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14. ABSTRACT (200 words) This is our annual report for the Sealing Penetrating Eye Injuries using Photoactivated Bonding project. Due to delays in renovations to the site the work is to take place, we have been unable to start research. We are now preparing by obtaining the green laser and starting to hire staff to conduct the research. The overall goal of this project is to develop a light-activated technology to improve the surgical closures of traumatic eye injuries, with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. The scope of the research includes evaluating two light-activated approaches- one where the amniotic membrane is stained with dyed and treated with green light, and another where the dye is applied to the wound walls before being activated by green light to close the wound.					
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INTRODUCTION

The overall goal of this research is to develop a light-activated technology to improve the surgical closures of traumatic eye injuries with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries.

Fragments and debris propelled at high velocity by improvised explosive devices (IEDs) have increased the incidence of penetrating eye injuries in the current conflicts compared to earlier wars. Rapid closure of penetrating eye wounds with formation of a water tight seal is critical to preventing infection and stabilizing the eye for further surgery, thus improving vision outcomes. Suturing the cornea, sclera and eyelid skin requires specialized training to precisely place hair-fine sutures and requires long surgery time. When flat objects enter the eye through the cornea it shears the corneal lamella making suture closure impossible. In this case adjuvants to sutures are used to ensure a watertight closure at the first surgical intervention.

Cyanoacrylate glues, frequently used, can complicate further surgery by sticking to sutures and possibly causing additional damage when removed. It breaks down to formaldehyde which can be tough on the cornea. Additionally it obscures the view to the retina and must be removed before reconstructive surgery can be accomplished. If the glue is stuck to sutures, then the sutures are likewise removed possibly reopening the original injury. Resulting in a much more complicated situation.

Our sutureless, glueless method (1-4) is rapid and uses currently FDA-allowed devices (clinical laser, light-activated dye, amniotic membrane) and thus may move rapidly to the deployment environment.

The scope of the research includes evaluating two light-activated approaches to closing penetrating injuries in the cornea and sclera of rabbit eyes. In one method, amniotic membrane is stained with the dye, placed over the wound and treated with green light; in the other, the dye is applied to the wound walls and activated by green light to directly close the wound.

BODY

This research project is a collaboration between military medical investigators who treat battlefield eye wounds, a laboratory team who developed the light-activated treatment and investigators who have extensive experience in laser eye safety. This Grant Agreement is a joint proposal with Col Anthony J. Johnson, MD at Brooke Army Medical Center (BAMC) who is PI on Grant Agreement # WB1WH-09-2-0069. The Statement of Work includes tasks to be carried at both the Massachusetts General Hospital (MGH) and the BAMC. Dr. Kochevar and Dr. Johnson have discussed this project frequently by phone and during Dr. Johnson's visit to Dr.

Kochevar's lab in Boston in April, he provided ophthalmologic expertise during an early in vivo study.

Task 1. Evaluate photoactivated bonding for sealing amniotic membrane over corneal lacerations

1.a. Obtain IACUC and ACURO approvals Approvals were obtained on July 31, 2009 (IACUC) and September 4, 2009 (ACURO) for studies at MGH and on March 6, 2009 (IACUC) and April 16, 2010 (ACURO) for studies at BAMC.

1.b. The Wellman Group has establish parameters for strong bonding of amniotic membrane to cornea of rabbit eyes

The model development phase outlined below has been delayed due to BRAC construction but will begin earnestly in Jan 2011.

The model development phase:

Experiments:

The experiments will occur in 2 phases, first a model development phase in which 6 rabbits will be used, and then an experimental phase involving 3 separate experiments in which a total of 130 rabbits will be used.

Model Development

Goal

1) Determine the amount of fibrin glue and the optimal technique of applying amniotic membrane for corneal sealing

2) Develop technique for making corneal laceration without damaging the rabbit's lens.

3) Develop technique to applying rose Bengal to the laceration while minimizing endothelial exposure

4) Verify our ability to reproduce the Saad staining technique, and ensure it does not adversely affect the pathologic evaluation of inflammation

This experiment will require 6 rabbits/group x 1 group for a total of 6 rabbits.

The rabbit will be anesthetized with isoflurane, (masked with isoflurane until jaw tone is relaxed, then endotracheal intubation will be accomplished with a surgical plane of anesthesia using isoflurane 1-3%, delivered in room air), then prepped and draped in a sterile fashion. Additional drops of topical tetravisc will be placed for additional anesthesia. A lid speculum will be placed in one eye. A template will be placed on the cornea and outlined with a skin-marking pen. The cornea thickness will be verified by pachymeter (ultrasonic device to measure corneal thickness- uses sound waves bounced off the layers of the cornea), then a V shaped laceration with a diamond knife will be placed in the central cornea.

If the rabbits anterior chamber collapses (loses fluid from front of eye between cornea and iris (colored portion surrounding the pupil)) rapidly leading to lenticular damage with the diamond blade, the technique will be varied to include use of viscoelastic (gel like space maintainer composed of chondroitin sulfate and hyaluronate) to deepen the anterior chamber (fluid filled chamber in the front of the eye- above the iris) after one arm of the corneal laceration is placed. (The viscoelastic gel comes in a syringe with a fine cannula. The cannula is placed in the wound and the viscoelastic is injected into the eyeball. The placement of the second arm of the X or V shaped laceration will then be placed under viscoelastic protection. (The X or V laceration is made by 2 linear cuts on the cornea, the mobility in the cornea caused by the effects of the incision may allow fluid to escape causing the front of the eye to collapse, if this occurs a space maintainer (viscoelastic) may be needed after the first incision to maintain eyeball integrity so the second incision does not collapse part of the eye. Fine linear lacerations of the cornea with a diamond blade are frequently self sealing if there is no pressure on the globe, more complex lacerations are more likely to leak.) After the laceration is place the viscoelastic will be rinsed out of the eye to ensure it does not confound our eye pressure measurements, or cause pain to the animal from elevated intraocular pressure. (The thickness of the viscoelastic makes it exit from the eye slower causing fluid backup and elevated eye pressures which can be painful if significantly elevated, by clogging the manometer it can lead to artificially elevated IOP measurements.)

Then:

- 1) For 2 rabbits a 16mm unstained amniotic membrane disc stromal side down will be placed on the cornea that drapes over the limbus and sutured to the episclera.
- 2) For 2 rabbits, a micropipette will be attached to a TB syringe and a 30-gauge needle to deliver stromal rose bengal to the cornea. The technique will initially be perfected in cadaveric eyes, and then once perfected will be used in live rabbits (we will require live animals to evaluated the Saad technique described above)
- 3) For 2 rabbits 10mm disc of amniotic membrane will be placed over the cornea and sutured in place with a purse-string suture

In the above experiments, if we are unable to obtain a watertight seal with minimal pressure with an amniotic membrane sutured to the eye, then 2 drops of fibrin glue will be placed on the cornea followed by the amniotic membrane disc. The amniotic membrane will be flattened with a muscle hook prior to suture placement.

After the amniotic membrane is in place, a 27 G needle is inserted parallel to the iris, 2 mm above the limbus into clear cornea and positioned above the lens. The needle is connected to both a calibrated blood pressure transducer and an infuser via a T-coupler. IOP is gradually increased by infusion of saline (containing a blue dye for visualization) into the anterior chamber. The signal generated by the transducer-amplifier combination is proportional to the IOP, and is calibrated using a manometer. The IOP is increased until either leakage from the sealed amnion is apparent or the maximum pressure reading of 100 mm Hg is attained. This is recorded as the leak pressure (IOPL).

After the endpoint is reached the iop will be lowered to physiologic pressure. This can be accomplished by removing the iop measuring apparatus and attaching a tuberculin syringe with the plunger removed. This will equilibrate the eye at 12 mmhg. The IOP will be verified by tonopen (portable device to measure eye pressure by it's ability to indent the cornea). Then sub-conjunctiva antibiotics consisting of 0.2 ml of ancef (50 mg/ml) and dexamethosone (4 mg/ml) will be injected sub-conjunctively. Then the speculum will be removed.

The rabbit will be allowed to awake from anesthesia.

The rabbit will be monitored that evening, Signs of pain and/or distress to be monitored will include, but not be limited to, the following parameters: activity level (e.g., restlessness, hunched posture, huddled, lethargy, etc.), vocalization, self-trauma, change in behavior from preoperative observations, body condition (e.g., loss of body weight/emaciation, dehydration, lack of grooming, congested mucous membranes, etc.), lameness, neurological signs (e.g., tremors, convulsion, paralysis/paresis, head tilt, etc.), or dyspnea. If observed, we will consult with the staff veterinarian.

Topical antibiotics will be placed 3x per day. The rabbits will be examined every day for infection and pain.

Every other day for the first 2 weeks, the rabbits will be removed from their cages and taken to the procedure room. The rabbits will be placed in the photo apparatus and examined using a portable slit lamp and photos to evaluate the presence of epithelial cells over the amniotic membrane. The re-epithelialized (unstained) area will be analyzed from digital camera images (area calculated with NIH image software). Photographs with digital camera will be taken, every other day for 2 weeks and at weeks 3, and 4.

The rabbits will be monitored throughout the process for stress. (Restlessness, vocalization, dyspnea if observed will be reported to the veterinarian and IM ketamine will be administered to ensure the rabbit does not experience undue stress.



(Photos provided by Dr. Mah- University of Pittsburgh)

The rabbit will then be returned to its cage.

After 28 days, the amniotic membrane will be removed for analysis, and the cornea will be evaluated with Fluorescein staining and photographed. The corneal button will be removed and evaluated for endothelial staining and photographed, then submitted for pathologic evaluation of inflammation.

Experimental phase

Experiment #1a

Goals:

Compare corneal crosslinking with amniotic membrane and four alternate therapies, in their ability to repair a simple corneal laceration in a watertight fashion. We will compare each modalities ability to maintain a normal eye pressure, minimize eye inflammation and minimize toxicity to the endothelial cells.

We will need 5 groups of rabbits, 15 rabbits/group for a total of 75 rabbits.

The rabbit will be anesthetized with isoflurane, then prepped and draped in a sterile fashion. Additional drops of topical tetravisc will be placed for additional anesthesia. A lid speculum will be placed in one eye. A template will be placed on the cornea and outlined with a skin marking pen. The cornea thickness will be verified by pachymeter, then a V shaped laceration with a diamond knife will be placed in the central cornea.

Then:

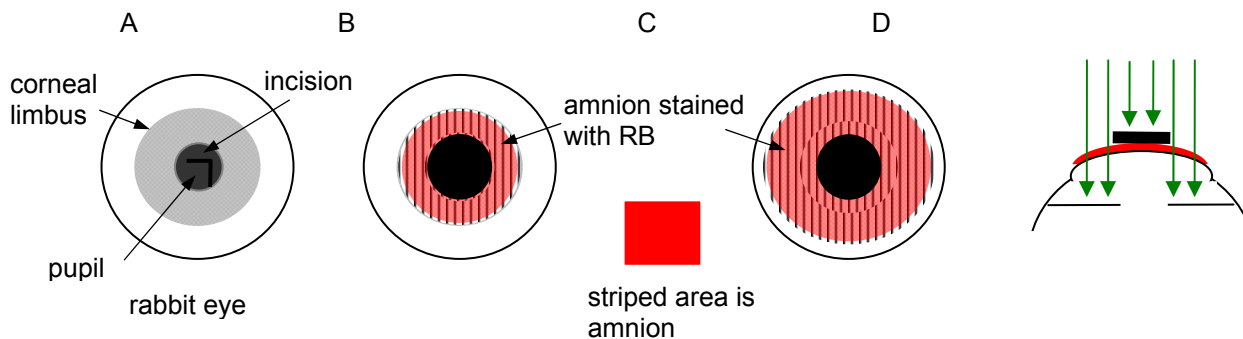
Group 1: 2 drops of fibrin glue and 10mm disc of amniotic membrane will be placed over the cornea, and sutured in place with a purse-string suture

Group 2: (A dispersive viscoelastic will be placed into the anterior chamber), then a 10mm disc of amniotic membrane will be soaked with rose bengal and placed on the cornea. A 4mm protective disc will be placed centrally over the area of pupil, and the cornea will be irradiated with the laser settings established at MGH.

Group 3:, 2 drops of fibrin glue and a 16mm unstained amniotic membrane disc will be placed on the cornea, which drapes over the limbus and sutured to the episclera.

Group 4: A dispersive viscoelastic will be placed into the anterior chamber, then a 16mm disc of the amniotic membrane stained with rose bengal dye. A 4mm opaque disc placed centrally over the pupil, amniotic membrane will be draped over the limbus and the cornea will be irradiated with the laser with the settings established at MGH.

Group 5: the incision will be sealed with 10-0 nylon sutures.



After the amniotic membrane is in place, a 27 G needle is inserted parallel to the iris, 2 mm above the limbus into clear cornea and positioned above the lens. The needle is connected to both a calibrated blood pressure transducer and an infuser via a T-coupler. IOP is gradually increased by infusion of saline (containing a blue dye for visualization) into the anterior chamber. The signal generated by the transducer-amplifier combination is proportional to the IOP, and is calibrated using a manometer. The IOP is increased until either leakage from the sealed amnion is apparent or a pressure reading of 100 mm Hg is attained. This is recorded as the leak pressure (IOPL).

After the endpoint is reached the iop will be lowered to physiologic pressure, defined as under 22 for this experiment. This can be accomplished by removing the iop measuring apparatus and attaching a tuberculin syringe with the plunger removed. This will equilibrate the eye at 12 mmhg. The IOP will be verified by tonopen (a handheld apparatus designed to measure intraocular pressure based on it's ability to indent the

cornea). Then sub-conjunctiva antibiotics consisting of 0.2 ml of ancef (50 mg/ml) and dexamethosone (4 mg/ml) will be injected sub-conjunctivally. Then the speculum will be removed.

The rabbit will be allowed to awake from anesthesia.

The rabbit will be monitored that evening, Signs of pain and/or distress to be monitored will include, but not be limited to, the following parameters: activity level (e.g., restlessness, hunched posture, huddled, lethargy, etc.), vocalization, self-trauma, change in behavior from preoperative observations, body condition (e.g., loss of body weight/emaciation, dehydration, lack of grooming, congested mucous membranes, etc.), lameness, neurological signs (e.g., tremors, convulsion, paralysis/paresis, head tilt, etc.), or dyspnea. If observed, we will consult with the staff veterinarian.

Topical antibiotics will be placed 3x per day. The rabbits will be examined every day for infection and pain.

Every other day for the first 2 weeks, the rabbits will be removed from their cages and taken to the procedure room. The rabbits will be placed in the photo apparatus and examined using a portable slit lamp and photos to evaluate the presence of epithelial cells over the amniotic membrane. The re-epithelialized (unstained) area will be analyzed from digital camera images (area calculated with NIH image software). Photographs with digital camera will be taken, every other day for 2 weeks and at weeks 3, and 4.

The rabbits will be monitored throughout the process for stress. (Restlessness, vocalization, dyspnea if observed will be reported to the veterinarian and IM ketamine will be administered to ensure the rabbit does not experience undue stress.

Six parameters will be used to compare the animals in the treatment groups:

- 1) Ability to achieve a watertight closure with a supra-physiologic pressure.
- 2) re-epithelialization over the amniotic membrane,
- 3) retention of the graft,
- 4) inflammation (evaluated by physical exam- the appearance of redness, ulceration or erosions of the ocular surface will be noted)
- 5) appearance of neovascularization (in and towards the wound)
- 6) retention of endothelial cells

5 rabbits from each group will be euthanized on days 3 and 7, and at the end of the study, day 28, to determine the amount of inflammation, neovascularization, and evidence of endothelial toxicity. The amniotic membrane will be removed for analysis, and the cornea will be evaluated with Fluorescein staining and photographed. The corneal button will be removed and evaluated for endothelial staining and photographed, then submitted for pathologic evaluation of inflammation.

- 1) Inflammation will be assessed by infiltration of inflammatory cells into the cornea and cytokine assay on corneal tissue. To evaluate inflammation all 15 rabbits will be euthanized with phenobarbital. The

corneoscleral button of tissue will be removed and divided into portions for paraffin and frozen sections. Polymorphonuclear leukocytes and macrophages will be counted after immunohistochemical staining with antibodies against myeloperoxidase (MPO) and CD14. Inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8, and IL-10) will be measured by cytokine protein Q-plex™ array (Quansys Biosciences) on total protein extracts of cornea.

2) Endothelial toxicity will be assessed by staining the endothelial cells and taking digital photographs of the cornea. Then, using adobe photoshop software to determine amount of staining via pixel analysis using the technique pioneered by Hisham Saad MD (described above), a quantitative assessment of endothelial loss will be determined.

Experiment 1b:

Goals:

- 1) Compare direct corneal wound crosslinking of a V shaped laceration to standard therapy of suture closure, in their ability to repair a simple corneal laceration in a water tight fashion, while maintaining a normal eye pressure, minimizing eye inflammation and toxicity to the endothelial cells.
- 2) Compare direct corneal wound crosslinking of a stellate laceration (modeled by the X shaped laceration) to standard therapy of suture closure, in their ability to repair a simple corneal laceration in a water tight fashion, while maintaining a normal eye pressure, minimizing eye inflammation and toxicity to the endothelial cells

We will require 6 groups with 5 rabbits per group for a total of 30 rabbits.

The rabbit will be anesthetized with isoflurane, then prepped and draped in a sterile fashion. A lid speculum will be placed in one eye. Topical tetravisc will be placed for additional anesthesia. A template will be placed on the cornea and outlined with a skin marking pen. The cornea thickness will be verified by pachymeter, and then a V shaped laceration with a diamond knife will be placed in the central cornea. In-group 5 an X shaped laceration will be placed to model a more complex injury.

Then

Group I II, and III:

A small amount of dispersive viscoelastic will be placed between the cornea and iris, , then rose Bengal will be applied via syringe to the vertical 2/3's of the walls of the laceration with care to minimize excess dye getting into the anterior chamber. The wounds will be approximated with direct pressure and then irradiated with a laser. Groups I-III will differ only in the laser settings used. (*in complex lacerations the wounds may gape, gentle pressure at the edge of the cornea will be placed so that the corneal surfaces are touching when the laser is applied, and any gap is removed).

Group IV

The V shaped incision will be sealed with 10-0 nylon sutures and an operating microscope in the normal fashion of laceration repairs. Then 27-gauge needle with BSS will be used to reform the chamber.

Group V

Instead of a V incision, an X incision will be placed in the cornea. The incision will be perpendicular to the corneal surface. A dispersive viscoelastic will be placed to protect the endothelium, then 0.1% Rose Bengal will be placed in each incision with a 27 gauge needle in a similar fashion to experiments I-III, and then the sides will be manually approximated with pressure on the cornea while it is being irradiated with a green laser with a 4mm diameter beam. (*in complex lacerations the wounds may gape, gentle pressure at the edge of the cornea will be placed so that the corneal surfaces are touching when the laser is applied, and any gap is removed).

Group VI-

The X shaped incision will be sealed with 10-0 nylon sutures and an operating microscope. Then 27-gauge needle with BSS will be used to reform the chamber.

After the wounds are closed, a 27 G needle is inserted parallel to the iris, 2 mm above the limbus into clear cornea and positioned above the lens. The needle is connected to both a calibrated blood pressure transducer and an infuser via a T-coupler. IOP is gradually increased by infusion of saline (containing a blue dye for visualization) into the anterior chamber. The signal generated by the transducer-amplifier combination is proportional to the IOP, and is calibrated using a manometer. The IOP is increased until either leakage from the sealed amnion is apparent or the maximum pressure reading of 100 mm Hg is attained. This is recorded as the leak pressure (IOPL).

After the endpoint is reached the iop will be lowered to physiologic. This can be accomplished by removing the iop measuring apparatus and attaching a tb syringe with the plunger removed. This will equilibrate the eye at 12 mmhg. The IOP will be verified by tonopen. Then subconjunctival antibiotics consisting of 0.2 ml of ancef and dexamethosone will be injected subconjunctivally. Then the speculum will be removed. The bunny will be allowed to awake from anesthesia.

The bunny will be monitored that evening for pain. Signs of pain and/or distress to be monitored will include, but not be limited to, the following parameters: activity level (e.g., restlessness, hunched posture, huddled, lethargy, etc.), vocalization, self-trauma, change in behavior from preoperative observations, body condition (e.g., loss of body weight/emaciation, dehydration, lack of grooming, congested mucous membranes, etc.), lameness, neurological signs (e.g., tremors, convulsion, paralysis/paresis, head tilt, etc.), or dyspnea. If observed, we will consult with the staff veterinarian.

Every other day for the first 2 weeks, the rabbits will be removed from their cages and taken to the procedure room. The rabbits will be placed in the photo apparatus and examined using a portable slit lamp and photos to evaluate the presence of epithelial cells over the amniotic membrane. The re-epithelialized (unstained) area will be analyzed from digital camera images (area calculated with NIH image software). Photographs with digital camera will be taken, every other day for 2 weeks and at weeks 3, and 4.

The rabbits will be monitored throughout the process for stress. (Restlessness, vocalization, dyspnea if observed will be reported to the veterinarian and IM ketamine will be administered to ensure the rabbit does not experience undue stress.

Four parameters will be used to compare the animals in the treatment groups:

- 1) Ability to achieve a water tight closure with a supraphysiologic pressure.

- 2) inflammation (evaluated by physical exam- the appearance of redness, ulceration or erosions of the ocular surface will be noted)
- 3) , appearance of neovascularization (in and towards the wound)
- 4) retention of the endothelial cells

5 rabbits from each group will be euthanized on days 3 and 7 and on day 28, to determine the amount of inflammation and evidence of endothelial toxicity.

1) Inflammation will be assessed by infiltration of inflammatory cells into the cornea and cytokine assay on corneal tissue. To evaluate inflammation all 15 rabbits will be euthanized with phenobarbital. The corneoscleral button of tissue will be removed and divided into portions for paraffin and frozen sections. Polymorphonuclear leukocytes and macrophages will be counted after immunohistochemical staining with antibodies against myeloperoxidase (MPO) and CD14. Inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8, and IL-10) will be measured by cytokine protein Q-plexTM array (Quansys Biosciences) on total protein extracts of cornea.

2) Endothelial toxicity will be assessed by staining the endothelial cells and taking digital photographs of the cornea. Then, using adobe photoshop software to determine amount of staining via pixel analysis using the technique pioneered by Hisham Saad MD (described above), a quantitative assessment of endothelial loss will be determined.

KEY RESEARCH ACCOMPLISHMENTS TO DATE

None- Research Delayed due to BRAC

REPORTABLE OUTCOMES:

None to date

CONCLUSIONS:

In this reporting period, the initial stages of our experimentation have been delayed due to construction delays at our facility. The initial facility was due to finish in March. The facility is now open and we are beginning to transfer activity there.

All items needed to start experiment are in their final procurement stage. The Wellman group has constructed a fiber optic handpiece to be used in this portion of the experiment- delivered to BAMC Dec 2010. Plan to initiate research in Jan 2011.

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