PATHOLOGY ENDPOINTS: EVALUATION OF THYROTOXICANTS IN ANIMAL STUDIES

HESI Thyroid Workshop. May 9-10, 2019. Washington, DC
ENDOCRINE SYSTEM

Organs with endocrine function

- Hypothalamus
- Pituitary gland
- Thyroid gland
- Adrenal glands
- Heart
- Thymus
- Adipose tissue
- Pancreas
- Pineal gland
- Parathyroid glands
- Gonads
- Digestive tract
- Kidneys

https://www.emedicinehealth.com/anatomy_of_the_endocrine_system/article_em.htm#hypothalamus
HYPOTHALAMIC-PITUITARY-THYROID (HPT) AXIS

Thyroid hormones = $T_4$ and $T_3$

https://slideplayer.com/slide/9234885/
THYROID HORMONE FUNCTION

↑ CHO utilization
↑ Protein catabolism
↑ Nitrogen excretion
↑ Fat oxidation

https://www.researchgate.net/figure/Specific-functions-of-thyroid-hormones-on-target-organs_fig3_330902320
• Half-life of $T_4$ in rats is 0.5-1 day
• Rat maintains relatively high TSH levels which gives the thyroid gland a slightly more cellular appearance
(HISTO)PATHOLOGIC ENDPOINTS OF THYROTOXICITY IN ADULTS

Metabolic
- ↓ Basal metabolic rate
  • ↑ Body weight w/o increased appetite
  • ↓ lipid metabolism

Cardiovascular
- ↓ Heart rate
- Atherosclerosis

Internal organs
- Liver: lipid deposition and hepatomegaly
- Kidney: lipid accumulation in glomeruli

Reproduction
- ↓ libido
- ↓ sperm count
- Abnormal/absence of estrous cycles

Thyroid gland (rats)
- Decreased follicular size
- Decreased colloid
- Increased epithelial cell height
- Degeneration/vacuolation of epithelium

Elevated blood cholesterol, decreased cholesterol excretion (clinical pathology)
Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption\textsuperscript{\#}

M.E. Gill\textsuperscript{b,c}, J.H. Goodman\textsuperscript{b,c}, J. Gomez\textsuperscript{b}, A.F.M. Johnstone\textsuperscript{a}, R.L. Ramos\textsuperscript{d}

- Brain and hippocampus weights ↓ in 10 ppm group as well
- Study demonstrated a method of quantitative assessment of neuronal development in rats

\textsuperscript{*} Gilbert et al., 2017
HISTOPATHOLOGIC ENDPOINTS
Gilbert et al., 2017

- Neurogenesis in dentate gyrus continues throughout life
- Severe hypothyroidism in adults ↓ new neuron survival

**B** Experiment 2: Adult Neurogenesis

- 0 ppm PTU GD6-PN21
- 3 ppm PTU GD6-PN21
- 0 ppm PN21-PN60
- 3 ppm PTU PN60-93/120 – PTU-CON
- 0 ppm PN60-93/120 – PTU-CON
- 3 ppm PTU PN60-93/120 – PTU-PTU

**Legend:**
- PN4 Cell Litter
- PN24 Sacrifice: Pup-serum
- PN21 Sacrifice: Dam-serum
- PN60 PTU Re-Exposure
- BRDU PN8-92
- Mean PN21
- Untreated (normal) rat
- Rat: ↓ T₄ during fetal-early PND
- Rat with adult onset ↓ T₄
- Rat: ↓ T₄ during fetal-early PND + adult

BrdU labeling for cell proliferation

- Delay in neurologic lesions in adults after mild hypothyroidism in late gestational/early neonatal rats
Developmental Thyroid Hormone Insufficiency Induces a Cortical Brain Malformation and Learning Impairments: A Cross-Fostering Study
Katherine L. O'Shaughnessy,† Patricia A. Kosian,Jermaine L. Ford,W Wendy M. Oshiro, Sigmund J. Degitz, and Mary E. Gilbert

• PTU exposure resulted in permanent cortical heterotopia in rats
• Lesion is bilateral to the corpus callosum
• Composed of glutamatergic and GABAergic neurons
• Heterotopic neurons connected structurally and functionally to cortical neurons
• Rats also exhibit increased seizure sensitivity along with learning and memory deficits
HISTOPATHOLOGIC ENDPOINTS

Developmental delay and unstable state of the testes in the rdw rat with congenital hypothyroidism

Yasuhiro Sakai, Shohei Yamashina and Sen-ichi Furudate

1Department of Anatomy and 2Department of Laboratory Animal Science, Kitasato University School of Medicine, Sagamihara 228-8555, Japan

- RDW rat: decreased serum T4
- Delay in full development of the normal structures
- After full development, degeneration of testes began
- Partially reversed by T4 treatment
- These findings, along with other studies, suggests thyroid hormones have a role in both development and maintenance of testicular function

A-D: control rat at 2, 4, and 8 weeks
E-H: rdw (hypothyroid) rat at 4, 8, 22 weeks
Upregulation of CYP enzymes in the liver
- Xenobiotic administration
  - Phenobarbital
  - Rifampicin
- Pesticide exposure
- Increased expression of sulfo- and glucuronyl-transferases in liver

Increased metabolism of $T_4$ (and $T_3$)
Decreased $T_4$ levels stimulates TSH production/release

Consequences
- Thyroid gland hypertrophy + ↑ thyroid weight (+/-)
  - Follicular hyperplasia (early)
  - Follicular neoplasia (late)
- Thyroid hyperplasia/neoplasia
- Liver hypertrophy
- Pituitary hypertrophy
HISTOPATHOLOGIC LESIONS IN THYROTOXICOSIS

Neurologic
- Altered neurologic development
  - Decreased cell numbers
  - Decreased dendritic arborization
  - Decreased axonal myelination
  - Decreased synaptogenesis
- Decreased hippocampal neurogenesis in adult
  - Decreased granule cell layer area
  - Decreased granule cell layer volume
- Increased apoptosis of cerebellar granular neurons

Reproductive
- Altered spermatogenesis

Inhibit development of the hypothalamo-pituitary-adrenal axis in the rat*

Thyroidectomy in utero on sheep fetus
- Smaller type II muscle fibers
- Delayed epiphyseal closure
- Delayed bone maturation
- Thin skin, abnormal or no wool (follicle) development
- Fewer type II pneumocytes
- Abnormal neonatal cardiovascular adaptations

Forhead and Fowden, 2014

Inhibit growth of the kidney, liver, and diaphragm*
**THYROID GLAND/HORMONE DEVELOPMENT**

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Rat (days)</th>
<th>Human (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at term</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>Colloid production</td>
<td>17</td>
<td>13-14</td>
</tr>
<tr>
<td>Follicular cell organogenesis</td>
<td>18 days-3 weeks postnatally</td>
<td>&gt;14</td>
</tr>
<tr>
<td>Synthesis/ssecretion hormones</td>
<td>17.5</td>
<td>16-18</td>
</tr>
<tr>
<td>Rise in plasma $T_3$</td>
<td>Birth to 3 weeks postnatally</td>
<td>30 weeks to birth</td>
</tr>
<tr>
<td>H-T-P maturation</td>
<td>Postnatally</td>
<td>Last trimester+postnatally</td>
</tr>
</tbody>
</table>

- Thyroid gland is histologically mature by PND 21 in the rat
- Thyroid gland and HTP axis not fully developed until PND 28 in the rat

Modified from Forhead and Fowden, 2014
THYROID GLAND HISTOLOGY

- Follicular epithelial cells
- Follicles-colloid
- C-cells
- Vasculature
- Stroma
- Capsule
- Parathyroid (when present)
- Ectopic tissue

Photomicrograph courtesy of Cathy Picut

Hatakeyama et al 2011
THYROID-RELATED END POINTS OF THE EPA PUBERTAL ASSAYS

Guidance for Thyroid Assays in Pregnant Animals, Fetuses and Postnatal Animals, and Adult Animals (2005)

• Maternal, fetal (GD 20), and F1 offspring (PNS 4 and 21) thyroids should be collected...for pathologic analyses

• The thyroids/parathyroids, attached to a section of the trachea, of all maternal, fetal, and offspring samples should be excised and immersion fixed immediately after collection in 10% neutral buffered formalin or other appropriate fixative. Following fixation, the thyroid/parathyroid tissue samples should be carefully trimmed and weighed.

• Hormonal analyses should be conducted on GD 20 fetuses and dams, PND 4 pups, and PND 21 pups and dams.

Thyroid gland weights

• 2-4 week exposure required to see weight changes

• Male assay (4~4.5 weeks) more sensitive than female assay

• Technical considerations when collecting weighing thyroid gland
  • Need entire gland/remove extraneous tissues
  • Post fixation weighing recommended

Hormone evaluation (T4, T3, TSH)

• T4 measurements complicated by circadian rhythms, short half-life, inter-animal variability, body weight

• Advances in assay technology allow for quantifying T3 and T4 in GD20 and PND 4 rats
THYROID-RELATED END POINTS OF THE EPA PUBERTAL ASSAYS

**Histologic evaluation**
- Most reliable indicator of thyrotoxicosis
- Interpretation is subjective
- Follicular epithelial cell height males > females
- Variation in cell height and colloid area in different regions
- ↓ follicular colloid can occur within days

**EPA Endocrine Disruptor Screening Program Test Guidelines:**

**Examination of colloid area and follicular cell height**

**Colloid content**
- C1 to C5 (5 point scale) with C5=normal*

**Follicular epithelial cell height**
- F1 to F5 (5 point scale) with F1=normal*

**Generally colloid content decreases while follicular cell height increases**
- Follows a pattern
  - F1C5 (normal) → F2C4 → F3C3 → F4C2 → F5C1
  - Not set in stone
Control adult rat: Grade 2F 4C

Photomicrograph courtesy of Cathy Picut
PTU treated adult rat: Grade 3F 3C

Photomicrograph courtesy of Cathy Picut
PTU treated adult rat: Grade 4F 2C

Photomicrograph courtesy of Cathy Picut
PRENATAL AND EARLY POSTNATAL HISTOLOGY OF THE THYROID GLAND IN THE RAT

Picut et al., 2017
Female Sprague Dawley rats administered propylthiouracil (6-PTU) in the diet at a concentration of 0 and 3 parts per million (ppm) daily from Gestation Day (GD) 6 through Postnatal Day (PND) 21

Microscopic examination of the thyroid gland was performed on GD 20 fetuses, PND4 pups, PND 21 pups, and adult females

Thyroid hormone levels and thyroid weights also measured
GD 20 MALE FETUS

Control

PTU
PND 4 MALE

Control

PTU
PND 4 FEMALE

Control

PTU
## RESULTS

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Histopathology</th>
<th>Organ weight</th>
<th>Hormone levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD 20 Fetuses</td>
<td>No follicular lumen or No colloid formation Follicular cell hypertrophy</td>
<td>↑ thyroid weight</td>
<td>↓ total T₄ ↓ total T₃ ↑ TSH</td>
</tr>
<tr>
<td>PND 4 Pups</td>
<td>↑ follicular height ↓ colloid area</td>
<td>↑ thyroid weight</td>
<td>↓ total T₄ ↑ TSH</td>
</tr>
<tr>
<td>PND 21 Pups</td>
<td>↑ follicular height ↓ colloid area</td>
<td>↑ thyroid weight</td>
<td>↓ total T₄ ↑ TSH</td>
</tr>
<tr>
<td>GD 20 Dams</td>
<td>↑ follicular height ↓ colloid area</td>
<td>↑ thyroid weight</td>
<td>↓ total T₄ ↑ TSH</td>
</tr>
<tr>
<td>LD 21 Dams</td>
<td>↑ follicular height ↓ colloid area</td>
<td>↑ thyroid weight</td>
<td>No change</td>
</tr>
</tbody>
</table>

- EPA guidelines applied to PND 4 & 21 pups and to Dams
- Establish the criteria for each grading scheme in each cohort
- For GD 20 fetuses, there were limitations on applying EPA guidelines for histopathology as written
- Modified grading scheme accurately identified PTU-related histopathologic lesions
DIGITAL SLIDE SCANNING AND ANALYSIS

Leicabiosystems.com
WHOLE TISSUE ANALYSIS

GD 20

PND 30

PND 3-4

Picut et al., 2017

Zabka et al., 2011
INDIVIDUAL CELL ANALYSIS
Deep Learning Workflow Example

Visiopharm Artificial Intelligence

Slide courtesy of Dan Rudmann
Objective of Collaboration:

1. Develop code that advances QuPath capability to measure colloid area and follicular cell size in the thyroid
2. Build partnership for long term machine learning approach for specific diagnostic endpoints common for toxicologic pathologists
CONCLUSIONS

• Disruption of the HPT axis can affect numerous physiologic systems in the fetus, adolescent, and adult animals which can result in adverse and often permanent outcomes and which produce corresponding histopathologic lesions

• With the exception of the brain and, to a lesser extent, the reproductive system, very little published research has included histopathologic endpoints in the results

• DNT studies with earlier time points including histopathology would help characterize early hypothyroid-related lesions

• ...not to mention all of the other organ systems...

• Histopathologic guidelines established by the EPA for the evaluation of thyroid function in rats to determine if a pesticide, chemical, or other substance may pose a risk to humans due to disruption of the endocrine system are sensitive and repeatable. Additionally, they can be applied to immature rat pups and rat fetuses as early as gestational day 20.

• Digital imaging and image analysis technology has the potential to enhance histopathologic evaluation of the thyroid gland
THANK YOU

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