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University of Pennsylvania
Center for Sensor Technologies
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DEVELOPMENT OF A NOVEL THERAPY FOR OCULAR NEOVASCULARIZATION

NSF Summer Undergraduate Fellowship in Sensor Technologies
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ABSTRACT

Age-related macular degeneration (AMD), the leading cause of blindness in the developed Western World, affects over 10 million Americans. Intravitreal injection of triamcinolone acetonide (TA) has been successfully used in the clinic to treat age-related macular degeneration and other similar neovascular diseases, but its mechanism of action is still unclear. However, this experiment sheds light on the subject and leads to a better understanding of the investigational therapy for human disease. It is hypothesized that TA inhibits development of neovascularization (NV) by reducing Vascular Endothelial Growth Factor (VEGF). The goal of this project is to determine if TA decreases VEGF, a protein associated with the development of NV in AMD and other retinopathies.

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1. INTRODUCTION

Drawing from earlier research findings, it has already been demonstrated that vascular endothelial growth factor (VEGF) plays a pivotal role in the development of age-related macular degeneration, one of the most common causes of blindness in developed Western world. A number of investigators have demonstrated that VEGF is sufficient and necessary to produce ocular changes seen in both non-proliferative and proliferative diabetic retinopathy. In particular, VEGF induces neovascularization, the blinding complication of diabetic retinopathy. Several companies have developed anti-VEGF compounds that are in clinical trials for the treatment of neovascular retinal diseases.

One novel strategy for targeting VEGF is through intravitreal injection of triamcinolone acetonide. Triamcinolone acetonide inhibits choroidal neovascularization in a rat model that mimics neovascular retinal disease. The mechanism of this is unclear. One hypothesis is that it downregulates VEGF. We intend to test this hypothesis in vitro.

Also, since intravitreal injections of triamcinolone have been used clinically to treat age-related macular degeneration and other neovascular retinopathies, we would like to test the toxicity of triamcinolone acetonide on human cells. We will implement an Alamar Blue Cytotoxicity Test. Alamar Blue is a dark blue dye added to the cells' media. Healthy cells will oxidize the dye to violet or pink tint. Unhealthy or deceased cells would be unable to oxidize the dye and their surrounding solution would remain dark blue. This test would therefore serve as an appropriate gauge of TA toxicity.

The purpose of this project is to develop a treatment for age-related macular eye degeneration and other neovascular retinopathies, using intravitreal injection of triamcinolone acetonide directed against vascular endothelial growth factor to the retina.

2. MATERIALS AND METHODS

2.1 Testing the Effectiveness of Triamcinolone

2.1.1 Cell Culture

To determine if triamcinolone is indeed inhibiting VEGF production, human ARPE-19 cells were exposed to varying doses of triamcinolone in addition to various controls. The cell cultures were then challenged with 130 uM deferoxamine, a hypoxic stimulus. The cells were incubated in a plate as follows:

–	+ **CtrlT**	+ **1 TA**	+ **6 TA**	+ **Ctrl5**	+ **C5**
–	+ **CtrlT**	+ **1 TA**	+ **6 TA**	+ **Ctrl5**	+ **C5**
–	+ **CtrlT**	+ **1 TA**	+ **6 TA**	+ **Ctrl5**	+ **C5**
Just Cell	**Just Cell**	**Just Cell**			

Each group of cells was tested in triplicates to obviate well-to-well deviations.

The "-" groups of cells were not treated with deferoxamine. Therefore, they would experience no hypoxia, be healthy, and express healthy levels of VEGF. They acted as negative controls for the "+ ctrl TA" and "+ ctrl C5" cells.

The "+ ctrl TA" group of cells represented cells treated with deferoxamine to induce hypoxia so as to generate abnormally high levels of VEGF. However, the cells were bathed in media without TA, and thus acted as negative controls for the "1 mg/mL TA" and "6 mg/mL TA" cells.

The "1 mg/mL TA" cells were treated with deferoxamine to induce hypoxia and with 1 mg/mL TA suspension at a dosage similar to the lower range of dosages used clinically for human. This constituted the first of two TA dosages tested.

The "6 mg/mL TA" cells were treated with deferoxamine to induce hypoxia and with 6 mg/mL TA, at a dosage similar to the higher range of dosages used clinically in humans. This constituted the second of the two TA dosages tested.

The "+ ctrl C5" cells were treated with deferoxamine to induce hypoxia and with siRNA Candidate 5's solvent, aqueous OptiMEM. This acted as a negative control for "+ C5".

The "+ C5" cells were treated with deferoxamine to induce hypoxia and with siRNA Candidate 5. The agent siRNA Candidate 5 had already been demonstrated in previous studies to be capable of suppressing the VEGF gene. It therefore serves as a positive control for the "1 mg/mL TA" and "6 mg/mL TA" cells.

The "Just cells" group of cells was incubated along with the other groups for the purpose of establishing a standard curve to measure the RNA levels.

2.1.2 ELISA

To determine if TA decreases cellular concentrations of VEGF protein, a VEGF Enzyme-linked Immunosorbent Assay (ELISA, R&D Systems) was used. Briefly, ELISA is a colorimetric method of detecting VEGF that has been bound by a VEGF-specific

antibody. By comparing results to a standard curve, the concentration of VEGF in unknown samples can be determined.

–	–	–
+ **CtrlT**	+ **CtrlT**	+ **CtrlT**
+ **1 TA**	+ **1 TA**	+ **1 TA**
+ **6 TA**	+ **6 TA**	+ **6 TA**
+ **ctrl C5**	+ **ctrl C5**	+ **ctrl C5**
+ **C5**	+ **C5**	+ **C5**
2000 pg/mL	**1000 pg/mL**	**250 pg/mL**
125 pg/mL	**62.5 pg/mL**	**31.25 pg/mL**

Supernatant was obtained from each treatment and control group of cell culture that had been spun down in a centrifuge at 13,000 rpm for 2 minutes. In order to reduce TA's interference with the ELISA measurement of VEGF, the supernatant of the "1 mg/mL TA" and "6 mg/mL TA" cell cultures had to be centrifuged to spin down the TA particles suspended in the supernatant. This was done by first transferring the supernatants of all control and treated cell cultures to the low retention 1.5 mL tubes for centrifuge. After centrifuge, 200 uL of each tube was transferred to the ELISA plate. All tests were performed in triplicates to counter potential slight deviation of results between wells.

A standard curve was constructed from a stock solution of human VEGF protein, with concentrations varying from 31.25 to 2000 pg/mL. Readings from different concentrations of VEGF protein were later used to create a standard curve to assay the concentration of VEGF protein in each of the treatment and control samples.

2.1.3 Quantitative Real Time RT-PCR

A real time Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used to determine if TA decreased VEGF mRNA. Because real time RT-PCR was performed in real time, an additional set of controls was needed. As the amounts of loaded RNA may differ, it is vital to normalize all VEGF results relative to a housekeeping gene, 18s srRNA. Thus, the real time RT-PCR was performed on a 96-well plate as follows:

VEGF						18s srRNA					
100	25	6.25	- hyp	+ C5	+ 1 TA	100	25	6.25	- Hyp	+ C5	+ 1 TA
100	25	6.25	- hyp	+ C5	+ 1 TA	100	25	6.25	- Hyp	+ C5	+ 1 TA
100	25	6.25	- hyp	+ C5	+ 1 TA	100	25	6.25	- Hyp	+ C5	+ 1 TA
50	12.5		+ctl C5	+ctl TA	+ 6 TA	50	12.5		+ctl C5	+ctl TA	+ 6 TA
50	12.5		+ctl C5	+ctl TA	+ 6 TA	50	12.5		+ctl C5	+ctl TA	+ 6 TA
50	12.5	NTC	+ctl C5	+ctl TA	+ 6 TA	50	12.5	NTC	+ctl C5	+ctl TA	+ 6 TA

After the supernatant was removed for ELISA, total RNA was isolated from cells using RNeasy (Qiagen) and treated with DNase (Ambion) to remove contaminating DNA. Total RNA was then reversed transcribed into cDNAs using reverse transcriptase (ABI). VEGF and 18s srRNA cDNAs were amplified using gene-specific Taqman probes on a real time cycler. Non-template control (NTC) wells were included. They received no RNA and therefore served as negative control. Standard curves for VEGF and 18s srRNA were generated with serial dilutions of RNA (100, 50, 25, 12.5, and 6.25 ng/uL). Results were samples were extrapolated to the corresponding standard curve.

2.2 Preliminary Cytotoxicity Test

One of the theories to account for the unexpected results in 2.1 prompted a cytotoxicity test for triamcinolone.

2.2.1 Cell Culture

Human ARPE-19 cells were plated in the following manner.

–	+ CtrlT	+ 1 TA	+ 6 TA	+ Ctrl5	+ C5
–	+ CtrlT	+ 1 TA	+ 6 TA	+ Ctrl5	+ C5
–	+ CtrlT	+ 1 TA	+ 6 TA	+ Ctrl5	+ C5
Media Only	Media Only	Media Only	Just Cell	Just Cell	Just Cell

1 TA only	**6 TA only**	**Media +AB**			
1 TA only	**6 TA only**	**Media +AB**			
1 TA only	**6 TA only**	**Media +AB**			
Media Only	**Media Only**	**Media Only**			

Since triamcinolone acetonide has a solubility of 21 mg/L, we were concerned that some triamcinolone might remain in solution and thereby interfered with the oxidation process. Hence, we included "1 mg/mL TA only" and "6 mg/mL TA only" controls, which were free of cells and contained only triamcinolone and growth media.

On Day 0, we plated the human ARPE-19 cells. On Day 1, we added the TA and transfected the cells with siRNA Candidate 5. On Day 2, we added the deferoxamine to induce hypoxia. On Day 3, the cells were harvested to be tested.

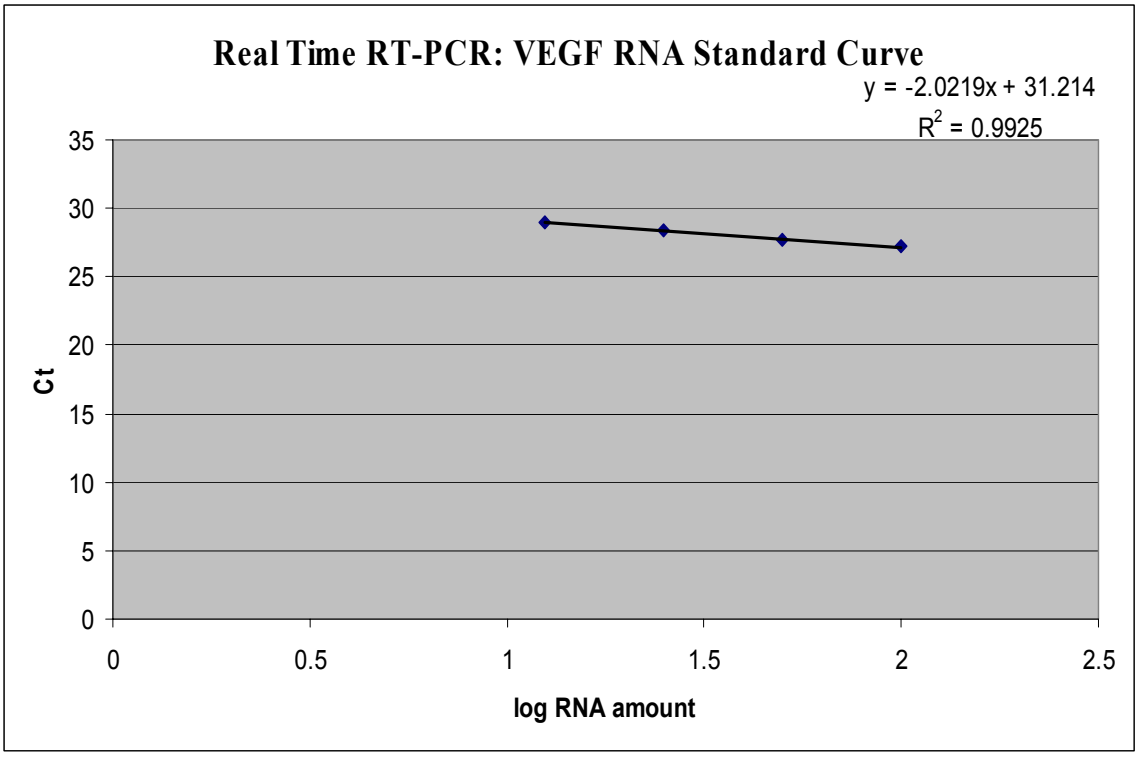
2.2.2 Alamar Blue Cytotoxicity Test

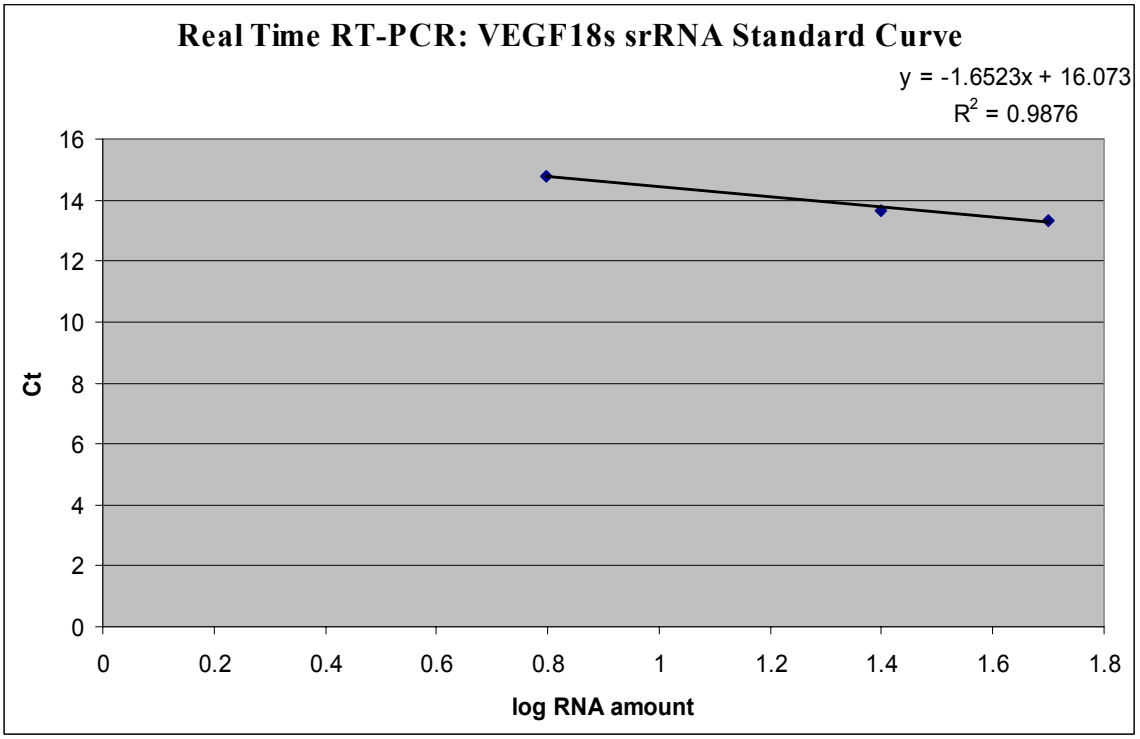
On Day 3, 12 mL of 10% Alamar Blue (Biosource) growth media was prepared by mixing 1.2 mL of alamar Blue with 10.8 mL of regular growth media. Then the growth media was removed from all groups except "media only" cells, and replaced with 300 uL Alamar Blue media in each well. Afterwards, the cells were allowed to incubate for 4 hours at 37 degrees C. At the end of the incubation, the supernatant was centrifuged to remove the suspended triamcinolone particles. The supernatant from each well was then placed into individual square vials and tested by a DU-640 spectrophotometer set at wavelengths 570 and 600 nm.

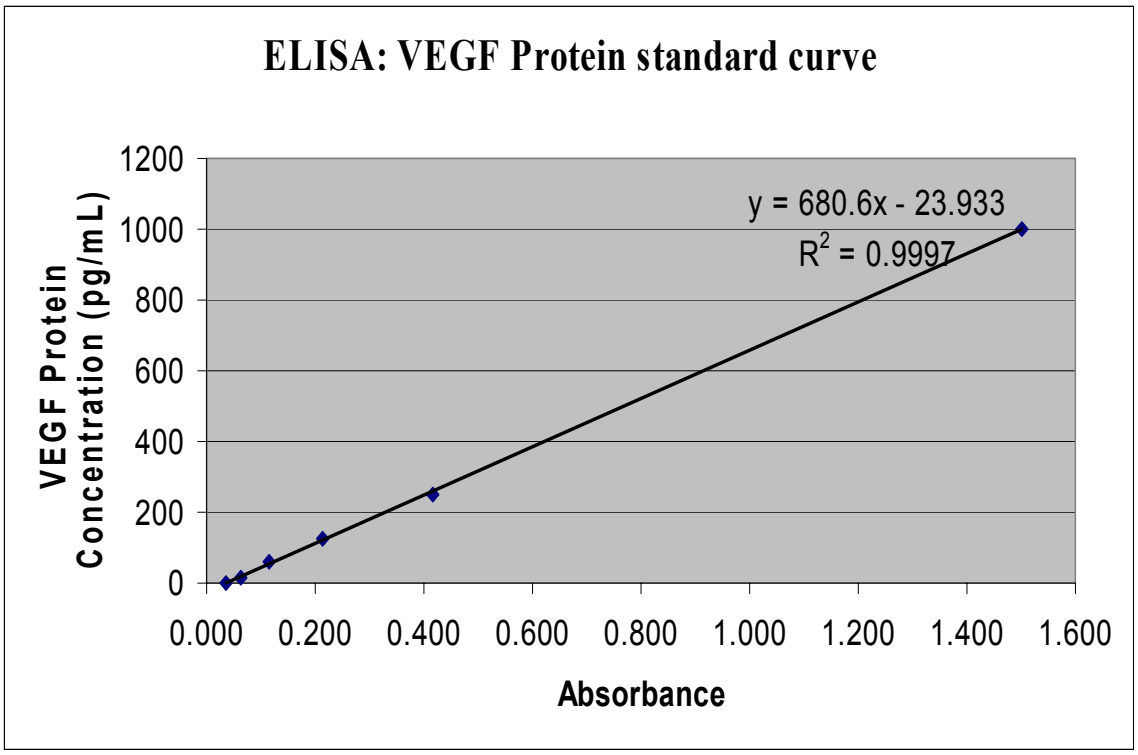
3. RESULTS AND DISCUSSION

3.1 Testing the Effectiveness of Triamcinolone

In order to accurately measure the VEGF RNA and protein levels expressed by the cells, the following standard curves were constructed.

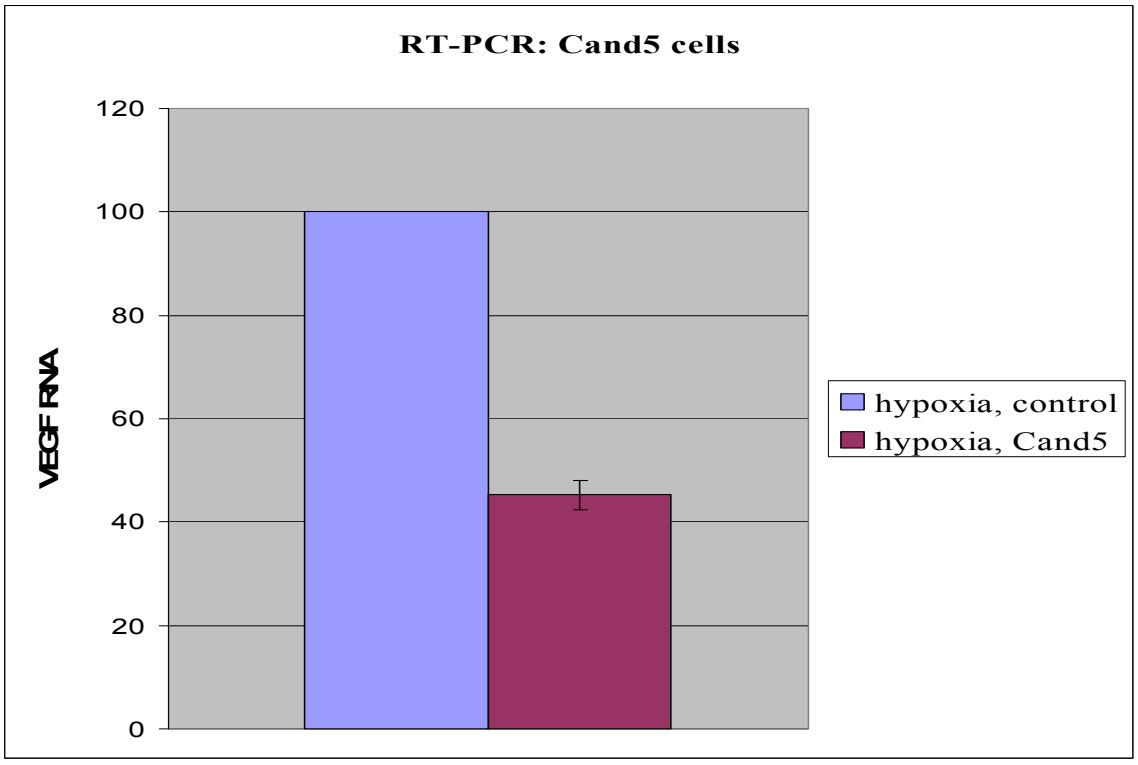

Real Time RT-PCR: VEGF RNA Standard Curve
y = -2.0219x + 31.214
R2 = 0.9925
Ct
log RNA amount
0
5
10
15
20
25
30
35
0
0.5
1
1.5
2
2.5

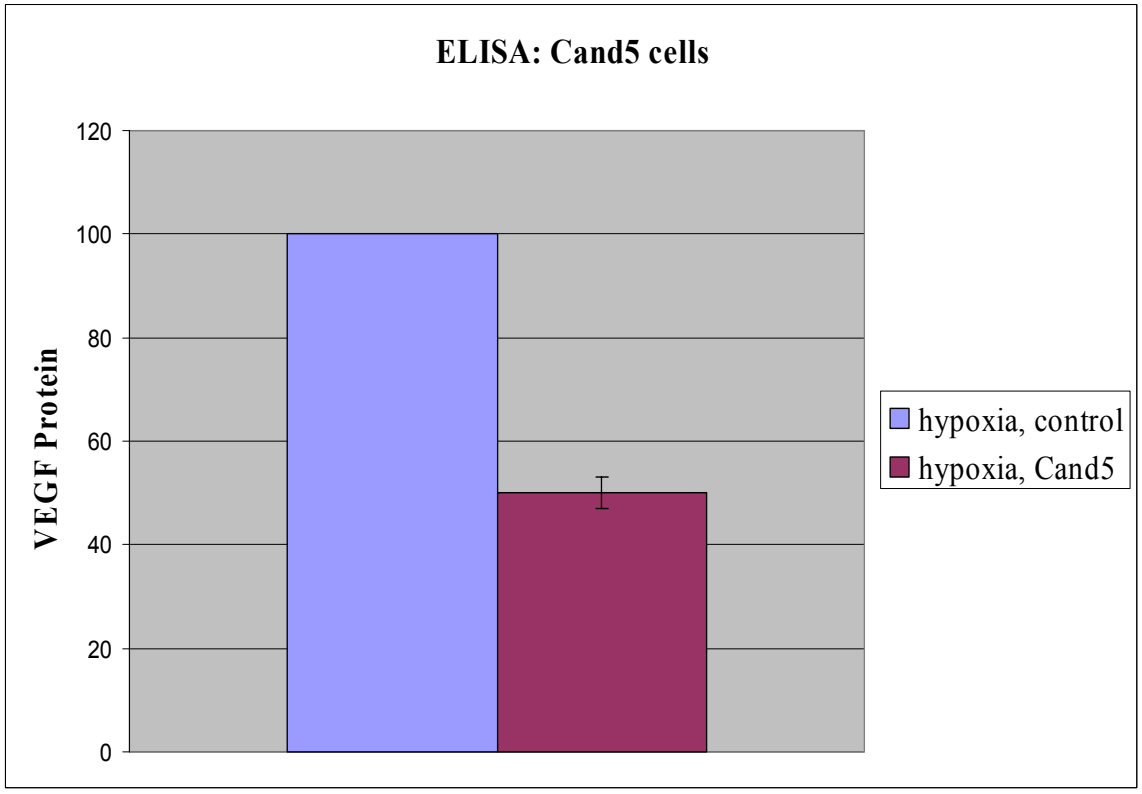

Real Time RT-PCR: VEGF18s srRNA Standard Curve
y = -1.6523x + 16.073
R2 = 0.9876
Ct
log RNA amount
0
2
4
6
8
10
12
14
16
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8



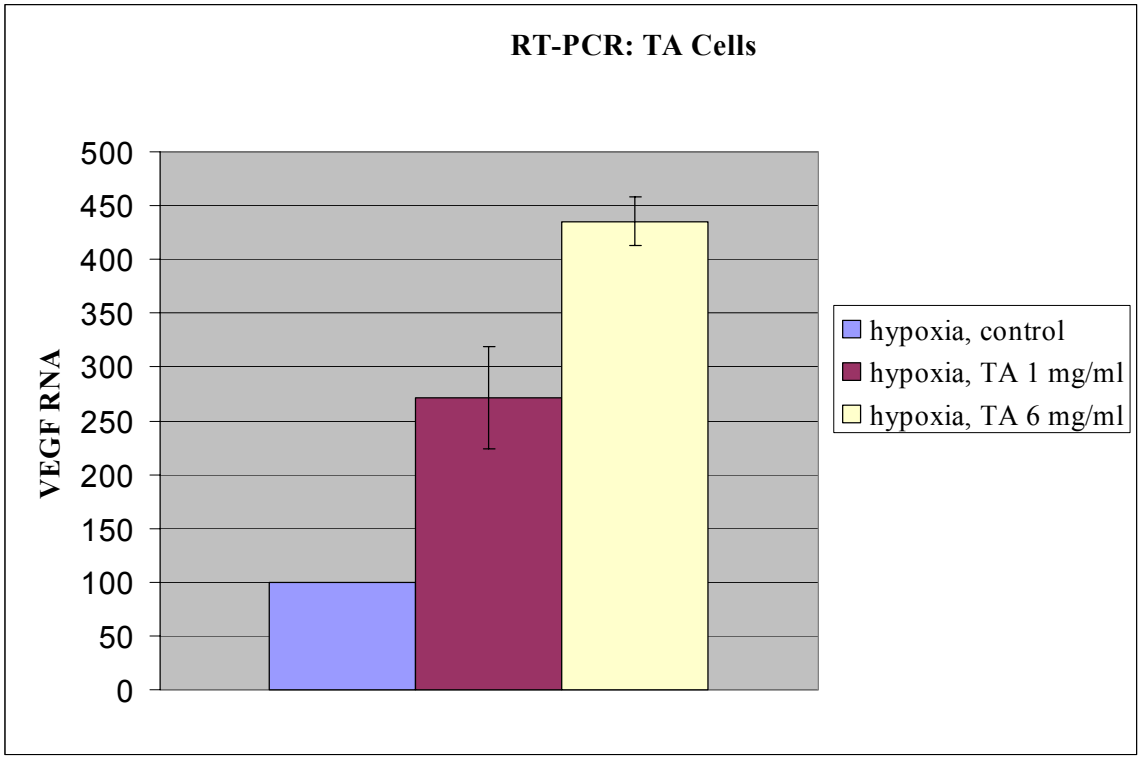
Determination of the concentrations of VEGF RNA and protein from the standard curves above allowed subsequent comparisons of the data obtained from the treatment and control groups.

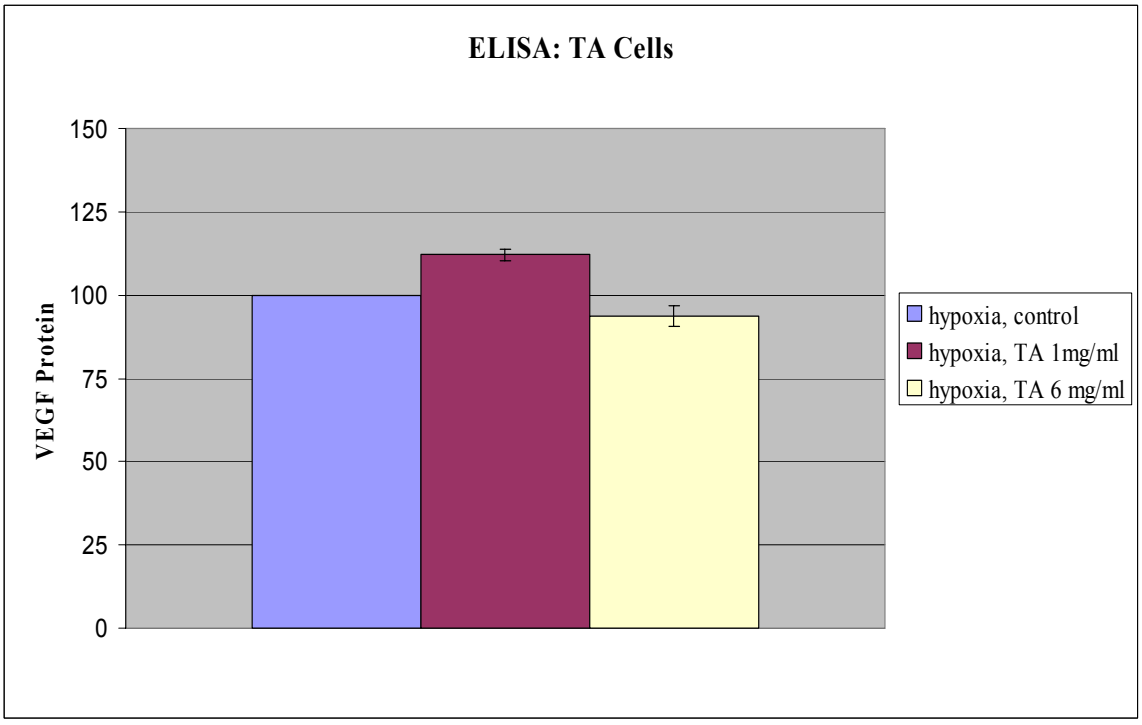
The positive control, siRNA Candidate 5, decreased both VEGF RNA and protein levels. Both measurements had p-values less than 0.05 and were therefore statistically significant.


RT-PCR: Cand5 cells
VEGF RNA
120
100
80
60
40
20
0
hypoxia, control
hypoxia, Cand5


ELISA: Cand5 cells
VEGF Protein
120
100
80
60
40
20
0
hypoxia, control
hypoxia, Cand5

Initially, the TA treatments were expected to mimic Candidate 5's ability to lower VEGF protein and RNA levels. However, the cells treated with TA did not behave as expected. The TA increased VEGF RNA significantly, but had minimal effect on VEGF protein.





It was observed that TA did not reduce and might actually increase VEGF RNA 24 hours after treatment. Also, among the TA treated cells, the ELISA result did not mimic that of RT-PCR.

There are several possible explanations for this unexpected result. First, TA may be interfering with ELISA reading. As it has a solubility of 21 mg/L, a small amount of TA would still remain in solution after removal of suspended TA particles by centrifugation. Second, the triamcinolone may need more than 24 hours to affect the cellular RNA and protein expressions. Third, TA particles might settle toward the bottom of the wells, thus increasing the TA concentration that the cells are exposed to and consequently enhanced its cellular effects. Lastly, TA's cytotoxicity and its reaction to heat may affect the experimental results.

3.2 Preliminary Cytotoxicity Test

Suspecting that triamcinolone might be cytotoxic[11] and that this property might be responsible for the unexpected results noted in 3.1, a preliminary cytotoxicity test was performed on TA. Calculations were made using the formulas described by BioSource, the manufacturer of the Alamar Blue used.

The calculation of % Reduced is as follows when the samples are read at

$\lambda_1 = 570$ nm

$\lambda_2 = 600$ nm

$$\% \text{ Reduced} = \frac{(\varepsilon_{ox}\ \lambda_2)\ (A\ \lambda_1) - (\varepsilon_{ox}\ \lambda_1)\ (A\ \lambda_2)}{(\varepsilon_{red}\ \lambda_1)\ (A'\ \lambda_2) - (\varepsilon_{red}\ \lambda_2)\ (A'\ \lambda_1)} \times 100$$

Where:

$(\varepsilon_{red}\ \lambda_1)$ = 155,677 (Molar extinction coefficient of reduced alamarBlue™ at 570 nm)

$(\varepsilon_{red}\ \lambda_2)$ = 14,652 (Molar extinction coefficient of reduced alamarBlue™ at 600 nm)

$(\varepsilon_{ox}\ \lambda_1)$ = 80,586 (Molar extinction coefficient of oxidized alamarBlue™ at 570 nm)

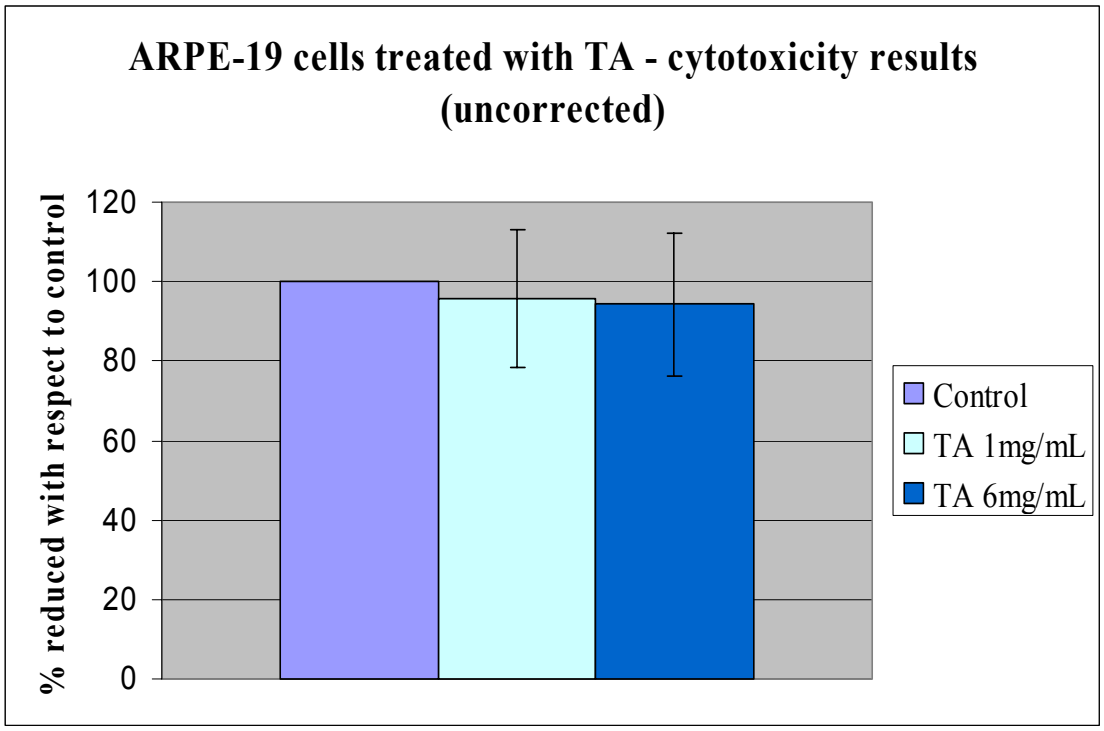
$(\varepsilon_{ox}\ \lambda_2)$ = 117,216 (Molar extinction coefficient of oxidized alamarBlue™ at 600 nm)

$(A\ \lambda_1)$ = Absorbance of test wells at 570 nm

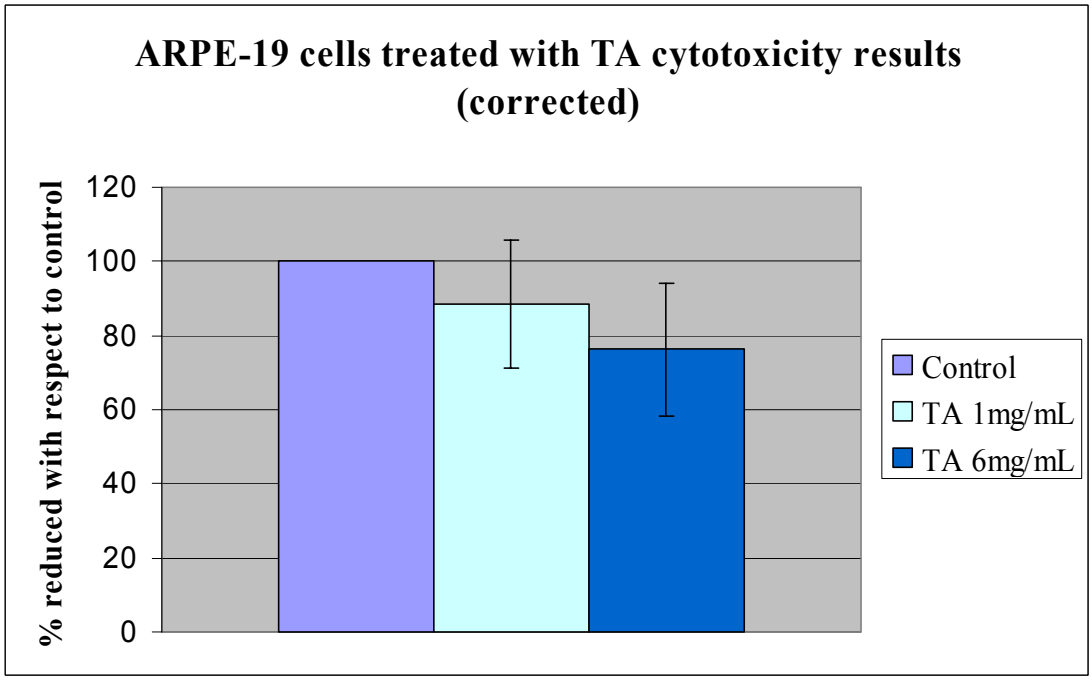
$(A\ \lambda_2)$ = Absorbance of test wells at 600 nm

$(A'\lambda_1)$ = Absorbance of negative control wells which contain medium plus alamarBlue™ but to which no cells have been added at 570 nm.

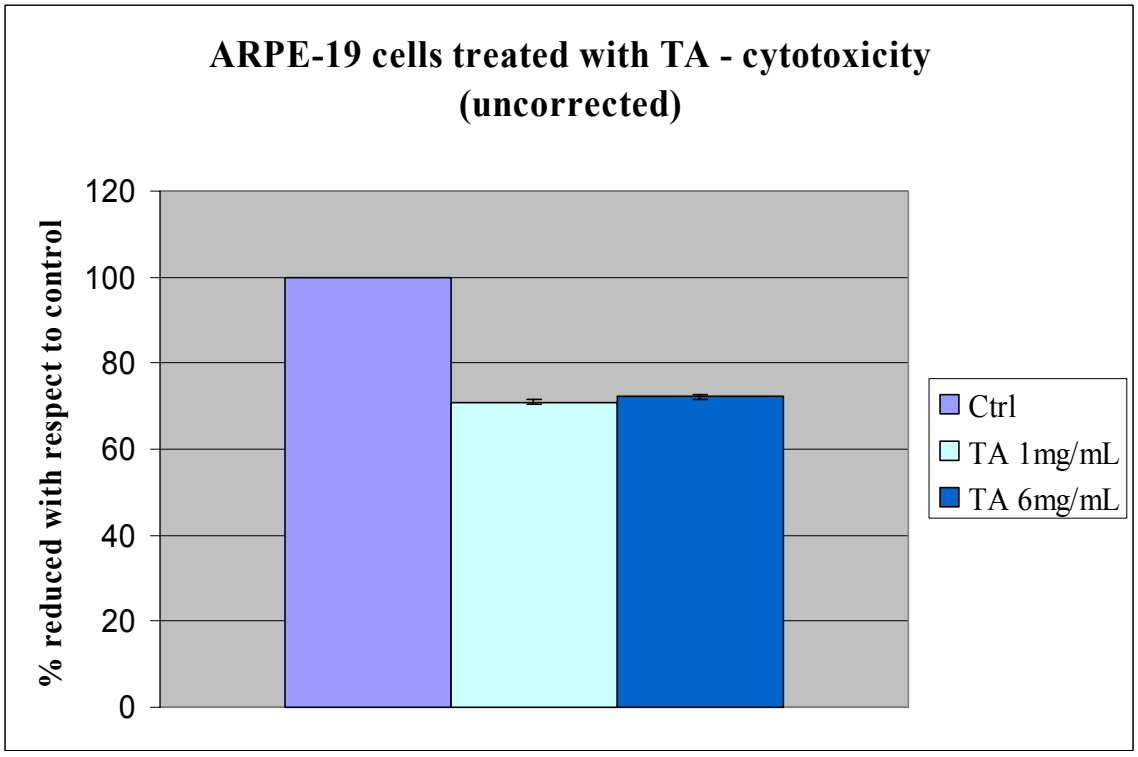
$(A'\lambda_2)$ = Absorbance of negative control wells which contain medium plus alamarBlue™ but to which no cells have been added at 600 nm.

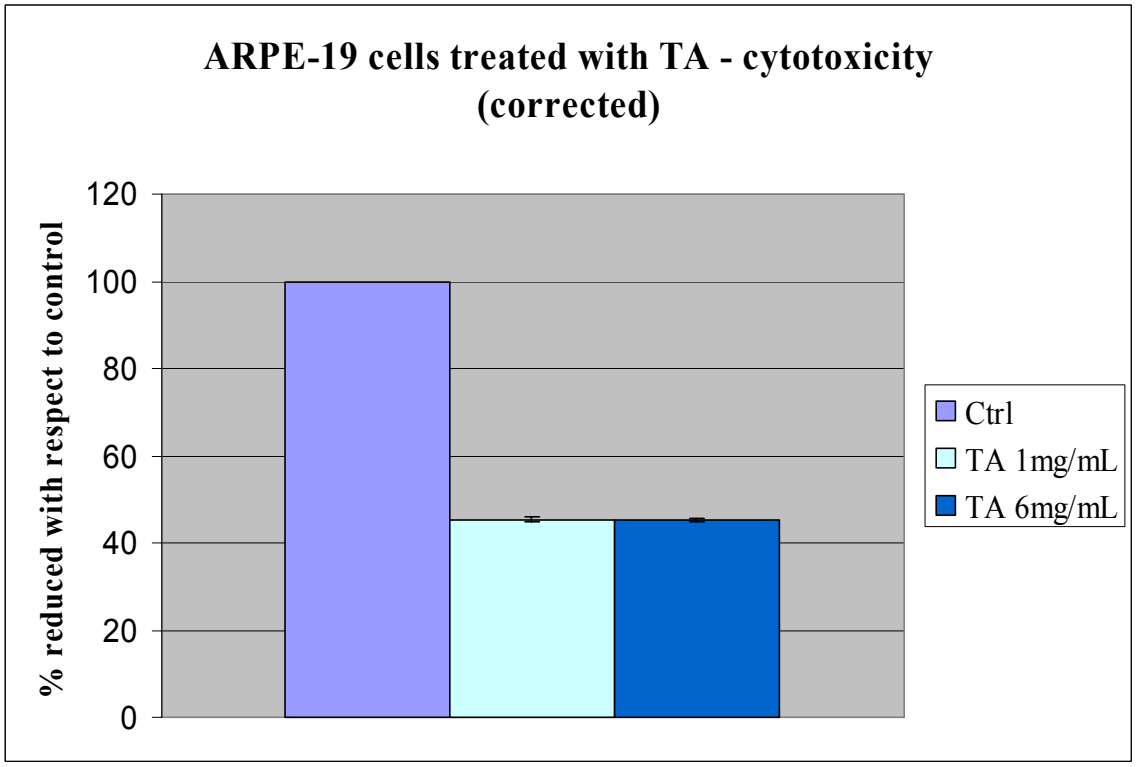


The TA cytotoxicity readings were then corrected using the cytotoxicity readings for the "TA and media only".



Due to the high variance in the data, the experiment was repeated to confirm the results.


ARPE-19 cells treated with TA - cytotoxicity (uncorrected)
% reduced with respect to control
120
100
80
60
40
20
0
Ctrl
TA 1mg/mL
TA 6mg/mL


ARPE-19 cells treated with TA - cytotoxicity (corrected)
% reduced with respect to control
120
100
80
60
40
20
0
Ctrl
TA 1mg/mL
TA 6mg/mL

The percent of Alamar Blue reduced by the TA treated cells was significantly smaller than the percent reduced by the control cells. Thus, TA was determined to be significantly cytotoxic.

4. CONCLUSIONS

The hypothesis that TA, at its clinical dose of 1 mg/mL, inhibits choroidal neovascularization by downregulating VEGF was refuted in this experiment. At 1 mg/mL, triamcinolone acetonide significantly increases VEGF RNA and protein levels.

Further investigation addressed the cytotoxicity of TA. Overall, at 1 mg/mL and 6 mg/mL doses, TA appears to be toxic. Additionally, such doses also correlate with a significant increase in VEGF level. This increase is likely a stress response to TA's cytotoxicity.

It was observed that TA at concentration of 6 mg/mL did not appear to kill more cells or increase VEGF more than TA at concentration of 1mg/mL. This may be because all the intracellular receptors for TA are completely saturated at a TA concentration of 1 mg/mL. Therefore, higher concentrations of TA suspension could not further increase TA binding to its intracellular receptors, and thus, there were no further increase in VEGF expression nor enhancement of its cytotoxicity at concentrations higher than 1 mg/ml.

Intravitreal injection of triamcinolone acetonide has been successfully used clinically in the treatment of age-related macular degeneration, but its mechanism of action still remains unclear. However, these experiments have refuted the theory of attributing triamcinolone's clinical efficacy to possible reduction of VEGF expression.

5. FUTURE WORK

Further investigation is recommended to address the effects of TA over a wider range of doses, ranging from .01 to 6 mg/mL. Also, it would be helpful to determine if there is any correlation of VEGF levels to TA's dose-toxicity curve. Additionally, it would be constructive to determine whether or not there is still a significant increase in VEGF level after normalizing it to total protein level, cell count, and total RNA level.

This project is part of a larger, long-term initiative to test various treatments for age-related macular degeneration and other similar neovascular retinopathies. Other alternatives have been proposed, such as the transfection of short hairpin RNA. Each option will be compared and the one which proves itself the most effective will be chosen for clinical testing. One of the approaches is to apply sensor technology to non-invasively measure the clinical effects of various treatment modalities, thus facilitating the search for the optimal therapy and the monitoring of the patients' clinical response to treatment. Examples of such potential senor technology include surface measurement of electrical activities in the visual cortex during the treatment and convalescent phases.

6. ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my advisors, Elaine Wu and Professor Michael Tolentino, for their guidance, encouragement, support, and supervision. I extend a special note of thanks to Elaine for introducing me to sensor technologies critical in this project, including the ABI Prism 7000 Sequence Detection System, Wallac Victor 1420 Multilabel Counter, and PTC-400 Thermocycler. I would also like to thank the National Science Foundation for their support through the Research Experience for Undergraduates Grant that made the Summer Undergraduate Fellowship in Sensor Technologies program possible.

7. REFERENECES

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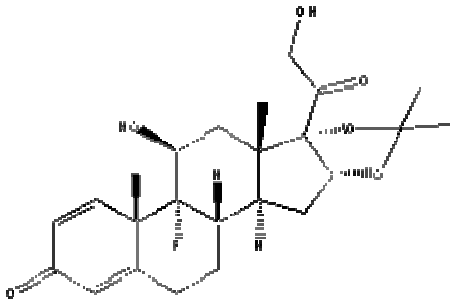
8. APPENDICES

APPENDIX 1: Triamcinolone Acetonide



APPENDIX 2: Sensors

1. **ABI Prism 7000 Sequence Detection System**
2. **Wallac Victor 1420 Multilabel Counter**
3. **PTC-400 Thermocycler**