Identifying and Justifying Stress in Preclinical Toxicity Studies

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Overview

- What do we mean by stress and what causes a “stress response”
- Common stress related changes in endocrine organs, reproductive organs, lymphoid organs, clinical pathology
- Problems distinguishing stress from immunotoxicity in lymphoid tissues
- Identifying and justifying stress related changes: case studies
What is Stress?

Definition

- Physiological responses to alterations of homeostasis that are the result of actual or perceived stimuli
- Physical, chemical or emotional
- Good (eustress) and bad (distress) stress

Stress is generally unavoidable in toxicity studies due to experimental design (MTD)

Variable responses to stressors and duration of stress

- Acute versus chronic stressors (adaptation)
- Type of stressor (food reduction vs restraint vs social)
Acute versus chronic stress

- **Acute stress**
  - Single blood sample or dosing procedure or 2hr restraint
  - Results in “classic” primary, neural and endocrine stress response
  - Most stress research uses acute stressors

- **Chronic intermittent or chronic sustained stress**
  - Repetitive procedures, long term restraint, social pressures, chronic disease/morbidity
  - Results in habituation/adaptation of the acute response
  - Hormonal response is often the opposite to that seen with acute stress (e.g. corticosterone or LH release)
  - Stress response in toxicity studies is generally “chronic “
Primary Response to Stress

Neural response
- mainly sympathetic pathway activation in brain and adrenal medulla (catecholamine-mediated)

Endocrine response
- mainly activation of the HPA axis (glucocorticoid-mediated)
Response is influenced by sex, species as well as the severity of the stress.
Responses To Chronic Stress: Endocrine Organs

- **Adrenal**: increased weight, decreased lipid vacuoles, hypertrophy and hyperplasia of ZF and ZR. Also medullary hypertrophy, hyperplasia and increased pheochromocytomas (rats). Chronic stressed rats may have normal basal ACTH and cortisol levels but increased response to CRH/ACTH stimulation.

- **Pituitary**: Increased weight, cellular hypertrophy and hyperplasia (corticotrophs).

- **Thyroid**: decreased T3 and T4 with no change in TSH. No detectable morphologic changes.
Responses To Chronic Stress: Male Reproductive Organs

- Chronic activation of HPA is associated with inhibition of HPG axis.
- Decreased GnRH (hypothalamus), decreased LH, FSH (pituitary), decreased steroidogenesis (gonads)
- Reduced weight of prostate and seminal vesicles (with no morphological change)
- No effects on testes or sperm parameters (except in mice).
Responses To Chronic Stress: Female Reproductive Organs

- Decreased GnRH (hypothalamus), decreased LH, FSH (pituitary), decreased steroidogenesis (gonads)
- Disruption of estrous cyclicity (prolonged diestrous/anestrous)
- Reduced weight of ovaries and uterus
- Generalized “atrophic changes” in female reproductive tissues
  - Reduced CLs, increased follicular atresia
  - Diestrous uterus
  - Vaginal atrophy/mucification
Stress can affect ovulation

Normal mouse

Nutrient stressed
Disrupted estrous cycle: detectable by vaginal morphology

Normal proestrous

Abnormal mucification
Responses To Chronic Stress: Clinical Pathology

- Leukocyte parameters are most sensitive.
  - Decreased lymphocytes and eosinophils
  - Increased neutrophils
- Effects may also be seen on red blood cell parameters
  - Decreased red blood cell mass (count, Hb conc, hematocrit) with decreased or unchanged reticulocytes.
  - Bone marrow hypocellularity
- Parameters with increased but variable responses
  - Acute phase proteins, platelets, serum enzymes (LD, CK, AST, ALT), serum constituents (glucose, urea, cholesterol)
Responses To Chronic Stress: Lymphoid organs

- Thymus, spleen and bone marrow are the most sensitive of the lymphoid tissues
- Lymphoid depletion is due to effects on cell trafficking/ redistribution (adrenergic), and effects on cell survival (glucocorticoid). Adaptation occurs.
  - Thymus: cortical decreased lymphocytes/ apoptosis and decreased weight
  - Spleen: decreased/apoptotic B lymphocytes (marginal zone and periarteriolar sheaths). Decreased spleen weight (acute stress), maybe increased spleen weight (chronic stress) due to increased macrophages and adaptation
  - Bone marrow: decreased lymphocytes, increased granulocytes
Lymphocyte apoptosis in the thymus (monkey)
Lymphoid depletion of splenic marginal zone: rat

Normal marginal zone

Marginal zone lymphoid depletion
Stress vs. Immunotoxicity

- The International Committee of Harmonization (ICH) guidelines stipulate that if alterations of the immune system are thought to be attributable to stress, evidence to support this interpretation must be provided.

- ICH S8 Immunotoxicity Testing Guidelines state that evidence of immunotoxicity in nonclinical safety assessments may dictate the requirement for additional evaluations of immunotoxicity.
Problems for Identifying Stress Effects on the Immune System

- Morphology of the lymphoid organs is very variable under normal physiologic conditions.
- Additional variation due to age and health status as well as tissue sampling makes differentiation between direct (immunotoxic) and indirect (including stress related) effects challenging.
- Thymic involution begins at sexual maturity and progresses to total atrophy: cortical depletion and increased apoptosis. Age, species, sex and strain dependent.
- In general mild stressors stimulate the immune system while severe stressors suppress immune function.
- Often no correlation between morphologic appearance and organ weight or cell number (on an individual animal basis).
Physiologic variability in lymph node size/cellularity in control rats

Mesenteric

Mediastinal
Thymic involution

Control monkey

Control monkey
Spleen: lymphoid follicular and germinal center variability

Control monkey

Control monkey
Identifying stress in routine preclinical safety studies

- Hallmarks
  - Adverse clinical signs and morbidity
  - Decreased body weights
  - Decreased food consumption
  - Decreased thymic weights
  - Lymphoid depletion/apoptosis in the thymus and spleen
  - Increased adrenal gland weights, cortical hypertrophy
  - Decreased circulating lymphocytes and eosinophils
  - Increased circulating neutrophils
  - Decreased weight of prostate and seminal vesicles
Justifying stress mediated responses

- Weight of evidence approach: look at all the parameters that would point to a stress response, including clinical data (body weight, food intake, clinical signs)
- If it all correlates ..fine, more often it does not, then what?
- Look at the pharmacology/target receptors of the molecule….is there any indication that the pathology seen could be attributable to the therapeutic target or to that class of compound?
- Look at previous studies and look at subsequent studies for consistency of the effect.
- Consider doing hormonal assessment for stress hormones (cortisol/corticosterone and ACTH, including ACTH response test)
Case Study 1

3 month oral (capsule) study in mature male dogs with an androgenic steroid
Histopathology

- Thymus
  - Lymphoid depletion (all doses, flat dose response)
- Spleen
  - Increased size/number germinal centers (mid and high dose)
- Reproductive organs
  - Testicular degeneration/atrophy and oligospermia (inverse dose response)
  - Prostatic hypertrophy/hyperplasia (dose related)
- Adrenal
  - Cortical thinning, mostly ZR, (flat dose response)
Thymic lymphoid depletion

- Increased severity of lymphoid depletion all doses
- Decreased thymic weight all dose levels
**Spleen: Increased size/number germinal centers**

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>cont</th>
<th>low</th>
<th>mid</th>
<th>high</th>
</tr>
</thead>
<tbody>
<tr>
<td># animals examined</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Spleen: Germina center size/cellularity**

<table>
<thead>
<tr>
<th>Grade</th>
<th>cont</th>
<th>low</th>
<th>mid</th>
<th>high</th>
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</thead>
<tbody>
<tr>
<td>Grade 1</td>
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<td>2</td>
<td>1</td>
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<tr>
<td>Grade 2</td>
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<tr>
<td>Total</td>
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</tbody>
</table>

**Control**

**High dose (grade 2)**
Testis: tubular degeneration atrophy

- Grade 5 germ cell loss in low dose
- Grade 2-3 in high and mid dose
- Correlated with organ weight loss
Adrenal: cortical thinning

- Cortical thinning Grades 3-4 all dose levels
- Decreased adrenal weights
## ORGAN WEIGHTS

<table>
<thead>
<tr>
<th>Sex</th>
<th>Males</th>
<th>Dose group</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymic weight</td>
<td>-56%*</td>
<td>-41%*</td>
<td>-55%*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testes weight</td>
<td>-68%*</td>
<td>-56%*</td>
<td>-47%*</td>
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<tr>
<td>Prostate weight</td>
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<td>+4.5%*</td>
<td>+5%*</td>
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<tr>
<td>Adrenal weight</td>
<td>-42%*</td>
<td>-47%*</td>
<td>-47%*</td>
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</table>
Clinical Pathology

- Decreases in RBC, Hb, MCHC (slight)
- Increased neutrophils (+60%)
- No change in lymphocytes
- Effects in high dose only
Body weight and clinical signs

- No mortality
- Increased incidence of vomiting
- Body weight gain in treated animals
- Mild diarrhea in control and test article treated groups (due to vehicle)
- No other organ toxicity
<table>
<thead>
<tr>
<th>Changes</th>
<th>Possible Interpretations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymic atrophy</td>
<td>Stress related</td>
</tr>
<tr>
<td>Splenic increased germinal centers</td>
<td>Direct “toxicity”</td>
</tr>
<tr>
<td>Testicular degeneration/atrophy</td>
<td>Pharmacologically mediated</td>
</tr>
<tr>
<td>Adrenal atrophy</td>
<td>Within normal limits</td>
</tr>
</tbody>
</table>
Conclusions Case study 1

- Testicular degeneration and adrenal atrophy
  - Pharmacologically mediated, through negative feedback by the exogenous androgen on the HPG and HPA axis

- Thymic atrophy
  - Pharmacologically mediated effect of androgens on thymic T lymphocytes

- Splenic GC activation
  - May be a direct effect of the drug
Case Study 2

3 month oral gavage study in monkeys with an anti viral drug
Histopathology

- Thymic lymphoid hypocellularity
- Splenic germinal center lymphoid hypocellularity
Thymus: lymphoid hypocellularity

- Decreased lymphocytes
- Loss of corticomedullary distinction
- Correlated with decreased weight and decreased circulating lymphocytes
**Histopathology: Thymus**

<table>
<thead>
<tr>
<th>Dose Group</th>
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<th>high</th>
</tr>
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<tbody>
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<td># animals examined</td>
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<td>3</td>
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**Thymus: lymphoid hypocellularity**

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<th>Females</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>grade 1</td>
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**Organ weights**

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<td>low</td>
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<td>High</td>
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<tr>
<td></td>
<td>Thymic weight</td>
<td>-</td>
<td>-52%*</td>
<td>-73%*</td>
</tr>
</tbody>
</table>
Spleen: decreased size/cellularity germinal centers

- Increased incidence of spleens with small germinal centers
- No decrease in spleen weight
- Similar change seen in 28 day study with decrease in spleen weight
## Histopathology: Spleen

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<tr>
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### Spleen: GC lymphoid hypocellularity

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<td>2</td>
</tr>
</tbody>
</table>

| total | 2 | 3 | 2 | 3 | 2 | 3 | 3 | 3 |

No effect on spleen organ weight (but decrease seen at 28 days)
**Clinical pathology**

Decrees (minimal but stat signif) in RBC count and Hb
Sporadic decreases in neutrophil counts in low and high
dose group animals

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<tr>
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<td>Low</td>
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* indicates statistical significance.
Body weight and clinical signs

- No mortality
- No significant body weight loss
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Conclusion for Case Study 2

- Lymphoid depletion in the thymus and spleen and circulating lymphocytes were considered test article related because:
  - No significant clinical signs or body weight loss
  - No increase in adrenal weight
  - No consistent correlative evidence of stress in hematological parameters

- Functional assays of immune parameters initiated
Summary

- The response to stress varies with the type and duration of the stressor, as well as with the sex and species under study.
- Most stress in toxicity studies is chronic stress, resulting in adaptation of HPA hormones.
  - No easy way of testing for a stress response.
- Attributing changes to stress depends on a weight of evidence approach.
  - How many “hallmarks of stress” are present?
  - Correlation of hallmarks of stress with dose response of “questionable” effects.
  - Pharmacology/therapeutic class of the test compound.
  - Putting all the pieces of the puzzle together.
Acknowledgements

Co-authors of the STP Draft Manuscript on Stress Responses in Toxicity Studies (coming soon!)

- Nancy Everds
- Tom Rosol
- Paul Snyder