·Basic Research·

Acellular porcine corneal matrix as a carrier scaffold for cultivating human corneal epithelial cells and fibroblasts *in vitro*

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Abstract

• **AIM: To investigate the feasibility of corneal anterior lamellar reconstruction with human corneal epithelial cells and fibroblasts, and an acellular porcine cornea matrix (APCM) *in vitro*.**

• **METHODS: The scaffold was prepared from fresh porcine corneas which were treated with 0.5% sodium dodecyl sulfate (SDS) solution and the complete removal of corneal cells was confirmed by hematoxylin-eosin (HE) staining and 4′, 6-diamidino-2-phenylindole (DAPI) staining. Human corneal fibroblasts and epithelial cells were cultured with leaching liquid extracted from APCM, and then cell proliferative ability was evaluated by MTT assay. To construct a human corneal anterior lamellar replacement, corneal fibroblasts were injected into the APCM and cultured for 3d, followed by culturing corneal epithelial cells on the stroma construction surface for another 10d. The corneal replacement was analyzed by HE staining, and immunofluorescence staining.**

• **RESULTS: Histological examination indicated that there were no cells in the APCM by HE staining, and DAPI staining did not detect any residual DNA. The leaching liquid from APCM had little influence on the proliferation ability of human corneal fibroblasts and epithelial cells. At 10d, a continuous 3 to 5 layers of human corneal epithelial cells covering the surface of the APCM was observed, and the injected corneal fibroblasts distributed within the scaffold. The phenotype of the construction was similar to normal human corneas, with high expression of cytokeratin 12 in the epithelial cell layer and high expression of Vimentin in the stroma.**

• **CONCLUSION: Corneal anterior lamellar replacement can be reconstructed *in vitro* by cultivating human corneal epithelial cells and fibroblasts with an acellular porcine cornea matrix. This laid the foundation for the further transplantation *in viva*.**

• **KEYWORDS:** corneal epithelial cells; corneal keratocytes; acellular porcine cornea matrix; corneal tissue engineering; limbal epithelial cells
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INTRODUCTION

Once corneal tissue is damaged by trauma, inflammation, or degeneration, the cornea loses its normal function, as the major element in the optical pathway of the eye and as a physical barrier to the outside environment, leading to visual impairment and even blindness. Currently, more than 10 million individuals worldwide suffer from corneal blindness, and corneal blindness ranks second in eye diseases that cause blindness[1-2]. Corneal transplantation, using human donor tissue, is the only effective treatment for corneal blindness. However, a severe shortage of corneal donors because of an increased use of corrective laser surgery and high risk for rejection after transplantation has led to the lack of timely and effective treatment for some of patients[3-4]. Therefore, efforts have been made to fabricate biological human corneal equivalents by employing tissue engineering principles to overcome the present disadvantages of allografts. While partial thickness lamellar keratoplasties have been attempted in animal models and even in human clinical trials, no clinically viable full-thickness human corneal equivalents have been produced by tissue engineering methods [5-6]. The construction of tissue engineering of the cornea is a promising opportunity for the treatment of corneal blindness. Many researchers have constructed full thickness corneal substitutes similar to the native cornea by using natural or synthetic polymers with corneal cells, but these corneal replacements have been limited to *in vitro* applications[7-9].

An ideal scaffold for a tissue-engineered cornea should have good biocompatibility, high optical clarity, toughness to withstand surgical procedures, and non-immunogenicitic properties [10]. Three-dimensional scaffolds for corneal equivalents have most often been fabricated from collagen and fibrin [10-16]. Porcine collagen is well tolerated after heterogeneous implantation, and shares similar anatomic characteristics with the human cornea [17-20]. We had showed that an acellular porcine cornea matrix (APCM) which was developed by 0.5% sodium dodecyl sulfate (SDS) could preserve the key features of the cornea, including the intact basement membrane, and support the growth of rabbit corneal epithelial cells and fibroblasts or human embryonic stem cells-limbal stem cells (hESCs-LSCs) *in vitro* [21-25].

Our present study for the first time explored that human corneal epithelial cells and fibroblasts could be cultured with the same APCM *in vitro* to construct a corneal anterior lamellar replacement for further human clinical trials such as lamellar keratoplasty (LKP).

MATERIALS AND METHODS

Corneal Cell Culture Limbal epithelial cells were obtained from human donor limbal rims which were not adequate for transplantation. Excessive sclera, iris, corneal endothelium, conjunctiva, and Tenon's capsule was mechanically separated from the corneal stroma. The remaining tissue specimens were incubated in 2.4 U/mL dispase II (Roche, Basel, Switzerland) for 1h at 37℃, followed by mechanical separation of the corneal epithelium from the underlying stroma under a dissecting microscope using two fine forceps, and further digestion with 0.25% trypsin/0.02% EDTA (Sigma, St. Louis., MO, USA) at 37℃ for 5min to isolate single cells. Then the corneal epithelium cells were incubated at 37℃ under 5% CO_2 and fed with fresh DMEM/F12 medium containing 10% fetal bovine serum every other day. To obtain corneal fibroblasts, the corneal stroma deprived of epithelial and endothelial cells was cut into small pieces, and 2×2-mm^2 explants were digested by 0.5 mg/mL collagenase I at 37℃ for 3h to isolate single cells, and then incubated with fresh DMEM/F12 medium containing 10% fetal bovine serum.

Preparation of the Scaffold Before the decellularization process, fresh porcine corneas were washed 3 times in sterile phosphate buffered saline (PBS; Beyotime, Nantong, Jiangsu Province, China), and then trimmed to 10 mm diameter sections with a pair of curved scissors. Porcine corneas were subsequently incubated in 0.5% (wt/vol) SDS at 4℃ with agitation for 24h. Then the APCM was washed 10 times in sterile PBS supplemented with 200 U/mL penicillin and 200 U/mL streptomycin for 24h. Finally, the scaffolds were stored at -20℃ before use. All steps were conducted under sterile conditions.

Cytotoxicity Assay of the Acellular Porcine Cornea Matrix To determine whether the extracts from the APCM would cause cytotoxicity, each scaffold (10 mm diameter) was extracted using 3 mL 1:1 mixture of Dulbecco's minimal essential medium and Ham's F12 medium containing 10% fetal bovine serum at 37℃ for 48h. Human corneal fibroblasts were seeded into each well of 96-well plates and cultured with the leaching liquid extracted from the APCM (the experimental group, n =6) or the normal medium (control group, n=6), respectively. So were human epithelial cells. The proliferation activity of the cells was quantitatively determined at 1, 3, 5, and 7d by a MTT assay. The optical density (OD) value of absorbance at 490 nm was measured by a microplate reader (InTec Reader 2010, USA). Differences in the OD value between experimental and control groups were statistically analyzed using t-test. All experiments were repeated at least 6 times. Data are reported as mean± standard deviation. Statistical significance was set at P<0.05. All statistical analyses involved use of SPSS 19.0.

Construction of Corneal Anterior Lamellar Replacement with the Acellular Porcine Cornea Matrix The APCM was soaked in culture medium at 37℃ for 24h before cell seeding. Cultured human corneal fibroblasts from passage 3 were trypsinized and re-suspended at a final concentration of 5×10^5 cells/mL. Parallel to the surface of the scaffold, 1 mL of cell suspension was gently injected into the prepared APCM at eight different sectors using a 1 mL insulin syringe, and cultured for 3d. After 3d, cultured human corneal epithelial cells from passage 1 for each scaffold (5×10^3/ mm^2) were gently seeded onto the surface of the reconstructed stroma, and cultured for anther 10d. All constructions were cultured at 37℃ in a humidified atmosphere containing 5% CO_2. The culture medium was exchanged every two days.

Microscopy and Histology For light microscopy, APCMs and constructed corneal anterior lamellas were fixed in 4% formaldehyde, and embedded in paraffin. Sections of 5 μm were stained with HE staining, and viewed under a light microscope. For transmission electron microscopy (TEM), dehydrated APCMs were embedded in propylene araldite and sections stained with saturated uranyl acetate and reynolds lead citrate. Sections were examined using a TEM (Phillips CM10, Munich, Germany).

Immunofluorescent Staining Immunofluorescent staining was performed as in a previously reported method. In brief, the rabbit monoclonal antibody for anti-cytokeratin 12 (CK12; Abcam, San Francisco, CA, USA), a marker of corneal epithelial cells, and anti-vimentin (Abcam, San Francisco, CA, USA), a marker of corneal fibroblasts, were placed on the constructed corneal replacement and normal human anterior cornea, and incubated at 4℃ overnight (PBS

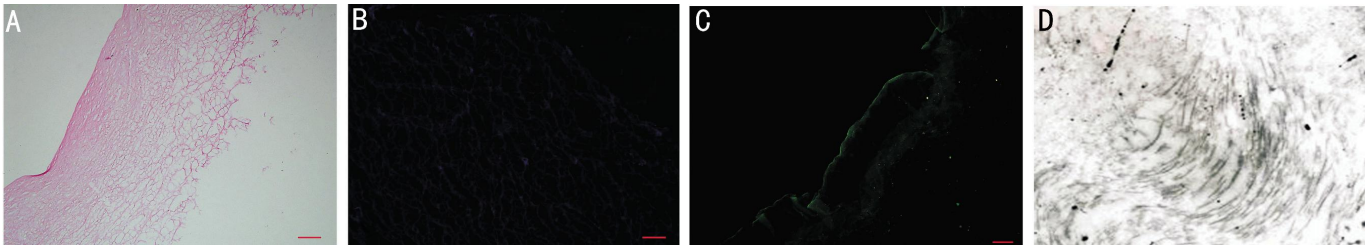

Figure 1 Histological examination of the acellular porcine cornea matrix A: There were no cells in the APCM by HE staining; B: DAPI staining did not detect any residual DNA within the APCM; C: Immunofluorescent staining showed laminin was abundantly present; D: The transmission electron microscopy examination showed that the collagen fibrils were regularly arranged. A, B: Scar bar: 100 μm; C: Scar bar: 50 μm.

as negative control), respectively. The final working concentrations for anti-CK12 or anti-vimentin were 1:50 and 1:100 dilutions, respectively. The secondary antibody, fluorescein-conjugated affinipure goat anti-rabbit IgG (Zhongshan Goldbridge Biotechnology Co., Ltd., Beijing, China), was applied for 1h in a dark incubation chamber at room temperature. After washing in PBS, the all specimens were mounted with 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining and examined under a fluorescent microscope. Moreover, immunofluorescent staining for laminin (LN) was be performed in the APCM.

RESULTS

Characterization of the Acellular Porcine Cornea Matrix Histological examination indicated that there were no cells in the APCM by HE staining (Figure 1A) and DAPI staining did not detect any residual DNA within the APCM (Figure 1B). Immunofluorescent staining showed LN was abundantly present, one of basement membrane (BM) components (Figure 1C). The TEM examination showed that cell components were removed and the collagen fibrils were regularly arranged (Figure 1D). The APCM was opaque due to the decellularized process (Figure 2A), and transparency was restored when soaked in 100% sterile glycerol for 5min (Figure 2B).

Cytotoxicity Assay of the Acellular Porcine Cornea Matrix There were no significant differences in the proliferation of human corneal epithelial cells between the experimental and control groups (0.6375±0.43851 *vs* 0.6375± 0.43805, P>0.05). And there were no significant differences in the proliferation of human corneal fibroblasts between the experimental and control groups (0.4575±0.32745 *vs* 0.4638± 0.33345, P>0.05) (Figure 3). The results suggested that the APCMs did not retard cell proliferation and had no cytotoxic effects.

Human Corneal Cells Morphology with the Acellular Porcine Cornea Matrix The seeded human corneal epithelial cells adhered to the APCM surface within 2h. HE staining showed that corneal epithelial cells formed a mono-layer covering the surface of the APCM at 3d (Figure 4A1, 4A2), formed closely arranged 2 or 3 layers of cells at

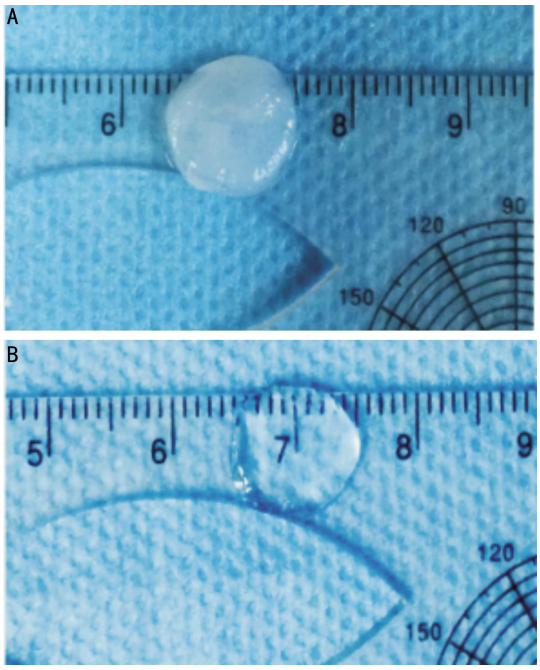

Figure 2 Transparency of the acellular porcine cornea matrix A: The APCM was opaque due to the decellularized process; B: Transparency of the APCM was restored when soaked in 100% sterile glycerol for 5min.

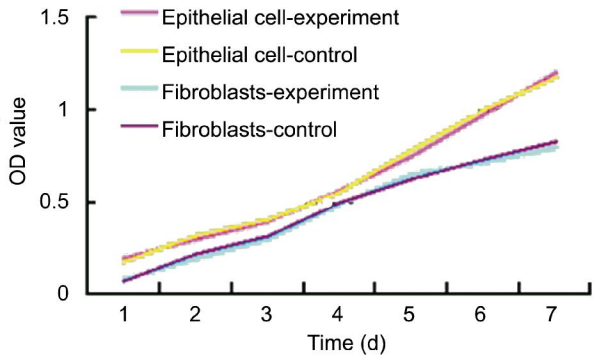

Figure 3 MTT assay for cytotoxicity determination of the extracts from the acellular porcine cornea matrix Data represent mean ±standard deviation in each sample from 6 independent experiments. There were no significant differences in the proliferation of human corneal fibroblasts between the experimental and control groups (n=6, P>0.05), and there were no significant differences in the proliferation of human corneal epithelial cells between the experimental and control groups (n=6, P>0.05).

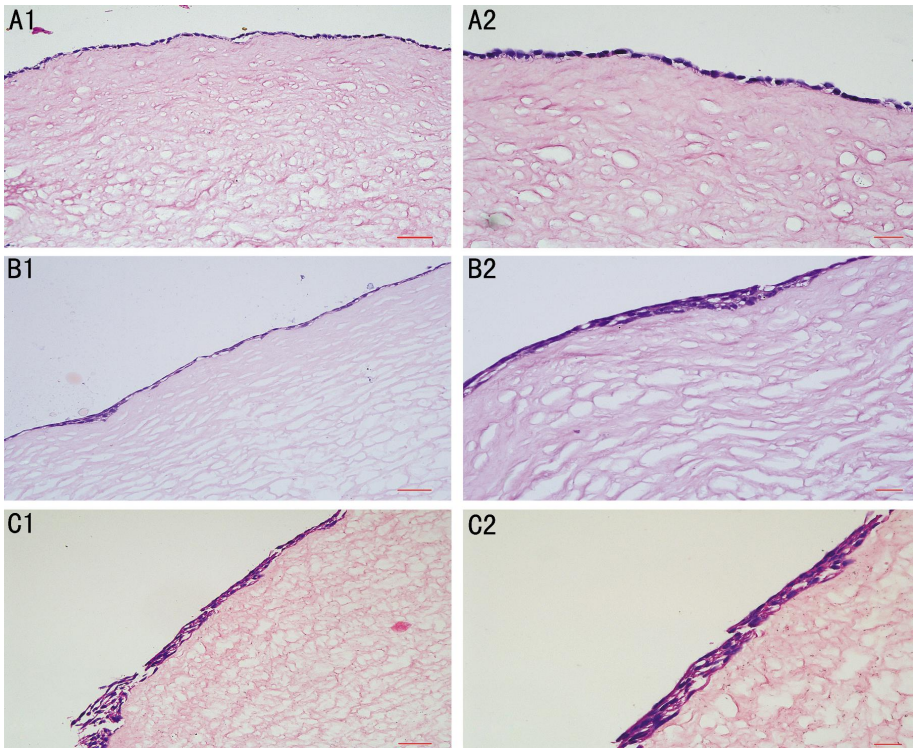

Figure 4 HE staining of human corneal epithelial cells on the acellular porcine cornea matrix A1, A2: At 3d, corneal epithelial cells formed a mono-layer covering the surface of the APCM; B1, B2: At 5d, formed closely arranged 2 or 3 layers of cells; C1, C2: At 10d, formed arranged 3 to 5 layers of cells. A1, B1, C1: Scar bar: 50 μm; A2, B2, C2: Scar bar: 20 μm.

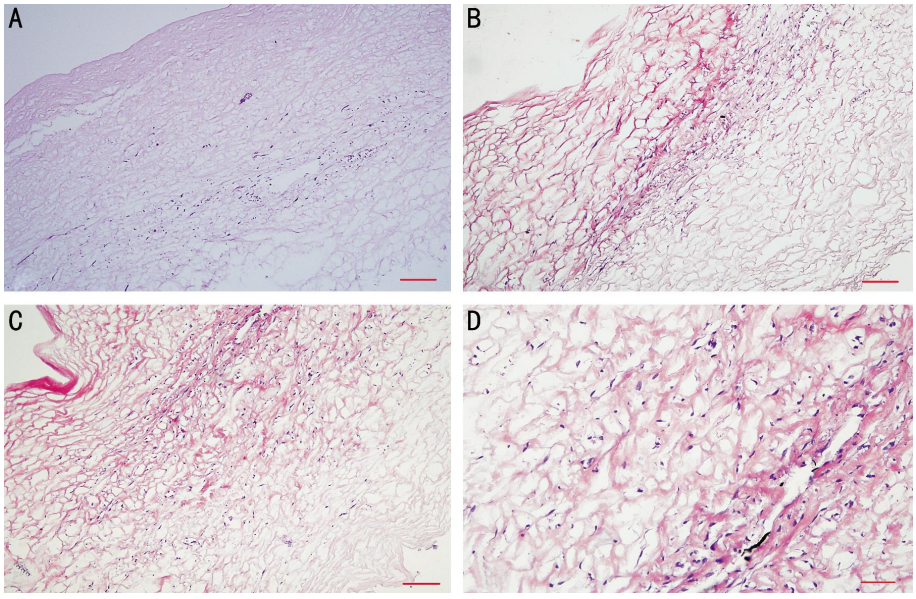

Figure 5 HE staining of human corneal fibroblasts in the acellular porcine cornea matrix A: Scattered fibroblasts within the APCM at 3d, some of the collagen fibers were fractured; B: Small amount of fibroblasts within the APCM at 5d; C, D: Many fibroblasts within the APCM at 10d, most along the insulin syringe injection region. A, B, C: Scar bar: 100 μm; D: Scar bar: 50 μm.

5d (Figure 4B1, 4B2), and then formed arranged 3 to 5 layers of cells at 10d (Figure 4C1, 4C2).

There were a lot number of fibroblasts within the APCM at 3, 5, 10d (Figure 5A, 5B, 5C, respectively), most along the insulin syringe injection region. Some of the collagen fibers were fractured (Figure 5).

Characterization of the Corneal Anterior Lamellar Replacement The human corneal anterior lamellar replacement was efficiently constructed with the scaffold of the APCM. After the epithelial cells were seeded on top of the stromal substitutes within fibroblasts, a growing number of corneal cells were observed at 3, 5, 10d by HE staining (Figure 6A, 6B, 6C, respectively). Epithelial cells formed a mono-layer epithelial cells covering the surface of the APCM at 3d (Figure 6A1, 6A2), formed closely arranged 2 or 3 layers of cells at 5d (Figure 6B1, 6B2). At 10d, a continuous 3 to 5 layers of human corneal epithelial cells covering the surface of the acellular porcine cornea matrix

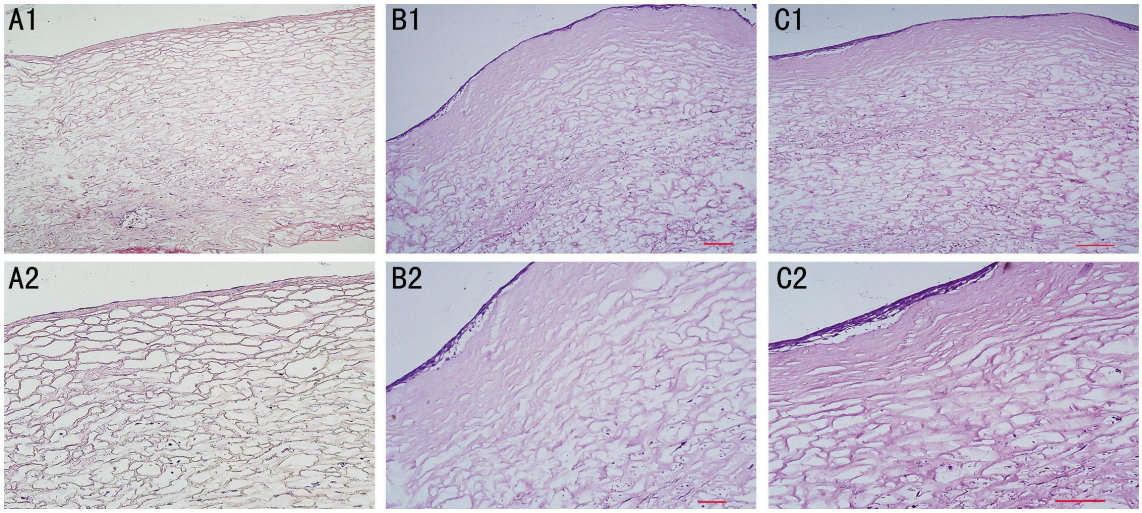

Figure 6 HE staining of human corneal anterior lamellar replacement A1, A2: Epithelial cells formed a mono-layer epithelial cells covering the surface of the APCM at 3d, the injected corneal fibroblasts distributed within the same scaffold; B1, B2: Formed closely arranged 2 or 3 layers of cells at 5d, the injected corneal fibroblasts distributed within the same scaffold; C1, C2: At 10d, a continuous 3 to 5 layers of human corneal epithelial cells covering the surface of the acellular porcine cornea matrix was observed. The injected corneal fibroblasts distributed within the same scaffold. A1, B1, C1: Scar bar: 100 μm; A2, B2, C2: Scar bar: 50 μm.

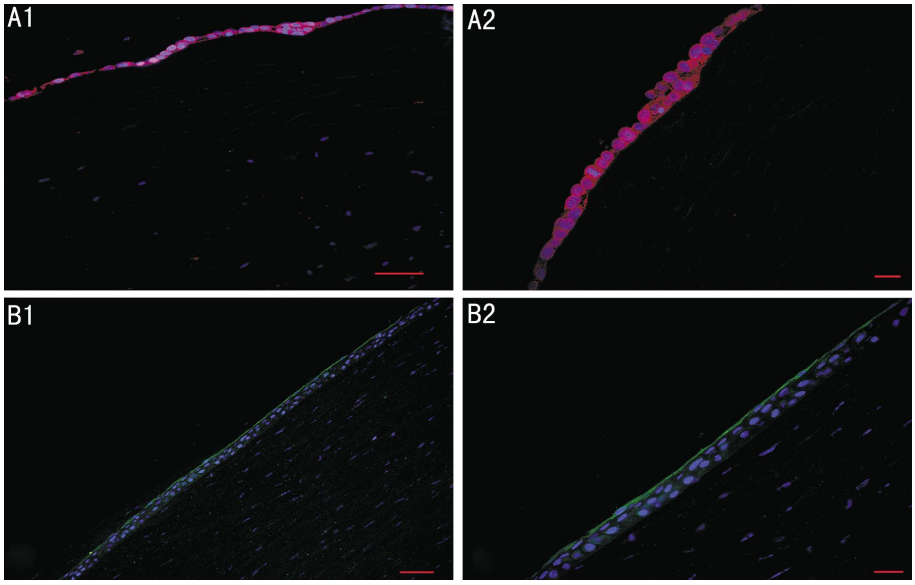

Figure 7 Immunofluorescent staining showed that the expression of CK12 of the construction and normal human anterior cornea A: The construction highly expressed CK12 in the epithelial cell layer; B: Normal human anterior cornea expressed CK12 restricted to the superficial layers. A1, B1: Scar bar: 50 μm; A2, B2: Scar bar: 20 μm.

was observed (Figure 6C1, 6C2). And the injected corneal fibroblasts distributed within the same scaffold (Figure 6).

The phenotype of the construction was similar to normal human corneas (Figures 7, 8), with high expression of CK12 in the epithelial cell layer (Figure 7A1, 7A2) and high expression of vimentin in the stroma (Figure 8A), as demonstrated by immunofluorescent staining.

The construction of the corneal anterior lamellar replacement was opaque due to the decellularized process (Figure 9A), and transparency was restored when soaked in 100% sterile glycerol for 5min (Figure 9B).

These findings showed that the APCM had good biocompatibility with human corneal epithelial cells and corneal fibroblasts.

DISCUSSION

One of the important elements of artificial cornea construction is the source of cells which can be seeded on or in the scaffolds. Researchers had explored potential cell sources, including cultured oral mucosal epithelia, human adipose tissue mesenchymal stem cells, marrow mesenchymal stem cells, and human embryonic stem cells (hESCs) [26-28,21]. However, all these sources have their limitations, such as low induction efficiency, the unknown induction mechanism and tumorigenicity [29]. Using a small amount of tissue thereby minimizing damage to the donor and depletion of its stem cell reserve, limbal stem cells (LSCs) may be obtained from the fellow eye (autograft), a cadaver (allograft) or a living relative (allograft). LSCs

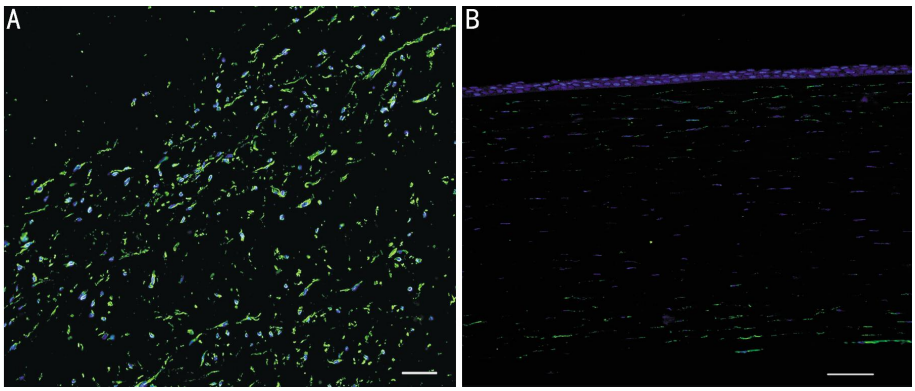

Figure 8 Immunofluorescent staining showed that the expression of Vimentin of the construction and normal human anterior cornea A: The construction highly expressed vimentin in the stroma; B: The expression of vimentin in normal human anterior cornea. Scar bar: 50 μm.

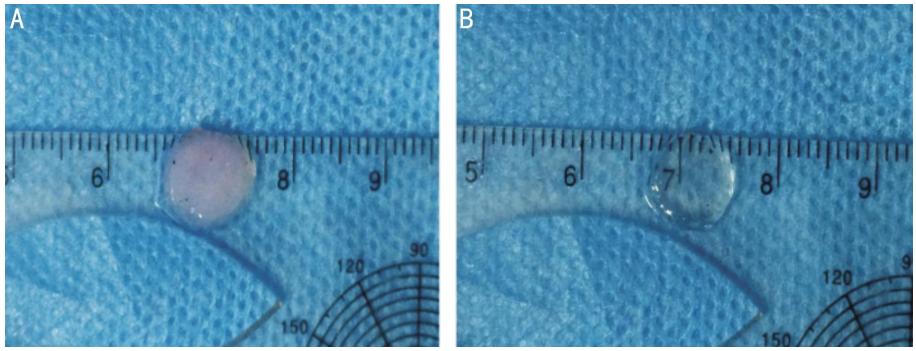

Figure 9 Transparency of the corneal anterior lamellar replacement A: The construction was opaque due to the decellularized process; B: Transparency of the construction was restored when soaked in 100% sterile glycerol for 5min.

located in the limbus, are known as the basis of corneal epithelial self-renewal and repair, have higher proliferative potential in cell cultures than central and peripheral corneal epithelial cells [30]. We found exhibited LSCs a typical cobblestone appearance during *in vitro* expansion for 10d with expressing CK12.

Keratocytes are native resident cells of the corneal stroma, principally responsible for the maintenance of the unique transparent stromal tissue by secreting a spectrum of unique matrix molecules. However, these cells inevitably differentiate into corneal fibroblasts during expansion *in vitro* under serum-containing culture medium [31]. Corneal fibroblasts exhibit a wound-healing phenotype and secrete disorganized extracellular matrix (ECM) typically found in corneal scars. Corneal fibroblasts did down-regulate expression of keratocyte marker genes (keratocan, corneal N-acetylglucosamine-6-O-sulfotransferase, prostaglandin D2 synthase), and up-regulate expression of smooth muscle alpha actin (a-SMA) and extra-domain A fibronectin (EDA-FN) [32]. But the fibroblasts and keratocytes all expressed vimentin, confirming their mesenchymal origin, and fibroblasts had strong proliferation capacity and spread to passage 15 *in vitro* [33]. ECM secreted from keratocytes *in vivo* plays an important role in cell growth, proliferation, and migration , promotes the attachment of epithelial cells to the basal lamina and also involves in the migration and growth of these cells [34-35]. However, this cross-talk occurs *in vivo* but not seem to be the case here. Comparison of the APCM with corneal epithelial cells group and the APCM with corneal epithelial cells and fibroblasts group, these all formed a mono-layer epithelial cells covering the surface of the scaffold at 3d, formed closely arranged 2 or 3 layers of cells at 5d, and then formed arranged 3 to 5 layers of cells at 10d. There are no obviously differences between them. Many studies had shown that corneal fibroblasts secreted an ECM lacking cornea-specific components (collagen type I, collagen type V, collagen type VI, keratan sulfate, lumican and keratocan), these are the major fibrillar collagens of cornea and constitute the major structural components of stromal tissue [31-32]. Therefore, loss of these adhesion molecules secreted from keratocytes may not promote migration and growth of corneal epithelial cells.

An ideal scaffold for a tissue-engineered cornea should have good biocompatibility, high optical clarity, toughness to withstand surgical procedures, and non-immunogenicity properties [10]. Decellularized biological scaffolds have been successfully used in clinical applications including heart valves, blood vessels, skin, nerves, skeletal muscle, tendons, ligaments, small intestinal submucosa, urinary bladder, and liver for tissue engineering and regenerative medical applications[5,36-40]. Fu *et al* [20] constructed a tissue-engineered cornea with acellular porcine corneal stroma developed by

Triton X-100, but if the scaffold had enough toughness to withstand surgical procedures for transplantation was unknown. And the world's first artificial biological cornea-acellular porcine corneal stroma-has been successfully used in clinics by many chinese scientists, and has been reviewed by the State Food and Drug Administration. This product as an ocular surface substitute, can be used to treat a variety of corneal disease. Our decellularization method was good and resulted in no cells or cell nuclear material within the APCM by HE staining and DAPI staining. Immunofluorescent staining showed LN was abundantly present, one of basement membrane components. The TEM examination showed that cell components were removed and the collagen fibrils were regularly arranged. And the APCM was opaque due to the decellularized process, and transparency was restored when soaked in 100% sterile glycerol for 5min. The cytotoxicity assay of the APCM demonstrated that human corneal epithelial cells and corneal fibroblasts had an obvious proliferation tendency, suggesting that the APCMs did not retard cell proliferation and had no cytotoxic effects. Moreover, our previous studies also showed that between the fresh and acellular corneas, no loss of collagen type I, fibronectin, and LN was observed, no significant change in the hydroxyproline content and the uniaxial mechanical test[23-24].

In clinics, deep lamellar keratoplasty (DLK) has been found to be a safe alternative to penetrating keratoplasty (PK) for common diseases such as keratoconus, because the best corrected visual acuity, refractive results, and complication rates are similar after DLK and PK and the risk of endothelial rejection can be avoided [41-42]. As research continues, tissue-engineered lamellar keratoplasty promises to be an increasingly important option for the corneal surgery, solves the problem of high risk for rejection after the traditional donor-derived corneal transplatation, will bring a bright hope for corneal blindness. In the present study, we have constructed human corneal anterior lamellar replacement with the scaffold of APCM. The phenotype of the construction was similar to normal human corneas, with high expression of CK12 in the epithelial cell layer and high expression of Vimentin in the stroma. In our study, LSCs differentiated terminal corneal-type epithelium when culturing for 10d *in vitro*, with expressing CK12. Therefore, as a step toward clinical testing, further study of construction technique with stem cells and reconstructed anterior cornea equivalents in animals with diseased corneas will be evaluated.

In this study, the APCM with a basement membrane facilitated the growth of human corneal cells *in vitro*, suggesting that the APCM is an suitable scaffold material for tissue-engineered corneas and a potential therapeutic tool for reconstructing a functional ocular surface. Furthermore, the present study demonstrated for the first time the use of human corneal epithelial cells and fibroblasts for reconstructing human anterior corneal lamellar replacement. Although the phenotype of the construction was similar to normal human corneas, the effect of current human anterior corneal lamellar replacement in animal models and even in human clinical trials needs to be further elucidated.

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Conflicts of Interest: Zhang J, None; **Zhang CW,** None; **Du LQ,** None; **Wu XY,** None.

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