Clinical Pathology Aspects of Stress

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Review Paper

- Interpreting Stress Responses during Routine Toxicology Studies: A Review of the Biology, Impact, and Assessment (*Toxicologic Pathology*, 2013)

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- Paul Snyder
- Keith Bailey
- Brad Bolon
- Dianne Creasy
- George Foley
- Thomas Rosol
- Teresa Sellers

www.toxpath.org
Why worry about stress?

- The 3Rs (reduce, refine, and replace)
- Kinder, gentler experiments
- Reduce erroneous data
  - Minimize impact of preanalytical influences
- Increase consistency in data
  - Potential for reduction in animal numbers
- Correctly attribute changes to stress vs. test article
Stress in routine preclinical safety studies

- **Hallmarks**
  - Decreased body weights or body wt gain
  - Decreased food consumption
  - Decreased thymic weights and/or cellularity
  - Increased adrenal gland weights
  - Increased neutrophil counts
  - Decreased lymphocyte and eosinophil counts

- **Additional findings**

- **Weight-of-evidence approach**
Literature Review for Rats: Sensitivity of Various Systems to Stress

- No consistency in sensitivity—depends on what is measured and type of stress
- Body weight effects fairly sensitive
- Usually effects on peripheral blood lymphocytes earlier than thymic lymphocytes
- Sensitivity of organ weights:
  - Thymus = adrenal
  - Thymus > adrenal
  - Adrenal > thymus
  - Thymus and spleen > lymph node
- Corticosterone sensitive for acute stress
- Histological changes attributable to stress among animals in the same group variable
Outline of Talk

- Introduction to Stressors and Evaluation of Stress
- Stress of Preanalytical Procedures
- Effect of Stress on Hematology Parameters
- Effect of Stress on Clinical Chemistry Parameters
Stress: Interrelated pathways

Stress literature does not separate excitement vs. glucocortic stress

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Nature Reviews Immunology 5 243-251
Experiments Evaluating Stress Responses

- Surgical or pharmacologic
  - Administration of stress-related hormones
  - Blocking glucocorticoid or adrenergic receptors
  - Adrenalectomy, etc.

- Stressors
  - Acute: minutes to hours
  - Chronic models: usually intermittent for days, not weeks/months

- Mostly “preanalytical”-type of effects
  - Informative, but not representative of effects observed on toxicity studies
Corticosterone can be measured in urine, feces, or serum/plasma

- Serum or plasma glucocorticoids
  - Measure in AM because response to stress or ACTH greatest at trough of CORT (7-11 am)

- Restraint and collection of blood is stressful
  - Dogma—decapitation or catheterization required for blood collection for stress hormone measurements
  - Current research—other methods are preferred if samples collected within ~3 minutes

Outline of Talk

- Introduction to Stressors and Evaluation of Stress
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Laboratory conditions affect stress levels

- Caging
- Fasting
- Time of day
- Repetition
Housing density and stress

- Environmental enrichment
  - Increased CORT, larger adrenals, but considered “good stress”
- Density: Living alone or crowded not good
- Social order: Being a bully or wimp is best

Serra et al (2005) Stress 8:259-64
Stress of Novel Housing

- Novel caging transiently increases CORT in rats

Morning vs. Evening Stress

- Stressors have most impact on CORT early in day (rats)
- Stress at beginning of light phase vs beginning of dark phase

Morning vs. Evening Stress and HPA Axis: Chronic Food Restriction in Rats

- Diet restriction of 60% of ad lib amount
- Restricted diet available in morning
  - Disrupts diurnal rhythm of ACTH and CORT
    - >10x increase in AM CORT
- Restricted diet available in evening
  - Maintenance of diurnal rhythm
    - Increased PM ACTH
    - Increased CORT in AM and PM
- Rodent diet restriction with food access in evening probably most physiological and relevant

Belda et al (2005) Pharmacol, Biochem and Behav 41-6
Stress of Repetitive Procedures

- Hyperresponsiveness may occur early (2-5 days)
  - Especially with more severe or unpredictable stressors

- Habituation generally occurs later
  - Usually after 5~10 days
  - More likely with predictable and/or controllable stressors

Kant et al (1992) Physiol Behav 51: 1285-8
Preanalytical procedures involved in blood collection increase CORT

- Transportation of animals
- Anesthetic used
- Frequency of blood collection
- Amount and speed of blood collected
- Handling and method of collection
Animal Transport: Effect of Cage Movement

Mice divided into 3 groups
- Control (stayed in animal room)
- Simulated stress (ACTH injection)
- Traveling mice (cage moved up and down elevator for 15 minutes)

Drozdowicz et al AJVR 51 1841(1990)
Animal Transport: Effect of Cage Movement

- Thymus weights (mg) after 12 minutes of cage movements and elevator rides

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Animal Transport: Effect of Cage Movement

Corticosterone

Time

Corticosterone (ng/mL)

0900 1400 1900 2400 2900 3400

Control
ACTH
Transport
Animal Transport: Effect of Cage Movement

Cage Transport

- ▲ Control-Cort
- ■ Transport-Cort
- ▲ Control-Lym
- □ Transport-Lym

Corticosterone (ng/mL) vs. Lymphocytes (x10^3/uL) vs. Hours after Transport
Stress of Anesthetics/Blood Collection in Rats

- **Anesthetics**
  - Isoflurane minimally stressful
  - CO₂ and pentobarbital more stressful

- **Blood collection**
  - Change in CORT depends on method and speed of collection
    - 10% of blood volume: minimal effects
    - 12-16% of blood volume: release of epinephrine, norepinephrine, ACTH, and CORT
    - Hyperresponsiveness during subsequent collections

Stress of Blood Collection in Canines

- Effect of venipuncture and location on laboratory canines acclimated to their handlers
  - Collected blood via indwelling catheter and venipuncture
  - Collected blood in kennel and treatment room
  - No effect on luteinizing hormone, testosterone, or cortisol

- Effect of walking to unfamiliar area outside of kennel
  - Approximately 3x increase in cortisol

Stress of Blood Collection in Non-human Primates

- Ways to decrease stress of blood collection
  - Training (positive reinforcement training)
  - Voluntary blood collection (lower glucose, total leukocytes, and neutrophils)
  - Pair-house NHPs (only slightly less stressed)
  - Minimize room disturbance (disturbance affects lymphocyte counts, but not ACTH or CORT concentrations)
  - Awareness of advanced cognition

J Applied Animal Welfare Science 6 (3) 2003
Outline of Talk

- Introduction
- Methods for Evaluation of Stress Response
- Stress of Preanalytical Procedures
- Effect of Stress on Hematology Parameters
- Effect of Stress on Clinical Chemistry Parameters
Stressors affect red cell parameters

- **Acute stressors: transiently increased red cell mass**
  - Epinephrine infusion, transportation, exercise, restraint
    - 5-10% increase in red cell mass
    - 2-6x increase in reticulocytes
  - **Mechanisms**
    - Splenic contraction
    - Release of reticulocytes from bone marrow

- **Chronic stressors: decreased red cell mass**
  - Severe dietary restriction
  - Severe traumatic injuries
  - Chronic diseases or chronic catheterization

Hematologic Effects of Severely Restricted Diet (Rats)

- Severity of effects proportional to degree of diet restriction
- Decreased peripheral blood cell counts and bone marrow cellularity
- Markedly decreased peripheral blood cell counts in rats fed 25% of ad lib (% of ad lib group)
  - Reticulocytes and neutrophils ~20-25%
  - Lymphocytes and monocytes ~10%
  - Platelets ~40%
  - Necrosis of bone marrow
- Similar effects not as consistently observed in other species
  - Some human patients with anorexia nervosa with bone marrow hypocellularity and peripheral cytopenias
  - Not reported in mice or dogs

Levin et al ToxPath 21 (1993) 1-14 and unpublished data
Diurnal Rhythm of Corticosterone Affects Peripheral Leukocyte Counts

- **Lights on (morning)**
  - WBC: 44%
  - Lymphocytes: 49%
  - Monocytes: 80%
  - Neutrophils: 15%

- **Lights off (evening)**

Stressors have cell-specific effects on leukocytes (infusion studies)

- **Epinephrine effects: early (within minutes)**
  - ↑ neutrophils (mostly mediated by α-adrenergic receptors)
  - ↑ lymphocytes (mediated by β2-adrenergic receptors)
  - Mostly in proportion to circulating cells
    - Demargination: ↑ blood flow, ↓ adhesion
  - Contributions from spleen and lungs

- **Glucocorticoid effects: later (within 20-30 minutes to few hours)**
  - ↑ neutrophils
    - ↑ half-life, distribution into circulating pool
  - ↓ lymphocytes
    - Apoptosis, altered trafficking
  - ↓ eosinophils (generally most specific and sensitive)--apoptosis
  - variable changes in monocytes (usually track with lymphocytes)

Stress affects leukocytes rapidly

- Neutrophils increased minimally
- Lymphocytes and monocytes decreased
- Recovery occurs rapidly

Emotional Stress affects leukocytes (humans)

Treatment
- 83-minute horror movie
- Educational pamphlets

▲▲ Movie
□□□ Pamphlets

Toxicology correlation
- Stress in NHPs: sensitive to extraneous procedures

Reported Effects of Stress on Serum/Plasma Clinical Chemistry and Urine Parameters

- **Acute Stress**
  - Inc. or dec. glucose
  - Inc. CK, LD, AST, ALT
  - Inc. urea nitrogen and creatinine
  - Acute phase protein response
  - Inc. urine volume

- **Chronic stress (greater than 4 days)**
  - Effects variable depending on model
  - Usually no change in glucose
  - Dec. triglycerides
  - Changes in proteins
Effect of Stress Hormone Infusions on Serum Glucose

- Requires epinephrine (hepatic glycogenolysis and gluconeogenesis)
  - No increase in glucose with either glucagon or glucocorticoids alone

- Fed/fasting status

Harazi et al J Neuroimmunol (Epub; 2007)
Effect of Immobilization Stress on Glucose in Rats and Mice

- Stress increases glucose in fed but not fasted rats
  - C57BL/6>DBA2 mice but not BALC/c mice
  - Maximum effect with intact adrenal cortex and medulla

*Eigler et al (1979) J Clin Invest 63: 114-123*
*Harazi et al J Neuroimmunol (Epub; 2007)*
Acute Stress and Serum/Plasma Enzyme Activities

- Psychological stress in rats (water immersion for 6 hours)
  - Increased LD, CK, AST, and ALT (blocked by β-adrenergic blockers)
  - Also increased urea nitrogen, creatinine, and glucose

- β-adrenergic agonists cause similar increases in LD, CK, AST, and ALT (canines and rats)

- Repeated stress causes habituation of increased enzymes at day 5

Strain Differences in Acute and Chronic Stress

- Strain sensitivity to stressors in rats and mice
- Lewis < SD < F344
  - Acute stress
  - Adaptation to repeated one-hour stress
  - Exposure to novel stress

Summary

- Preanalytical procedures are stressful
  - Minimize stress
  - Maximize consistency in procedures

- Effects of stressors dependent on species, stimulus, duration, timing, magnitude, habituation, etc.

- Clinical pathology changes can provide support for stressor-related effects (weight-of-evidence approach)
  - Leukocytes most sensitive clinpath parameters

- No single parameter pointing to stress—use a weight of evidence approach