·Basic Research·

Evaluation of novel decellularizing corneal stroma for cornea tissue engineering applications

Yi Shao [1], Yao Yu [2], Chong-Gang Pei [1], Qiong Zhou [1], Qiu-Ping Liu [3], Gang Tan [4], Jing-Ming Li [3], Gui-Ping Gao [1], Lu Yang [1]

Foundation items: National Natural Science Foundation of China (No.81160118, 81100648, 81101858, 81100649); Natural Science Foundation of Jiangxi Province, China (No. 20114BAB215029); Technology Foundation of Jiangxi Province, China (No.20111BBG70026-2); Health Department Science and Technology Foundation, China (No. 20121026); Education Department Youth Scientific Research Foundation, China (No.JJJ12158); National High Technology Research of China (863 project)(No. 2006AA02A131)

[1]Department of Ophthalmology, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

[2]Department of Endocrinology, the Third Hospital of Nanchang, the Affiliated Nanchang Hospital of Southern Medical University, Nanchang 330009, Jiangxi Province, China

[3]Department of Physiology, Endocrinology Institute of Oklahoma University, Oklahoma City, OK 73104-5020, USA

[4]Department of Ophthalmology, the First Affiliated Hospital of Nanhua University, Hengyang 421001, Hunan Province, China

Correspondence to: Yi Shao. Department of Ophthalmology, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China. freebee992296 @yahoo.com.cn

Received: 2012-05-22 Accepted: 2012-07-17

Abstract

• AIM: To develop a new decellularization method depended upon the natural corneal structure and to harvest an ideal scaffold with good biocompatibilities for corneal reconstruction.

• METHODS: The acellular cornea matrix (ACM) were prepared from de-epithelium fresh porcine corneas (DFPCs) by incubation with 100% fresh human sera and additional electrophoresis at 4℃. Human corneal epithelial cells (HCEs) were used for the cytotoxicity tests of ACM. ACM were implanted into the Enhanced Green Fluorecence Protein (eGFP) transgenic mouse anterior chamber for evaluation of histocompatibility.

• RESULTS: HE and GSIB4 results showed fresh porcine cornea matrix with 100% human sera and electrophoresis could entirely decellularize stromal cell without reducing its transparency. ACM has no cytotoxic effect *ex viva*. Animal test showed there was no rejection for one month after surgery.

• CONCLUSION: These results provide a decellularizing approach for the study of corneal tissue engineering and had the broader implications for the field of biological tissue engineering in other engineered organ or tissue matrix.

• KEYWORDS: cornea; human sera; electrophoresis; tissue engineering; decellularization; biocompatibility
DOI:10.3980/j.issn.2222-3959.2012.04.02

Shao Y, Yu Y, Pei CG, Zhou Q, Liu QP, Tan G, Li JM, Gao GP, Yang L. Evaluation of novel decellularizing corneal stroma for cornea tissue engineering applications. *Int J Ophthalmol* 2012;5(4):415-418

INTRODUCTION

Cornea disease is a major cause of vision loss. Although corneal transplantation using human cornea stroma is the most appropriate therapeutic method for keratoconus[1], its supply is in a grave shortage in China. These facts have prompted various efforts to develop several corneal substitutes, including keratoprosthesis (KPro), natural and synthetic corneal replacement[2-4]. The physial properties and safety of these substitutes were improved to some degree but were still remain many problems, which have restricted their clinical applications [5-8]. The method of decellularization in porcine cornea has been widely used, because of its advantages in preserving the natural extracellular matrix, which is difficult to imitate using synthetic methods. Therefore, we examined the efficacy of a novel method of decellularizing porcine corneal stroma and the feasibility of using them *in vivo* and *ex viva*. To our best knowledge, a study of this novel decellularizing method has not been previously reported.

MATERIALS AND METHODS

Materials

Preparation of fresh human sera and decellularized porcine cornea All studies using human sera were in

accordance with the tenets of the Declaration of Helsinki and the policies of the institutional review board for human subjects from Xiamen Eye Center (Xiamen, China). Under aseptic conditions, 90mL of human blood was collected from healthy volunteers and centrifuged at 2000r/min for 10 minutes, the supernatant sera which preserved complement activity was then harvested and stored in -20℃ before use. Sera from blood hemotype A was applied in this study.

Porcine cornea was immediately transported to the laboratory in moist chambers at 4℃. Cornea was washed in the phosphate buffer saline (PBS, pH 7.4) containing antibiotics (penicillin 60mg/L, streptomycin 100mg/L, Invitrogen, USA) three times. Corneal epithelium and endothelium was then removed by cell scraper, and the epithelium side was marked by using 10-0 nylon (Alcon, USA). Cornea lamellar stroma (about 300μm in thickness) was incubated with 100% sterile human sera for duration up to 1 day. After washed 3 times with PBS for 15 minutes, lamellar stroma (including DNA fragmentation with electric charge) were subjected to electrophoresis (150V/cm,) in sterilized buffer (pH 7.4, 320 mosmol/kg) containing 40mmol/L Tris-base, 18mmol/L glacial acetic acid and antibiotics for 1 hour at 4℃, followed by 3 times washes with PBS for 20 minutes at 4℃ with continuous shaking in thermostat-controlled water bath. Cornea stroma went through such procedure were defined as acellular corneal matrix (ACM).

Methods

α −Gal epitopes binging assay GSIB4 staining was performed to detect α-Gal epitopes as previously described[9]. In brief, ACM section were reacted with Alexa fluor 568 conjugated GSIB4 (5μg/mL in PBS, Invitrogen, USA) for 10 minutes at room temperature. After 3 times washes with PBS for 15 minutes, the nuclei were counterstained with DAPI (Vector Laboratories, USA). The samples were investigated and photographed with laser confocal microscopy (Fluoview 1000, Olympus, Japan).

Extract cytotoxicity assay The ACM were tested for their potential cytotoxicity (cytotoxicity test: ISO 1099317) to the proliferation of human corneal epithelial cells (HCE, ADL Company, USA; culture medium: DMEM supplemented with 10% Fetal Bovine Serum). The scaffolds were extracted at 37℃ for 24 hours in 10mL medium consisting of HCE culture medium under constant shaking in thermostat-controlled water bath. HCE (1.0×10^4/cm^2) were seeded onto 96-well plates and incubated for 24 hours at 37℃ in 5% CO_2 and air, after that the culture medium was aspirated, and 100μL of culture medium accordingly plus 100μL of the aseptic scaffolds extracts (pH 7.2) were added to five replicate wells, respectively. Controls included 40% (v/v) di-methyl sulfoxide (positive control) in media and media alone (negative control). The plates were incubated for further 12, 24, 36 and 48 hours. The effect of the extracts was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) following the manufacturer's instructions. Within 2 hours, the optical density (OD) at 450nm was determined with a microplate reader (ELX800, BIO-TEK Corporation, USA).

Mouse anterior chamber implant assay Animal experiments were performed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the Guidelines of the Animal Experimental Committee of Xiamen University (Fujian, China). Enhanced Green Fluorecence Protein (eGFP) transgenic mouse (20g-30g, 6 weeks old, female) was anesthetized with intraperitoneal pentobarbital sodium (30mg/kg) and topical 0.5% proparacaine hydrochloride. After sterilization, The square ACM (1mm×1mm, n=5) were implanted into the anterior chamber in the left eye of mice. One month after the transplantation, the left eye was enucleated and the cornea was stained with HE for histological study.

Histology analysis Tissue samples (6μm) were prepared and fixed, HE staining and DAPI staining were performed as described in previous report [5]. Sections were observed under light microscope and Eclipse epi-fluorescence microscope (TE-2000U, Nikon, Japan) and photographed.

Statistical Analysis Data were expressed as mean±SD and analyzed statistically by Student's t-test. $P<0.05$ was regarded as statistically significant. The statistical analysis was conducted with commercial software (GraphPad Prism for Windows, ver. 5.00; GraphPad Software. Inc., USA).

RESULTS

Morphology of ACM Compared the fresh porce cornea (Figure 1B, D), HE staining revealed the maintenance the excellent transparence and uniform structure of collagen fibrils in the ACM (Figure 1A, C). The GSIB4 binding assay and DAPI staining showed the absence of cells and α-Gal in ACM (Figure 1E) but fresh porce cornea(Figure 1F).

***Ex vivo* evaluation of cytotoxicity** Evaluation of the safety of ACM was performed *in vitro* by cells seeding on leaching liquor of ACM. The OD values after 12, 24, 48 and 72 hours of culture were showed in Figure 2A. There were no significant differences of the proliferation of HCE between ACM extract and negative control at predetermined time points ($P>0.05$).

***In vivo* evaluation of histocompatibility** One month postoperatively, ACM did not invoke an adverse immune response by the host in anterior chamber (Figure 2B). HE staining showed that the transplanted ACM could be recognized in the mouse anterior chamber and that there were no eGFP marker cells infiltrated the transplanted ACM (Figure 2C).

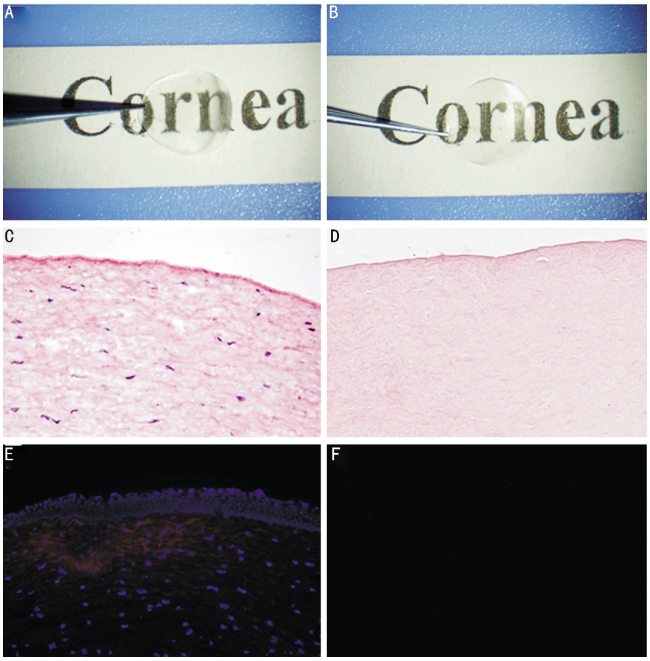

Figure 1 Representative photographs, histology -stained sections of the fresh porcine cornea and ACM (GSIB4, red; DAPI, blue;(C-F), magnification, ×200) The left column shows the transparency (A), HE staining (C), GSIB4 and DAPI staining (E) of fresh porcine cornea. The right column shows the transparency (B), HE staining (D), GSIB4 and DAPI staining (F) of ACM.

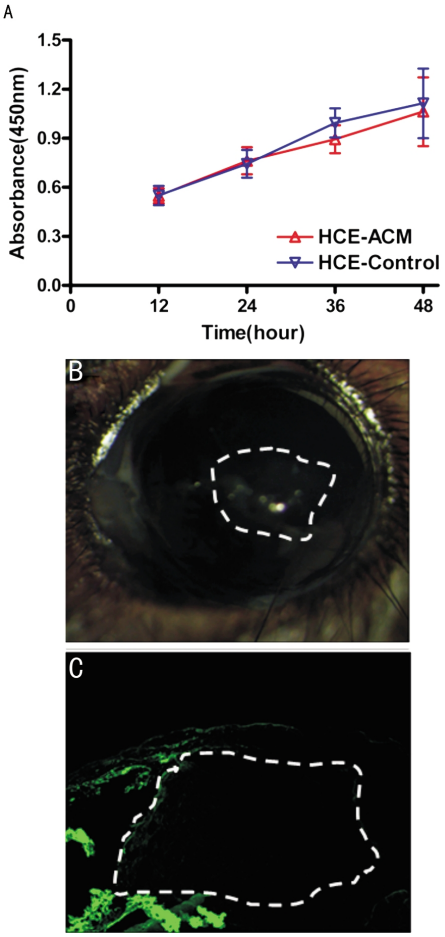

Figure 2 CCK-8 assay for proliferation of HCE in ACM extract/control medium after *in vitro* culture for different hours (A). There are no significant differences in the proliferation of HCE between ACM extract and negative control (P>0.05). One month postoperatively, ACM did not invoke an immune response in anterior chamber in eGFP mouse anterior chamber (B). HE staining showed that there were no eGFP marker cells (bright dot) infiltrated the transplanted ACM (C). (n=5 for A. dash line: ACM).

DISCUSSION

This study has identified that our method of decellularization using with 100% sterile human sera and electrophoresis entirely decellularize the porcine cornea stromal cell without reducing its transparency and inducing rejection in an anterior chamber implant models. But its mechanism was not detected since the study was focused on developing a new decellularization method.

The α-Gal epitope is one of the most abundant carbohydrate epitopes on cells of non-primate mammals and New World monkeys, where it is synthesized by expressing a functional α1, 3 galactosyltransferase enzyme. On the contrary, in humans, apes and Old World monkeys, this epitope is absent. However, the gastrointestinal bacterium that express α-Gal can induce anti-Gal antibodies in humans [10]. It is known that the major immunologic barrier in xenotransplantation is the interaction between anti-Gal IgM and IgG antibodies and α-Gal epitope. Previous studies have shown that the hyperacute rejection of vascularized xenografts in the *in vitro* porcine-to-human combination is triggered by the binding of human preformed natural antibodies (PNAbs) to the α-Gal epitope in pig endothelium and the subsequent activation of complement [11] and it is induced by the combining of the complement and antibody dependent cell mediated cytotoxicity process *in vivo*[12,13]. However, serum support the colloid osmotic pressure, the antiprotease component (trypsin inhibitor), the growth factor (fibroblast growth factor, epidermal growth factors and platelet growth factor), and the nutrient substances (amino acid, vitamin, lipoid and inorganic substance). Serum also protects the fiber tissue and cell to away from the damage of mechanic and enzymatic treatment. Since α-Gal epitope is expressed in several keratocytes in the most anterior corneal stroma [14,15], our study was to use human serum, which contains anti-Gal, to decellularize the porcine corneal stromal cells.

As we know, 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 medicine applications [16,17]. However, transparency is an essential property of the cornea. Most of decellularized procedure use

detergents or snap freezing to remove cells from tissue[18,19]. Detergents are generally toxic and may lead to the tissue denaturation and cornea opacification[20]. Freezing alone did not decellularize the corneal stroma[21]. Our decellularization method resulted in no cell residual in the corneal stroma and the transparency was well preserved. The extract cytotoxicity was demonstrated that HCE cells had obvious proliferation tendency. These results suggest that the ACM do not retard the cell proliferation and have no cytotoxic effects *ex vivo*. Histologic evaluation revealed that few cells entered the ACM 1 month after they were transplanted into the mouse anterior chamber. Since keratocytes may play some role in metabolism of the corneal matrix, the degradation of ACM must be observed for a longer period.

In conclusion, we found the acellular method without destroying stroma structure, and the nontoxic ACM that was transplanted into the mouse anterior chamber for one month. This decellularization method may be valuable for preparation of xenogenic corneal matrix equivalent for clinical application.

Acknowledgments: We thank Dr Yue-Ping Zhou (Eye Institute and Affiliated Ophthalmic Centre, Xiamen University, Fujian, China) and Luo-Wa Quyang(Cullen Eye Institute of Baylor University, Texas, USA) for correcting the English in the manuscript for this article.

REFERENCES

1 George AJ, Larkin DF. Corneal transplantation: the forgotten graft. *Am J Transplant* 2004;4(5):678-685

2 Chirila TV, Chirila M, Ikada Y, Eguhi H, Shiota H. A historical review of artificial cornea research. *Jpn J Ophthalmol* 2005;49(1):S1-13

3 Liu Y, Gan L, Carlsson DJ, Fagerholm P, Lagali N, Watsky MA, Munger R, Hodge WG, Priest D, Griffith M. A simple, cross-linked collagen tissue substitute for corneal implantation. *Invest Ophthalmol Vis Sci* 2006;47(5): 1869-1875

4 Chirila TV. An overview of the development of artificial corneas with porous skirts and the use of PHEMA for such an application. *Biomaterials* 2001;22(24): 3311-3317

5 Chen J, Li Q, Xu J, Huang Y, Ding Y, Deng H, Zhao S, Chen R. Study on biocompatibility of complexes of collagen-chitosan-sodium hyaluronate and cornea. *Artif Organs* 2005;29(2):104-113

6 Zhang Y, Zhang M. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. *J Biomed Mater Res* 2001;55(3):304-312

7 Teixeira AI, Abrams GA, Bertics PJ, Murphy CJ, Nealey PF. Epithelial contact guidance on well-defined micro- and nano-structured substrates. *J Cell Sci* 2003;116(Pt 10):1881-1892

8 Natu MV, Sardinha JP, Correia IJ, Gil MH. Controlled release gelatin hydrogels and lyophilisates with potential application as ocular inserts. *Biomed Mater* 2007;2(4):241-249

9 Shao Y, Quyang L, Zhou Y, Tang J, Tan Y, Liu Q, Lin Z, Yin T, Qiu F, Liu Z. Preparation and physical properties of a novel biocompatible porcine corneal acellularized matrix. *In Vitro Cell Dev Biol Anim* 2010;46 (7):600-605

10 Wang H, Tao T, Tang J, Mao YH, Li W, Peng J, Tan G, Zhou YP, Zhong JX, Tseng SC, Kawakita T, Zhao YX, Liu ZG. Importin 13 serves as a potential marker for corneal epithelial progenitor cells. *Stem Cells* 2009; 27(10) :2516-2526

11 Galili U, Mandrell RE, Hamadeh RM, Shohet SB, Griffiss JM. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect Immun* 1988;56(7):1730-1737

12 Kin T, Nakajima Y, Aomatsu Y, Kanehiro H, Hisanaga M, Ko S, Ohyama T, Nakano H. Humoral human xenoreactivity against isolated pig pancreatic islets. *Surg Today* 2000;30(9):821-826

13 Watier H, Guillaumin JM, Vallée I, Thibault G, Gruel Y, Lebranchu Y, Bardos P. Human NK cell mediated direct and IgG dependent cytotoxicity against xenogeneic porcine endothelial cells. *Transplant Immunol* 1996;4 (4):293-299

14 Galili U. Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotranplantation in humans. *Immunol Today* 1993;14(10):480-482

15 Amano S, Shimomura N, Kaji Y, Ishii K, Yamagami S, Araie M. Antigenicity of porcine cornea as xenograft. *Curr Eye Res* 2003;26 (6): 313-318

16 Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft *via* optimized chemical processing. *Tissue Eng* 2004;10(9-10): 1346-1358

17 Mertsching H, Walles T, Hofmann M, Schanz J, Knapp WH. Engineering of a vascularized scaffold for artificial tissue and organ generation. *Biomaterials* 2005;26(33):6610-6617

18 Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. *Biomaterials* 2006;27(19): 3675-3683

19 Zhang C, Jin Y, Nie X, Liu Y, Lei J, Hu D. A comparative study on biocompatibility of acellular corneal stroma materials prepared by serial digestion methods. *Zhongguo Xiufu Chongjian Waike Zazhi* 2006;20 (2): 185-188

20 Oh JY, Kim MK, Lee HJ, Ko JH, Wee WR, Lee JH. Processing porcine cornea for biomedical applications. *Tissue Eng Part C Methods* 2009;15 (4):635-645

21 Amano S, Shimomura N, Yokoo S, Araki-Sasaki K, Yamagami S. Decellularizing corneal stroma using N_2 gas. *Mol Vision* 2008;14:878-882