Triamcinolone:
Delaying Eye Deterioration

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Facts

- Age-related Macular Degeneration (AMD) is the leading cause of blindness in the developed Western World
- Affects over 10 million Americans
- Vascular Endothelial Growth Factor (VEGF) is a protein associated with the development of neovascularization (NV) in:
  - Age-related macular degeneration (AMD)
  - Other retinopathies
Project Goal

- To determine if triamcinolone acetonide (TA) decreases cellular concentrations of VEGF
VEGF & Retinopathy

DNA

VEGF RNA

VEGF Protein

Neovascularization

Retinopathy

Triamcinolone Acetonide

Other Factors
Hypothesis

- TA inhibits development of NV by reducing VEGF.

Expected Data:

- RT-PCR
- ELISA
Method Overview

Cell Culture

ELISA

RNA Isolation

DNase Treatment

Real Time RT-PCR
  Reverse Transcription
  PCR
Cell Culture

- ELISA
- RNA Isolation
  - DNase Treatment
  - Real Time RT-PCR
    - Reverse Transcription
    - PCR
## Cell Culture

<table>
<thead>
<tr>
<th>Condition</th>
<th>CtrlT</th>
<th>TA Level</th>
<th>C5 siRNA</th>
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<td>Hypoxia C5 siRNA</td>
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<tr>
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<td>Hypoxia 6mg/mL TA</td>
<td>Hypoxia Ctrl for C5</td>
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### Table

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### Legend
- \(-\) indicates absence of each condition.
Test for VEGF Protein

- Cell Culture
  - ELISA
  - RNA Isolation
    - DNase Treatment
      - Real Time RT-PCR
        - Reverse Transcription
        - PCR
ELISA

1. Prepare all reagents and standards as directed.

2. Add Assay Diluent RD1W to each well.

3. Add Standard, control or sample to each well.

4. Aspirate and wash 3 times.

5. Add 200 μL Conjugate to each well. Incubate 2 hrs. RT

6. Aspirate and wash 3 times.

7. Add 200 μL Substrate Solution to each well. Protect from light.

8. Add 50 μL Stop Solution to each well. Read at 450 nm within 30 min. 
λ correction 540 or 570 nm
Test for VEGF RNA

Cell Culture

ELISA

RNA Isolation

DNase Treatment

Real Time RT-PCR
Reverse Transcription
PCR
<table>
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<tr>
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<td>+1 TA</td>
<td>+ hyp C5</td>
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<tr>
<td>100 25 6.25</td>
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<tr>
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**Real Time RT-PCR**
Results: Cand5

Cand5 decreases VEGF RNA and protein levels as expected

* P ≤ 0.05

**P ≤ 0.01
Results: TA

TA increases VEGF RNA significantly, but has minimal effect on VEGF protein.

* $P \leq 0.05$
Discussion

- TA does not reduce (may increase) VEGF RNA 24 hours after treatment. The ELISA result does not parallel that of the RT-PCR.

- Possible explanations:
  - Need longer than 24 hours to see effect
  - TA sinks to bottom, increasing TA concentration
  - TA solubility, toxicity, and reaction to heat

FOR MORE INFO...

See The Toxic and Stress Responses of Cultured Human Retinal Pigment Epithelium (ARPE19) and Human Glial Cells (SVG) in the Presence of Triamcinolone
AlamarBlue Cytotoxicity Test

- AlamarBlue is a blue dye added to the cells media
- Healthy cells will oxidize it to violet or pink
- Gauge TA toxicity
AlamarBlue Cytotoxicity Test

- **Plate Design**

<table>
<thead>
<tr>
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<th>+ 6 TA</th>
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<td>+ CtrlT</td>
<td>+ 1 TA</td>
<td>+ 6 TA</td>
<td>+ Ctrl5</td>
<td>+ C5</td>
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- **1st trial:** TA background reading too high
- **2nd trial:** Variance too high
- **3rd trial:** Background and variance low
Cytotoxicity Results

- TA is toxic.
## Normalized Cytotoxicity

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<th>+ .1 TA</th>
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<tr>
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<td>Media +AB</td>
<td>Media +AB</td>
<td>Just Cell</td>
<td>Just Cell</td>
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### RT-PCR
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only

### ELISA
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only

### Total Protein Assay
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only

### Cytotoxicity
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only

### Cell Count
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
- .01TA only
Normalized Cytotoxicity

- Cytotoxicity: Testing lower TA doses
  - 0.01 mg/mL
  - 0.1 mg/mL

- Cell Count: Z1 Particle Counter
  - Normalize VEGF to number of cells per vial

- RT-PCR

- ELISA

- Total Protein Assay: BCA Assay
  - Normalize VEGF to Total protein found instead of srRNA housekeeping gene
Normalized Cytotoxicity

- **Cytotoxicity**: Testing lower TA doses
  - 0.01 mg/mL
  - 0.1 mg/mL

- **BCA Total Protein Assay**
  - Normalize VEGF to Total protein found instead of srRNA housekeeping gene

- **Z1 Particle Counter**
  - Normalize VEGF to number of cells per vial
Normalized to Total Protein

- ELISA & Total Protein Assay:
  TA increases VEGF Protein

![Graph showing VEGF levels in ARPE-19 cells treated with TA](image)
Further Investigations

- TA vs. VEGF normalized to RNA
  - RT-PCR
- TA vs. VEGF normalized to Cell Count
  - Z1 Particle Counter
Closing Thoughts

- Age Related Macular Degeneration will cause blindness in 10 million Americans
- And we here at the University of Pennsylvania are working towards its solution.
The End