Dityrosine: A Non-invasive Biomarker to Monitor Aging?

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Studies in The Biochemistry of Aging Laboratory

• 1. Mitochondrial Oxidative Stress and Aging
• 2. Mitochondrial-induced Apoptosis and Aging
• 3. Toxicity of Doxorubicin on the Heart

Human Studies on Aging, Nutrition, Exercise/Inflammation

• Inflammation in healthy humans
• Nutritional interventions
• Finding non-invasive markers of oxidative stress to monitor aging and disease states in humans

Clinical Research
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Outline

- **Animal Studies**
  - Aging, Caloric Restriction and Dityrosine
  - Antioxidant Therapy and Dityrosine

- **Human Studies**
  - Kwashiorkor and Dityrosine
  - Pilot data on Aging Humans and Urinary levels of Dityrosine

Now also found in the inter-membrane space of mitochondria

[Diagram of cellular processes involving dityrosine and reactive oxygen species]
Quantification of Oxidized Amino acids by Gas Chromatography-Mass Spectrometry

- Acid Stable $^{13}$C Labeled Internal Standards
- HCl-Propanol / Heptafluorobutyric Anhydride.
- Negative Chemical Ionization (Methane)
- DB-1 capillary column 12 meter
- Selected Ion Monitoring
- Quantification: Ratio of Authentic and Corresponding Labeled Standard
**In vitro**

- Gas Chromatography Mass Spectrometry is used to detect oxidative stress “fingerprints”

- Hydroxyl radicals, tyrosyl radicals, and reactive nitrogen increase levels of o-tyrosine, o,o’-dityrosine, and 3-nitrotyrosine *in vitro*

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What is the oxidants production in a day?

- Using 3.5ml O₂/kg/min gives at Rest
- O₂ consumption of 352.8L/day (70kg; male)
- 14.7 moles oxygen a day
- If 1% of oxygen becomes a superoxide radical
- Hypothetically 0.147 moles of superoxide is produced in a day and half of this would form hydrogen peroxide.

- Has this ever been measured in mitochondria?

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Aging, oxidant production, antioxidant defenses and detection of oxidative damage

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There is a chronic exposure to oxidants during a life-span

- Phaneuf & Leeuwenburgh (Unpublished Data)


Leeuwenburgh, C. et al. Oxidative Damage in Heart and Skeletal Muscle is Increased with Aging and is Reduced by Caloric Restriction.

Leeuwenburgh, C. et al. Mitochondrial Deletions.
Mitochondrial Dysfunction

- ↑ Oxidants
- ↑ Calcium levels
- ↑ mtDNA damage/deletions
- ↑ Protein Oxidation

Does this lead to a reduction in ATP?

Conclusions

- Aging Results in an increase in oxidant production (heart, brain, liver)
- Antioxidant enzymes (SOD, GPX) and GSH increase in response to the chronic oxidative stress
- In addition, MtDNA deletions increase in several tissues
  - Was this may mean for mitochondrial function is yet unclear
- There is a decline in maximum rate of ATP production
- Despite the adaptive response against oxidative stress, there is oxidative damage to lipid, DNA, and proteins.

Can we attenuate oxidative damage with (additional) antioxidants?

Drew, B., A. Dirks, & C. Leeuwenburgh (unpublished data)
Animals and Diet

Animals:
• Female Colony-bred Long Evans/Wistar
• Age 24 Months
• At 5 months of age animals began the antioxidant diet and life-long voluntary wheel running

Antioxidant Diet: Harlan-Tekland
• Ascorbic acid
• α-Tocopherol
• BHT
• β-Carotene

Hypothesis

• Antioxidant therapy will reduce both tissue and urine level of oxidized amino acids providing markers to monitor protein oxidation non-invasively in vivo

Conclusions

- Oxidized amino acids may be recognized by proteolytic enzymes degraded, released, and excreted into the urine
- Quantification of the levels of oxidized amino acids in urine may thus serve as an integrated, noninvasive measure of oxidative stress in vivo

Clinical Studies: Increased Oxidative Stress in Kwashiorkor

**Kwashiorkor**

- Characteristics:
  - Edema
  - ↓ Vitamin E
  - ↓ Glutathione
  - ↓ β-carotene

**Question?**

- Is oxidative stress involved in Kwashiorkor

- Non-invasive markers
  - $o-o'$-Dityrosine as well as *ortho*-Tyrosine in the urine of children with Kwashiorkor

**Methods**

- Children approximately three years old were classified as either
  - Well nourished (n = 5)
  - Cerebral malaria (n = 6)
  - Kwashiorkor (n = 8)
  - Kwashiorkor with infection (n=17)
Methods

• Urine was collected using a sterile catheter

• Samples were mixed with antioxidant buffer containing 0.1% phenol and diethylenetriaminepentaacetic acid (DTPA)

• Immediately frozen in dry ice and stored at −80 °C until analysis

• Oxidized amino acids were measured in the urine using isotope dilution negative-ion electron capture gas chromatography/mass spectrometry with selected ion monitoring

• Amino acids were normalized to amino acid precursors and to creatinine in order to correct for differences in glomerular filtration rates

Isolation of Oxidized Amino Acids from Tissue and Urine

**Isolation of Oxidized Amino Acids from Tissue and Urine**

**Tissue**
- 5 mg protein
- Dialysis
- HCl Hydrolysis
- Amino Acid Isolation (Supelco Column)
- Derivatization
- GC-MS analysis

**Urine**
- 0.5-1 ml urine (13C standard)
- TCA precipitation
- Amino Acid Isolation (Supelco Column C18)
- Derivatization
- GC-MS analysis

**Conclusions**

- Kwashiorkor involves oxidative stress and oxidative damage to proteins
- This disease may be monitored using non-invasive markers in the urine
- Therapies which include antioxidant supplementation may decrease the incidence of Kwashiorkor and the number of malnutrition-related deaths

**Design of Small Pilot Study (Aging Humans)**

- Urine Collected from:
  - Young males and females (25-35y; n = 5, each)
  - Old females and one male (80-85y; n = 11)

- Healthy subjects with no indication of diabetes and cardiovascular disease
No difference in precursor amino acids and creatinine between young and old humans

Significant changes in both dityrosine and o-tyrosine with age

Clinical Studies

- In humans, urinary levels of both dityrosine and o-tyrosine increase by 100% and 40%, respectively, comparing young and old subjects
- Kwashiorkor (disease characterized by protein and antioxidant deficiency) showed a 200-600% increase in urinary oxidized amino acids compared to well-nourished children
- Therefore, markers for oxidative stress in urine may be useful for non-invasive assessment of aging and several disease states.

Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. Sell et al. PNAS, 93, 485-490, 1996
(pentosidine/pmol mg collagen)
Future Studies

• Large scale clinical trials are needed to determine range of dityrosine in aging humans before it could function as a marker to monitor aging.


The End