In Vivo Confocal Microscopic Observation of Lamellar Corneal Transplantation in the Rabbit Using Xenogenic Acellular Corneal Scaffolds as a Substitute

Yun Feng, Wei Wang

Department of Ophthalmology, Peking University Third Hospital, Peking University Eye Center, Beijing 100191, China

Abstract

Background: The limiting factor to corneal transplantation is the availability of donors. Research has suggested that xenogenic acellular corneal scaffolds (XACS) may be a possible alternative to transplantation. This study aimed to investigate the viability of performing lamellar corneal transplantation (LCT) in rabbits using canine XACS.

Methods: Fresh dog corneas were decellularized by serial digestion, and LCT was performed on rabbit eyes using xenogeneic decellularized corneal matrix. Cellular and morphological changes were observed by slit-lamp, light, and scanning electron microscopy at 7, 30 and 90 days postoperatively. Immunocytochemical staining for specific markers such as keratin 3, vimentin and MUC5AC, was used to identify cells in the graft.

Results: Decellularized xenogenic corneal matrix remained transparent for about 1-month after LCT. The recipient cells were able to survive and proliferate into the grafts. Three months after transplantation, grafts had merged with host tissue, and graft epithelialization and vascularization had occurred. Corneal nerve fibers were able to grow into the graft in rabbits transplanted with XACS.

Conclusions: Xenogenic acellular corneal scaffolds can maintain the transparency of corneal grafts about 1-month and permit growth of cells and nerve fibers, and is, therefore, a potential substitute or carrier for a replacement cornea.

Key words: Corneal Substitute; Decellularization; Lamellar Corneal Transplantation; Xenogenic Acellular Corneal Scaffolds

INTRODUCTION

Cornea transplantation is the main useful surgery in treating corneal blindness worldwide. More and more scientists are interested in finding a cornea substitute. There is renewed interest in the possibility of using xenogeneic tissues for transplantation. The use of xenogeneic donors may potentially be a way to alleviate the shortage of donor corneas, because of the unique characteristics of the cornea compared with other tissues and organs. Early studies of corneal xenografts indicated that cellular immunity plays a significant role in the development of xenograft failure. The major problem associated with xenotransplantation is the risk from unknown potential pathogens and the rejection of the xenografts.[1-3] The dog or porcine cornea is similar to the human cornea with the advantage of broad supplement and similar ultra-structure. They have been shown to help reconstruct the corneal stroma after transplantation. A significant immune response to the xenogenic antigen occurred, however, after the xenogeneic corneal matrix was implanted into the host tissue. This limited the use of the xenogeneic tissue in clinical trials.[4]

Previous research has indicated that corneal xenografts ordinarily suffer severe rejection, mediated mostly by xenoreactive T-cells.[5-7] Studies of corneal acellular matrice from allogeneic or xenogeneic resources serving as scaffolds for tissue engineering,[8,9] have indicated that the porcine corneal acellular matrix is a promising scaffold for bioengineered cornea due to good three dimension structure, biocompatibility, and mechanical function. No research has been performed using the acellular corneal matrix as a corneal lamellar matrix. In our study, we used the decellularized dog cornea to isolate the matrix alone as the cornea substitute for lamellar transplantation. The aim of our study was to explore the efficacy and limitations of transplanting the decellularized corneal matrix derived from xenogeneic corneas.

Address for correspondence: Dr. Wei Wang,
Department of Ophthalmology, Peking University Third Hospital,
Peking University Eye Center, Beijing 100191, China
E-Mail: puh3_ww@bjmu.edu.cn

Methods

Animals

Experiments were performed on the right eyes of 20 New Zealand white rabbits of either sex, weighing 2–3 kg. Animals were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/kg) and xylazine (5 mg/kg). The rabbits were provided by the Experimental Animal Department of Peking University Health Science Center. Fresh canine eyes were provided by Experimental Department of Peking University First Hospital. Animals involved in this study were cared for and used in accordance with the Association for Research in Vision and Ophthalmology Resolution on Use of Animals in Research.

Decellularization procedure of the donor canine cornea

Bilateral enucleation was performed within 1 h of death. The average time from death to enucleation was 0.6 h for dogs, which were ready to be sacrificed after the clinical operation. Eyes were aseptically removed from their orbits by incising the periorbital connective tissue and muscle, as well as the optic nerve. This incision was extended along the entire limbus. The corneoscleral tissue was placed in 1 mol/L NaOH at 4°C for 2 h and then with 0.1% dispase at 4°C for 16 h. Corneoscleral tissue was then rinsed with balanced salt solution for 30 min to remove any small particles such as those originating from the iris and cell debris. Stromal discs were soaked in 1 mmol/L tris-HCL for 12 h, treated with a 1% Triton X-100 (Sigma, St. Louis, Mo., USA) solution at 4°C for 12 h, digested with 0.25% trypsin (Sigma) at 37°C for 30 min and treated with DNAase (Sigma) and RNAase (Sigma) at 4°C for 16 h to remove hereditary material. Discs were washed twice with 5 mmol/L tris-HCl for 10 min between the treatment steps. Finally, the scaffold material was freeze-dried at 20°C for 8 h, 0°C for 8 h, and 20°C for 4 h, and then sterilized with gamma irradiation. The corneoscleral tissue was stored at 4°C in a sterile pocket with 0.1% gentamycin solution. A random selection of the corneoscleral tissue was fixed for 3 h in 2% glutaraldehyde for microscopic evaluation. The structure and biocompatibility of this tissue were observed by light and electron microscope. Another random selection of the decellularized corneal tissue was cultured for examination of bacterial contamination. The graft became transparent when the decellularized corneal matrix was washed in phosphate-buffer saline (PBS, pH = 7.4) and placed into 90% glycerin for 30 min before surgery.

Physical and mechanical characterization of xenogenic acellular corneal scaffolds

Optical properties

Primary xenogenic acellular corneal scaffolds (XACS) derived from canine tissue were placed in 35-mm culture dishes (Corning, Acton, MA, USA), containing 90% glycerol. XACS were dehydrated for periods of 30 min and 1 h at room temperature (RT). Light transparency and absorption of primary cornea, dehydrated XACS, and the normal canine cornea were measured using a spectral photometer (DR5000; Hach, Loveland, CA, USA).[8,10,11]

Mechanical properties

Xenogenic acellular corneal scaffolds were tested for mechanical strength, expansion and ability to retain water. Mechanical strength was investigated using a precise chest developer (BAT1000; Aikoh, Tokyo, Japan) to elongate the dehydrated XAC matrix. The initial length (L1) and area (A1) of the normal canine cornea and primary XAC and the final length (L2) and area (A2) of the normal canine cornea and dehydrated XAC were measured. The rate of strength was measured using the following formula: (L2–L1)/(A2–A1). The rate of expansion was measured using a counting cup (VC305; Branluebbe, Norderstedt, Germany) with the following equation: (V1–V)/V1 × 100%, where V was the volume after dehydration for 2 h at 65°C, V1 was the volume of the primary condition of the normal dog cornea and XACS. The overall experimental sizes of the specimen areas were 1 cm × 1 cm.[8,10,12,13] The ability of the XACS to retain water was investigated using an electronic scale (AG135; Mettler Toledo, Schwerzenbach, Switzerland) by the following formula: (G2–G1)/G2 × 100%, where G1 was the weight of the initial sample of XAC or the normal dog cornea and G2 was the weight after being dried for 2 h at 65°C.

Hematoxylin and eosin staining and Masson staining

A standard procedure for hematoxylin and eosin staining and Masson staining was performed.

In vivo biocompatibility study: Transplantation of xenogenic acellular corneal scaffolds into the rabbit cornea pocket

Five rabbits were anesthetized with 100 mg/kg ketamine hydrochloride and 5 mg/kg xylazine delivered by intramuscular injection. The topical anesthesia, proparacaine was applied to the eye. Following the protocol described by Xu *et al.*,[8] a slice of decellularized canine corneal stroma was transplanted into the rabbit corneal stromal pockets to determine their biocompatibility. The intrastromal pocket was made with lamellar dissection using a cataract knife. The XACS (0.2 mm thick and with a diameter of 5 mm) was inserted into the pocket, and the incision was closed with three sutures of 10–0 nylon (Alcon, USA).

Technique of corneal transplantation (lamellar transplantation)

Lamellar corneal transplantation (LCT) with XACS was performed. The surgical procedure for LCT involved the use of an 8 mm trephine to score the donor cornea and the donor graft was removed using curved corneal scissors to remove tissue above and immediately adjacent to the deep area of the lamellar. The recipient cornea was similarly scored with a 7.5 mm trephine, and a central 7.5 mm area two-thirds of the depth of the whole cornea was removed. The donor graft was then sutured in place using 12 interrupted sutures of 10–0 nylon. The rabbits were sacrificed, and morphology structures of cornea grafts were studied by light microscopy (LM) and transmission electron microscopy

at 7 days, 1-month and 3 months after the transplantation. Fresh canine cornea group (fixed dose combination) ($n = 4$): The canine corneal lamellar piece was put into the cornea pocket and rabbit cornea group (RT) were used as the control group ($n = 4$), performing the same procedure.

Treatment

No immunosuppressant agents were administered at any time during the study. Antibiotic eye drops were performed daily after operation during the 1st month.

Clinical observation

Grafts were observed every other day for the 1st week and at least twice a week thereafter. A fluorescein sodium solution was used to check the integrity of corneal epithelial cells. Grafts were graded for clarity, inflammation, and vascularization. The clinical signs observed by slit-lamp examination in each group included: (1) Central graft transparency: Grade 0: Cornea complete transparency; Grade 1: Cornea was not transparent, iris was not clear, the pupil was clear to see; Grade 2: Pupil was not clear to see; Grade 3: Pupil cannot be seen; (2) neovascularization on the cornea surface: Grade 0: No neovascularization; Grade 1: New vessels appear on the recipient cornea but no new vessels were seen on the graft; Grade 2: New vessels appear on graft but not in the center of the graft; Grade 3: New vessels on the center of the graft; (3) intraocular inflammation graded by Kp: Grade 1: Several; Grade 2: 1/3 post of cornea Kp with mild flare; Grade 3: 2/3 post of the cornea with severe flare or exudates.

In vivo study after transplantation – Heidelberg Retina Tomograph-II confocal study

In vivo confocal microscopy (Heidelberg Retina Tomograph-II [HRT-II] in combination with the Rostock Cornea Module [RCM]) was performed on each eye, at central and peripheral regions. Anesthetic oxybuprocaine hydrochloride was applied topically into the lower conjunctival fornix of the eye. A coupling medium, carbomer gel (Visidic; Dr. Mann Pharma, Berlin, Germany) was added dropwise. Images were obtained at sufficient resolution using a combination of the HRT-II with the RCM, a computer-controlled hydraulic linear scanning device (Nikon Hydraulic Micromanipulator, Tokyo, Japan) equipped with a water contact objective (Zeiss, 63×/0.95W, 670 nm, ∞/0, Jena, Germany). Examining a total of four sections per corneal epithelium, numbers of cells were counted manually in one visual field (250 μm × 250 μm) per epithelial section using a grid system with a 50-μm grid width and are presented as cells per square millimeter.

Immunofluorescence assessment of corneal markers at 3 months postoperatively

After 3 months postoperatively, the rabbits were sacrificed for the immunohistochemical staining study for corneal epithelial marker keratin 3 (K3), corneal keratocyte marker vimentin and conjunctival epithelial marker MUC5AC. Corneal tissues were frozen in an optimal cutting temperature (OCT; Sakura Finetek, Torrance, CA,

USA) compound and sectioned with a cryostat. Sections were blocked with 2% bovine serum albumin in PBS, and primary antibodies, and fluorescein conjugated-goat and anti-mouse IgG secondary antibodies (1:100 dilution) were applied overnight at 4°C in a moist chamber. The primary antibodies used were anti-K3 (1:100 dilution; Chemicon, Temecula, CA, USA) and anti-vimentin (1:100 dilution; Sigma). Negative controls were prepared by incubation with only the secondary antibodies. Fluorescently labeled cells and tissues were visualized using a confocal laser microscope (LSM 510; Zeiss, Oberkochen, Germany) and epifluorescence and LM were used to obtain corresponding differential interference contrast images. Nuclei were counterstained with Hoechst dye.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 for windows (SPSS Inc., Chicago, IL, USA). Data were shown as mean ± standard deviation (SD). A $P < 0.05$ was considered as statistically significant.

RESULTS

Physical and mechanical characterization of xenogenic acellular corneal scaffolds

Xenogenic acellular corneal scaffolds had similar physical and mechanical characteristics to normal canine corneal tissue. As shown in Figure 1, the XAC matrix was opaque/ivory white because the corneal tissue was significantly swollen after the decellularization procedure. The XAC matrix curvature, however, remained similar to that of a normal cornea. The matrix properties included elasticity as well as tenacity. Comparison in terms of strength, expansion, and ratio of water content in initial XACS with dehydrated XACS and normal canine cornea showed no statistical significance of the differences ($P > 0.05$), while the ratio of light transparency of initial XACS compared to other groups showed a statistical significance of the differences (all $P < 0.05$). The mechanical strength, rate of expansion and ability to retain water of the XAC were similar to that of a normal dog cornea ($P > 0.05$) [Table 1].

Histological characterization of xenogenic acellular corneal scaffolds

Hematoxylin and eosin staining found [Figure1a-1d] no blue-stain cell nucleus or cell debris in the matrix and no microbial contamination was observed [Figure 1e and 1f]. Collagens were arranged in a loose formation that was similar to the normal corneal matrix fiber [Figure 2].

Biocompatibility tests of xenogenic acellular corneal scaffolds

Slit-lamp observation was performed 1-week, 1-month and 3 months after surgery on eight rabbits [Figure 3]. Fresh canine corneal stroma was implanted as a control in four rabbit eyes. Although a mild haze was initially observed, none of the XACS implants showed any inflammation reactions, signs of rejection or neovascular invasion over this period. Haze became severe in the fresh canine cornea

Figure 1: (a) Xenogenic acellular corneal scaffolds (XACS) was ivory white after the decellularized procedure; (b) Plastic cup as the control; (c) XACS was turned to be transparent after the dehydration, the under character could be seen clearly; (d) XACS became swellen after 1 h rehydration; (e-f) H and E staining of XACS (original magnification, ×10), no blue-stain cell nucleus or cell debris was found in the matrix for H and E staining by LM. The collagens were lined up in a loose formation but similar to the normal cornea matrix fiber; (e) Surface is the original corneal epithelium layer. (f) Surface is the original corneal endothelium layner; (g-h) Masson staining of XACS (original magnification, ×20).

Table 1: Physical and mechanical characterization of dog acellular corneal scaffold (mean ± SD)

Items	XACS (initial)	XACS (dehydrated 30 min)	XACS (dehydrated 1h)	Normal canine cornea
Strength	3.12 ± 0.23	3.28 ± 0.75	3.35 ± 0.84	3.52 ± 0.11
Expansion	86.36 ± 2.13	78.74 ± 0.83	73.61 ± 1.03	75.22 ± 0.89
Ratio of water content	87.46 ± 0.43	75.68 ± 0.31	66.21 ± 0.42	72.36 ± 0.41
Ratio of light transparency	1.7 ± 0.02	45.23 ± 0.23	49.43 ± 0.21	56.25 ± 0.37

SD: Standard deviation; XACS: Xenogenic acellular corneal scaffolds.

group at 1-month and neovascularization appeared at 3 months [Figure 3].

Clinical observation after lamellar corneal transplantation

After the lamellar cornea transplantation with dehydrated XACS, all rabbits were alive with normal activity and normal feeding habits. Grafts did not melt or shed during the observation period. XACS grafts remained transparent for 1-month (28.15 ± 4.24 days) using slit lam microscope [Figure 4]. Neovascularization appeared 4–5 weeks after LCT. Immunochemistry study of XACS group at different postoperative period were observed: K3 positive staining cells in the graft's surface and vimentin positive cells in the graft stroma were noted, while there were all MUC5AC negative [Figure 5].

Confocal study in xenogenic acellular corneal scaffolds transplantation *in vivo*

Corneal epithelial cells were found in 6 of 8 rabbit eyes (75%) 14 days postoperatively. Keratocyte-like cells were found in the grafts in 5 of 8 rabbit eyes 7 days after the operation and were noted in all 8 rabbit eyes 14 days postoperatively. Nerve fiber structures were not detected in the graft 1-week postoperatively. Small nerve fibers were observed in the graft interface 1-month postoperatively [Figure 6a and 6b]. The majority of new nerve fibers were single branches. Abnormal regeneration of stromal nerves was observed in 3 rabbit eyes 90 days postoperatively. The stromal nerves showed prolific regeneration after a coiled course with an abnormal branching pattern. Nerve fibers within the graft were twisted in shape and formed smaller branches, and there were fewer nerve fibers formed than the normal cornea [Figure 6c-6p].

Discussion

Corneal tissue has unique mechanical properties, cell and tissue organization. Several studies have been performed using acellular matrix as a scaffold in tissue engineering because it possesses fine biocompatibility and can be easily prepared with sufficient resources.[14-20] The acellular matrices were made by biological or chemical methods to remove the cellular elements and maintain the normal extracellular matrix (ECM) structure.[14-16] There are also some studies of corneal acellular matrice from allogeneic or xenogeneic resources serving as scaffolds for tissue engineering.[8,18-20] Recently a research has been conducted showing that decellularized corneas could be useful as a corneal scaffold for tissue regeneration.[18-20]
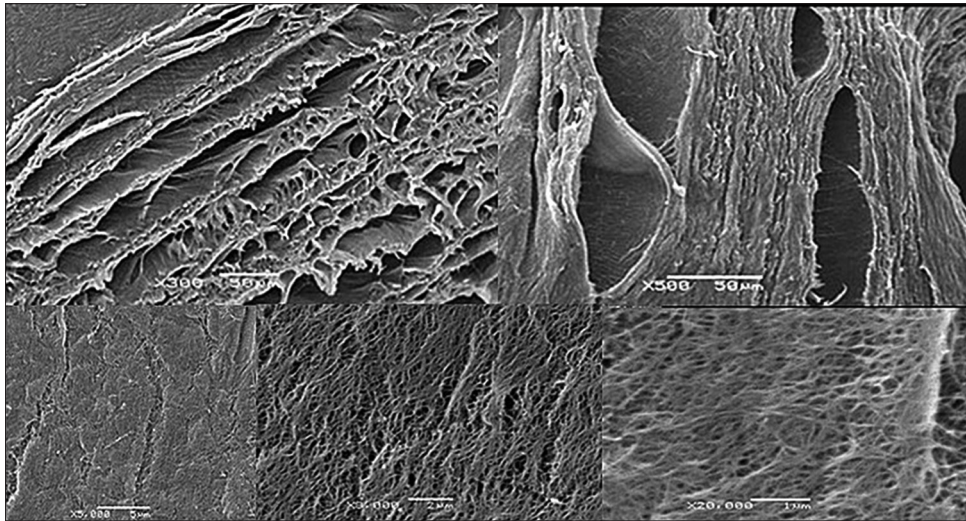
Figure 2: The results of scanning electron microscopic examination showed that the collagen fiber diameter similar to normal corneal fiber and inter-connected to network, formed collagen bundle regular and parallel to the corneal surface.
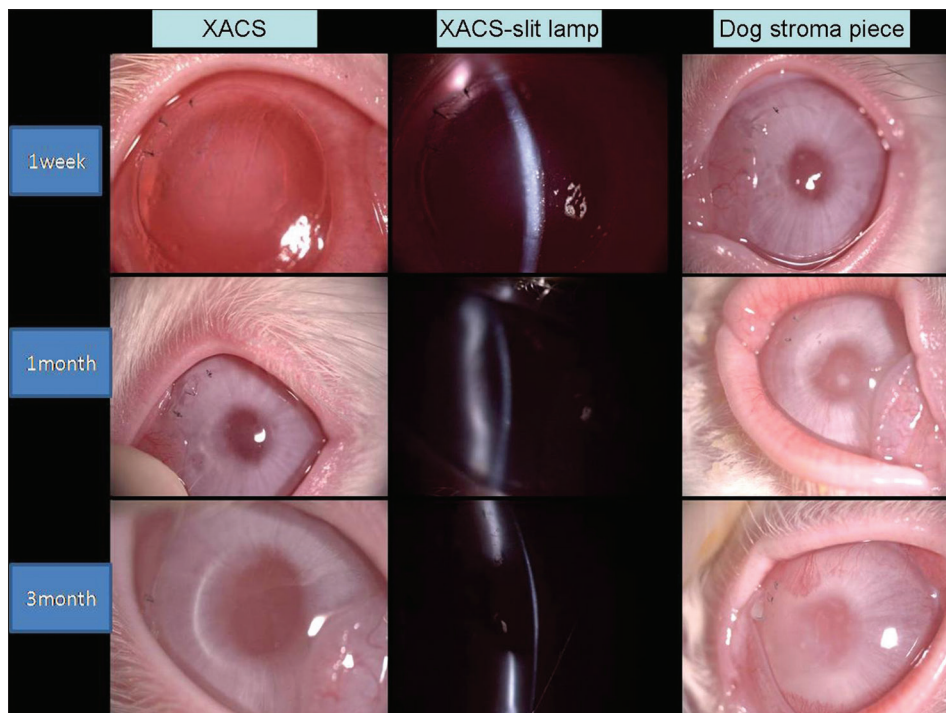

Figure 3: Investigate the biocompatibility of xenogenic acellular corneal scaffolds (XACS) for rabbit cornea. The XACS was inserted into a rabbit corneal stromal pocket and observed at 1 week, 1 month and 3 months of postsurgical period. The cornea showed a mild haze at 1 week, optically clear slit-lamp images at 1 month and 3 months. The fresh dog cornea stroma is presented in the left column as a control, which is similar to XACS group at 1-week. Haze became severe in fixed dose combination group at 1 month and neovascularization appeared at 3 months.

Research has not been performed; however, using XACS as the lamellar transplantation substitute. The majority of previous research has demonstrated that XACS implanted into a pocket of the cornea and displayed good biocompatibility,[8,18-20] but as a bioengineered cornea, this matrix cannot be restricted to the lamellar pocket.

We demonstrated that XACS can be processed using techniques that can maintain the unique ECM structure. In this study, treatments were selected which were compatible with corneal tissue. According to this experiment, the XACS used in rabbits for lamellar keratoplasty can maintain transparency for approximately 4 weeks, recipient cells can grow into XACS and regenerated nerve fiber can also grow into these matrices. New vessels appeared about 4–5 weeks postoperatively; superficial new vessels on the ocular surface may be due to the long-term corneal epithelium defect and suture stimulation. The XACS graft was completely merged into the host cornea 3 months after LCT with epithelialization and neovascularization. Those results showed that the XACS may possibly be a new corneal replacement material, which
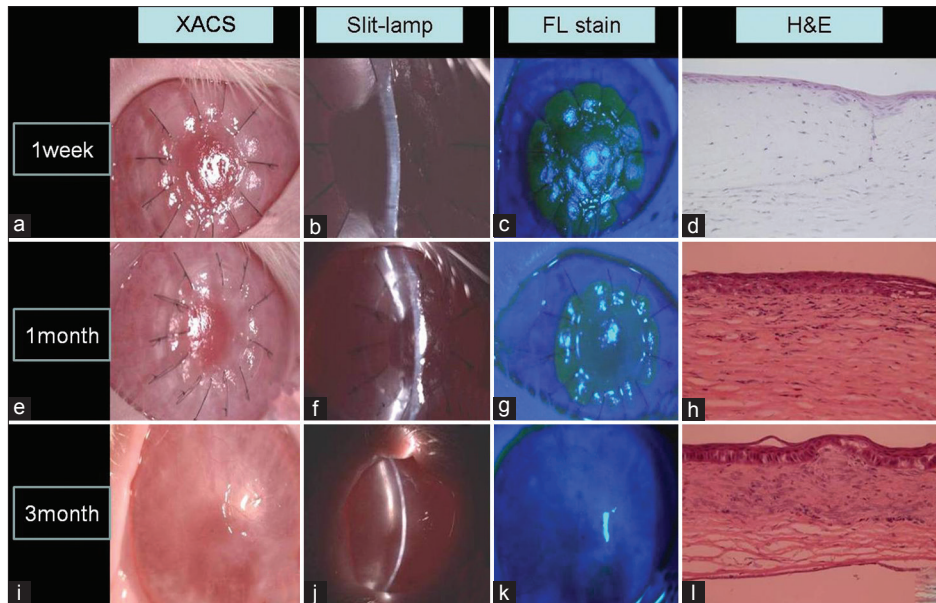
Figure 4: Clinical observation: investigate xenogenic acellular corneal scaffolds (XACS) as a lamellar cornea substitute in rabbit. Corneal grafts following the lamellar corneal transplantation using XACS were observed by slit-lamp microscopy with or without fluorescein staining at 1 week (a-c), 1 month (e-g) and 3 months (i-k) after operation, respectively. Morphology of the rabbit cornea in different time points after the transplantation (Hematoxylin and eosin staining, ×20) (d, h, l): (d) A thin layer of corneal epithelium cells were noted. Few spindle-like cells in matrix were seen; (h) 1 month after operation. Cobble stone-like cells were located at the surface of the cornea; (l) 3 months after the operation, new vessels were noted in the corneal matrix in graft.
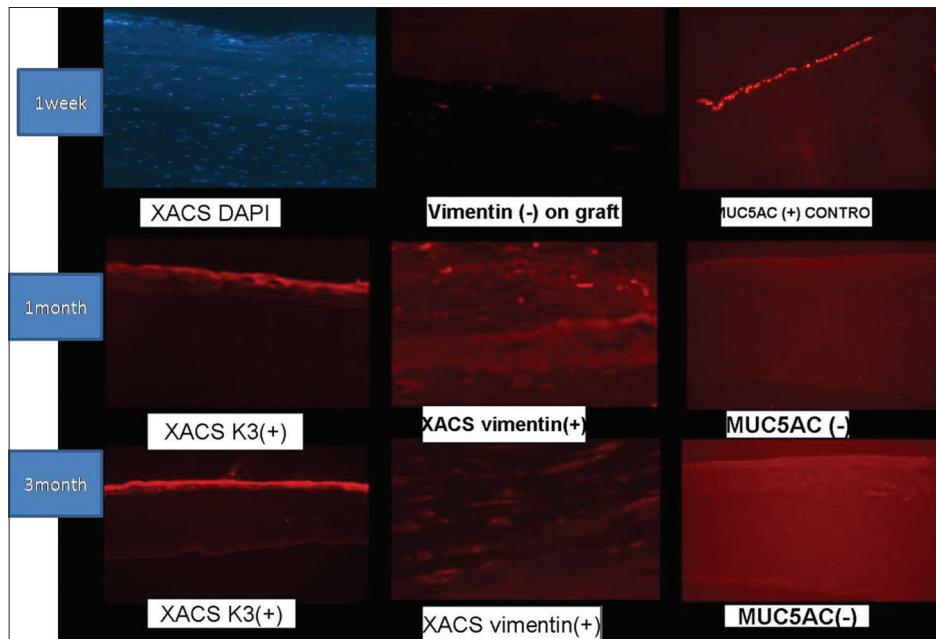

Figure 5: Immunochemistry study of xenogenic acellular corneal scaffolds group at different postoperative period: Keratin 3 positive staining cells were noted in the graft surface and vimentin positive cells were observed in the graft stroma. All epithelium cells were observed MUC5AC negative (original magnification, ×10).

can be directly used in the short term. The emergence of new vessels, after a 5-week transplantation period, however, the emergence of new vessels also creates a restrictive effect on the prospects for the use of this matrix.

Biocompatibility is the most important thing to achieve clinical application for a bioengineered cornea substitute. In our previous study, we placed the decellularized cornea matrix into the lamellar cornea pocket and noticed that the graft was well merged into the host cornea and remained transparent.[8] The reason for this phenomenon was because the immunogenicity of cornea matrix is the lowest in all three layers of the cornea. It was only 1.62% in total immunogenicity of the cornea. After the decellularized procedure, the immunogenicity became lower.[17-18]
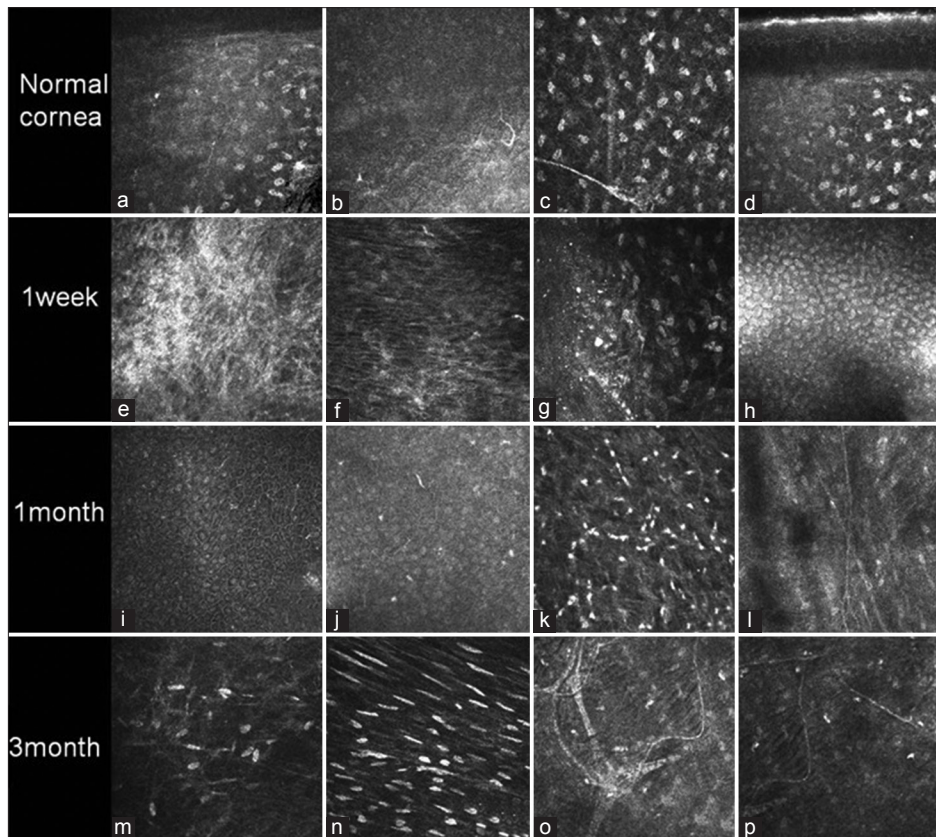
Figure 6: *In vivo* Heidelberg Retina Tomograph-II confocal microscopy in normal rabbit cornea (a-d): (a) Subepithelium nerve fiber; (b) Langhan's cell with high reflection; (c) Cornea nerve fiber in the corneal stroma; (d) The epithelium layer and anterior stroma layer. One week postoperatively in xenogenic acellular corneal scaffolds (XACS) group (e-h): (e) No cells were detected in the anterior stroma, which was in a loose network formation; (f) Few long spindle like cells were in the posterior of the stroma of the graft; (g) There was no space between the interface of the XACS graft and the recipient rabbit cornea. Some high reflection dots were detected in the graft; (h) The endothelium layer was similar to the normal rabbit cornea. One month postoperatively in XACS group (i-l): (i) Corneal epithelial cells on the graft; (j) Activated langhan cells in the subepithelial layer without any corneal nerve detected; (k) High reflex cells with small nuclei in the anterior part of the graft, especially around the suture area; (l) Regenerated corneal nerve fiber grew into the graft in anterior stroma. Cells were less than the normal cornea. Three months postoperatively in XACS group (m-p): (m) Spindle-like cells were detected in the posterior part of the graft; (n) Cells in the anterior part of the graft in a parallel formation; (o) Tortuous corneal nerve in the graft matrix; (p) Tortuous corneal nerve.

Our study showed that the structure of the xenogeneic cornea matrix was similar to that of a normal human cornea after the decellularization procedure. The graft maintained transparency after dehydration and after LCT (>4 weeks) in identical clinical conditions as a corneal graft in allograft transplantation. The XACS is a very promising xenogeneic corneal matrix for clinical applications, especially in some emergency conditions such as corneal perforation due to ulcer when the availability of donor corneas is limited. Recipient cornea cells could migrate into the graft matrix, where they could remain viable and proliferate. The transitional keratocyte and epithelium cells could be seen in the graft 1-week after operation.

According to current perspectives concerning the xenotransplantation, we cannot completely rule out the possibility of immune rejection reactions. It has been reported that the majority of patients treated with bovine collagen do not suffer from adverse immune responses, but in a small number of patients' immune responses do occur.[9] According to our previously study, the XACS put in the innercapsular cornea, there were no inflammatory cells in the decellularized corneal graft.[8] In the XACS lamellar transplant group, however, we detected inflammatory cells around the graft and the suture area for 1-week after transplantation. Inflammatory was not detected in the graft. When the suture was removed after 1 and 3 months, numbers of inflammatory cells were not significant. So, we suspected that the presence of inflammatory cells may be related to suture stimulation.

According to our experiments, 40 days after transplantation, all corneas in the XACS group presented different degrees of angiogenesis. At the latter stage in the XACS group, corneal neovascularization clearly formed on the surface of the cornea. This may be due to rejection induced by heterogeneous collagen. So, we believed that the main reasons associated with the neovascularization may be as the following reasons: (1) The stimulation of the suture in the cornea, as new vessels began to regress when sutures were removed and fewer new vessels were observed after early removal of sutures than late removal; (2) the epithelium

defected persistently.

In vivo confocal microscopy was used to study the structure of the cornea. By *in vivo* confocal microscopy, we found that regenerated nerve fibers can grow into the graft, which indicated that the XACS had a favorable biocompatibility with the host. Besides, the XACS also showed a promoted effect on nerve regeneration. Those results showed that the XACS can possibly become a new corneal replacement material that could be used directly in the short term. However, after transplantation for 5-week, the emergence of new vessels restricted its use clinically. The possibility of the hetergenous collagen or the other antigens remains unclear after the decellurized procedure.

The reason for corneal noevascularization which appeared at the latter stage of transplantation was not further explored in this study, it may be related to stimulation of suture, continuous epithelial defection or the hetergenous collagen rejection. Application of decellularization protocols to corneal tissue provides a gateway toward developing new materials for tissue engineering applications, including, but not limited to, corneal reconstruction. At the same time, our work indicated that there is still a long way for the xenotransplantation in clinical application. Further researches should be explored and urgent in the future.

In conclusion, we have detected that XACS can maintain the transparency of corneal grafts over a period and recipient cells and nerves could grow into the grafts, which indicated that these matrices may be used candidate substitutes for the cornea. Corneal neovascularization that appeared at the latter stage of transplantation indicated that the further immunological study should be explored for the decellurized xenogic corneas.

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