Triamcinolone: Delaying Eye Deterioration

Dominique Low
Advisors: Elaine Wu and Mike Tolentino, M.D.
University of Pennsylvania
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**

- VEGF RN:
  - 0
  - 20
  - 40
  - 60
  - 80
  - 100
  - 120

**ELISA**

- VEGF Protein:
  - 0
  - 20
  - 40
  - 60
  - 80
  - 100
  - 120
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></th>
<th>CtrlT</th>
<th>1 TA</th>
<th>6 TA</th>
<th>Ctrl5</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Hypoxia</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td>+</td>
<td>CtrlT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/mL TA</td>
<td></td>
<td>+</td>
<td>1 TA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
<td>+</td>
<td>Ctrl5</td>
<td>C5</td>
</tr>
<tr>
<td>6 mg/mL TA</td>
<td></td>
<td>+</td>
<td>Ctrl5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
<td>+</td>
<td>Ctrl5</td>
<td>C5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>Ctrl5</td>
<td>C5</td>
<td></td>
</tr>
</tbody>
</table>

*Just Cell*

*Just Cell*

*Just Cell*
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
# Real Time RT-PCR

<table>
<thead>
<tr>
<th>VEGF</th>
<th>18s srRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>- hyp</td>
<td>- hyp</td>
</tr>
<tr>
<td>+ C5</td>
<td>+ C5</td>
</tr>
<tr>
<td>+ 1 TA</td>
<td>+ 1 TA</td>
</tr>
<tr>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>- hyp</td>
<td>- hyp</td>
</tr>
<tr>
<td>+ C5</td>
<td>+ C5</td>
</tr>
<tr>
<td>+ 1 TA</td>
<td>+ 1 TA</td>
</tr>
<tr>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>- hyp</td>
<td>- hyp</td>
</tr>
<tr>
<td>+ C5</td>
<td>+ C5</td>
</tr>
<tr>
<td>+ 1 TA</td>
<td>+ 1 TA</td>
</tr>
<tr>
<td><strong>50</strong></td>
<td><strong>50</strong></td>
</tr>
<tr>
<td><strong>12.5</strong></td>
<td><strong>12.5</strong></td>
</tr>
<tr>
<td>+ ctl C5</td>
<td>+ ctl C5</td>
</tr>
<tr>
<td>+ TA</td>
<td>+ TA</td>
</tr>
<tr>
<td>+ 6 TA</td>
<td>+ 6 TA</td>
</tr>
<tr>
<td><strong>50</strong></td>
<td><strong>50</strong></td>
</tr>
<tr>
<td><strong>12.5</strong></td>
<td><strong>12.5</strong></td>
</tr>
<tr>
<td>+ ctl C5</td>
<td>+ ctl C5</td>
</tr>
<tr>
<td>+ TA</td>
<td>+ TA</td>
</tr>
<tr>
<td>+ 6 TA</td>
<td>+ 6 TA</td>
</tr>
<tr>
<td><strong>50</strong></td>
<td><strong>50</strong></td>
</tr>
<tr>
<td><strong>12.5</strong></td>
<td><strong>12.5</strong></td>
</tr>
<tr>
<td>+ctl C5</td>
<td>+ctl C5</td>
</tr>
<tr>
<td>+TA</td>
<td>+TA</td>
</tr>
<tr>
<td>+ 6 TA</td>
<td>+ 6 TA</td>
</tr>
<tr>
<td><strong>50</strong></td>
<td><strong>50</strong></td>
</tr>
<tr>
<td><strong>12.5</strong></td>
<td><strong>12.5</strong></td>
</tr>
<tr>
<td>+ctl C5</td>
<td>+ctl C5</td>
</tr>
<tr>
<td>+TA</td>
<td>+TA</td>
</tr>
<tr>
<td>+ 6 TA</td>
<td>+ 6 TA</td>
</tr>
</tbody>
</table>

**NTC** (No Template Control)
Results: Cand5
Cand5 decreases VEGF RNA and protein levels as expected

* P ≤ 0.05
Results: TA

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

* P ≤ 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>
<th></th>
<th>Media Only</th>
<th>Media Only</th>
<th>Media Only</th>
<th>Just Cell</th>
<th>Just Cell</th>
<th>Just Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st trial:</td>
<td>-</td>
<td>+ CtrlT</td>
<td>+ CtrlT</td>
<td>+ Ctrl5</td>
<td>+ C5</td>
<td></td>
</tr>
<tr>
<td>2nd trial:</td>
<td>-</td>
<td>+ CtrlT</td>
<td>+ CtrlT</td>
<td>+ Ctrl5</td>
<td>+ C5</td>
<td></td>
</tr>
<tr>
<td>3rd trial:</td>
<td>-</td>
<td>+ CtrlT</td>
<td>+ CtrlT</td>
<td>+ Ctrl5</td>
<td>+ C5</td>
<td>Media Only</td>
</tr>
</tbody>
</table>

- 1st trial: TA background reading too high
- 2nd trial: Variance too high
- 3rd trial: Background and variance low
Cytotoxicity Results

- TA is toxic.

![Bar Graph](image_url)

**Cytotoxicity: ARPE-19 Cells with TA**

- % reduced vs. different concentrations of TA.

Legend:
- Ctrl
- TA 1mg/mL
- TA 6mg/mL
## Normalized Cytotoxicity

<table>
<thead>
<tr>
<th></th>
<th>CtrlT</th>
<th>+ .01TA</th>
<th>+ .1 TA</th>
<th>+ 1 TA</th>
<th>+ 6 TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>_</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>_</td>
<td>+</td>
<td>+ .01TA</td>
<td>+ .1 TA</td>
<td>+ 1 TA</td>
<td>+ 6 TA</td>
</tr>
<tr>
<td>_</td>
<td></td>
<td>+</td>
<td>+ .1 TA</td>
<td>+ 1 TA</td>
<td>+ 6 TA</td>
</tr>
<tr>
<td>Media</td>
<td>CtrlT</td>
<td>Media</td>
<td>Media</td>
<td>Just</td>
<td>Just</td>
</tr>
<tr>
<td>+AB</td>
<td>+</td>
<td>+AB</td>
<td>+AB</td>
<td>Cell</td>
<td>Cell</td>
</tr>
</tbody>
</table>

- Cytotoxicity
- Cell Count
- RT-PCR
- ELISA
- Total Protein Assay
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
ELISA & Total Protein Assay:
TA increases VEGF Protein
Further Investigations

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