pediatric Drug Products, US FDA, Feb 2006 (dose juveniles, but evaluate when reach sexual maturity). **No requirement.**
- **Nonclinical Safety Testing in Support of Development of Paediatric Medicines** (ICH Harmonized Guidelines) S11, 2018 (evaluate testes at sexual maturity). **No requirement.**
- **Extended One-Generation (and Two-Generation) Reproductive Toxicity Studies** OECD 443 and 416. **No requirement.**
- **Rat Pre- and Postnatal Developmental Toxicity Study** ICH Guidelines S5R(3); Section 9.4.2 (exposed GD 6 through sexual maturation). **No requirement.**
- **Reproduction/Developmental Toxicity Screening Test** (OECD 421), 2016 (only gross examination of tissues at PND 13). **No requirement.**
- **NTP's Modified One-Generation (MOG) Reproduction Study**—dosed GD6–PND 90+. **No requirement.**

# Introduction

## "Juvenile" Testes Are More Commonly Evaluated in Investigatory Studies

- Most of what we have learned about toxic effects on the juvenile testis comes from investigatory studies done by academia and government research departments (e.g., NIH, NIEHS)
  - Phthalates
  - Bisphenol A
  - Other endocrine disruptors (DDT, organochlorines)

# Introduction

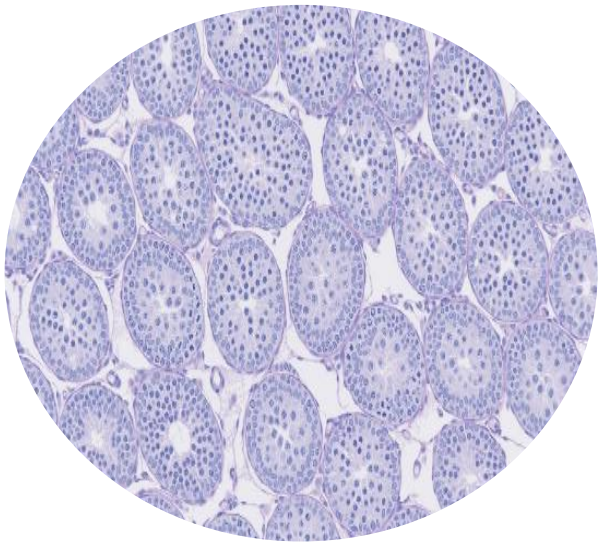
**Goal:**

- To advocate use of a juvenile-stage endpoint in preclinical and environmental safety studies

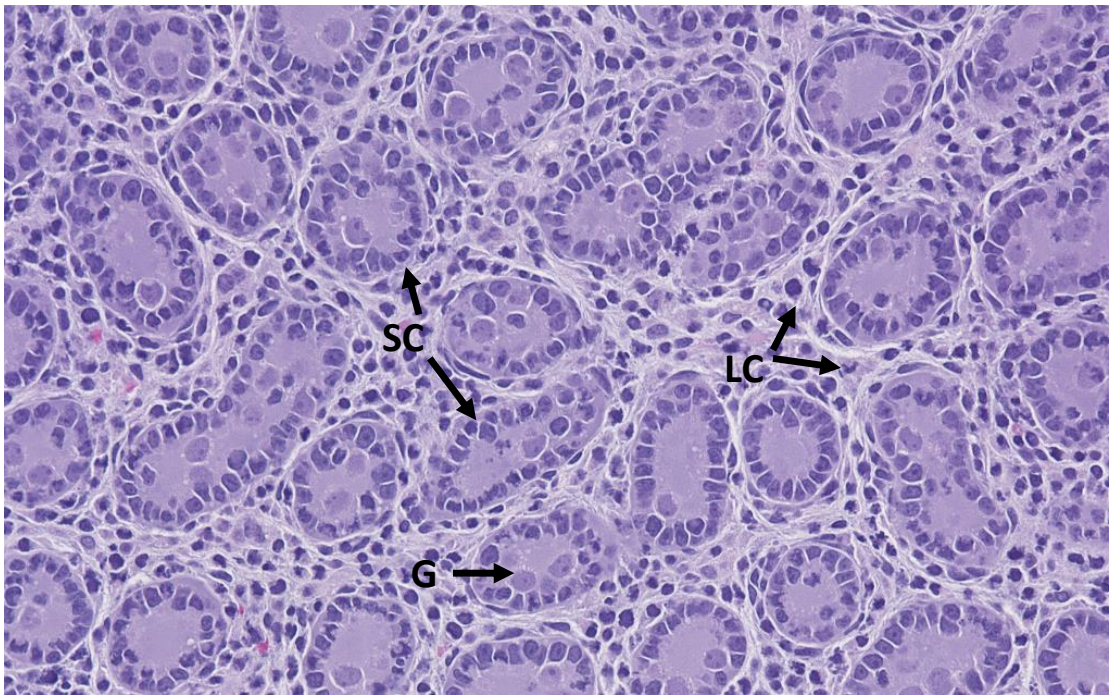
**Agenda:**

- Microscopic features of the "juvenile" rat testis
- Advantages of using "juvenile" testes as an endpoint
  - -- Testicular Dysgenesis Syndrome --
  - -- Case Study Examples --
- Limitations of using "juvenile" testes as an endpoint
- Special procedures

# Microscopic Features of "Juvenile" Testes



# Rat Testis PND 1 (Neonatal)




**Cell types:**

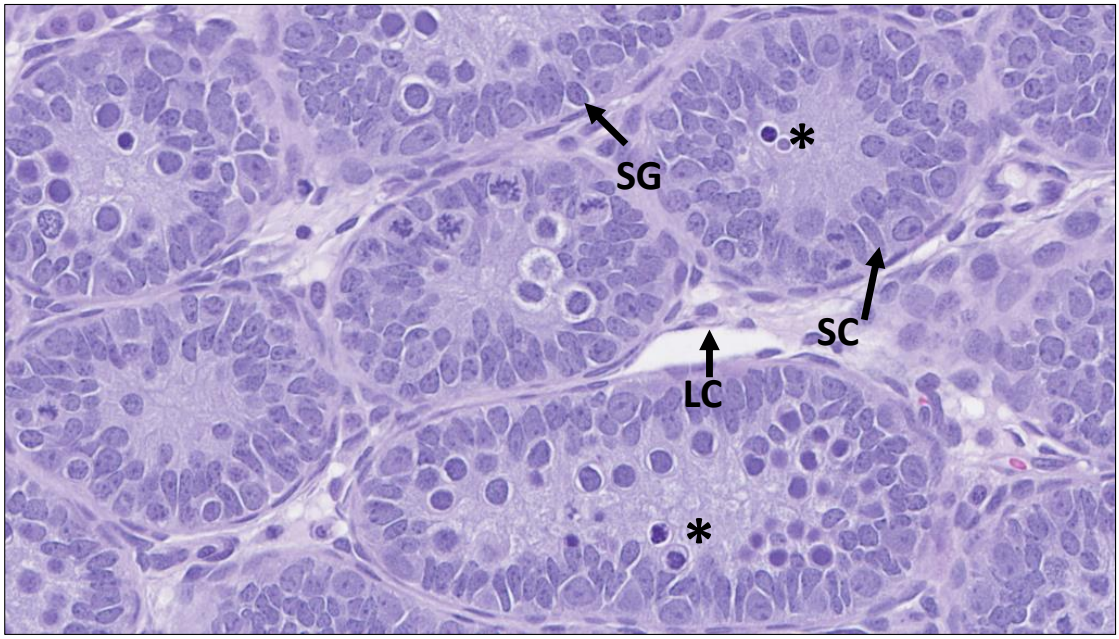
- Immature Sertoli cells (SC)
- Gonocytes (G)
- Fetal Leydig cells (LC)

**Uniform tubules:**

**No Blood-Testis Barrier**

**High Testosterone Levels**

# Rat Testis PND 14 (Infantile)




**Cell Types:**

Immature Sertoli cells (SC)

Spermatogonia (SG)

Progenitor Leydig cells (LC)

Apoptosis (*)

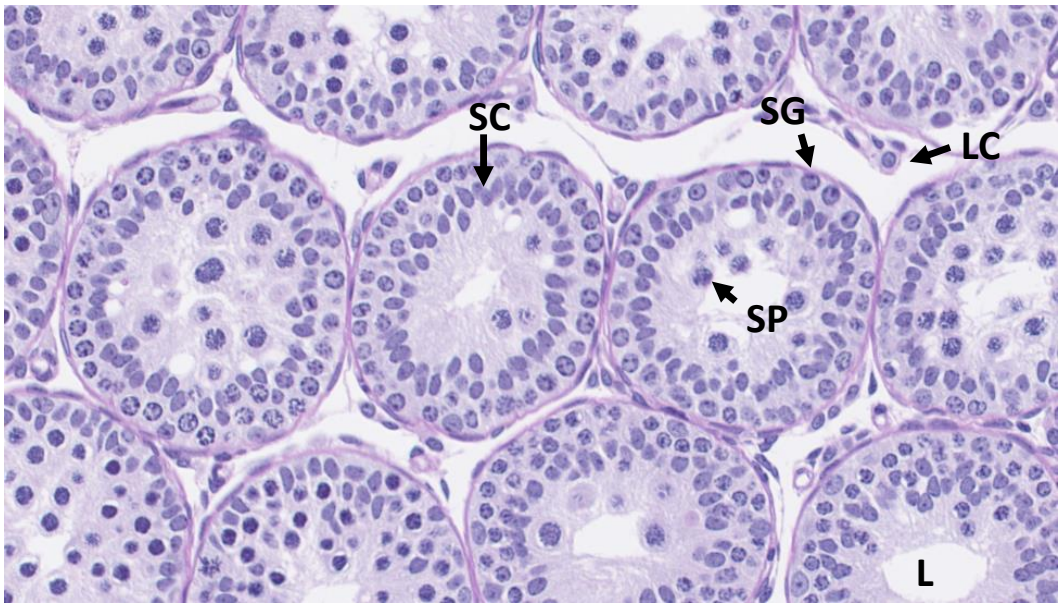
**High Mitotic Rate**

**Uniform Tubules:**

**No Blood-Testis Barrier**

**Low Testosterone Levels**

# Rat Testis PND 18 (Late Infantile)

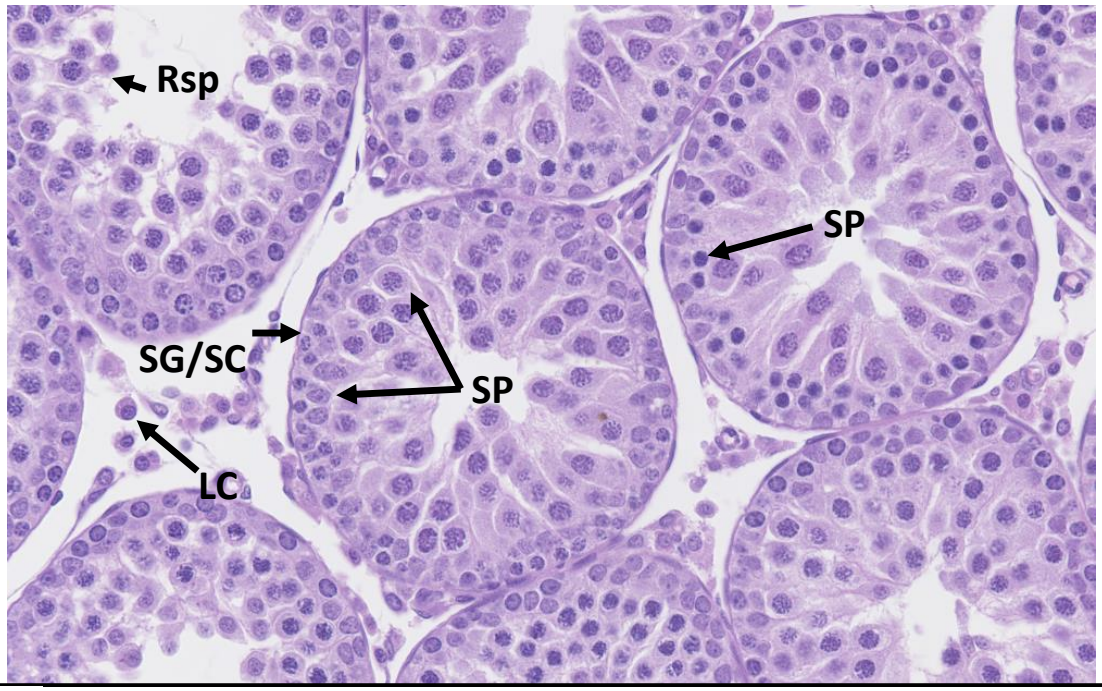



**Cell Types:**

- **Sertoli cells (SC):** postmitotic, mature inner layer of rosette
- **Spermatogonia (SG)** baseline mitotic rate (M) outer layer of rosette
- **Spermatocytes (SP)**
- **Immature Leydig cells (LC)**

**Non-uniform Tubules**
**Lumen forms (L)**
**Blood-Testis Barrier formed**
**Low Testosterone Levels**

# Rat Testis PND 27 (Juvenile)




**Cell Types:**

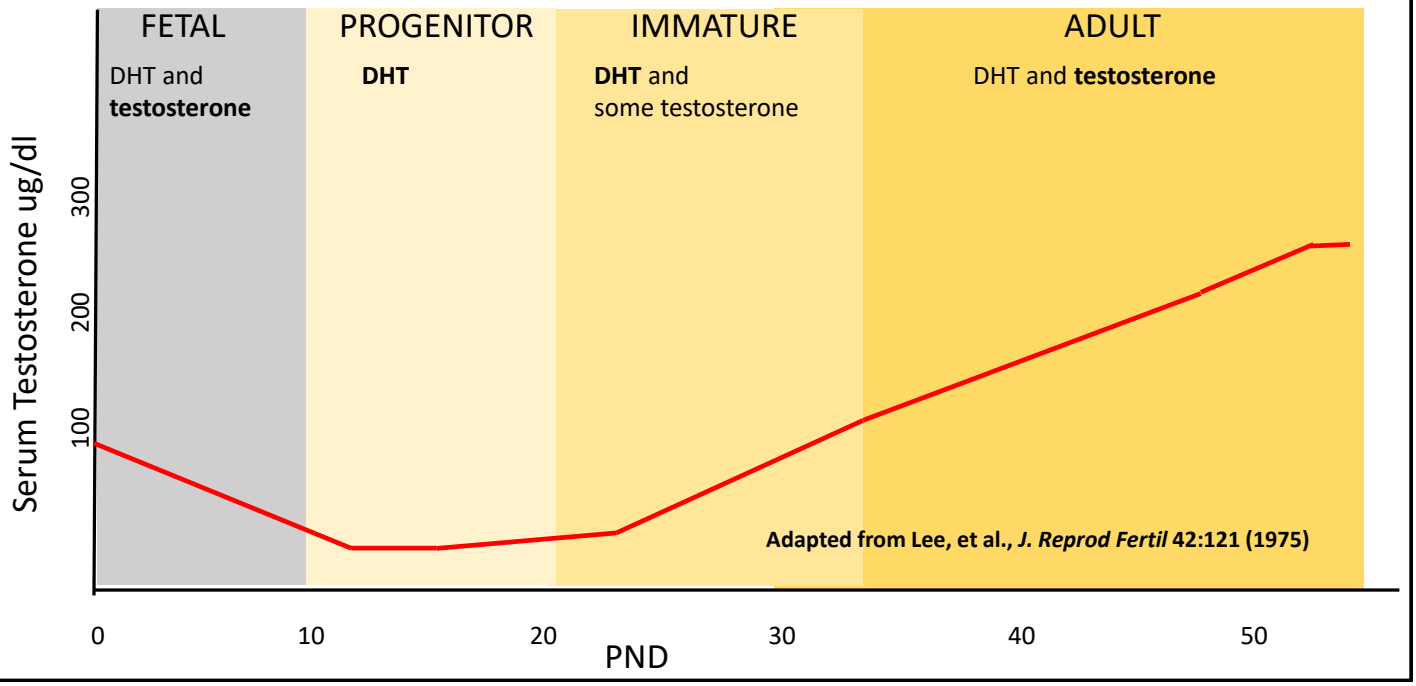
- **Sertoli cells (SC)**
- **Spermatogonia (SG)**
- **Spermatocytes (SP)**
- **Spermatids (Rsp only)**
- **Adult Leydig cells (LC)**

**Non-Uniform Tubules**

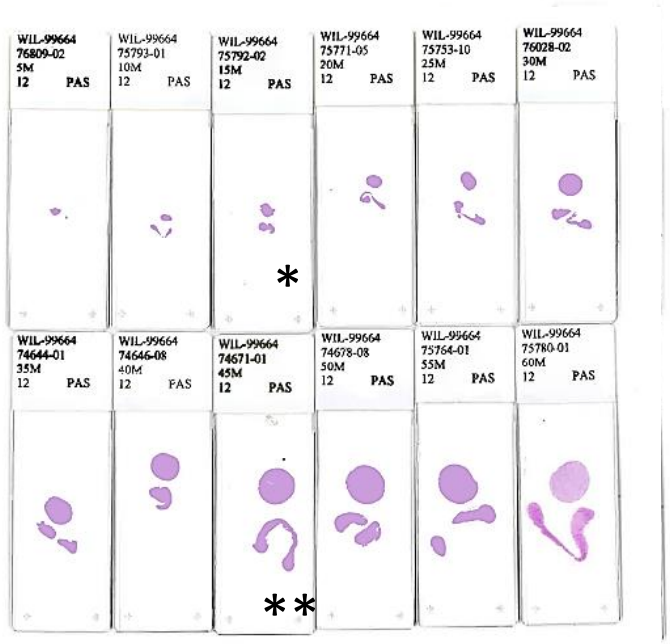
**Blood-Testis Barrier**

**Rising Testosterone Levels**

# Postnatal Development of Leydig Cells

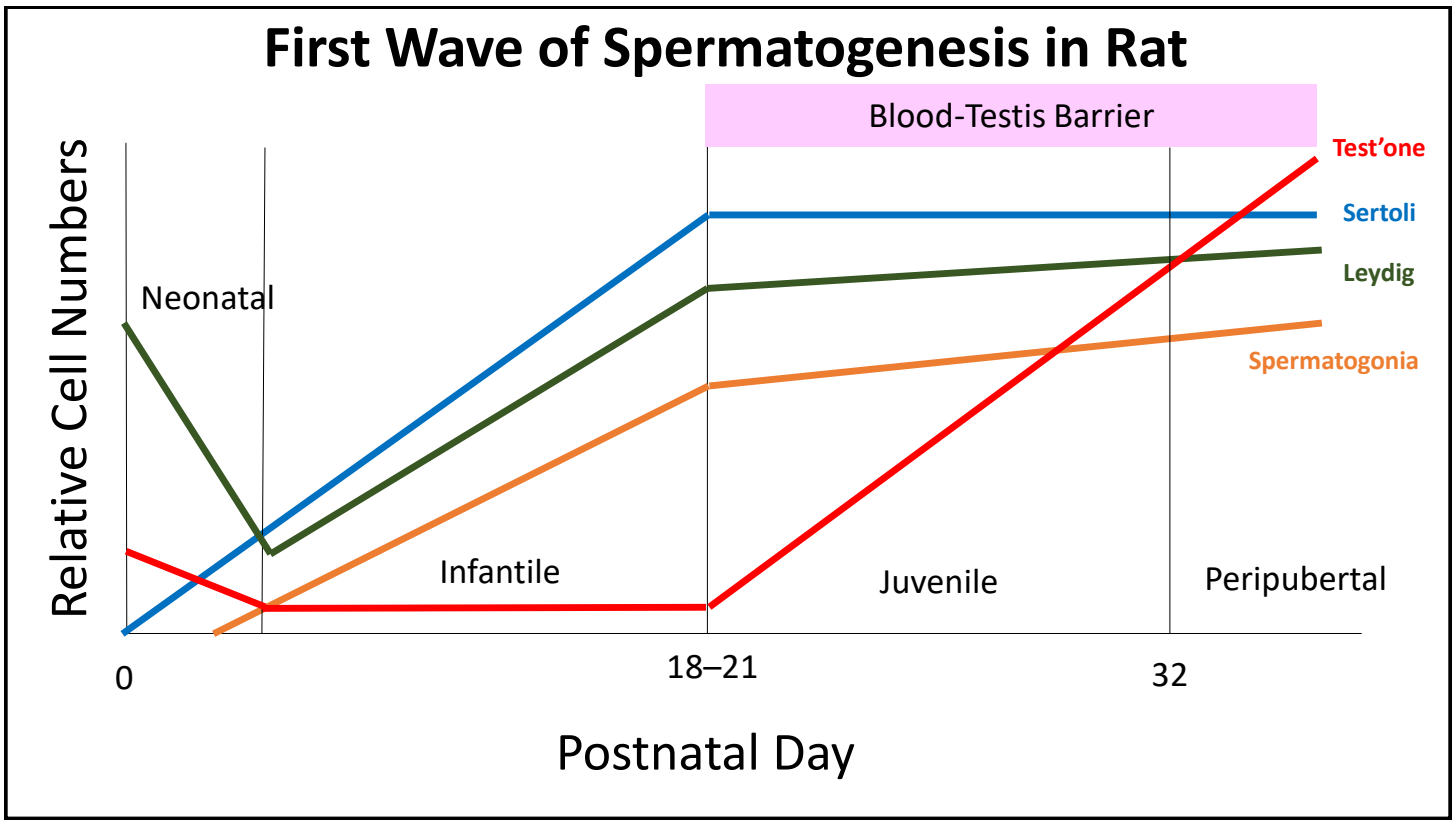



# ↑ Testosterone → Increase Tubule/Testes Size (Rat)




- Dramatic increase in testis size from PND 15 (*)–PND 45 (**)
- Tubule diameter increases 3.5x from PND 15–PND 45

Gaytan et al., (1986)



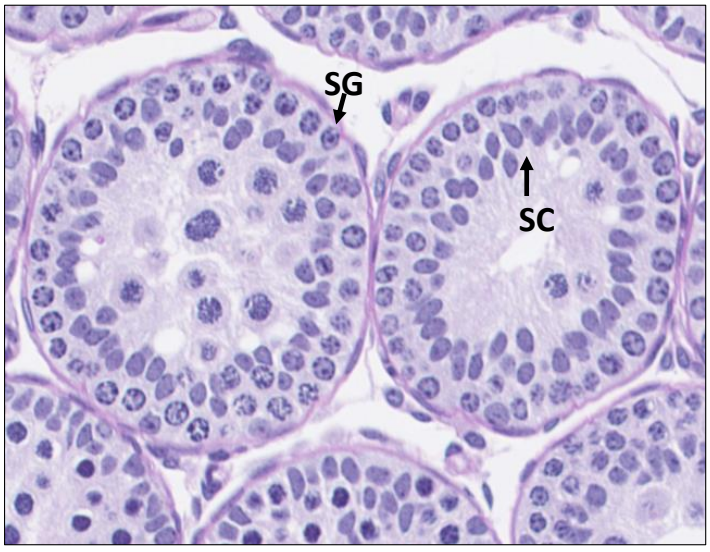

## Advantages in Evaluating "Juvenile" Testes

## Advantages in Evaluating "Juvenile" Testes for Potential Toxicants

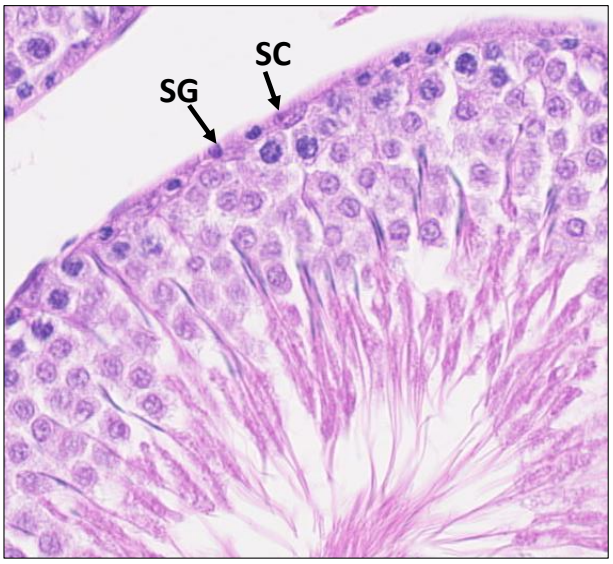
### From Birth–PND 18

- Easier to visualize spermatogonia and Sertoli cells
- Uniform tubules—uncomplicated by stages
- Unique/transient findings that might go unrecognized if there is no "juvenile" endpoint
- High susceptibility of immature, rapidly dividing SC, SG, and LCs

## Easily Visible Cells <PND 21




PND 18

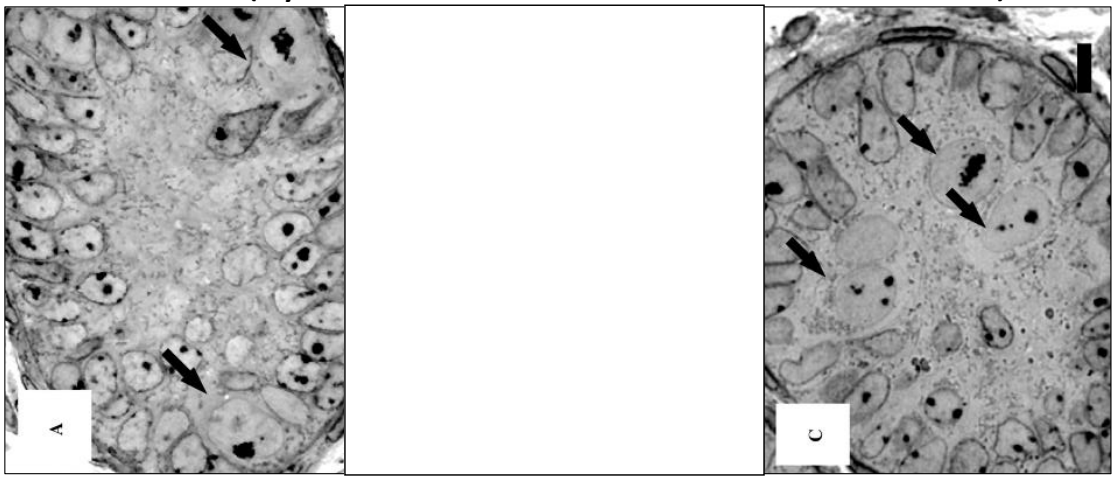



Adult

# Unique Findings

## Persistent Central Gonocytes in Rat

(Tyrosine Kinase Inhibitor Given PND 4)



Control
at PND 8

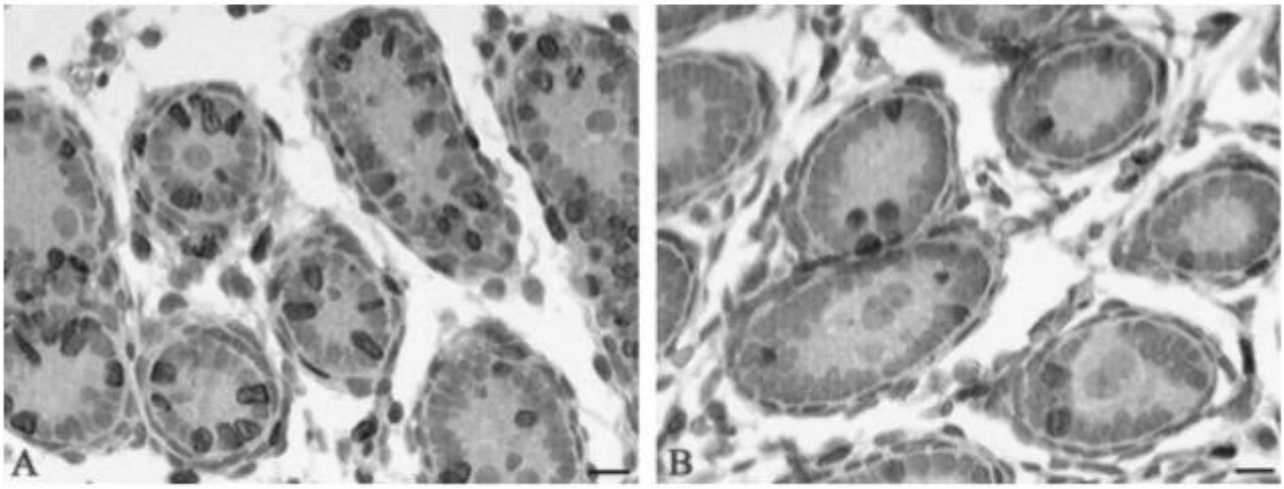
Imatinib 150 mg/kg
at PND 8

Nurmio et al., 2007

# Unique/Transient Findings

## Decreased Proliferation of Sertoli Cells

Phthalates Given at PND 3 (DEHP)



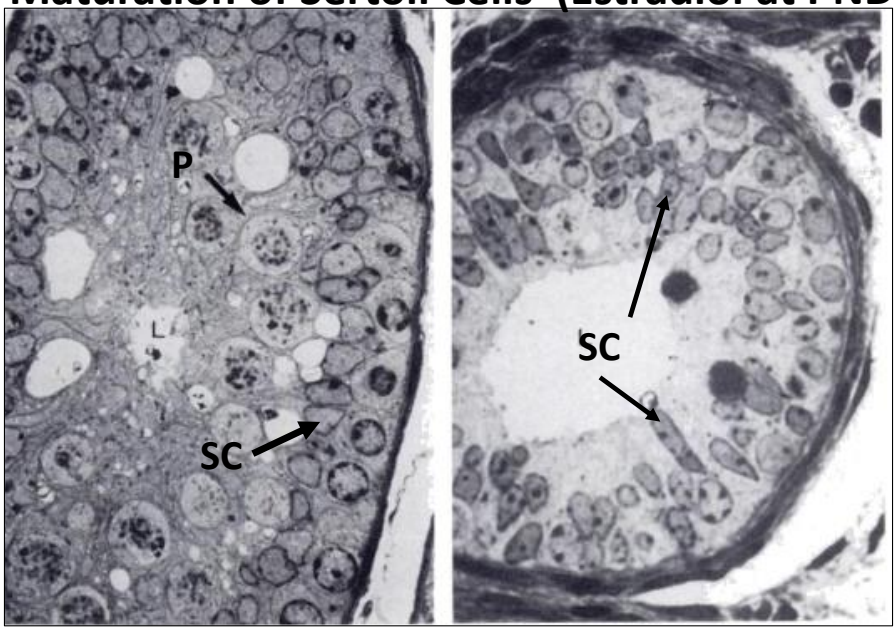

BrDU labeling Control Rat PND 3

BrDU labeling DEHP PND 3

Li et al., 2000

# Unique Findings

## Delayed Maturation of Sertoli Cells (Estradiol at PND 1)



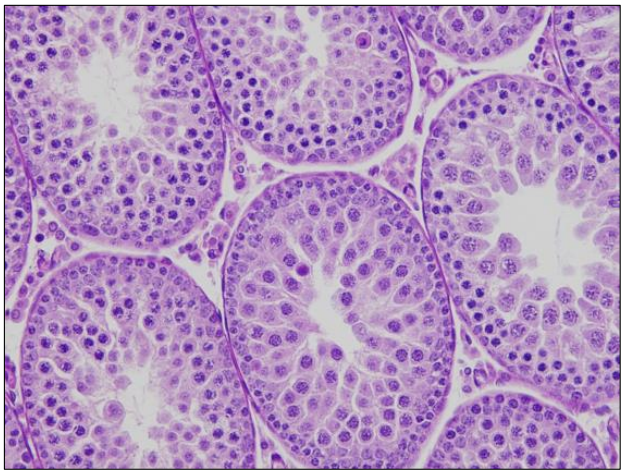

Gaytan et al., 1986

Control ≈PND 20 500 ug Estradiol ≈PND 20

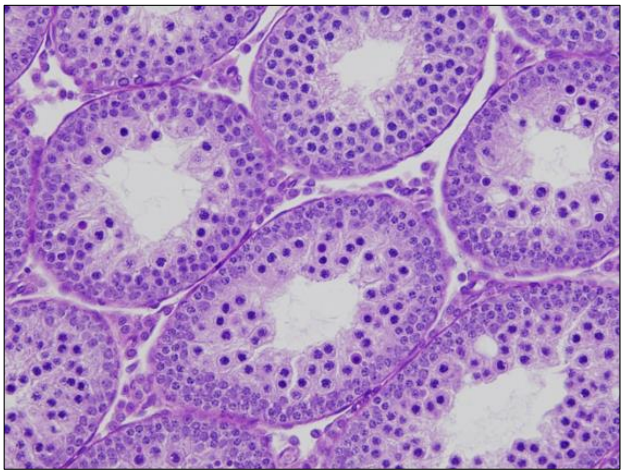
# Unique Findings

## Delayed Maturation of Primary Spermatocytes

Metronidazole—PND 7–PND 22



Control PND 22
Pachytenes plentiful

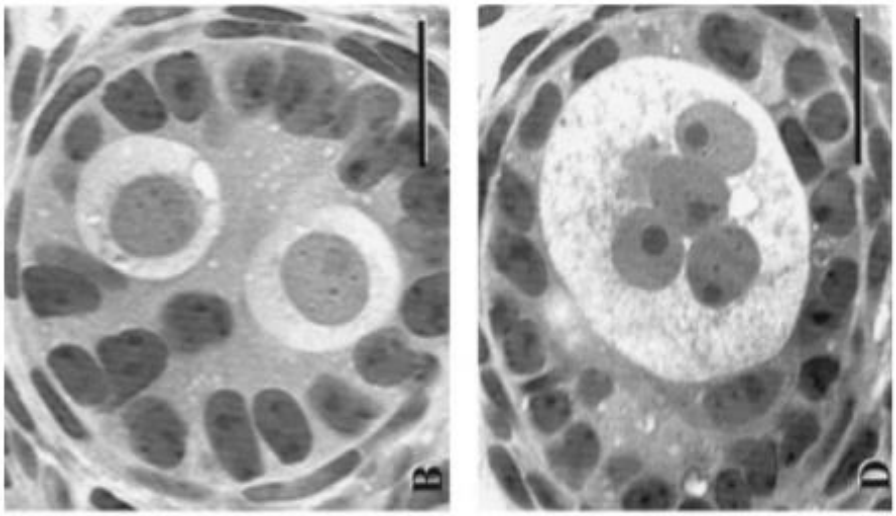


High-Dose PND 22
Leptotene/zygotene and smaller diameter tubules

# Unique Findings

## Multinucleated Gonocytes in Rat

Prenatal Phthalate Exposure



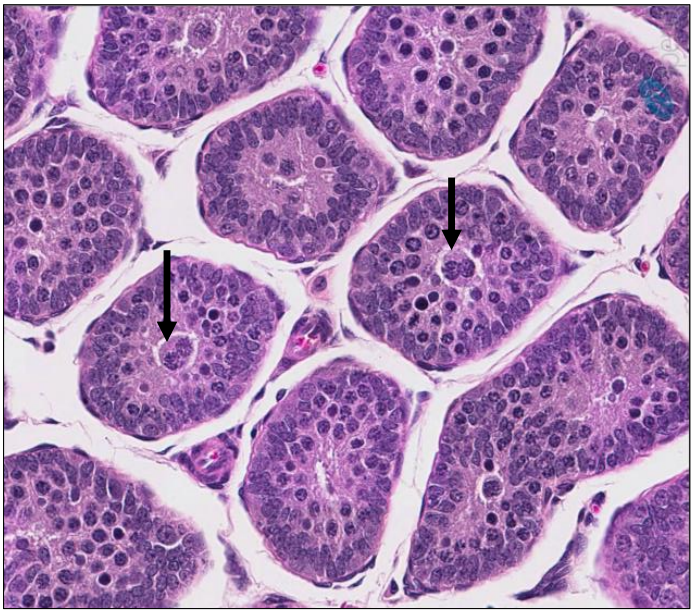
PND 4 Control

PND 4 500 mg/kg DEHP

Li et al., 2000

# Unique Findings

## Multinucleated Gonocytes in PND 23 Rat

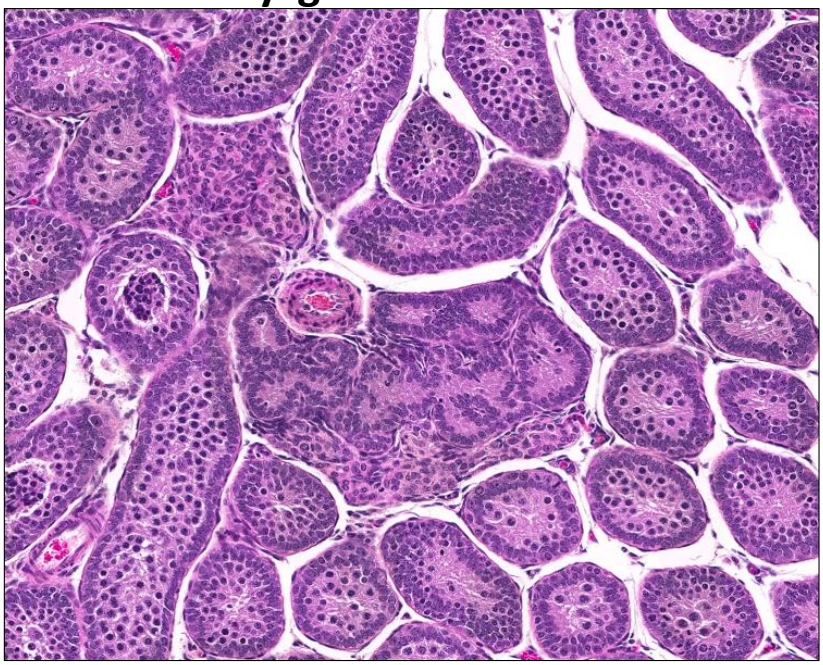


Due to pre-/postnatal phthalate exposure

Image provided by Dr. Cynthia Willson

# Unique Findings

## Tubule Dysgenesis in PND 23 Rat

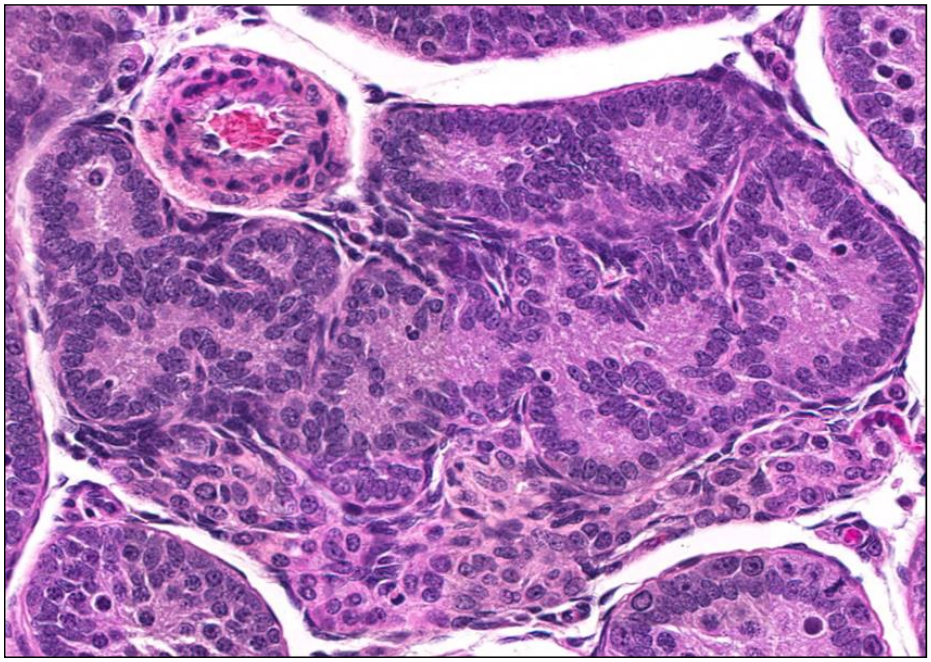


Due to pre-/postnatal phthalate exposure

Image provided by Dr. Cynthia Willson

# Unique Findings

## Tubule Dysgenesis in PND 23 Rat



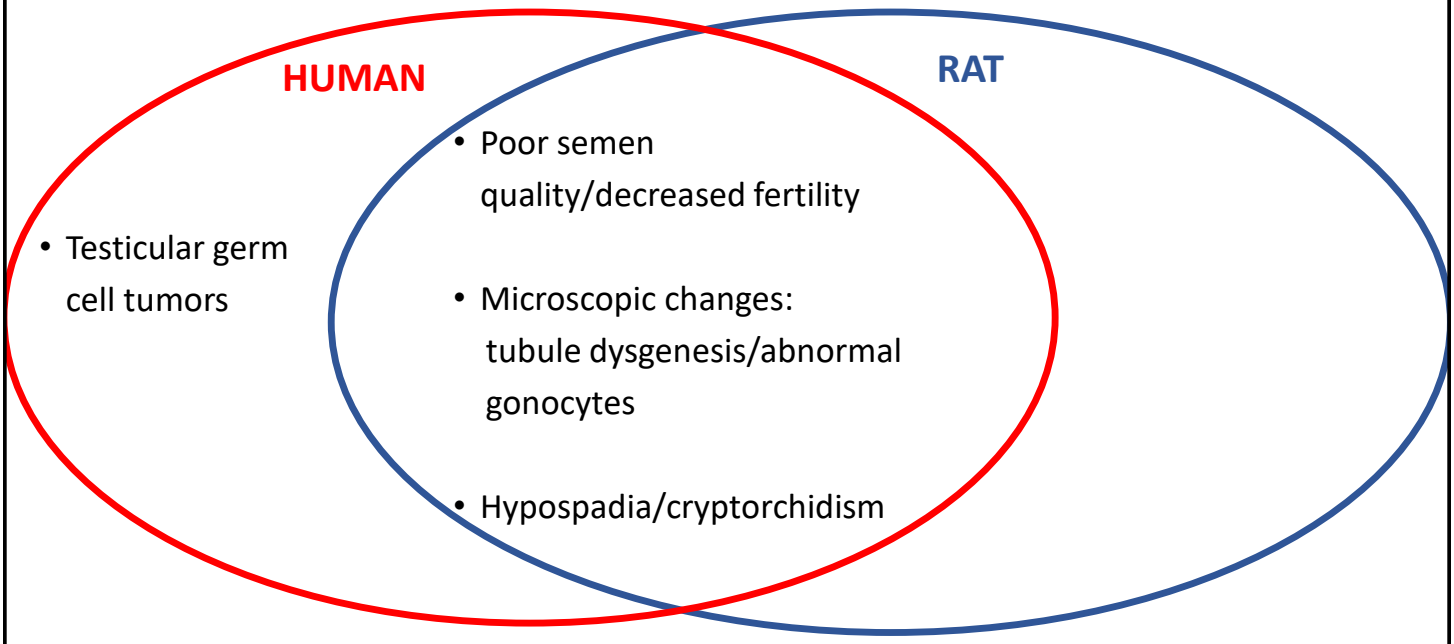
Anastomosing abnormally shaped tubules

Clusters of Leydig cells

Image provided by Dr. Cynthia Willson

# Testicular Dysgenesis Syndrome

**---Increasingly common in man and likely due to environmental endocrine disruptors---**




# Microscopic Features of Testicular Dysgenesis Syndrome

## Human

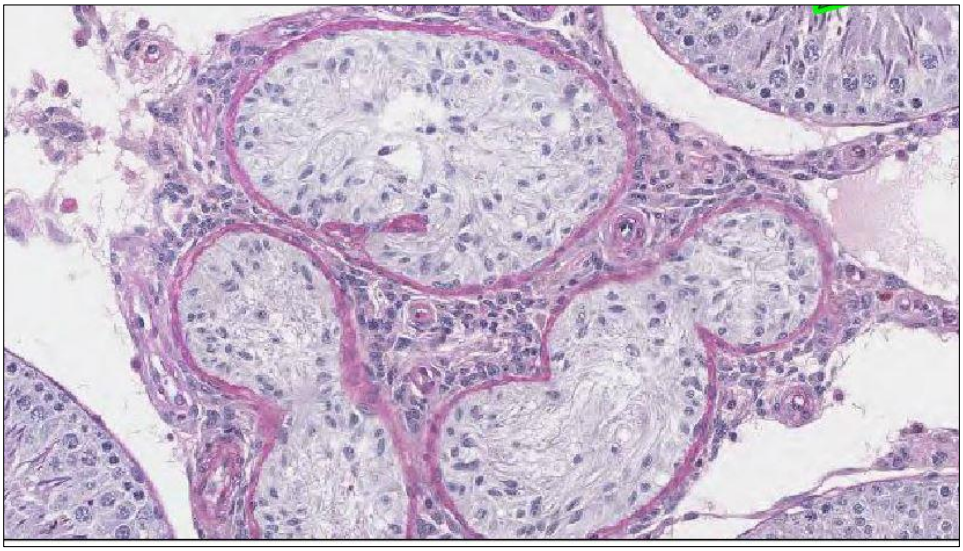
- Abnormal Gonocytes
  - Carcinoma *in situ* cells (transformed gonocytes)
  - Germ Cell Tumors
- Tubule Dysgenesis
  - Disfigured tubules
  - Thickened peritubular areas
  - Immature Sertoli cells
  - Leydig cell aggregates
  - Microlithiasis

## Rat

- Abnormal Gonocytes
  - Multinucleated gonocytes seen up to PND 23
- Tubule Dysgenesis
  - Disfigured tubules
  - Thickened peritubular areas
  - Immature Sertoli cells
  - Leydig cell aggregates

# Tubule Dysgenesis in Rat (and Man)

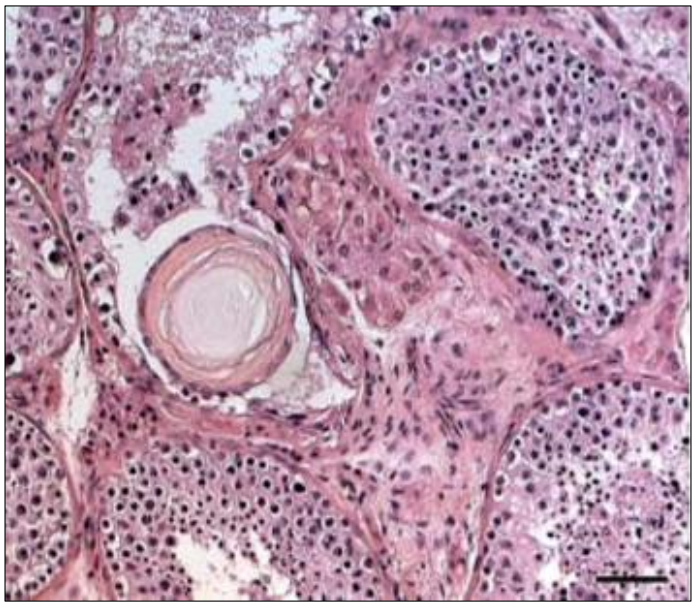
(10-week old rat—prenatally and postnatal exposure to phthalates)



Hyalinized tubules with peritubular myocytes, Leydig cell aggregates, immature Sertoli cells (nuclei haphazardly arranged), and distorted tubule shape; most common in central regions of testis near the rete

# Tubule Dysgenesis—Adult Human Testis

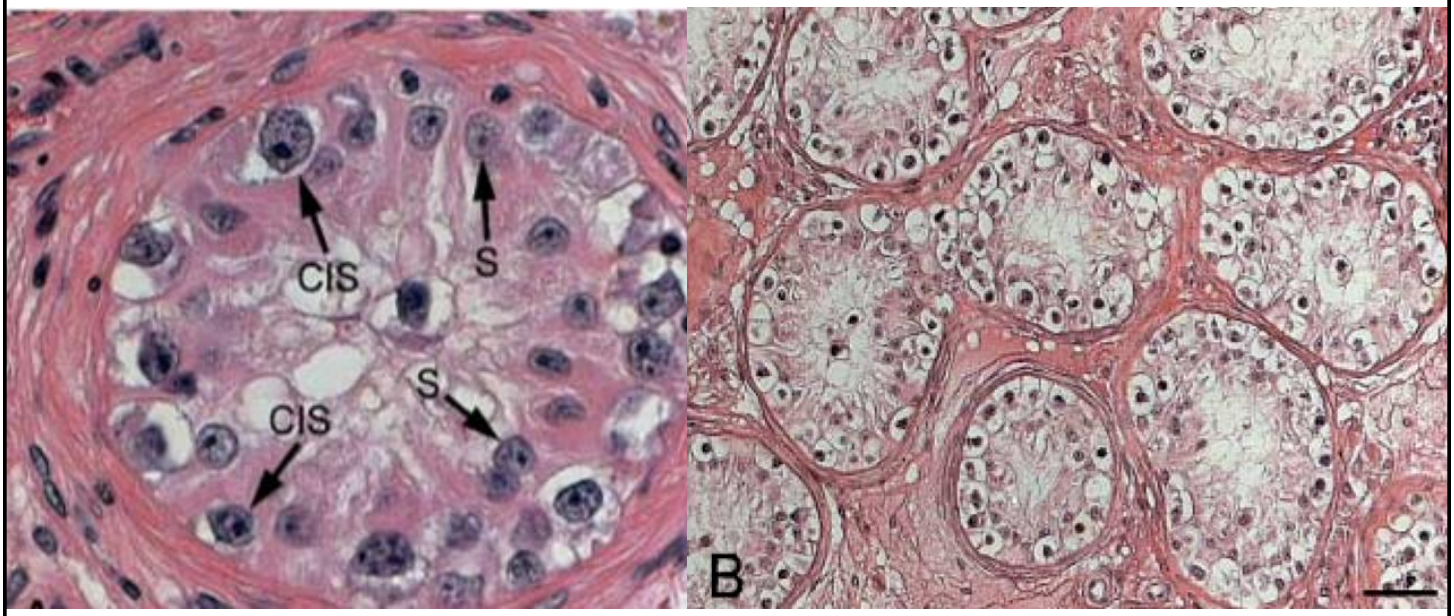
Added Feature: Microlithiasis



Hoei-Hansen et al., 2005

# Testicular Dysgenesis Syndrome: Abnormal Gonocytes

Carcinoma *In Situ* Cells in Adult Human Testis




Carcinoma *in situ* cells (arrows) in 8.7% of patients with contralateral germ cell cancer

Hoei-Hansen et al., 2005

## Pathogenesis of Testicular Dysgenesis Syndrome

Estrogens and Antiandrogens (Prenatal during MPW)

**ABNORMAL DEVELOPMENT OF MALE TISSUES**

Genetic Predisposition (45X/46XY)

Disturbed Sertoli Cell Function → Impaired Gonocyte Differentiation → **Poor semen quality**; **Abnormal Gonocytes → germ cell tumors (man)**; **Tubule dysgenesis**

Decreased Leydig Cell Function → Androgen Insufficiency → **Hypospadia**

Decreased Leydig Cell Function → InsL3 Deficiency → **Cryptorchidism**

MPW=Masculinization Programming Window (GD 15–17 in rat; 9–14 weeks in man)

# Why Are Some of the Unique Findings in a Juvenile Rat Testis "Transient"?

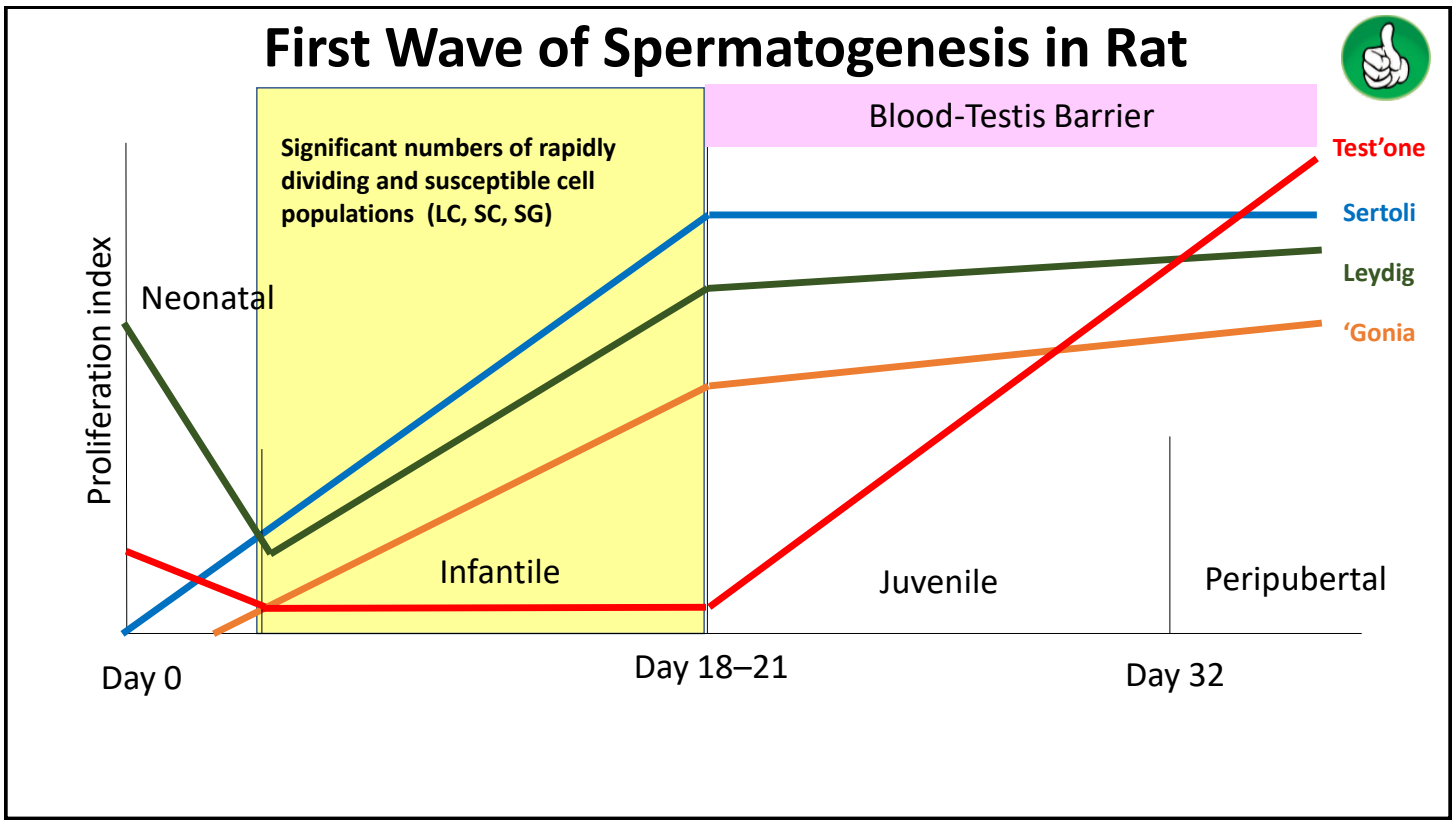
Rodent testes have high plasticity and lesions may resolve if wait until the adult endpoint

- Phthalates to neonatal rats—recover by adult stage (Li et al., 2000)
- Tyrosine Kinase inhibitor to neonatal rats—recover by adult (Nurmio, 2007)
- Neonatal exposure to endocrine disruptors suppresses juvenile testis weight and spermatogenesis at PND 21, but the testes recover by PND 50 (benzophenone, bis(2-ethylhexyl)phthalate, methoxychlor, styrene, tributyltin) (Kuwada et al., 2002)
- Unclear if human testes have high plasticity

# Advantages in Evaluating "Juvenile" Testes for Potential Toxicants

## From Birth–PND 18

- Easier to visualize spermatogonia and Sertoli cells
- Uniform tubules—uncomplicated by stages
- Unique/transient findings that might go unrecognized if there is no juvenile endpoint
- High susceptibility of immature, rapidly dividing SC, SG, and LCs

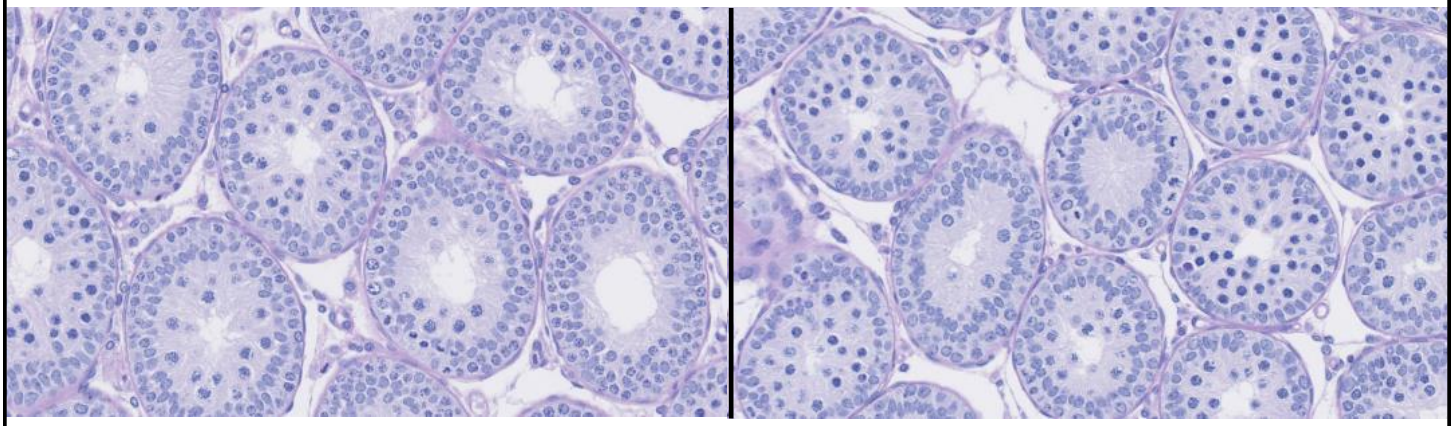



## Case Study: Mineral X

- Mineral X is ubiquitous in groundwater
- Mineral X was given to SD rats from GD 6–PND 118
- Looked at testes at PND 18 and at PND 118
- At PND 18, there was treatment-related:
  - Testis lesion—all dose groups
  - Reduced absolute testis weight and relative testis weights—all dose groups
  - No change in body weight
- At PND 118, there was:
  - No treatment-related testis lesion

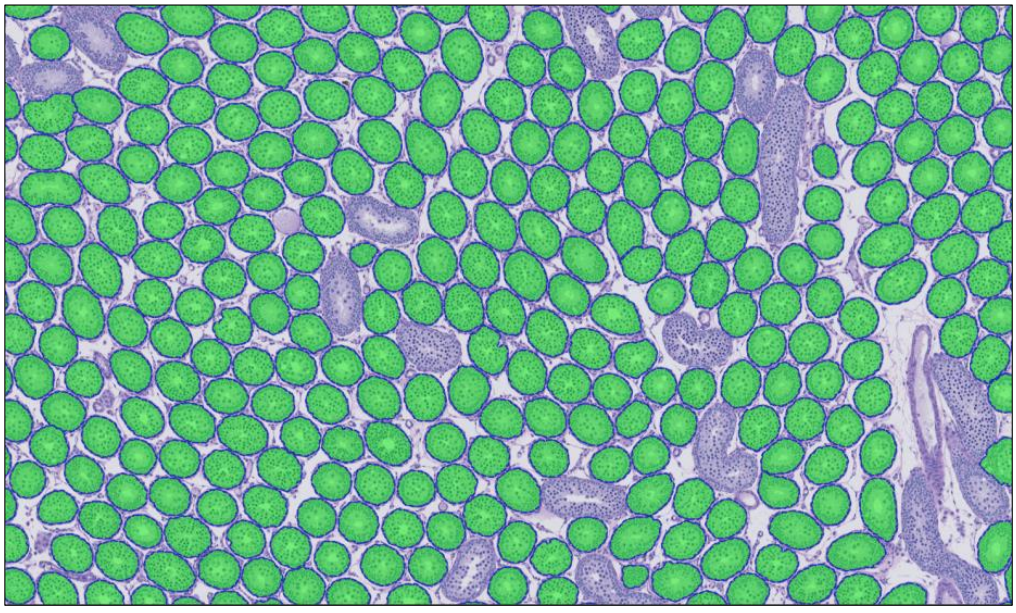
## Rat Testes
## PND 18

**Control** **"Mineral X"**

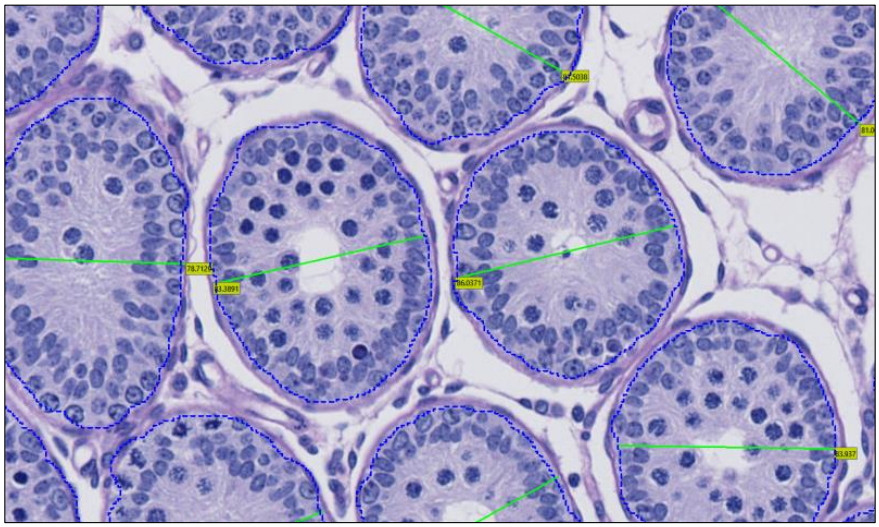


Decreased tubule size—minimal to mild

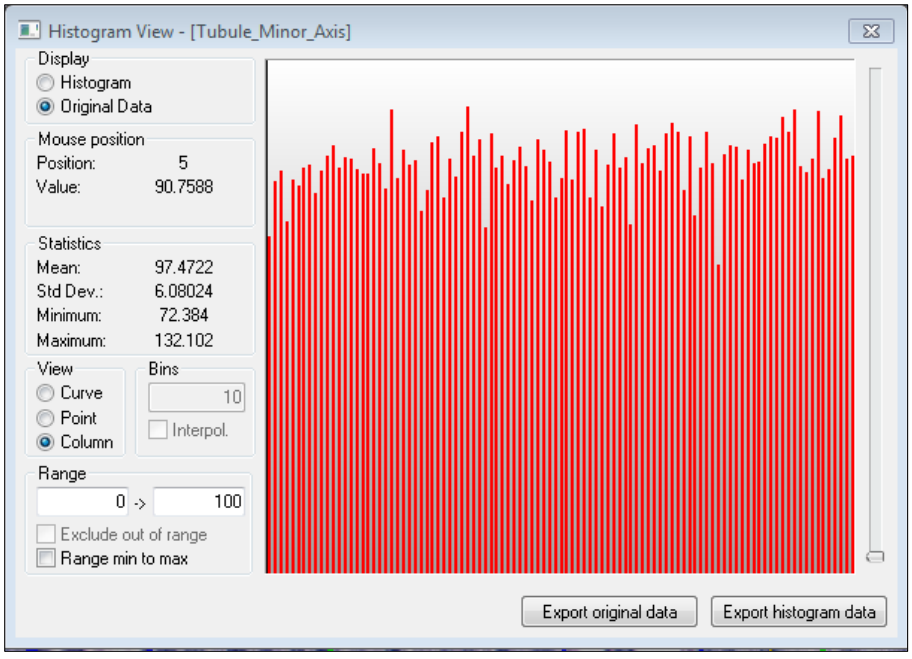
## First Label Region of Interest (ROI)
Control Rat Testis



# Next, Measure Diameter (Minor Axis) 40x

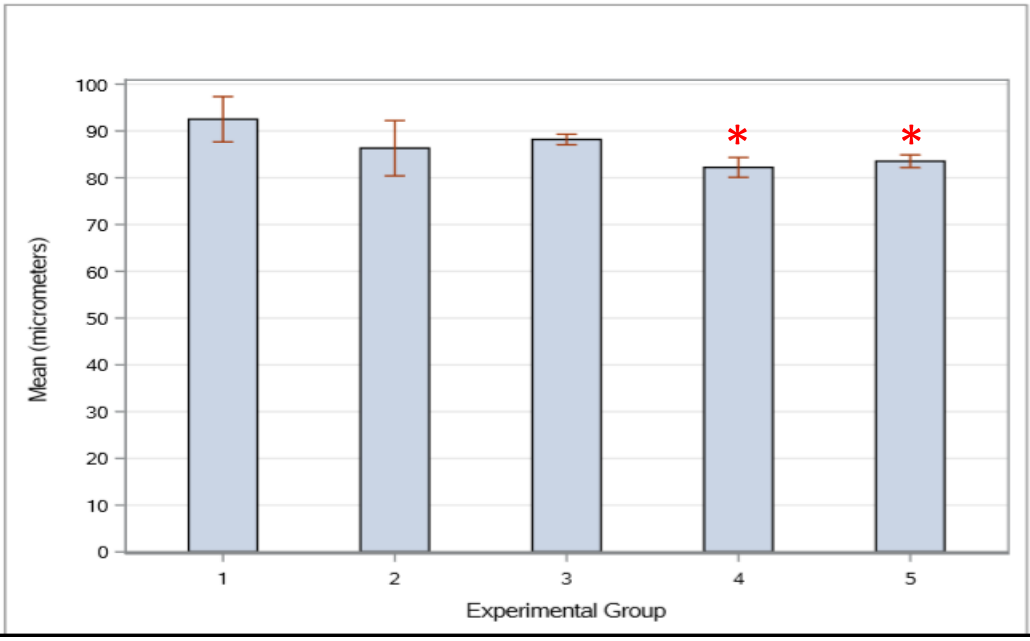


# Next, Chart the Diameter of Each Tubule in Testis



# Last, Make a Bar Graph for Mean of Each Group

PND 18 Male Rats




Results: two highest dose groups have lower tubule diameters, $p<0.05$

# Conclusion of Mineral X Effect on Testis

- There is no developmental delay caused by Mineral X
- The smaller tubule size was real (microscopy confirmed by image analysis)
- The finding was considered a direct "Mineral X"–related effect, because there was no effect on body weight
- The effect of "Mineral X" on the testes was transient and no longer present at PND 118, so including a "juvenile" endpoint allowed us to identify toxicity

Mechanisms of action?? Speculative—
possible effect on proliferation of immature Sertoli cells

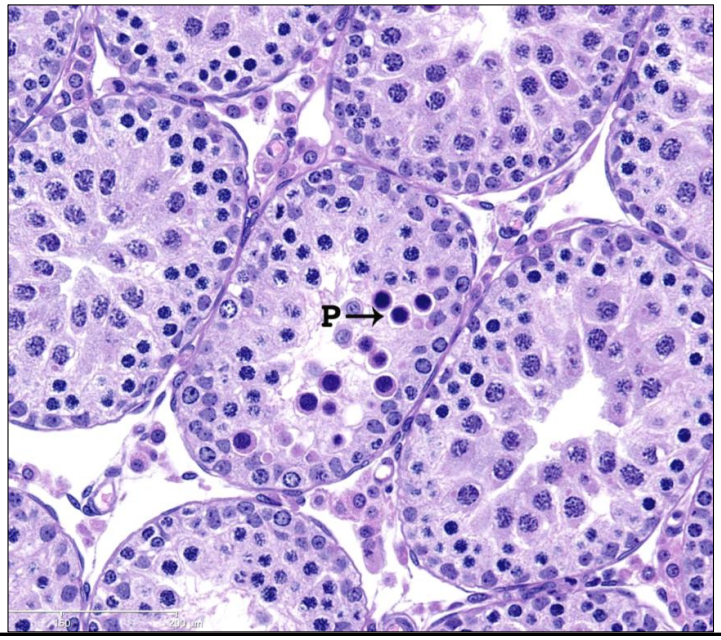
# Limitations in Evaluating "Juvenile" Testes

## Limitations in Evaluating Juvenile Testes

- Background findings that mimic toxicity
  - "Degenerative" change in prepuberty
  - "Deficient" spermatogenesis in peripubertal phase
- Don't have all the cell populations available to you
- Body weight changes will effect absolute testis weights (unlike in the adult)
- Translational issues
  - "Juvenile" rat is *allegedly* not a good model for the infant/childhood stage of man

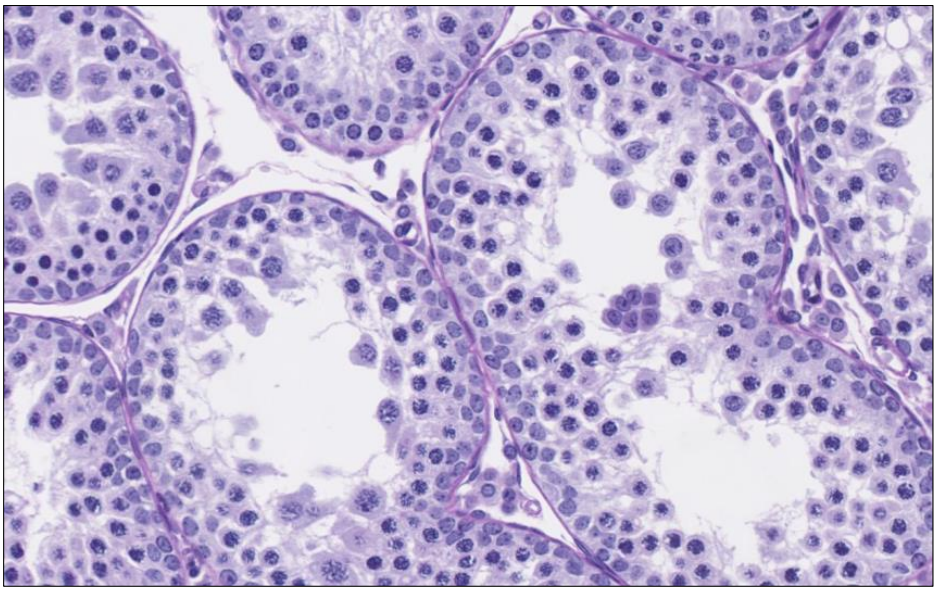
## Background Degenerative Findings Mimic Toxicity

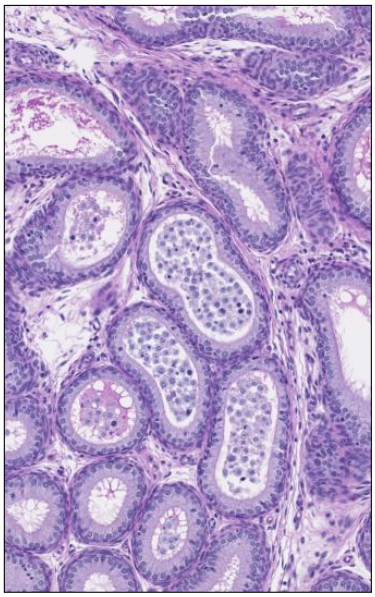
Apoptosis of Spermatocytes (P) Rat PND 28 (Normal)




## Background "Degenerative" Findings Mimic Toxicity

Paucity of Germinal Epithelium and Sloughing
Rat PND 28 (Normal)





# Limitations: Testes Weights Change with Body Weight in "Juvenile" Male Rat

- In ADULT rats:
  - When there is TA-related loss of body weight gain:
    - There **IS NO** decrease in absolute testes weights when there is loss of body weight gain
    - There **IS** an increase in relative testis weights
  - Therefore, use absolute testes and epididymides weights as indication of toxicity
  - Lower absolute testis weights generally means testicular toxicity

- In IMMATURE rats:
  - When there is TA-related loss of body weight gain:
    - There **IS** a decrease in absolute testis weights
    - There **IS NO** decrease in relative testis weights
  - Therefore, use relative testes and epididymides weights as indication of toxicity
  - Lower relative testis weights generally means testicular toxicity

## Poor Translation between Rat and Human?

| Stage | Rat | | Human | |
|---|---|---|---|---|
| | Age | Event | Age | Event |
| **Neonatal** | Days 0–7 | **Minipuberty** (2–8 hours) | Days 0–28 | |
| **Infantile** | Days 8–21 | **Rapid proliferation of LC, SC, SG (diffuse)** | 1 Month–2 Years | **Minipuberty** (2–6 Months)<br>**Quiescence** (6 Months–11 Years) |
| **Juvenile/ Childhood** | Days 22–32 | | Years 2–11 | |
| **Peripubertal** | Days 33–55 | | Years 11–14 | **Rapid proliferation of LC, SC, SG (lobule by lobule)** |

## Are Other Species More Translatable to Man?

- Marmosets have:
  - Quiescent period 4–11 months of age
  - Rapid proliferation (age unknown) that is lobule by lobule
- Dogs have:
  - Quiescent period 3–18 weeks of age
  - Rapid proliferation (18–20 weeks) that is diffuse

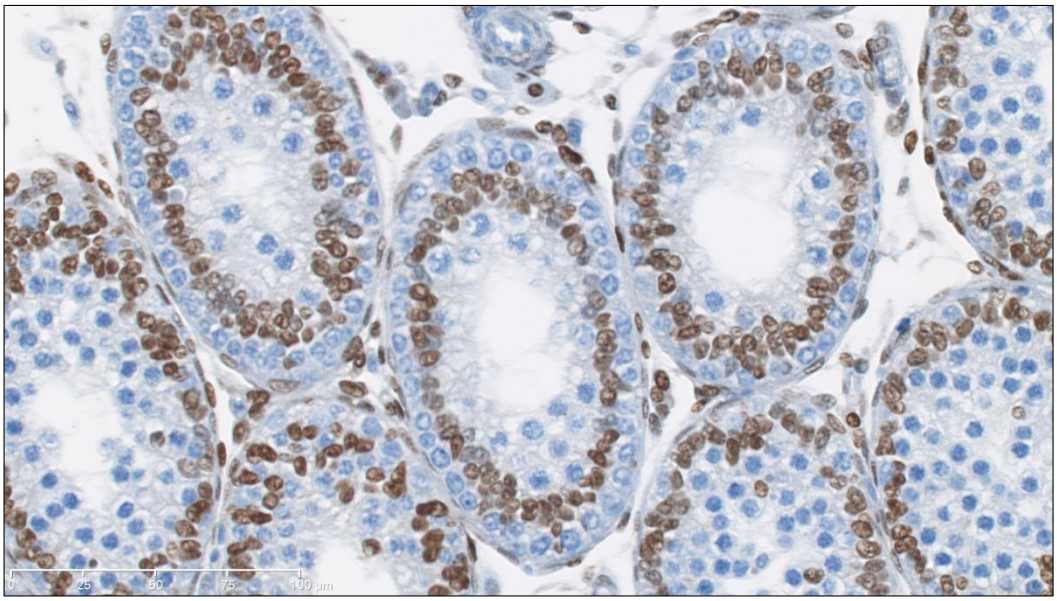
## Special Procedures

- Immunohistochemistry

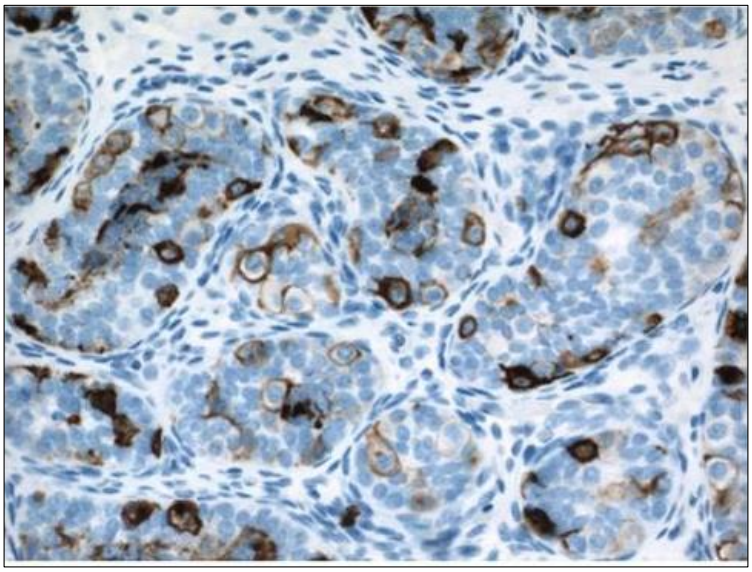
| Cell of Testis | Immunohistochemical Stains |
|---|---|
| Gonocyte | PLAP; OCT-4; AP2γ; VASA (weak) |
| Immature Sertoli Cells | GATA-4; AMH |
| Mature Sertoli Cell | GATA-4 |
| Leydig Cell | GATA-4; 3β-HSD |
| Spermatogonia | MAGE-A4; VASA (weak) |
| Spermatocytes | MAGE-A4; VASA (strong) |

- Image Analysis
  - TUNEL assay or Caspase—three for apoptosis
  - PCNA and BrDU labeling to quantify proliferation

# Rat Testis—PND 18 GATA-4 IHC

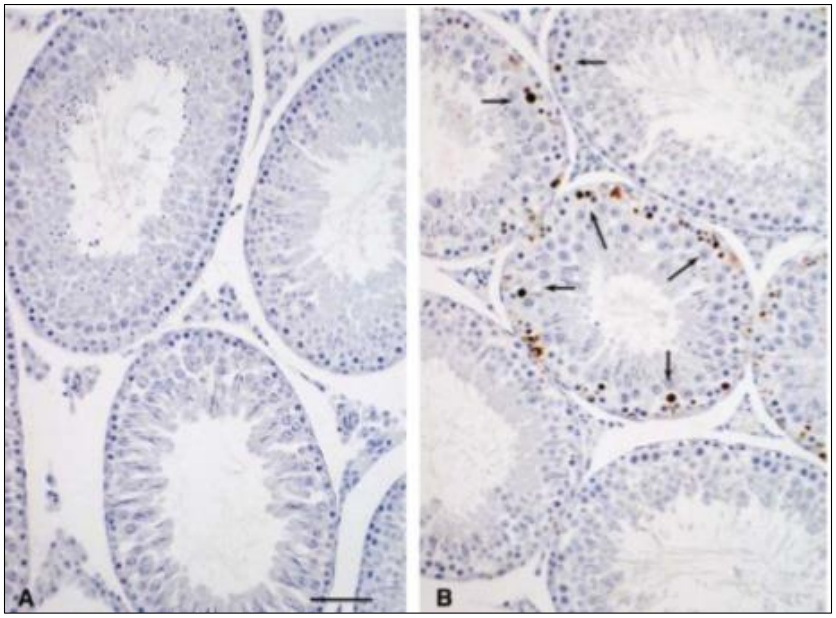


# Abnormal Gonocytes—Human Testis (12 Years Old): PLAP Stain



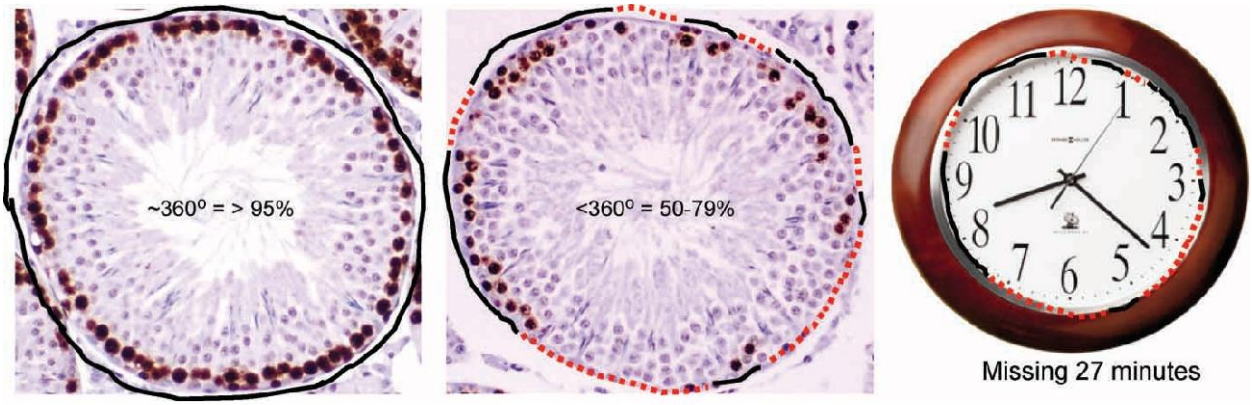
Hutson, JM , *Front Endocrinol* (Lausanne) 2012, 3, 176

# TUNEL Positive (Apoptosis) Germ Cells




D'Andrea et al., 2010

# PCNA Staining for Proliferating Germ Cells



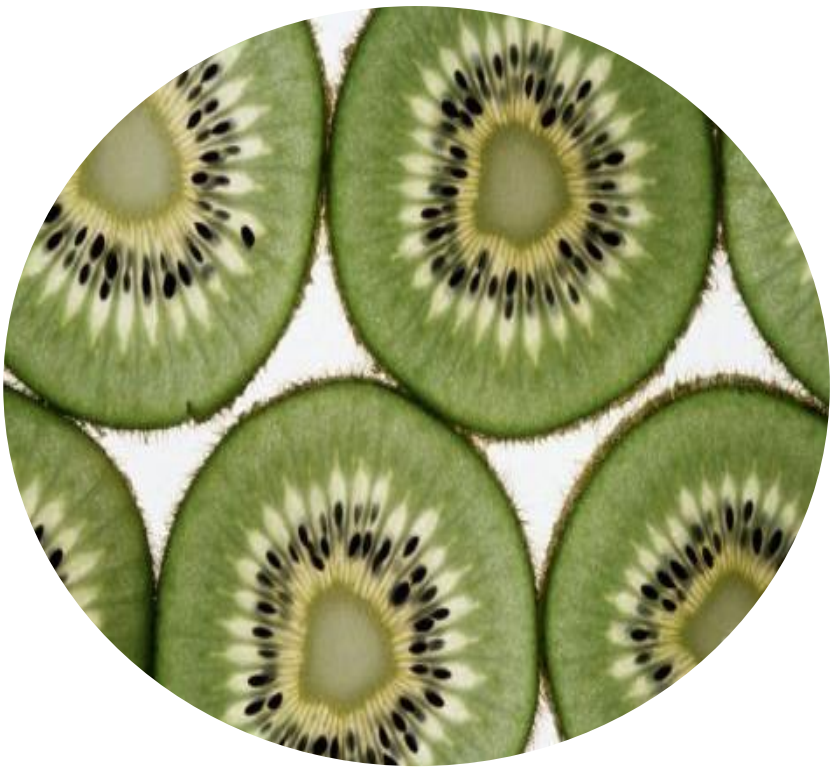

D'Andrea et al., 2008

## Summary and Conclusion

- Neonatal/infantile time points offer unique cell types, unique lesions, and increased sensitivity to certain toxicants
- Incorporating a pathology endpoint at PND 18–20 may reveal target cell toxicity that may otherwise go unrecognized
- Advantages outweigh limitations for including "juvenile" endpoint

 > 

- PND 5–15 in rat translates to years 11–13 in human: there is no rat correlate to the human quiescent period 6 months–11 years
- Special staining in conjunction with image analysis are useful techniques for evaluating "juvenile" testes



The End

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# Pathology in Reproductive Toxicology Assessments and the Role of Stage-Awareness for Testis Evaluation

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## Conflict of Interest Statement

The author declares no conflict of interest.

## Abbreviations

- ASG: accessory sex glands
- H&E: hematoxylin and eosin
- HRS: homogenization resistant spermatid count
- MDF: Modified Davidson's fixative
- NBF: neutral buffered formalin
- PAS/H: periodic acid Schiff/hematoxylin
- RB: residual body
- RT: room temperature
- T: testosterone

## Overview

- Holistic assessment of reproductive endpoints
  - Organ weights
- Proper fixation and trimming of testis
- Stage-awareness
  - Stages—basics
  - Why it's important—something's missing, something's added, and stage-specific lesions
- Patterns of toxicity in testis histopathology

# Holistic Assessment of Reproductive Endpoints

- Fertility/functional assessments—may be most sensitive for:
  - Changes in mating behavior (libido, mounting, erection, and ejaculation)
  - Genotoxic effects/transmissible genetic alterations
  - Presence of agent/drug in seminal fluid
  - Epigenetic changes
- Sperm parameters
  - Big decrease (up to 90% in some strains) in sperm production in rodents may have no detectable effect on fertility
  - In contrast, small decrement in human sperm count may cause infertility
- **Good** histopathology assessment is the most sensitive endpoint for the determination of testicular toxicity
  - Should be done in conjunction with epididymis, ASG, and mammary gland
  - Endocrine organs, brain—also affect reproductive function
- Organ weights—*NEXT*

# Absolute or Relative Organ Weights?

| Tissue | Use absolute or relative? | Notes | Effect of ↓ body weight |
|---|---|---|---|
| Testis | Absolute | If absolute weight is ↓, should see histopathology. Can have significant histopath findings without a testis weight change. | In adult rats, no changes in the weight or histopathology of the testes or epididymides with ↓ in body weight gain of ≤ 30% of controls. Mice and young rats, however, are more sensitive to ↓ body weight. |
| Epididymis | Absolute and relative | If weight ↓ by 15% or more, should see ↓ sperm on histopathology. | |
| Prostate | Relative | Organ weight often more sensitive than histopathology. Very sensitive indicator of insufficient androgens. Weigh carefully with secretions. | ↓ Body weight gains of ≥ 10% of controls result in ↓ size and weight of the seminal vesicles and prostate. |
| Seminal Vesicle | Relative | | |

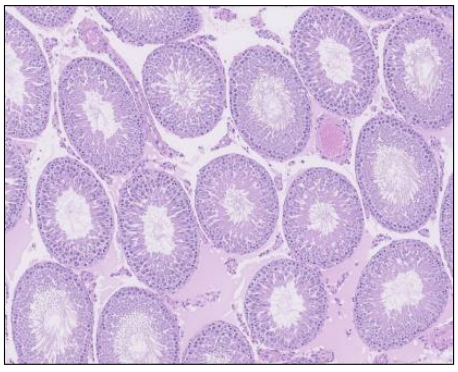
Chapin et al., 1993 and Rehm et al., 2008

# Cheat Sheet to Changes in Organ Weights

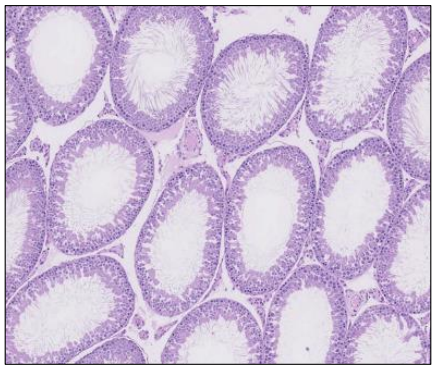
| Finding | Look for | Possible causes |
|---|---|---|
| ↑ Testis weight | Dilation of seminiferous tubules | Outflow obstruction (e.g., in rete testis, efferent ducts, epididymal sperm granuloma); decreased resorption in rete/epididymis; increased production by Sertoli cell |
| | ↑ Interstitial fluid (edema) | Vascular/hemodynamic/lymphatic alteration |
| ↓ Testis weight | Loss of germ cells, and/or contraction of tubular profiles d/t ↓ tubular fluid | Decreased spermatogenesis: germ cell, Sertoli cell, or hormonal disturbance. Tubular contraction due to decreased elongate spermatids +/- decreased T |
| ↑ Epididymis weight | ↑ Ductular fluid (edema) | ↓ Resorption by rete, efferent ducts, initial segment |
| | ↑ Interstitial fluid (edema) | Vascular/hemodynamic/lymphatic alteration |
| | Sperm granulomas | Spontaneous or induced by epithelial damage |
| ↓ Epididymis weight | Hypospermia, contraction of lumen profiles | Decreased spermatogenesis +/- ↓ seminiferous fluid production by testis |
| ↓ Prostate or seminal vesicle weight | Atrophy; decreased epithelial height/secretions | ↓ Serum T, steroidogenesis inhibition, androgen receptor disruption |

Lanning et al., 2002

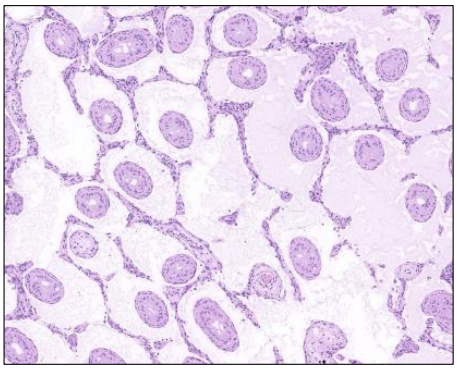
# INCREASED Testis Weight: Disturbance of Fluid Balance



**Normal**



**Seminiferous tubule dilation**
↑ Fluid in the seminiferous tubules
Look for: outflow obstruction
Possible sequela: atrophy
Reported for: fungicide carbendazim, a PDE4 inhibitor, a leukotriene inhibitor, and endothelin antagonists



**Interstitial edema**
↑ Fluid in the interstitium
Cause: vascular/hemodynamic alteration, ↓ lymphatic drainage

**DECREASED testis weight: disturbance of *spermatogenesis* (↓) +/- ↓ fluid production**

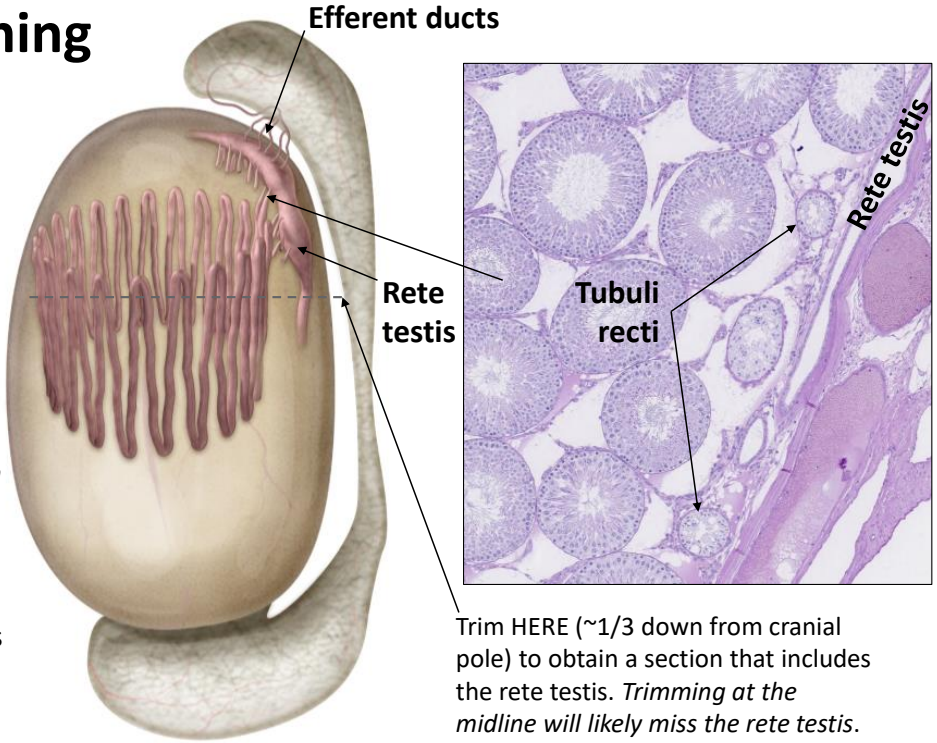
# Appropriate Fixation, Trimming, and Staining

| Tissue | Fixation | Trimming | Staining |
|---|---|---|---|
| Testis | Modified Davidson's fixative for 48 hr at RT. Can then transfer to 10% NBF for storage. | Transverse section through the rete testis (~1/3 down the testis from the cranial pole). Midline may miss the rete. Don't squeeze! | PAS/H for rodent studies up to 28 days. H&E >28 days. |
| Epididymis | 10% NBF for ≥48 hr at RT. Can be fixed in Modified Davidson's. | Longitudinal section through the epididymis to include the caput, corpus, and cauda | H&E (or PAS/H if testis w/PAS/H) |
| Prostate | 10% NBF for ≥48 hr at RT | Mid-transverse section through the prostate to include dorsolateral and ventral lobes | H&E |
| Seminal Vesicles and Coagulating Gland | 10% NBF for ≥48 hr at RT | Transverse section through each seminal vesicle and its coagulating gland | H&E |

Lanning et al., 2002

# Appropriate Trimming of the Testis

- 1 of the ~20–30 seminiferous tubules in the rat testis; arranged in longitudinally oriented coils stacked within each other.
- Both ends of the seminiferous tubules join the **rete testis** via a transition through the **tubuli recti** (straight tubules). *Don't confuse these with peripheral tubules with atrophic tubules.*
- It is important to include the rete testis, as it can be a site of treatment-related lesions. Lesions here can help with interpreting testicular changes.




Trim HERE (~1/3 down from cranial pole) to obtain a section that includes the rete testis. *Trimming at the midline will likely miss the rete testis.*

## "Stage-Awareness" in Microscopic Evaluation

- Enables the detection of the most common morphological consequence of injury to male reproductive system: DISTURBANCE OF SPERMATOGENESIS
  - Regardless of target of toxicity
    - Sertoli cell, any one of the germ cell populations, secondary response to altered hormone levels, altered vascular supply, or altered fluid balance
  - Although most important for shorter-term studies, the testis, regardless of study type, should always be examined with an awareness of the spermatogenic cycle
- What is "stage-awareness"?
  - **Qualitative, not quantitative,** evaluation based on familiarity with what cell associations are supposed to be in what stages
  - Relies on **pattern recognition** rather than counting stages or cells
- Requires some use of high magnification (≥20X)

## Why Is "Stage-Awareness" Important?

- Testicular histopathology is considered the most sensitive parameter for the detection of testicular toxicity
  - Sensitivity increases if the pathologist assesses testicular tissue in a stage-aware manner
- Must have sufficient knowledge of the GENERAL appearance of the germ cell types within different stage tubules
  - ***Must know normal to appreciate abnormal spermatogenesis***
  - Otherwise, subtle, but important, lesions may be missed
    - Relatively easy to identify when there are changes to the cells present
    - More difficult to know when something is missing or shouldn't be there!
- "Stage-awareness" allows for the ability to recognize:
  - #1: when an **appropriate** cell type/layer is **missing**
  - #2: when an **inappropriate** cell type is **present**
  - #3: **stage- and/or cell-specific** lesions

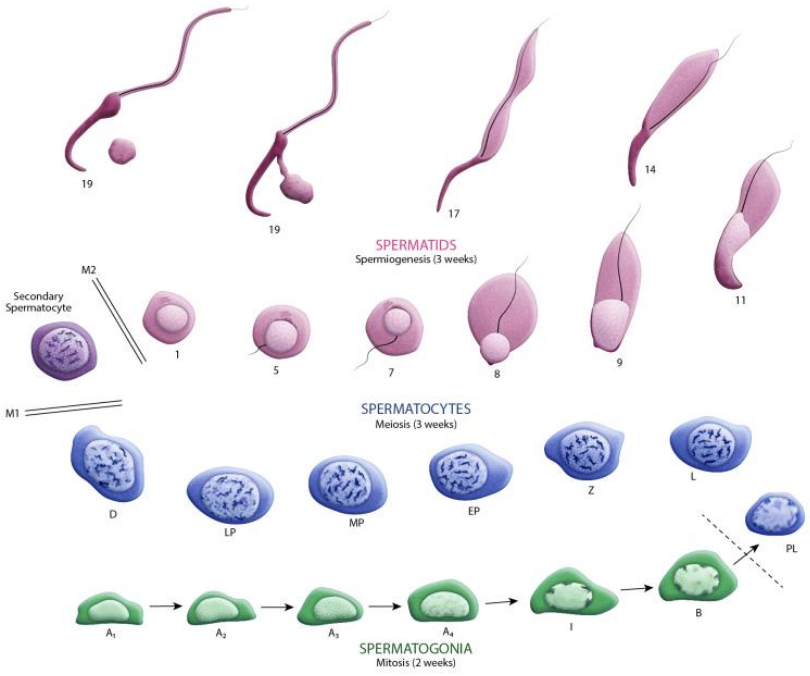
# How Do You Know If Stage-Awareness Was Implemented in a Screening Study?

- Pathologist should be requested to use "stage-awareness," not to "do testicular staging"
  - Should not be counting the number of profiles in each stage
- Look for pertinent language in the study protocol or report:
  - Testes were evaluated in a "stage-aware" manner . . .
  - A detailed qualitative examination of testes was made, taking into account stages of the spermatogenic cycle . . .
  - Microscopic evaluation was conducted to identify . . . missing germ cell layers, retained spermatids . . .
  - Any cell- or stage-specificity of testicular lesions were noted . . .

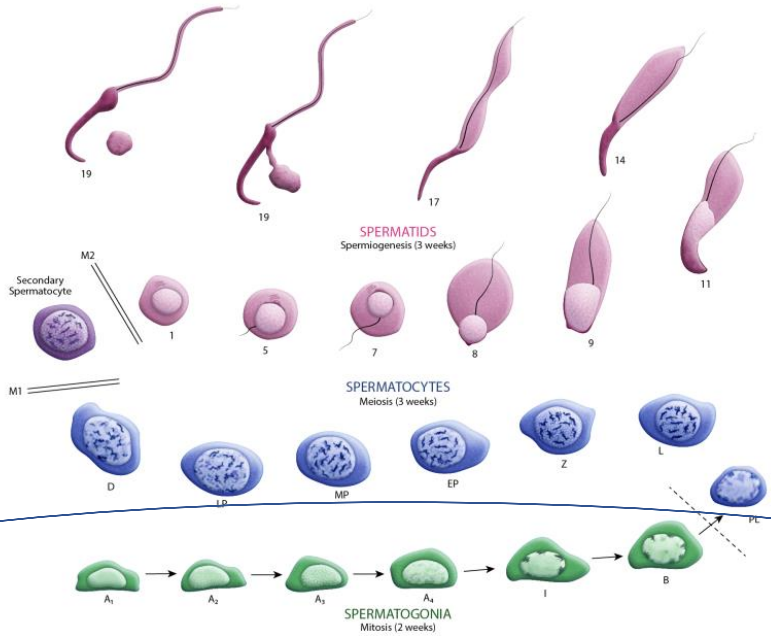
Lanning et al., 2002

# Spermatogenesis

**Cycle of SPERMATOGENESIS**: A complete and ordered series of germ cell associations (**STAGES**) in a given cross-section of seminiferous epithelium over time. Cycle is ~8 weeks in rats.



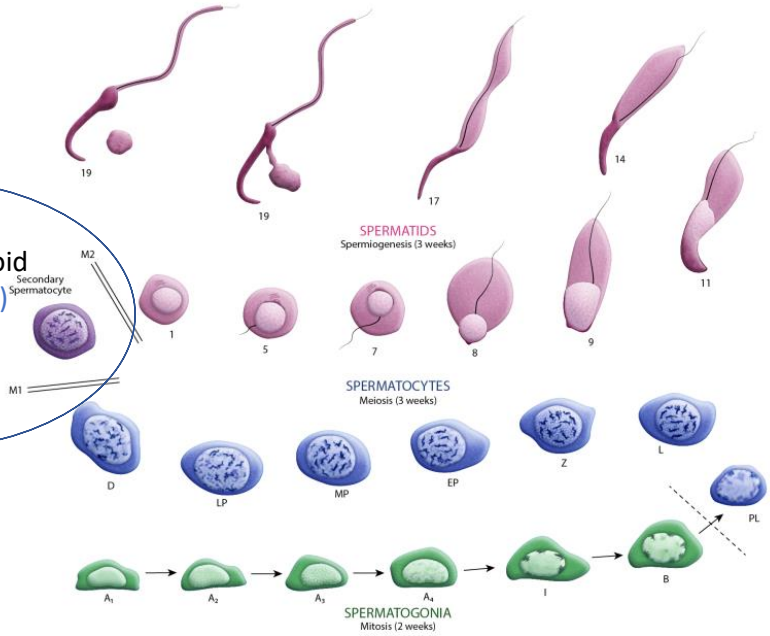
# Spermatogenesis




#1: **Spermatogoniogenesis**: Mitoses and differentiation of diploid spermatogonia.

# Spermatogenesis

#2: **Meiosis**: Tetraploid spermatocytes (blue) into haploid round spermatids (pink).

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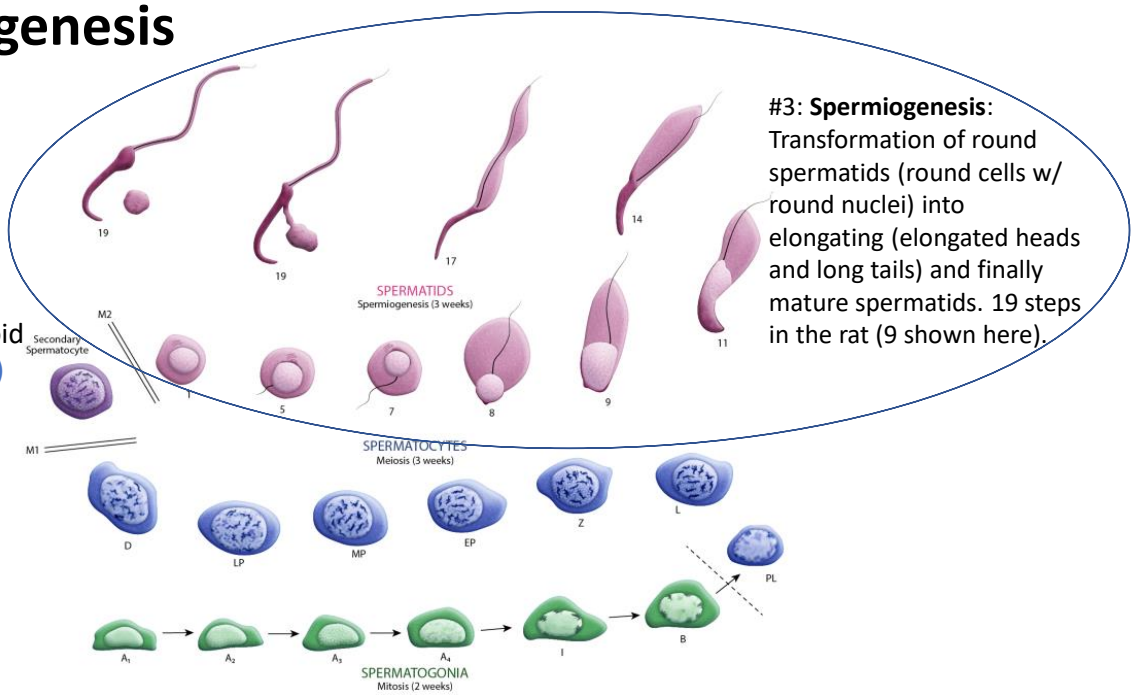


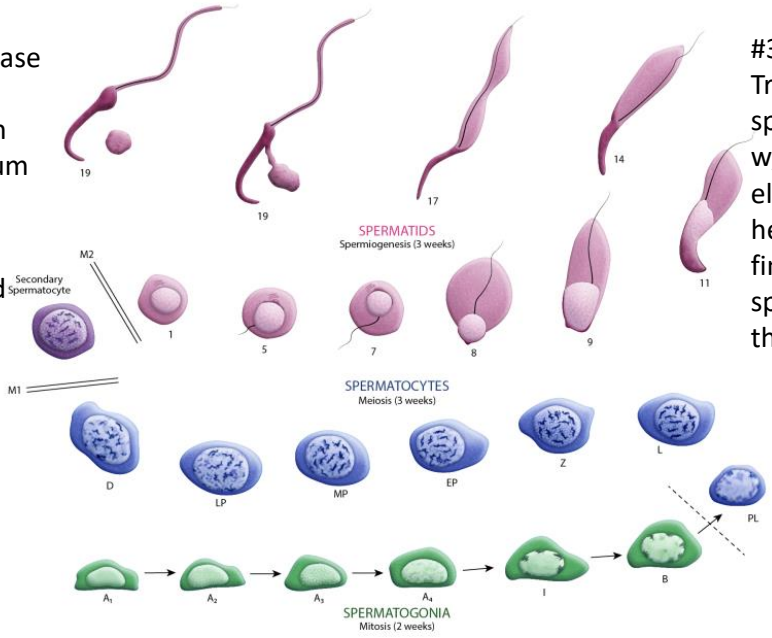

#3: **Spermiogenesis**: Transformation of round spermatids (round cells w/ round nuclei) into elongating (elongated heads and long tails) and finally mature spermatids. 19 steps in the rat (9 shown here).

#1: **Spermatogoniogenesis**: Mitoses and differentiation of diploid spermatogonia.

# Spermatogenesis

#4: **Spermiation**: Release of mature (step 19) elongated sperm from the germinal epithelium into the lumen.

#2: **Meiosis**: Tetraploid spermatocytes (blue) into haploid round spermatids (pink).

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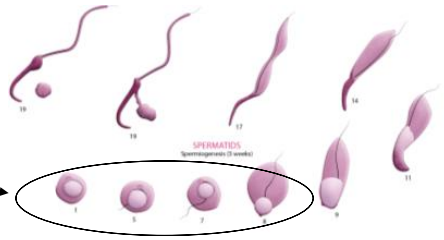
#1: **Spermatogoniogenesis**: Mitoses and differentiation of diploid spermatogonia.

# Stages of the Cycle of Spermatogenesis

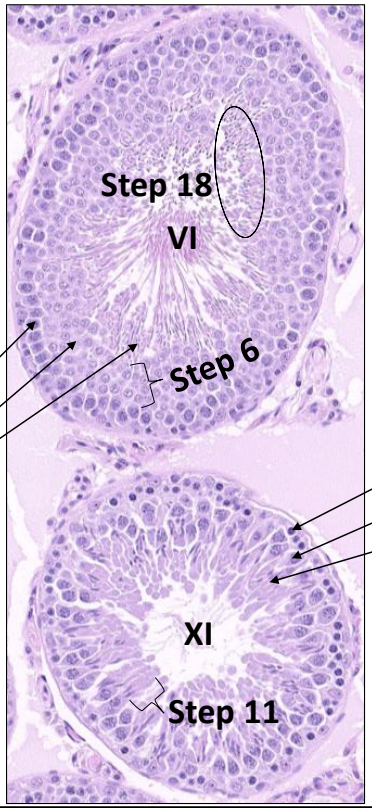
- **STAGES**: associations of germ cells from different generations in concentric layers that are always seen together when looking at cross sections of tubules
  - These stages succeed each other over **time** and repeat to make up the cycle
  - # of stages varies with species. **Roman numeral stages I–XIV** in the rat (I–XII in mouse).
- **STEP**: a classification system of spermatids based on the progression of changes in spermiogenesis
  - The steps are based on the form and shape of the acrosome (from the Golgi) and, to a lesser extent, the spermatid head shape and degree of chromatin condensation
  - **Arabic numeral steps 1–19 in the rat**
- A **stage** is defined by the youngest **step** of spermatid, either **round** or **elongating**



# Stages and Steps? Oh My!

STAGES **I–VIII** have both **round** and **elongating** spermatids, so spermatids of two different steps

- *Generally, four generations:*
  - spermatogonia,
  - pachytene spermatocytes,
  - layers of round spermatids,
  - elongating spermatids
- **Steps 1–8** PLUS steps **15–19**
  - The stage is named for the round spermatid, which is a generation younger than the elongating spermatid



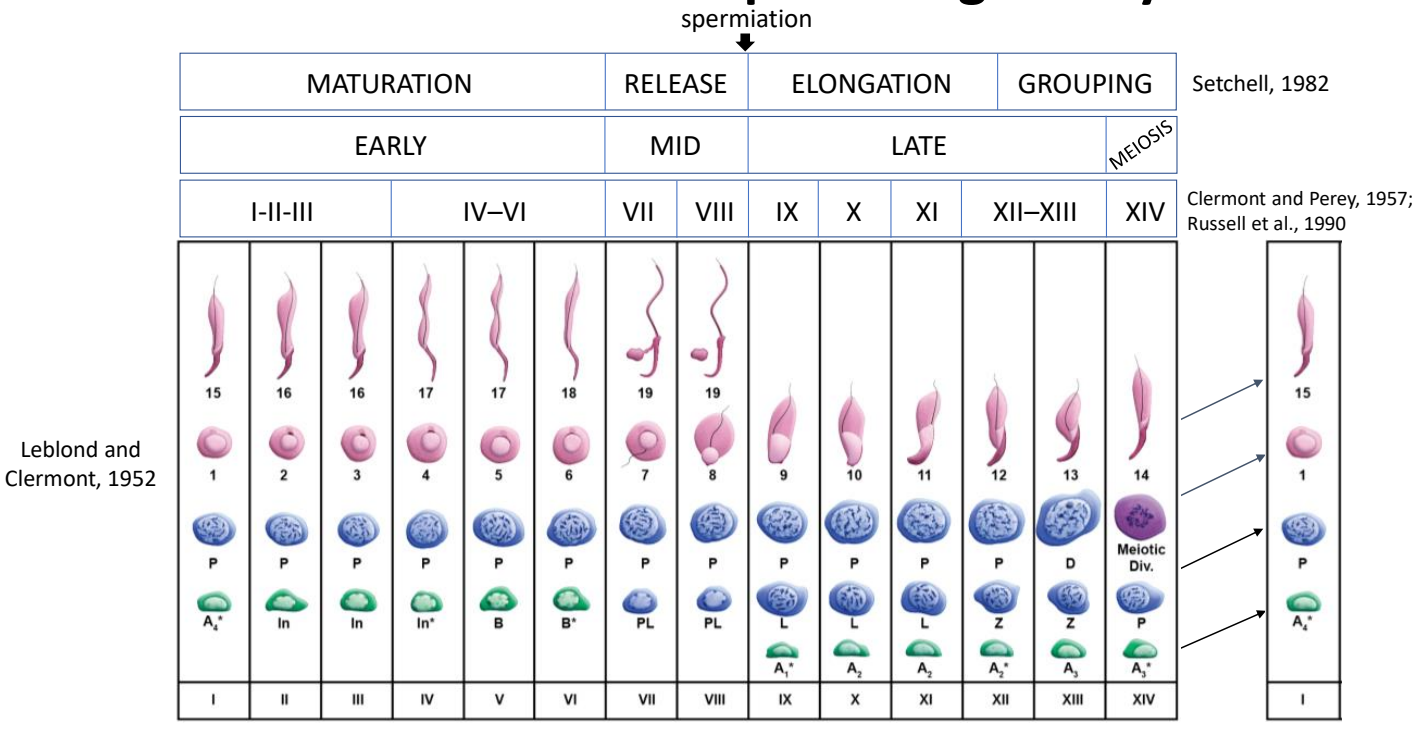

STAGES **IX–XIV** have only **elongating** spermatids, so spermatids of only one step

- *Generally, four generations:*
  - spermatogonia,
  - prepachytene spermatocytes,
  - late pachytene spermatocytes,
  - elongating spermatids
- Steps **9–14**
  - Stage # = Step # (but Roman stage versus Arabic step)

# Classification of the Spermatogenic Cycle

- For the rat, most use the classic Leblond and Clermont (1952) scheme of 14 stages (I–XIV) in the rat *(I–XII in the mouse)*
  - Other schemes have fewer or even more
  - Fourteen stages are based on acrosome features in stages I–VI, which requires certain fixation/embedding/staining to distinguish
- For many applications, can use coarser groupings that can be applied to any species
  - Early, Mid, Late, and Meiosis
  - Maturation, Release, Elongation, Grouping (Setchell 1982)

# Classifications of the Spermatogenic Cycle



# Identifying Tubular Stages: First Question to Ask

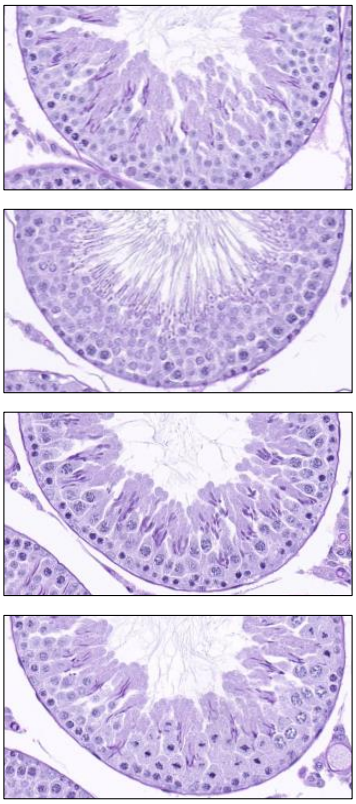
Are there **two generations of spermatids** (round and elongating) present?

YES → Stages I–VIII

- Stages I–VI: Maturation (EARLY)
- Stages VII–VIII: Release (MID)

NO, just elongating → Stages IX–XIV

- Stages IX–XIII: Elongation (LATE)
- Stage XIV: Grouping (MEIOSIS)



# Maturation (EARLY) Stages

Are there **two generations** of spermatids (**round** and **elongating**) present?

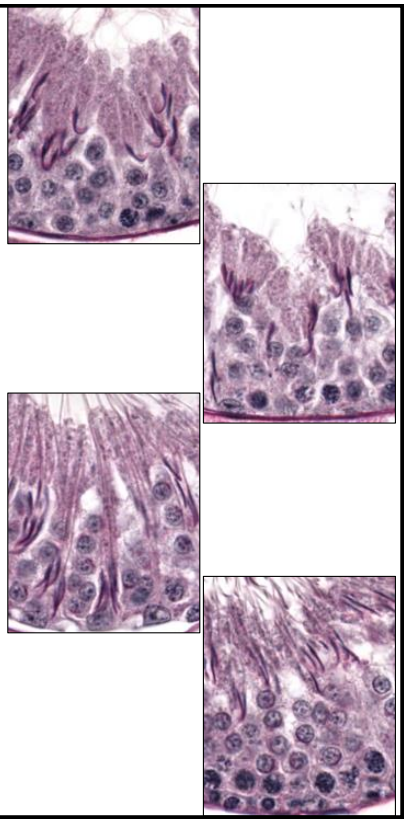
**YES** → Do the elongated spermatids line the luminal surface with long, thin tails and cytoplasmic droplets?

YES → Stages VII–VIII: Release (MID)

**NO** → **Stages I–VI: Maturation (EARLY)**

- **Shortest, fat, chunky tails with heads at lumen? Stage I**
- **Short, chunky tails, heads starting to descend? Stages II–III**
- **Heads close to base, may "kiss" the Sertoli cell nucleus (wagon wheel)? Stages IV–V**
- **Heads returning to lumen, finer tails, but not as fine as in stages VII/VIII? Stage VI**

NO → Stages IX–XIV: Elongation (LATE) or Grouping (MEIOSIS)



# Release (MID) Stages

Are there **two generations** of spermatids (**round** and **elongating**) present?

YES I–VIII

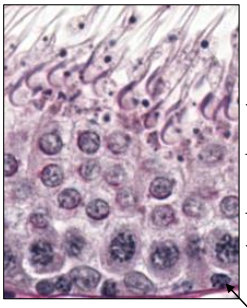
Do the elongated spermatids line the luminal surface; with long, thin tails and cytoplasmic droplets?

YES

**Cytoplasmic droplets small, fine on elongated spermatids,**

**nuclei in center of cytoplasm in round spermatids**

**Stage VII**



Step 19

**Step 7**

Pachytene spermatocyte

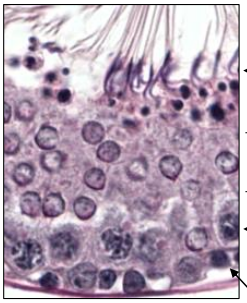
Preleptotene spermatocyte

YES

**Cytoplasmic droplets large, coarse on elongated spermatids,**

**nuclei at basal pole of cytoplasm in round spermatids**

**Stage VIII**



Step 19

**Step 8**

Pachytene spermatocyte

Preleptotene spermatocyte

NO

IX–XIV

Elongation (LATE) or Grouping (MEIOSIS)

NO

I–VI

Matur-ation (EARLY)

*Important to ID for:* ***germ cell apoptosis,*** *degeneration/depletion of germ cells, abnormal spermiation*

# Elongation (LATE) + Grouping Stages

Are there two generations of spermatids (round and elongating) present?

YES

Stages I–VIII (Maturation or Release)

NO

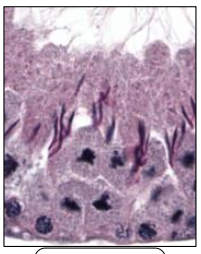
**Only elongating spermatids are present** (no round).

Are meiotic figures and/or 2' spermatocytes present?

YES

**Stage XIV**

**Grouping (MEIOSIS)**



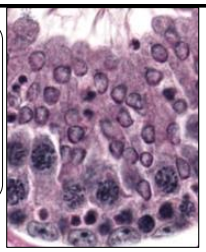
NO

**Stages IX–XIII**

**Elongation (LATE)**

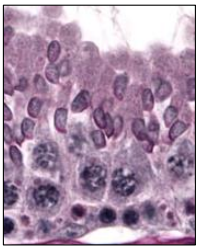
**Nucleus with open chromatin, flat on one side, round on other ("D-shaped"), RBs near lumen and/or descending?**

**Stage IX**



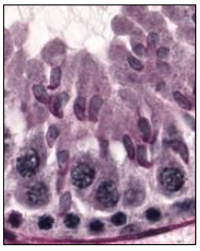
**Nucleus with open chromatin, both sides curved, ventral angle, and apex ("sesame seed"), RBs present near base or disappearing?**

**Stage X**



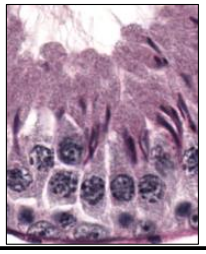
**Nucleus with ventral and dorsal angle, chromatin condensing ("banana")?**

**Stage XI**



**Skinnier nucleus, dense chromatin ("bent rod/ hockey stick")?**

**Stage XII/XIII**



*Important to ID these post-spermiation stages for:* ***spermatid retention, abnormal RBs****, degeneration of elongating spermatids, missing layers, increased apoptosis*

# Cheat Sheet to Stages of the Cycle

- Stages I–VI ("Maturation")
  - PAS/H: using **round** spermatids, look at acrosome (may be difficult to see)
  - H&E: using **elongating** spermatids, look at depth of spermatid heads in epithelium + their tail width and length
- Stages VII and VIII ("Release")—easily recognizable by location and key to know
  - Elongated spermatid heads **line the lumen with attached residual bodies**; **thinnest and "whorled" tails**
    - Bigger RBs on step 19 spermatids in VIII than in VII
    - Step 7 spermatids have central nucleus; step 8 spermatids have nucleus oriented toward basement membrane
    - Spermiation occurs at end of stage VIII

# Cheat Sheet to Stages of the Cycle

- Stages IX–XII ("Elongation")—most are easily recognizable by nucleus shape
  - Spermiation has occurred, so the spermatids that were previously round in stage VIII now start to elongate
  - Look at **shape of the elongating spermatid head** and condensation of chromatin
    - Stage XI: nucleus asymmetric "D-shaped" (flat on one side); open, pale chromatin + RBs present in epithelium
    - Stage X: nucleus "sesame seed" shaped with ventral "V"; open, pale chromatin; RBs gone or basal
    - Stage XI: nucleus "banana" shaped, chromatin condensing at base of nucleus
    - Stages XII and XIII: thinning "bent rod" or "hockey stick" nucleus, dense chromatin
- Stages XIII and XIV ("Grouping")
  - Either **huge diplotene spermatocytes** (XIII) or **any meiotic figures/secondary spermatocytes** (XIV)
  - Secondary spermatocytes (XIV) are a little bigger than step 1 spermatids and seen with very low frequency

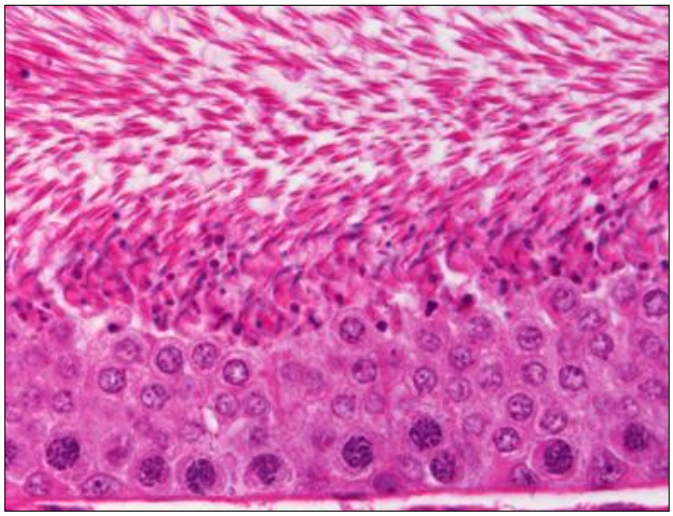
## Look for the "Helpers"

- The testis is ***not*** like Vegas
  - What happens in the testis usually does NOT stay in the testis
- Changes in sperm parameters often will be linked to changes in histopathology
  - ↑ in HRS? Look for spermatid retention in the testis.
  - ↓ in HRS? Look for ↓ elongated spermatids in the testis.
- Testicular toxicity is often reflected in cellular, fluid, or other changes in downstream, androgen-dependent, or endocrine tissues
  - E.g., debris/exfoliated germ cells in the epididymis? Figure out where they came from.
- Look for weight and/or histopathological changes in:
  - Epididymis, accessory sex glands, pituitary gland, mammary gland (male rats)

## Three Reasons Why Stage-Awareness Is Important

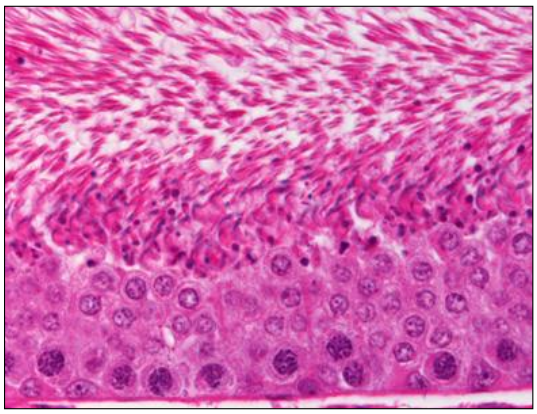
- (#1) When an **appropriate** cell type/layer is **missing**
  - It is relatively easy to identify when there are changes to the cells present
  - More difficult to detect when something is missing
  - Examples: depletion of spermatocytes, depletion of elongating spermatids
- (#2) When an **inappropriate** cell type is **present**
  - Example: spermatid retention in stages IX–XII (also, a stage-specific lesion)
- (#3) To identify **stage- and/or cell-specific** lesions
  - Example: germ cell apoptosis in stages VII/VIII

# Is It Normal?

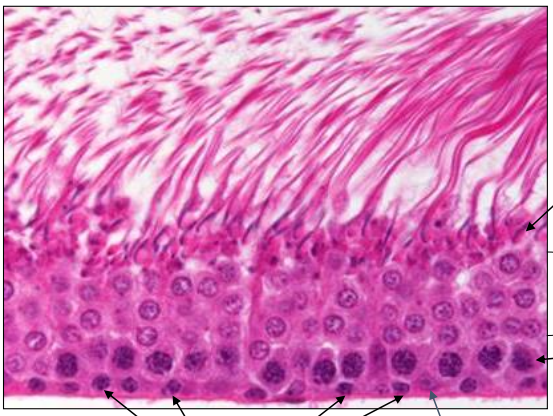


# (#1a) When an Appropriate Cell Type/Layer Is Missing: Depletion of Spermatocytes

Depletion, preleptotene spermatocytes, VII/VIII



Control, VII/VIII



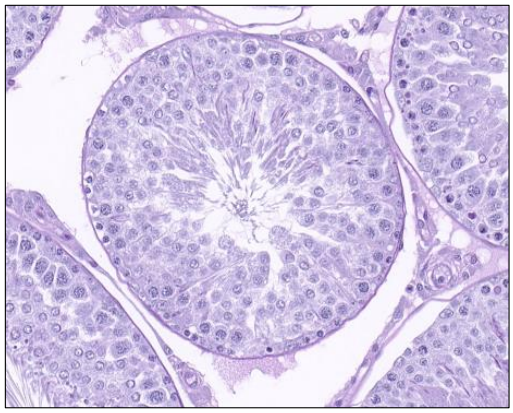

- Loss of early spermatocytes (preleptotene) after two weeks of treatment with a spermatogonia mitotic inhibitor
- At this early point, difficult to detect the initial loss of spermatogonia
- Eventually, there will be loss of more mature cell types

Courtesy of Dr. Justin Vidal

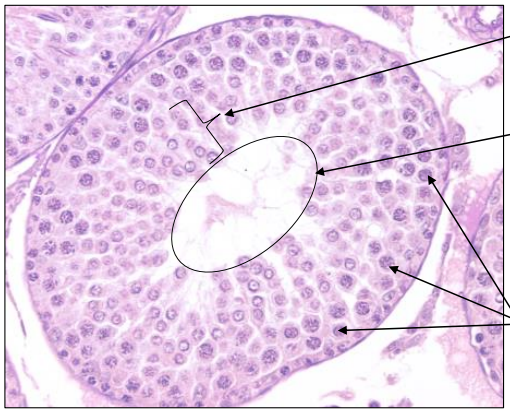
# (#1b) When an Appropriate Cell Type/Layer Is Missing: Depletion of Elongating Spermatids

*Note: this can be difficult to detect at low power and without knowing what should be present*

Partial depletion, elongating spermatids



Depletion, elongating spermatids



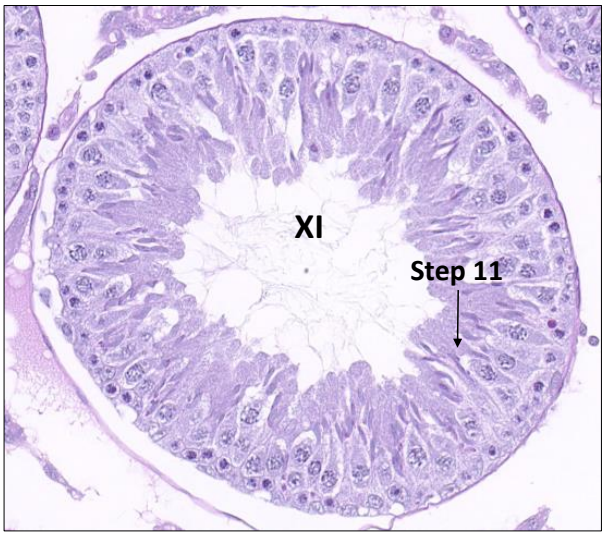

Partial (left) and complete (right) loss of elongating spermatids in two maturation or release stage tubules with prolonged reduction in testosterone due to treatment with a steroidogenesis inhibitor. The tubules are also contracted in diameter (due to loss of elongating spermatids and likely due to ↓ T). Germ cell depletion is the most common sequel to spermatogenic disturbance.
"Helpers": ↓HRS; may see ↓ testis weight (↓ spermatids and fluid) and ↓ epididymis weight/sperm/fluid.

# (#2) When an Inappropriate Cell Type Is Present: Spermatid Retention—A "Hallmark" Stage-Specific Lesion

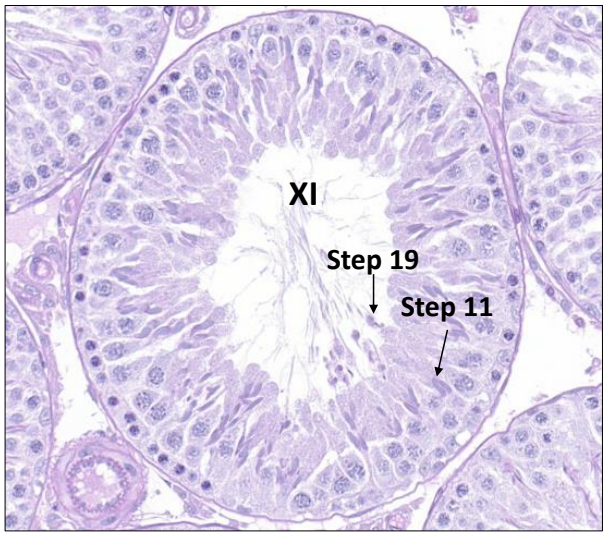
- Failure of mature (step 19) spermatids to be released from the Sertoli cell during spermiation at the end of stage VIII
  - Seen in stages IX–XII as the inappropriate presence of mature spermatids
  - At luminal surface, within epithelium, or being phagocytosed in basal Sertoli cell cytoplasm
- A sensitive, but not specific, finding seen with a number of chemicals or hormonal disturbance, due to either:
  - Sertoli cell injury
  - Reduction in testosterone levels (direct or indirect)
    - May be a direct effect on Leydig cells causing decreased T production
    - Hormonal: severe/chronic reduction of androgens, exogenous androgens, GnRH antagonism, estrogens
- "Helpers"
  - *This microscopic change is far more sensitive than changes in testis weight; may be only lesion with no change in weight*
  - Sperm data: spermatid retention is usually associated with one or more sperm parameter abnormalities (morphology, motility, sperm count, spermatid head count)
  - May be associated with decreased fertility
  - If ↑ HRS, be sure to look for spermatid retention!

Lanning et al., 2002; Vidal and Whitney 2014

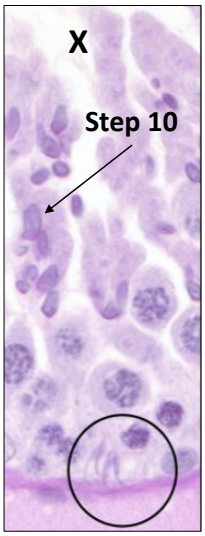
# (#2) Spermatid Retention: A Stage-Specific Lesion (IX–XII)




Normal stage XI tubule with step 11 spermatids from a control animal. Spermiation occurred back in stage VIII.




Stage XI tubule with retained step 19 spermatids at the lumen, in addition to the appropriate step 11 spermatids, after treatment with a steroidogenesis inhibitor.




Stage X with step 19 spermatids deep in the epithelium.

# Examples of Early Cell- and Stage-Specific Toxicity

| Chemical | Primary cell target | Germ cells affected | Stage specificity | References |
|---|---|---|---|---|
| 2-methoxyethanol | Pachytene spermatocyte | Pachytene spermatocyte (death) | I–III, XIV @ low dose<br>I–V, XIII–XIV @ mid dose<br>All stages @ high dose | Creasy and Foster 1984; Creasy et al., 1985 |
| Dinitrobenzene | Sertoli cell | Pachytenes (death);<br>Spermatid retention | II–V, VI–XI (death)<br>IX–XIV (retention) | Blackburn et al., 1988; Hess et al., 1988 |
| 2,5-Hexanedione | Sertoli cell | Pachytenes (death);<br>Round spermatids (death);<br>Elongating s'tids (death) | I–VIII | Boekelheide 1988; Chapin et al., 1983 |
| Ethane dimethane sulphonate | Leydig cell | Pachytenes (death);<br>Step 7 spermatids (death) | VII | Bartlett et al., 1986; Kerr et al., 1993 |

- Stage specificity can vary with dose and duration.
- Different cell targets can result in pachytene spermatocyte death (apoptosis).
- Note that some chemicals result in pachytene and round spermatid apoptosis that is limited to stage VII (and VIII). *This is important to be able to detect (may be low numbers of cells and tubules affected).*

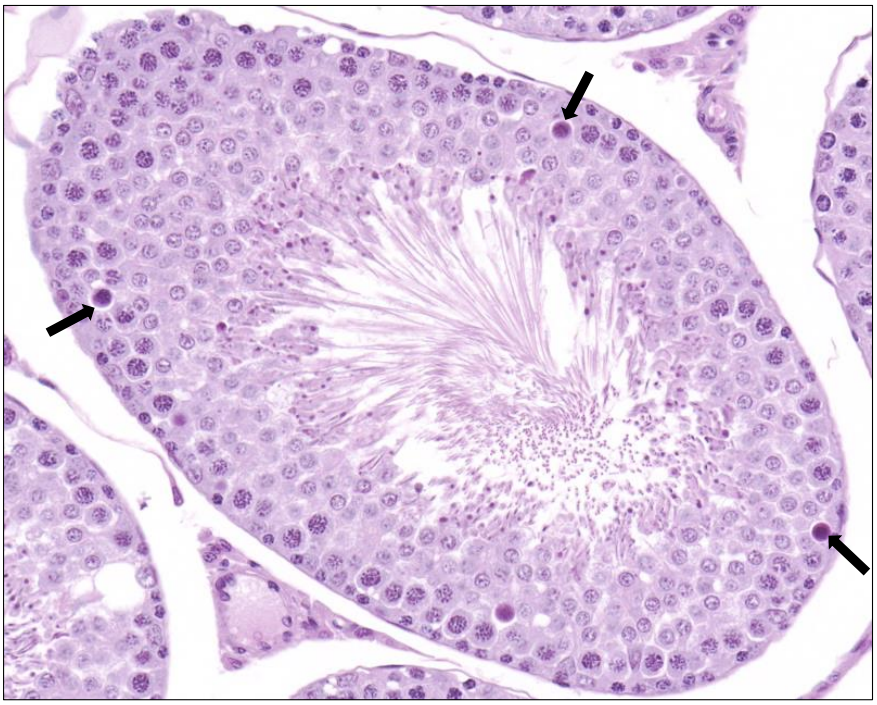
Redrawn from Creasy 1997

# (#3) To Identify Stage- and/or Cell-Specific Lesions: Germ Cell Apoptosis in Stages VII/VIII (Release)

- Features:
  - Cytoplasmic eosinophilia and contraction
  - Chromatin condensation (esp. spermatocytes) or margination (esp. round spermatids)
  - *Note: don't confuse with residual bodies in stage VIII–X, or low-level background attrition (XII, XIV)*
- Germ cell apoptosis can occur directly (damage to germ cells) or indirectly (damage to somatic cells [Sertoli or Leydig]) (Boekelheide, 2005)
  - **Pachytene spermatocytes (+/- round spermatids) in stages VII, VIII (Release)**
    - **Specific: due to low intratesticular testosterone** (intratesticular T normally 50x serum T)
    - When present, seen in VERY low numbers of tubules/cells, so requires careful examination
    - A single IP injection of ethane dimethane sulfonate: after three days, Leydig cell obliteration and undetectable testicular T, but still only low numbers of stage VII/VIII tubules with apoptotic spermatocytes (Bartlett et al., 1986)
    - Considered the most sensitive marker of reduced T and an early effect of androgen deficiency (Russell et al., 1990)

Creasy 2001; Creasy et al., 2012

# (#3) Germ Cell Apoptosis in VII/VIII: A Stage-Specific Lesion



Stage VII tubule from a rat demonstrating numerous apoptotic pachytene spermatocytes (arrows) consistent with reduced testosterone (H&E)

*Note: this lesion has also been observed in stage VII pachytene spermatocytes in young rats in short-term food restriction studies* (Rehm et al., 2008)

# Patterns of Toxicity

- Some changes are non-stage-specific
  - Sertoli cell toxicity often features several nonspecific degenerative lesions
    - Lesions may start out specific and end in nonspecific degeneration and depletion → atrophy
- Two examples featuring stage- and/or cell-specific changes
  - Androgen deprivation
    - Early changes
      - Two stage-specific lesions! Pachytene apoptosis stage VII/VIII, spermatid retention
    - Progressive changes—depletion of elongating spermatids
    - "Helpers"—ASG, pituitary gland, male rat mammary gland
  - Estrogenic compounds in male rats
    - Similar lesions as decreased testosterone in the testis
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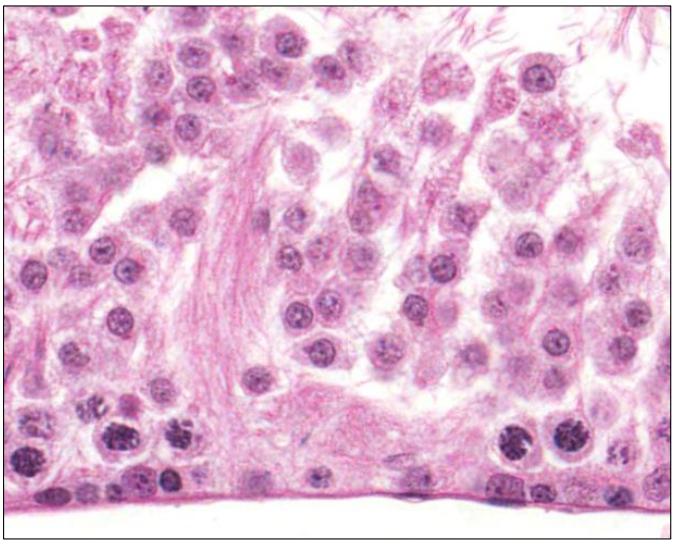
    - Similar lesions as decreased testosterone in the testis
    - "Helpers"—ASG, male rat mammary gland

# Patterns of Toxicity: Sertoli Cell Injury

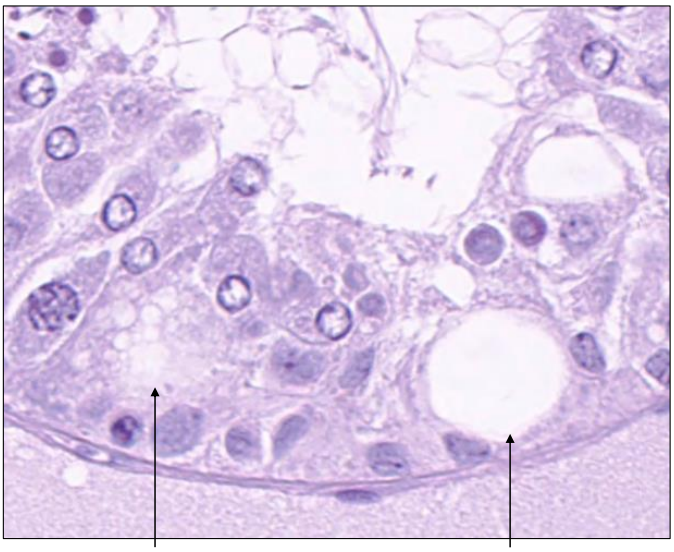
- Loss of function rather than death—"easy to injure, hard to kill"
- Early indicators
  - **Vacuolation**
    - "micro-" or "macrovacuolation"
    - E.g., 2,5-hexanedione, phthalate esters
  - **Focal germ cell "dropout"** of cohort supported by that Sertoli cell
    - Often manifests as germ cell loss (focal, then segmental or extensive)
  - **Germ cell exfoliation**
    - E.g., carbendazim, a microtubule disruptor
    - The exfoliated cells look rounded and "normal" and lack degenerative features
  - **Spermatid retention/disordered spermiation**—very SENSITIVE, but not specific
    - Often a very early indicator of Sertoli cell disturbance; may be the only change
    - But also an early feature of testosterone reduction
- Progressive changes
  - **Disorganization** of germ cell layers in the epithelium
  - Progressive **degeneration**, **depletion**, or **exfoliation** of germ cells
  - End stage: Sertoli cell only tubules—germinal epithelium **atrophy**

Vidal and Whitney 2014

# Early Effects of Sertoli Cell Injury



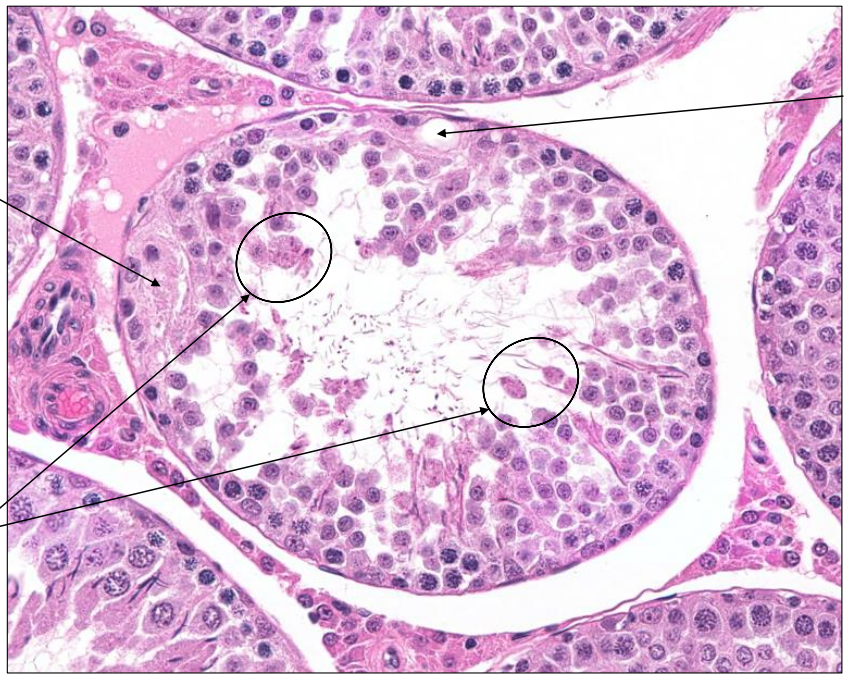
Focal germ cell dropout—loss of germ cell cohort from Sertoli cell



Microvacuolation (multiple, small vacuoles)

Macrovacuolation (single, large vacuole)

# Progressive Effects of Sertoli Cell Injury

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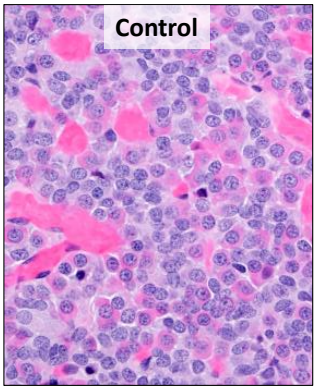
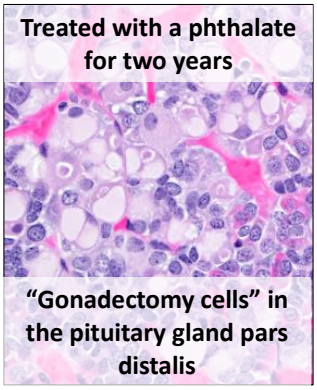
    - Early changes
      - Two stage-specific lesions! Pachytene apoptosis stage VII/VIII, spermatid retention
    - Progressive changes—depletion of elongating spermatids
    - "Helpers"—ASG, pituitary gland, male rat mammary gland
  - Estrogenic compounds in male rats
    - Similar lesions as decreased testosterone in the testis
    - "Helpers"—ASG, male rat mammary gland

# Patterns of Toxicity: Low T

- Androgen deprivation/decreased T occurs via a variety of mechanisms. Findings depend somewhat on the mechanism.
- Sensitive, early, or slightly low:
  - Degeneration (**apoptosis**) of **pachytene spermatocytes** and occasional round spermatids in **stage VII/VIII** tubules (sensitive and specific)
  - **Spermatid retention** (sensitive, but also seen with Sertoli cell toxicity)
  - Epididymis may be normal weight and histology
- Prolonged or very low:
  - **Decreased testis weight**
  - **Progressive degeneration and depletion of elongating spermatids**
  - Presence of elongated spermatids stimulates the Sertoli cell to produce fluid, so depletion of elongating spermatids → decreased fluid → **decreased tubular diameter**
  - **Reduced epididymis weight and hypospermia**
  - **Decreased prostate, seminal vesicle weights +/- histological atrophy**
  - +/- Pituitary gland pars distalis "castration cells"

Creasy, 2008

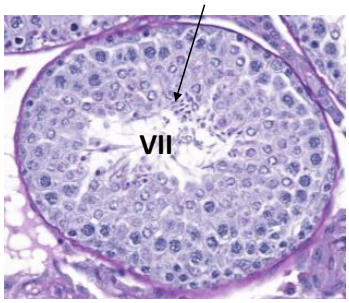



"Gonadectomy cells" in the pituitary gland pars distalis

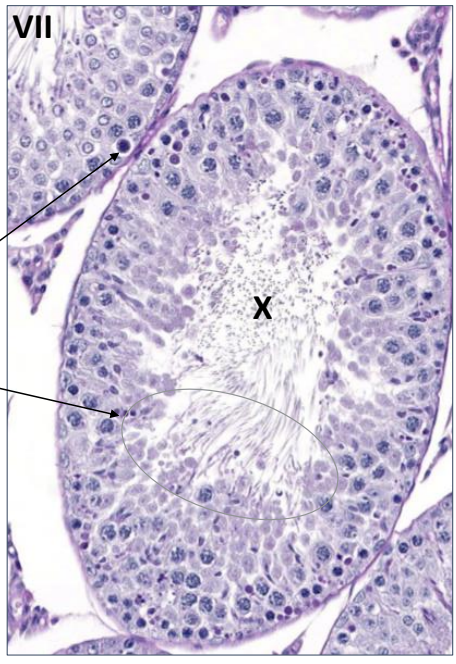
# Patterns of Toxicity: Estrogens

- In part, directly inhibit T secretion from Leydig cell
- Also, "mimic" T in the negative feedback on GnRH and LH release → ↓ LH → ↓ intratesticular T production by Leydig cells
- **Effects of estrogens in testis can look similar to low T**
  - Two stage-specific lesions: stage VII/VIII apoptosis of spermatocytes
  - Stage IX–XII spermatid retention
  - Decreased ASG weights
  - Progressive depletion of elongating spermatids




*Left*: depletion of elongating spermatids in a contracted stage VII tubule

*Right*: apoptosis of pachytene spermatocyte in a stage VII tubule (*top left*) and spermatid retention in a stage X tubule (*center*)

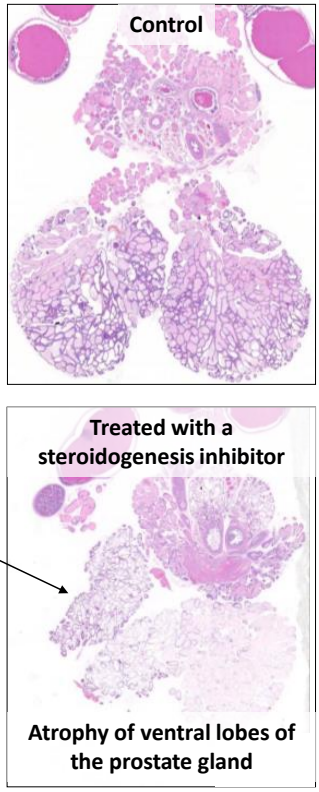



Cook et al., 1998; Creasy, 2008; Yamasaki et al., 2002

## Patterns of Toxicity: Low T versus Estrogens: Look for the "Helpers"

- Estrogenic agent "helpers":
  - **Dorsolateral prostate response to estrogen: acute (neutrophilic) inflammation**
  - +/- Male rat mammary gland—male-to-female differentiation
- Low T "helpers":
  - **Ventral prostate response to low androgen: atrophy** (lobe most sensitive to circulating androgen levels)
  - Decreased weights +/- microscopic atrophy: epididymis, prostate, seminal vesicle
    - ↓ weight may be more sensitive than histopathology
  - +/- Male rat mammary gland atrophy

Creasy, 2008; O'Connor et al., 2002; Tangbanluekal and Robinette, 1993




## Summary and Conclusion

- Testes should be evaluated in a stage-aware manner
  - To detect what's missing, what's there that shouldn't be there, or stage-specific lesions
- Take the holistic view
  - Look for the "helpers"
  - Integrate the endpoints available (weights, histopathology of reproductive and endocrine tissues, sperm parameters, fertility)
  - Examine reproductive and endocrine organs as a unit to evaluate patterns of change

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