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corneas were examined histologically by haematoxylin and eosin staining and immunohistochemistry against vimentin and α-smooth muscle actin (α-SMA).

The Dohlman-Doane layer may lead to pigment deposition or extrusion of the anterior surface. Although satisfactory vision is achieved with a successful Kpro implant, a discontinuous epithelial tissue intact beneath the epithelium, such a surgical technique may allow for a long standing artificial device. In order to achieve this architecture, we combined the use of a microkeratome with a D surgical technique already in clinical use. The technique is similar to the microkeratome assisted posterior keratoplasty described by two different groups without penetrating into the anterior chamber. In this report, we show the macroscopic and histological results of poly(vinyl alcohol) (PVA) discs transplanted in rabbits using this “microkeratome assisted deep lamellar Kpro” technique, which showed promising mid-term results.

**Aims:** To establish a keratoprosthesis (Kpro) surgical technique that maintains an intact superficial corneal layer.

**Methods:** A manual microkeratome (Moria LSK-1) was used to create a 130 μm flap of approximately 10 mm diameter in the right eye of Japanese white rabbits. The stoma beneath the flap area was dissected before the removal of a 5.0 mm stromal disc. A 5.0 mm collagen I immobilised poly(vinyl alcohol) (COL-PVA) disc was placed on the exposed posterior stroma close to Descemet’s membrane. The flap was repositioned and fixed using 10-0 nylon sutures, which were removed 2 days following surgery. The corneas were followed clinically by slit lamp microscopy and photographs. Rabbits were sacrificed after 6 months, and the transplanted corneas were examined histologically by haematoxylin and eosin staining and immunohistochemistry against vimentin and α-SMA staining at levels comparable to lamellar keratoplasty control.

**Conclusions:** Microkeratome assisted deep lamellar keratoprosthesis may be a safe technique for the transplantation of artificial hydrogels for therapeutic purposes.

The search for an ideal artificial cornea has a long history, which has led to the development of several keratoprosthesis (Kpro), some of which are already in clinical use. The Dohlman-Doane and AlphaCor Kpros are the two models currently approved by the Food and Drug Administration, and clinical studies are accumulating. The material used for Kpros should be biocompatible to the extent that excessive inflammation and scarring do not occur at the anterior and posterior interfaces of the visual axis. In order to preserve an optically clear interface, several Kpros are designed so that the corneal epithelium does not cover the anterior surface. Although satisfactory vision is achieved with a successful Kpro implant, a discontinuous epithelial layer may lead to pigment deposition or extrusion of the Kpro. In order to circumvent such complications, a strategy to integrate biological components to increase biocompatibility was adopted in the development of corneal equivalents. Corneal equivalents show characteristics similar to the natural cornea in vitro, however, a prototype that lasts indefinitely in vivo is yet to be designed.

The need for corneal equivalents is based on the assumption that healthy components of the epithelium, stroma, and corneal nerves are required to reconstitute the cornea.

However, several studies have shown that artificial materials are stable when placed in stromal pockets, as long as stromal tissue and nerves are left intact in the remaining cornea. The presence of healthy stromal keratocytes is of importance in maintaining a healthy epithelial layer because of the many soluble factors exchanged by both tissue in an elaborate epithelial stromal “cross talk.” An intact epithelium with an underlying stromal layer in laser in situ keratomileusis (LASIK) flaps may explain the fewer incidences of haze observed in LASIK compared with photorefractive keratectomy (PRK).

Opacification of the posterior stromal interface is also occasionally observed in Kpro surgery. Retrocorneal membranes are associated with Kpros that have posterior optics projecting directly into the anterior chamber; and retro-prosthetic membranes occur in approximately 9% of patients with the AlphaCor Kpro. In either case, opacification can be minimised if activation of fibroblasts can be suppressed by physical or pharmacological means. From our experience with deep lamellar keratoplasty (Dro), we have found that by dissecting directly down to the Descemet’s membrane (DM), interlamellar opacification following surgery is minimal. We therefore hypothesised that if Kpros can be transplanted directly onto the deep stroma, while leaving anterior stromal tissue intact beneath the epithelium, such a surgical technique may allow for a long standing artificial device. In order to achieve this architecture, we combined the use of a microkeratome with a D surgical technique already in clinical use. The technique is similar to the microkeratome assisted posterior keratoplasty described by two different groups without penetrating into the anterior chamber. In this report, we show the macroscopic and histological results of poly(vinyl alcohol) (PVA) discs transplanted in rabbits using this “microkeratome assisted deep lamellar Kpro” technique, which showed promising mid-term results.

**MATERIAL AND METHODS**

**PVA implants**

Collagen I was immobilised to PVA (PVA-COL) as previously described. In brief, PVA powder was dissolved in a mixture of dimethyl sulfoxide (DMSO) water solvent and was allowed to stand at −20°C for 24 hours to form a gel. The surface of the gel was modified with hexamethylenedisocyanate (HMDI), which was then immersed in type I collagen solution (porcine skin, 0.5 mg/ml, Nitta Gelatin Co Ltd, Osaka, Japan). PVA-COL was prepared as a non-porous hydrogel, with a water content of 78% to 80%, which is similar to the host cornea. PVA-COL discs (200 μm thickness, 5.0 mm diameter) were sterilised before surgery.

**Abbreviations:** α-SMA, α-smooth muscle actin; BSA, bovine serum albumin; COL-PVA, collagen I immobilised poly(vinyl alcohol); DLPKro, deep lamellar keratoprosthesis; DM, Descemet’s membrane; DMSO, dimethyl sulfoxide; HE, haematoxylin and eosin; HMDI, hexamethylenedisocyanate; Kpro, keratoprosthesis; LKP, lamellar keratoplasty; LASIK, laser in situ keratomileusis; PBS, phosphate buffered saline; PRK, photorefractive keratectomy; PVA, poly(vinyl alcohol)
All animals were treated according to the ARVO statement for the use of animals in ophthalmology and vision research. Female Japanese white rabbits (n = 6, 3 kg body weight, Shiraishi experimental animal breeding farm, Tokyo, Japan) were anaesthetised with 4 ml intramuscular ketamine and xylazine (1:7 mixture), as well as topical xylocaine at the start of surgery. Two rabbits were used as lamellar keratoplasty (LK) control and four rabbits underwent the study procedure. A 130 mm flap with a nasal hinge was created using the LSK-1 microkeratome (Moria, Antony, France). After the flap was lifted, a manual trephine (5.0 mm diameter) was used to create an incision extending into the deep stroma without perforating the DM. Air was injected into the anterior chamber to allow visualisation of Descemet’s membrane (DM). A 5.0 mm PVA disc (stained with trypan blue to enhance visibility) was inserted into the excised wound, and the flap was repositioned and fixed using three to five 10-0 nylon sutures. The PVA-COL disc was then placed into the circular space without any suture fixation. The flap was repositioned and fixed with 5 10-0 nylon sutures. A summary of the surgical procedure is presented in figure 1.

Flap sutures were removed 2 days following surgery after confirming that the corneal epithelium was intact. Topical antibiotics and steroids were applied twice daily for 1 week. One of the rabbits in the study group experienced flap complications (thin flaps, suture related infections), therefore, a total of three rabbits were observed and photographed for up to 6 months following surgery. One rabbit each was sacrificed after 1 month, 3 months, and 6 months with an overdose of pentobarbital, and the corneas with implants were excised and fixed for histological and immunohistochemical analysis.

**Surgical procedure**

All animals were treated according to the ARVO statement for the use of animals in ophthalmology and vision research. Female Japanese white rabbits (n = 6, 3 kg body weight, Shiraishi experimental animal breeding farm, Tokyo, Japan) were anaesthetised with 4 ml intramuscular ketamine and xylazine (1:7 mixture), as well as topical xylocaine at the start of surgery. Two rabbits were used as lamellar keratoplasty (LK) control, and four rabbits underwent the study procedure. A 130 mm flap with a nasal hinge was created using the LSK-1 microkeratome (Moria, Antony, France). After the flap was lifted, a manual trephine (5.0 mm diameter) was used to create an incision extending into the deep stroma without perforating the DM. Air was injected into the anterior chamber, and the DM was dissociated from the posterior stromal surface using a blunt spatula as previously described for deep lamellar keratoplasty (DLKP). Air injection into the anterior chamber was first described by Melles et al, which allows visualisation of incision depth reducing the risk of perforating DM. The trephined stromal disc was then removed using micro scissors and forceps to remove any remaining stromal strands. The PVA-COL disc was then placed into the circular space without any suture fixation. The flap was repositioned and fixed with 5 10-0 nylon sutures. A summary of the surgical procedure is presented in figure 1.

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**Histological analysis**

Corneas were fixed in 10% formalin neutral buffer solution (Mildform 10N, Wako Pure Chemical Industries, Osaka, Japan) at 4°C overnight, and then embedded in paraffin. Four μm sections were stained with haematoxylin and eosin (HE) or immunostained with primary antibodies against α-smooth muscle actin (α-SMA, clone 1A4, 0.2 μg/ml, Neomarkers, Lab Vision Corporation, Fremont, CA, USA) and vimentin (clone V9, 0.2 μg/ml, Neomarkers). In brief, sections were rehydrated, treated with 3% H2O2, blocked with phosphate buffered saline (PBS) containing 10% normal goat serum and 1% bovine serum albumin (BSA), and treated with primary antibody at 4°C overnight. After washing, sections were incubated with biotinylated goat anti-mouse IgG1 (0.5 μg/ml, Southern Biotechnology Associates Inc, Birmingham, AL, USA) or anti-mouse IgG2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), treated with ABC kit (Vector Laboratories Inc, Burlingame, CA, USA), and ABC is detected by diaminobenzidine (DAB) kit (Vector Laboratories Inc).

**Figure 1** Demonstration of DLKP surgical technique using an enucleated rabbit eye. (A) A manual microkeratome is used to create a hinged flap. (B) A 5 mm incision (arrow) is trephined under the lifted flap (*). Arrowheads indicate the margin of the flap bed. Air is injected into the anterior chamber to allow visualisation of Descemet’s membrane (see text for details). (C, D) Stromal tissue is removed using a lamellar knife to reveal Descemet’s membrane (DM). (E–H) A 5.0 mm PVA disc (stained with trypan blue to enhance visibility) is inserted into the excised wound, and the flap is repositioned and fixed using three to five 10-0 nylon sutures.

**Figure 2** Surgical results of DLKP. Postoperative slit photographs 2 days (A, ×22) and 6 months (B, ×16) following surgery. HE staining of the same cornea shows an intact disc within the stroma with minimal cellular infiltration (C). High magnification of the flap (D) and posterior stroma (E) shows no cellular infiltration. The space between stroma and implant is an artefact of tissue fixation. PVA = PVA implant.
An intact ocular surface may be a way to prevent these complications; however, long-term maintenance of a stratified epithelial layer over an artificial hydrogel has proved difficult for extended periods in vivo. The reason why such polymers, which often support stratified epithelium in vitro, fail to maintain a healthy epithelium in vivo can be explained by a lack of stromal cells that are involved in the homeostasis of the epithelium. The surgical technique described in this report makes use of residual stroma within the flap to function both as a mechanical barrier against polymer extrusion, as well as a reservoir of keratocytes for the maintenance of the overlying epithelium. The structure of the anterior stroma is similar to the AlphaCor implant before the stage II procedure in cases without the Gunderson conjunctival flap. As shown from the histology of the implanted cornea after 6 months, keratocytes are observed between the polymer and epithelium, and the appearance of the overlying epithelium is normal without signs of mechanical stress or malnutrition. The COL-PVA hydrogel used for the implant has already been shown to maintain a normal glycogen content in overlying epithelium demonstrated by PAS staining.

Another vital property of keratoprosthesis is the optical clarity achieved along the visual axis after implantation. Therefore, conventional full thickness keratoprosthesis may offer better vision when successfully managed. The advantage of the material and technique used in this study is higher biocompatibility of the keratoprosthesis that is completely implanted within the stroma. Figures 2 and 3 show that opacification of the interface is minimal, with scarring observed only along the edge of the implanted disc. Such scarring does not hinder the refraction of the cornea, and may serve to secure the polymer in position within the stroma. Figure 2 also shows residual stroma between the polymer and endothelium. Since there was no apparent opacification at the posterior surface of the implant, complete exposure of DM may not be necessary. This may be beneficial since exposing DM may be difficult in clinical cases.

Clinical indications for this procedure may be limited because of the requirement of a relatively clear anterior stroma. One such indication may be keratoconus patients with relative thickness of the central cornea. Although there may be an ethical issue with using keratoprosthesis in keratoconus patients, DLKPro may be an option before PKP using donor tissue. However, we think that another broader indication may be patients with recurring bullous keratopathy. Although a healthy endothelium is required to maintain clarity in a full thickness cornea, decreased vision as a result of stromal oedema can be substantially decreased by replacing 300–400 μm of the swelled stroma with a transparent polymer. Although visual acuity may be sub-optimal, functional vision may be restored in patients who otherwise may require multiple grafts because of rejection or endothelial decompensation. Animal studies to investigate this possibility are under way.

Another advantage of this technique is the simple design of the implant, which may be constructed from modified contact lens moulds. Although the disc used in this study was flat, a curved design fit for the human cornea may be more compatible for clinical use. Custom designed discs may be used modulate postoperative refraction as well, since the overall refraction of the cornea can be modified by the curvature of the disc implanted beneath the flap. The concept of refractive correction using corneal inlays has been studied, with positive clinical results. Although the results are only preliminarily, improving surgical technique may compensate for some of the disadvantages of using non-biological materials for artificial corneas. The technique that we have described combining the use of a microkeratome with DLKP procedures offers a safe and reliable method to implant Kpros.
into the deep stroma. An intact Descemet’s membrane will act as a barrier to prevent dislocation of the Kpro into the anterior chamber, and may also minimise interface opacification because of the formation of retroprosthetic membranes.

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