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 (DSAEK) grafts. **Methods:** Thirty rhesus monkeys were randomly divided into three groups: control group (C) and only Descemet's membrane (DM) stripping, DSAEK 1 (D1) and DSAEK 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 DSAEK surgery, most cor-

neas 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 DSAEK groups were $2,715.83 \pm 516.20/\text{mm}^2$ (D1) and $2,220.00 \pm 565.13/\text{mm}^2$ (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.

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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

Table 1. Donor's number, age, ECD, GT, and the corresponding recipient's number and group

Donor (WZSP)				Recipient (rhesus monkey)		
number ^a	age, months	right (R) or left (L) eye	ECD after pre-cut, cells/mm ²	GT, μ m	number	groups
P01	6	R	2,911	134	MM07	D1
		L	3,459	137	MM13	D2
P02	7	R	3,289	132	MM12	D2
		L	3,245	176	MM10	D1
P03	13	R	3,133	181	MM08	D1
		L	3,285	137	MM11	D2
P04	10	R	3,289	144	MM14	D2
		L	2,925	180	MM15	D2
P05	10	R	3,300	115	MM09	D1
		L	2,901	137	MM06	D1
P06	11	R	3,141	183	MF12	D2
P07	18	R	3,030	187	MF08	D1
		L	3,632	100	MF11	D2
P08	8	L	3,367	181	MF13	D2
P09	7	R	3,759	191	MF14	D2
P10	6	R	4,049	124	MF10	D1
		L	3,217	180	MF06	D1
P11	7	R	3,093	193	MF09	D1
		L	3,278	186	MF07	D1
P12	8	R	3,379	188	MF15	D2

MM, Monkey (male); MF, Monkey (female). ^a P01: WZSP, no. 1.

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 xenopetrating 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 xenoantigens' presence, we tried replacing xeno-PKP with xeno-Descemet'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 post-operative 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 xeno-DSAEK 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

Table 2. Groups and postoperative treatments

Groups	Recipients, N	Surgery	Treatment	
			medication	usage
Control	MM01~MM05 MF01~MF05 (10)	DM stripping	Cravit [®]	Q2d × 2 weeks, then BIW × 2 weeks
			Tobradex [®] (ointment)	
			Diprospan [®] 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	Same as the control group
D2	MM11~MM15 MF11~MF15 (10)	DM stripping and DSAEK	Tobradex [®] (eye drop)	After DSAEK, QID × 15 days and then stop
			Pred Forte [®]	After 15 days, post-DSAEK
			Cravit [®]	QID × 2 months, TID × 4 months, and BID × 3 months
			TALYMUS [®]	Within 3 days, post-DSAEK QID × 2 months, TID × 2 months, and BID × 5 months
			Hialid [®]	TID, if necessary

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); Diprospan[®]: 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).

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 3. Slit-lamp evaluations

Grade	Criteria	Grade	Criteria
<i>Opacity</i>		<i>Conjunctival congestion</i>	
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
<i>Edema</i>		<i>Epithelial defection</i>	
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 stainless than 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
<i>Neovascularization</i>			
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		

Groups

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.

Surgeries 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® 0.5% levofloxacin eye drops (Santen, Osaka, Japan) 3 times a day. We used a 6.25-mm trephine to make an epithelial mark in the cornea center, followed by two corneal incisions before stripping the Descemet's membrane (DM) along with the epithelial mark by a reverse hook. The animals in groups D1 and D2 subsequently received the xeno-DSAEK transplant. The graft's diameter was 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 and held the face-up position for at least 30 min.

Ten days after the surgery, the recipients in group D1 and the control group received betamethasone (Diprosan®; 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, Ja-

pan) 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.

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

Fig. 1. Typical slit-lamp photos at different time points after the surgery. This figure shows 1 case in the control group (MM05), 1 case in group D1 (MF09), and 1 case in group D2 (MF15) 3 days, 14 days, 1 month, 3 months, 6 months, and 9 months after the DSAEK surgery or DM stripping. The cornea became edematous subsequently after DM stripping and sustained edema until 9

months; D1 and D2 received xenotransplants after DM stripping. The corneas became clear in 2 weeks and remained clear. P3D, postoperative 3 days; P14D, postoperative 14 days; P1M, postoperative 1 month; P3M, postoperative 3 months; P6M, postoperative 6 months; P9M, postoperative 9 months.

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 χ^2 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 $159.27 \pm 45.83 \mu\text{m}$ 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 synechiolysis 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

Fig. 2. Imageological examinations of rejected xenotransplant slit-lamp photos. MF10 is a case of reversible rejection in group D1. This case showed RCM and early rejection on P11D. After a more intensive steroid treatment, the cornea returned to transparency. MF13 is a case of late rejection in group D2. This case had a well-attached graft and a completely transparent cornea until 9 months after the DSAEK surgery. Mild inflammatory response occurred on the endothelium without corneal edema. Nine months after the DSAEK surgery, we can observe significant edema and keratoplasties on the OCT image. The LSCM image shows that the endothelial cells of MF13 were well-shaped and preserved 6 months after the DSAEK surgery. However, when the rejection reaction occurred on P9M, inflammatory cells appeared among the corneal endothelium of xenotransplants. P9M, postoperative 9 months.

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 sub-

conjunctival 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 $461.50 \pm 19.74 \mu\text{m}$ before the surgery and increased after the surgery in three groups. The CT of the control group increased to

Fig. 3. Survival curve of the DSAEK groups. Three months post-DSAEK, the group D2 had 1 case of graft failure. Six months post-operatively, the group D1 had 1 case of graft failure. Nine months after the DSAEK, there was 1 case of graft failure in the D2 group. *p* value = 0.457 (log-rank test).

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		<i>p</i> value
	control		control		control		
P14D	3.10±0.74	0.63±0.52	0.67±0.54	0.80	0.00	0.00	0.19
P1M	3.40±0.52	0.62±0.74	0.71±1.11	0.86	0.00	0.00	0.35
P3M	3.11±0.33	0.63±0.74	0.67±0.82	0.92	0.00	0.25±0.71	0.71
P6M	2.80±0.45	0.43±0.54	0.54±0.76	0.54	0.00	0.00	0.87
P9M	2.67±0.50	0.57±0.79	0.69±0.86	0.87	0.00	0.17±0.41	0.19
							0.36

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.

Fig. 4. OCT images of rhesus monkey cornea and xenotransplant at different periods (months). Typical OCT images of the control groups and groups D1 and D2. After DM stripping, the cornea became edematous in the control group. Groups D1 and D2 show clear xenotransplants in a good position. The host's corneas also showed a normal thickness.

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 value = 0.724) and 1 month (p value = 0.642), 3 months (p value = 0.682), 6 months (p value = 0.644), and 9 months (p 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 $3,215.25 \pm 501.92$ cells/mm² and $3,352.86 \pm 273.63$ cells/mm², respectively. Subsequently, the ECDs decreased after the DSAEK surgery. After 9 months, the ECDs of groups D1 and D2 were $2,715.83 \pm 516.20$ cells/mm² and $2,220.00 \pm 565.13$ cells/mm², 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 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).

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. $*p = 0.025$, $**p = 0.011$, $^{\dagger}p = 0.007$, $^{\ddagger}p = 0.047$, $^{\S}p = 0.011$, $^{\S\S}p = 0.023$, $^{\#}p = 0.025$, $^{\#\#}p = 0.034$. 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.

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,

Fig. 6. LSCM images of the rhesus monkey cornea and xenotransplant at different periods (months). Typical vivo LSCM images of all groups. The corneal layer showed edema after DM stripping in the control group. The stromal tissue near the DM became indistinct, and the endothelium could not be seen. Groups D1 and D2 displayed normal monkey's epithelium and superficial stromal structures and xenotransplants: pig original stroma and endothelium and the regions of the grafts and recipient's stroma gap.

37]. 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 endothe-

lial 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.

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	RCM existed persistently. Graft's clarity reduced
			MM09	P6D	None	RCM existed persistently. Graft's clarity reduced
			MF10	P6D	None	RCM began to disappear on P18d. Graft's clarity recovered
	D2	1	MF12	P11D	None	RCM existed persistently. Graft's clarity reduced
Anterior synechiae	D1	2	MM06	P3D	Surgery ^a	Low IOP, AC disappeared, anterior synechiae, and graft failure
			MF08	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. ^a Surgery included synechiolysis, AC habitation, and peripheral iridectomy.

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

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 maintain for a more extended period of time.

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 μ m. 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. Yang Zhang, BS: 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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