

# CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY REPRODUCTIVE SYSTEM



ORGANIZED BY SOCIETY FOR TOXICOLOGIC PATHOLOGY IN INDIA (STPI)

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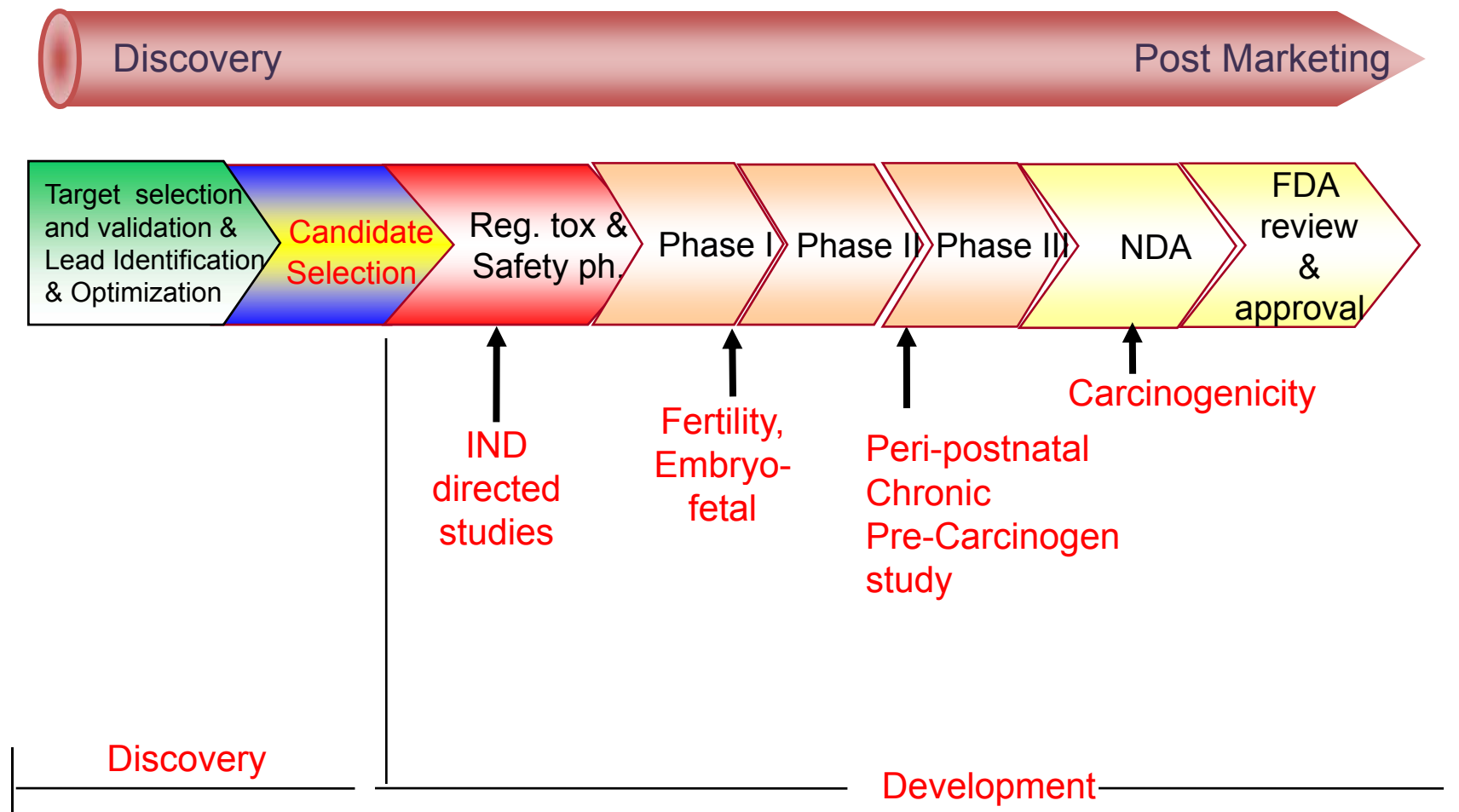
# **Assessment of Female Reproductive Toxicity in Routine Toxicological Studies**

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# New Drug Development Process



Development of a new molecule is very lengthy and expensive process

# Prediction of Toxicology

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- **Structure-Based Prediction of Toxicity**

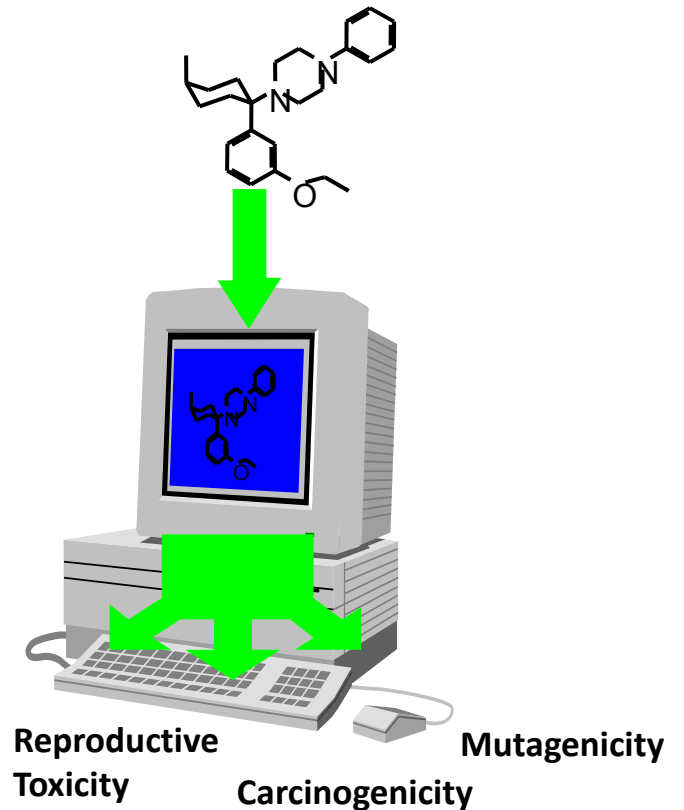
- In-Silico Prediction
- Ex: TOPKAT, MULTICASE, DEREK

- **Mechanism-Based Prediction of Toxicity**

- In Vitro Prediction
- Ex: Cell-lines to predict various organ toxicities

- **Toxicology In Silico**

- Genomics, Toxicogenomics
  - Ex: DNA microarray.
- Proteomics



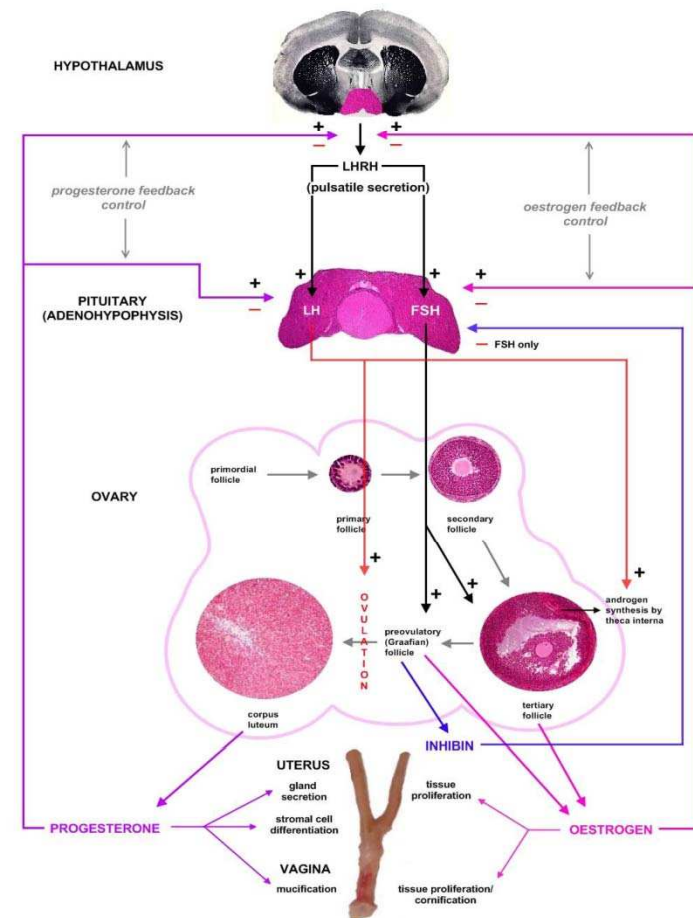
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Expensive, less predictive, difficult to correlate and not regulated currently

**Assessment of Female  
Reproductive toxicity in Routine  
Toxicological Studies**

# Introduction

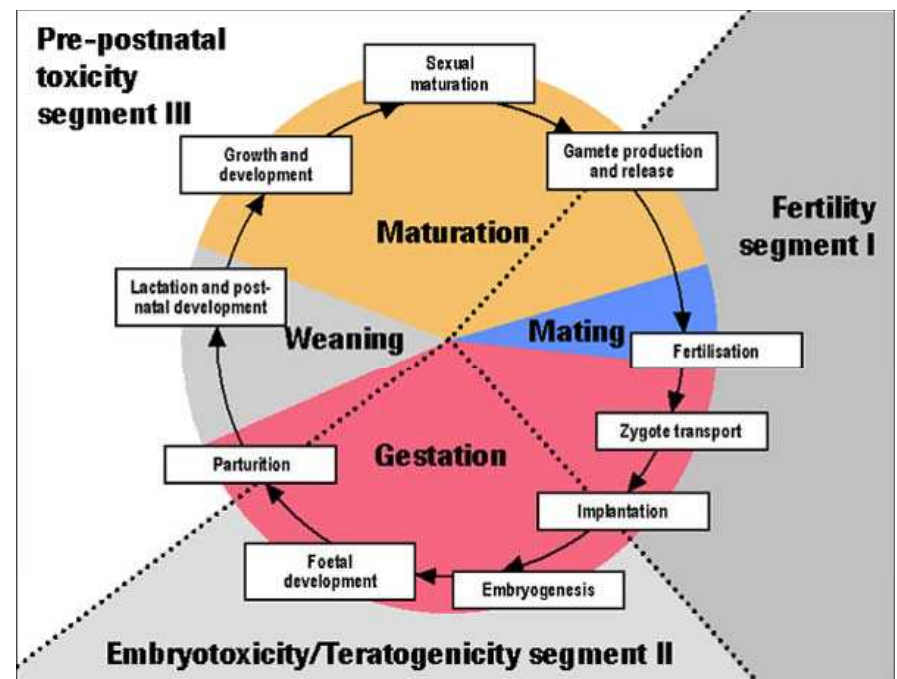
- Female reproductive functions are very complex and involve set of independent and interdependent processes.
- Factors affecting evaluation of female reproductive functions are;
  - Hormonal regulation
  - Morphological changes occurring during normal estrus cycle.
  - Age related changes.
  - Species differences.
  - Effects that occur in combination with other endocrine organs.



Female's role in reproduction is more complicated than male's

# Introduction

- Concern for the unintentional exposure of an embryo or fetus.
- Effects of xenobiotics on the female reproductive system dictate strict regulatory requirements.
- The pre-clinical reproductive safety studies conducted before inclusion of WOCBP are;
  - Segment I- Reproduction and fertility
  - Segment II- Teratology
  - Segment III- Pre & postnatal study



These three segments cover entire reproductive cycle

# Introduction

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Regional differences - Timing of reproduction toxicity studies.

| Japan  | EU  | USA  |
|--|---|--|
| Female fertility and EFD prior to inclusion of WOCBP | EFD- Prior to Phase I and female fertility prior to Phase III | WOCBP –included. Female fertility and EFD – Prior to Phase III |

ICH M3 GL- WOCBP can be included in repeated dose phase I and II trials without reproductive toxicity studies provided;

- The female reproductive organs should be evaluated in the repeated-dose toxicity studies.
- Knowledge of the mechanism of action of the agent.



# Introduction

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- The reproductive studies examine functional perturbations of reproductive performance. However in repeated dose toxicity studies the morphological assessment of reproductive organs is studied.
- Getting maximum information from repeated dose toxicity studies will certainly compliment the succeeding reproductive toxicity studies and help in interpretation of results.

Whether the parameters used in repeat dose toxicity studies can detect female reproductive toxicity?

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# Outline of Presentation

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- **Potential mechanisms of female reproductive toxicity**
  - Directly reproductive toxicants
  - Indirectly acting reproductive toxicants
  - Site of action of reproductive toxicants
- **End points used in evaluation of Female reproductive toxicity**
  - Clinical chemistry
  - Organ weights
  - Tissue sampling
  - Microscopic evaluation
- **Morphological patterns (Type I, II and III)**

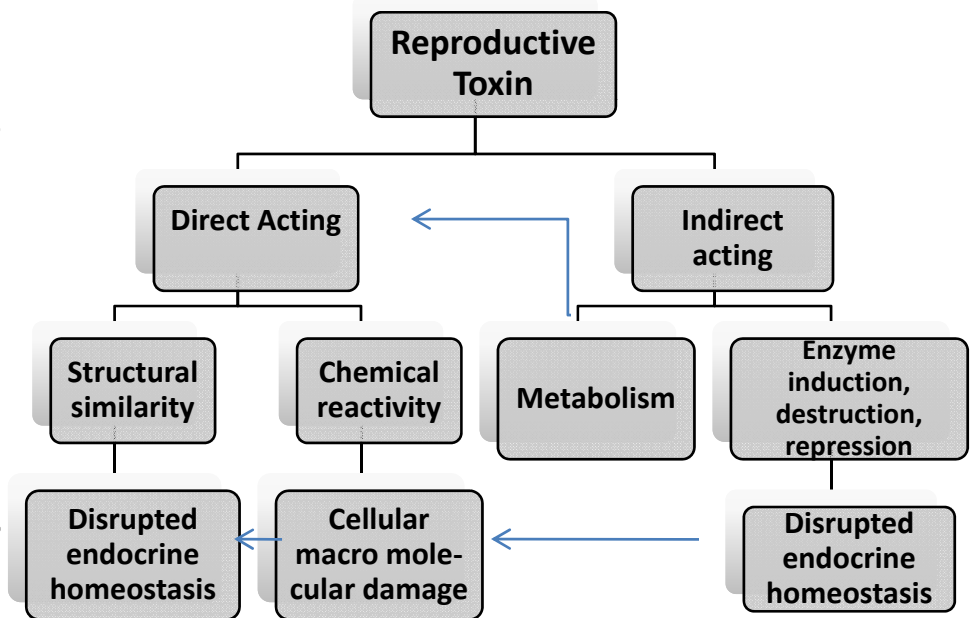
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As reproduction is essential for continuation of any species it is important to understand mechanism of action and site of action of reproductive toxicants

# Potential Mechanisms of Female Reproductive Toxicity

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- Toxicity to reproductive organs- Direct or indirect.
- Toxicant either parent or metabolite disrupts normal events required for reproduction.
- Toxic insult may be specific affecting single cell/ event or non-specific affecting multiple sites, many organs.



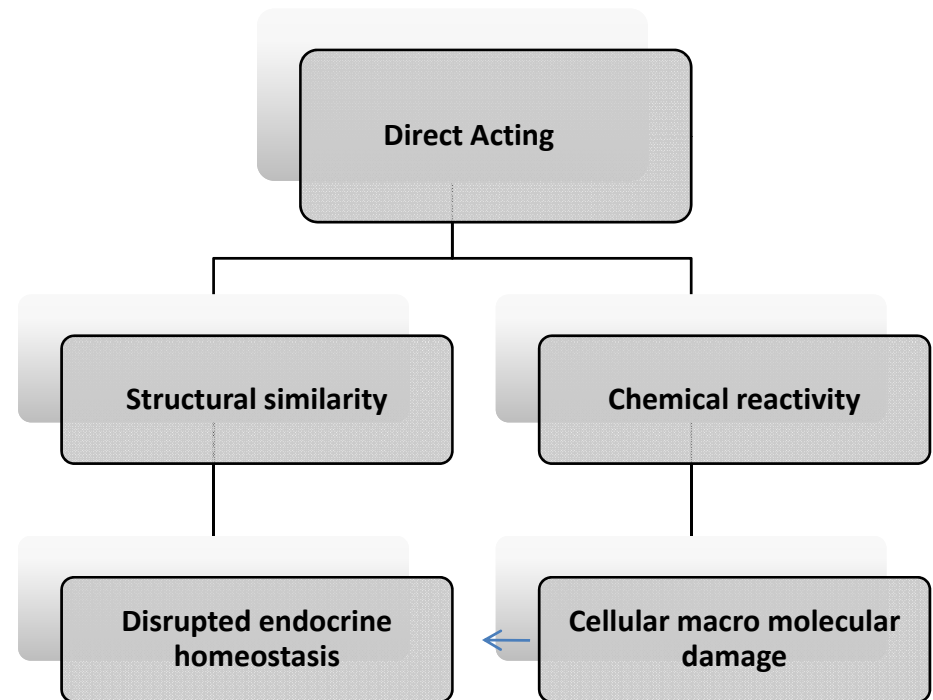
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The toxicological mechanisms underlying female reproductive systems are complex

# Potential Mechanisms of Female Reproductive Toxicity- Directly acting toxicants

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- Multiple layers of granulosa cells - restricts the access toxicants to oocytes .
- Structural similarity- endogenous compounds. Ex. hormones, vitamins.
  - Easy access to target site.
  - Mislead normal processes
  - Act either as agonist or antagonist
- Chemical reactivity - Ex. Alkylating agents , metals



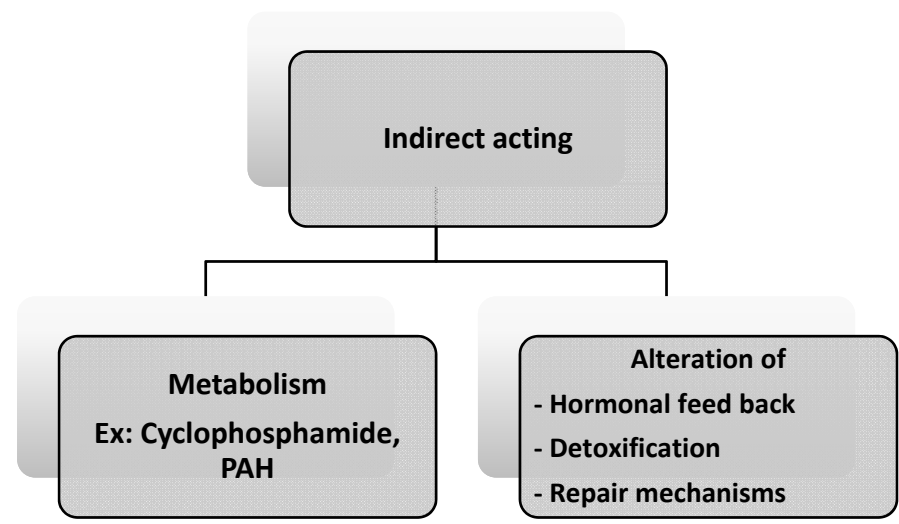
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The toxins interact with sub-cellular components and disrupts the events necessary for normal reproduction.

# Potential Mechanisms of Female Reproductive Toxicity- Indirectly acting toxicants

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- Indirectly acting toxicants - metabolic activation or disrupt the physiological control.
- Ovaries -metabolic activation – reactive metabolites.
  - Metabolic activation - Ex:- Cyclophosphamide, PAH.
  - Alteration in hormonal feedback (rate of steroidogenesis or clearance) Ex:- DDT, PCB, PBB.
  - Alteration of detoxification (enzyme deficiency or damaging organ)
  - Impairing repair mechanisms.



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The toxic effects may be very specific affecting single cell or nonspecific with multiple sites of toxicity

# Potential Mechanisms of Female Reproductive Toxicity- Sites of actions

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Several sites of actions - hypothalamus - pituitary – gonadal axis.

| Site of action     | Function   | Mechanism of action of toxicant -Examples  |
|--------------------|--|--|
| Hypothalamus       | <ul style="list-style-type: none"> <li>• Synthesis and secretion of GnRH.</li> <li>• Have receptors for FSH, LH and Prolactin</li> </ul>                       | <ol style="list-style-type: none"> <li>1. Affecting neurotransmitter synthesis</li> <li>2. Affecting neuropeptide regulation of hypothalamic releasing factors.</li> </ol>                               |
| Anterior pituitary | <ul style="list-style-type: none"> <li>• Synthesis and secretion of FSH, LH and prolactin.</li> <li>• Have receptors for GnRH, FSH, LH and steroids</li> </ul> | <ol style="list-style-type: none"> <li>1. Modification of pituitary hormone secretion- DES</li> <li>2. Acting on pituitary membrane receptors- Bromocriptine inhibition of prolactine release</li> </ol> |

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**Compounds targeting central regulation of neuroendocrine axis affect fertility by altering LH surge, delaying ovulation and modifying normal feedback**

# Potential Mechanisms of Female Reproductive Toxicity- Sites of actions

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- Ovary takes care of set of processes independently and interdependently.

| Site of action | Function  | Mechanism of action of toxicant -Examples   |
|----------------|---|---|
| Ovaries        | <ul style="list-style-type: none"> <li>• Synthesis and secretion of estrogen and progesterone</li> <li>• Development of follicles (ovum, granulosa cells).</li> <li>• Metabolic processes.</li> <li>• Endocrine functions.</li> </ul> | <ol style="list-style-type: none"> <li>1. Hormone analogues- mifepristone, tamoxifen, medroxyprogesterone acetate (MPA) .</li> <li>2. Primordial follicle damaging agent- busulfan, cisplatin, 4-venylcyclohexene diepoxide (VCD-1 &amp;2), cyclophosphamide.</li> <li>3. Metabolite imbalance inducers- inhibition of aromatase enzyme by Anastrozole and prostaglandin synthesis by indomethacin.</li> <li>4. Endocrine imbalance inducers- inhibition of LH surge by chlorpromazine hydrochloride, hypoprolactinemia by Bromocriptine or hyperprolactinemia by sulpiride.</li> </ol> |

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**Ovary is the important organ while evaluating the female reproductive capacity.**



# Potential Mechanisms of Female Reproductive Toxicity- Sites of actions

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| Site of action                  | Function  | Mechanism of action of toxicant -Examples  |
|---------------------------------|---|--|
| Uterus,<br>Vagina and<br>cervix | <ul style="list-style-type: none"> <li>• Sperm transport.</li> <li>• Initiation and maintenance of pregnancy</li> <li>• Have estrogen and progesterone receptors</li> </ul> | <ol style="list-style-type: none"> <li>1. Alteration of endometrial estrogen receptors- lead acetate.</li> <li>2. Damage to uterine microcirculation - Cadmium chloride</li> <li>3. Uterine atrophy by loss or suppression of ovarian sex hormone - Tamoxifen, toremifene, Butyrophenones.</li> <li>4. Endometrial hyperplasia - oral contraceptives.</li> </ol> |
|                                 |   | <ol style="list-style-type: none"> <li>1. Hyperkeratosis and hyperplasia of vagina and cervix - Estrogenic compounds.</li> <li>2. Increased mucus secretion by endo-cervical epithelium-Progestational compounds.</li> </ol>   |

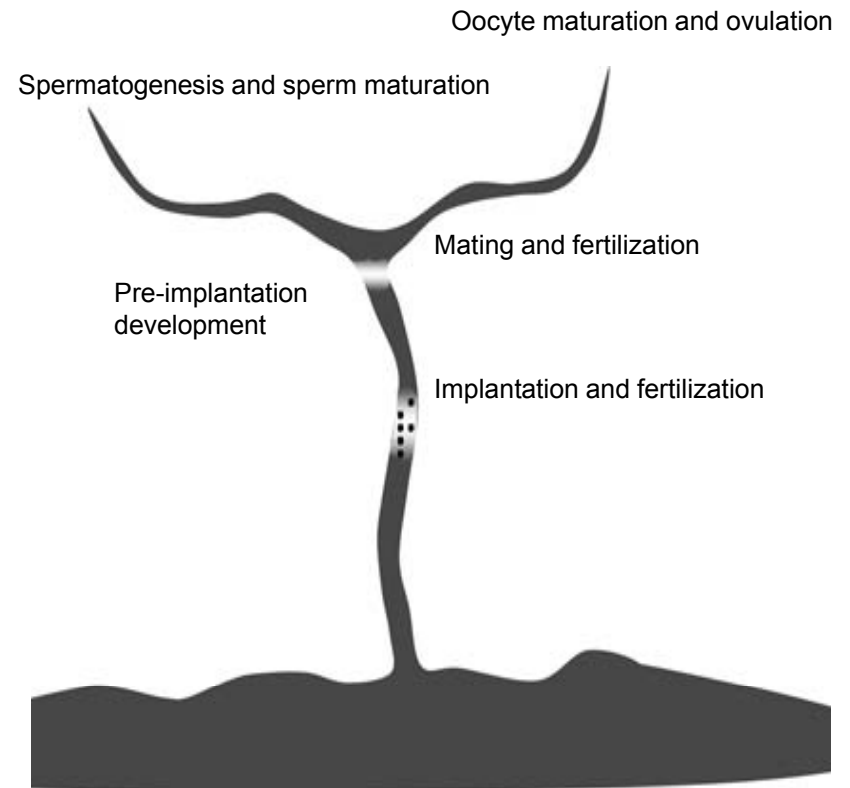
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**The reproductive toxicants produce their effects in variety of ways and at multiple sites**

# Evaluation of Female Reproductive Toxicity

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- The purpose of reproductive studies are to determine whether the test compound has adverse effects on reproductive system and to determine reproductive NOAEL.
- Segment I – reproduction and fertility studies covers periods of premating, co habilitation and mating and early pregnancy through implantation.
- For females these studies detects effects on libido, estrous cycle, ovulation, mating behavior, oviductal transport and implantation.



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**Various tests are designed to examine every stage of female reproduction.**

# Evaluation of Female Reproductive Toxicity

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| Observations | Parameters evaluated                             |   |
|--------------|--|---|
|              | fertility study                                  | Repeat dose studies                           |
| In life      | Clinical signs                                   | Clinical signs                                |
|              | Body weights                                     | Body weights and feed intake                  |
|              | <b>Estrous cyclicity</b>                         | Detailed clinical observations                |
| At necropsy  | Mating index, Fertility index<br>pregnancy index | Hematology, Clinical<br>Chemistry, Urinalysis |
|              | Gross pathology,<br>Organ weights                | Gross pathology,<br>Organ weights             |
| Post-life    | Histopathology                                   | Histopathology                                |

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**Evaluation of various endpoints can generate sufficient information to detect toxicity to Female reproductive system**

# Evaluation of Female Reproductive Toxicity

## Clinical signs

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- **Viability check-**
  - Daily cage-side observations– determine mortality and morbidity - twice a day .
- **Detailed clinical observations –**
  - Changes in the skin fur, mucus membranes, secretions and excretions.
- **Functional Observation Battery –**
  - Extended detailed clinical observations
  - Grip strength, foot splay, body temperature etc – weekly or as per protocol.

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Clinical sign evaluation will detect physical and behavioral characteristics of animal

# Evaluation of Female Reproductive Toxicity

## Body weights and food consumption

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- Provides an index of the general health status of the animal may be relevant in the interpretation of reproductive effects.
- Progesterone and estrogen influence- fluctuation in BW and FI
- Reduced BW and FI is sensitive indicator of estrogenic compound.
- Other factors to be considered are – stress, systemic toxicity, anorexia, palatability.
- Unless a direct causal link between the observed female reproductive effect and decreased BW or BWG can be established, female reproductive system alterations are considered an adverse effect and not secondary to the occurrence of systemic toxicity.
- Mild to moderate BW changes - alteration in female reproductive measure are an adverse female reproductive effects.
- Severe BW changes – adverse effects on female

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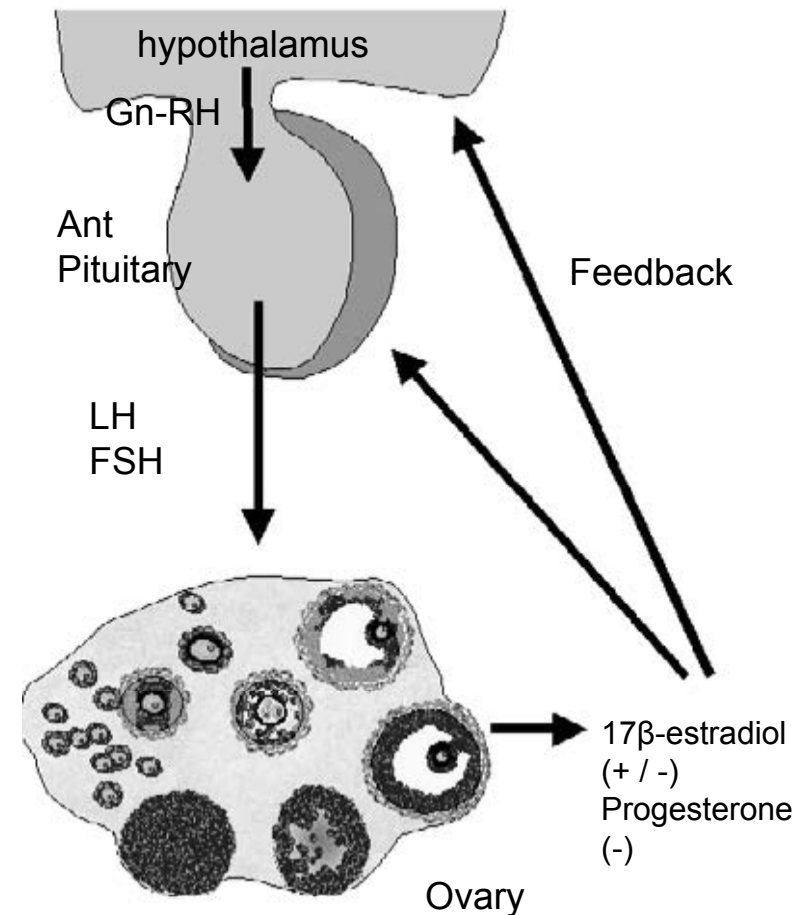
**Monitoring female body weight during treatment provides an index of the general health status and help in interpretation of reproductive effects.**

# Evaluation of Female Reproductive Toxicity

## Clinical Pathology

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- Measurement of hormonal changes – damage to reproductive organs
- Primary hormones measured include- LH, Gn-RH, FSH, Estradiol, progesterone and prolactin.
- Effects on ovaries -  $17\beta$ -estradiol and progesterone (Hoyer and Devine, 2002).
- CK, isoenzyme BB – reversible or irreversible damage to endometrium (Lanning, 2006).



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**Clinical chemistry and hematology used to assess organ damage- has limited usefulness in assessment of damage to reproductive organs**

# Evaluation of Female Reproductive Toxicity

## Organ weights

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- Uterus and ovaries weighed routinely.
- Pituitary, vagina and oviduct weights not evaluated routinely.
- STP recommends- weighing the ovaries in studies of less than 6 month durations.
- Ovaries – common target organ of toxicity – weight variation shows correlation with histological observations.
- The decrement of 10% or greater in the organ weight measures are usually of Toxicologic significance

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**Organ weight changes are very sensitive indicators of chemically induced changes**

# Evaluation of Female Reproductive Toxicity-

## Organ weights- Ovaries

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- Ovarian weight in the normal rat does not show significant fluctuations throughout the estrous cycle.
- Alterations in the ovarian weights indicates;
  - Persistent polycystic ovaries,
  - Oocyte depletion,
  - Decreased corpus luteum formation,
  - Luteal cysts
  - Altered pituitary-hypothalamic function or
  - Reproductive senescence

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**Ovarian Wt indicates ovarian dysfunction and help in establishing a NOEL even in absence of a morphologic correlate.**

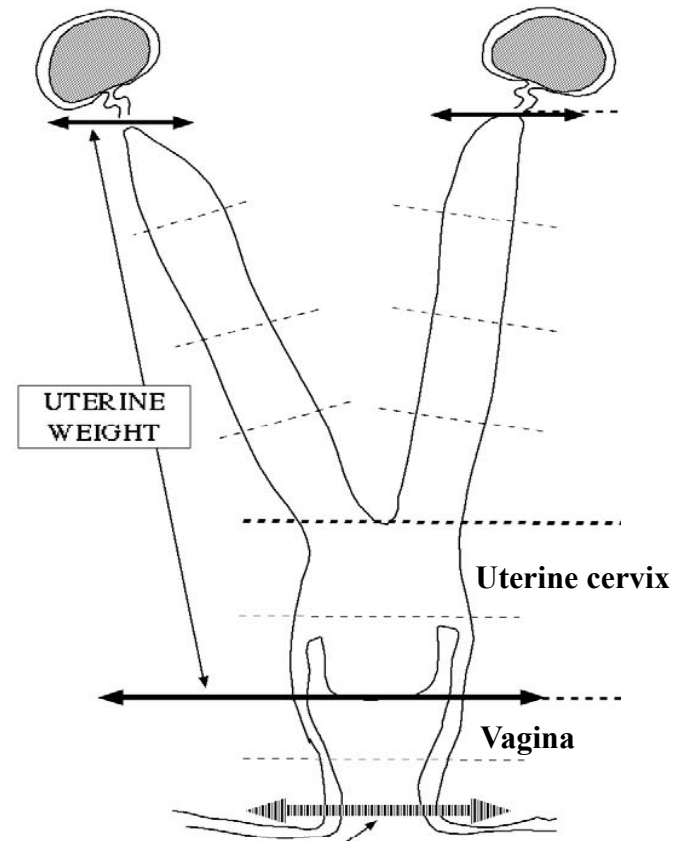


# Evaluation of Female Reproductive Toxicity

## Organ weights- Uterus and Oviduct

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- Uterine weight fluctuates throughout the estrous cycle.
- **Increase in uterine weight**
  - Proestrus- max weight- distended with watery fluid- estrogen secretion.
- **Decrease in uterine weight**
  - Compounds that inhibit steroidogenesis and cyclicity – small and atrophic uterus.
- Estrogen agonists- increase uterine weight
- Estrogen antagonists- decrease uterine weight
- Oviduct - not routinely weighed
- Oviduct weight fluctuates with estrous cycle.
- Contains fluid during metestrous and during period of ovulation.



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**The uterus is under the control of estrogens to stimulate and to maintain growth**

# Evaluation of Female Reproductive Toxicity-

## Organ weights- Pituitary

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- Pituitary weight increase with age.
- Increase in pituitary weight:
  - Induced by estrogenic compounds.
- Decrease in pituitary weight:
  - Decreased estrogenic stimulation
- Pituitary contains other cell types- histology with special stains to confirm the changes associated with reproductive functions .

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**The Pituitary gland weight can provide information into the reproductive status of the female**

# Evaluation of Female Reproductive Toxicity

## Tissue sampling and trimming

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- **Guides and published literature:**

- Registry Nomenclature Information System (RENI)

- <http://www.goreni.org/> or <http://reni.item.fraunhofer.de/reni/trimming/index.php>

- **Publications:**

- *Ruehl-Fehlert C, et al. (2003) Revised guides for organ sampling and trimming in rats and mice - Part 1. Exp Toxicol Pathol 55: 91–106*

- *Kittel B, et al. (2004) Revised guides for organ sampling and trimming in rats and mice - Part 2. Exp Toxicol Pathol 55: 413–431*

- *Morawietz G, et al. (2004) Revised guides for organ sampling and trimming in rats and mice - Part 3. Exp Toxicol Pathol 55: 433–449.*

- [http://www.epa.gov/endo/pubs/edmvac/uterotrophic\\_oecd\\_rodent\\_validation\\_p1\\_3\\_22\\_2001.pdf](http://www.epa.gov/endo/pubs/edmvac/uterotrophic_oecd_rodent_validation_p1_3_22_2001.pdf)

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Fixation of tissues, sampling of specific structures and tissue orientation are important from histological evaluation

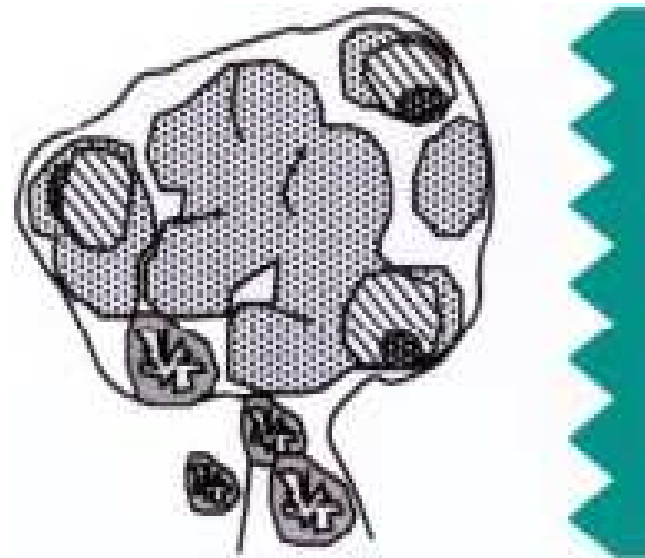
# Evaluation of Female Reproductive Toxicity

## Tissue Sampling and Trimming

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### Ovaries:

- **Ovaries if not weighed –**
  - Ovary processed along with the oviduct.
  - Ovaries - Longitudinal sections.
  - Oviduct - Transverse sections.
- **Ovaries if weighed:**
  - Ovaries and oviducts are separated at necropsy.
  - Oviduct taken with tip of uterine horn and embedded.
- **If Uterus is also weighed:**
  - Oviduct separated from ovaries and uterus and put into cassette with ovaries.



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For safety assessment of drug, detection of ovarian toxicity in preclinical toxicity is very important

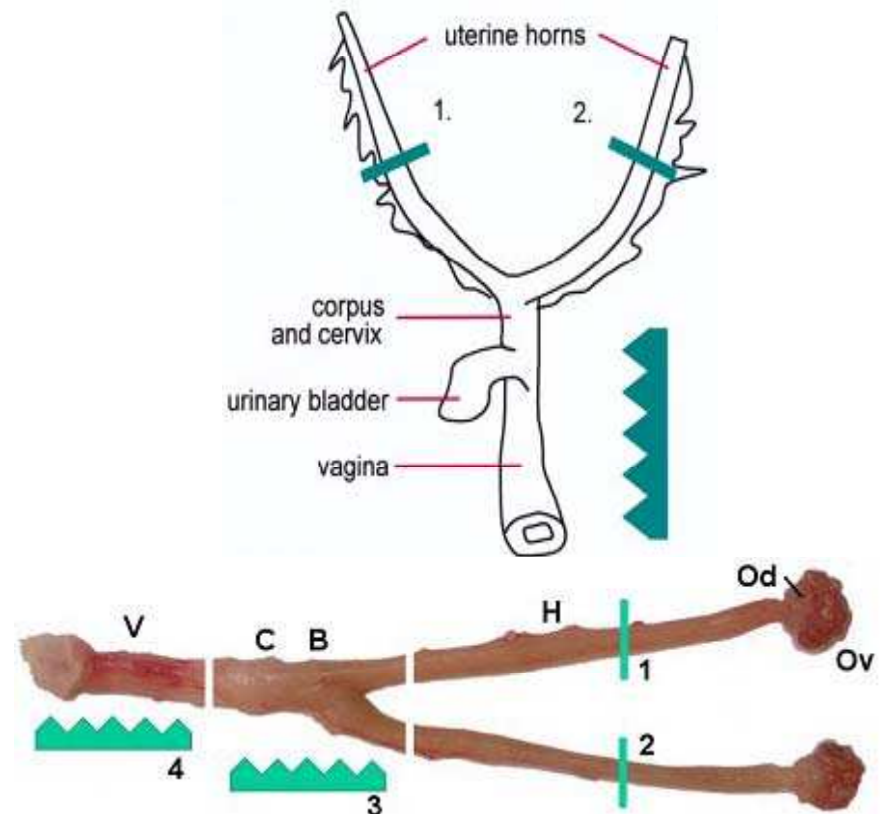
# Evaluation of Female Reproductive Toxicity

## Tissue Sampling and Trimming

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### Uterus, Cervix and Vagina:

- **Uterine horns:**
  - Transverse sections middle portion of both uterine horns.
- **Uterine body and cervix:**
  - Horizontal section made through uterine body, cervix and vagina.
- **If Uterus is weighed:**
  - Uterine cervix and vagina are two separate specimen and are cut longitudinally.



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These sections cover the relevant anatomical and functional structures of female reproductive organs

# Evaluation of Female Reproductive Toxicity

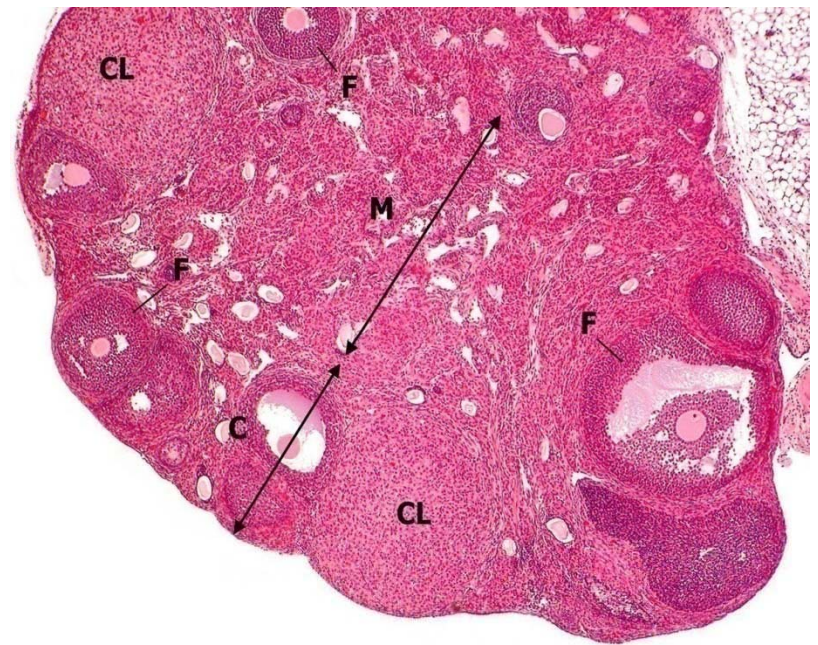
## Microscopic Evaluation

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- The alterations caused by the chemicals in female reproductive tract, can be identified by histological changes in ovaries, uterus and vagina.

### Ovaries:

- STP recommends 2-tier approach for evaluation of rodent ovary
- **First tier / Qualitative assessment:**
- Evaluation of follicles, CL, stroma, interstitium and vasculature.
- Special attention to primary and primordial follicles.
- In conjunction with other data (BW, organ wt, cyclicity and histology of other reproductive organs).



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The ovary serves a number of functions that are critical to reproductive activity

# Evaluation of Female Reproductive Toxicity

## Microscopic Evaluation

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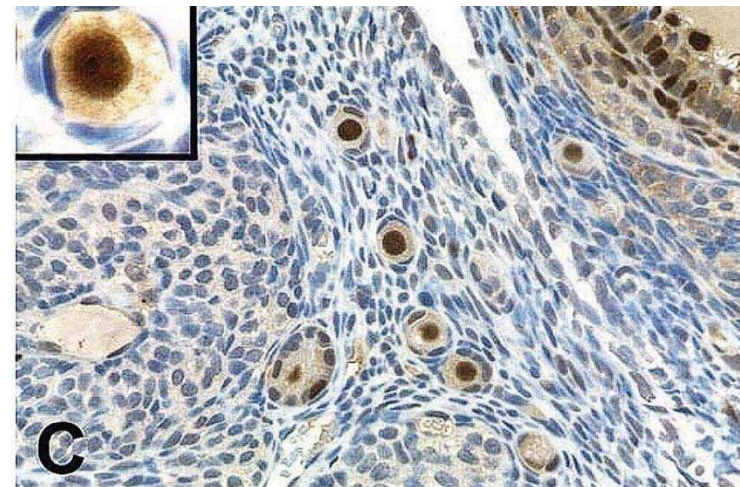
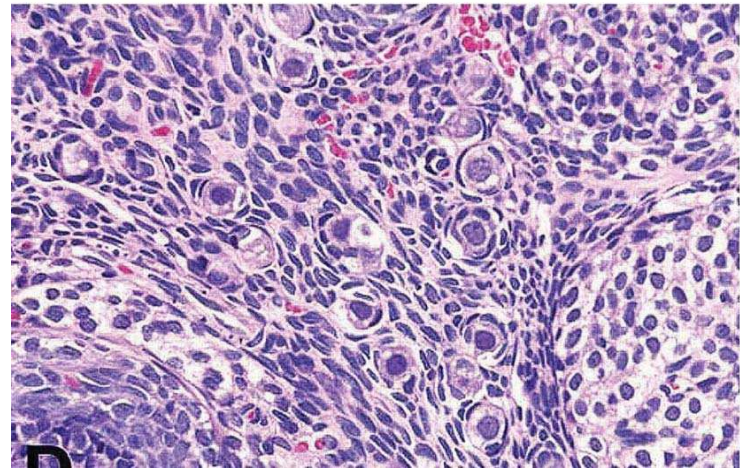
### Ovaries :

- **Second tier / Quantitative assessment:**
- Regulatory guidelines - do not require ovarian follicle counting in general toxicity studies.
- Quantification of primary and premordial follicles
- Immuno-histochemical methods - PCNA human CYP1B1 enhance visibility.
- Further characterize ovarian toxicants and understand their mechanism of action.

*Oocyte staging will be covered by other speakers.*

*Regan KS, et al. (2005). Toxicol Pathol 33: 409–412.*

*Picut CA, et al. (2008). Toxicol Pathol 36: 674–679.*



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**Ovarian follicle count appears to be more sensitive endpoint than ovarian weight**

# Morphological Patterns of Female Reproductive Toxicity

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- Disruption of hormonal imbalance will dictate morphological features of lesions.
- Yuan (1998) – Classification – morphological response – combined histological appearance of ovary, uterus and vagina.
- 3 types of morphological response:
  - Type I – Atrophic ovary, uterus and vagina
  - Type II - Atrophic ovary, hypertrophic / hyperplastic uterus and vagina
  - Type III - hypertrophic / hyperplastic ovary, uterus and vagina

*Yuan Y (1998). Fundamentals of Toxicologic Pathology, Academic Press, 485-514.*

*OECD (2008). Guidance Document. Part3: Section 5.*

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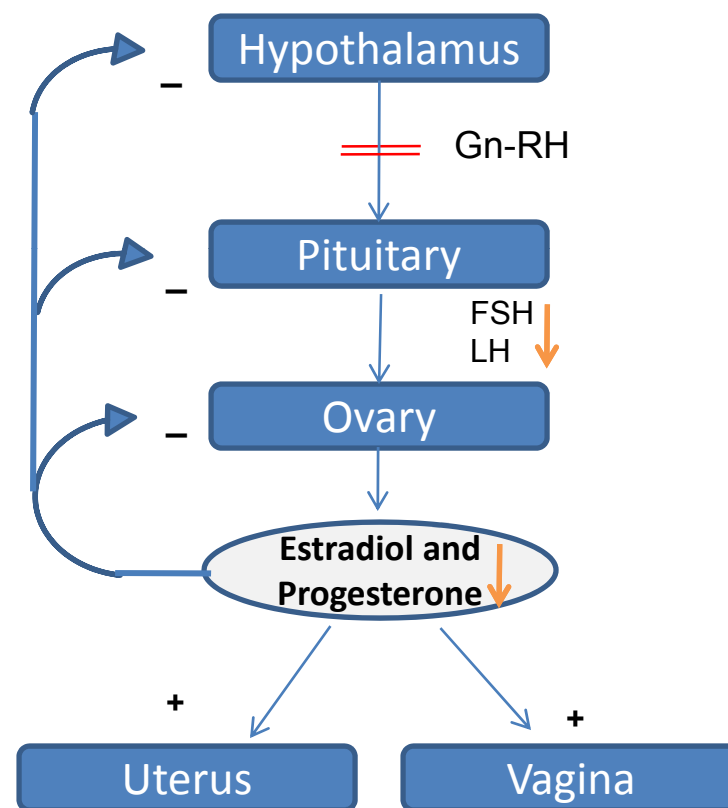
**Endocrine disruptors cause overt histological changes in vagina, uterus & ovary that are easy to identify.**



# Morphological Patterns of Female Reproductive Toxicity

## Type I pattern – Atrophic ovary, uterus and vagina

- **Reduce gonadotrophin secretion:**
  - Decreased Gn-RH, LH and FSH  
Ex: Stress, reduced FI, BW loss, Morphine, opiates, gasoline byproduct, heavy metals.
- **Impaired follicular development:**
  - Direct toxicity to oocyte  
Ex: radiation, cytotoxic agents as cyclophosphamide, cadmium
  - Quantitative follicular assessment.
- **Decreased steroidogenesis in ovary:**
  - Inhibition of estrogen production
    - ✓ Reduced precursor (Cholesterol)
    - ✓ Aromatase inhibition (Ex- DEHP)
    - ✓ Inhibition of steroidogenic dehydrogenases (Spironolactone)

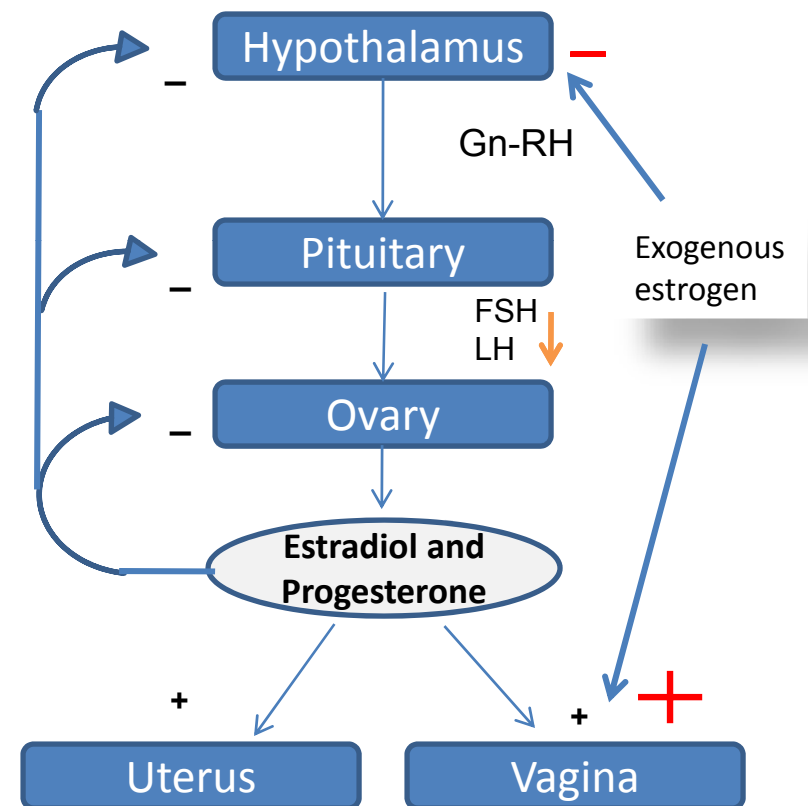


The atrophy of uterus and vagina are secondary to reduced ovarian steroid hormones.

# Morphological Patterns of Female Reproductive Toxicity

## Type II pattern– Atrophic ovary, Hypertrophic uterus and vagina

- **Estrogenic effects:**
  - Uterus - cystic endometrial hyperplasia, squamous metaplasia, neutrophilic infiltrate.
  - Vagina - Squamous hyperplasia, persistent estrous.
- **Combined effects of estrogen /progesterone:**
  - Uterus- cystic endometrial hyperplasia, inflammation.
  - Vagina – mucinous hyperplasia.



Type II response is seen when circulating levels of endogenous/ synthetic sex steroids are increased.

# Morphological Patterns of Female Reproductive Toxicity

Species specific effects of estrogen agonist on reproductive tissues

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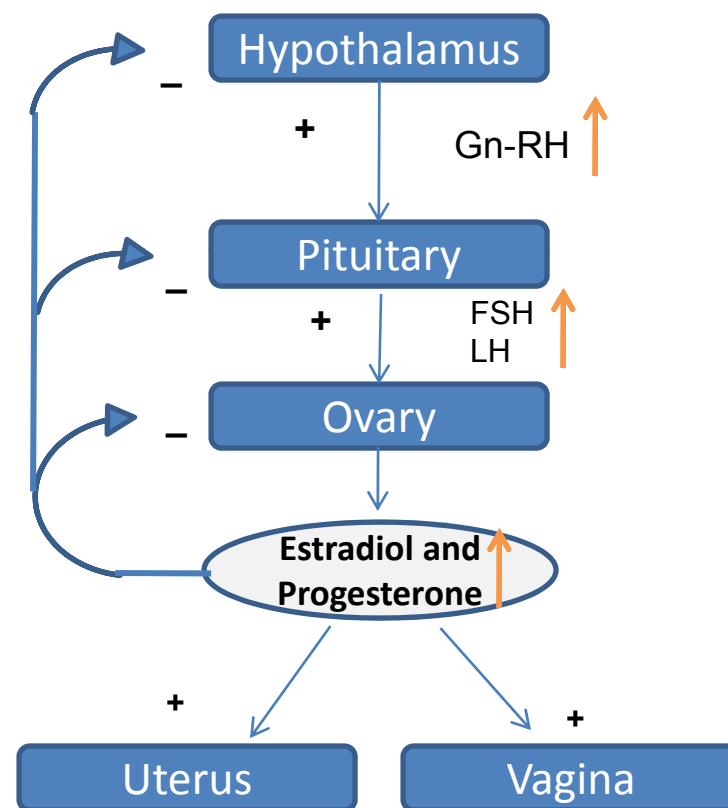
|         | Ovary   | Uterus             | Vagina             |
|---------|---------|--------------------|--------------------|
| Mouse   | Atrophy | <b>Hypertrophy</b> | <b>Hypertrophy</b> |
| Rat     | Atrophy | Atrophy            | <b>Hypertrophy</b> |
| Primate | Atrophy | <b>Hypertrophy</b> | Atrophy            |

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# Morphological Patterns of Female Reproductive Toxicity

## Type III pattern– Hypertrophic ovary, uterus and vagina

- **Increased gonadotrophin or Prolactin:**
  - Continuous follicle maturation and CL formation
  - Uterus and Vagina – hypertrophy and hyperplasia.
- **Effects depend on predominance of hormone (FSH/ LH/ Prl)**
  - High FSH and LH – hypertrophy and hyperplasia of uterus, vagina, continuous development of follicles and CL.
  - High prolactine – Persistent CL and mammary gland hyperplasia.



The compounds with gonadotropic activity trigger this type of response.

- We have seen;
    - **Potential mechanisms of female reproductive toxicity**
    - **Various parameters used in evaluation of Female reproductive toxicity and**
    - **Morphological patterns of toxicity to female reproductive system**
-

# Is it possible to detect the female reproductive toxicity in general repeat dose studies?

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- Collaborative work - National Institute of Health Sciences (NIHS) and Japan Pharmaceutical Manufacturers Association (JPMA) (2009).
- Whether ovarian toxicities can be detected by 2- or 4- week repeated dose general toxicity studies in rats.
- 18 companies participated – conducted female fertility in comparison with 2 and 4- week repeat dose toxicity studies.
- Parameters evaluated;
  - During study: Clinical signs, Estrous Cycle, Body Weights
  - Pathology: Organ Weights (vagina and uterus), histopathology of ovaries, uterus, vagina and mammary gland.
  - Immunohistochemistry – stained with PCNA to identify primordial or primary follicles.

*Sanbuissho A, et al. (2009) Collaborative work on evaluation of ovarian toxicity by repeated-dose and fertility studies in female rats. J Toxicol Sci 34:SP1-SP22.*

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**The reproductive toxicants produce their effects in variety of ways and at multiple sites**

# Observations of Validation Study

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| Sr. No | Category                            | Pathological observations  | Examples   |
|--------|-------------------------------------|--|--|
| 1      | Hormone analogues                   | Increased no. of large atretic follicles, absence/ decreased no. of CL, enlarged previous CL                     | Mifepristone, Tamoxifen                                      |
| 2      | Primordial follicle damaging agents | increase in no of atretic follicles, Decrease no. of follicles, atrophy of CL                                    | Busulfan, cisplatin, cyclophosphamide                        |
| 3      | Metabolite imbalance inducers       | Increased no. of large atretic follicles, follicular cysts, decreased no. of CL, interstitial gland hyperplasia. | Anastrozole, Indomethacin, PPAR $\alpha/\gamma$ dual agonist |
| 4      | Endocrine imbalance inducers        | Increased no. of large atretic follicles, increased no. of CL, enlarged previous CL                              | Atrazine, Bromocriptine, Sulpride                            |

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**The reproductive toxicants produce their effects in variety of ways and at multiple sites**

# Observations of Validation Study

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- *Common findings and significance.*
- Increased number of large sized atretic follicles - indicate disturbance of ovulation and large follicle development.
- Follicular cysts- metabolite imbalance inducers and endocrine imbalance inducers .
- Decreased no of follicles- compounds damaging small follicles.
- Non-ovulated follicles, luteal cysts, unruptured follicles- hormonal analogues and metabolite imbalance inducers.

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**Ovarian toxicity could be detected by careful qualitative histopathological examination conducted in 2- or 4-week repeated dose toxicity studies**



# Recommendations

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- Use of sexually mature animals .
- Following technical information of tissue sampling, fixation, trimming and staining.
- Use of organ weight and clinical pathology estimations including hormonal analysis.
- Considering the estrous cyclicity and oocyte staging during microscopic examination.

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**The only assessment of female reproductive organs performed prior to FIM is examination of sex organs in repeated dose toxicity studies**

# Concluding Remarks

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- Toxic injury to the female reproductive system may be due to either direct or indirect effects of the toxicants.
  - Consideration of estrous cyclicity and oocyte staging during microscopic examination and
  - Meticulous use of different methods of pathology can generate sufficient information for evaluation of reproductive system during repeated dose toxicity.
-

*Thank you*