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14. ABSTRACT
Purpose: To develop a light-activated technology (called PTB) with the potential to decrease vision loss and ocular complications in warfighters sustaining penetrating eye injuries. **Scope:** To compare light-activated bonding of amnion to direct sealing of penetrating cornea wounds, to evaluate photobonding for puncture wounds in sclera, and to evaluate safety of photobonding to the iris. **Major findings:** Identified the treatment parameters for rapidly and strongly sealing amniotic membrane over scleral puncture wounds. Demonstrated that photobonding amion effectively sealed wounds spanning the cornea sclera. Determined that sealing penetrating cornea wounds using a dye-stained amniotic membrane patch and green light is superior to direct photo-activated bonding of the wound walls. Established that the green light parameters used to seal amnion over cornea wounds are below the threshold for thermal damage to the iris.

15. SUBJECT TERMS
cornea, sclera, eyelid skin, penetrating wound, laser, photochemistry

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INTRODUCTION

The overall goal of this research is to develop a light-activated technology 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. Cyanoacrylate glues can complicate further surgery by sticking to sutures and possibly causing additional damage when removed. Our sutureless, glueless method 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. The scope also includes developing a light-activated method for rapid closure of eyelid lacerations using hairless mouse skin as a model. Finally, the scope includes designing, constructing and evaluating a light delivery system that meets ANSI standards for retina and iris safety. Major tasks for Year 3 of the studies at Massachusetts General Hospital (MGH) were to evaluate the ability of light-activated bonding to directly seal irregular penetrating wounds in cornea, to evaluate photoactivated bonding for sealing puncture wounds in sclera and to determine if the radiant exposure levels of green light reaching the iris during photoactivated bonding are below the ANSI damage thresholds.

BODY

This research project is in collaboration with Col Anthony J. Johnson, MD at the US Army Institute for Surgical Research and Brooke Army Medical Center (BAMC). During year 3, Dr. Kochevar and Dr. Johnson continued to hold frequent phone discussions about the studies and discuss results. They also discussed the project status and planned experiments at the Association for Research in Vision and Ophthalmology national meeting in May 2012. This annual report describes the results of studies carried out at MGH; Dr. Johnson is submitting a separate annual report. A no-cost extension has been requested and a final report will be submitted when the studies are completed.

All of the studies in this annual report employed the same clinical laser that was used in previously reported studies (IRIDEX OcuLight OR KTP green laser, IRIDEX Corp., Mountain View CA), which emits green light (continuous wave, cw) at 532 nm. The amniotic membrane was also prepared, stored and stained with Rose Bengal (RB) as described in our Year 1 annual report.

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

Milestone 1. Analyze results and submit a manuscript describing results. A manuscript was submitted in year 2 and was published in Year 3 in *Investigative Ophthalmology and Visual Sciences,* the premier ophthalmology research journal. It is listed in the Reportable Outcomes section.

Task 2. Evaluate photoactivated bonding for direct sealing of corneal lacerations

2.a. Establish laser parameters for strong immediate water-tight seals
In addition to sealing amniotic membrane over a penetrating corneal injury with photochemical tissue bonding (PTB)(Task 1), we had proposed to evaluate the direct bonding, with PTB, of the edges of wounds with irregular shapes that mimic traumatic wounds. We had previously demonstrated that PTB effectively sealed linear incisional wounds in cornea (1, 2). For the current studies we used V-shaped incisions in the central cornea of ex vivo rabbit eyes. As noted in our Year 2 report, this approach was challenging because our group at MGH lacks the appropriate cornea surgical skills and experience. Our attempts were unsuccessful. To overcome this problem, we had proposed that Dr. Johnson, a highly skilled corneal surgeon, would develop the technique and then visit Boston to teach our group the appropriate technique. During this year, Dr. Johnson evaluated several approaches to directly sealing V-shaped incisions using photoactivated bonding; this is detailed in his report. Briefly, he reached the conclusion that the simple direct bonding of complex wounds, i.e., without sutures or other materials, is not feasible because the wound walls do not approximate well.

In summary, these observations and our previous studies indicate that light-activated sealing penetrating cornea wounds using a dye-stained amniotic membrane patch is superior to direct bonding of the wound walls for these complex injuries.

Task 3. Evaluate photoactivated bonding for sealing puncture wounds in sclera.

1.c. Photoactivated bonding for sealing amniotic membrane over penetrating wounds in the sclera.
Sealing amnion over penetrating wounds in the sclera differs from bonding amnion over cornea wounds since the surface composition of the two tissue differs, and consequently the photobonding efficiency may differ. The surface of de-epithelialized cornea is a basement membrane composed mainly of Type IV collagen and laminin. The surface of the sclera is Type I collagen. Similar to the studies we have reported previously for sealing corneal wounds, our studies were designed to evaluate the immediate strength of the seal, first ex vivo and then in vivo, after amnion is bonded over a puncture wound in the sclera. Dr. Johnson will then study the healing process in longer-term studies.

Studies were carried out using ex vivo rabbit eyes to establish the size and shape (circular vs rectangular) of the amnion patch to use for in vivo studies. For all studies, the conjunctiva was removed from the scleral area before wounding. The conjunctiva was removed using small surgical scissors and a blunt tip to undermine the conjunctiva, working outwards from the limbus.

The effect of circular patch size (7 mm vs 10 mm circle diameter) was evaluated for sealing 3-mm incisions made perpendicular to the limbus and 3-4 mm away from the limbus. The patch was stained with 0.1% Rose Bengal (RB) for 5 min, rinsed and allowed to partially dry before being placed over the incision. The entire patch was exposed to green laser light using an irradiance of 0.25 W/cm^2 to deliver 50 J/cm^2 (200 sec) or 100 J/cm^2 (400 sec). The control in all experiments was RB-stained amnion that is not irradiated.

The measurement of the strength of the bonding was somewhat modified from that described previously for bonding amnion over corneal wounds. The patch was moistened with a drop of water before insertion of a 19 gauge needle into the posterior segment ~ 1 cm from the incision site but still within ~ 5 mm of the limbus. The needle tip was placed directly behind the incision. An aqueous solution containing a blue dye (for visualization) was infused into the posterior segment and the pressure measured. The intraocular pressure (IOP) at which leakage from

underneath the amnion was detected (the leak intra ocular pressure, IOP_L) indicated the bonding strength.

The results are shown in Figs. 1A and 1B. Photobonding the 7 mm diameter amnion patch using 100 J/cm^2 produced an IOP_L of only 60 mm Hg (Fig. 1A). Photobonding the 10 mm diameter patch was more effective (Fig. 1B). The IOP_L increase in a light dose-dependent manner and reach almost 200 mm Hg when 100 J/cm^2 was used. Even 50 J/cm^2 produced moderately strong sealing compared to the normal IOP of about 20 mm Hg. The 7-mm amnion patch only allows a 2-mm rim of amnion between the end of the 3-mm incision and the edge of the amnion disc. This appears to be an insufficient area to provide a strong seal with photobonding.

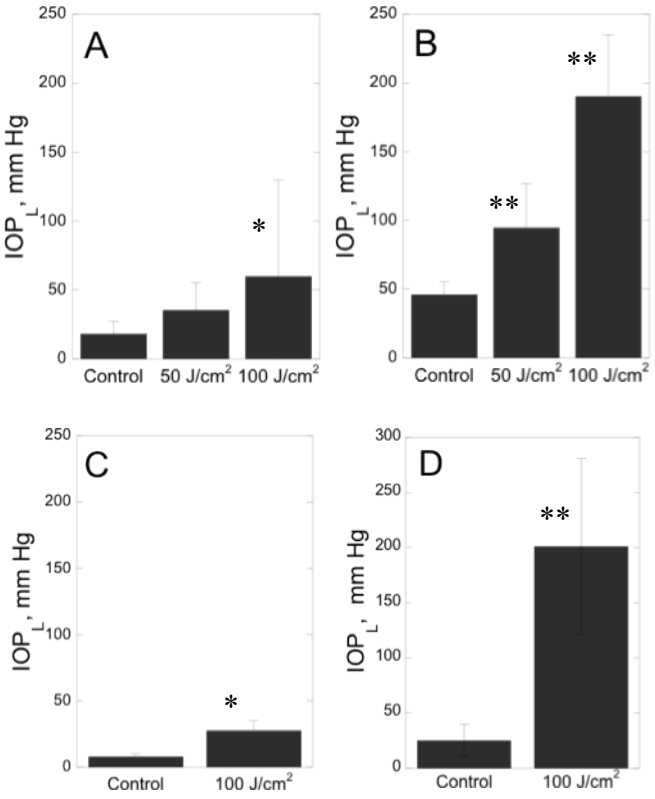


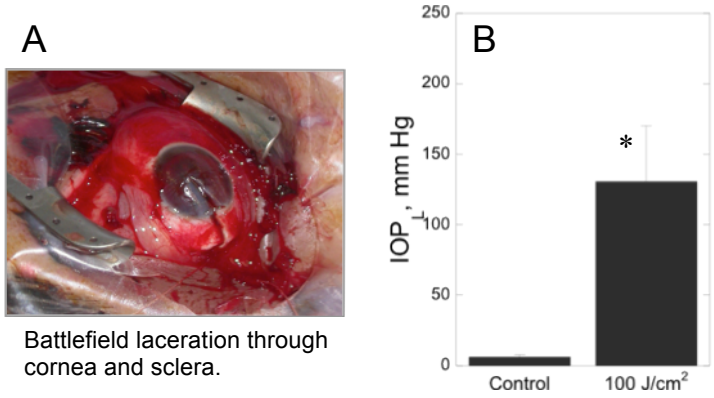
Figure 1. Photobonding amnion patches over linear incision wounds in rabbit sclera ex vivo. (A, B) 3-mm wounds perpendicular to the limbus were covered with Rose Bengal-stained circular amnion patches either 7 mm (A) or 10 mm diameter (B) and irradiated with green laser light. The IOP_L indicates the bonding strength. (C, D) 3.5 mm wounds perpendicular to the limbus were covered with RB-stained amnion either 4 x 8 mm rectangles (C) or 5 x 11 mm rectangles (D) and irradiated with green laser light.
* indicates $p < 0.05$ compared to unirradiated control.
** indicates $p < 0.01$ compared to unirradiated control.

The influence of the distance between the end of the incision to the edge of the amnion was investigated further by photobonding amnion over a 3.5 mm linear wound using rectangular patches, 4 mm x 8 mm or 5 mm x 11 mm. As shown in Fig. 1C, the smaller rectangular patch was not effective; the mean IOP_L was only 30 mm Hg. The larger rectangular patch produced strong bonding (Fig. 1D). The mean IOP_L, ~200 mm Hg, is the same as the value produced when a 10 mm circular disc patch is bonded (Fig. 1B). These results indicate that ~3.5 mm is required from the end of a wound to the edge of the patch (using either circular or rectangular amnion patches) to produce strong sealing of amnion over a wound in the sclera.

Interestingly, the IOP_L values for sealing amnion to sclera under the optimal conditions (Fig. 1A) are very similar to those we measured for bonding amnion to cornea (Year 1 & 2 reports and ref (3)). Values for IOP_L were in the range 200-250 mm Hg for both tissues. It wasn't apparent whether bonding to sclera would be as strong as to cornea because the molecules available for photo-crosslinking on surface of the two tissues differ, as noted above. This result suggests that

the covalent crosslinks between Type I collagen in the amnion and the tissue surface proteins is non-specific, i.e., isn't influenced by type of protein.

Photobonding amnion over a more challenging type of wound was also evaluated. Our initial motivation for photobonding amnion over penetrating eye injuries was wounds involving both the cornea and the sclera such as shown in Fig. 2A. Incisions (3.5 mm) were made perpendicular and across the limbus, covered with 10-mm RB-stained amnion disc patches and irradiated with 100 J/cm^2 or not irradiated. As shown in Fig. 2B, reasonably strong bonding was achieved (~130 mm Hg, measured in anterior chamber) indicating that our approach can be used for these severe wounds.



Battlefield laceration through cornea and sclera.

Figure 2. Photobonding amnion over corneal/sclera incisions in rabbit eyes ex vivo. (A) Cornea/sclera penetrating wound from battlefield trauma. (B) Photobonding amnion over 3.5-mm corneal/scleral incisions. 10 mm RB amnion patches and green laser used. The IOP$_L$ indicates the bonding strength. * indicates $p < 0.01$.

The energy of the laser light is absorbed by the RB dye on the amnion, but not all of the energy is used to photochemically link the amnion to the scleral surface. Some is converted to heat, which might damage the sclera if the temperature increase is too great. Consequently, we measured the temperature of the amnion surface during the irradiation using a non-contact infrared thermometer (model 572, Fluka; Mississauga, ON Canada). The results are shown in Fig. 3 (filled squares). The temperature increase was about 12°C after correcting for the control. (The eyes were cooler than room temperature at the beginning of the experiment). The normal cornea surface temperature is 33°C at ambient room temperature of 23°C. Thus, the cornea surface would reach about 45°C, which is approximately the temperature for non-permanent thermal damage to cornea. Cornea damage will be thoroughly evaluated in the long term studies of photobonding of amnion to rabbit eyes in vivo that Dr. Johnson is carrying out.

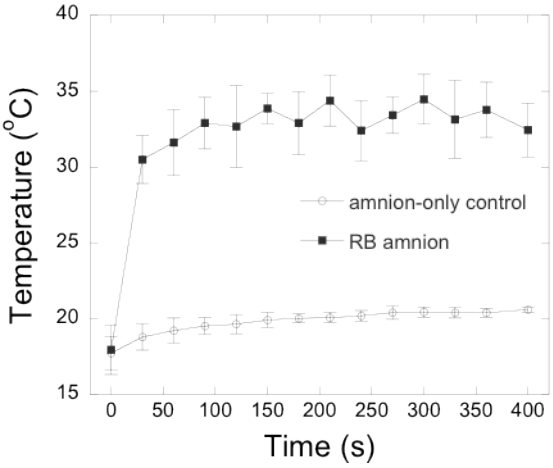


Figure 3. Temperature change during photobonding of RB-stained amnion over scleral wounds in ex vivo rabbit eyes. Irradiance = 0.25 W/cm^2

In summary, we have established the amnion patch size and shape that strongly seals RB-stained amnion over penetrating scleral wounds and wounds that span both the cornea and sclera. This information will be used for in vivo evaluation of this photobonding technique for sealing scleral wounds in rabbit eyes during the no-cost extension period.

Task 4. Identify best treatment parameters for sealing eyelid skin lacerations. These studies were completed in Year 2.

Milestone 3. The manuscript describing the technique developed for sealing eyelid lacerations with PTB was submitted in Year 2 and was published in Year 3 in *Lasers in Surgery and Medicine*. It is listed in the Reportable Outcomes section.

Task 5. Design, construct and test safe light delivery systems for direct bonding of corneal injuries.

In Years 1 and 2 the studies for this task focused on designing and evaluating a light delivery system that would provide sufficient light for photobonding on the cornea surface but would be safe for the retina according to ANSI (American National Standards Institute). In Year 3 our goal was to determine if the radiant exposure levels reaching the iris while photobonding amnion to the cornea are below the ANSI thresholds for damage. This information is needed because the small opaque disc that blocks green light from entering the eye through the pupil does not block this light from reaching the iris.

The human iris contains melanin in the stromal layer and in a pigmented epithelial layer on the posterior surface. When the melanin absorbs green light, the light (electromagnetic) energy is converted into thermal energy. If the rate of light energy absorption is greater than the rate of dissipation of the thermal energy, the temperature will rise. However, the temperature increase will be limited by blood flow through the iris, which will remove heat.

A threshold for thermal damage to the iris is not well defined. It is sometimes taken to be the same threshold as for skin, and sometimes estimated to be 3 to 5 times higher than the threshold for retina. Consequently, we decided to directly measure the iris surface temperature during photobonding.

We measured the change in iris temperature under the conditions used for bonding amnion to the cornea (irradiance = 0.25 W/cm^2, fluence = 100 J/cm^2.) Freshly obtained swine eyes were used for these measurements because the iris is darkly pigmented (high melanin content) whereas the albino rabbit eyes used in our studies lack melanin in their iris. The cornea, iris, lens and a rim of sclera were removed and placed in a specially constructed holder that allowed irradiation of the cornea from above and measurement of the iris temperature from below (Fig. 4A). The temperature was measured with a non-contact infrared thermometer (model 572, Fluka) that detects surface temperature.

The temperature increased slowly and reached approximately 4°C higher than the starting temperature (Fig. 4B). The eyes were below room temperature at the beginning of the measurements. A 4°C increase is an upper limit for the change in vivo because the ex vivo eyes do not have blood circulation in the iris to remove heat. Thus, our measurements indicate that photobonding amnion onto cornea will not cause thermal damage to the iris.

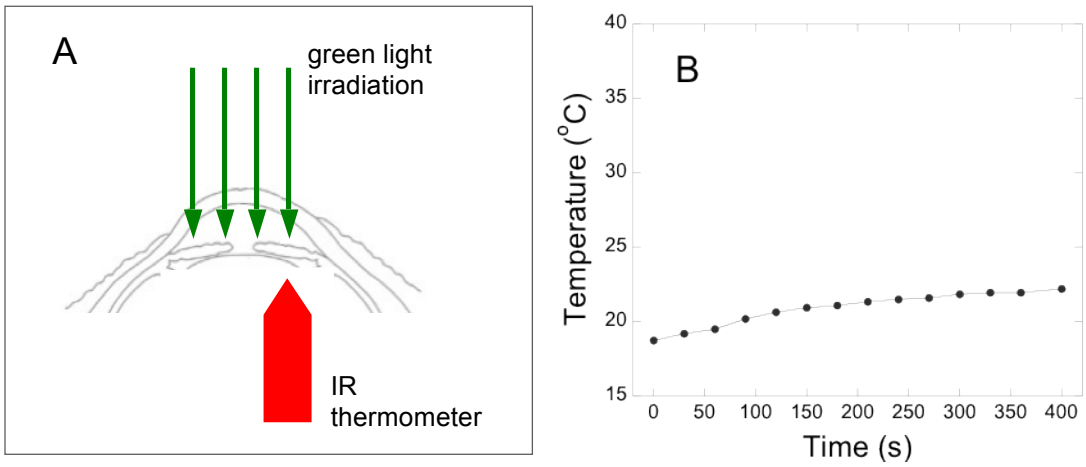


Figure 4. Measuring temperature of iris while irradiating cornea with green light. (A) Schematic diagram of experimental set up. (B) Temperature of posterior surface of iris measured with non-contact infrared thermometer during bonding amnion to cornea using green light.

KEY RESEARCH ACCOMPLISHMENTS

- Identified the treatment parameters for strongly sealing amniotic membrane over a puncture wound in sclera using light-activated bonding. The amnion patch size and shape, and light fluence were established for the subsequent in vivo study.

- Demonstrated that dye-stained amnion can effectively seal penetrating wounds that span the cornea and sclera.

- Determined that sealing penetrating cornea wounds using a dye-stained amniotic membrane patch and green light is superior to direct photo-activated bonding of the wound walls.

- Established that the irradiation parameters used to seal amnion over cornea wounds are below the threshold for thermal damage to the iris.

REPORTABLE OUTCOMES

Verter EE, Gisel TE, Yang P, Johnson AJ, Redmond RW, Kochevar IE. Light-initiated bonding of amniotic membrane to cornea. Invest Ophthalmol Vis Sci. 2011 Dec 9;52(13):9470-7.

Yang P, Yao M, DeMartelaere SL, Redmond RW, Kochevar IE. Light-activated sutureless closure of wounds in thin skin. Lasers Surg Med. 2012 Feb;44(2):163-167.

CONCLUSIONS

We further extended our development of a simple and rapid light-initiated technology for sealing penetrating eye injuries. The goal of applying this technology is to decrease vision loss and ocular complications after traumatic ocular injuries. Rapid closure of these wounds is critical to stabilizing the eye for further surgery and preventing infection. Our approach seals penetrating wounds by a rapid, sutureless, glueless attachment of a biological membrane over the wound or

by directly bonding the wound edges together.

In Year 3 we demonstrated that our technology could be extended beyond cornea penetrating wounds. We identified the amount of light, amount of dye, and size of the repair patch that strongly seal sceral puncture wounds. Wounds spanning the cornea and sclera were also successfully sealed.

Our studies indicated that light-activated bonding of amnion over a cornea wound is superior to directly photobonding the edges of a penetrating wound. The patch approach is substantially more rapid and simple; it does not require preliminary suturing or use of additional materials.

We demonstrated that the green light used for tissue bonding of corneal wounds does not thermally damage the iris, even though the light is absorbed by melanin in the iris. Thus, this potential safely concern has been eliminated.

Together with our results from previous years' studies, we now have a good understanding of the light treatment parameters for sealing a variety of penetrating eye wounds and have demonstrated that these repair procedures are safe to ocular tissues. Longer-term animal model studies are needed before translation of this technology to clinical application.

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3. Verter EE, Gisel TE, Yang P, Johnson AJ, Redmond RW, Kochevar IE. Light-initiated bonding of amniotic membrane to cornea. Investigative ophthalmology & visual science. 2011;52(13):9470-7.

Appendices

1. Verter EE, Gisel TE, Yang P, Johnson AJ, Redmond RW, Kochevar IE. Light-initiated bonding of amniotic membrane to cornea. Invest Ophthalmol Vis Sci. 2011 Dec 9;52(13):9470-7.

2. Yang P, Yao M, DeMartelaere SL, Redmond RW, Kochevar IE. Light-activated sutureless closure of wounds in thin skin. Lasers Surg Med. 2012 Feb;44(2):163-167.

Light-Initiated Bonding of Amniotic Membrane to Cornea

E. Eri Verter,[1] *Thomas E. Gisel,*[1] *Penggao Yang,*[1,2] *Anthony J. Johnson,*[3] *Robert W. Redmond,*[1] *and Irene E. Kochevar*[1]

Purpose. Suturing amniotic membrane to cornea during surgery is time consuming, and sutures may further damage the eye. The authors introduce a novel sutureless, light-activated technique that securely attaches amnion to cornea through protein-protein crosslinks.

Methods. Cryopreserved human amniotic membrane, stained with Rose Bengal (RB), was placed over a full-thickness wound in deepithelialized rabbit cornea and was treated with green laser. The intraocular pressure that broke the seal (IOP_L) was measured, and adhesion was measured with a peel test. The influences on bonding strength of fluence, irradiance, RB concentration, and amnion surface bonded were measured. Epithelial cell migration on treated amnion and keratocyte viability after bonding were also measured. The involvement in the bonding mechanism of oxygen, singlet oxygen, and association of RB with stromal collagen was investigated.

Results. Sealing amniotic membrane over cornea using 0.1% RB and 150 J/cm^2 at 532 nm produced an IOP_L of 261 $\pm$ 77 mm Hg ex vivo and 448 mm $\pm$ 212 mm Hg in vivo. The ex vivo IOP_L increased with increasing fluence (50–150 J/cm^2). Equivalent IOP_L was produced for bonding basement membrane or stromal amnion surfaces. The bonding treatment was not toxic to keratocytes but slightly reduced the migration of corneal epithelial cells on amnion ex vivo. Mechanism studies indicated that RB forms two complexes with amnion stromal collagen, that bonding requires oxygen, and that singlet oxygen mediates protein crosslinking.

Conclusions. A rapid, light-activated technique produces strong, immediate bonding between amnion and cornea and merits further evaluation for ocular surface surgeries. (*Invest Ophthalmol Vis Sci.* 2011;52:9470–9477) DOI:10.1167/iovs.11-7248

Amniotic membrane is frequently used in corneal and scleral surgery as a temporary patch or a reconstructive graft.[1] Uses of amniotic membrane transplantation (AMT) include covering persistent epithelial defects, pterygium surgery, and ocular surface reconstruction in stem cell deficiency. Amniotic membrane is composed of a single layer of epithelial cells attached to a basement membrane that lies over a stromal layer containing primarily types I and III collagen, proteoglycans, and fibroblasts. Currently amnion is sutured to the cornea, a time-consuming process requiring high skill to place hair-fine sutures. In addition, suturing may injure the eye and, because sutures act as a foreign body, can lead to persistent inflammation, infection, and granuloma.[1,2]

We have evaluated an additional application of AMT, namely, sealing amniotic membrane over penetrating corneal wounds using a novel light-activated technology called photochemical tissue bonding (PTB). PTB produces an immediate seal between tissue surfaces without additional glues or proteins. Covalent crosslinks are formed that bridge proteins between the tissue surfaces by a photochemical, nonthermal mechanism.[3–12] After applying a photoactive dye, the tissue surfaces are placed in contact, and the area is treated with a green laser to activate the dye and initiate the bonding chemistry. The dye, Rose Bengal (RB), is approved by the US Food and Drug Administration for the diagnosis of ocular surface damage.

A rabbit eye model of penetrating eye injury was chosen to challenge the ability of PTB to form a strong and secure seal between amniotic membrane and the corneal surface. In this study we identified the PTB treatment conditions that produced strong bonding, evaluated potential side effects, and investigated mechanisms for crosslink formation between amnion and corneal surface proteins.

Materials and Methods

Materials

Frozen albino rabbit eyes from Pel-Freeze Biologicals (Rogers AR) were used at room temperature. Rose Bengal (95%; Sigma-Aldrich, St. Louis, MO) was a 0.1% wt/vol solution in phosphate-buffered saline (PBS; Sigma-Aldrich). Fibrin sealant (Tisseel) was from Baxter (Deerfield, IL). Nitrogen (Ultra High Purity) and oxygen (99.5%) were from Airgas (Cambridge, MA). New Zealand White rabbits (weight range, 2–2.5 kg) were purchased from Charles River Laboratories (Wilmington MA). The in vivo study was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Amniotic membrane was obtained from scheduled caesarean deliveries, as we have done previously.[9] This research adhered to the tenets of the Declaration of Helsinki. The amnion epithelium was removed with trypsin (0.25%, 90 minutes, 37°C) and light rubbing. Amnion was stored on nitrocellulose paper at −80°C in 1:1 glycerol/Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin-streptomycin and 0.05% amphotericin B (Sigma-Aldrich).

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Disclosure: **E.E. Verter**, None; **T.E. Gisel**, None; **P. Yang**, None; **A.J. Johnson**, None; **R.W. Redmond**, Aura Medsystems (C), P; **I.E. Kochevar**, Aura Medsystems (C), P

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Absorption Spectra

Spectra of RB on amnion or on amnion bonded to cornea were measured on glass slides using a microplate reader (Spectramax M5; Molecular Devices, Sunnyvale CA). To correct for scattering from these tissues, spectra of amnion or cornea (without RB) were subtracted.

Association of Rose Bengal with Amnion

Amnion samples (1.1–1.3 mg) were stained on the stromal surface with 0.1% RB (5 minutes) and were briefly washed with PBS before absorption spectra were measured. Samples were then placed individually in 1 mL PBS and kept in the dark at room temperature until absorption spectra were measured. After each measurement, the samples were placed in fresh PBS.

Preparation of Amnion Patch

Amniotic membrane was first rinsed in PBS for 45 minutes to remove glycerol from the storage medium, then transferred from the nitrocellulose backing to Parafilm with the stromal surface upward and allowed to dry. A 13-mm diameter circle was cut from the amnion and Parafilm backing (Fig. 1A). RB solution (0.1% in PBS, ~200 μL) was placed on the amnion stromal surface for 5 minutes (Fig. 1B), and excess was removed to produce slightly moist, but not wet, amnion. The dye-stained amnion was peeled from the backing (Fig. 1C).

Bonding Amniotic Membrane to Rabbit Cornea

The same procedure for photobonding amnion to cornea was followed for both ex vivo and in vivo rabbit eyes, except for the differences noted. In vivo, the nictitating membrane was displaced and held with a suture (Fig. 1D). The epithelial layer was removed with 70% ethanol (10–15 seconds), and a full-thickness incision was made in the central cornea (ex vivo: V-shaped, 90° angle, 2-mm arms; in vivo, linear 3 mm) (Fig. 1E). The RB-stained amnion was placed with its stromal surface in contact with the cornea, and wrinkles were removed (Figs. 1G, 1H). A 4-mm diameter pupil-blocking opaque white disc was placed over the center cornea (Fig. 1I) before irradiation (Fig. 1J). A green laser (Ocu-Light OR KTP; Iridex Corp., Mountain View, CA) emitting cw 532 nm radiation was used for irradiances = 0.25 W/cm^2. A 532 nm cw Nd/YAG laser (Aura i; Laserscope, San Jose, CA) was used to deliver 0.5 W/cm^2. The beam was transmitted through a 600-μm optical fiber and passed through a microscope objective (20× or 40×) to produce a homogeneous beam at the corneal surface. Laser power was measured with a spectroradiometer (SPR-01; Luzchem, Ottawa, ON, Canada). The amnion surface was lightly misted with water every 90 seconds during irradiations.

To evaluate the influence of oxygen on photobonding, the cornea was stained instead of the amnion because RB photobleaches more rapidly in the presence of oxygen. Thus, more green light would have reached the cornea-amnion interface through the RB-stained amnion in the presence of oxygen. The deepithelialized corneal surface was

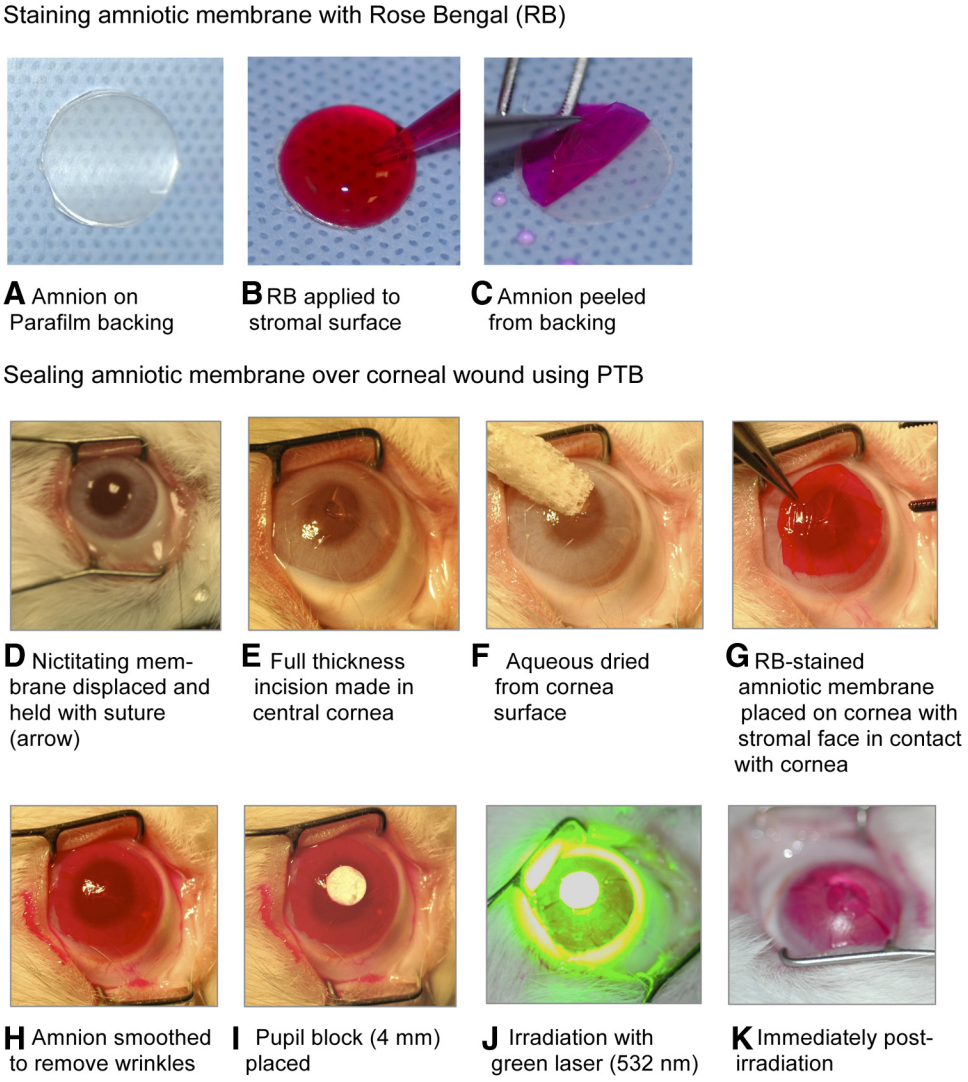


Figure 1. (**A–C**) Preparation of RB-stained amniotic membrane and (**D–K**) use of PTB to seal amnion over full-thickness incisional wounds in rabbit cornea.

placed in 1% RB for 2 minutes and then briefly washed. After an incision was made and the amnion patch was placed, the eye was placed in a 3-cm diameter, 7.5-cm tall plastic cylinder. To maintain humidity, water-saturated gases were used to purge the cylinder before (10-minute) and during irradiation. The amnion-covered cornea was irradiated through the plastic top (150 J/cm^2, 0.25 W/cm^2).

When H_2O and D_2O were compared, the corneal surface was immersed in either D_2O or H_2O for 30 minutes before the incision. The amnion was treated for 5 minutes with 0.1% RB prepared in either D_2O or H_2O.

For bonding with fibrin sealant (Tisseel; Baxter), 11 μL thrombin solution was spread on the amniotic membrane and 11 μL fibrinogen solution was spread on the cornea, as described previously.[13] The amnion was then placed over the V-shaped wound in the cornea, and any wrinkles were removed. The eye was allowed to stand at least 15 minutes before bonding strength was measured.

Measurement of Bonding Strength

Bonding strength between the amnion and cornea was determined by slowly infusing PBS into the anterior chamber through a 22-gauge needle inserted into the cornea ~1 mm anterior of the limbus and parallel to the iris. A mini-infuser (Genie Plus Infusion/Withdrawal Pump, Kent Scientific, Torrington, CT) and a pressure transducer (Isotec; Harvard Apparatus, Holliston, MA) were connected by a T-couple to the needle. The increase in IOP was measured immediately after photobonding by the method used previously.[8,12] The amnion surface was wetted before measurement to ensure that drying of the amnion on the cornea did not contribute to the bonding strength. The pressure attained immediately before fluid leaks from under the amnion, the leak pressure (IOP_L), is a measure of bonding strength. For in vivo studies, the animals were euthanized immediately after the measurement.

Adhesion was also measured using a peel test. A 5-mm wide, 20-mm long strip of amnion was bonded to a 6-mm wide and 10-mm long strip of cornea using PTB or fibrin sealant (Tisseel; Baxter). The bonded overlap area measured 5 mm × 10 mm (Supplementary Material and Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental). The PTB bonding procedure mimicked that used to seal amnion to the intact cornea as described. A previously described procedure was followed for bonding amnion to cornea with fibrin sealant.[13] Equal volumes (7.5 μL) of thrombin and fibrinogen solutions were applied to the amnion and corneal surfaces, respectively, which were then placed in tight contact. After at least 1 hour, the force generated while peeling amnion from the cornea was measured using a universal testing system (Nano UTM; Surface Systems and Technology GmbH, Hueckelhoven, Germany) with a separation rate of 5.0 mm/min. The mean force (milliNewtons [mN]) generated while peeling amnion from the cornea, after the initial peak, was taken as the adhesion strength.

Keratocyte Viability

RB-stained amnion and a 4-mm opaque disc were placed on the central corneas of freshly harvested rabbit eyes, which were then exposed to either 100 or 200 J/cm^2. The amnion was removed, and the eye was maintained in organ culture in DMEM at 37°C and 5% CO_2.[14] After 24 hours, the corneas were fixed in 10% formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Vertical sections (5 μm) contained both the irradiated peripheral and the light-blocked central areas. The keratocytes in 12 areas, each measuring 0.25-mm^2, adjacent to the anterior corneal surface were counted in both the non–light-treated area and the surrounding irradiated areas.

Migration of Corneal Epithelial Cells on Photocrosslinked Amnion

Amnion was treated with 0.1% RB for 5 minutes before brief washing and irradiation with fluence of 532 nm between 0 and 150 J/cm^2. Excess RB was removed by soaking amnion in PBS for 18 hours. Immortalized human corneal-limbal epithelial cells[15] (4×10^4) were placed on the basement membrane of deepithelialized amnion within a 6-mm cloning ring for 3 hours. At 24 to 96 hours after the ring was removed, the distance from the ring to the edge of the migrating cells was measured at six evenly separated locations on the circumference of the circle.

Statistical Analysis

Student's *t*-test for unpaired data was used to compare groups; significance was set at $P < 0.05$.

Results

Rose Bengal Associates with Amniotic Membrane Components

The amount of RB associated with amnion was calculated from the absorbance of RB at 532 nm after treating the stromal surface with RB (0.05% and 0.1%, ~200 μL) for 5 or 10 minutes and briefly washing. As shown in Figures 2A and 2B, the RB absorption approximately doubled between 5 and 10 minutes of staining. The mean RB concentration was estimated using an absorption coefficient for RB at 532 of $3.9 \times 10^4\ M^{-1} \cdot cm^{-1} \cdot nm$ (measured for RB bound to type I collagen in solution, (Y. Tang, unpublished result, 2010). Because amnion demonstrates a location-dependent variation in thickness, the absorption by RB is expected to vary.[16] Using a mean amnion thickness of 50 μm, the stromal RB was calculated to be 2.56 and 5.12 mM after 5 minutes of staining with 0.05% and 0.10% RB, respectively. These RB concentrations are approximately fivefold greater than the RB staining solutions (0.05% and 0.10% correspond to 0.5 and 1 mM RB), indicating that RB complexes with components in amnion.

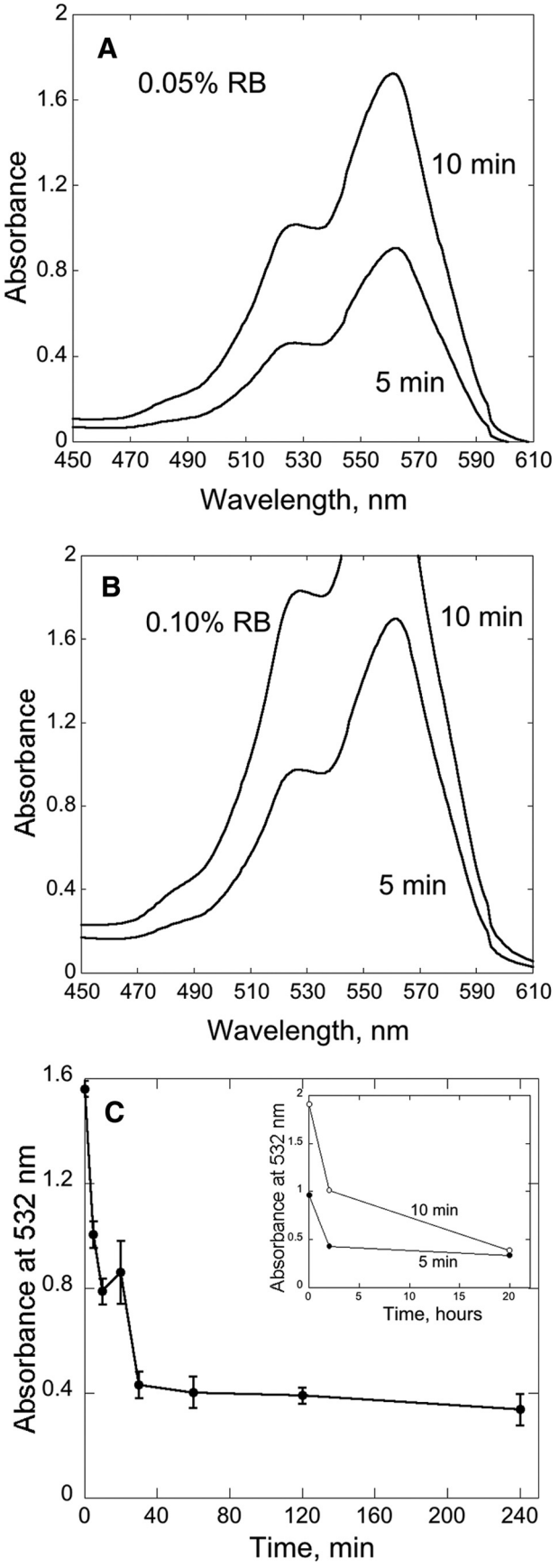
To further investigate this association, amnion was stained with 0.1% RB for 5 minutes and then was incubated in PBS for varying times. The decrease in RB absorption over time indicated that a portion of the RB diffused from the membrane (Fig. 2C). After 30 minutes, only approximately 25% of the RB was retained in the membrane. When the staining period was 10 minutes, the initial absorbance was higher, but the final amount of RB retained was the same (Fig. 3C, inset). Thus, RB appears to associate with amnion collagen in tight-binding sites that retain RB even after extended washing and in a larger number of loose-binding sites.

Bond Strength Increases with Fluence

The relationship between fluence and bond strength was measured using three fluences, 50, 100, and 150 J/cm^2, delivered in 3.3, 6.7, and 10 minutes, respectively, to amnion stained with 0.1% RB. Controls were amnion stained with RB but not irradiated, unstained amnion treated with 150 J/cm^2, and amnion sutured to the cornea with eight nylon sutures. As shown in Figure 3A, strong bonding was produced using all three fluences; the mean IOP_L values of 95 to 261 mm Hg are significantly higher than the normal IOP_L of human eyes (~20 mm Hg). The IOP_L produced by 100 and 150 J/cm^2 differed significantly from the control ($P < 0.001$) and from each other ($P = 0.03$). Bonding strength for the controls were all <20 mm Hg. Fibrin sealant produced an IOP_L of 66.6 ± 18.8 mm Hg, significantly different from the control ($P < 0.05$).

The adhesion of amnion to cornea was also measured using a 180° peel test (Fig. 3B). The force generated while peeling amnion from cornea after bonding with PTB was greater than that for the control using either 100 or 50 J/cm^2 ($P < 0.0005$). The adhesion strength after bonding amnion to cornea with fibrin sealant did not differ significantly from the control ($P = 0.085$).

Although the trend in the results in Figure 3A suggests that fluences higher than 150 J/cm^2 might produce even stronger



bonding, RB was destroyed (i.e., photobleached) during the irradiation as seen by the decrease in the RB absorption (Fig. 3C), suggesting that higher fluences would not proportionally increase the IOP_L.

Relationships among Irradiance, Temperature, and Bonding

Higher irradiance delivers the same fluence in a shorter time according to the relationship: Irradiance (W/cm^2) × Time (s) = Fluence (J/cm^2). However, it might also produce a damaging temperature increase. We measured IOP_L after delivering 100 J/cm^2 at irradiances varying by a factor of 4, which required 13.3, 6.7, and 3.3 minutes of irradiation, respectively. Surface temperature was measured during the irradiations with an infrared thermometer (model 572; Fluka, Mississauga, ON, Canada). As shown in Figure 3D, the mean IOP_L for the three irradiances were 206 to 304 mm Hg, with no significant differences between these values.

The maximum temperatures attained using 0.125 , 0.250, and 0.500 W/cm^2 were 22.2°C, 27.8°C, and 36.7°C, respectively (Fig. 3D); thus, a substantial increase over the control (18.3°C) was produced only by the highest irradiance. However, even 36.7°C is much lower than what is used for thermal laser welding (~75°C).[17,18] To eliminate any potential thermal effect, 0.25 W/cm^2 was used throughout these studies.

Other Factors Influencing Bond Strength

A fixed fluence (100 J/cm^2) and irradiance (0.25 W/cm^2) were used to test variables that might affect the IOP_L. A higher RB concentration might increase bond strength because of greater light absorption (Fig. 2) and more photocrosslinking sites. As shown in Figure 4A, the IOP_L using 0.10% or 0.05% RB did not differ ($P > 0.05$). The amnion surface in contact with the cornea might also have affected the IOP_L. RB was applied to the stromal surface of deepithelialized amnion, and either the stromal or the basement membrane surface was in contact with the deepithelialized cornea. The IOP_L did not differ (Fig. 4B, second and third bars), indicating that RB applied to the stromal surface penetrates the basement membrane surface. When the epithelial layer was not removed, applying RB to the epithelial face and placing the epithelial face in contact with the cornea during irradiation produced an IOP_L that did not differ from the control (Fig. 4B, fourth bar). Finally, RB-stained amnion was washed for 1 hour to remove loosely associated RB before irradiation. The IOP_L did not differ from the control IOP_L (Fig. 4B, fifth bar), indicating that photoactivation of the tightly associated RB in the amnion was not sufficient to produce strong bonding. Further studies are required to assess the effect on photobonding of the known variation in amnion thickness and transparency.[16]

In Vivo Bonding of Amniotic Membrane to Rabbit Cornea

The bonding procedure shown in Figure 1 was used with 50, 100, or 150 J/cm^2 (3.3, 6.7, and 10 minutes' irradiation, respec-

FIGURE 2. Association of RB with amniotic membrane. Absorption spectra of amniotic membrane after staining with (**A**) 0.05% or (**B**) 0.10% RB for 5 and 10 minutes. (**C**) RB-stained amnion (0.1%, min) was suspended in PBS at room temperature for varying times, and the absorption at 532 nm RB retained in the membrane was measured (mean ± SD). *Inset*: amnion was stained with RB for 5 or 10 minutes, then suspended in PBS, and absorption was measured at times up to 20 hours.

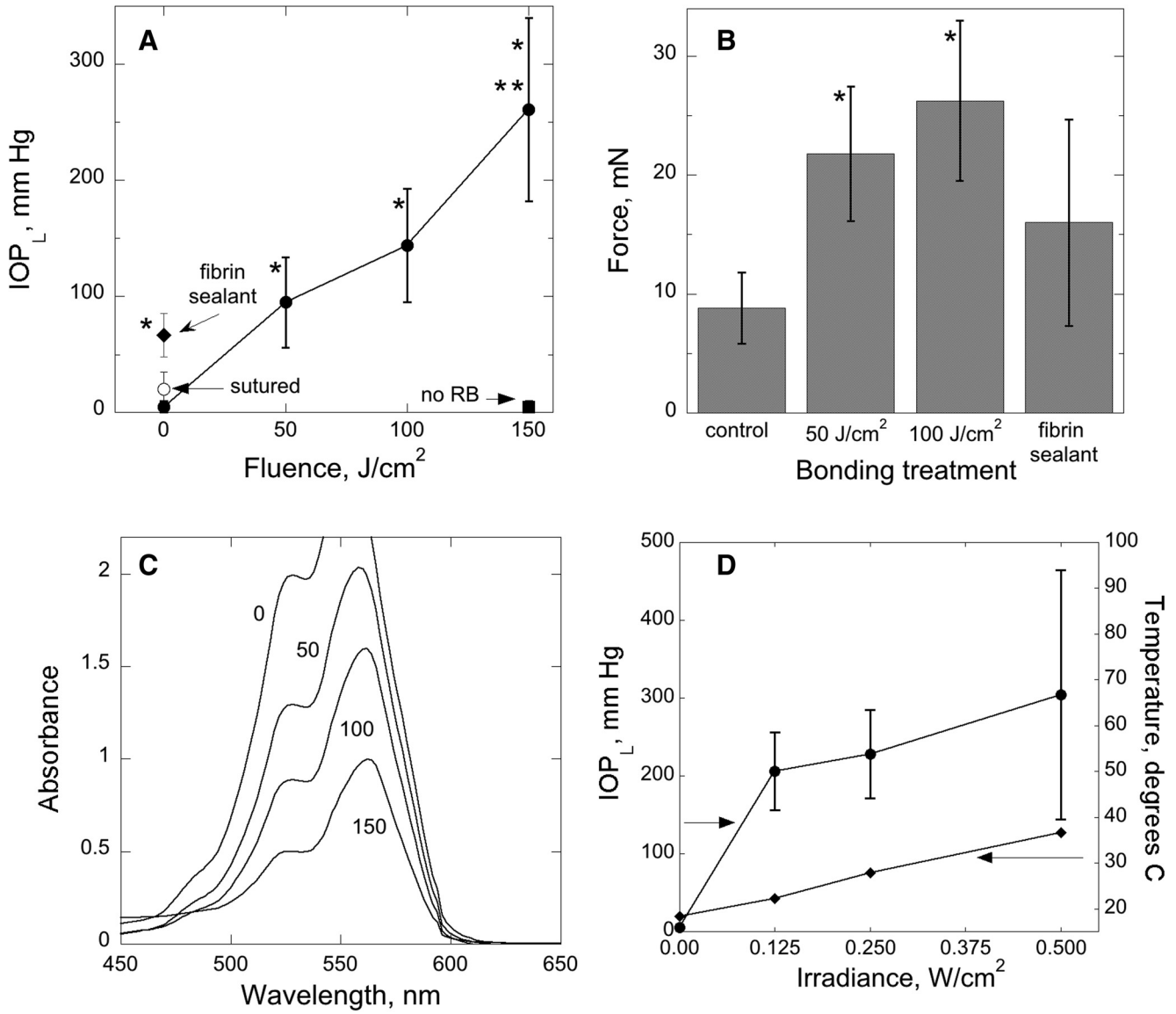


FIGURE 3. Bonding RB-stained amnion to ex vivo rabbit eyes. (**A**) Fluence was varied and irradiance was constant at 0.25 W/cm^2. Comparison was made with amnion sutured to cornea and amnion sealed to cornea with fibrin sealant. IOP$_L$ was measured immediately after bonding treatment. Mean ± SD; $n = 6$–8. $^*P < 0.05$ versus the unirradiated control. $^{**}P < 0.05$ versus the 100 J/cm^2 group. (**B**) Adhesion was measured as the force (mN) generated while peeling amnion that had been bonded to the cornea with PTB or with fibrin sealant. $n = 6$–8. $^*P < 0.05$ versus the unirradiated control. (**C**) Photobleaching of RB-stained amnion on cornea after irradiation with fluences used for photobonding. (**D**) Irradiance was varied and fluence was constant at 100 J/cm^2. IOP$_L$ (mean ± SD) is shown on the left y-axis. $n = 6$. Temperatures measured during the irradiation are shown for irradiance on the right y-axis.

tively). Strong immediate bonding was produced at all fluences, and the bond strength increased with fluence (Fig. 5). Controls (RB-stained amnion not irradiated or unstained amnion irradiated with 150 J/cm^2) did not bond. Photobonding amnion to cornea was not toxic to keratocytes (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental).

Corneal Epithelial Cell Migration on Photocrosslinked Amnion

Because RB diffuses the stroma, green light may crosslink multiple stromal proteins, including those in the basement membrane. The migration of corneal epithelial cells involves interaction with basement membrane proteins and is influenced by the properties of the surface[19,20]; therefore, we tested whether PTB treatment might alter the migration of these cells on amnion. As shown in Figure 6, the PTB treatment conditions used for bonding, 100 and 150 J/cm^2, decreased the extent of migration by approximately 15% to 30%.

Mechanism for Photobonding Amnion to Cornea

Photoactivated RB generates singlet oxygen ($^1O_2$), a reactive oxygen species that initiates protein-protein crosslinking by oxidizing amino acid side chains, especially histidine.[21–23] Oxidized histidine then reacts with certain amino acids, mainly lysine, to form protein-protein crosslinks. Photoexcited RB may also transfer an electron to or from certain amino acids (AA) to form radical ions.[24] Crosslinks may form without oxygen when the protonated AA$\cdot^-$ combines or when AA$\cdot^-$ reacts with oxygen to form products that subsequently lead to pro-

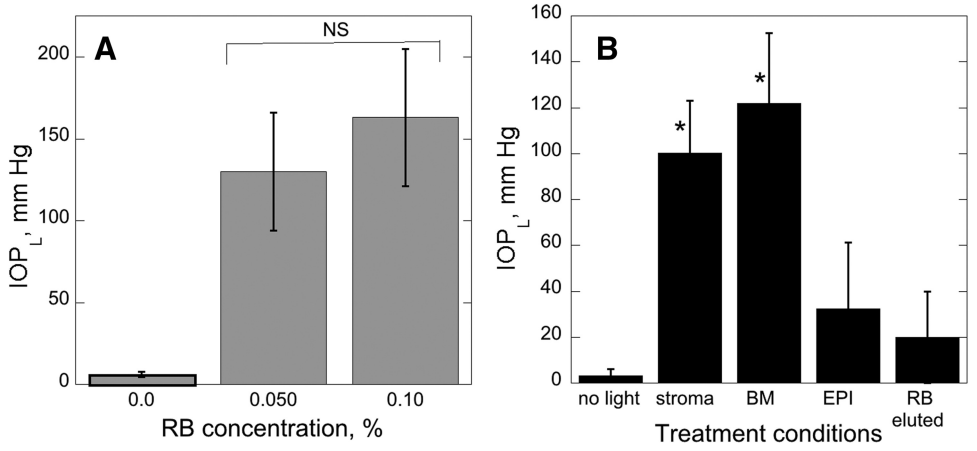


FIGURE 4. Factors potentially influencing seal strength for bonding amnion to rabbit eyes ex vivo. (**A**) RB concentration used to stain amnion for 5 minutes was varied. NS = $P > 0.05$. (**B**) Influence on IOP_L of the surface stained with RB, the surface bonded to the cornea, and removal of loosely bound RB. Amnion-covered corneas treated with 100 J/cm^2 and 0.25 W/cm^2 in all experimental groups. S, stromal surface stained, then bonded; BM, stromal surface stained but basement membrane surface bonded; EPI, epithelial layer stained, then bonded; RB eluted, loosely bound RB removed by soaking in PBS for 1 hour, then stromal surface bonded. Mean ± SD shown; $n = 5$. *$P < 0.05$ compared with no light group.

tein-protein crosslinks. These processes are shown in Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental.

To determine whether photobonding requires oxygen, irradiation was carried out in air, oxygen, or nitrogen atmospheres. Control corneas were irradiated in oxygen but were not stained with RB. Irradiations in air and oxygen produced IOP_L of 178 ± 12 and 208 ± 81 mm Hg, respectively (Fig. 7A). Irradiation in nitrogen produced a substantially lower IOP_L (66 ± 44 mm Hg) that did not differ from the control, indicating that oxygen participated in at least a portion of the reactions leading to covalent crosslinks.

To test for the involvement of $^1O_2$, we used the inherently longer lifetime of $^1O_2$ in D_2O than in H_2O, which will lead to increased crosslink formation.[25] As shown in Figure 7B, using D_2O produced a higher IOP_L irradiation than using H_2O. Thus, the protein photocrosslinking responsible for bonding between amnion and cornea is mediated, at least partially, by $^1O_2$.

DISCUSSION

These studies demonstrate that a light-activated technology that joins tissue surfaces by forming molecular crosslinks between proteins can securely attach amniotic membrane to the corneal surface. The seal formed was immediate and strong enough to seal penetrating eye wounds. The seal strength

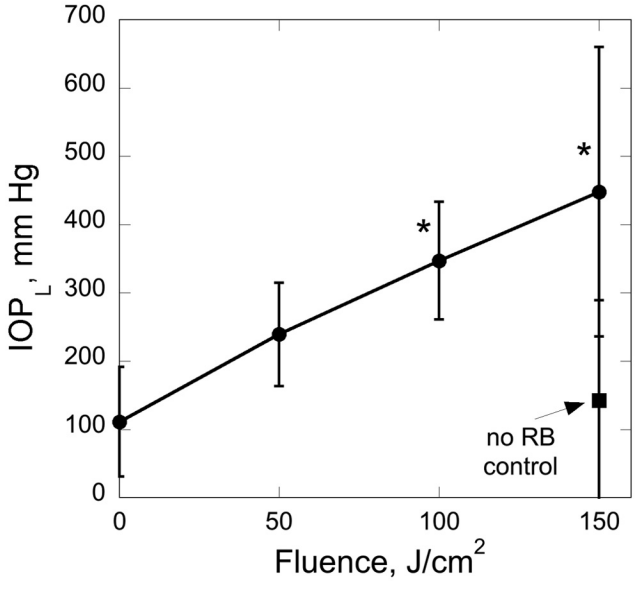


FIGURE 5. In vivo bonding of RB-stained amnion to corneal surface of rabbit eyes. Varying fluences were delivered at 0.25 W/cm^2 after staining with 0.1% RB for 5 minutes. No RB control, unstained amnion irradiated with 150 J/cm^2; $n = 5$. *$P < 0.05$ compared with no RB control.

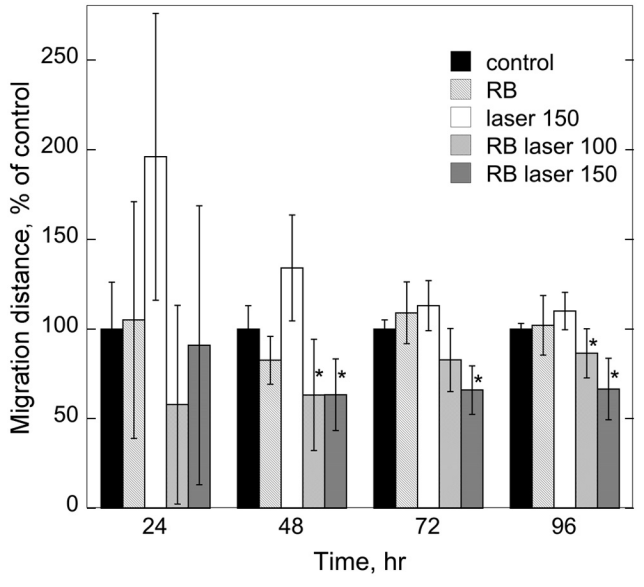


FIGURE 6. Migration of corneal epithelial cells on RB- and light-treated amniotic membrane. Cells attached to irradiated (0.1% RB, 100 and 150 J/cm^2) and control amnions were allowed to migrate away from a circle, and the migration distance was measured. Mean ± SD, cumulative results from three experiments with duplicate samples. *$P < 0.05$.

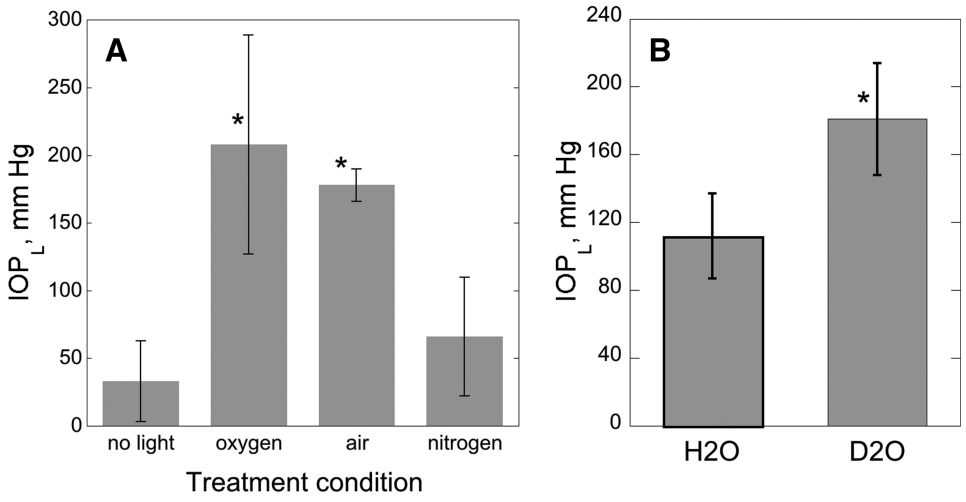


FIGURE 7. (**A**) Comparison of oxygen and nitrogen atmospheres on bond strength after PTB using ex vivo rabbit eyes. After conditioning eyes in oxygen, air, or nitrogen atmospheres, RB-stained amnion patches on corneas were irradiated with 100 J/cm^2. n = 4/group. $^*P < 0.05$ compared with no light control. (**B**) Effect of D_2O on IOP_L. RB was prepared in H_2O or D_2O, and eyes were incubated in H_2O or D_2O before bonding using 100 J/cm^2; n = 5/group. $^*P < 0.05$ compared with samples treated in H_2O. (**B**) Effect of D_2O on IOP_L. RB was prepared in H_2O or D_2O, and eyes were incubated in H_2O or D_2O before bonding using 100 J/cm^2; n = 5/group. $^*P < 0.05$ compared with samples treated in H_2O.

increased with increasing fluence and required a short irradiation time.

Sutureless attachment of amnion to cornea has the advantages of being rapid, forming an immediate water-tight seal and not causing additional damage to the cornea compared with the use of sutures. Although fibrin glue has been used for sealing amnion to the ocular surface[26,27] and is used for low-tension applications, clinical experience indicates that it has insufficient bonding strength required for large lacerations and stellate lacerations. The results of this study using a V-shaped incision mimicking a large irregular laceration support this experience. Fibrin sealant produced a lower bonding strength (Fig. 3A) and adhesion strength (Fig. 3B) than PTB. In addition, fibrin adhesive is sticky, difficult to use on cornea, and must be prepared immediately before use. PTB can be simplified for clinical application by using prestained, dry-stored amnion discs and a compact, inexpensive, non–laser light source (e.g., LED) that can deliver higher irradiances that shorten the irradiation time.

Photosensitized crosslinking of collagen is well established and is under evaluation for the treatment of keratoconus using riboflavin-5-phosphate (RF-5P).[28] RB also photosensitizes crosslink formation in collagen gels and scaffolds.[29,30] Our results (Fig. 6) indicate that RB, like RF-5P,[31] initiates protein crosslinks by a mechanism involving $^1O_2$. However, riboflavin has been shown to be phototoxic to keratocytes,[14] whereas our results indicate that PTB will not damage keratocytes (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental). We have previously shown that PTB was not phototoxic to dermal cells in vivo when PTB was used to seal skin wounds.[32] A small decrease in the extent of corneal epithelial cell migration was observed (Fig. 6). Whether this response will be observed in vivo must be tested. The opaque disc over the central cornea effectively blocked light from entering the pupil, but other treatment geometries may not allow this approach. We are testing optical delivery devices that prevent focusing on the retina and thus keep the irradiance below established damage thresholds.[33]

Collagens type I and III provide multiple positively charged lysines and arginines that may be sites for ionic bonding with negatively charged RB. Sequence-specific and hydrophobic interactions[34] also contribute because other negatively charged dyes (i.e., riboflavin-5-phosphate) do not bind in amnion (unpublished results, 2010). Tight RB-collagen complexes might involve sites within the collagen fibers, and loosely bound RB may be associated with the fiber surfaces. This model is consistent with the observation (Fig. 4B) that amnion containing only tightly bound RB does not photocrosslink to the cornea because, to bond the tissue surfaces, the protein-protein crosslinks must form between amino acids on the external surface of collagen fibers.

The full-thickness wounds used for this study are frequent in both civilian and military populations, in which they constitute approximately 50% of the eye wounds in current wars.[35,36] Sealing amnion with PTB over these difficult-to-suture wounds has distinct advantages. In addition, our results suggest that PTB can be used for sealing amnion in corneal surgery, including pterygium excision, forniceal reconstruction, corneal melting syndromes, and attachment of composite limbal stem cell and amnion grafts.[37] We have initiated a detailed study of longer term biological responses to photobonding amnion to cornea.

Acknowledgments

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Light-Activated Sutureless Closure of Wounds in Thin Skin

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Background and Objectives: Closing lacerations in thin eyelid and periorbital skin is time consuming and requires high skill for optimal results. In this study we evaluate the outcomes after single layer closure of wounds in thin skin with a sutureless, light-activated photochemical technique called PTB.

Study Design/Materials and Methods: Dorsal skin of the SKH-1 hairless mouse was used as a model for eyelid skin. Incisions (1.2 cm) were treated with 0.1% Rose Bengal dye followed by exposure to 532 nm radiation (25, 50, or 100 J/cm^2; 0.25 W/cm^2) for PTB. Other incisions were sutured (five 10-0 monofilament), exposed only to 532 nm (100 J/cm^2), or not treated. Outcomes were immediate seal strength (pressure causing leakage through incision of saline infused under wound), skin strength at 1, 3, and 7 days (measured by tensiometry), inflammatory infiltrate at 1, 3, and 7 days (histological assessment), and procedure time.

Results: The immediate seal strength, as measured by leak pressure, was equivalent for all PTB fluences and for sutures (27–32 mmHg); these pressures were significantly greater than for the controls (untreated incisions or laser only treatment; $P < 0.001$). The ultimate strength of PTB-sealed incisions was greater than the controls at day 1 ($P < 0.05$) and day 3 ($P < 0.025$) and all groups were equivalent at day 7. Sutures produced greater inflammatory infiltrate at day 1 than observed in other groups ($P = 0.019$). The average procedure time for sutured closure (311 seconds) was longer than for the PTB group treated with 25 J/cm^2 (160 seconds) but shorter than the group treated with 100 J/cm^2 (460 seconds).

Conclusion: PTB produces an immediate seal of incisions in thin, delicate skin that heals well, is more rapid than suturing, does not require painful suture removal and is easy to apply. Lasers Surg. Med.

Key words: eyelid; incision; laceration; laser welding; photochemical tissue bonding; Rose Bengal; wound healing; wound closure

INTRODUCTION

Repairing lacerations and closing surgical incisions in eyelid skin and the adjacent periorbita is particularly problematic because the tissue is very thin and delicate. Sutures are the current gold standard for closure of these wounds, but are time-consuming, especially for long or multiple lacerations, and require skilled placement of fine sutures. Suture marks may be caused by epidermal ingrowth along the suture track when the sutures are tied too tightly or remain in place too long [1]. In addition, suture removal is painful, generally requiring sedation in pediatric patients.

A sutureless light-activated technique has been introduced for wound repair that reconnects extracellular matrix proteins to form a continuous molecular seal. With this technique, called photochemical tissue bonding (PTB), a photoactive dye is applied to the tissue surfaces that are then placed in contact and irradiated with green light. The visible light does not thermally damage the tissue [2,3]. PTB is a light-activated chemical process that produces molecular bridges (crosslinks) between tissue surface proteins. We have already demonstrated in porcine skin [4] and in a clinical study (ClinicalTrials.gov, NCT00586040) that PTB is an excellent replacement treatment for superficial interrupted sutures in a layered closure of full thickness surgical wounds. After deep sutures were used to approximate the wound edges, PTB produced excellent healing and less scarring than epidermal sutures.

Eyelid skin is generally less than 1 mm thick and lacerations require only superficial sutures, not a layered closure. PTB may substantially reduce the time required for closing eyelid skin lacerations compared to sutured closure and has the advantages of allowing normal mobility of this skin compared to the stiffness produced by cyanoacrylate glues or Steri-Strips™ (3M, St. Paul, MN). However, forming a strong seal edge-to-edge in thin eyelid skin may be a challenge for PTB, because the strength of

Conflict of Interest: The PTB technology has been licensed by the Massachusetts General Hospital to Aura Medsystems, Inc. I.E.K. and R.W.R. have consulted for Aura Medsystems and will share in any income received by the hospital from the license agreement in accordance with hospital policy.

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the bond is dependent on the size of the tissue areas in contact. In the present study, we used dorsal skin of the SKH-1 hairless mouse as a model for eyelid skin. This mouse is albino, hairless, and immunocompetent, and the back skin is 0.4–0.5 mm thick [5]. The processes of wound healing and inflammation are well characterized and readily observed in this strain of mice [6].

The broad aim of this study was to evaluate the efficacy of PTB for the single layer repair of incisions in very thin skin. Outcome measures were initial seal strength, adherence at 1, 3, and 7 days, inflammatory infiltrate, and procedure time.

MATERIALS AND METHODS

Surgical Procedure

The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures in this study. A total of 32 female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA), 7–8 weeks old, weighting 17–19 g, were anesthetized with ketamine (90 mg/kg) and xylazine (9 mg/kg) by intraperitoneal injection. The skin area for incision was cleaned with a 10% solution of povidone iodine (Clinipad Corporation, Guilford, CT) and rinsed with sterile saline solution before surgery. Four full-thickness incisions (1.2 cm long) were made in the skin on the back of each mouse, two on the upper flank of each side. All incisions were made perpendicular to the spine (Fig. 1A). After surgery, the incisions were dressed with Tegaderm^{TM} film (3M Health Care) and observed once each day for 3 days.

Photochemical Tissue Bonding

A solution of 0.1% (w/v) Rose Bengal (Aldrich Chemical Co., Milwaukee, WI) in phosphate buffered saline was applied to the walls of the incision with a cotton swab and allowed to absorb for 1 min. A cw KTP laser (Oculight, IRIDEX Corporation, Mountain View, CA) was used to produce 532 nm radiation, which is strongly absorbed by Rose Bengal (absorption coefficient ~30,000 M^{-1} cm^{-1} at 532 nm). The laser irradiation was delivered with a 0.6 mm diameter fiber to a 1.13 cm^2 circular area. The irradiance was 0.25 W/cm^2. PTB was evaluated in three groups ($n = 5$ each) using laser fluences of 25, 50, or 100 J/cm^2 (100-, 200-, and 400- second exposures, respectively) to close the incisions. For the first minute of the irradiation, the wound was very gently held closed using forceps with slight eversion of the wound edges.

Control groups ($n = 5$ each) received either no treatment or laser only (100 J/cm^2, 532 nm). In another group ($n = 5$) the incisions were closed using black monofilament 10-0 nylon (Ethilon, Ethicon, Somerville, NJ). Five sutures, perpendicular to the wound line, were evenly spaced (0.2 cm) and of equal length (0.2 cm) and closed with 2-1-1 knots. The incisions were randomized, using a prescribed order for treatments that did not follow an obvious pattern. Thus, the quality of the incision was not related to the treatment group.

Leak Pressure (LP) Measurement

The integrity of the tissue seal was determined at day 0 (immediately after treatment) by infusing saline into a compartment between the dermis and subcutaneous layers and measuring the pressure required to cause leakage of saline through the incision. This procedure is shown in Figure 1B. Before treatment, an angiocath (IV catheter needle, 24 GA, Becton Dickinson, Franklin Lakes, NJ) was inserted through normal skin 3 mm from one end of the incision and placed between the dermis and subcutaneous layer to the middle-point of the incision. The inner metal needle was

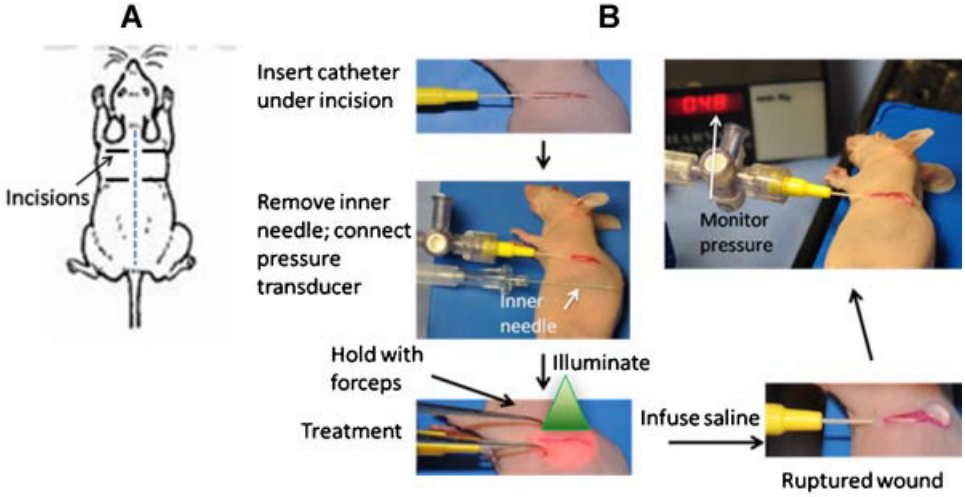

Fig. 1. Schematic diagrams of incisions and procedure for measurement of seal strength. **A**: Sites of 1.2 cm incisions on dorsal skin of mouse. **B**: Steps in measurement of leak pressure. After insertion of catheter (top left) and connecting to pressure transducer, the incision is closed with PTB, sutures, laser only, or untreated (lower left). After treatment, saline is infused under the incision (lower right) and the pressure that causes rupture is recorded.

removed so that the remaining flatheaded plastic hollow needle did not penetrate into the surrounding tissue. The needle was then connected to both a calibrated blood pressure transducer (Harvard Apparatus, South Natick, MA) and a mini-infuser (Model 400; Bard Harvard) through a T-coupler. The pressure was gradually increased by infusion of saline (0.2 ml/min) through the angiocath. The signal generated by the transducer-amplifier combination indicates the pressure. The pressure was increased until either the incision opened or fluid leaked from the incision. For groups that were not closed with PTB or sutures, each incision was held together with forceps for 400 seconds allow for formation of a natural fibrin seal.

Adherence Test

After euthanization on days 1, 3, 7, and 14, two strips (0.3 cm wide and ~0.8 cm long) were made across the treated incisions, one for tissue strength measurements and one for histology. The force needed to break the skin at the incision was measured with a tensiometer coupled to a digital force gauge (Zwick Roell Z010, Kennesaw, GA) with a 10 N load cell. The peak force for rupture is divided by the cross sectional area of the skin strip (width × thickness) to determine the ultimate stress. The data were recorded by computer software (testXpert II V3.1, Zwick Roell).

Procedure Time

For the PTB groups, the time from applying RB to the wound edges to the time for completion of illumination was recorded. For the suture group, the time from opening the suture package to the time for completion of suturing was recorded.

Histology

A total of 65 specimens were obtained postoperatively: 5 specimens for the untreated, laser only, PTB (25) and PTB (100) groups were taken at day 1 and 3 and for all groups at day 7 and 14. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Five micrometer vertical sections were cut to include the incision site and stained with H&E. All slides were coded and evaluated in a blinded manner by four researchers. Severity of skin inflammation was semi-quantitatively analyzed and scored on a three value scale: grade 0, normal; grade 1 (infiltrating inflammatory cells were present in <10% in the 200× histology image), grade 2 (10–50%), grade 3 (>50%).

Statistics

The level of significance between different groups was analyzed by ANOVA with SPSS 13.0 software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Acute Bond Strength

The influence of closure method on the pressure required to open the wound immediately after sealing (leak pressure, LP) was evaluated. The three PTB groups were treated with laser fluences of 25, 50, or 100 J/cm^2 delivered over 100, 200, and 400 seconds, respectively, at a constant irradiance of 0.25 W/cm^2. Control incisions were untreated or treated with laser only (100 J/cm^2). One group was closed with 10-0 suture. The results are shown in Figure 2. The LP for incisions treated with laser only was not significantly different from the untreated control. The LP was ~3-fold greater for all PTB-treated incisions than for the untreated or laser only group ($P < 0.001$). There was no significant difference of LP among PTB-treated groups ($P > 0.05$). The saline infusion endpoint for all sutured incisions was leakage between the sutures. None of the sutured incisions dehisced. The LP for all PTB groups was equivalent to that for the suture group ($P > 0.05$). No signs of thermal damage, such as tissue shrinkage, were observed under the irradiation conditions used.

Adherence Strength

Incision adherence was assessed by tensiometry to determine ultimate strength (MPa), elongation (%), and Young's Modulus (MPa). One-way ANOVA showed that incision ultimate strength and Young's modulus increased with time after closure (Fig. 3). Ultimate strength for the PTB (25) and PTB (100) groups on days 1 and 3 were significantly higher than those for the untreated and laser only groups ($P < 0.05$). The suture group could not be tested on days 1 and 3 because removing the sutures separated the incision. There was no significant difference in ultimate strength amongst all groups on day 7. Elongation and Young's modulus were equivalent in all groups at all treatment times except for a difference in elongation

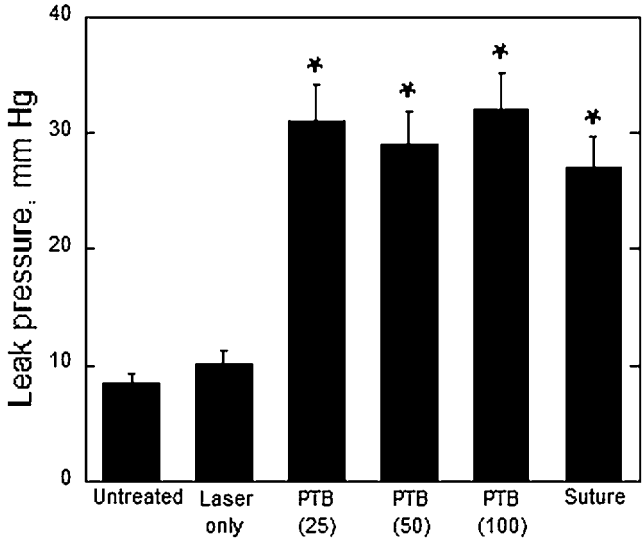

Fig. 2. Relationship between closure methods and leak pressure. Immediately after treatment, saline was infused under incisions and the pressure causing leakage through the incision recorded. $^*P < 0.001$ compared to the untreated and laser only groups.

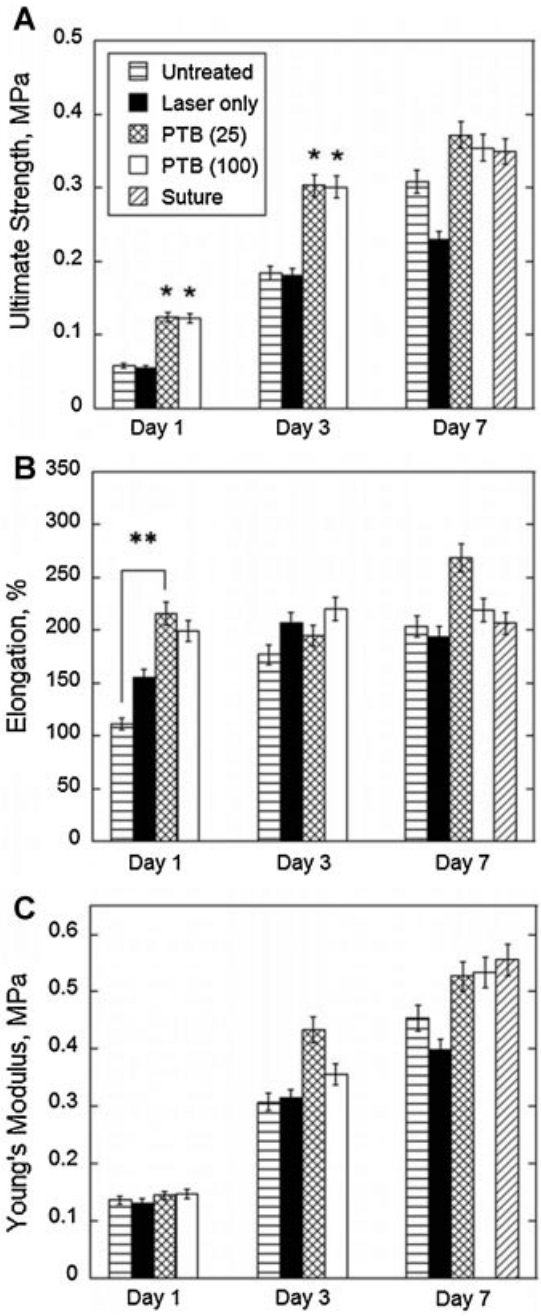

Fig. 3. Measurements of physical properties of incision site on days 1, 3, and 7 after closure with PTB, sutures, laser only, or no treatment. Measurements were made on skin strips 0.3 cm wide × 0.8 cm long. $N = 3$ or 4. **A**: Ultimate strength. **B**: Percent elongation. **C**: Young's modulus. $^{*}P < 0.05$ compared with the untreated and laser only groups. $^{**}P < 0.05$ compared with the untreated group.

between untreated and PTB (25) groups on day 1 ($P > 0.05$). The results for samples taken at day 14 could not be analyzed because a decrease in the measured force occurred before the skin broke at the incision.

Procedure Time

The time required for incision closure in PTB (25), PTB (100) and the suture group was measured. For the PTB (25) and PTB (100) groups, the average times required were 160 and 460 seconds, respectively. The average time required in the suture group (311 seconds) was shorter than for the PTB (100) group ($P < 0.05$), but is about two times longer than the PTB (25) group ($P < 0.05$).

Histology

We assessed inflammation by scoring the severity of infiltration of leukocytes into the dermis. One day after surgery, we observed relatively more inflammatory cell infiltrate in the suture group (Table 1). Many fewer leukocytes were observed in the other groups. From day 3 to 14, there were no significant differences in the amount of infiltrating cells amongst all the groups. Although the suture group showed the greatest infiltrate, this was not statistically significant after day 1.

DISCUSSION

In this study, the PTB technique was found to generate immediate adherence and good healing in a mouse skin incision model that mimicked an eyelid skin wound. Moreover, this technique produced less inflammation and required less time than conventional suture wound closure. These results suggest that PTB may be applicable to single layer skin wound closure in emergency wound treatment and cosmetic surgeries.

The formation of an immediate and continuous seal is a significant feature of eyelid wound closure with PTB. Since it is difficult to apply an occlusive water-tight dressing to eyelids after suture closure of wounds, sealing the incisions with PTB would allow patients to wash and shower. In addition, an immediate wound seal may reduce infections. The protein–protein crosslinks that create the immediate seal are expected to form through the entire thickness in mouse skin and at least 0.5 mm of human eyelid skin based on our previous modeling of PTB for skin incision closure [7]. This deep seal accounts for the greater pressure required to open PTB-closed wounds than those of the control (untreated and laser only) groups (Fig. 2). Sutures, however, are significantly stronger that the PTB seal. They remained intact during the strength measurements made immediately after repair with leakage occurring only between sutures.

Healing after PTB and sutured closure of incisions appeared to be similar with an increase in inflammatory cells one day after surgery and a decrease over the following 2 weeks in all groups (Table 1). The PTB groups did not show greater levels of inflammatory cells than the other groups, consistent with our previous study demonstrating that PTB was not toxic to skin cells *in vivo* [7]. In fact, on day 1 more inflammatory cells were observed in the suture group, likely due to a foreign body reaction to the suture. This result suggests that PTB will not cause greater scarring than sutured closure. Scarring cannot be assessed in a mouse skin model, but in a pilot clinical

TABLE 1. Evaluation of Inflammation After Closure of Incisions in Hairless Mouse Skin

	Leukocyte infiltration			
Groups	Day 1	Day 3	Day 7	Day 14
Untreated	1.38 ± 0.52*	1.50 ± 0.51	1.38 ± 0.51	1.08 ± 0.29
Laser (100)	1.25 ± 0.46*	1.65 ± 0.49	1.45 ± 0.52	1.09 ± 0.30
PTB (25)	1.17 ± 0.39*	1.35 ± 0.49	1.36 ± 0.50	1.08 ± 0.29
PTB (100)	1.13 ± 0.35*	1.35 ± 0.49	1.13 ± 0.35	1.00 ± 0.00
Suture	2.00 ± 0.71	1.67 ± 0.89	1.50 ± 0.53	1.25 ± 0.46

Values are mean ± SE of five samples per group.
*$P < 0.05$ compared to suture group at day 1.

study PTB caused less scarring than superficial sutures for closure of skin excisions (unpublished results). An increase in the strength of the skin at the wound site over the first 7 days was also observed, indicating deposition and organization of new collagen (Fig. 3A). Although sutures are stronger than the PTB seal immediately and probably at early times, by 7 days the skin strength was the same for PTB-treated and sutured (with sutures removed) wounds.

A shorter procedure time is an advantage of PTB over sutured repair. The PTB (25) group, which produced equivalent wound strength to the PTB (100) group (Figs. 2 and 3), required one-half the time as the suture group. The ratio of time required for PTB compared to suturing would be even greater for longer incisions because the beam size can be lengthened and the laser power increased to treat a longer incision in the same time used for the 1.2-cm incision in this study. Another time saving step for PTB may be reducing the 1 min delay between applying RB and irradiation. A caveat for comparing procedure times is, however, that the 10-0 sutures we used to close the very thin and soft mouse skin took longer to place than the 6-0 to 8-0 sutures generally used for patient eyelid skin.

Other comparisons of PTB and sutures can be made. Closure of eyelid wounds with PTB eliminates the need for painful suture removal, which in children generally requires sedation. Other sutureless methods, namely, cyanoacrylate glues and skin tapes, restrict opening and closing of the eyelids compromising vision [8]. Potential suture-related complications are also eliminated with PTB. These include placing sutures too deeply and incorporating the orbital septum during closure. This is particularly a problem in traumatic injuries and may lead to cicatricial ectropion and cicatricial lagophthalmos [9,10]. PTB is also very simple to apply, only requiring application of a dye solution to the wound edges, apposition of the edges, and exposure of the site to green light. The need for careful and evenly spaced placement of sutures is eliminated.

Since PTB utilizes a bright green light, a contact lens-like nonreflective corneal shield would be placed on the patient's eye, and the surgeon and operative team would wear eye protection. A small green clinical laser (Oculight, IRIDEX Corp., Mountain View, CA) was used in this study. Closure of eyelid incisions only required 25 J/cm^2 suggesting that small compact light sources might be used in the future. As blood also absorbs green light, the PTB irradiation should not be carried out in the presence of active bleeding.

In summary, these results demonstrate the potential of PTB to close full-thickness incisions in thin skin. The repair procedure produces an immediate watertight seal that heals equivalently to sutured closure, requires a shorter time than suturing, does not require painful suture removal, and is easy to apply. PTB merits further evaluation for closing wounds to eyelid and periorbital skin and may have particular application to traumatic injuries and to pediatric patients.

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