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BRIEF COMMUNICATION

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Comprehensive evaluation of decellularized porcine corneal after clinical transplantation

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Abstract

Corneal disease is the second most common blinding disease in the world. The shortage of cornea donors has become the greatest challenge in curing corneal disease. Decellularized porcine corneas have the potential to be clinically applied as a substitute for human cornea in lamellar keratoplasty. Porcine corneas will help relieve the cornea donor shortage. To comprehensively evaluate the characteristics of the grafts and the effect of the decellularized porcine cornea on the host cornea after clinical transplantation, we assessed the microstructure of the transplanted decellularized porcine corneal tissues. Through the analysis of the microstructure of the tissues by H&E staining, TEM and immunofluorescence of anti-human vimentin, anti-pig vimentin,IL-1, IL-2, IL-3, IL-6, IL-8, INF-γ, and TNF-α immunofluorescence staining, we found that despite the slight rejection that occurred, the porcine cornea has good biocompatibility and can provide a scaffold for cell growth. Genetic analysis using Solexa sequencing of the samples showed that decellularized porcine corneas cannot affect genes in patients' corneas. Decellularized porcine corneas are effective biological materials for use in corneal transplantation.

KEYWORDS
cornea, decellularized porcine cornea, transplantation and microstructure

1 | INTRODUCTION

Corneal disease ranks second among blinding diseases in China, with corneal blindness accounting for one-fourth of the blind population. The prevalence rate of corneal disease is 2.49% in China and that of corneal blindness is 0.225%, together representing approximately 3-5.00 million patients.[1] Corneal transplantation remains the main method for visual rehabilitation once the disease has affected corneal clarity. In the USA, more than 40 000 corneal transplantations are performed per year,[2] whereas in China, fewer than 10 000 corneal transplantations are performed each year.[3] The low transplantation rate in China prevents most patients with corneal blindness from receiving treatment. The lack of cornea donors has become the main limitation to potentially curing corneal disease.[4]

Recent advances in the field of tissue engineering have prompted researchers to attempt to create tissue-engineered corneas made of functional biological materials for use in corneal transplantation.[5,6] As the rate of corneal rejection is low due to the lack of blood vessels on the cornea, xenocorneal transplantation is possible. Decellularized porcine corneas, which eliminate the major immunogenic cellular components involved in immune rejection, are promising candidates for reconstructing corneal tissues. These corneas demonstrate physiological and biochemical functional equivalence to normal corneal tissues.[7,8] As scaffolds, porcine corneal extracellular matrices play an increasingly important role in cell attachment, migration, and proliferation during regeneration without inducing inflammatory and immune rejection events.[9,10]

Researchers have developed a method for decellularizing porcine corneas, and they have reported the survival of porcine corneal grafts

in monkeys and rabbits for more than 6 months.[11] The results indicate that decellularized porcine corneas encourage keratocyte ingrowth and show good biological stability when used as a matrix in tissue engineering.[12] Decellularized porcine corneas have been used in human corneal transplantation since 2012.[13] Zhang et al[14] treated fungal corneal ulcers with decellularized porcine corneal stroma and observed patients for 3 years after transplantation; no obvious rejections or other systemic or local side effects were observed. However, the study only verified that there was no obvious impact on the patients based on clinical observation and did not investigate whether decellularized porcine corneas affected the microstructure of the patient or whether mutual fusion occurred. To investigate these possibilities, we evaluated the biological, biochemical, and genetic characteristics of patient-porcine corneal microstructure at 2 months after transplantation.

2 | MATERIAL AND METHODS

This study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. The patient provided written informed consent. All of the procedures were performed following the tenets of the Helsinki Declaration, if applicable.

2.1 | Materials

The decellularized porcine corneas used in our study were prepared by the Acornea Corneal Engineering Co., LTD (Shenzhen, China). Briefly, corneas were harvested from pigs that were certified by the Animal Quarantine Bureau of China. Following the immersion of the corneas in 2M NaCl and deionized water, they were dehydrated in glycerol and sterilized by Co60 irradiation. Following this procedure, we obtained dry corneas that were smaller than native corneas.

A 40-year-old male patient had a 1.5-mm-diameter corneal ulcer caused by bacterial keratitis in the center of his left cornea, and his vision had rapidly decreased. The patient underwent corneal reparation with a decellularized porcine cornea. In short, the anterior lamellae were incised with a 2-mm-diameter trephine and manually dissected until the healthy stromal tissues were exposed. Decellularized porcine corneas (Acornea, Product, 350 μm thick) were adjusted to the same size as the patient's cornea defect. A lamellar graft was placed in the patient's bed and secured (as shown in Figure 1a). After surgery, the patient received steroids, namely prednisone acetate suspension, 4 times per day. Within the 2-month follow-up period, the corneal ulcer transplant turns transparent, and his visual acuity improved from 0.01 to 0.1. The patient's cornea was imaged using AS-OCT (anterior segment optical coherence tomography) (as shown in Figure 1b). The patient's corneal ulcer was in the pupil area (as shown in Figure 1c). Although we treated the corneal ulcer with a decellularized porcine cornea, the improvement in the patient's visual acuity was insufficient. To improve his eyesight, the patient underwent a second deep lamellar keratoplasty using a human donor's cornea. Then, we obtained an 8-mm-diameter patient cornea sample along with the graft for subsequent examination. The harvested tissues were cut into 3 parts (as shown in Figure 1d) for H&E staining, immunofluorescence, TEM, and gene sequencing.

2.2 | In vivo confocal microscopy

The patient's cornea was examined using in vivo confocal microscopy (Heidelberg Engineering, Heidelberg, Germany) to compare the differences between the preoperative corneal ulcer and postoperative the transplanted decellularized porcine cornea. In the normal cornea, polygonal-shaped corneal epithelial cells are closely arranged; linear nerve fibers can be seen in the subepithelial layer; and many fused keratocytes are in the corneal stroma, but no inflammatory cells, which appear as highly reflective white dots, or dendritic Langerhans cells are present.

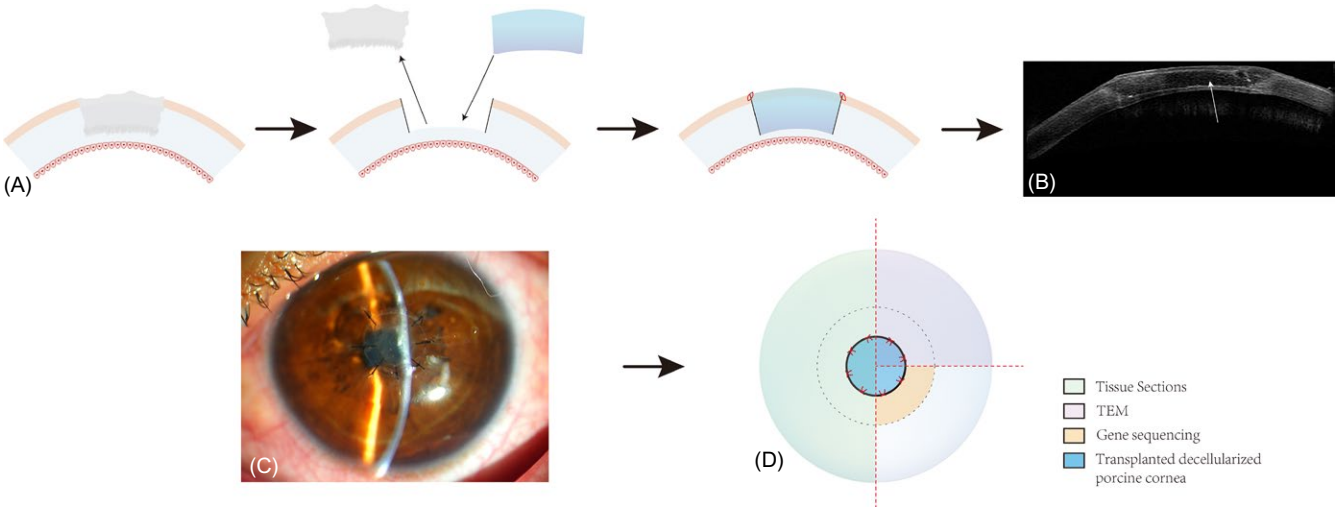

FIGURE 1 Operative process and images of the patient's postoperative the transplanted decellularized porcine cornea. (A) Operative process. (B) An AS-OCT image of the cornea after transplantation of the porcine implant (white arrow). (C) The transplanted decellularized porcine cornea 2 months postoperatively. (D) Corneal tissue segmentation diagram

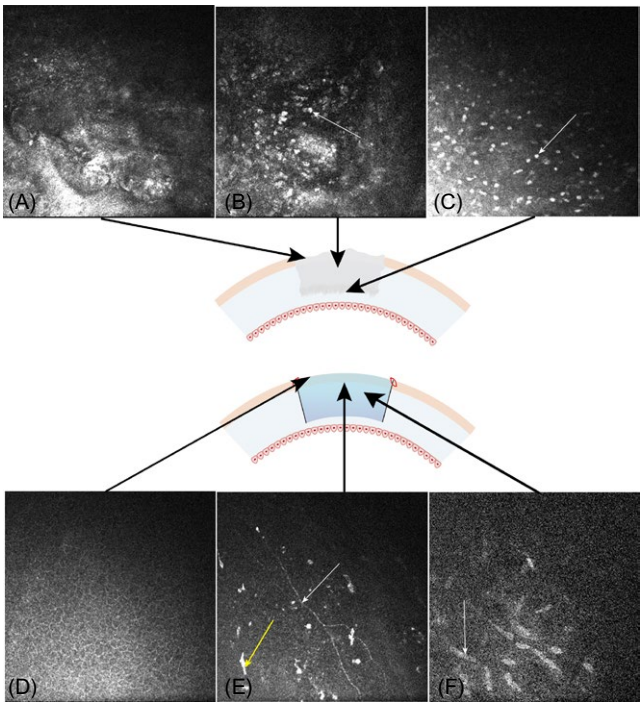

FIGURE 2 Confocal microscopic images of the patient's preoperative cornea and postoperative cornea. (A) Corneal epithelium of the patient preoperation. (B, C) Corneal stromal layer of the patient preoperation. (A, B, C) Corneal lesions were observed, as well as loss of normal structure, and substantial inflammatory cell (white arrow) infiltration preoperation. (D) Polygonal corneal epithelium was observed to grow onto the decellularized graft. (E) Nerve fibers (white arrow) grew into the subepithelial layer postoperation, and Langerhans cells (yellow arrow) were apparent. (F) Fused stroma cells (white arrow) but no inflammatory cells were observed in the decellularized graft. Scale bars: 100 mm

2.3 | Histological examination

The corneal tissues were fixed at 4°C in 4% paraformaldehyde (Keyu, China) overnight, dehydrated, and embedded in paraffin (Keyu, China) wax. Transverse sections (4 μm) were placed on JK-5-coated slides for standard histological staining with H&E. Standard H&E staining was used to evaluate the general microarchitecture of the tissues.

2.4 | Transmission electron microscopy

The sections of the cornea were fixed in glutaraldehyde (Di Ke Chemical Plant, Keyu, China). The dehydrated specimens were embedded in Araldite (HUNTSMAN, Salt Lake City, UT, USA), and the sections were stained with saturated uranyl acetate (Keyu, China) and lead citrate (Keyu, China). The sections were examined using a Philips CM-120 transmission electron microscope (Philips, Amsterdam, the Netherlands).

2.5 | Immunofluorescence

Immunofluorescence was preceded by autoclave antigen retrieval in citrate buffer (pH 6.0). Immunofluorescence staining was performed using monoclonal antibodies anti-human vimentin (Abcam, England, Rabbit), anti-pig vimentin (LSBIo, USA, Mouse), IL-1 (Cloud-Clone, USA, Mouse), IL-2 (Cloud-Clone, USA, Mouse), IL-3 (Abcam, England, Rabbit), IL-6 (Abcam, England, Rabbit), IL-8 (Boster, USA, Rabbit), INF-γ (Cloud-Clone, USA, Mouse), and TNF-α (Cloud-Clone, USA, Mouse). Tissue sections were incubated with secondary antibody Alexa Fluor (Life Technologies, New York, NY, USA) for 30 minutes at room temperature, followed by DAPI (Sigma, San Francisco, CA, USA) nuclear staining. The images were acquired using a CX-21 fluorescence microscope (Olympus, Tokyo, Japan).

2.6 | DNA library preparation and Solexa sequencing

Porcine-patient corneal samples were frozen in liquid nitrogen and then used for DNA extraction with the GenElute™ DNA Kit (Sigma, San Francisco, CA, USA). DNA was treated with RNase A and then purified through the Genomic DNA Clean & Concentrator Kit column (Zymo, USA). The library was constructed using the Covaris S220 System (Applied Biosystems, New York, NY, USA) and PCR. A TruSeq Rapid PE Kit (Illumina, San Diego, CA, USA) was used to cluster the flow cell and library on a HiSeq 2500 system (Illumina). The samples for transcriptome analysis were prepared using Illumina kits following the manufacturer's recommendations. The sequences were then compared with that of Homo sapiens and Sus scrofa genomic sequences were obtained from the gene pool using Picard software (Travis CI, GmbH, Berlin, Germany).

3 | RESULTS

3.1 | In vivo confocal imaging evaluation of graft tissues

In vivo confocal microscopic images indicated serious infection that the preoperative cornea with ulcer as shown was damaged and exhibited loss of normal structure with highly reflective, white-dotted inflammatory cell (white arrow) infiltration in the stroma layer (Figure 2a,b,c). Postoperatively, closely arranged polygonal corneal epithelial cells can be seen grown onto the decellularized graft (Figure 2d). Linear nerve fibers had grown into the subepithelial layer and a few quiescent, dendritic Langerhans cells were apparent (Figure 2e); fused keratocytes had grown into the decellularized graft, but no inflammatory cells were detected (Figure 2f).

3.2 | Histomorphological evaluation: H&E, TEM, and immunofluorescence

Similar to the in vivo confocal findings, the porcine acellular corneal matrix was well integrated with the host cornea and keratocytes had grown into porcine acellular matrices 2 months after transplantation (Figure 3c). Compared with the keratocytes in native cornea (Figure 3b), the keratocytes that had grown into the porcine implant exhibited no significant difference in morphology.

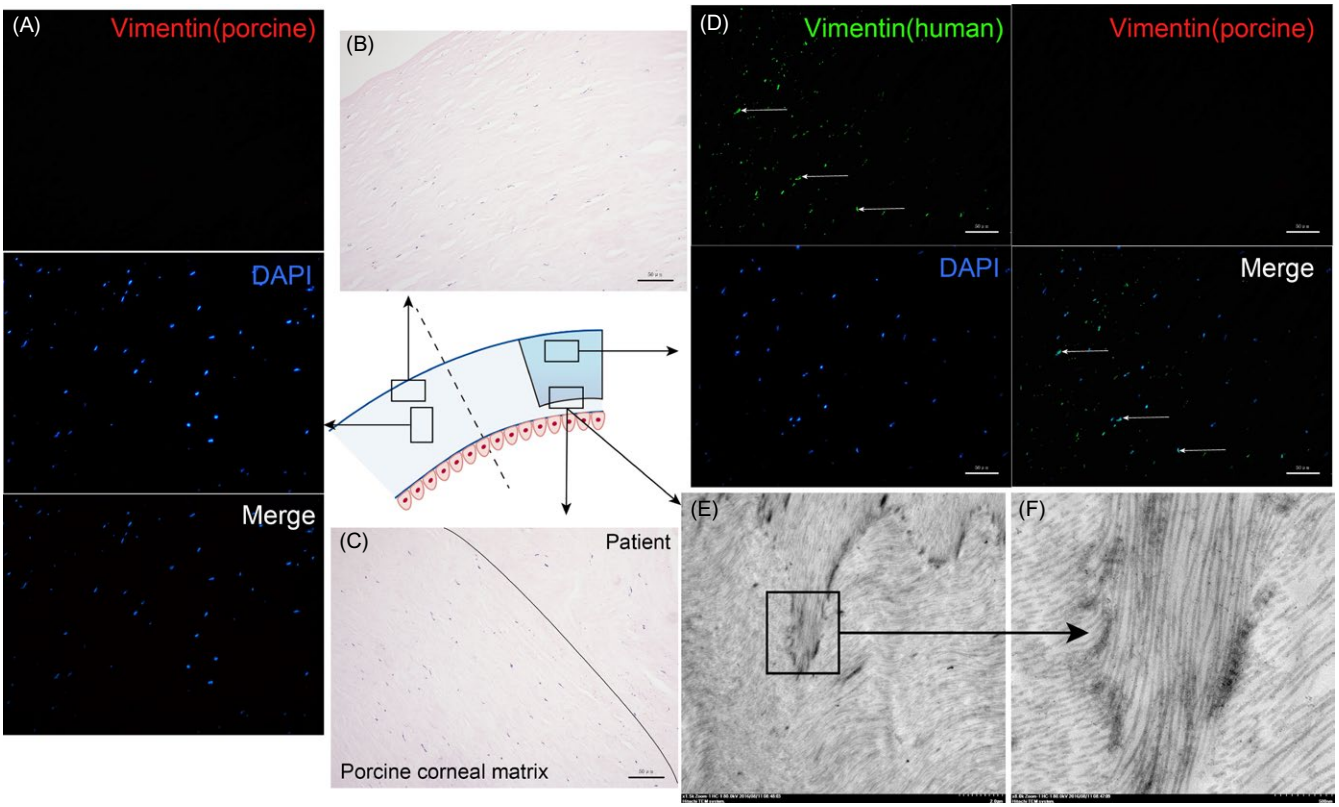

FIGURE 3 H&E staining, TEM, and vimentin immunofluorescent staining of the human-porcine junction of the porcine cornea and the patient's native cornea. (A) Immunofluorescence of human corneal tissue stained with vimentin for a control. Sections stained with porcine vimentin showed negative results (white arrow). (B) The corneal stroma layer and keratocytes in the corneal matrix are shown. Scale bar: 200 μm. (C) The collagen fibers in the patient's cornea were disorganized, with scattered keratocytes. In the porcine corneal portion, the collagen fibers were organized, and many keratocytes grew into them. Scale bar: 200 μm. (D) Immunofluorescence of the transplanted decellularized porcine corneal tissue for vimentin. Sections stained with human vimentin showed positive results (white arrow). Sections stained with porcine vimentin showed negative results. (E) The collagen fibrils were intact, and the morphology of the collagen was maintained. The porcine and patient corneas were integrated. (F) A magnified view of the region outlined in panel E

We also observed the distribution and morphology of collagen fibers in the junction of porcine-patient sections using TEM. The microarchitecture of the decellularized porcine corneal matrix portion had been retained, even though the collagen fibers were disorganized, and the decellularized porcine cornea and patient's cornea collagens exhibited cross-growth and mutual integration (Figure 3e,f).

The transplanted decellularized porcine corneal sections were examined with immunofluorescence to determine the sources and species of cells that had grown into the porcine acellular corneal matrix. Immunofluorescence demonstrated positive constitutive anti-human vimentin staining in the keratocytes of the transplanted decellularized porcine cornea but no anti-pig vimentin staining (Figure 3d). We also checked the anti-pig vimentin antibody in part of the patient's normal cornea for a control, which appeared similar to no staining (Figure 3a).

3.3 | Biocompatibility evaluation: inflammatory response and rejection

Immunofluorescence staining of corneal sections was performed using anti-IL-1, anti-IL-2, anti-IL-8, anti-INF-γ, and anti-TNF-α antibodies. We detected negative fluorescence for IL-1, IL-8, and TNF-α in the corneal implant. The cytokine-stained corneal implant exhibited no or weak red immunofluorescence staining of the corneal stroma surrounding the stromal cells. However, immunereactive IL-2 and INF-γ were significantly increased as indicated by the positive fluorescence, which showed red staining after corneal exposure to anti-IL-2 and anti-INF-γ, antibodies for 24 hours (Figure 4).

3.4 | Genetic evaluation

NGSQC Toolkit 2.3.3 software (National Institute of Plant Genome Research, New Delhi, India) was used to filter low-quality reads and primer dirty reads. High-quality reads accounted for 95.55% of the reads. The clean reads of all the samples were reattached to the reference gene with the use of BWA software, which was used for sequence contrast. Picard software was used for quality control of the sequence results. The statistical results showed that the genes of the samples showed 98.3% similarity to the Homo sapiens genome and only a 1.2% overlap with the Sus scrofa genome (Table 1).

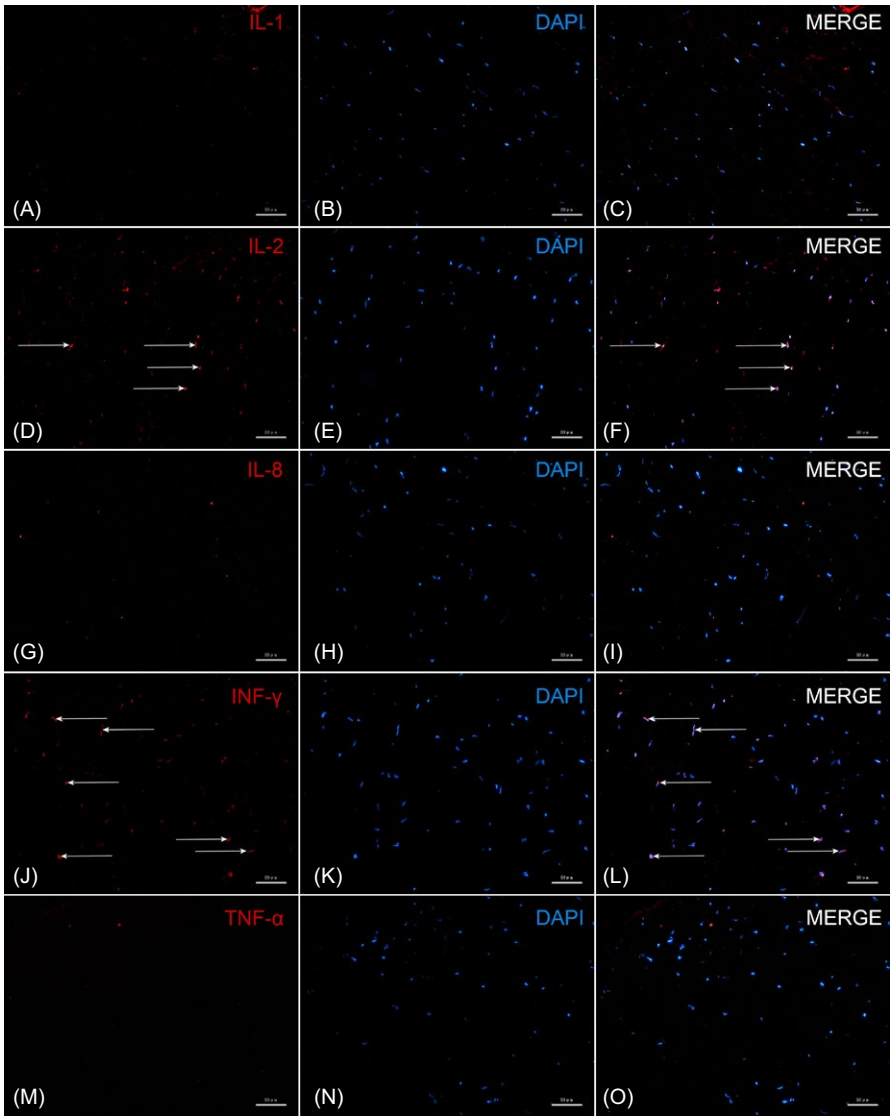

FIGURE 4 Immunofluorescent staining of porcine-patient corneal tissue for IL-1, IL-2, IL-8, INF-γ, and TNF-α. (A) The section showed negative fluorescence for IL-1. (D) The section showed IL-2 expression (white arrow). (G) The section showed negative fluorescence for IL-8. (J) The section showed INF-γ expression (white arrow). (M) The section showed negative fluorescence for TNF-α. (A, G, M) The red staining spots were impurities that had no DAPI staining. (B, E, H, K, N) Nuclei were stained with DAPI. (C, F, I, L, O) Merged images. Scale bar: 50 μm

4 | DISCUSSION

In 2003, Amano et al[15] proposed that porcine corneal stromata might be ideal alternatives to human corneas because of their lower antigenicity, and they used them in animal experiments to heal corneal injury. In another study, Wang et al[16] found that the immunogenicity of the endothelia, epithelia, and stroma was 70.75%, 27.63%, and 1.62%, respectively, by analyzing cellular immunity for the three cell layers of porcine corneas. Cellular components of the cornea are thought to be the main source of antigens responsible for xenograft rejection; therefore, decellularized cornea has been investigated. The decellularized porcine cornea used in the present study was the first clinically tested tissue-engineered cornea product in the world. Lou et al performed H&E staining and TEM and showed that the porcine decellularized cornea was completely extracted and left no cell membrane or nuclear structures within the tissue.[12]

Researchers have performed decellularized porcine cornea transplantation in animal experiments as well as in human beings. However, for transplantation in humans, most of the results are based on clinical observations rather than on examination of the microstructure as performed in our research. In our study, in vivo confocal microscopic images confirmed that there was no obvious inflammatory response after implantation (Figure 2e). H&E staining and TEM results showed that the porcine acellular corneal matrix implant had uniformly thin collagen fibril organization that was similar to the patient's own cornea. The decellularized porcine graft and the patient's cornea were mutually fused, and there were no inflammatory cells in the tissue, indicating good biocompatibility (Figure 3). Decellularized porcine cornea transplantation avoids vision-threatening complications, such as infection, iridoptosis, low intraocular pressure, and retinal detachment, and provides the patient with more time to wait for a donor cornea.

To verify that the cells in the matrix of the decellularized porcine cornea were human corneal stromal cells that had proliferated and migrated from the host, vimentin immunofluorescence was performed. Vimentin is a major structural intermediate filament (type III) protein of mesenchymal cells; keratocytes, the only mesenchymal cells in cornea, maintain corneal transparency. Based on cytomorphological aspects in histology, vimentin was considered a specific marker for

TABLE 1 The results of comparative analysis between the sample and the genome

Sample	Genome	Total reads	Mapped reads	HQ Mapped reads
DNA	*Homo sapiens*	782564516	769260619	98.3%
DNA	*Sus scrofa*	782564516	9390774	1.2%

keratocytes in the cornea. Vimentin is expressed in keratocytes.[17–19] In our study, positive anti-human vimentin staining and negative anti-pig vimentin staining indicated that the cells within the porcine corneal graft were from the host patient. After the cell removal and dehydration process, the decellularized porcine cornea maintained its original structure and physiological features. As a scaffold, decellularized porcine corneal stromata have been confirmed by other researchers to also be suitable for cell attachment, migration, and proliferation.[9] The keratocytes were able to penetrate into the scaffold due to the porosity of the decellularized porcine cornea, which is favorable for the diffusion of nutrients for cell metabolism and the migration of keratocytes into the pores.[20] After transplantation, the tissues were repopulated with human recipient cells.

Although the cornea is an avascular tissue, humoral and cellular immune responses might be involved in corneal xenograft rejection. Recent research suggested the inflammatory cytokines secreted by tissue-based cells, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), can directly adjust the tropism of leukocyte chemotaxis both in vitro and in vivo. Other immunomodulatory factors, such as IL-2, IL-8, INF-γ, and TNF-α, also regulate corneal inflammation and immunity.[21,22] In our study, quiescent Langerhans cells were present as shown by confocal microscopy, although the graft remained transparent 2 months after transplantation (Figure 2d). Moreover, there was positive immunofluorescent staining of IL-2 and TNF-α in the peripheral area of the graft (Figure 4). These two results revealed a non-specific inflammatory-type reaction in the xenograft. Combined with clinical point of view, despite these responses, the decellularized graft was still transparent that showed good survival, suggesting that a partial reduction in the antigen load is beneficial. And after the second surgery, the xenograft did not affect the survival of the second graft which was transparent with no rejection. The patient's visual acuity was improved to 0.8.

However, other animal studies and clinical observations have also showed that decellularized porcine corneas can minimize immune reactions.[12,14]

We also assessed the effect of decellularized xenocorneal transplantation on human genes. We sequenced the patient's corneal tissue near the graft and compared the gene sequence with both the human genome and the pig genome. The results showed 98.3 similarity with *Homo sapiens* over the entire sequence and showed that the porcine decellularized corneal graft had few undesired effects on the recipient (Table 1).

In view of the variable immunologic reactions, a larger sample with long-term follow-up is necessary before solid conclusions can be drawn. Due to the difficulty in obtaining human tissues for testing, we had only one eligible patient from which the valuable tissue 2 months after xenotransplantation could be obtained. Another limitation of our study is that there was no genetic control. As it is impossible to obtain the corneal tissues preoperatively, we compared the gene sequences using gene pool data. Although this method cannot accurately determine the corneal gene changes, it can provide an indication of a trend of genetic change.

In conclusion, the decellularized porcine cornea has the potential to provide a natural microenvironment for host keratocyte migration to regenerate corneal tissue and replace human donor corneas for the treatment of corneal diseases. Our results support that xenotransplantation and use of viable pig organs may continue to function after clinical transplantation.

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