Local Immunosuppression in Wuzhishan Pig to Rhesus Monkey Descemet’s Stripping Automated Endothelial Keratoplasty: An Innovative Method to Promote the Survival of Xenografts

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Keywords
Corneal xenotransplantation · Descemet’s stripping automated endothelial keratoplasty · Immunosuppressor · Nonhuman primate

Abstract
Introduction: Corneal xenotransplantation is an effective solution for human corneal shortage. We investigated the feasibility and efficacy of different postoperative protocols on xeno-Descemet’s stripping automated endothelial keratoplasty (DSEAK) grafts. Methods: Thirty rhesus monkeys were randomly divided into three groups: control group (C) and only Descemet’s membrane (DM) stripping, DSEAK 1 (D1) and DSEAK 2 (D2) groups, DM stripping followed by endothelial keratoplasty. Betamethasone 3.5 mg was subconjunctivally injected in groups control and D1 postoperatively, whereas rhesus monkeys in group D2 received topical 0.1% tacrolimus and topical steroids. All groups were evaluated by slit lamp, anterior segment optical coherence tomography, and laser scanning confocal microscopy for at least 9 months. Results: Twenty-four monkeys met the inclusion criteria. Nine months after the DSEAK surgery, most corneas were transparent. Graft rejection was observed in 25% and 28.57% of the cases in group D1 and group D2 (\(p > 0.05\)), respectively. Corneal endothelium densities in DSEAK groups were 2,715.83 ± 516.20/mm\(^2\) (D1) and 2,220.00 ± 565.13/mm\(^2\) (D2) (\(p > 0.05\)). Conclusions: Xenogeneic corneal endothelial grafts can survive and function in rhesus monkey eyes for a prolonged period of time with subconjunctival steroid or topical tacrolimus and steroid treatment. Furthermore, topical drugs are more suitable for clinical use.

Introduction

Corneal transplantation is a relatively mature therapeutic procedure that is considered the only effective treatment for corneal blindness, which is the second-largest treatable blindness. However, only less than 10,000 cases of corneal transplantation are performed annually in China [1, 2]. Due to religious and customary reasons, there is a substantial imbalance between the number of corneal donations and the actual demand for corneas in Asia, especially in China [3]. The worldwide shortage of
allogeneic corneas has necessitated the use of corneal replacements. The use of xenogeneic corneas, especially the porcine cornea (PC), has recently gained attention worldwide due to its sufficient donor supplies and similarity to the human cornea [4–7]. However, the PC is significantly thicker than human and rhesus monkey corneas [4–7]. This difference in corneal thickness (CT) can increase the failure rate of xenopenetrating keratoplasty (PKP) and increase the incidence of complications such as anterior synechiae and retrocorneal membrane (RCM) formation [8–10]. To reduce corneal transplantation’s failure because of thickness differences and xenoa antigens’ presence, we tried replacing xeno-PKP with xeno-Des- cemet’s stripping automated endothelial keratoplasty (xeno-DSAEK). This approach is consistent with the broader clinical trend, wherein endothelial keratoplasty (EK) based on DSAEK is gradually replacing PKP as a surgical treatment for corneal endothelial lesions such as bullous keratopathy and Fuchs’ corneal dystrophy because of the low rates of rejection, favorable visual outcome, and uncomplicated postoperative course.

A recent pig to nonhuman primate (NHP) corneal PK model has shown that xenotransplant survival could be prolonged by administering systemic steroids, immunosuppressants, intravenous immunoglobulin, or anti-CD40/anti-CD154 antibodies [11–13]. However, long-term systemic treatment is not practical in clinical use. In contrast, because of the low antigen load associated with DSAEK grafts, the incidence of immune rejection after allogeneic DSAEK is only 3.5%–13.9% [14–17]. This low rejection rate indicates the feasibility of topical medication. Liu et al. [18] had found that xenodSAEK grafts can survive over 180 days with only subconjunctival betamethasone injections. However, a continuous subconjunctival injection may cause pain, subconjunctival hemorrhages, and conjunctival scarring. Patients need to travel between the hospital and

| Donor (WZSP) | Recipient (rhesus monkey) |
|-------------|---------------------------|
| number³ | age, months | right (R) or left (L) eye | ECD after pre-cut, cells/mm² | GT, μm | number | groups |
| P01 | 6 | R | 2,911 | 3,459 | 134 | MM07 | D1 |
| | | | 132 | 137 | MM13 | D2 |
| P02 | 7 | R | 3,289 | 3,245 | 132 | MM12 | D2 |
| | | | 176 | 176 | MM10 | D1 |
| P03 | 13 | R | 3,133 | 3,285 | 181 | MM08 | D1 |
| | | | 137 | 137 | MM11 | D1 |
| P04 | 10 | R | 3,289 | 2,925 | 144 | MM14 | D2 |
| | | | 180 | 180 | MM15 | D2 |
| P05 | 10 | R | 3,300 | 2,901 | 115 | MM09 | D1 |
| | | | 137 | 137 | MM06 | D1 |
| P06 | 11 | R | 3,141 | 2,901 | 115 | MM09 | D1 |
| | | | 137 | 137 | MM06 | D1 |
| P07 | 18 | R | 3,030 | 3,632 | 187 | MF08 | D1 |
| | | | 100 | 100 | MF11 | D2 |
| P08 | 8 | L | 3,367 | 181 | 100 | MF13 | D2 |
| P09 | 7 | R | 3,759 | 191 | 180 | MF14 | D2 |
| P10 | 6 | R | 4,049 | 3,217 | 180 | MF10 | D1 |
| | | | 124 | 124 | MF06 | D1 |
| P11 | 7 | R | 3,093 | 3,278 | 193 | MF09 | D1 |
| | | | 186 | 186 | MF07 | D1 |
| P12 | 8 | R | 3,379 | 188 | 188 | MF15 | D2 |

MM, Monkey (male); MF, Monkey (female). *P01: WZSP, no. 1.
home for several times, which is expensive and inconvenient. Therefore, patients are more likely to receive topical eye drops for long-term treatments. Fortunately, 0.1% tacrolimus eye drop (TALYMUS®; Senju, Osaka, Japan) is routinely used to prevent and treat immune rejection after corneal transplantation in China. We had previously shown that 94.5% of the high-risk [19–23] PKP cases treated by topical steroids combined with 0.1% tacrolimus eye drop could survive for more than 1 year [24].

However, topical tacrolimus has not been used after porcine to NHP xeno-keratoplasty, especially after xeno-DSAEK in previous studies. Our study aimed to investigate the feasibility and efficacy of using topical steroids combined with tacrolimus therapy on xeno-DSAEK grafts. We also refined the previous methods using anterior chamber (AC) optical coherence tomography (OCT) and vivo laser scanning confocal microscopy (LSCM) to prepare xenotransplants and observe the outcomes continuously and dynamically after the DSAEK surgery. We hope that the outcomes of this study facilitate the clinical use of porcine xenotransplants.

**Materials and Methods**

**Donors and Recipients**

Thirteen porcine endogenous retrovirus noninfectious strains of Wuzhishan miniature pigs (WZSPs) aged 6–18 months that passed microbiological testing [25] were selected as the donors. As recipients, 30 healthy rhesus monkeys aged 24–36 months, including 15 male and 15 female monkeys that showed no apparent abnormalities in the ophthalmologic examination and passed the microbiological examinations [26–29] were selected. The basic information of the donors and the recipients is shown in Table 1.

**Donor’s Graft Preparation**

Before cutting the WZSP corneas, we used the slit lamp to check for edema, scarring, punctate lesions, and other abnormalities that might make the cornea inappropriate for DSAEK. Corneal endothelial cell density (ECD) was examined using the specular microscope (Konan, Tokyo, Japan); AC-OCT (TOMEY, Nagoya, Japan) was used to measure the thickness of the central area of the corneal graft for tissue slice preparation. The method used for obtaining corneal tissue and preparing DSAEK grafts is as described previously [18] and was refined further. In brief, we used the 500–550-μm blade of an automatic microkeratome (Moria, Antony, France) to obtain an appropriate graft thickness (GT) (approximately 100–200 μm). We checked the ECD and the GT again before storing the grafts in Optisol-GS (Bausch & Lomb, St. Louis, MO, USA) at 4°C for use.

**Table 2. Groups and postoperative treatments**

| Groups       | Recipients, N | Surgery            | Treatment                                                                 |
|--------------|---------------|--------------------|---------------------------------------------------------------------------|
| Control      | MM01~MM05, MF01~MF05 (10) | DM stripping       | Medication usage: Cravit®, Q2d x 2 weeks, then BIW x 2 weeks                |
|              |               |                    | Tobradex® (ointment): Subconjunctival, 5 injections every 10 days and then once a month |
|              |               |                    | Diprosran® 3.5 mg: Subconjunctival, 5 injections every 10 days and then once a month |
|              |               |                    | Hialid®: TID, if necessary                                                  |
| D1           | MM06~MM10, MF06~MF10 (10) | DM stripping and DSAEK | Same as the control group                                                  |
| D2           | MM11~MM15, MF11~MF15 (10) | DM stripping and DSAEK | Same as the control group                                                  |

| Medication and usage: | |
|-----------------------| |
| Tobradex® (eye drop): | tobramycin and dexamethasone ophthalmic eye drops (Alcon, Belgium); Tobradex® (ointment): tobramycin and dexamethasone eye ointment (Alcon, Belgium); Pred Forte®: prednisolone acetate ophthalmic suspension 0.1% (Allergan, Ireland); Cravit®: 0.5% levofloxacin eye drops (Santen, Japan); Diprosran®: betamethasone 3.5 mg (Schering-Plough, USA); TALYMUS® 0.1%: tacrolimus eye drops (Senju, Japan); Hialid®: 0.3% sodium hyaluronate eye drops (Santen, Japan). MM, monkey (male) No.06; MF, monkey (female). |
Local Immunosuppression in Xeno-DSAEK

The 30 rhesus monkeys were randomly divided into 3 groups, with each group containing an equal number of males (MM01–MM15) and females (MF01–MF15). Only the monkeys’ right eyes received the surgeries, treatments, and observations. The bullous keratopathy model [18] was established in the 3 groups: DSAEK 1 (D1), DSAEK 2 (D2), and control (C) groups.

Surgical Procedures and Treatments

Xeno-DSAEK was subsequently performed in groups D1 and D2. All surgical procedures in this study were performed by one experienced surgeon (Dr. Pan). Three days before the surgery, all recipients received Cravit® 3 times a day. We used a 6.25-mm trephine to make a graft’s diameter of 6.50 mm. An AC maintainer was used to maintain the chamber structure. The porcine DSAEK graft was placed at a proper position by pulling a 10-0 Nylon suture stitched at the graft’s edge into the AC. Finally, the limbal incisions were closed with 10-0 nylon sutures. Subsequently, we filled the AC with sterile air. All rhesus monkeys received betamethasone 3.5 mg subconjunctivally and tobramycin and dexamethasone eye ointment (Tobradex®; Alcon, Puurs, Belgium) in the right eye after the surgery.

Ten days after the surgery, the recipients in group D1 and the control group received betamethasone (Diprospan®; Schering-Plough, Kenilworth, NJ, USA) 3.5 mg subconjunctivally every 10 days for 5 times and then changed to once a month until the observation ended. Group D2 received steroids (Pred Forte®; Allergan, Ireland) combined with 0.1% tacrolimus (TALYMUS®; Senju, Japan) topically on the first day after the surgery. The frequency of medication decreased gradually from 4 times a day to twice a day. The specific grouping and treatment plan are shown in Table 2.

Table 3. Slit-lamp evaluations

| Grade | Criteria | Grade | Criteria |
|-------|----------|-------|----------|
| **Opacity** | | **Conjunctival congestion** | |
| 0 | Clear cornea | 0 | No conjunctival congestion |
| 1 | Slight haze | 1 | Slight congestion |
| 2 | Increased haze, but AC structures still clear | 2 | Dark red congestion around the limbus, 2–3 mm wide |
| 3 | Advanced haze with difficult view of the AC | 3 | Dark red congestion around the limbus, more than 3 mm wide |
| 4 | Opaque cornea without view of the AC | 4 | Extensive congestion |

| **Edema** | | **Epithelial defect** | |
| 0 | No stromal or epithelial edema | 0 | Complete corneal epithelium |
| 1 | Slight stromal edema | 1 | Punctate fluorescein staining on epithelium |
| 2 | Diffuse stromal edema | 2 | Fluorescein stain exceeding 1/4 of the epithelium |
| 3 | Diffuse stromal edema with microcystic edema of epithelium | 3 | Fluorescein stain exceeding 1/4 of the epithelium |
| 4 | Bullous keratopathy | 4 | Significant defect |

| **Neovascularization** | | |
| 0 | No vascularization | |
| 1 | Vascularization of the peripheral native cornea | |
| 2 | Vascularization of the graft periphery | |
| 3 | Vessels appearing in the graft to 2 mm | |
| 4 | Vascularization of the entire graft | |

Postoperative Clinical Evaluations

Rhesus monkeys that met the following criteria were included for further evaluation: (1) grafts were at the proper position 7 days after the surgery or could be relocated after air injection in the AC; (2) the cornea began to return to transparency 24 h after the surgery or the corneal edema gradually decreased after the surgery and the cornea became completely transparent within 7 days; (3) no infection was present in the cornea and the AC within 7 days after the surgery; and (4) no significant complications, such as intraocular hypertension or expulsive choroidal hemorrhage, were observed for 7 days after the surgery. Cases that did not meet these criteria were considered to indicate primary graft failure [30, 31] and excluded in the following observation.

Grafts were evaluated twice a week for the first month after the surgery, then weekly for 2 months, and then twice a month until 9 months after the surgery by slit-lamp assessments (CSO, Scandicci, Italy), which included the evaluation of the conjunctiva, corneal epithelial defects, corneal clarity, edema, and neovascularization. A rejection index was determined based on the sum of the grades for clarity, edema, and neovascularization (Table 3) [32, 33]. Grafts with a rejection index score ≥6 or grade of clarity ≥2 were considered to be rejected [34, 35]. Intraocular pressure (IOP) was measured by a Tono-Pen (Reichert, Depew, NY, USA) simultaneously accompanied by the slit-lamp examination. The mean IOP of the rhesus monkeys was 20.70 ± 3.88 mm Hg through multiple measurements at different times in the preoperative period. Therefore, we defined IOP ≤10 mm Hg as low IOP and IOP ≥25 mm Hg as high IOP. On postoperative months 1, 3, 6, 9, and 12, anterior segment OCT (AS-OCT) was...
used to measure the thickness of the cornea and the graft, meanwhile LSCM was used to assess each layer of the cornea and the ECD.

**Statistical Analyses**

Statistical analyses were performed using the Statistical Package for the Social Sciences version 21.0 (IBM, Armonk, NY, USA). The differences in the survival rate between groups D1 and D2 were compared by Pearson χ² and Fisher's exact test. Repeated measures ANOVA was used to compare ECD and thickness of the cornea and graft at different time points. The slit-lamp examination data (edema, opacity, and neovascularization) were analyzed by a nonparametric Mann-Whitney U test. p values <0.05 were considered statistically significant. IOP in different groups was compared using the t test.

**Results**

Before the surgery, the endothelial graft’s average thickness was 159.27 ± 45.83 μm as measured by AS-OCT. The endothelial cells were well-organized and hexagon-shaped and showed no noticeable swelling or loss. No microorganisms were detected in the preservative solution after the surgery.

All monkeys maintained healthy conditions during the experimental period, except one in the control group that died due to anesthetic intolerance. After the DSAEK surgery, 5 of 20 cases showed severe anterior synechiae and shallow AC within 3 days, which persisted after synchecomlysis and AC rehabilitation. These 5 cases were confirmed to experience primary graft failure because the corneas remained edematous 7 days after the DSAEK surgery and were thus excluded in the following observation. Thus, the success rate of the surgery was 75%, and a total of 24 monkeys (24 eyes) met the inclusion criteria, including 11 males (MM) and 13 females (MF): 9 (MM 4/9) in the control group; 8 (MM 4/8) in group D1; and 7 (MM 3/7) in group D2.

Nine months after the DM stripping surgery, all corneas in the control group remained edematous and opaque. The average IOP in all three groups remained constant. Almost all cases showed an IOP of 10–25 mm Hg. Some cases showed a transient IOP increase to more
than 25 mm Hg, which subsequently normalized without any treatment.

**WZSP-Rhesus Monkey Xeno-DSAEK Graft Survival**

Nine months after the DSAEK surgery, all the xenotransplants were well-attached, and most of the corneas were transparent. The typical postoperative slit-lamp photos are shown in Figure 1. In group D1, two graft rejections appeared, but one returned transparency through fortified steroid treatment. In group D2, two grafts showed persistent opacity because of irreversible rejection (Fig. 2). The xenograft survival curve is shown in Figure 3.

We observed rejection reaction in 4 cases within 9 months after the DSAEK surgery, including three irreversible rejections and one reversible rejection. In group D1, two grafts (MM06 and MF10) showed edema and opacity on day 96 and day 11 after the surgery, respectively. One graft (MF10) showed early rejection, but the cornea returned to transparency after an additional subconjunctival steroid injection (Fig. 2). In group D2, two grafts (MM14 and MF13) showed graft rejection action on day 79 and day 252 after the surgery (Fig. 2). These two grafts remained edematous and opaque and experienced graft failure, although subconjunctival steroid injection was also administered. The graft rejection rates of groups D1 and D2 were 25.00% (2/8) and 28.57% (2/7), respectively, but their graft survival rates were 87.5% and 71.43%, respectively. No statistically significant differences ($p$ value >0.05) in corneal opacity, edema, and neovascularization were found between groups D1 and D2 during the 9-month observation period (Table 4).

**Observations of Xenotransplant and Recipient CT by AS-OCT**

All xenotransplants attached well after the DSAEK surgery. No dislocation was observed in AS-OCT (Fig. 4). The CT (Fig. 5a) of the rhesus monkey was 461.50 ± 19.74 μm before the surgery and increased after the surgery in three groups. The CT of the control group increased to
905.44 ± 171.51 μm on 3 months after the surgery and then decreased to 835.22 ± 179.26 μm on 9 months after the surgery. The CT of the monkey’s cornea and the xenotransplant of D1 and D2 increased to 750.25 ± 87.72 μm and 733.38 ± 73.26 μm, respectively, 1 month after the DSAEK surgery. Subsequently, the CT decreased to 690.43 ± 97.23 μm and 685.20 ± 120.44 μm, respectively, 9 months after the DSAEK surgery. There was no significant difference between groups D1 and D2 at 1 month, 3 months, 6 months, and 9 months after the surgery (p value = 0.378, 0.446, 0.213, 0.358) in CT assessments. However, the CT in the control group was significantly higher, showing significant differences, compared with those in groups D1 and D2 at 1 month (p value = 0.025, 0.011), 3 months (p value = 0.007, 0.047), 6 months (p value = 0.011, 0.023), and 9 months (p value = 0.025, 0.034) after the surgery.

The GT of groups D1 and D2 increased from 158.63 ± 40.07 μm and 165.00 ± 27.92 μm before the surgery to 244.86 ± 61.53 μm and 228.40 ± 56.31 μm 1 month after the surgery. Finally, it decreased to 205.29 ± 64.12 μm and 199.82 ± 48.76 μm 9 months after the surgery. The average GTs in group D1 increased by 54.36%, 48.50%, 28.88%, and 29.41% on P1M, P3M, P6M, and P9M after

| Table 4. Scores of slit-lamp findings in groups D1 and D2 and the control group at different time points after the surgery (mean±standard deviation) |
|-----------------------------------------------------|
| Edema | Opacity | Neovascularization |
| control | D1 | D2 | p-value | control | D1 | D2 | p-value | control | D1 | D2 | p-value |
| P14D | 3.10±0.74 | 0.63±0.52 | 0.67±0.54 | 0.80 | 1.50±0.53 | 0.71±0.76 | 0.71±0.54 | 0.80 | 1.50±0.53 | 0.71±0.76 | 0.71±0.54 | 0.80 |
| P1M | 3.40±0.52 | 0.63±0.74 | 0.67±0.82 | 0.92 | 1.33±0.65 | 0.83±0.79 | 0.83±0.98 | 0.71 | 1.33±0.65 | 0.83±0.79 | 0.83±0.98 | 0.71 |
| P3M | 3.11±0.33 | 0.63±0.74 | 0.67±0.82 | 0.92 | 1.11±0.33 | 0.71±0.76 | 0.83±0.98 | 0.71 | 1.11±0.33 | 0.71±0.76 | 0.83±0.98 | 0.71 |
| P6M | 2.80±0.45 | 0.63±0.74 | 0.67±0.82 | 0.92 | 0.54±0.76 | 0.26±0.42 | 0.26±0.42 | 0.80 | 0.54±0.76 | 0.26±0.42 | 0.26±0.42 | 0.80 |
| P9M | 2.67±0.50 | 0.57±0.79 | 0.69±0.86 | 0.87 | 0.66±0.75 | 0.29±0.49 | 0.33±0.52 | 0.87 | 0.66±0.75 | 0.29±0.49 | 0.33±0.52 | 0.87 |
| P14D, postoperative 14 days; P1M, postoperative 1 month; P3M, postoperative 3 months; P6M, postoperative 6 months; P9M, postoperative 9 months. * p-value between D1 and D2.
the DSAEK surgery compared to those before the DSAEK surgery. The average GTs increased in group D2 were 38.42%, 32.48%, 15.21%, and 21.10%, respectively (Fig. 5b). There was no statistically significant difference in xenotransplant thickness between the 2 groups before the surgery \((p \text{ value } = 0.642)\), 3 months \((p \text{ value } = 0.682)\), 6 months \((p \text{ value } = 0.644)\), and 9 months \((p \text{ value } = 0.518)\) after the DSAEK.

Examinations of Xenotransplant and Recipient Cornea by LSCM

On 1 month, 3 months, 6 months, and 9 months after the DSAEK surgery, we performed LSCM examination on all 30 rhesus monkeys. We also examined ten normal rhesus monkeys to compare the effect of DM stripping and xeno-DSAEK on the cornea of rhesus monkeys. LSCM found that subepithelial blisters and stromal edema with corneal thickening were observed in the control group 1 month after the DM stripping, and the posterior stroma and the rest of cornea could not be visualized in LSCM.

We found that most of the rhesus monkeys in groups D1 and D2 had the same images of the epithelium, Bowman’s membrane, and superficial stroma with normal NHPs. The corneal epithelium kept intact, with a tight and regular arrangement of epithelial cells. Besides, the density of the subepithelial nerves did not decrease, and edema was not observed in the stroma. LSCM also showed that the junctional zone between the host and the xenotransplant was not easily distinguishable.

Endothelial cells were well-preserved in most grafts (Fig. 6). Before the DSAEK surgery, the xenotransplant’s ECDs in groups D1 and D2 were \(3,215.25 \pm 501.92 \text{ cells/mm}^2\) and \(3,352.86 \pm 273.63 \text{ cells/mm}^2\), respectively. Subsequently, the ECDs decreased after the DSAEK surgery. After 9 months, the ECDs of groups D1 and D2 were \(2,715.83 \pm 516.20 \text{ cells/mm}^2\) and \(2,220.00 \pm 565.13 \text{ cells/mm}^2\), respectively (Fig. 5c). On 1 month, 3 months, 6 months, and 9 months after the DSAEK surgery, the endothelial cell losses in groups D1 and D2 were 26.12%, 25.31%, 25.87%, and 29.91% and 26.16%, 35.16%, 31.19%, and 35.60%, respectively. There was no statistical difference between the DSAEK groups \((p \text{ value } = 0.378, 0.364, 0.359, 0.253)\).

Other Complications of Xeno-DSAEK

Similar to allogeneic corneal transplantation, postoperative complications cannot be completely avoided in xeno-DSAEK surgery. Table 5 lists the complications observed in the present study. Early complications such as anterior synechiae and RCM were predominant. We observed 4 cases of partial anterior synechiae 9 months after the DSAEK surgery. A total of 4 cases of RCM were observed 2 weeks after the DSAEK surgery, and the RCM showed a distinct thickening of the graft caused by retraction of the RCM (Fig. 7).
Discussion

Corneal xenotransplantation is a promising choice for corneal blindness in most developing countries, especially in China [1, 2]. Corneal component transplantation procedures, including anterior lamellar keratoplasty, deep anterior lamellar keratoplasty, DSAEK, and DMEK, are overtaking PK as the most common keratoplasty procedures [3, 35]. Moreover, EK can overcome obstacles caused by inconsistency in the graft and host thickness in PKP. Our previous porcine-rhesus monkey model has proven that xenotransplantation was feasible [4, 9, 18, 36, 36].

Fig. 5. Change of CT (a), GT (b), and graft ECD (c). The CT (a) in the control group was significantly higher, showing significant differences, compared with those in groups D1 and D2 at 1 month, 3 months, 6 months, and 9 months after the DSAEK surgery. \*p = 0.025, \*\*p = 0.011, \*\*\*p = 0.007, \*\*\*\*p = 0.047, \*\*\*\*p = 0.011, \*\*\*\*\*p = 0.023, \*p = 0.025, \*\*p = 0.034. GT and ECD have no statistic difference between D1 and D2 at 1 month, 3 months, 6 months, and 9 months after the DSAEK surgery.
The xeno-DSAEK grafts could survive more than 180 days with local steroid administration, and the average ECD of the xenotransplants remained greater than 2,000 cells/mm² 6 months after surgery. Pathological assessments showed that there were no inflammatory cell infiltrations in the grafts and the host corneas.

In this study, the 9-month results confirmed that xeno-DSAEK is significantly useful in solving corneal edema and bullous keratopathy caused by endothelial lesions. Our findings showed that 80% of corneal endothelial grafts could survive for longer than 270 days. Xenotransplants treated with topical tacrolimus and steroids showed almost the same survival rate as those treated by conjunctival steroid injections. We believe that this result is associated with the lower antigen load by DSAEK surgery and the AC-associated immune deviation. Previous studies did not find immune rejection-related factors in the rhesus monkey’s blood [18]. We believe that the immune rejection reaction occurred locally; thus, topical treatment effectively prevents immune rejection.
We used AS-OCT and LSCM to observe the xeno-transplants and the host corneas at different time points after the DSAEK surgery dynamically and continuously and found that the cornea returned to transparency 3~7 days after the DSAEK surgery. Although the cornea appeared to thicken temporarily, CT decreased after reaching the peak 3 months after the surgery. The ECD of xenotransplants decreased significantly by the first LSCM.

Fig. 7. Slit-lamp images and OCT scans of the RCM. Four cases of RCM appeared 6 days and 11 days after DSAEK. Except for 1 RCM that disappeared on P18D (MF10) without any treatment, the RCMs existed for 9 months. The corneas were slightly edematous, but showed no signs of rejection.

Table 5. Complications after xeno-DSAEK

| Complications       | Groups | Numbers | Monkeys | Time | Treatments | Results                                                                 |
|---------------------|--------|---------|---------|------|------------|-------------------------------------------------------------------------|
| RCM                 | D1     | 3       | MM07    | P6D  | None       | RC existing persistently. Graft’s clarity reduced                       |
|                     |        |         | MM09    | P6D  | None       | RC existing persistently. Graft’s clarity reduced                       |
|                     |        |         | MF12    | P6D  | None       | RC began to disappear on P18d. Graft’s clarity recovered                |
|                     | D2     | 1       | MF12    | P11D | None       | RC existing persistently. Graft’s clarity reduced                       |
| Anterior synechiae  | D1     | 2       | MM06    | P3D  | Surgerya   | Low IOP, AC disappeared, anterior synechiae, and graft failure          |
|                     |        |         | MM08    | P3D  | Surgery    | Anterior synechiae, corneal edema, and opacity                          |
|                     | D2     | 2       | MM14    | P3D  | Surgery    | Hyphema, AC disappeared, high IOP, and rejection                       |
|                     |        |         | MF14    | P3D  | Surgery    | Anterior synechiae, corneal edema, and opacity                          |

P3D, postoperative 3 days. *Surgery included synechiolysis, AC habitation, and peripheral iridectomy.
examination, which was probably due to the surgical operation, and xenotransplants could not immediately adapt the recipient’s immune environment. However, subsequently, the ECD was maintained at a satisfactory level 9 months after the surgery. We hypothesize that immune tolerance is gradually formed accompanied by local immunosuppression, but more evidences should be explored further.

Considering the insufficient studies assessing the rate of corneal endothelial cell loss after xeno-DSAEK, we refer to the literature of clinical allogeneic DSAEK and found that the ECD loss rates are 25.67%, 31.15%, and 33.98% at 1, 3, and 6 months after allogeneic DSAEK [35–37]. This was similar to the loss rate of ECD found in xeno-DSAEK. We propose that the xenotransplant might experience fewer immune attacks than allografts. LSCM also showed signs of early rejection, such as presence of dendritic cells and KP, which were also observed on slit-lamp assessments. Notably, these traces of rejection were observed not only in cases of eventual rejection but also in cases of nonrejection. We attribute these phenomena to the use of steroids and immunosuppressants and the resultant inhibition of most of the host’s immune response to the xenotransplant.

Moreover, we also found that one of the two rejected grafts in group D1 could be revived by a more intense subconjunctival steroid injection protocol and that it became transparent again. The inhibition of immune rejection could be attributable to prompt detection of early signs of immune reaction and timely steroid treatment adjustment. However, in group D2, different from the frequent slit-lamp examination in the early postoperative period, the twice-a-month observations 9 months after the DSAEK surgery might delay early intervention, since 1 case showed rapid development of rejection 9 months after the surgery, and irreversible rejection occurred despite intensive subconjunctival steroid injection treatment. We believe that a lower frequency of eye drops might be another cause for this late rejection. In the future, immunosuppressant should be tapered slowly and appropriately to reduce surgical operations and protect corneal endothelial cells. Second, recipients could not keep the facing-up position after the surgery, which easily caused complications such as synechia or malignant glaucoma. Therefore, the smaller xenotransplants might help reduce surgery-related complications. In future clinical applications, we should strictly control the thickness of the porcine graft within 150 μm. Moreover, the postoperative position can also be guaranteed, and a larger diameter xenotransplant is possible.

After the anesthesia wore off, the monkey’s activity increased, and the head position was difficult to control. As a result, some complications occurred. Some studies [38–42] have shown that in allograft surgery, the probability of rejection with one or two quadrants of anterior synechiae is 29%, whereas the rejection rate with three to four quadrants is 34%. Thus, we propose that anterior synechiae may affect the success rate and prognosis of the xenotransplant. RCM is another complication that affects corneal transparency and occurs after allogeneic PKP [43], DSAEK [44, 45], and DMEK, with an incidence ranging from 17% to 83% [46–49]. RCM develops from hemorrhage, exudative precipitates with entrapped inflammatory cells that undergo organization or induce endothelial fibrous metaplasia, displaced proliferative iris stromal melanocytes, or adherent proliferating lens epithelial remnants [50–53]. Lee et al. [53] used pigs aged 1–3 months for porcine-rhesus monkey PKP. Three months after the surgery, a total of 4 cases (4/4) developed RCM mainly derived from corneal donor cells within 3–11 days. We found that 4 cases (26.67%) in the xeno-DSAEK groups showed RCM within 11 days after the surgery. Although RCM may reduce corneal transparency and endanger the visual quality, we found that the occurrence of RCM did not cause corneal rejection or severe corneal edema. We hypothesize the RCM might block the immune or inflammatory factors in the aqueous humor from attacking the xenotransplant. Thus, RCM formed a barrier to protect the xenotransplants from immune rejection.

We used xenotransplants that were slightly smaller than the allografts in clinical settings because the following advantages: first, the xenografts were thicker than the theoretical value due to the structure and hardness of the PC, which made the implantation process more difficult. Therefore, it was possible to reduce the grafts’ diameter appropriately to reduce surgical operations and protect corneal endothelial cells. Second, recipients could not keep the facing-up position after the surgery, which easily caused complications such as synechia or malignant glaucoma. Therefore, the smaller xenotransplants might help reduce surgery-related complications. In future clinical applications, we should strictly control the thickness of the porcine graft within 150 μm. Moreover, the postoperative position can also be guaranteed, and a larger diameter xenotransplant is possible.

In general, based on the observations obtained over 9 months after WZSP-rhesus monkey xeno-DSAEK, we determined that xenogeneic corneal DSAEK graft could survive for a long period of time and minimize corneal edema while maintaining corneal transparency. Thus, WZSP corneal DSAEK grafts might be used clinically. Furthermore, we predict that RCM and iris anterior synechiae incidence should be relatively low from previous clinical experience, but the postoperative immune rejection action is still the main challenge.
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Statement of Ethics

All procedures performed in this study strictly complied with the ARVO Statement regarding ethics and animal welfare in ophthalmic and visual research. This study was also approved by the Animal Ethics Committee of Institutional Animal Care and Use Committee of NCSED (approval reference no. IACUC-2019-034).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Xu Li, MD: main author of the paper, chief operator of the research, and chief data analyst. Ying Huang, PhD: participated in the research design and participated in the conduct of the study. Qingfeng Liang, MD: participated in the conduct of the study and contributed new reagents or analytic tools. Shutang Feng, PhD: participated in the conduct of the study. Guoping Li, MS: participated in organizing the study and contributed new reagents or analytic tools. Yaowen Song, MD: participated in the conduct of the study. Li Wang, Coll: participated in the conduct of the study. Ying Jie, MD: participated in the conduct of the study. Zhiqing Pan, MD: corresponding author, participated in the conduct of the study, participated in organizing the study, and participated in the writing of the paper.

Data Availability Statement

The data included in this study are not available because the study is ongoing.
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