A Simple, Cross-linked Collagen Tissue Substitute for Corneal Implantation

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Purpose. To develop a simple corneal substitute from cross-linked collagen.

Methods. Porcine type I collagen (10%; pH 5), was mixed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The final homogenous solution was molded to corneal dimensions, cured, and then implanted into rabbits and minipigs by lamellar keratoplasty. The implants were followed for up to 6 months after surgery. Clinical examinations of the cornea included detailed slit lamp biomicroscopy, in vivo confocal microscopy, topography and esthesiometry for nerve function. Histopathologic examinations were also performed on rabbit corneas harvested after 6 months.

Results. Cross-linked collagen (refractive index, 1.35) had optical clarity superior to human corneas. Implanted into rabbit and porcine corneas, only 1 of 24 of the surgical corneas showed a slight haze at 6 months after surgery. All other implants showed no adverse reactions and remained optically clear. Topography showed a smooth surface and a profile similar to that of the contralateral nonsurgical eye. The implanted matrices promoted regeneration of corneal cells, tear film, and nerves. Touch sensitivity was restored, indicating some restoration of function. The corneas with implants showed no significant loss of thickness and demonstrated stable host–graft integration.

Conclusions. Collagen can be adequately stabilized, using water soluble carbodiimides as protein cross-linking reagents, in the fabrication of corneal matrix substitutes for implantation. The simple cross-linking methodology would allow for easy fabrication of matrices for transplantation in centers where there is a shortage of corneas, or where there is need for temporary patches to repair perforations in emergency situations. (*Invest Ophthalmol Vis Sci.* 2006;47:1869–1875) DOI:10.1167/iovs.05-1339

Corneal opacification from disease or trauma is estimated to affect more than 10 million people worldwide[1] and is generally treated by transplantation with grafts from donor human corneas. Current and projected shortage of acceptable corneas for transplantation worldwide driven by age demographics, increases in incidence of transmissible diseases (like HIV, hepatitis, and Creutzfeldt-Jakob disease) and the increasing use of laser vision corrective surgery (which renders corneas unsuitable for grafting) have led to the need to develop viable alternatives to human donor tissue. Although survival of first time grafts is very high, at 90% at 5 years and 82% at 10 years,[2] regrafts have significantly lower survival rates of 53% and 41% for these same time points, respectively. Furthermore, reinnervation is very slow and can lead to loss of sensitivity.[3]

The cornea is the main refractive element of the visual system and also serves as a protective barrier. As such, corneas have several key properties that must be replicated in any artificial replacement. These include high optical clarity, appropriate refractive index, toughness to withstand surgical procedures, and nontoxicity, nonimmunogenicity and noninflammatory properties. A variety of synthetic and naturally derived materials has been used to form hydrogels for cornea tissue engineering scaffolds.[4] We recently reported on corneal replacements based on collagen and the copolymer poly(*N*-isopropylacrylamide-co-acrylic acid-co-acryloxylsuccinimide; designated TERP).[5,6] These tissue-engineered matrices were optically clear, moldable into the dimensions of human corneas, and adequately robust for implantation. When implanted into porcine corneas, they promoted regeneration of corneal cells as well as nerves. Although such matrices have the potential to be useful as corneal implants, custom synthesis of TERP would be necessary. However, collagen-TERP implant performance confirmed that collagen-based matrices are viable alternatives to donor tissues.

The human cornea comprises lamellae of mainly type I collagen (~70% dry weight) interspersed with glycosaminoglycans. Collagen I is widely available from bovine, porcine or, more recently, recombinant sources. Collagen forms robust hydrogels as a result of its semirigid-rod, triple-helix structure. However, collagen is susceptible to biodegradation by collagenases and for use in tissue repair, requires stabilization. Collagen biodegradation is retarded by chemical cross-linking with water-soluble carbodiimides (WSCs), a family of protein cross-linking reagents.[7] WSC's themselves do not become incorporated as part of the final cross-links in these hydrogels, so there is no possibility of toxic substance release into tissues from subsequent cross-link breakdown.[7]

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The objective of this study was to develop simple, cell-free, cornea-shaped matrices, based on high concentrations of collagen I cross-linked with a WSC, and to implant them so that progressive recruitment of autologous host cells would occur to make the implanted matrix both functional and integrated with the recipient's tissue.

METHODS

Fabrication of Corneal Implants

Corneal implants contained 9% (wt/wt) collagen in the final gels and used an EDC-to-collagen primary amine group ratio of 0.5:1 equivalent/equivalent. Briefly, 0.5- to 1-mL aliquots of 10 (wt/wt) porcine type I atelocollagen in acidic solutions (Nippon Ham; Tskuba Japan, Koken, Japan) were loaded into a syringe mixing system (Fig. 1A).[5] The collagen solution was adjusted to pH 5 ± 0.5 with microliter quantities of 1.0 M aqueous NaOH, followed by thorough mixing. Calculated volumes of aqueous solutions of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and its coreagent *N*-hydroxysuccinimide (NHS; both at 10% [wt/vol]; Sigma-Aldrich, Oakville, Ontario, Canada) were added to give a 2:1 molar ratio of EDC to NHS and mixed with the collagen solution. The final homogenous solution was dispensed into contact lens molds and cured at 100% humidity. Curing (21°C for 24 hours and then at 37°C for 24 hours) gave clear, cross-linked, cornea-shaped hydrogels after removal from the molds. After they were washed with PBS at 4°C, the implants were stored in PBS containing 1% chloroform to maintain sterility.

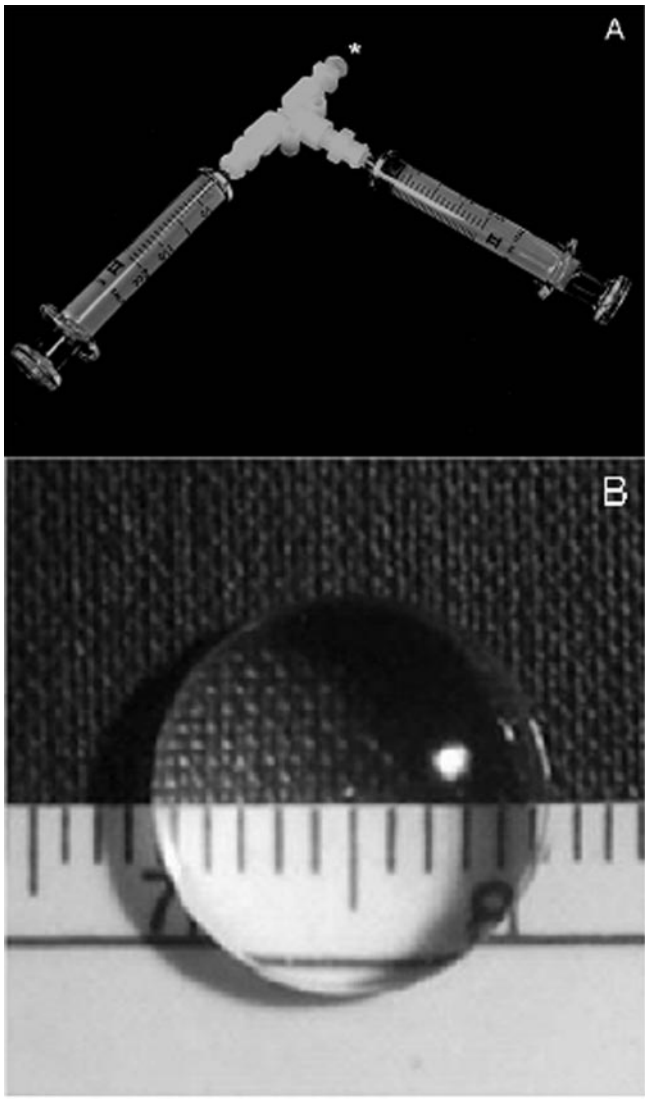

FIGURE 1. Syringe mixing system and resultant cornea-shaped implant. (**A**) Collagen, placed into one syringe, is coupled to a second, empty syringe through a T connector. To obtain the desired pH, microliter quantities of NaOH solution were injected through the septum in the connector (✶) and complete mixing effected by pumping the combined solutions between the two main syringes shown. NHS and EDC solutions were then added sequentially from syringes via the septum, followed again by complete mixing. (**B**) Cornea-shaped implant: 500 μm thickness, matrix concave side down and PBS filled in plan view (half over a black fabric and half over a white rule scaled in millimeters).

Physical, Chemical, and Mechanical Characterization

Optical Properties. Transmission and backscattering measurements were made at 21°C, both for white light (quartz-halogen lamp source) and for narrow spectral regions (centered at 450, 500, 550, 600, and 650 nm) for implants and human eye bank corneas, as previously described.[6] Refractive indices of hydrogel samples, fully hydrated in PBS, were measured on a refractometer (Abbé; Bellingham and Stanley, UK).

Mechanical Properties. Suturability of the cornea-shaped implants to human corneal rims was evaluated in vitro by determining the ability of the implants to tolerate placement of 16 polyamide, monofilament sutures (Ethicon, 10-0, 33 μm), including knotting and burial of knots, without shearing or tearing. This is the same surgical procedure as that used for transplantation. Evaluation of surgical suturability in vivo on mice was determined with continuous sutures (11-0 monofilament).

For comparison with this surgical performance, hydrogel properties were measured on an Instron Tensile Testing Machine by the "suture pull out" method described previously[6] also known as "suture retention testing."[8]

Diffusion Permeability. Glucose diffusion permeability was determined by using the procedure of Liu and Sheardown.[9] Measurements were made at 35°C (the cornea's normal, physiological temperature) using a modified Ussing chamber (Warner Instruments, Hamden, CT) with air-lift mixing. Hydrogel implants (440-μm thick) were placed between the glucose permeate chamber (8 mL of 0.05 g/mL glucose in PBS) and the receptor (PBS) chamber. The receptor chamber was sampled periodically and colorimetrically analyzed at 540 nm, using a glucose assay kit (GAGO-20; glucose oxidase/peroxidase reagent and *O*-dianisidine dihydrochloride reagent; Sigma-Aldrich) with a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan).

For albumin diffusion permeability measurements at 35°C, a simple side-by side diffusion chamber with magnetic stirring in each chamber (PermeGear, Bethlehem, PA) was used to avoid albumin-induced foaming. FITC-labeled bovine albumin (66 kDa; Sigma-Aldrich, St. Louis, MO) was used as the tracer molecule. Both the receptor and permeate chambers were 3 mL in volume, with 50 μM albumin used in the latter chamber. Sampling was performed as in the glucose measurements. Albumin concentrations in the receptor chamber were determined by measuring the fluorescence of each sample in a fluorophotometer (Fluoro IV, Gilford, Oberlin, OH) and fitting the values to a regression line formed with standards of known concentration.

In Vitro Biocompatibility Tests

Toxicity tests were performed by NAMSA (North American Science Associates, Inc., Norwood, OH) in accordance with International Organization for Standardization (ISO) tests.[10–12]

Agarose Overlay for Cytotoxicity. A 1-cm^2 piece of EDC/NHS cross-linked collagen hydrogel, a negative control, and a positive control, were placed on each of triplicate vital stain-containing agarose

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surfaces directly overlying confluent monolayers of L-929 mouse fibroblast cells in 10-cm^2 wells.[10] After 24 hours of incubation at 37°C in a 5% CO_2 environment, the cultures were evaluated for toxicity. Dead cells do not take up vital stain. Hence, the extent of unstained areas under and around the test sample gave an indication of toxicity. Cells were also examined for abnormal morphology. Cytotoxicity was graded on a scale of 0 (nonreactive or nontoxic) to 4 (severe reactivity). A positive control (latex rubber) was graded at ≥4, whereas a negative control (polyethylene) was graded at 0.

Genotoxicity Tests. Bacterial reverse mutation tests were used as rapid screening procedures for determination of mutagenic and carcinogenic potential.[11] Briefly, dimethyl sulfoxide (DMSO) was used to extract any leachables from the hydrogels that might cause mutagenic changes. The DMSO vehicle served as the negative control, whereas paradimethylaminobenzene diazosulphonic acid sodium (dexon), a known mutagen, served as a positive control. The extracts were tested to determine whether they was inhibitory to the growth of established histidine-dependent *Salmonella typhimurium* and tryptophan-dependent *Escherichia coli* strains. Briefly, separate tubes were loaded with 2 mL of molten agar, cooled, and supplemented with histidine-biotin solution for *S. typhimurium* and with tryptophan for *E. coli*. Separate tubes were inoculated with 0.1 mL each of five tester bacterial stains and 0.1 mL of the DMSO extract. A sterile S9 homogenate (0.5 mL) prepared from PCB (Aroclor 1254; Monsanto, St. Louis, MO)-tainted rat livers was added to each plate to induce metabolic activation. DMSO extracts and negative and positive controls were tested in triplicate, with each strain of tester bacteria.

Cytotoxicity of Extractable Materials. To determine whether leachables extracted from collagen hydrogels would cause cytotoxicity, hydrogels were extracted using DMEM containing 5% serum and 2% antibiotics.[9] Each extract was placed on a prepared L-929 cell monolayer grown in 10-cm^2 well and incubated for 48 hours. Negative controls comprised extracts from polyethylene and DMEM extraction vehicle alone. Tin-stabilized polyvinylchloride served as a positive control. Toxic leachables would be expected to cause cell lysis. Grading of reactivity was done from 0, no lysis, to 4, severe lysis (i.e., causing greater than 70% lysis).

Systemic Toxicity Tests. Collagen hydrogels were rinsed with PBS and extracted in either 0.9% sodium chloride or sesame oil. Crl:CF-1 BR mice, 18 to 22 g body weight, were injected with each extract at a dose of 50 mL/kg.[12] Sodium chloride extracts were injected intravenously while sesame oil extracts were given intraperitoneally. The mice were observed for toxicity immediately after administration and at 4, 24, 48, and 72 hours.

In Vitro Performance. Immortalized human corneal epithelial cells (HCECs)[13] were used to evaluate epithelial coverage. HCECs were seeded on top of 1.5-cm^2 hydrogel pieces and supplemented with a serum-free medium containing epidermal growth factor (keratinocyte serum-free medium [KSFM]; Life Technologies, Burlington, Ontario, Canada) until confluence. The medium was then switched to a serum-containing modified supplemented hormonal epithelial medium (SHEM)[14] for 2 days, followed by maintenance at an air–liquid interface. At 2 weeks, constructs were fixed in 4% paraformaldehyde in 0.1 M PBS and were processed for routine hematoxylin and eosin (H&E) staining. As an internal control for HCEC viability,[15] growth rates of cells from each HCEC batch were also measured on tissue culture dishes (plasma-treated polystyrene) under identical culture conditions. The epithelial stratification on hydrogels was also evaluated.

To determine the ability of the hydrogels to support nerve growth, dorsal root ganglia from chick embryos (E 8.0) were attached to the surface of washed gel pieces with a collagen-based adhesive. Neurite growth was observed for up to 7 days and then fixed in 4% paraformaldehyde in PBS (pH 7.2–7.4) and stained at 4°C overnight for the presence of neurofilaments, by using mouse anti-NF200 antibody. Neurofilaments were visualized the following day by using donkey anti-mouse-Cy2 secondary antibody. Wholemounts were imaged by microscope (Axiovert; Carl Zeiss Meditec, Inc., Dublin, CA).

Implantation and Evaluation

Implantation. In accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with ethics approval from both Linköping University (Protocol 47-03) and the University of Ottawa (Protocol EI-5), matrices (350-μm-thick and 5-mm diameter, trephined from the cornea-shaped hydrogels; Fig. 1B) were implanted into the corneas of 16 New Zealand White rabbits, 3 kg in weight, by deep lamellar keratoplasty (DLKP) with overlying sutures (Zirm retention bridge suturing).[16] Before surgery and at all examinations, animals were anesthetized with intramuscular (IM) xylazine (5 mg/kg; Rompun; Bayer Leverkusen, Germany) and ketamine (30 mg/kg; Ketalar; Parke-Davis, Barcelona, Spain). Only one eye was operated on for each animal—the nonsurgical, contralateral eye being used as a positive control. DLKP was also performed on eight Göttingen minipigs using the same protocol as for rabbits, except that implants were 500-μm-thick and 6-mm in diameter. Preliminary studies of penetrating keratoplasty on mice were performed with continuous running 11-0 sutures. Corneal thicknesses were approximately 380 (rabbits), 700 (mini-pigs), and 100 (mice) μm. Animals were not given steroids, only antibiotics and analgesics during the first week after surgery. Sutures were removed at 3 weeks after surgery.

Clinical Evaluation. Follow-ups were performed daily on each rabbit and pig for 7 days after surgery and then weekly. Examinations included slit lamp examination to ensure that corneas were optically clear, Schirmer's test to assess tear film regeneration, and sodium fluorescein staining to assess integrity and barrier function. Intraocular pressure measurements were taken to ensure that implants were not blocking aqueous humor flow.

Corneal topographies were measured with a fluorescence profilometer (Par Vision Systems, New Hartford, NY) on both control and surgical eyes in the pigs before surgery and at 6 months after surgery. Average refractive powers were derived from the measured topographies of each cornea. The average power (P_{ave}) was calculated by transforming the radius of curvature (R in meters) of the best-fit sphere from the topography into dioptric power with $P_{ave} = (n - 1)/R$ using a refractive index (n) of 1.337.

In vivo confocal microscopic examination (ConfoScan3; Nidek, Tokyo, Japan) was used to assess cell and nerve ingrowth, as well as to measure corneal thickness in live animals. Corneal touch sensitivity was measured with a Cochet-Bonnet esthesiometer (Handaya Co., Tokyo, Japan).

Histopathologic Evaluation. Pairs of rabbits were killed sequentially after three days, 1 week, and 1, 2, 3, and 5 months after implantation, with four animals killed at 6 months after surgery. Corneas with implants and control, nonsurgical corneas were processed for routine histopathologic examination after hematoxylin and eosin (H&E) staining.

Statistics

We tested the two-tailed hypothesis that there is either thinning or swelling of the implanted cornea over the nonsurgical contralateral controls using a paired two sample t-test for means. Statistical significance was set at $P \leq 0.05$.

Results

Physical and Mechanical Characterization

Optical Properties. Visually, implants were very clear (Fig. 1B). Transmission of white light was 99%, with a backscatter of 0.2% for hydrogel samples of 500-μm thickness. These values and those across the visible spectrum (Fig. 2A) are superior to

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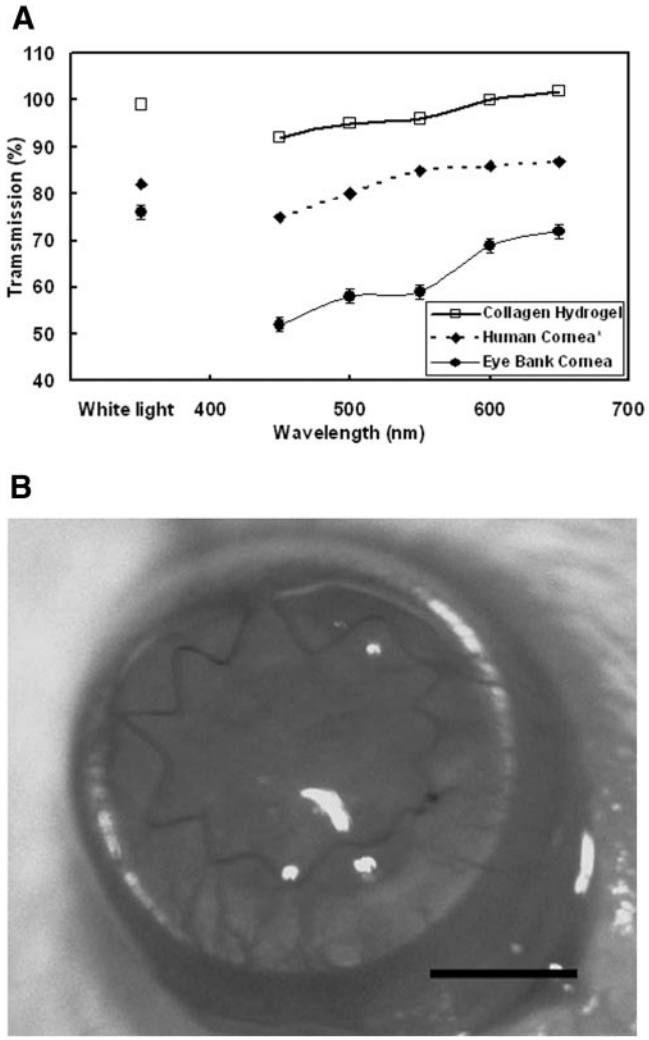

FIGURE 2. Physical and mechanical characteristics of corneal implant. (**A**) Optical properties at 21°C. Eye bank cornea data are the average of five healthy, white corneas. Published human data (Ref. 17) were from a healthy cornea of a 61-year-old donor.[17] (**B**) Suturability of a mouse cornea implanted with a full-thickness implant secured with continuous sutures, at 1 month after surgery. Bar, 1 mm.

those found in eye bank corneas (550 ± 5-μm corneal thickness from confocal microscopy), using the same equipment, and superior to value in the literature.[17] The EDC/NHS cross-linked hydrogels had refractive indices of 1.35 compared with 1.38 for the human cornea stroma.[18]

Mechanical Properties. The corneal implants tolerated 13 of 16 partial-thickness stitches that were inserted around the implant-to-rim junction. Each stitch was individually knotted, and the knots were buried in the implant material. For penetrating keratoplasty on mice, continuous 11-bite running sutures were made and knotted off in full-thickness implants (Fig. 2B). Surgical suturability performance correlated well with mechanical suture pull-out measurements.

Diffusion Permeability. The glucose diffusion permeability coefficient for EDC/NHS cross-linked collagen hydrogels was $(2.7 \pm 0.8) \times 10^{-6}$ cm^2/s ($n = 3$ samples), whereas that of the corneal stroma was estimated to be 2.5×10^{-6} cm^2/s.[19] The albumin diffusion permeability coefficient was measured as $(1.6 \pm 0.6) \times 10^{-7}$ cm^2/s ($n = 4$ samples).

Biocompatibility Tests

Hydrogel Cytotoxicity. Agarose overlay testing for cytotoxicity showed no zones of unstained cells and were graded as 0 or unreactive, compared with the positive control (latex rubber) that had average zones of unstained cells of 5 mm and a grade of 3 or moderate reactivity ($n = 3$ for each of the experimental, negative and positive controls). Genotoxicity tests were negative compared with the tin stabilized PVC, the negative control ($n = 3$ for each of experimental and negative and positive controls).

Extracted leachables showed no cytotoxicity, in contrast to the positive controls that gave severe grade-4 reactions causing lysis in 90% of cells ($n = 3$ per test group).

None of the mice tested with sodium chloride or sesame oil extracts of hydrogels or vehicle blanks ($n = 5$ per group) showed any mortality or evidence of systemic toxicity.

In Vitro Performance. The hydrogels supported attachment and proliferation of corneal epithelial cells, as well as stratification. Under identical culture conditions, HCEC growth rates were identical (within 5%) on hydrogels and control tissue culture plate surfaces, reaching confluence within 4 days. Nerve overgrowth and ingrowth were also observed (not shown).

Implantation and Clinical Evaluation. Sutures were removed at 3 weeks after surgery. Slit lamp examination of both rabbit and porcine corneas showed re-epithelialization within the first week. Although a mild haze was initially observed, only one of the total of 24 surgical corneas showed a slight haze at 6 months after surgery and none of the implants showed any sign of inflammation or rejection over this period. In the rabbit series, at 1 week after surgery, the epithelium was beginning to stratify but had not reached full thickness. At 1 month after surgery (Figs. 3A, 3B), rabbit H&E-stained sections showed a stratified epithelium over the implant (Fig. 3B). Sodium fluorescein showed no staining, indicating the presence of an intact epithelial barrier. By 3 months after surgery (Figs. 3C, 3D), stromal cells had migrated into the implant region (Fig. 3D). At 6 months after surgery (Figs. 3E, 3F), sections through an implant showed a normal histologic appearance (cf. Fig. 3F), when compared to the nonsurgical contralateral corneas (not shown). The implants were well-integrated within the host corneas. IOPs were normal throughout, and the nonsurgical endothelium showed no pathologic changes.

In pigs (Fig. 4), the healing process occurred over the same time course as for rabbits. The absence of sodium fluorescein staining indicated restoration of an intact epithelial barrier. Schirmer's tests indicated the presence of a tear film comparable to the contralateral nonsurgical eyes. From preoperative topographical measurements, the P_{ave} of the control and surgical eyes were not significantly different, whereas after surgery, the calculated P_{ave} of both the control and surgical eyes had significantly decreased but the postoperative P_{ave} of the control and surgical eyes was not significantly different.

From in vivo confocal microscopy on surgically treated and control porcine corneas, nerves within the implant area were visible at 3 months after surgery. At 6 months after surgery, a subepithelial nerve plexus was visible within the implant (Fig. 4C) as in the contralateral cornea (Fig. 4D). Large stromal nerves were visible at 6 months in corresponding regions both in contralateral and surgical corneas (Figs. 4E, 4F). Esthesiometry showed a return of touch sensitivity that was extinguished when lidocaine was applied topically to the corneas.

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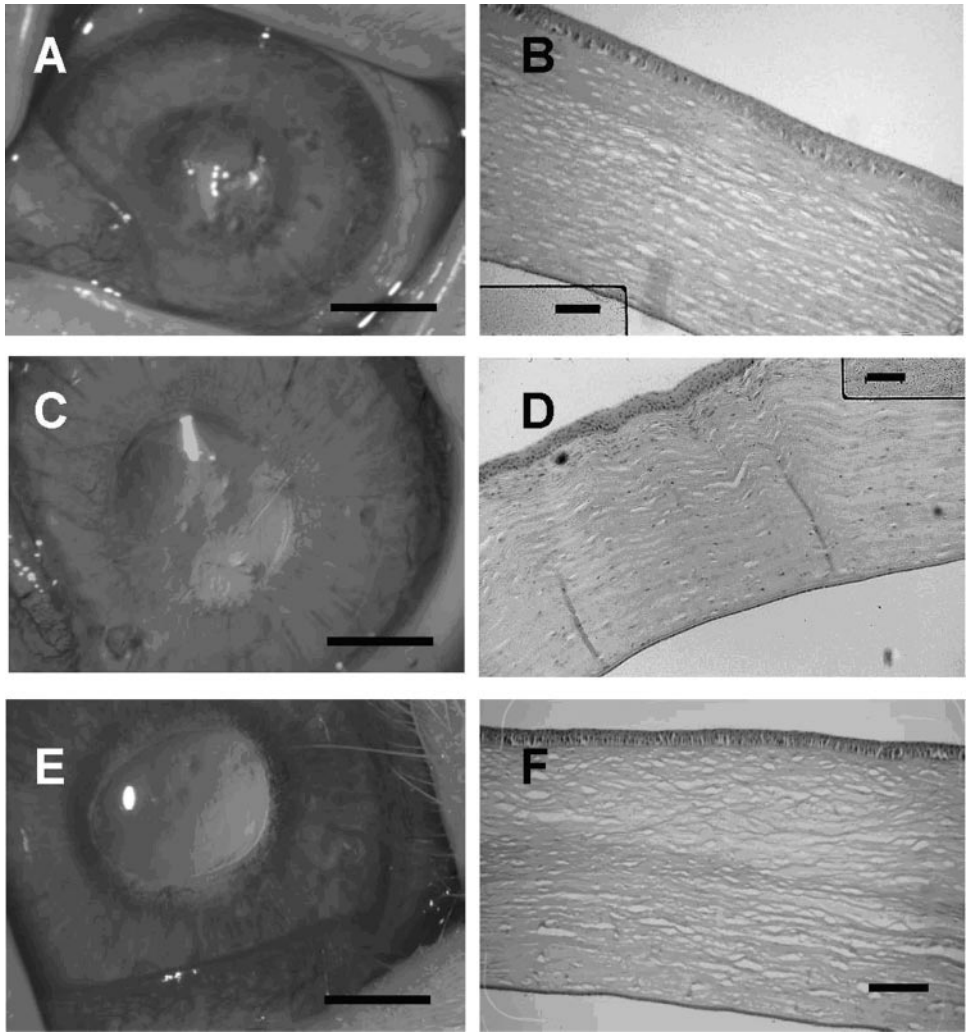

FIGURE 3. Rabbit corneas observed over a 6-month postsurgical period, showing optically clear slit lamp images at 1 (**A**), 3 (**C**) and 6 (**E**) months after LKP surgery. Corresponding 1-, 3-, and 6-month H&E-stained histologic sections through the region of the implants are shown in (**B**), (**D**), and (**F**), respectively. Bar: (**A**, **C**, **E**) 5 mm; (**B**, **D**, **F**) 50 μm.

Measurements of corneal thickness, with or without implants, showed no statistical significance ($P < 0.05$) between the surgical and nonsurgical corneas, indicating that the implants were not thinning or swelling within the 6-month test period.

DISCUSSION

Collagen-based biomaterials are widely used for tissue engineering. Collagen turnover in the eye is very slow, but in response to injury, production of matrix metalloproteinases, including collagenases and stromelysins,[20] occurs and functions to remodel the collagen, which is laid down as opaque scar tissue. Corneal substitutes designed for transplantation should be resistant to such natural biodegradation, to provide a sufficient implant lifetime. WSCs, in combination with NHS are very effective collagen cross-linkers.[21] Details of the physical characterization of WSC cross-linked collagen and property dependence on reaction conditions have been described recently (Liu et al., manuscript submitted). From the pigs and rabbits in the present study, we removed and replaced only each anterior cornea (epithelium and part of the stroma) in a surgical technique (LKP) analogous to that used to treat humans with superficial corneal damage. Nonpenetration of the anterior chamber keeps the inner contents of the eye intact, reducing the rate of rejection and after surgery complications, thus improving long-term graft stability[22] and, in this study, minimized the potential confounding factor of postsurgical intraocular infection. In clinics, deep LKP has been found to be a safe alternative to penetrating keratoplasty (PK) for common indications such as keratoconus, because best corrected visual acuity, refractive results, and complication rates are similar after DLK and PK.[23] Furthermore, the risk of endothelial rejection can be avoided.[22]

Our EDC/NHS cross-linked collagen implants allowed ingrowth of host corneal cells and nerves as with our previous collagen-TERP hydrogels.[5] The EDC/NHS cross-linked collagen implants were indistinguishable from the host stroma, as measured by haze, topographical differences and thickness changes. Hence, each implant was seamlessly integrated into the host stroma where it served as a biostable scaffold for ingrowing cells and nerves. To determine whether remodeling had occurred or not, and, if so, to what extent under different postsurgical care conditions (e.g., with or without steroid treatment), is the subject of an ongoing follow-up study. Whatever the case may be, we found no statistically significant differences in corneal stromal thickness between the surgical corneas and contralateral nonsurgical ones at 6 months after surgery in the healthy rabbits and pigs studied. As a step toward clinical testing, implants in animals with diseased corneas will be evaluated next.

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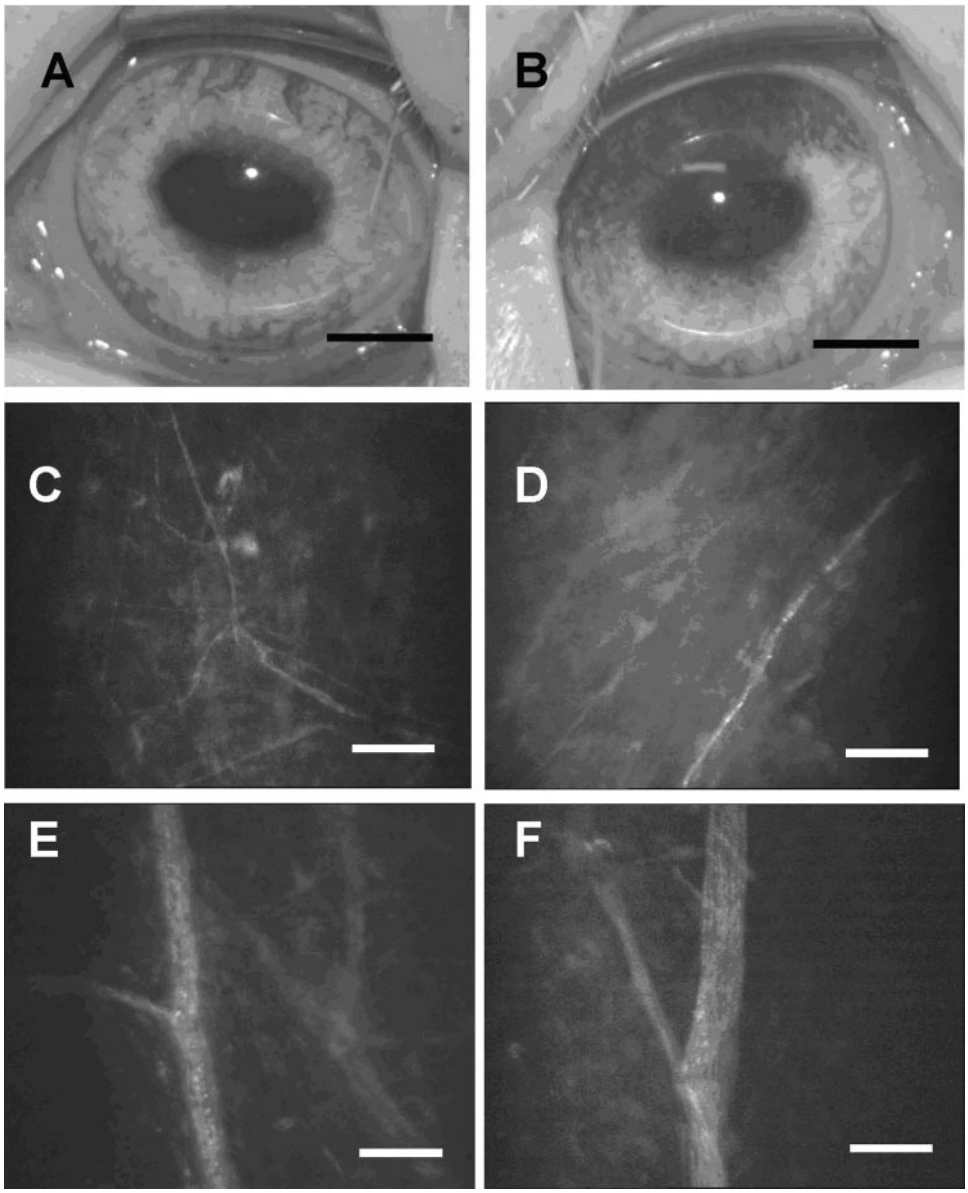

FIGURE 4. Porcine corneas at 6 months. (**A**) Optically clear, cornea with surgical implant. (**B**) Nonsurgical, contralateral cornea. (**D–F**) In vivo confocal images. Regenerated fine subepithelial nerves (**C**) and large stromal nerves (**E**) in the implant. (**D**, **F**) Innervation was visible in corresponding areas in the nonsurgical control cornea. Bar: (**A**, **B**) 5 mm; (**C–F**) 100 μm.

Although there have been many previous attempts at development of corneal implants,[4] only AlphaCor devices (CooperVision Surgical, Inc., Lake Forest, CA) are in clinical use.[24] These pHEMA-based, core-skirt keratoprostheses support neither epithelialization nor innervation.[24] All other implants to date have this same limitation.[4] In contrast, our WSC cross-linked porcine collagen implants offer a simple methodology for easy fabrication of implants, or for production of temporary patches to repair perforations.

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