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A Photosealed Cap Prevents Disorganized Axonal Regeneration and Neuroma following Nerve Transection in Rats

Benjamin B. Scott, MD*†
Ruby C. Wu, BA*
Viviane Nietlispach, MD†
Mark A. Randolph, MAS†
Robert W. Redmond, PhD*

Background: Neuroma is a common sequela of traumatic peripheral nerve injury that can result in pain and decreased quality of life for patients. Neuromas result from axonal outgrowth in an attempt to reestablish continuity with the disrupted distal nerve end. Photosealing is a light-activated technique whereby tissues can be securely isolated in a strong and secure manner. This study investigated whether photosealing of autologous vein and crosslinked human amniotic membrane (xHAM) to cap the proximal stump of transected sciatic nerve would prevent disorganized axonal regeneration and neuroma in a rat model.

Methods: The right sciatic nerve of Lewis rats (n = 27, 300–350 g) was transected 1 cm proximal to the trifurcation. Animals were randomized to one of three groups (n = 9): no further intervention (Group 1), photosealing with xHAM (Group 2), or photosealing with vein (Group 3). After 60 days, rats were euthanized and their right hindlimbs were re-explored for evidence of disorganized axonal regeneration and/or bulbous neuroma.

Results: All untreated control animals were found to have protruding nerve fibers, often invading the adjacent muscle, and 33% of these control animals exhibited a bulbous neuroma. Photosealing with xHAM successfully capped 100% of nerves, with no observable axonal outgrowth. Photosealing with vein prevented axonal outgrowth in eight of nine nerves. No bulbous neuroma was found in any photosealed nerves.

Conclusion: Nerve capping with photosealed xHAM or autologous vein can prevent axonal outgrowth in transected nerves, therefore decreasing the likelihood of symptomatic neuroma formation following nerve transection injury or surgical intervention. *(Plast Reconstr Surg Glob Open 2022;10:e4168; doi: 10.1097/GOX.0000000000004168; Published online 7 March 2022.)*

INTRODUCTION

Formation of a neuroma is a common sequela of peripheral nerve injury, often leading to functional deficits and decreased quality of life for the patient. Neuromas are benign growths of nerve tissue, containing all elements of the nerve sheath and nerve fibers, resulting from disorganized axonal regrowth following nerve injury.[1] Neuroma formation after nerve injury has been estimated to occur in 5%–10% of patients who sustain a peripheral nerve injury and as frequently as 15%–32% of lower extremity amputations.[2–4] Axonal nerve damage after nerve injury/amputation initiates an inflammatory response and regenerative process in an attempt to recreate nerve continuity. Neuroma can result from disorganized axonal outgrowth that fails to reach the target tissue, instead forming a bulbous mass of tangled axons.[5] When no viable distal nerve target is available (eg, amputation), the surgical approach to prevent neuroma formation often focuses on inhibiting axonal regrowth from the injured proximal nerve stump. Mechanisms studied to prevent disorganized axonal regrowth include implantation of the proximal nerve stump into adjacent tissue,[6–9] nerve coaptation,[10–13] nerve conduits,[14–18] and nerve capping,[19–24] with mixed results.

Nerve conduits and caps have historically been sutured into place at the proximal end of the transected nerve. The epineurial suture technique potentially results in trauma

*From the *Wellman Center for Photomedicine, Harvard Medical School, Massachusetts General Hospital, Boston, Mass.; and †Plastic Surgery Research Laboratory, Department of Surgery, Harvard Medical School, Massachusetts General Hospital, Boston, Mass.*

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to the nerve and foreign body response, including inflammation and fibrosis,[25] and gaps between sutures can result in escape of regenerating axonal fibers. Photochemical tissue bonding (PTB) harnesses light and a photoactive dye to chemically crosslink and seal tissue interfaces in a watertight manner,[26] with obvious advantages over suture fixation of nerve cap materials to isolate the regenerating nerve environment. This technology has previously demonstrated efficacy in sealing a variety of tissues in animal models,[27] including human amniotic membrane (HAM) as an epineurial wrap in peripheral nerve repair, where it was associated with improved functional recovery and histomorphometric parameters when compared with standard suture repair techniques.[28–32] The photosealed environment was hypothesized to isolate the regenerating nerve from the surrounding environment and increase the concentration of growth factors at the repair site, promoting nerve regeneration and improved function.

We chose to investigate two potential biomaterials of clinical relevance for nerve photosealing to prevent disorganized axonal outgrowth and/or neuroma. HAM is a commercially available, immunoprivileged material with demonstrated antiinflammatory properties,[33] used in wound healing. However, HAM has been found to be susceptible to proteolytic degradation in some settings[34]; thus, to promote increased longevity, the HAM was biocompatibly crosslinked before photosealing (vide infra). On the other hand, autologous vein is often readily available and easily harvested in the clinical setting,[20] with no concern regarding immunoreactivity. Additionally, vein could also be harvested from an amputated limb, resulting in no additional morbidity to the patient. We tested the hypothesis that photosealing would inhibit axonal regeneration after nerve transection by utilizing PTB to seal either crosslinked HAM (xHAM) or harvested donor vein from isogeneic Lewis rats over the proximal end of a transected sciatic nerve. The ultimate goal of this study was to develop a potential method to prevent the formation of neuroma after peripheral nerve injury and amputation.

Takeaways

Question: Could a photosealed cap of either human amniotic membrane or autologous vein prevent disorganized axonal regeneration and neuroma after nerve transection?

Findings: Axonal regeneration and neuroma can be prevented by a photosealed cap of either human amniotic membrane or autologous vein in a rat model of sciatic nerve transection.

Meaning: This simple technology has potential to improve outcomes following nerve transection and amputation.

METHODS

Preparation of Crosslinked Human Amnion

HAM was obtained from the placenta of healthy donors following Cesarian delivery with the approval of the institutional review board at the Massachusetts General Hospital (Boston, Mass.). Following removal of the chorion, the HAM was washed with sterile phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, Mo.), placed on sterile parafilm, and then stored in a 1:1 solution of 100% glycerol (Sigma-Aldrich)/Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) with 1% penicillin-streptomycin (Gibco) at −80°C. On the day of use, the HAM was defrosted and washed three times with sterile PBS to remove glycerol. Chemical crosslinking was performed with a solution of 4 mM EDC [N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride] (Sigma-Aldrich), 1 mM NHS (N-hydroxysuccinimide) (Sigma-Aldrich), and 9 mM MES [2-(N-morpholino) ethanesulfonic acid] (Sigma-Aldrich) on a HS 260 rocker (IKA Works Inc., Wilmington, NC) at 10 Hz for 1 hour. The crosslinked HAM (xHAM) was then washed repeatedly with PBS to completely remove crosslinking solution and placed in sterile PBS before use.

Animals and Operative Procedures

All experiments and animal care conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. All animals had free access to standard rat chow and water.

Harvest of Lewis Rat Donor Femoral Vein

To reduce morbidity to the recipient, isogeneic Lewis rats (Charles River Laboratories International Inc., Wilmington, Mass.) were used to provide donor vein for use in photosealed capping without risk of immunoreactivity, analogous to an autologous tissue. Donor Lewis rats (n = 5, 300–350 grams) were anesthetized with isoflurane (1%–5% titrated to effect, i.h.) (Patterson Veterinary, Devens, Mass.). The bilateral inguinal folds were then clipped of hair and prepared in a sterile fashion. Bilaterally, the femoral vasculature was exposed with a 2-cm incision in the inguinal fold and retraction of muscular structures. The femoral veins were carefully dissected from surrounding structures under a dissecting microscope. Branching vessels from the femoral vein were doubly ligated with 9-0 nylon suture and divided. The femoral veins were ligated proximally and distally and ~1 cm segment of femoral vein was sharply excised. The animal was then euthanized with pentobarbital (100 mg/kg, i.p.) (Euthasol, Virbac AH, Inc., Fort Worth, Tex.). The harvested femoral veins were then opened sharply in a longitudinal fashion and thoroughly irrigated with heparinized saline. The opened vein was then placed on parafilm and covered with a sterile PBS-soaked gauze until use for nerve capping.

Nerve Transection and Photosealing

Lewis rats (n = 27, 300–350 g, Charles River Laboratories International Inc.) were acclimatized for 3 days before surgery in standard animal facilities at the Massachusetts General Hospital. Rats were anesthetized with isoflurane (1%–5% titrated to effect, i.h.) (Patterson Veterinary),

and given preoperative doses of buprenorphine (0.01 mg/kg, s.c.) (Buprenex, Reckitt Benckiser, Slough, UK) and carprofen (5 mg/kg, s.c.) (Rimadyl, Zoetis Inc., Parsippany-Troy Hills, N.J.). The right hindlimb was clipped, prepared with betadine, and draped in a sterile fashion. A 1-cm oblique incision was made on the posterior right thigh with a No. 15 scalpel. The fascial plane between the gluteus maximus and the anterior head of the biceps femoris was opened, revealing the sciatic nerve. The nerve was exposed from the sciatic notch to the distal trifurcation and the diameter measured with a micro caliper before transection 1 cm proximal to the trifurcation (Fig. 1A). The distal sciatic nerve stump was buried into adjacent muscle and secured with two 8-0 nylon sutures to prevent any possibility of reestablishment of nerve continuity (Fig. 1B).

Animals were randomized into one of three groups: no further intervention (Group 1, n = 9) to serve as a control group, photosealing (vide infra) with xHAM (Group 2, n = 9), or photosealing with harvested donor vein (Group 3, n = 9) (Fig. 2). In all groups, the wound bed was then irrigated with sterile saline, muscle was sutured closed with 4-0 Vicryl (Ethicon, Somerville, N.J.), and the skin incision closed with 5-0 Monocryl (Ethicon). All animals received carprofen (5 mg/kg, s.c., Rimadyl, Zoetis Inc., Parsippany-Troy Hills, N.J.) analgesia daily for 3 days postoperatively.

Photosealing

A sterile solution of 0.1% rose bengal (RB) (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) was prepared by dissolving RB (Sigma-Aldrich) in sterile PBS (Sigma-Aldrich) and filtering through a 0.22 µm filter (EMD Millipore, Burlington, Mass.). The dye solution was applied to the nerve capping material for 1 minute and excess removed with a sterile gauze. The nerve capping material was trimmed to size in an approximately $5 \times 5\ mm^2$ while flat on parafilm, large enough to ensure a 2–3 mm circumferential interface with the epineurium of the proximal nerve stump. The central portion of the square was then applied directly over the transected nerve stump (Fig. 1C, D). Microforceps were used to apply the edges in direct contact with the epineurium, forming the cap (Fig. 1E). Great care was taken to ensure that the stained nerve capping material was in intimate contact with the epineurium in a 360 degree fashion (Fig. 2, cap preillumination). The nerve capping material was bonded over the nerve stump by illumination at 532 nm from a continuous wave KTP laser (Laserscope Aura-i, San Jose, Calif.; irradiance of 0.5 W/cm^2) in two increments of 180 degrees around the nerve stump for 60 seconds each (Fig. 1F). Postillumination photobleaching of RB dye indicated that photosealing had occurred (Fig. 1G).

Histology

Animals (n = 27) were euthanized postoperatively at day 60 by injection of pentobarbital (100 mg/kg, i.p.) (Euthasol, Virbac AH, Inc., Fort Worth, Tex.). The right dorsal hindlimb incision was reopened, and dissection to the transected right sciatic nerve was performed. The gross appearance of the nerve, including evidence of adhesion to adjacent muscle, regenerating nerve fibers, and diameter of the terminal end of the proximal nerve stump, was recorded. The sciatic nerve was harvested (if adherent to adjacent muscle, the nerve was harvested en bloc with muscle) and secured longitudinally to a nonsterile wooden tongue depressor (Medline Industries, Northfield, Ill.). The nerve was submerged in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde buffered PBS (Sigma-Aldrich) for 20 minutes. A single edged razor blade (Stanley Black and Decker, New Britain, Conn.) was used to divide the stiffened nerve into either longitudinal or transverse sections. These individual sections were fixed in 10% formalin buffered PBS for 72 hours before paraffin embedding. From all paraffin blocks, 5-µm sections were cut, and staining was performed with Masson's trichrome.

RESULTS

All animals (n = 27) survived to end of study (60 days) without complication. No signs of autotomy were present in any animal. All Group 1 rats (control) were found to have outgrowth of axonal fibers emanating from the transected proximal nerve stump, often invading adjacent muscle (Fig. 2, end of study). Three of nine Group 1 rats were found to have a proximal nerve stump with a diameter at least twice that of sciatic nerve 1 cm proximal to the transection point. Longitudinal sections of control nerves demonstrated nerve infiltrating muscle fibers, with nervous tissue intimately intermixed with striated muscle fibers (Fig. 3). A transverse section through a bulbous end neuroma of a control nerve demonstrated intermixing of nerve tissue in

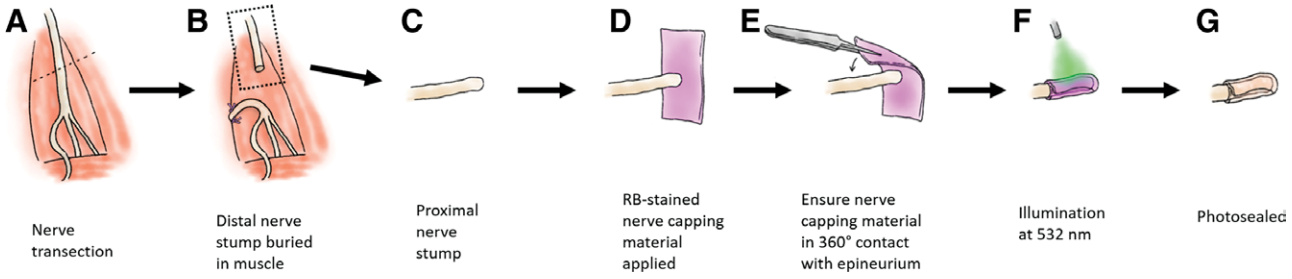

Fig. 1. Schematic of photosealing procedure. A, Nerve transected 1 cm proximal to trifurcation. B, Box around proximal nerve stump, with distal nerve stump buried in adjacent muscle and sutured in place. C, Proximal nerve stump posttransection. D, Nerve capping material placed directly over proximal nerve stump. E, Nerve capping material placed around epineurium to ensure 360 degree contact. F, Illumination with 532 nm light. G, Photobleaching of the RB dye demonstrating photocrosslinking has been successfully performed.

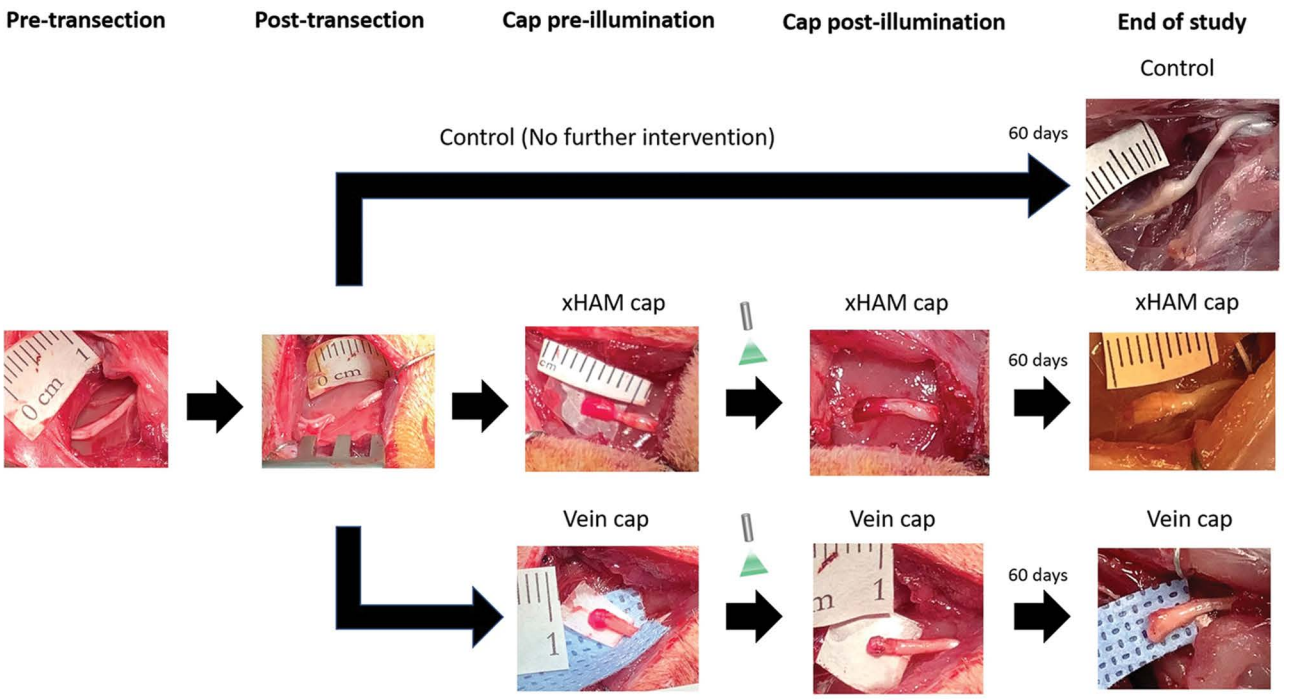

Fig. 2. Overview of each arm of the study. Pretransection: Demonstrating dissection of sciatic nerve of the right hindlimb free from surrounding tissues. Posttransection: Sciatic nerve is transected 1 cm proximal to trifurcation. Cap preillumination: xHAM or vein cap stained with RB, in place with 360 degree contact with epineurium. Cap postillumination: xHAM or vein cap in place after illumination. End of study: proximal nerve stump at 60 days posttransection. Control nerve demonstrating bulbous neuroma with regenerating axons invading adjacent muscle, xHAM, and vein caps in place without axonal regeneration or neuroma.

complete disarray with collagen bundles: histologic evidence of a neuroma (Fig. 4A, B). This is in contrast to a transverse section from the same sciatic nerve at a point 1 cm proximal to the transection point, which demonstrates a small amount of endoneurial fibrosis in an otherwise normal appearing nerve (Fig. 4C, D). The photosealed xHAM cap remained in place in all treated nerves in Group 2, with no evidence of axonal regeneration or neuroma (Fig. 2, end of study). Longitudinal sections demonstrate an intact, albeit folded with redundant layers of amnion, cap and absent axonal outgrowth, without evidence of invasion of adjacent muscle tissue or neuroma (Fig. 5). The photosealed vein cap remained in place in all treated nerves in Group 3; however, upon histological examination, one partial vein cap failure was noted, with axonal fibers escaping laterally around part of the vein cap with invasion of the adjacent muscle resulting in intermixing of nerve tissue and muscle fibers, similar to controls (Fig. 6). All other longitudinal sections of Group

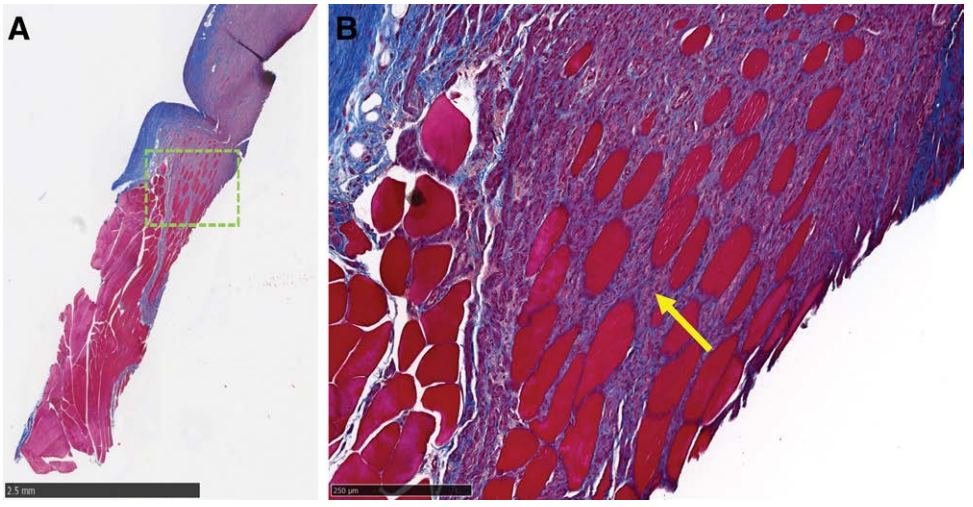

Fig. 3. A, Longitudinal section of control nerve with regenerating axons invading adjacent striated muscle (scale bar: 2.5 mm). B, Higher magnification of longitudinal section of control nerve revealing intimate intermixing of nerve tissue (arrow) with muscle fibers (scale bar: 250 μm).

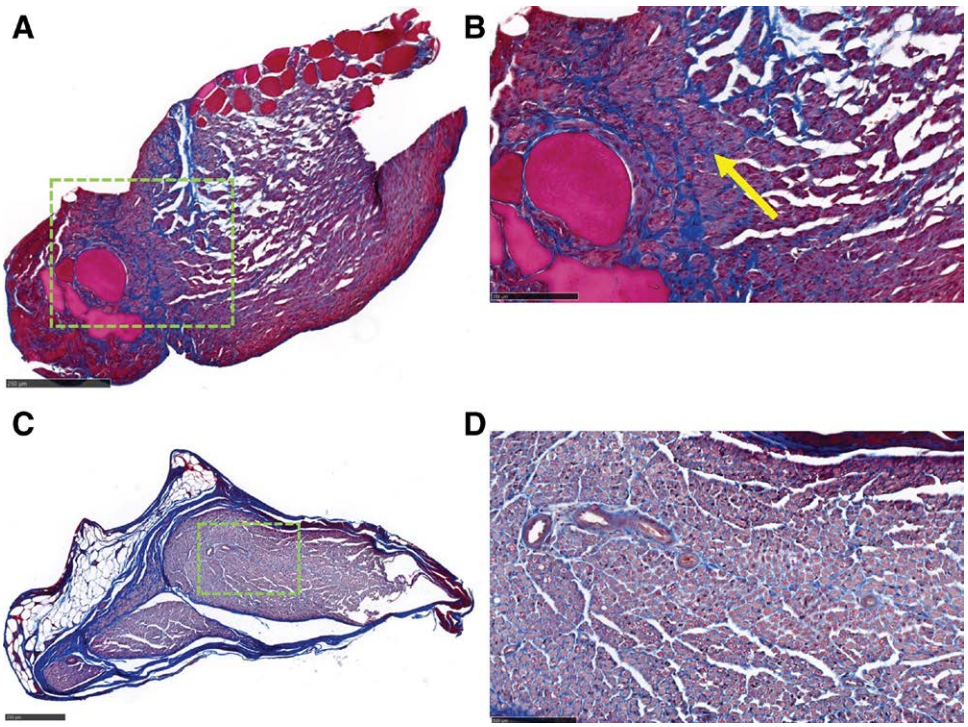

Fig. 4. A, Transverse section through bulbous neuroma of a control nerve (scale bar: 250 μm). B, Higher magnification of transverse section through control neuroma, demonstrating nerve tissue in disarray with collagen bundles (arrow) consistent with neuroma (scale bar: 100 μm). C, Transverse section from 1 cm proximal to bulbous neuroma from the same control nerve, revealing normal nerve tissue (scale bar: 250 μm). D, Higher magnification of transverse section through proximal control nerve demonstrating organized nerve fibers (scale bar: 250 μm).

3 nerves revealed intact vein caps without breaching of the perineurium by regenerating axons (Fig. 7).

DISCUSSION

Symptomatic neuroma after peripheral nerve injury can lead to pain and suffering, inability to wear prosthesis, and may require surgical revision and other health care concerns and expenditures.[35] Preventing and managing symptomatic neuroma remains a challenge for the reconstructive surgeon and although numerous approaches to prevent axonal regrowth have been studied, there is no universally accepted clinical method to prevent neuroma.

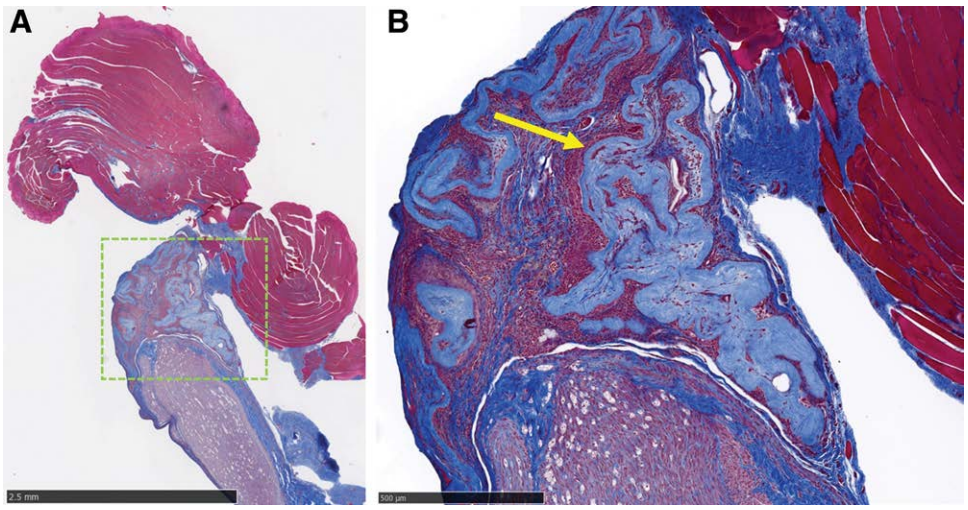

Fig. 5. A, Longitudinal section of transected nerve with xHAM cap intact without evidence of axonal regeneration (scale bar: 2.5 mm). B, Higher magnification of longitudinal section with xHAM nerve cap intact (arrow); no evidence of axonal regeneration into adjacent muscle (scale bar: 500 μm).

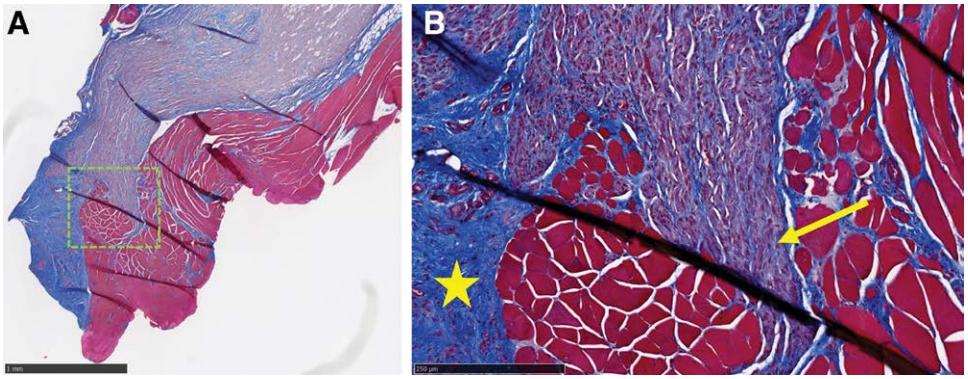

Fig. 6. A, Longitudinal section of vein cap failure, demonstrating incomplete coverage of vein cap resulting in regenerating axons escaping into adjacent muscle (scale bar: 1 mm). B, Higher magnification of vein cap failure revealing intimate intermixing of nervous tissue with striated muscle (arrow), similar to control nerves, demonstrating failure of vein cap (star) (scale bar: 250 μm).

Nerve caps, nerve conduits, and implantation into adjacent tissue are well-studied methods to prevent neuroma formation.[36] Reconstructive options such as targeted muscle reinnervation and regenerative peripheral nerve interface, initially developed to optimize prosthetic function, have recently demonstrated efficacy in reducing neuroma pain.[37,38] These examples of "active" reconstruction provide a new purpose for the nerve stump, and are more technically challenging than the "passive" nerve capping technique.[36] However, a widely agreed upon principle is that preventing uncontrolled axonal regrowth from the proximal stump may prevent neuroma formation and accompanying sequelae.

This study tested this principle utilizing two widely available materials (HAM and autologous vein) that photobond well to the nervous tissue. Autologous vein is often readily available at the time of injury with no additional financial cost. Additionally, there are many commercial forms of HAM currently available for clinical use.[39] In this study we demonstrated that photosealing with xHAM and vein results in a durable cap, as all 18 caps remained in place at 60 days after placement. This durable cap, photosealed circumferentially to the epineurium of a transected peripheral nerve, significantly hinders axonal regeneration as demonstrated by an absence of regenerative nerve fibers in 17 of 18 photosealed nerves. The photosealed cap not only provides a physical barrier to hinder axonal outgrowth but successful photosealing isolates the transected nerve from the surrounding environment, which often contains cytokines and other growth factors that promote axonal regeneration.[40–43] By isolating the transected nerve environment from extraneural growth factors and acting as a physical barrier to axonal outgrowth, the photosealed nerve cap prevents axonal regeneration. As neuromas form after disorganized axonal regrowth, one can postulate that the successful prevention of axonal regrowth at 60 days, as shown here, could significantly reduce the incidence of neuroma.

An additional benefit of photosealing is the absence of epineurial suture. Placing sutures results in inevitable needle trauma to the epineurium. Electron microscopy has demonstrated blood vessel wall defects twice the diameter of the needle after suture placement,[44] and it is reasonable to assume similar size defects in the epineurium. Traumatic needle defects to the epineurium may allow

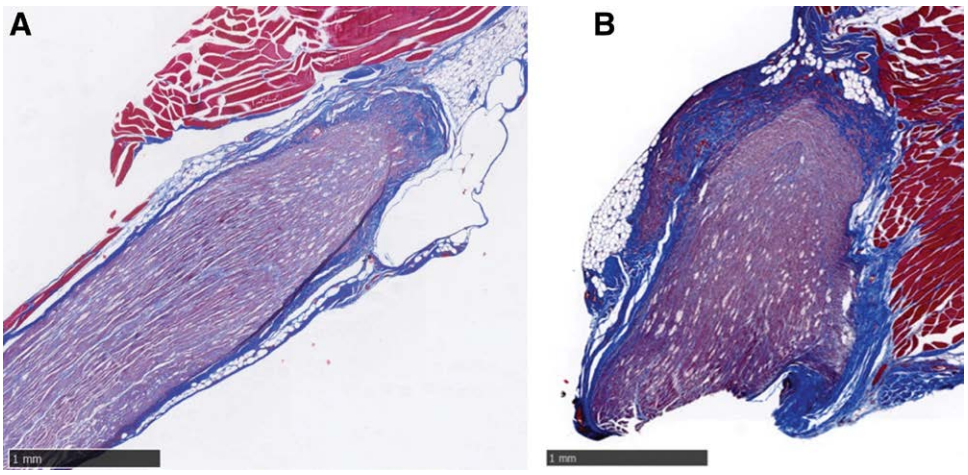

Fig. 7. A, B, Longitudinal sections of successful vein caps as evidenced by complete coverage with absence of regenerating nerve fibers (scale bar: 1 mm).

for axonal escape and regeneration, resulting in failure of the capping process. Suture may also lead to foreign body response, resulting in increased inflammation and fibrosis—two factors that are known to induce neuralgia.[45]

This was a pilot study with a hypothesis based on previously validated studies utilizing PTB in peripheral nerve injury and repair[29–32] with several limitations. Our study duration was relatively short (60 days). However, although neuromas may occur months after nerve injury, disorganized axonal regrowth and neuroma formation is often evident 28 days after amputation in rats.[5] Our study was designed to mimic nerve injury that occurs during extremity amputation without actually amputating the rat hindlimb. Although there was no evidence of any functional recovery in any animal in our study, this study did not assess for symptomatic neuroma, but only for histologic evidence of aberrant axonal regrowth and neuroma 60 days postinjury. Future studies in large animal models will be of longer duration and will assess for neuroma-induced pain, and true amputation models may be applicable. Furthermore, while this pilot study focused on preventing the formation of neuroma at time of nerve injury, the photosealing technique is easily applicable in the setting of revision neurectomy for symptomatic neuroma. Additional studies investigating the efficacy of a photosealed cap preventing the recurrence of neuroma after neurectomy are warranted.

Photosealing does require a low power light source, but light sources are readily available and a laser is not essential. Other low-cost light sources such as light-emitting diodes are suitable for the process. We did not include studies of xHAM or vein caps sutured in place in this pilot study due to prior studies from our laboratory[31,32] demonstrating that the intimate seal that results from the PTB process is required for desired effect. Although we only studied xHAM and vein in this pilot study, our technique is potentially applicable to other biological tissues with extracellular protein matrix, as long as they are fairly transparent (to allow light penetration to the interface with epineurium) and not too thick to be unwieldy in approximating to nerve sheath.

In this rat sciatic nerve amputation model, photobonding biologic nerve caps over the proximal sciatic nerve stump after transection and burying of the distal stump proved to be efficacious in preventing disorganized axonal regeneration from the proximal stump. It is reasonable to postulate that photosealed nerve caps of HAM or harvested autologous vein may prevent traumatic neuroma and adverse sequelae. By creating an intimate circumferential seal with the epineurium of the transected nerve, regenerating axons are prevented from extending distally, as demonstrated by their absence in this pilot study. Further study in large animals with a longer-term follow-up is required, but this technique shows great promise in preventing neuroma after peripheral nerve injury.

CONCLUSIONS

Nerve capping with photosealed HAM or autologous material such as vein results in a durable cap that remains in place 60 days after placement, isolating the site of nerve transection and preventing axonal regeneration of transected nerves. Without disorganized axonal regrowth, the formation of neuroma may be prevented.

Robert W. Redmond, PhD
Wellman Center for Photomedicine
Harvard Medical School
Massachusetts General Hospital, Thier 218
Boston, MA 02114
E-mail: redmond@helix.mgh.harvard.edu

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