Innervation of Tissue-Engineered Recombinant Human Collagen-Based Corneal Substitutes: A Comparative In Vivo Confocal Microscopy Study

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PURPOSE. To compare reinnervation in recombinant human collagen-based corneal substitutes with allografts during a 1-year postimplantation follow-up period in pigs. A retrospective comparison to innervation in porcine collagen-based biosynthetic grafts was also performed.

METHODS. Pigs received a corneal allograft or a substitute made of either recombinant human type-I or -III collagen. In vivo confocal microscopic examination of the central cornea of surgical and untouched control eyes before surgery and at 2, 6, and 12 months after surgery was performed to quantify the number, density, and diameter of nerves at various corneal depths.

RESULTS. By 12 months after surgery, the number and density of regenerated nerves in the anterior and deep anterior corneal stroma recovered to preoperative and control levels in both types of substitute grafts and in the allografts. In the subepithelial and subbasal regions, however, significantly fewer nerves were detected relative to those in control subjects at 12 months, regardless of graft type ($P < 0.05$), similar to the behavior of porcine collagen-based biosynthetic grafts. An absence of thick stromal nerve trunks (diameter, >10 μm) in all grafts, irrespective of material type, indicated that nerve regeneration in grafts was accompanied by persistent morphologic changes.

CONCLUSIONS. Nerve regeneration in recombinant human collagen-based biosynthetic corneal grafts proceeded similarly to that in allograft tissue, demonstrating the suitability of recombinant human collagen constructs as nerve-friendly corneal substitutes. Furthermore, only minor differences were noted between type-I and -III collagen grafts, indicating an insensitivity of nerve regeneration to initial collagen type. (*Invest Ophthalmol Vis Sci.* 2008;49:3895–3902) DOI:10.1167/iovs.07-1354

Many mechanisms, including disease, scarring, or trauma of the cornea, can lead to significant vision loss or even blindness that can be treated only by transplantation with a corneal graft. A worldwide shortage of acceptable human donor corneas, however, has necessitated the development of corneal substitutes suitable for implantation.[1,2] Recently, we reported successful implantation of cornea stromal matrices composed of cross-linked porcine collagen that, when transplanted into pigs, allowed regeneration of the corneal epithelium, stroma, and nerves over a 12-month postoperative observation period.[3,4] Although good implant-to-host graft integration was observed, there is still a concern that the animal-derived collagen used to construct the graft can stimulate immunologic or allergic reactions within the host. Moreover, the potential transmission of infectious agents (especially viral and prion-based) via animal-derived materials after implantation is of particular concern.[5] To address these possible problems, we replaced animal-derived collagen with recombinant human collagen (RHC) in new biosynthetic cornea formulations and report fabrication details and in vitro/in vivo test results with these new materials in detail in a companion paper.[6]

The cornea is one of the most densely innervated tissues in the human body. Corneal nerves provide the basis for a protective aversion reflex to external stimuli at the ocular surface,[7] they regulate corneal epithelial integrity and wound healing,[8] and they are necessary for normal tear film secretion and maintenance of physiologic balance in the cornea.[7] Accordingly, nerve regeneration plays a critical role in the restoration of normal corneal function after corneal surgery. It is then imperative that as biosynthetic corneal substitutes continue to develop and improve, the detailed nerve regeneration characteristics within the grafts are quantified in vivo in an objective manner. As such, the purpose of the present study was to evaluate nerve regeneration objectively within new RHC corneal grafts in vivo, to determine the feasibility of using this new class of biosynthetic graft as a substitute for donor human corneas for transplantation. In the present study, innervation within the RHC-based corneal grafts implanted into porcine hosts has been quantified longitudinally over a 12-month postoperative period by in vivo confocal microscopy. Specifically, for the first time, innervation was investigated quantitatively in three graft types: porcine allograft, RHC-I biosynthetic graft (composed of cross-linked recombinant human type I collagen), and RHC-III biosynthetic graft (composed of cross-linked recombinant human type-III collagen). Finally, we compared the new material performance to innervation within cross-linked, porcine collagen-based biosynthetic grafts reported in a previous study.[4]

METHODS

Fabrication of Corneal Substitutes

Biosynthetic corneal substitutes fabricated in this study were based on a previously reported cross-linked collagen model,[3] with RHC used in

place of porcine collagen. Full details of the fabrication are reported by Merrett et al.,[6] and only a summary is given here. Briefly, 13.7% (wt/wt) recombinant human (type-I or -III) collagen was cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and its co-reagent *N*-hydroxysuccinimide (NHS). An EDC/NHS weight ratio of 1 was used with an EDC/collagen ratio of 0.5 (for type I) or 0.4 (for type III). The final solutions were dispensed into plastic molds and cured at 100% humidity (at 21°C for up to 24 hours and then at 37°C for up to 24 hours). After curing and before implantation, the corneal constructs were stored in a phosphate-buffered saline solution containing 1% chloroform.

Surgical Procedure and Postoperative Treatment

According to animal use guidelines set by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with ethics approval from the University of Ottawa (Protocol EI-5), an allograft or corneal substitute (500-μm-thick, 6-mm-diameter) was implanted in one eye each of twelve 6-month-old Göttingen minipigs (Marshall Farms, North Rose, NY) by deep lamellar keratoplasty and held in place with overlying sutures. Contralateral corneas served as nonsurgical control eyes, and animals were given analgesics and antibiotics, as previously reported.[4] In addition, two drops of a corticosteroid (10 mg/mL prednisolone acetate; Sandoz-Prednisolone, Boucherville, QC, Canada) was administered four times daily for 1 week after surgery. Sutures were removed 1 month after surgery.

Clinical Follow-up

Postoperative clinical examination included slit lamp biomicroscopy, corneal touch sensitivity testing, and photography. Touch sensitivity was assessed with a Chochet-Bonnet aesthesiometer (Handaya Co., Ltd., Tokyo, Japan) using a modified method to compensate for the sedation required to immobilize the pigs. The aesthesiometer filament was kept fixed at 0.5 mm, and the time necessary to react to the touch was recorded. To confirm touch sensitivity, a drop of topical anesthetic (proparacaine hydrochloride 0.5%, Alcaine; Alcon, Mississauga, ON, Canada), was then placed onto each cornea, and the test was repeated. Only corneas in which the anesthetic extinguished the reaction were recorded as having a positive response. Nonsurgical contralateral corneas and preoperative baselines served as control samples.

Confocal Microscopy and Image Analysis

All corneas and control eyes were examined with a clinical in vivo confocal microscope (Confoscan3; Nidek Technologies Srl., Padova, Italy) before surgery and at 2, 6, and 12 months thereafter. The confocal examination procedure has been described[4] and is only briefly summarized here. A drop of transparent gel (Tear-gel; Novartis, Mississauga, ON, Canada) was placed on the tip of the objective lens, which was then brought into contact with the corneal apical region. In each confocal examination, the focal plane of the microscope objective lens was translated two to three passes through the full thickness of the cornea to yield 350 images (at an image capture rate of 25 frames/s). Images from adjacent depths were separated by an increment of 10 μm and presented a cross-sectional area of 440 $\times$ 330 μm (horizontal $\times$ vertical). Typically, two to three examinations were made of the same cornea successively, retracting the objective lens from corneal contact and realigning between each examination. Of these repeated examinations, only the single examination with the most uniformly illuminated, in-focus images was retained for further analysis.

For each examination retained for analysis, a composite full-thickness scan was constructed as described previously[4] to minimize the effect of eye motion along the optical axis and provide an accurate depth location of each image relative to the corneal surface. Within each composite scan, each unique image with nerves was extracted and assigned a random code indexed to the animal, eye, time point, and corneal depth, to mask the image source from the observers performing the nerve analysis. A total of 740 images were extracted in

this manner and stored for analysis. For the purposes of this study, nerves were defined as long, slender objects, with or without branches, substantially uniform in width and brightness, and having an increased brightness and contrast relative to the background. For each image within which a nerve was identified, all visible nerves in the image were traced along their length and at multiple locations across their diameter (diameter was traced approximately every 200 μm along the nerve) using widely available freeware.[9,10] From the tracings, the number of nerves (each branch with a length >50 μm was counted as a separate nerve[11]), length of each nerve, nerve diameter, and total nerve length with in the image were recorded. All nerves were traced by a single observer (NL); however, to assess the degree of interobserver variation in nerve parameter quantification from the confocal images, a second, blinded, independent observer (MH) randomly selected 25% of the images (185 images) for nerve tracing.

After all measurements were recorded, image source data was not blinded, and the following measures were compiled: number of nerves, nerve density, and nerve diameter. Although the number of distinct nerves observed in a given corneal volume serves as a measure of the vigor of new nerve sprouting and regeneration, nerve density expresses the total nerve length of all nerves in a given volume, indicating the extent and robustness of innervation. Because of the fine depth increment between adjacent confocal images in the stroma, nerves were not detected in all corneas for a given depth location. To enable a more robust nerve analysis, nerve parameters in each cornea were therefore grouped according to three corneal depth zones, as previously defined[4]:

> Zone 1: subepithelial region (20–50 μm below the epithelial surface);
>
> Zone 2: anterior stroma (60–100 μm below the epithelial surface; sequence of five frames below zone 1);
>
> Zone 3: deep anterior stroma (110–150 μm below the epithelial surface; sequence of five frames below zone 2).

These depth zones spanned the most anterior 25% of the grafted corneas, where over 85% of the nerves in this study were observed. Nerve density was calculated by dividing the total length of all nerves in a given corneal depth zone by the zone volume[4,11] (image cross-sectional area $\times$ zone depth of field), expressed in micrometers per cubic millimeters. The zone depth of field represented the total effective depth of field from a series of adjacent confocal images comprising a given depth zone and is the sum of a single-image depth of field and the depth interval between the first and last images in a given depth zone. The image depth of field was measured on our particular confocal microscope according to the method described by McLaren et al.[12] Briefly, 14 unique scans of the specular reflection peak from a microscope slide were obtained in seven separate confocal examinations. Confocal images were acquired at 1.1-μm depth increments, and the mean pixel intensity within two rectangular regions of interest across all images was plotted versus the image depth. The full-width at half-maximum intensity of the 14 peaks was averaged for each region of interest, and the mean of these two values was taken to be the image depth of field. For our instrument, the resulting depth of field was determined to be 27.4 $\pm$ 1.8 μm (mean $\pm$ SD). Rounding this value to 27 μm, the total zone depth of field was 57, 67, and 67 μm for zones 1, 2, and 3, respectively.

Statistical Analysis

Nerve data were sorted according to eye, time of examination, corneal depth zone, and graft type. To assess changes in the number and density of nerves in each corneal graft type over time, we used analysis of variance (ANOVA) with results stratified by depth zone and eye. We used one-way ANOVA on means, except when normality and equal variance criteria failed, in which case the Kruskal-Wallis one-way ANOVA on ranks was used. When ANOVA indicated significant differences, multiple pair-wise comparison tests were performed to isolate differences (the Tukey test for ranks or Student-Newman-Keuls

means). In addition, at each examination period, the degree of innervation in surgical corneas was compared to the corresponding control corneas by using a *t*-test on means (for normally distributed data) or a Mann-Whitney U test on ranks (for non-normally distributed data), with the number and density of nerves as measures. Finally, changes in the distribution of nerve diameter with corneal depth zone at the end of the study were assessed across the various graft types using Kruskal-Wallis one-way ANOVA on ranks with Dunn's method used to isolate pair-wise differences. For all comparisons in this study, normality criteria were determined by using the Kolmogorov-Smirnov test, and for all statistical tests, $P < 0.05$ was considered statistically significant. All statistics were calculated with commercial software (SigmaPlot ver. 9.0, with SigmaStat integration; Systat Software Inc., Point Richmond, CA).

RESULTS

Corneal touch sensitivity varied with the amount of sedation given. In general, however, corneas were sensitive to touch before surgery. Postoperative touch sensitivity was positive in corneas implanted with both RHC-I and -III grafts at 2 months after surgery, whereas allografts remained insensitive. By 6 months, however, both RHC-based and allograft-implanted corneas responded to touch.

The median number of nerves and nerve density per central cornea are summarized in Table 1 and Figure 1, respectively. Reinnervation of the central corneal graft in zones 2 and 3

occurred early, with the number and density of nerves restored to preoperative levels within the first 2 months after surgery in both allografts and biosynthetic grafts. The same reinnervation of zones 2 and 3 was noted in biosynthetic grafts fabricated by using porcine collagen in an earlier study.[4] Reinnervation within the shallower zone 1 was slower than in deeper zones, as the first zone 1 nerves were detected only after 6 months after surgery in both allografts and biosynthetic grafts, with a more vigorous growth of new nerves observed in allografts. By 12 months, the number of nerves in zone 1 had recovered to preoperative levels in all grafts. The same pattern was observed for nerve density, with the exception that zone 1 density in RHC-III grafts remained significantly less than preoperative levels at 12 months. Within porcine collagen-based biosynthetic grafts, an initial zone 1 nerve depletion was also observed, followed by recovery to preoperative levels 10 months after surgery.[4] Innervation differences across graft type were investigated by performing two-way ANOVA with time and graft type as independent variables, and no significant differences in nerve density between graft types was found at any time, nor was there any significant interaction between graft type and time ($P > 0.05$ for both). At the conclusion of the study, therefore, no significant difference in nerve density was detected in any depth zone in allografts relative to RHC-based biosynthetic grafts or between RHC-I and RHC-III-based grafts.

On comparison of operated with control corneas, it was found that at the end of the study the number and density of

TABLE 1. Number of Nerves Per Central Corneal Scan

| Cornea Type | Depth Zone | Cornea | Preop | Months after Surgery | | | ANOVA *P*† |
				2	6	12	
Allograft	1	TE	9.5 (4.8, 13.0)	0.0 (0.0, 0.0)	5.5 (0.8, 11.8)	2.0 (1.5, 3.0)	0.06†
		Control	4.5 (3.8, 7.5)	3.0 (2.0, 5.5)	5.0 (3.8, 7.0)	9.0 (8.8, 9.5)	0.29†
		*P**	0.72	**0.03***	0.78	**0.03***	
	2	TE	4.0 (2.3, 5.0)	1.0 (0.0, 3.3)	4.0 (3.8, 4.5)	5.5 (2.0, 10.8)	0.36
		Control	4.0 (2.8, 5.5)	3.5 (2.5, 5.5)	7.0 (6.3, 7.0)	4.0 (3.0, 6.8)	0.54†
		P	0.56	0.41	0.09	0.72	
	3	TE	2.0 (1.8, 2.0)	0.0 (0.0, 2.8)	0.0 (0.0, 2.8)	2.5 (1.5, 3.3)	0.55†
		Control	1.0 (0.8, 1.3)	1.0 (0.8, 3.0)	3.0 (1.8, 4.5)	3.5 (2.0, 5.3)	0.50
		P	0.17	0.49*	0.87	0.31	
RHC-I	1	TE	3.5 (1.5, 5.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 1.3)	0.07†
		Control	5.0 (1.5, 8.3)	7.5 (6.0, 8.3)	9.5 (5.5, 13.5)	9.0 (7.3, 14.0)	0.35
		P	0.69*	**0.03***	**0.03***	**0.03***	
	2	TE	4.5 (3.5, 5.3)	4.5 (2.0, 8.8)	7.0 (3.8, 10.3)	4.0 (3.3, 5.8)	0.84
		Control	4.0 (2.0, 6.8)	3.0 (2.3, 4.0)	5.5 (3.8, 6.5)	4.0 (2.5, 5.0)	0.83
		P	0.80	0.38	0.54	0.54	
	3	TE	1.5 (0.8, 2.0)	2.5 (1.8, 4.3)	7.5 (5.3, 8.0)	4.5 (3.0, 5.8)	0.25
		Control	0.5 (0.0, 1.0)	1.5 (0.8, 2.0)	1.0 (1.0, 1.5)	2.5 (0.8, 4.3)	0.40†
		P	0.23	0.22	0.34*	0.42	
RHC-III	1	TE	7.0‡ (6.0, 9.0)	0.0§ (0.0, 0.0)	0.0‡ (0.0, 1.3)	1.0‡ (0.0, 2.5)	**0.01†**
		Control	7.0 (5.0, 9.5)	7.5 (6.8, 9.3)	9.0 (4.8, 14.3)	9.5 (6.5, 11.3)	0.89
		P	0.82	**0.03***	0.06*	**0.03**	
	2	TE	2.0 (1.5, 2.8)	0.5 (0.0, 2.0)	4.5 (2.8, 6.5)	8.0 (6.8, 10.5)	0.05†
		Control	1.0‡ (0.0, 3.5)	3.5‡§ (3.0, 5.0)	8.0§ (6.0, 10.3)	3.5‡ (2.8, 4.0)	**0.04**
		P	0.91	0.20*	0.12	0.11*	
	3	TE	1.0‡ (0.0, 2.0)	4.0§ (2.3, 6.0)	3.0‡§ (1.5, 4.5)	6.5§ (6.0, 7.3)	**0.03**
		Control	2.0 (1.5, 2.3)	0.0 (0.0, 0.5)	0.5 (0.0, 1.3)	1.0 (0.8, 2.5)	0.52

Data are the median (interquartile range: Q25, Q75). *n* = 4 (control), *n* = 4 (surgical) corneas, for each material type (*N* = 24 corneas). Bold type indicates a significant difference between TE and control corneas at the same time point (Mann-Whitney or *t*-test) or a significant difference among the same group of corneas (TE or control) over time (Kruskal-Wallis or one-way ANOVA). In all cases, differences were considered significant at the $P < 0.05$ level.

 * Mann-Whitney U test on ranks was used for non-normally distributed data. Where data were normally distributed, a *t*-test was used.

 † Kruskal-Wallis one-way ANOVA on ranks was used when data did not satisfy normality and equal variance criteria. When the criteria were met, one-way ANOVA was used.

 ‡§ When ANOVA indicated significance, median or mean values with the same symbol in each row were not significantly different from each other (Tukey test for ranks or Student-Newman-Keuls method for means).
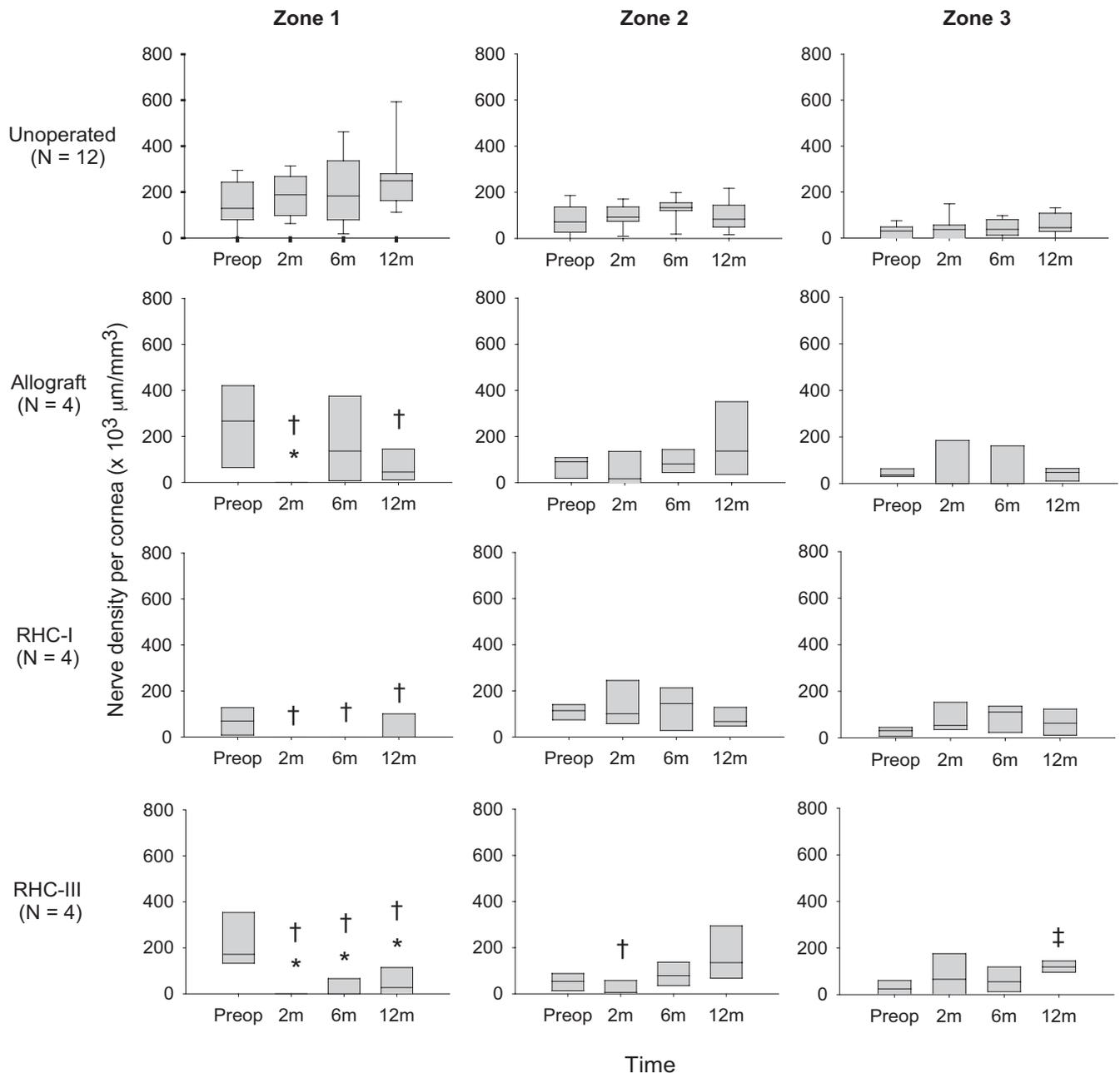
FIGURE 1. Changes in central corneal nerve density over time, corneal depth zone, and graft type. For control corneas (*1st row*), box plots represent medians, 1st and 3rd quartiles, and 5th and 95th percentiles (*whiskers*). In all surgical corneas (*2nd through 4th rows*) only medians and 1st and 3rd quartiles are shown. *Significant reduction from preoperative values; †significant reduction relative to control corneas; and ‡significant increase relative to control corneas.

nerves in zones 2 and 3 of grafts matched or significantly exceeded levels in control corneas, regardless of graft type. The same behavior of zone 2 and 3 nerves was observed in porcine collagen-based biosynthetic grafts.[4] In zone 1, the number and density of nerves was significantly reduced relative to control corneas 12 months after surgery in all graft types in this study; however, zone 1 was reinnervated to control levels after 12 months in the porcine collagen-based biosynthetic grafts.[4] Comparison of the absolute number and density of zone 1 nerves at 12 months, however, yielded no significant difference between porcine collagen-based biosynthetic grafts and any of the graft materials in this study (Kruskal-Wallis one-way ANOVA on ranks; $P = 0.78$ and 0.80 for number and density of nerves, respectively). Finally, no significant innerva-

tion differences were observed over time among the group of control corneas in any depth zone, indicating that no purely age-dependent change in corneal innervation was detected in this study.

The variation of nerve diameter with corneal depth was investigated by plotting all diameter measurements within a given image versus depth of the image from the corneal surface, in all images taken 12 months after surgery (Fig. 2). Thick stromal nerve trunks (>10 μm in diameter) found in nonsurgical corneas were absent in all grafts, for which nerve diameter remained below 10 μm regardless of corneal depth. In addition, innervation of deeper stromal regions was noted in allografts, whereas nerves in RHC materials were confined to the most anterior 200 μm of the cornea. Nerve diameter in
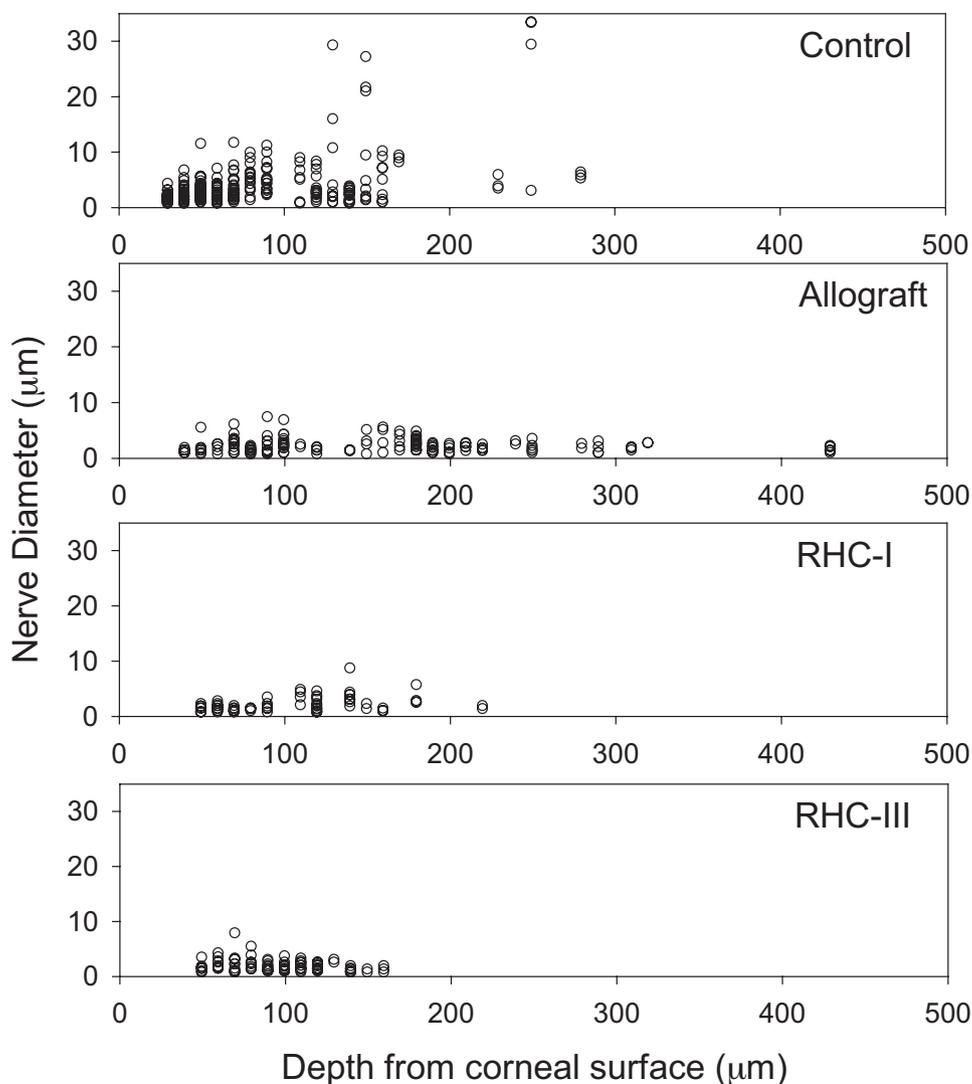
FIGURE 2. Nerve diameter depth distributions measured from in vivo confocal microscope images 12 months after surgery for various corneal graft types.

porcine collagen biosynthetic grafts followed the distribution within allografts in this study (innervation present at corneal depths in excess of 400 μm). Quantitative results of nerve diameter distribution with corneal depth zone 12 months after surgery are shown in Figure 3. In control corneas, a significant increase in diameter was found in depth zones 2 and 3 relative to zone 1 ($P < 0.001$, Kruskal-Wallis ANOVA on ranks; the Dunn multiple comparisons method). By contrast, no significant variation of nerve diameter with depth zone was found in allografts or in RHC-III or porcine collagen biosynthetic grafts ($P = 0.28$, 0.27, and 0.33, respectively; Kruskal-Wallis test). Only RHC-I biosynthetic grafts exhibited a significant increase in nerve diameter in depth zone 3 relative to zones 1 and 2 ($P < 0.001$, Kruskal-Wallis, Dunn method). A comparison across graft types revealed significantly larger nerve diameter in control corneas in depth zone 2, relative to all other graft types, and a significantly reduced diameter in RHC-III grafts in zone 3, relative to both control samples and RHC-I grafts ($P < 0.001$ for both, Kruskal-Wallis, Dunn method).

In the study, 1432 nerves were traced, and 2534 nerve diameter measurements were taken from 740 images extracted from 96 confocal examinations (24 corneas at each of four time points). Interobserver variability in the total nerve length traced from an image was 14.4% (mean difference; 95% confidence interval: −70%–97%), and the difference in the sum of nerve lengths across all 185 images was 14.5%. Interobserver

nerve diameter differences were assessed by grouping values from each observer into corneal depth zone, with no significant interobserver difference found ($P = 0.99$, 0.68, 0.30 for depth zones 1, 2, and 3, respectively, Mann-Whitney rank sum test).

DISCUSSION

In this study, we evaluated biosynthetic, tissue-engineered alternatives to donor human corneas from the perspective of nerve regeneration. To our knowledge, this is the first study to compare innervation in different corneal graft alternatives, with additional reporting of innervation in porcine allograft tissue as a benchmark.

Innervation of the subepithelial region (corneal depth zone 1) is an important indicator of the extent of nerve regeneration, as these nerves are the source of epithelial innervation and nerve endings at the ocular surface, which are ultimately responsible for environmental sensitivity, corneal epithelial viability, and a normal tear film. In this study, zone 1 nerves recovered to a similar extent in allografts, RHC-I, and RHC-III grafts, although they were reduced in number and density relative to the untouched control corneas 12 months after surgery. The absolute number and density of zone 1 nerves in all grafts at the end of this study, however, did not differ from
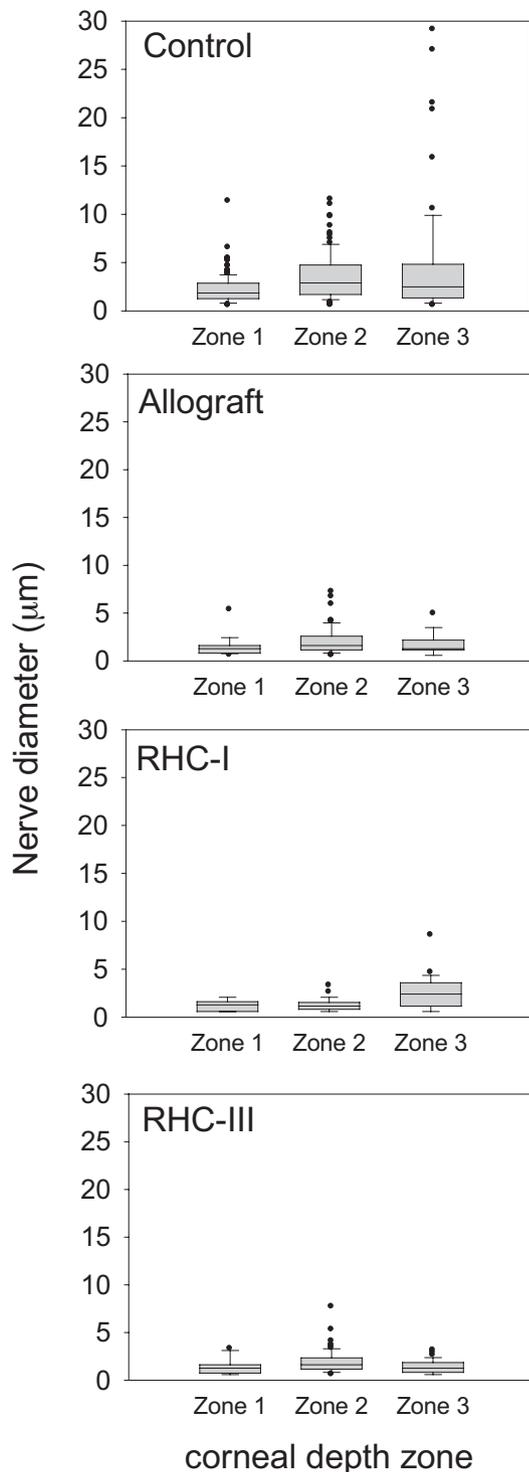
FIGURE 3. Nerve diameter distribution with corneal depth zone in three graft types and in control corneas at 12 months after surgery. Box plots indicate median, 1st and 3rd quartiles, 5th and 95th percentiles (*whiskers*), and outliers.

PRK and 5 years after LASIK.[15] The reduced zone 1 nerve density observed in the present study after 12 months has therefore been observed after other surgical procedures and is expected to improve over a longer postoperative period.

Although corneal touch sensitivity was noted in both types of RHC grafts as early as 2 months after surgery, the first subbasal nerves were not detected in confocal microscopy images until 6 months after surgery. It is unclear whether this discrepancy arose from the method used to assess touch sensitivity; failure to detect potentially short, faint, or sprouting subbasal nerves with the confocal microscope; or an increased sensitivity of nearby axons during the sprouting process. Although a trend toward an earlier re-establishment of touch sensitivity was observed in animals implanted with RHC grafts relative to allografts, it should be noted that the modified esthesiometry method used does not provide quantitative data and at best gives only an indication of possible return of sensitivity. Six months after surgery, however, a return of touch sensitivity in allografts and RHC grafts corresponded to the confocal microscopic visualization of the first subbasal nerves.

Stromal nerves below the subepithelial region (in zones 2 and 3) innervated all materials in this study equally rapidly and in numbers not significantly different from those found in the control corneas. This result suggests the RHC-based biosynthetic grafts possess as favorable an environment for neural regeneration as allograft or porcine collagen-based biosynthetic grafts, with the added benefit of being free from animal-derived components. Moreover, a further advantage of the biosynthetic grafts reported in this study is that their innervation proceeded naturally without incorporation of any nerve growth factor such as the bioactive peptide motif reported by Li et al.[1] in an earlier study. Li et al., however, observed regenerated subbasal nerves as early as 3 weeks after surgery when a growth factor was incorporated within a (differently formulated) biosynthetic graft,[1] whereas the first subbasal nerves in this study were observed 6 months after surgery in allografts and RHC-III grafts. Although this finding is evidence of a potential tradeoff between simplicity of graft fabrication and the speed of neural recovery, a more controlled, quantitative innervation study is needed to demonstrate the action of a nerve growth factor conclusively, as the earlier grafts were fabricated in a bovine collagen–copolymer material and were implanted to a shallower depth in a different species of pig.[1]

Several differences in nerve regeneration within RHC-I and RHC-III grafts were observed in this study. Zone 1 nerves in RHC-III grafts did not recover to preoperative levels, and zone 3 nerves in RHC-III grafts remained significantly thinner than in RHC-I grafts. It should be mentioned, however, that the absolute number and density of nerves in zone 1 of RHC-III grafts was not significantly different from RHC-I grafts 12 months after surgery. In addition, the functional consequences of a small reduction in stromal nerve diameter in depth zone 3 are believed to be minimal. The differences in collagen type observed in this study were therefore considered to be minor and indicated that in general, grafts composed initially of either collagen type provided an environment favorable to nerve growth. This is an important finding, as the native cornea consists primarily of type-I collagen. Type-III collagen, present only in a small amount in the native cornea, was found to produce cross-linked hydrogels with increased strength relative to type-I collagen[6] and could therefore be used to form more robust corneal substitutes. Gradual biodegradation of the collagen-based biosynthetic grafts by enzymes from the extracellular matrix, however, is expected,[5] as is the production of new collagen by host keratocytes observed within the grafts; both processes may have altered the collagen type within grafts in this study. Moreover, the collagen type may have also

the number in porcine collagen-based grafts.[4] For comparison, the first subbasal nerves take longer than 2 years to regenerate after corneal transplantation in humans,[13] and in most cases their density never recovers to preoperative levels.[14] Similarly, after refractive surgical procedures where a substantial portion of the corneal stroma remains intact, subbasal nerve density was reported to recover to preoperative levels 2 years after

been impacted by postsurgical scar tissue formation, as scar tissue is composed primarily of type-I collagen, and evidence of scarring from in vivo confocal images with a strongly light-scattering background haze as noted by others[16–18] was also observed in this study. The degree to which the collagen type was modified in vivo was therefore unclear and could be investigated in a future study through the use of a suitable marker.

The regenerated stromal nerves observed in allografts and in RHC-based biosynthetic grafts were significantly reduced in diameter from the thick nerve trunks observed in the anterior and mid stroma of the native cornea. This morphologic difference probably resulted from the severance of thick nerve trunks in the native cornea to the depth of the lamellar excision, with regenerated nerves (sprouting from the severed nerve or from a more peripheral location) preferentially adopting a thinner morphology, for reasons that are unclear. This behavior occurred regardless of graft type, as abnormally thin stromal nerves were observed in allografts and RHC materials, as well as in porcine collagen grafts in an earlier study. The functional implications of this altered stromal nerve morphology are unknown. Altered stromal nerve morphology has also been observed in transplanted human graft tissue after penetrating keratoplasty, where a marked scarcity and abnormality of stromal nerves and a reduced corneal surface sensitivity was reported.[13,14,19] Although abnormally curved or twisted stromal nerves have been observed after surgery in other studies,[13,14] the stromal nerves observed in this study did not exhibit such behavior. In addition, two studies of the cornea after penetrating keratoplasty[13,19] reported no visible connection of stromal nerves to more anterior nerves in the subepithelial region, suggesting a peripheral, limbal origin of the regenerated nerves in a direction predominantly parallel to the corneal surface.[19] The absence of thick stromal nerve trunks in the present study suggests that reinnervation may also have occurred from the graft periphery toward the center within the superficial stroma. In contrast to the scarcity of stromal nerves observed in transplanted human grafts, in this study, numerous anterior stromal nerves were observed in all graft types (even recovering to preoperative levels), a finding that may relate to the reduced surgical trauma of lamellar keratoplasty, compared with the penetrating procedure, or an increased affinity of regenerating nerves to the implanted graft materials. Although a paucity of nerve data after lamellar keratoplasty precludes a direct comparison of allografts and tissue-engineered grafts in this study with equivalent human graft tissue, it is noteworthy that the substantial innervation observed 12 months after surgery in the porcine allografts is in contrast to a sparse innervation observed in human allografts.[14,19] Whether this indicates a fundamental difference between the porcine and human model is unknown; however, it is plausible that this discrepancy may have arisen from differences in the clinical protocols used—for example, the allografts used in this study were excised from pigs and implanted into others in the same operating room and on the same day, thereby minimizing tissue handling and storage.

A characteristic reduction in the number of nerves with increasing corneal depth was observed in the preoperative porcine corneas in this study, similar to the distribution observed in the native human cornea.[8] Although the number of nerves in left and right corneas before surgery was not significantly different within any corneal depth zone, regardless of subject group (Table 1), an increased variability in the number of preoperative nerves was observed in depth zone 1. This variability probably originated from a variable contrast of the thin subbasal nerves in a confocal image relative to the background. As the relative displacement of the image focal plane from the thin subbasal nerve layer increased, subbasal nerves
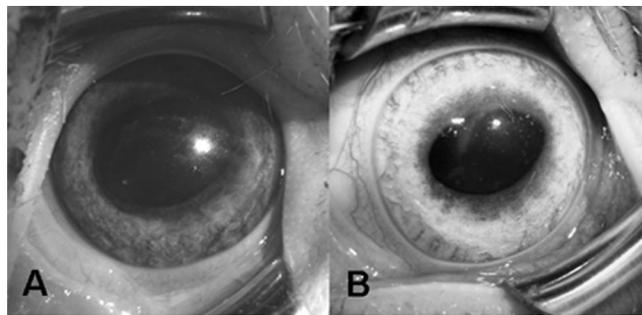

FIGURE 4. Typical RHC-I (**A**) and RHC-III (**B**) biosynthetic corneal substitutes, optically transparent in vivo 12 months after surgery.

lost their sharp contrast from the background, making their accurate quantification more difficult. Within each confocal examination, this relative displacement was random (due to a manual *z*-alignment procedure before image capture) and therefore presented a source of variability. Decreasing the depth interval between adjacent confocal images from 10 to a few micrometers (as in other studies examining subbasal nerves[14,15]) is expected to improve the consistency of nerve measurements in depth zone 1.

In vivo confocal microscopy enabled the quantification of nerve parameters in this study. However, as nerve identification and tracing is presently a manual process, some variability in the quantitative results is unavoidable. Interobserver repeatability in quantification of both nerve density and diameter was reported in one study to be within 12% in the subbasal nerve plexus layer in healthy human corneas, based on 28 images.[20] In another study, based on 22 images, nerve density was reported to be within ±16% in the subbasal nerve plexus in healthy and transplant-recipient human corneas.[14] Although the mean interobserver difference in nerve length within an image in this study (±14%) was similar to results reported by others, the relatively large variance we observed may have resulted from the larger number of images we chose to be examined by two observers, and the location of the images throughout the full corneal thickness (not confined to a specific depth region) where reduced contrast (particularly in the presence of postoperative background scatter) and feature interpretation may have played a role.

In conclusion, through the use of in vivo confocal microscopy, nerve regeneration was observed over a 12-month postoperative period in three different corneal graft types implanted into porcine hosts. Recombinant type-I and -III human collagen-based biosynthetic grafts both provided a suitable environment for neural regeneration throughout the anterior stroma and in the subepithelial corneal region, with nerves repopulating graft tissue in comparable levels to both porcine allograft and biosynthetic porcine collagen-based grafts after 12 months, while remaining optically transparent (Fig. 4). A robust innervation within these materials, the simplicity of their fabrication, and their human collagen-derived composition are strong indicators of their potential for use as allograft substitutes in human corneal transplantation.

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