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Endothelin-1 Promotes Bovine Corneal Endothelial Cell Proliferation via a MAPK Pathway: Implications for Keratopathy and Deturgescence

Brandon C. Farmer

Western Kentucky University, brandon.farmer396@topper.wku.edu

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ENDOTHELIN-1 PROMOTES BOVINE CORNEAL ENDOTHELIAL CELL
PROLIFERATION VIA A MAPK PATHWAY: IMPLICATIONS FOR
KERATOPATHY AND DETURGESCENT

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By:

Brandon C. Farmer

Western Kentucky University
2015

CE/T Committee:

Dr. Ken Crawford, Advisor

Dr. Nancy Rice

Dr. Matthew Nee

Approved by

Advisor
Department of Biology

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ABSTRACT

The corneal endothelium is vital in maintaining the functions of the cornea, namely hydration, thickness, and transparency. Diseases that impair the corneal endothelium are currently remedied solely via surgery. These surgeries present obstacles to patients of underserved areas due to cost, ineffectiveness, and lack of access. Trachoma, an infection which can cause corneal opacities, is the leading cause of infectious blindness in the world. In order to eradicate trachoma, epidemiological data is needed. Three villages in Kasigau, Kenya were studied to quantify the occurrence of trachoma in the region. The three villages were found to have adequate access to antibiotics and an unremarkable incidence of trachoma symptoms. An alternative therapy to surgery for corneal endothelial disease is needed. Endothelin-1 (ET-1) has been shown to induce cell proliferation in bovine corneal endothelial cells (BCEC). The pathway by which this occurs is not well understood. It was hypothesized that ET-1 induces proliferation through the mitogen-activated protein kinase (MAPK) pathway. Treatment of BCEC with 10 nM ET-1 for 15 min induced a 4.3 fold increase in pERK1/2 ($p < 0.001$). Furthermore, 30 min pre-treatment with a MAPK pathway inhibitor before ET-1 treatment significantly decreased pERK1/2 expression ($p < 0.05$). These results suggest that the MAPK pathway may be involved in BCEC ET-1 induced proliferation.

Keywords: cornea, corneal endothelium, endothelin-1, MAPK, trachoma, keratopathy, DSEK, Kenya, ERK

Dedicated to

The hilltop, where the spirit made me a master.

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VITA

- December 14, 1993.....Born – Lexington, Kentucky
- 2010.....Accepted to Carol Martin Gatton Academy of Mathematics and Science
- 2011.....Faculty-Led Study Abroad Program
Costa Rica
Cloudbridge Nature Preserve
- 2011.....NSF Research Experience for Undergraduates
Western Kentucky University
Department of Chemistry
- 2012.....Graduation from Gatton Academy
With Honors
- 2012.....Graduation from Bourbon County High School
Valedictorian
- 2012.....KBRIN Summer Research Program
University of Louisville
Division of Nephrology
- 2013.....KIIS Study Abroad Program
Merida, Mexico
- 2014.....Faculty-Led Study Abroad Program
Kasigau, Kenya
- 2014.....Undergraduate Clinical Research Internship Program
Vanderbilt University
Department of Pathology, Microbiology, and Immunology
- 2015.....L. Y. Lancaster Award for Excellence in Pre-Medical Studies
- 2015.....Graduation from Western Kentucky University
Summa Cum Laude
Honors College Graduate

PUBLICATIONS

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2. Brandon Farmer, Haichun Yang, MD/PhD, Agnes Fogo, MD. Fibroblast-Specific Knockdown of PAI-1 Attenuates Kidney Interstitial Fibrosis. 2014 Southeastern Medical Scientist Symposium. 23-24 Aug 2014. Atlanta, GA.
3. Brandon Farmer, Leah Frazier, BS, Akhila Bethi, MS, Ken Crawford, Ph. D. Endothelin-1 induces phosphorylation of ERK 1/2 in bovine corneal endothelial cells. 2014 Annual Meeting of the Association for Research in Vision and Ophthalmology; 4-8 Mar 2014; Orlando, FL.
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CHAPTER 1

INTRODUCTION

The Human Cornea

The cornea is the transparent tissue that forms the outermost layer of the eye, covering the iris, pupil, and anterior chamber. It is avascular but highly innervated by nerves. The cornea is essential for visual acuity as it provides 65-75% of the eye's focusing power.¹ Transparency of the cornea allows light to pass through while non-transparency (corneal cloudiness) will scatter light and decrease visual acuity. The cornea receives oxygen via passive diffusion at the cornea/atmosphere interface and nutrition from the tear film as well as the aqueous humor via the pump action of the corneal endothelium.²

Structure

The cornea is comprised of five layers. The most anterior layer is the corneal epithelium, made up of stratified squamous epithelial cells. This layer functions as the first line of defense for the eye from bacteria and external pathogens and is the site of oxygen diffusion. The corneal epithelium lies on a basement membrane known as Bowman's Membrane. This 8-12 μm thick layer is secreted by the epithelial cells and is made up of randomly oriented collagen fibrils. Immediately posterior to Bowman's membrane is the corneal stroma which comprises the majority of the cornea. The stroma is made up of regularly arranged collagen fibers along with sparsely distributed

interconnected keratocytes. The keratocytes function in wound repair and general corneal maintenance. Immediately posterior from the stroma lays Descemet's Membrane which is comprised of collagen type IV fibrils, serving as the corneal endothelium's basement membrane. Descemet's Membrane is acellular, secreted by the corneal endothelial cells, and 15-20 μm thick. Recently, a fourth caudal corneal layer, alleged anterior to Descemet's Membrane, was discovered by Dua *et al.*³ However, much controversy has surrounded this so called "Dua's Layer" as this study has not yet been replicated.

Corneal Endothelium

The corneal endothelium forms the fifth caudal layer of the cornea which rises from embryonic neural crest cells.⁴ The layer is comprised of a monolayer of simple squamous mitochondria-rich cells, hexagonal in shape, that form a barrier between the corneal stroma and the aqueous humor in the anterior chamber. The layer is contact inhibited *in vivo*, meaning that its cells will not proliferate naturally. Instead, for wound healing purposes, the endothelial cells will either enlarge or migrate to compensate for the loss of cells. As the cornea is an avascular organ, it relies on the endothelium for the transport of nutrients via pump action across the monolayer. The corneal endothelium also regulates the fluid capacity of the cornea.⁵ Fluid from the aqueous humor leaks into the stroma in a flux proportional to the integrity of the cell-to-cell tight junctions. Since the only method for wound healing is cell enlargement and migration, losing cells in the corneal endothelium causes irregular cell-cell connections. Less tight junctions and fewer pumps lead to an increase in bulk flow of fluid across the endothelium.⁶ This results in stromal swelling (corneal decompensation) and other corneal complications, such as

opacification. Therefore, maintaining a proper cell density in the corneal endothelium is vital for vision and corneal health.

CHAPTER 2

CORNEAL ENDOTHELIUM PATHOLOGY

Corneal Endothelial Pathology

In young individuals, the corneal endothelium is 4-6 μm thick and comprised of about 400,000 cells with a cellular diameter of 20 μm . The corneal endothelial cell density decreases gradually with age. At birth, density ranges from 3500-4000 cells/ mm^2 . Once an individual reaches adulthood, density ranges from 1400-2500 cells/ mm^2 . A cell density below 400-700 cells/ mm^2 leads to edema and loss of visual acuity.⁷ The leading cause of corneal endothelial cell loss is Fuchs' Endothelial Dystrophy (FED). FED is an autosomal dominant trait and is characterized initially by loss of visual acuity in the morning. This is due to high fluid content within the cornea that accumulates overnight without evaporative potential. This fluid will evaporate as the affected individual exposes their cornea to sunlight and air. As the disease worsens and the corneal endothelium becomes less dense, more fluid will leak through the corneal endothelium causing corneal edema, and vision will be further impaired gradually throughout the day.

Another cause for decreased corneal endothelial cell density is surgical error. Ultrasonic phacoemulsifiers are often used to degrade lenses in patients' eyes that present clouded or opaque areas called cataracts. Ophthalmologists use these probes in close proximity to the corneal endothelium and often inadvertently shear off endothelial cells. If a sufficient number of cells are lost, Pseudophakic Bullous Keratopathy will manifest

with corneal edema since the corneal endothelium cannot keep the cornea in a healthy and relatively dehydrated state, known as deturgescence.⁸

Trachoma

A third cause of corneal insult is trachoma, the result of an infection of *Chlamydia trachomatis* in the eye.⁹ This causes inflammation and scarring in the tarsal conjunctiva (eyelid) which, if left untreated, causes the eyelid to invert (entropion) often leading to inverted eyelashes that scrape the cornea (trichiasis). Late stage trachoma, called trachoma trichiasis, is characterized by entropion, but also corneal opacity. Today, trachoma is endemic in 51 countries affecting about 41 million people, mostly women and children, who have active trachoma infection and need treatment. It is estimated that 8.2 million people are blind from Trachoma trichiasis, making it the leading cause of infectious blindness worldwide. The bacterium can be spread via direct contact from an infected person and also via a fly vector. Therefore, communities and countries with poor sanitization practices often show the highest occurrences of trachoma.

CHAPTER 3

KENYA AND TRACHOMA

WHO & GET 2020

The World Health Organization (WHO) leads an international coalition known as the Alliance for the Global Elimination of Trachoma by the year 2020 (GET 2020). In order to properly eradicate trachoma, GET 2020 coordinates essential activities such as epidemiological assessment and mapping of affected regions, project planning, implementation, monitoring, disease surveillance, and resource mobilization. It is open to all parties - governments, international organizations and nongovernmental organizations - who are willing and ready to contribute to international efforts.¹⁰

The “SAFE” strategy was developed by the WHO as a set of methods to help fight Trachoma (Figure 3.1). SAFE is an acronym for surgery for trichiasis (inturned eyelids), antibiotics, facial cleanliness and environmental improvement.

These four interventions are necessary for trachoma therapy and prevention and have been proven to be effective. In 1998, Pfizer and the Edna McConnell Clark Foundation joined the global effort and co-established the International Trachoma Initiative (ITI), an independent not-for-profit organization dedicated to the elimination of blinding trachoma. Pfizer donates the Zithromax® needed to implement the “A” component of the SAFE strategy and reach the goal of elimination. To date, Pfizer has donated more than 444 million Zithromax® treatments to people in 21 countries.¹¹ Over

115 million doses of Zithromax® are approved for shipment in 2015, double that of last year's shipments.



Figure 3.1 – SAFE Method The WHO instituted the SAFE method (Surgery, Antibiotics, Facial cleanliness, Environmental change) as a set of methods to help combat trachoma. Source: (Pfizer)

Kenya and Trachoma

While the worldwide goal for trachoma eradication is 2020, specific countries have their own goals. Kenya has made 2015 as the year in which blinding trachoma should be completely eradicated from its country. The International Coalition for Trachoma Control estimates that 2.2 million Kenyans live in Trachoma endemic areas, with 67 thousand cases of actual blinding trachoma trichiasis.¹² As of 2010, Zithromax® had only been distributed to 3 districts of Kenya (Figure 3.2)

Partners in Caring: Medicine in Kenya

Each year a group from Western Kentucky University comprised of pre-professional students, volunteers, researchers, and medical professionals travels to

southern Kenya to offer medical attention to the underserved communities in the Kasigau community of the Taita Taveta district (Figure 3.3). A partnership has been built between the University and the people of Kasigau that has also incorporated studies including topics such as hypertension prevalence¹³, assessment of prenatal outcomes¹⁴, and human-wildlife conflict. In this study, a pilot survey of the occurrence of trachoma was conducted on patients in the clinics of three villages in Kasigau (Rukanga, Makwasinyi, Bhaguta). This survey was conducted in hopes to help direct efforts for Zithromax distribution in Kenya in order to reach the 2015 goal for the elimination of blinding trachoma.



Figure 3.2 – Antibiotic Distribution in Kenya as of 2010 Highlighted regions show districts of Kenya that received antibiotics from Pfizer as of the year 2010. Source: (International Coalition for Trachoma Control)



Figure 3.3 - Map of Kenya with a Pin at the Kasigau Region Source: (African Promise, 2014)

CHAPTER 4

TREATMENT FOR CORNEAL ENDOTHELIAL DYSTROPHY

Treatment for Corneal Endothelial Dystrophy

About 40,000 Americans receive a corneal transplant each year. Total corneal transplant, known as penetrating keratoplasty (PKP), was initially used to treat corneal complications, including trachoma trichiasis. This involved the excision of a patient's cornea and transplantation of a donor graft. The graft is sutured into the patient's eye with ultra-fine sutures which are removed a year post operation.

In 1998, G. Melles *et al.* developed Descemet's Stripping with Endothelial Keratoplasty (DSEK) which is now commonly used as the treatment for corneal opacities.¹⁵ This procedure calls for removal of the impaired corneal endothelium along with Descemet's Membrane through an incision in the sclera, followed by transplantation of a host corneal endothelium through the same incision. The ocular chamber is then pressurized and the patient lies supine for 24 hours to promote adherence of the corneal endothelium. DSEK is favored over PKP because it requires minimal restrictions in normal activities, provides faster visual recovery, does not require any sutures, and causes minimal change in glasses prescription.¹⁶

Issues with Surgical Correction of Corneal Opacities

Surgical intervention has many risk factors including infection, adverse reaction to anesthesia, and physician error. PKP involves complete excision of a whole graft while DSEK is less invasive and involves excision of the corneal endothelium only, making DSEK the apparent better candidate for surgical correction of corneal complications. Studies have been done to gauge the efficacy of DSEK compared to PKP by evaluating patient outcomes. Price *et al.* compared 173 DSEK patients to 410 PKP patients, and found that at one year post-transplantation, overall graft success was comparable for DSEK and PKP procedures but endothelial cell loss was higher with DSEK.¹⁷ Similarly, Lee *et al.* found that in terms of surgical risks, complication rates, graft survival (clarity), visual acuity, and endothelial cell loss, DSEK appears similar to PKP.¹⁸ DSEK seems to be superior to PKP only in terms of earlier visual recovery, refractive stability, postoperative refractive outcomes, wound and suture-related complications (as there are none), and intraoperative and late suprachoroidal hemorrhage risk. Therefore, even with the discovery of DSEK, outcomes are relatively similar to PKP, making the pressing need for alternative solutions to keratopathy still very relevant.

Furthermore, access to specialty surgical intervention in rural areas of the United States and underdeveloped countries is very poor. Not only are there geographic barriers that limit access, such as distance and transportation, but also the high cost of these procedures often leaves the impoverished without treatment. Frequently, those in developing countries where there are poor sanitization conditions due to lack of water and resources are the patients in greatest need of medical attention, yet they are least likely to be able to pay for such procedures due to their lack of affluence. Therefore, designing a non-surgical and cost effective alternative to corneal transplantation is

desirable in order to help treat those who do not have access to surgery, whether it is for geographic or monetary reasons.

CHAPTER 5

ENDOTHELIN-1

Endothelin-1

Endothelin-1 (ET-1) is a 21 amino acid protein that is found endogenously in the human body and has potent vasoconstrictive properties. It is predominantly released by vascular endothelial cells to regulate the tone of blood vessels, but it has also been found to be secreted by the ciliary epithelium which leaks into the aqueous humor of the eye. The aqueous humor is the chamber immediately posterior to the corneal endothelium. As the aqueous humor is not vascularized and drained very slowly by the Canal of Schlemm, ET-1 is found at concentrations several times higher in the aqueous humor than in blood plasma.¹⁹

Endothelin-1 has been shown to promote wound healing via proliferation in the bovine corneal endothelium (Figure 5.1).²⁰ It was shown that bovine corneal endothelial cells treated with 10 nM ET-1 for 72 hours showed significantly faster wound closure compared to control.

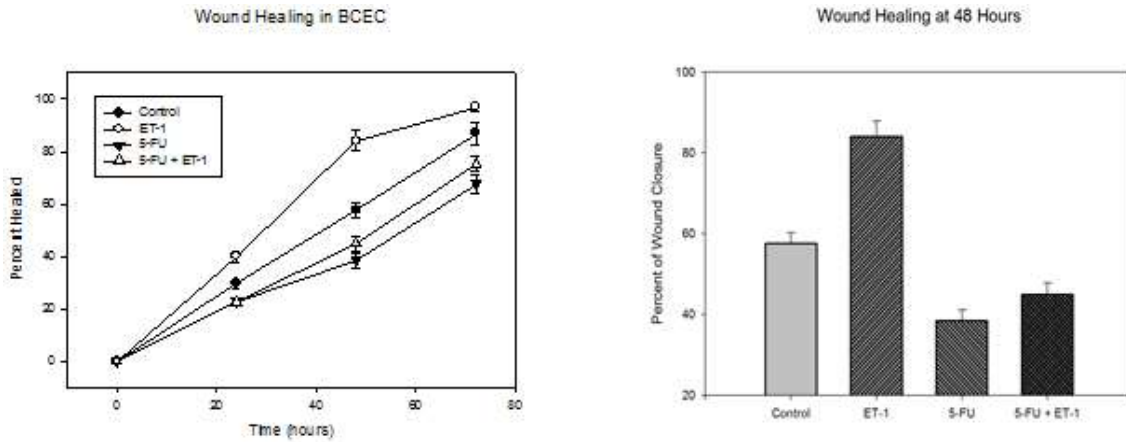


Figure 5.1 – Endothelin-1 Promotes Wound Closure in BCEC. Incubation with 10nM ET-1 for 72h shows enhanced wound healing in BCEC cultures. Error bars are SEM, n = 18 wells from 3 separate experiments. Confluent BCEC were mechanically wounded and allowed to heal for 48h in a serum-free medium in the presence or absence of ET-1 or 5-fluorouracil. ANOVA $p < 0.05$. (Source: Frazier, L.)

The intracellular pathway by which corneal cell proliferation occurs after treatment with ET-1 is not well understood. Wu *et. al* showed an increase in corneal endothelial proliferation to be linked to an increase in intracellular calcium concentration and stimulation of inositol phosphatases.^{21,22} It has also been shown that after ET-1 treatment there is a significant decrease in the expression of p27, a proliferative inhibitor that, unless degraded following phosphorylation, arrests BCECs in a G1 state.²³ The pathway through which these events happen has not been identified.

CHAPTER 6

MAPK & ERK1/2

MAPK Pathway

The mitogen activated protein kinase (MAPK) pathway is activated by mitogenic stimuli and is characterized by sequential phosphorylation of cytoplasmic protein kinases. The MAPK pathway has been found to play roles in inflammatory responses, differentiation, and proliferation among others. The MAPK pathway has also been found to lead to a decrease in p27 levels.²⁴ Therefore, for the present study, the MAPK pathway was selected as a potential candidate for ET-1 induced BCEC proliferation.

Erk 1/2

Extracellular regulated kinase 1 and 2 (Erk 1/2) are related protein-serine/threonine kinases. Erk 1/2 has been identified as a signal transducer in the MAPK pathway and is readily detectable in mammalian models. The phosphorylation of Erk 1/2 (pErk 1/2) at the tyrosine and threonine sites is required for enzyme activation.²⁵ Erk 1/2 has been identified as a MAPK playing a role in cell cycle regulation via the phosphorylation of p27, which was previously shown to be down regulated in ET-1 treated BCECs.²⁶ Therefore, we hypothesized that Erk 1/2, as a kinase in the MAPK pathway, plays a key role in ET-1 induced BCEC proliferation.

In this study, Erk 1/2 and pErk 1/2 are studied via western blot analysis after treatment of BCEC with ET-1 and a MAPK inhibitor. Three treatment groups are

established: control cells (untreated), ET-1 treated cells, and ET-1 + PD98059 (MAPK pathway inhibitor) treated cells. If ET-1 does indeed stimulate cell proliferation through the MAPK pathway, a decrease in ET-1 stimulated phosphorylation of Erk 1/2 will be observed after treatment with the MAPK inhibitor. If another pathway were to be responsible for the observed proliferation, there should be no change in the ET-1 stimulation of Erk 1/2 phosphorylation, even after MAPK inhibition.

CHAPTER 7

MATERIALS AND METHODS

Isolation and Culture of Bovine Corneal Endothelial Cells:

Bovine eyes were obtained from Kirby and Poe slaughterhouse in Alvaton, KY. Excess tissue (muscle, fat and fascia) around the globe were trimmed and the eye was then placed cornea side up in a shallow dish lined with sterile gauze. The eyeballs were covered with sterile gauze and soaked using 2X antibiotic solution (100X stock pen/strep/amphotericin; Invitrogen, Carlsbad, CA) for 30 minutes at room temperature. The cornea was then dissected leaving a 1-2 mm sclera rim and transferred to a sterile eyecup with the endothelium side up. The endothelium was incubated with Dispase (0.025g/mL, Roche) dissolved in EBSS (Earle's Balanced Salt Solution) for 90 min at 37°C. After incubation with the Dispase enzyme, endothelial cells were gently scraped off into solution using a silicon surgical rubber spatula. Cells were then aspirated with a sterile Pasteur pipette and transferred to a 15-mL tube containing 5 mL DMEM (Dulbecco's Modified Eagle Medium). The cells were pelleted by centrifugation at 600xg for 2 minutes. The supernatant was discarded and the pellet of cells was re-suspended with 5 mL of growth medium. Cells were transferred to T-25 flasks and fed three times per week with growth medium (DMEM) supplemented with 10% iron supplemented calf serum (HyClone) and 500 µg gentamicin (antibiotic) and 100 µL fungizone (antimycotic) for each 100mL of complete growth medium. Cultures were maintained in a CO₂

incubator at 37°C and 5% CO₂. Trypsin, 0.05% dissolved in calcium free EBSS, was used to subculture cells. The second passage cells were used for all experiments.

Endothelin-1 Stock Preparation

Endothelin-1 was obtained from Sigma Chemical, St. Louis, MO. The 20 μM ET-1 stock was prepared in dilute acetic acid. One drop of glacial acetic acid was added to 15 mL of nano-pure water, 100 μL of the diluted acetic acid was then added to 10 mL of nano-pure water and was sterile filtered. 1 mL of dilute acid was added to 50 μg of ET-1 and 25 μL ET-1 aliquots were prepared and stored at -20°C.

Antibodies

P44/42 MAPK (Erk 1/2) (catalog #9102) and phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) (catalog #9101) were purchased from Cell Signaling Technology. The source of p44/42 MAPK (Erk 1/2) antibody is rabbit. The source of the Phospho-p44/p42 MAPK antibody is rabbit and it detects the endogenous levels of p44 and p42 MAP kinases when phosphorylated either individually or dually at Thr 202 and Tyr204 of Erk 1 and Thr185 23 and Tyr187 of Erk 2. It does not cross react with non-phosphorylated Erk 1/2.

ET-1 and MAPK Inhibitor treatment in BCEC

BCECs were grown to 100% confluence in T-75 flasks and serum starved in DMEM for 24 hours to induce quiescence. BCECs were then treated with 10 nM ET-1 and incubated for 30 minutes. A MAPK pathway inhibitor (PD98059) was used 30 minutes prior to ET-1 treatment in one flask. Control cells were left untreated in serum-free DMEM. Protein samples were collected from control, ET-1, and MAPK Inhibitor treated cells.

Isolation of BCEC Protein

After appropriate treatment, cells were rinsed twice with cold EBSS for 5 min. 2 mL of ice cold RIPA buffer (25 mM Tris•HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor (1:500) and PMSF (1mM) was then added to the T-75 flasks. The flasks were then incubated on ice for 5 min. Flasks were swirled occasionally on ice during incubation. Using a rubber policeman, cells were scraped into a 15-mL conical blue cap tube. To increase yield, the pellet was sonicated for 10 seconds with 20% amplitude. Samples were then kept at -20°C for further experimentation.

Estimation of Protein Concentration:

Protein concentration was determined according to the Peterson modification of the Lowry protein determination assay. Samples and BSA standards (10-100 µg) were pipetted into cuvettes. Volumes of all samples and standards were brought up to 0.5 mL with dH₂O. 1 mL of reagent A (equal volumes of copper-tartrate-carbonate solution, 10% SDS, 0.8N NaOH and dH₂O) was added to all tubes, vortexed immediately, and left for 10 minutes at room temperature. Following the incubation with reagent A, 0.5 mL of reagent B (one part of Folin-Ciocalteu Phenol Reagent to 5 parts of dH₂O) was added to all tubes and incubated for 25 minutes. Absorbance of standards and samples was read at 750 nm wavelength. The protein concentration of samples was calculated from the standard curve of BSA.

SDS-PAGE and Western Blotting

A 4X sample loading buffer (BioRad) was added in a 1:3 ratio (1 part sample buffer: 3 parts sample) to each sample in 1.5-mL centrifuge tube and the mixture was

heated for 5 minutes with 2-mercaptoethanol (2%). An equal mass of sample (20 μ g) was loaded in each well of an Any kD mini protean pre-cast gel (Bio-Rad), and the proteins were separated at constant voltage (150 V) for 1 hour. 5 μ L of a protein molecular weight standard (Bio-Rad) was loaded in two wells of the gel. During electrophoresis, a sheet of PVDF was soaked for 30 minutes in methanol. After electrophoresis the gel was washed in 15 mL of 1X transfer buffer (30.3 g Tris-base, 144.1 g glycine, water to 1 liter for 10X) on a shaker at room temperature. The PVDF membrane and filter papers were wetted in transfer buffer and a blot sandwich was prepared in the following sequence from black side of the cassette to red side of the cassette: filter pad, filter paper, gel, PVDF membrane, filter paper and filter pad. Bubbles were removed by rolling a glass rod over the sandwich. The gel holder cassette was then closed and placed into the transfer tank filled with 1X transfer buffer. Proteins were transferred at a constant 100 V for 2.5 hours at 4°C.

A Pierce Fast Western Blotting kit was used for protein detection and quantification. After transfer, the PVDF was removed from the transfer apparatus, split into two symmetrical blots, and placed in a clean tray. The blot was incubated with primary antibody from Cell Signaling Technology p44/42 MAPK (Erk 1/2) Cat. No. 9101S and phospho-p44/42 MAPK (Erk 1/2) Cat. No. 9102S for 90 minutes at RT or overnight at 4°C. The primary antibodies Erk1/2 and pErk 1/2 were diluted (1:1000) in the Fast Western Antibody Diluent, and poured on respective halves of the blot in clean trays. The blots were then removed from the primary solution, and placed in clean trays with Fast Western Optimized HRP Reagent Secondary Working Dilution for a 15 minute incubation. The trays were shaken intermittently during this incubation period. The blots

were then removed from the secondary solution, placed in clean incubation trays, and washed 3 times with 20 mL of Fast Western 1X Wash Buffer with agitation. After the third wash, the blots were placed in a clean tray and 12 mL of the Detection Reagent Working Solution was added for a 5 minute incubation period. The blots were then removed and dried. Images of fluorescent bands were taken on an Alpha Innotech FlourChem HD2 (San Leandro, CA). Densitometry analysis for the bands was done using ImageJ.

Trachoma Occurrence Survey Methods

Institutional Review Board approval was obtained from Western Kentucky University on 12/5/13 (IRB 14-212) (Appendix 1). Three villages of the Kasigau region of Kenya, Rukanga, Bhugata, and Makwasinyi, were used as sample populations. Patients who arrived to the clinic at which the surveyor was stationed were directed to the surveyor. An informed consent document (Appendix 2) was read to each patient detailing the purpose of the project, the risks of the project, and the anonymity of the project. After the informed consent document, the patient was asked if they desired to continue. If so, the investigator gently everted the participant's upper eyelids by holding up the upper eyelid with eyelashes between thumb and forefinger, and bending the eyelid over a cotton-tipped applicator. Both upper tarsal regions of each eye were examined for symptoms of trachoma. Participant symptoms were compared to a grading card provided by the WHO illustrating each stage of trachoma (Figure 7.1). The grade of trachoma was noted on a unique specimen form for each participant.

**SPECIMEN FORM FOR REGISTRATION OF DATA
FOR THE EPIDEMIOLOGICAL SURVEY OF TRACHOMA**

EPIDEMIOLOGICAL SURVEY OF TRACHOMA

Examiner: _____

Village: _____
Household no.: _____
Sex: _____

ENTER ONE OF THE TWO FOLLOWING SYMBOLS FOR EACH SIGN

0 = Absent
1 = Present

	TT	CO	TF	TI	TS
Right eye					
Left eye					

Figure 7.1 Trachoma Survey Specimen Form A form proved by the World Health Organization to grade individuals participating in an epidemiological survey on the stage of trachoma which the participants present. (Source: WHO)

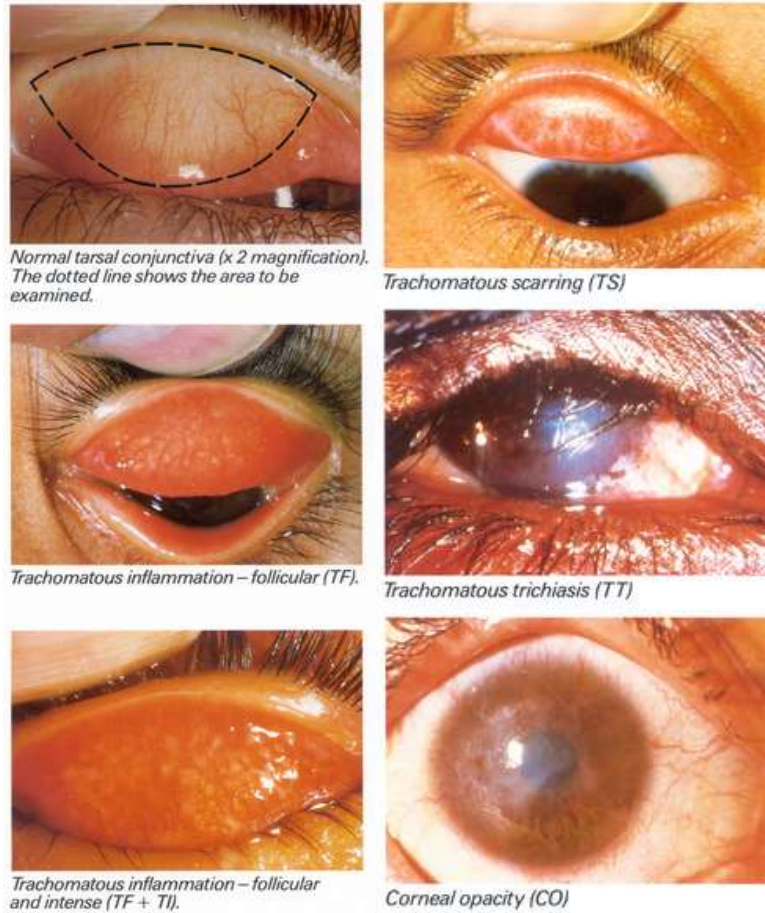


Figure 7.2 WHO Trachoma Grading Card A normal upper tarsal region is shown in conjunction with the five stages of trachoma, as developed by the WHO. Infected individuals will initially show minor inflammation, but this will progress to scarring and ultimately corneal cloudiness if untreated. (Source: WHO)

CHAPTER 8

RESULTS

Trachoma occurrence is non-remarkable in Kasigau, Kenya

Eighteen participants consented to the trachoma occurrence survey in three different villages, 5 from Makwasinyi, 9 from Rukanga, and 4 from Bhugata. Of the 18 participants, 9 were asymptomatic (Figure 8.1). Three participants showed corneal opacities.

Village	Mak.	Mak.	Mak.	Mak.	Mak.	Ruk.	Ruk.	Ruk.	Ruk.	Ruk.	Ruk.	Ruk.	Ruk.	Bhu.	Bhu.	Bhu.	Bhu.	
Participant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sex	M	M	F	F	M	F	M	M	M	M	M	F	F	M	F	F	F	F
OD																		
TS	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
TI	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1
TF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CO	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
TT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OS																		
TS	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
TI	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
TF	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
CO	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
TT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 8.1 Trachoma Occurrence Data from 3 Villages in Kasigau, Kenya. Makwasinyi (Mak), Rukanga (Ruk), and Bhugata (Bhu), were surveyed for trachoma. Trachomatous inflammation, follicular (TF)—Five or more follicles of >0.5 mm on the upper tarsal conjunctiva, Trachomatous inflammation, intense (TI)—Papillary hypertrophy and inflammatory thickening of the upper tarsal conjunctiva obscuring more than half the deep tarsal vessels, Trachomatous scarring (TS)—Presence of scarring in tarsal conjunctiva, Corneal opacity (CO)—Corneal opacity blurring part of the pupil margin, Trachomatous trichiasis (TT)—At least one ingrown eyelash touching the globe, or evidence of epilation (eyelash removal). OD-Right Eye, OS- Left Eye.

Endothelin-1 increases phosphorylation of ERK1/2

It was hypothesized that ET-1 induced cellular responses involves the MAPK pathway by phosphorylation of Erk 1/2. To determine whether ET-1 treated cells exhibit enhanced phosphorylation of Erk 1/2, serum-starved cells were treated with 10 nM ET-1 for 24 hrs. Protein samples were then prepared from untreated and ET-1 treated cells to analyze the phosphorylated Erk 1/2 levels through western blotting (Figure 8.2). Densitometry analysis revealed that ET-1 induced a significant 5.1-fold increase in phosphorylation of ERK compared to control ($p < 0.05$, $n = 3$).

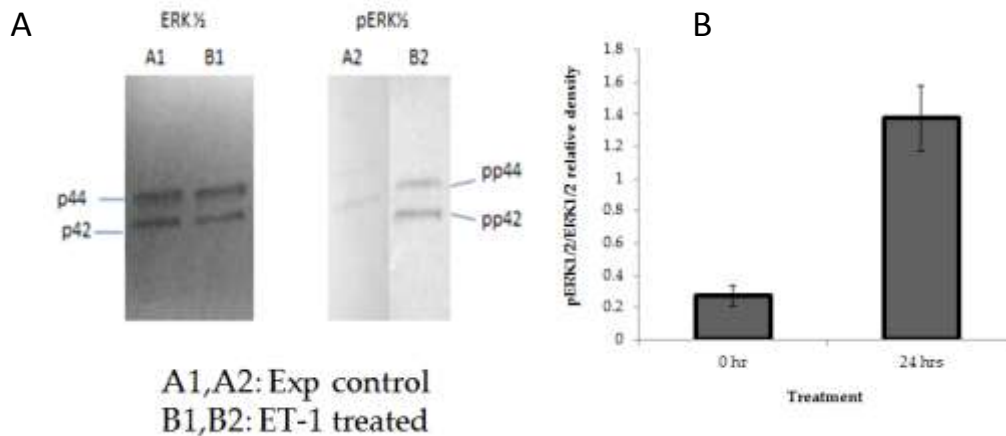


Figure 8.2 Endothelin-1 Increases pErk1/2 Expression A: Western blotting results of Control BCEC (A1, A2) and 10 nM ET-1 treated BCEC (B1, B2). B: Band densities were measured using the software (Alpha Innotech Imager) for Erk 1/2 and pErk 1/2. For each treatment we calculated the relative density of pErk to Erk .

pErk 1/2 expression is significantly attenuated after treatment with a MAPK pathway inhibitor.

It was hypothesized that inhibition of the MAPK pathway would lead to attenuation of expression in phosphorylated Erk 1/2. Treatment with 10nM PD98059

(Cell Signaling, Cat #9900, MAPK pathway inhibitor drug) led to a decrease by a factor of .37 in pErk1/2 expression (Figure 8.3) ($p < 0.05$, $n = 3$).



Figure 8.3 Inhibition of the MAPK Pathway Causes Attenuation of pErk1/2 Expression Western blotting results of Control BCEC, 10nM ET-1 treated BCEC, and 10nM PD98059 (MAPK Inhibitor) + 10nM ET-1 treated BCEC.

The ratios of Erk 1/2 expression from ET-1 treated cells and MAPK pathway inhibited cells were calculated and plotted for the three different experiments (Figure 8.4). The same was done for pErk 1/2 expression. pErk 1/2 expression shown as a ratio of its expression in MAPK inhibited cells to ET-1 treated cells was significantly lower than the ratio calculated for Erk 1/2 expression. ($p < 0.05$, $n = 3$).

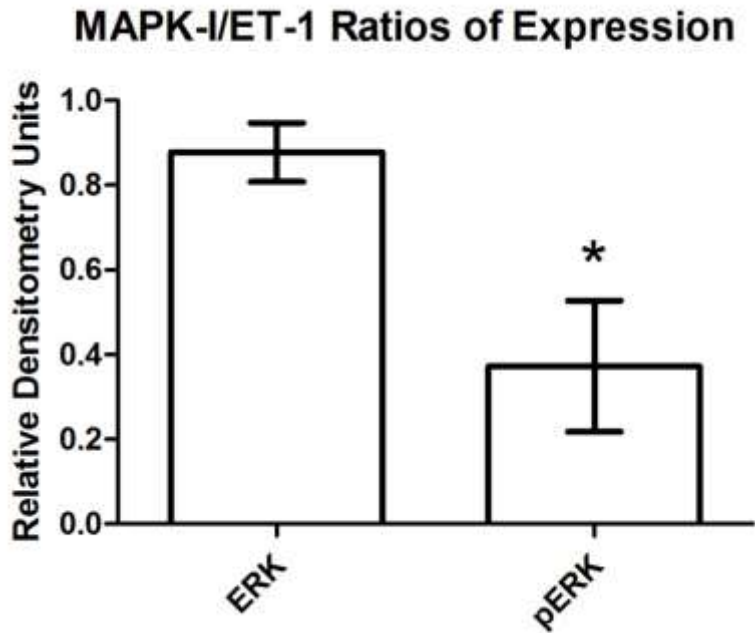


Figure 8.4 MAPK Pathway Inhibited Cells Express Significantly Less pERK1/2 than ET-1 Treated Cells Extra cellular regulated kinase 1 and 2 (ERK) and phosphorylated extra cellular regulated kinase 1 and 2 expression was quantified via western blot analysis. The ratios of ERK1/2 and pERK expression in MAPK Pathway inhibited (MAPK-I) cells and ET-1 treated (ET-1) cells was plotted. Unpaired t-test ($p=0.0203$). $n=3$.

Results were then normalized by dividing pErk 1/2 expression by Erk 1/2 expression (relative densitometry units) in each of the sample treatments (Figure 8.4). The MAPK pathway inhibited cells showed a significantly lower ratio of pErk 1/2 / Erk 1/2 expression compared to ET-1 treated cells. ($p<0.05$, $n=3$).

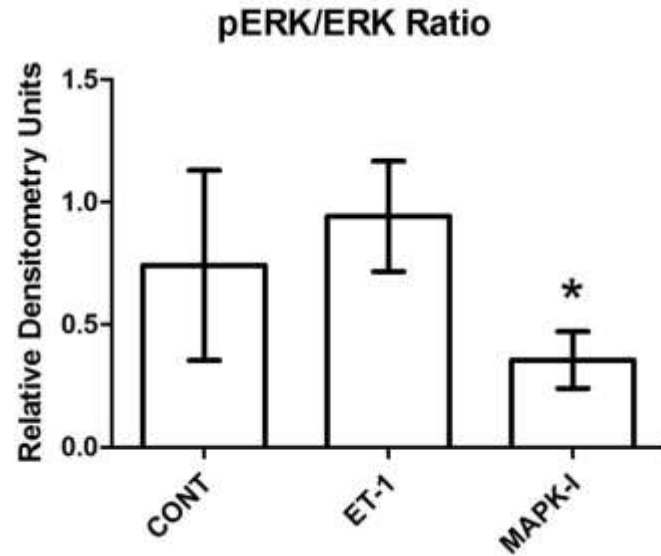


Figure 8.5 MAPK Inhibited Cells Express a Significantly Lower Ratio of pErk1/2/Erk1/2 than Control and ET-1 Treated Cells Levels of Erk1/2 and pErk1/2 expression were quantified via western blotting and analysis by ImageJ software. Cells treated with a MAPK Inhibitor (MAPK-I) showed a significantly lower ratio of pErk1/2 to Erk1/2 than cells treated with Endothelin-1 (ET-1). Unpaired t-test ($p=0.0408$). $n=3$.

CHAPTER 9

DISCUSSION

Erk 1/2 and the MAPK Pathway are Key Players in ET-1 induced BCEC Events

These results suggest that Erk 1/2 plays a key role in ET-1 induced BCEC intracellular events as it is seen to be phosphorylated, seen by the significant increase in pErk1/2 expression from the western blot. Similarly, the MAPK pathway plays a key role, since inhibition of the pathway causes attenuation of phosphorylation of Erk 1/2.

These results do not rule out the contribution of another pathway/pathways in ET-1 stimulation of cell proliferation. Further investigation should be done via proliferative assays after treatment with the MAPK inhibitor drug. If the current results and hypothesis hold true, a decrease in proliferative activity should be observed at the cell level after treatment with an ET-1 and MAPK pathway inhibitor cocktail.

Cause of Corneal Opacities in Trachoma Infected Individuals

Trachoma has been traditionally thought to cause corneal opacities via eyelash abrasion on the corneal surface after trichiasis. However, many studies have been done that show a biological rather than mechanical insult to the cornea. Oh and Tarizzo demonstrated that inoculation of rabbit eyes with trachoma in the anterior chamber led to diffuse lesion formation in the corneal endothelium (Figure 8.1), which was directly related to the rabbit corneal opacities that resulted after trachoma inoculation.²⁷ This

finding is in agreement with the understanding of the barrier function of the corneal endothelium. If lesions (irregular endothelial cell shape and size) in the corneal endothelium were to form, barrier function would be compromised and solutes would flow into the corneal stroma unimpeded causing corneal opacities, which is indeed what the authors observed.

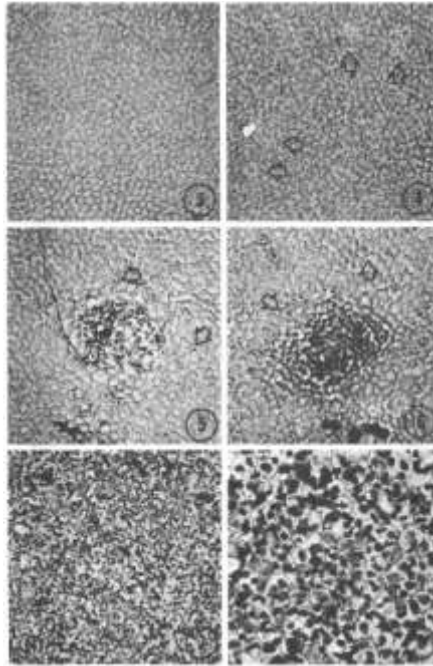


Figure 8.1 Corneal Endothelial Insults after Trachoma Inoculation 3) Corneal endothelium of normal rabbit. 4) Endothelial rosettes (arrows). 5-6) Discrete lesion. 7) Diffuse lesions. 8) Higher magnification of 7. All Silver and Giemsa Stained Source: (Oh & Tarizzo, 1969).

The mechanism by which these corneal endothelial insults occur has also been investigated. Shivanna *et. al* discovered that TNF- α , a proinflammatory cytokine, will decrease corneal endothelial barrier function. The authors saw a continual decrease in transendothelial electric resistance after TNF- α treatment of BCEC, showing the barrier dysfunction. The authors also saw a breakdown of the cytoskeletal proteins cadherin and ZO-1 after TNF- α treatment. Interestingly, these reactions were found to be mediated by a MAPK protein, p38. The authors were able to inhibit this TNF- α induced barrier

dysfunction after treatment with a p38 inhibitor. TNF- α was linked to Trachoma in a study done by Conway *et. al.* The authors found high TNF- α tear fluid levels to be highly associated with trachoma infection ($p < 0.001$) and suggested that TNF- α plays a major role in the pathogenesis of scarring trachoma. Other cytokines such as IL-1 β , IL-10, IFN- γ and MMP-9 have also been shown to be closely associated with trachoma as well as corneal endothelial apoptosis.^{28,29,30}

Therefore, it is plausible to hypothesize that corneal opacities in trachoma-infected individuals occur not only via the mechanical scrapping of eyelashes that results from trichiasis, but also via the degradation of the corneal endothelium by proinflammatory cytokines such as TNF- α that are released from the site of infection. As the barrier function of the corneal endothelium is indispensable in maintaining corneal clarity, this degradation would certainly lead to an increase in water and solute flow into the corneal stroma which would lead to the corneal opacities observed in untreated individuals with trachoma.

Potential for ET-1 Therapy in Corneal Endothelial Disease

As ET-1 has been shown to increase corneal endothelial density, it could be a likely candidate for an alternative therapy to surgery. Individuals in early stage trachoma infection may be able to take ET-1 treatments preventatively, in order to impede the infection from affecting the cornea via cytokine degradation. Similarly, individuals with a genetic predisposition for FED may be able to preventatively be treated with ET-1 to maintain a proper corneal endothelial density.

The mechanism for treatment of ET-1 is certainly a ripe topic for discussion and investigation. As ET-1 promotes cell division and vasoconstriction, specific targeting of the drug would be vital, as uncontrolled cell division could lead to cancerous activity. Luckily, the corneal endothelium is avascular. Therefore, treatments directly into the cornea or anterior chamber would be less hazardous in terms of getting into the vasculature than an intravenous treatment. Microinjection of ET-1 into an individual's cornea or anterior chamber may be a plausible route of treatment.

Small Incidence of Trachoma in Kasigau, Kenya

Of the 18 participants surveyed for trachoma in three villages of Kasigau, half were showed to be completely asymptomatic, while the other half showed minimal symptoms such as small inflammation and follicles. One reason for this may be that the participants have proper access to antibiotic treatment. All three villages were found to have tetracycline eye drops in stock at their respective medical dispensaries. Tetracycline has been shown to treat trachoma effectively.³¹ Therefore, it is likely that these villages are adequately equipped to treat trachoma and similar eye infections.

However, the study done was rather limited both in sample size and time spent at the sites. The participants were pseudo-selected, as all voluntarily came to the clinic, often for other reasons than an eye symptom. House to house paneling would be, and is used as, a more effective method for trachoma prevalence surveying. It is important to note that the survey done was simply a pilot study, and further surveying and epidemiological intervention should be put forth in order to adequately assess this region's need for antibiotic distribution.

CHAPTER 10

CONCLUSION

In conclusion, it was found that 10nM ET-1 treatment increases phosphorylation of Erk 1/2 in BCEC. This increase in pErk 1/2 expression occurs within the MAPK pathway, as inhibition of the pathway leads to an attenuation of pErk 1/2. As ET-1 increases BCEC proliferation, it is likely that the MAPK pathway is a key player in the reaction after ET-1 treatment. This work represents one more resolved piece of the puzzle of designing an alternative therapy to surgery for clouded corneas. These results may help to decrease the number of the some 40,000 Americans undergoing costly corneal transplantation each year. In the rural United States, and underprivileged countries, access to surgery is severely limited. Therefore, these results potentially could be used to develop a non-surgical and readily available solution to a compromised cornea.

In order to gauge the occurrence of trachoma in Kasigua, Kenya, an epidemiological survey was conducted in three villages in the area. Half of the participants in the survey showed no symptoms of trachoma whatsoever, while the other half displayed minor symptoms. Therefore, the occurrence of Trachoma in the Kasigau region of the Taita Taveta district of Kenya, according to this study, is unremarkable. This is likely due to the access to antibiotic therapies in the medical dispensaries of each village.

Appendix 1 : WKU IRB Approval



INSTITUTIONAL REVIEW BOARD
OFFICE OF RESEARCH INTEGRITY

DATE: December 5, 2013

TO: Brandon Farmer
FROM: Western Kentucky University (WKU) IRB

PROJECT TITLE: [544887-1] A Pilot Study of the Occurrence of Trachoma in Kasigau, Kenya
REFERENCE #: IRB 14-212
SUBMISSION TYPE: New Project

ACTION: APPROVED
APPROVAL DATE: December 5, 2013
EXPIRATION DATE: January 31, 2014
REVIEW TYPE: Expedited Review

Thank you for your submission of New Project materials for this project. The Western Kentucky University (WKU) IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a project design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding followed by an implied consent form. Informed consent must continue throughout the project via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All UNANTICIPATED PROBLEMS involving risks to subjects or others and SERIOUS and UNEXPECTED adverse events must be reported promptly to this office. Please use the appropriate reporting forms for this procedure. All FDA and sponsor reporting requirements should also be followed.

All NON-COMPLIANCE issues or COMPLAINTS regarding this project must be reported promptly to this office.

This project has been determined to be a Minimal Risk project. Based on the risks, this project requires continuing review by this committee on an annual basis. Please use the appropriate forms for this procedure. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of January 31, 2014.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Paul Mooney at (270) 745-2129 or irb@wku.edu. Please include your project title and reference number in all correspondence with this committee.

Appendix 2 : Informed Consent Document

INFORMED CONSENT DOCUMENT

Project Title: A Pilot Study of the Occurrence of Trachoma in Kasigau, Kenya

Investigator: Brandon C. Farmer, brandon.farmer396@topper.wku.edu or 859-230-0999

You are being asked to participate in a product conducted through Western Kentucky University. The University requires that you give your agreement to participate in this project. We will explain the project to you in detail including the purpose of the procedures to be used, and the potential benefits and possible risks of participation. You may ask any questions you have to help you understand the project.

This project is designed to note the occurrence of the eye disease Trachoma in the Kasigau aread of Kenya. The main objectives of this project is to determine if the majority of eye complications observed in the villages of Bhugata, Makwasinyi, and Rukanga are a result of the infections and preventable disease of Trachoma.

For this project you will be asked to give basic information including your village, age, and sex. The examiner will then examine your eye for symptoms of Trachoma. This will include the gentle eversion of both upper eyelids, as this is the site of infection. This may cause slight discomfort. The eyelid will be re-everted after the examination is complete. The information collected will be used to enhance the health and well-being of the people of Kasigau.

All participants will be assigned a number to maintain anonymity. No names will be recorded or presented in the data.

This survey will demonstrate the need for further surveying and involvement in this area, hopefully leading to the distribution of antibiotics and Trachoma prevention and cure.

Refusal to participate in this study will have no effect on any future services you may be entitled to from the University. Anyone who agrees to participate in this study is free to withdraw from the study at any time with no penalty.

You understand also that it is not possible to identify all potential risks in an experimental procedure, and you believe that reasonable safeguards have been taken to minimize both the known and potential but unknown risks.

*Your continued participation in the study will imply your consent.
Do you wish to continue?*

THE DATED APPROVAL ON THIS CONSENT FORM INDICATES THAT
THIS PROJECT HAS BEEN REVIEWED AND APPROVED BY
THE WESTERN KENTUCKY UNIVERSITY INSTITUTIONAL REVIEW BOARD
Paul Mooney, Human Protections Administrator
TELEPHONE: (270) 745-2129



WKU IRB# 14-212
Approval - 12/5/2013
End Date - 1/31/2014
Expedited
Original - 12/5/2013

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