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Xenotransplantation Using Lyophilized Acellular Porcine Cornea with Cells Grown *in vivo* and Stimulated with Substance-P

Jeong Kyu Lee, Seok Hyun Lee and Jae Chan Kim
Department of Ophthalmology, Chung-Ang University Hospital, Seoul,
Korea

1. Introduction

Corneal allograft transplantation has a high success rate, but the clinical use of corneal allografts is limited by an insufficient number of human donor corneas (Alldredge & Krachmer, 1981; Sedlakova & Filipec, 2007). Artificial substitutes may serve as an alternative to donor allograft use, but widely accepted substitutes are not currently available (Chen et al., 2001; Griffith et al., 1999; Trinkaus-Randall et al., 1988). Porcine corneas may serve as a reasonable alternative due to the ease of use and the potential for genetic engineering (Auchincloss, 1988; Insler & Lopez, 1991; Ross et al, 1993). In porcine organ xenografts, overcoming xenoantigene expression is of central importance to avoid graft rejection. Gal1 α -3Gal β 1-4GlcNAc (α -gal) on porcine tissues is one of the best known antigens involved in xenograft rejection (Amano et al., 2003; Collins et al., 1995; Good et al., 1992). We previously demonstrated that lyophilization of porcine corneas could eliminate the α -gal antigen by removing antigen-expressing cells, and lyophilized acellular porcine corneas (APCs) survived longer than fresh porcine corneas in pig-to-rat model (Lee et al., 2010). Though decellularization using lyophilization appear favorable in reducing graft rejection, but the early inflammation frequently encountered is another issue which requires resolution. The lyophilization process based on glycerol and surgical manipulation might be responsible for early inflammation, and the delayed healing caused by an acellular substrate graft is another concern.

Recently, several studies have reported that corneal transparency is highly dependent on corneal cells as well as the extracellular matrix (Meek et al., 2003; Mourant et al., 2000). Thus, adequate recellularization of acellular substitute might not only extend graft survival, but enhance optical transparency. Accordingly, we hypothesized that repopulation of lyophilized APCs with cell grown *in vivo* before transplantation can enhance the survival of the graft by reducing the damage caused by inflammation or apoptotic environment. To address this issue, lyophilized APCs were transplanted under the limbus of rabbit corneas in advance for repopulation with cells *in vivo*, then optical transparency and histologic findings were compared with controls over the follow-up period. Furthermore, lamellar keratoplasties were performed to evaluate the usefulness of lyophilized APCs with cells grown *in vivo* in a rabbit model.

2. Materials and methods

The animals in this study were treated according to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

2.1 Preparation of lyophilized acellular porcine cornea

Adult porcine corneas were obtained from a slaughterhouse within 2 hours death, then transported in a 4°C moist chamber to the laboratory. Using sterile techniques, the epithelium of each pig cornea was removed and a 4.0 mm sized stromal button with a thickness of 300 µm was created from the central pig cornea using a microkeratome (Automated Corneal Shaper®; Chiron Vision, Claremont, CA, USA). The corneal button was treated in a mixed solution consisting of 40 µ/ml Dnase and 40 µ/ml Rnase (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes, followed by distilled water for 2 hours, three freeze-thaw cycles (-196°C liquid nitrogen for 30 minutes, followed by rapid thawing at 37°C for 30 minutes), and centrifuged (15000 ×g, 7 minutes) to removed all of the cellular components. Then, the corneal button was treated in 100 % glycerol (Sigma-Aldrich) at 4 °C for 3 days, stored at -80 °C for 48 hours, then lyophilized using a lyophilizer (SFDSM06; Samwon Freezing Engineering Co., Busan, Korea) at -80 °C for 48 hrs. Finally, the corneal button was irradiated with γ-rays (25 kGy) for sterilization.

2.2 Surgical procedure for *in vivo* recellularization

Forty-eight New Zealand white rabbits of either sex, weighing 2-3 kg were used for this study. Rabbits were divided into 3 groups and anesthetized with an intramuscular injection of mixture of Tiletamine and Zolazepam (Zolatil®, 12.5mg/Kg; Virbac Lab, Carros Cedex, France) and xylazine (Rompun®, 12.5mg/kg, Bayer Korea, Ansan, Korea).

In 16 rabbits, the superior limbal conjunctivae of the left eyes were incised using Westcotts scissors after topical anesthesia, and lyophilized APCs were inserted under the superior conjunctivae. The conjunctivae were closed with 8-0 vicryl. One-half of the implants were treated with substance-P (50 nmol/kg) for 1 hour before grafting. As controls, collagen sheets (CSs) and 8 sheets of bovine amniotic membranes (AMs) were transplanted (4.0 mm diameter) under the superior limbal conjunctivae in 16 rabbits using the same procedure. One-half of the CSs and bovine AMs were also treated with substance-P for comparison. All rabbits received topical levofloxacin (Cravit®, Santen, Osaka, Japan) three times daily until the end of the study.

2.3 Assessment of lyophilized APC with *in vivo* recellularization

The implants were harvested, and transparency was assessed 3day, and 1, 2, and 3 weeks after grafting in 2 rabbits per each time.

2.3.1 Optical property

The harvested lyophilized APC was placed on a numeric panel. A 0-4+ scoring system was devised to describe the transparency semi-quantitatively according to the visibility of a figure through the implants. Scoring was as follows: 0, clear figure image compared with the next numeral; 1+, minimally blurred figure; 2+, half of the blurred figure compared with the next numeral; 3+, intense opacity with the blurred image; and 4+, complete opacification.

2.3.2 Histological examination

The harvested implants were fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin. Cross sections (2-4 μm) were made along the longitudinal axis, and serially-sectioned specimens were stained with hematoxylin and eosin. Sections were examined under light microscopy and photographed for analysis of infiltrating cells on graft. The number of infiltrating mononuclear cells and inflammatory cells per high power field (HPF; 40 \times objective) was counted from three different regions of each sample for comparison.

2.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The harvested implants were examined using RT-PCR to determine the ideal timing of implant removal from recipients. The harvested implants were cut into small pieces by blade, and total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacture's instruction. Briefly, harvested implants were homogenized in 1 ml of TRIZOL reagent, then 200 μl of chloroform were added. Samples were centrifuged at 12,000 $\times g$ for 15 min at 4 $^{\circ}\text{C}$, and the aqueous phase was transferred to fresh tubes. One ml of isopropanol was added and the mixture was placed at -20 $^{\circ}\text{C}$ for 8 hrs. Sedimentation was performed by centrifugation at 12,000 $\times g$ for 15 min at 4 $^{\circ}\text{C}$. The resulting RNA pellet was suspended in 75% EtOH and centrifuged at 8,000 $\times g$ for 10 min at 4 $^{\circ}\text{C}$. The RNA pellet was dissolved in DEPC-treated RNase-free water. Complementary DNA (cDNA) was made with AccuPower™ RT Premix (Bioneer Co., Daejeon, Korea) in a total volume of 20 μl containing 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl_2 , 50 mM DTT, 1 mM dNTP, 10 U RNasin, and 20 U RTase. Total RNA (2 μg) and random primer (0.5 μg) were added to the RT Premix. The RT reaction was started at 57 $^{\circ}\text{C}$ for 10 min to denature RNA and reverse transcription took place at 42 $^{\circ}\text{C}$ for 1 hr, followed by RTase inactivation at 94 $^{\circ}\text{C}$ for 5 min. The validity of the RT reaction was determined internally using rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. PCR amplification was performed with AccuPower™ PCR Premix (Bioneer Co.) using 5 μl of the cDNA product in a total volume of 20 μl , containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM MgCl_2 , 1 U DNA polymerase, 1 mM dNTP, and 10 pmole of each specific primers. The reactions were run using a GeneAmp PCR System 2400 (Perkin Elmer Co., Waltham, MA, USA). RT-PCR products were electrophoresed on 1.0% agarose gels in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, and photographed under UV transillumination.

2.4 Biocompatibility tests using lamellar keratoplasty

Five New Zealand white rabbits, weighing 2-3 kg, were used for this study. All rabbits were anesthetized with an intramuscular injection of mixture of Tiletamine and Zolazepam (Zolatil®, 12.5mg/Kg; Virbac Lab) and xylazine (Rompun®, 12.5mg/Kg, Bayer Korea). The superficial corneas of rabbits were excised with a 4-mm punch. Lyophilized APCs treated with substance-P, which were maintained under superior limbal conjunctivae of rabbits for 7 days, were transplanted and fixed with 10-0 nylon (8 sutures) into rabbit corneas. Therapeutic contact lens was applied and tarsorrhaphy was performed. All rabbits received topical levofloxacin (Cravit®; Santen) eye drops three times daily until the end of the study. Therapeutic lens and tarsorrhaphy were removed

after complete corneal epithelial regeneration, and the sutures were removed when they were loosened.

Slit lamp biomicroscopic examinations and photographs were done every week for 2 months for changes in transparency and neovascularization. A 0-4+ scoring system was devised to describe the extent of opacification semi-quantitatively (Fantès et al., 1990). Scoring was as follows: 0, totally clear; 0.5+, trace corneal haze seen only by indirect broad tangential illumination; 1+, haze of minimal density seen with difficulty with direct illumination; 2+, mild haze easily visible with direct focal illumination; 3+, moderate dense opacity that partially obscured the iris details; and 4+, severe dense opacity that obscured completely the details of intraocular structures. A similar scoring system was developed to assess the extent of neovascularization, as follows: 0, no vessels extending toward the graft; 1+, vessels reaching the graft margin; 2+, vessels invading the graft; 3+, many vessels traversing the grafts (Konya et al., 2005; Jeong et al., 2009).

At 4 and 8 weeks, the rabbits were sacrificed, and the corneas were harvested under anesthesia. H&E and vimentin staining were done to observe the stromal cells repopulated in lyophilized APCs.

2.5 Statistical analysis

Data are expressed as the means±standard deviation. Statistical analysis was performed using SPSS (version 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Intergroup comparisons were analyzed using the Mann-Whitney U test. Statistical significance was set at a $p < 0.05$.

3. Results

3.1 Assessment of lyophilized APC

3.1.1 Optical transparency

The lyophilized APCs were visually opaque initially. After subconjunctival implantation, the lyophilized APCs were cleared with a transparency of 1+ by POD 7th; subsequently, the APCs gradually became opaque over 21 days. However the CSs and 8 sheets of bovine AMs were opaque, with scores of 3+~4+ over the entire examination point (Fig. 1). There was no difference in visual transparency of each implant whether or not treated with substance-P.

3.1.2 Histologic characterization

H&E staining of lyophilized APCs that were kept subconjunctivally for 7 days showed a number of rounds or spindle-shaped mononuclear cells, suggestive of corneal stroma-like cells, with an occasional inflammatory cell infiltration. Histologic analysis of lyophilized APCs that were kept for 3 days subconjunctivally revealed rare corneal stroma-like cells inside the graft, while APCs that were kept for 14 and 21 days subconjunctivally showed significant inflammatory cell infiltration, including polymorphonuclear leukocytes, and some monocytes (data not shown). Thus, lyophilized APCs that were kept for 7 days subconjunctivally were chosen as the ideal implants with cells grown *in vivo*, and the histologic appearance was compared with controls (Fig. 2).

The number of corneal stroma-like cells in lyophilized APCs and bovine AMs were comparable (average, 16.2 ± 2.1 cells per field for lyophilized APCs vs. 18 ± 4.0 cells per field

for bovine AMs; $p=0.24$), and were significantly greater than CSs (average, 9.6 ± 1.7 cells per field; $p<0.05$). In implants treated with substance-P, increased infiltration of corneal stroma-like cells was observed, but there was no significant difference following treatment with substance-P ($p=0.23$).

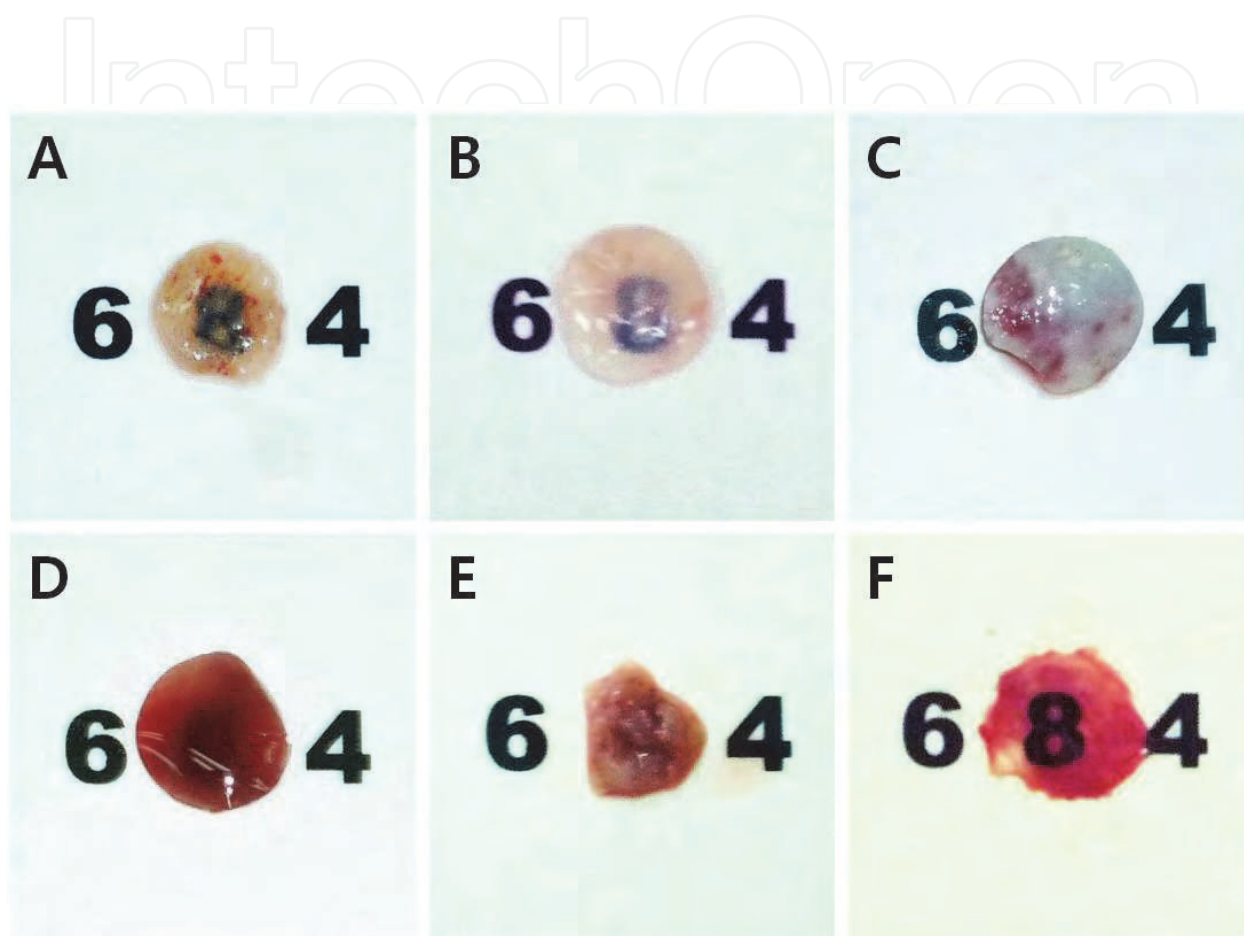


Fig. 1. Optical transparency of subconjunctival implants 7 days postoperatively.

The lyophilized acellular porcine cornea (A) and lyophilized acellular porcine cornea soaked in substance-P (B) showed approximately one-third transparency compared to the surrounding letter. Collagen sheet (C) and collagen sheet soaked in substance-P (D) were visually opaque. Eight sheets of amniotic membrane were also visually opaque (E), but single sheets of amniotic membrane was clear (F).

The average number of inflammatory cell in the lyophilized APC infiltrate was 11.4 ± 1.1 cells per field, which was much less than bovine AM and CS infiltrates (34.8 ± 5.5 cells per field for bovine AMs and 61.8 ± 8.3 cells for CSs). Treatment with substance-P did not have an effect on the inflammatory cell infiltrate in implants.

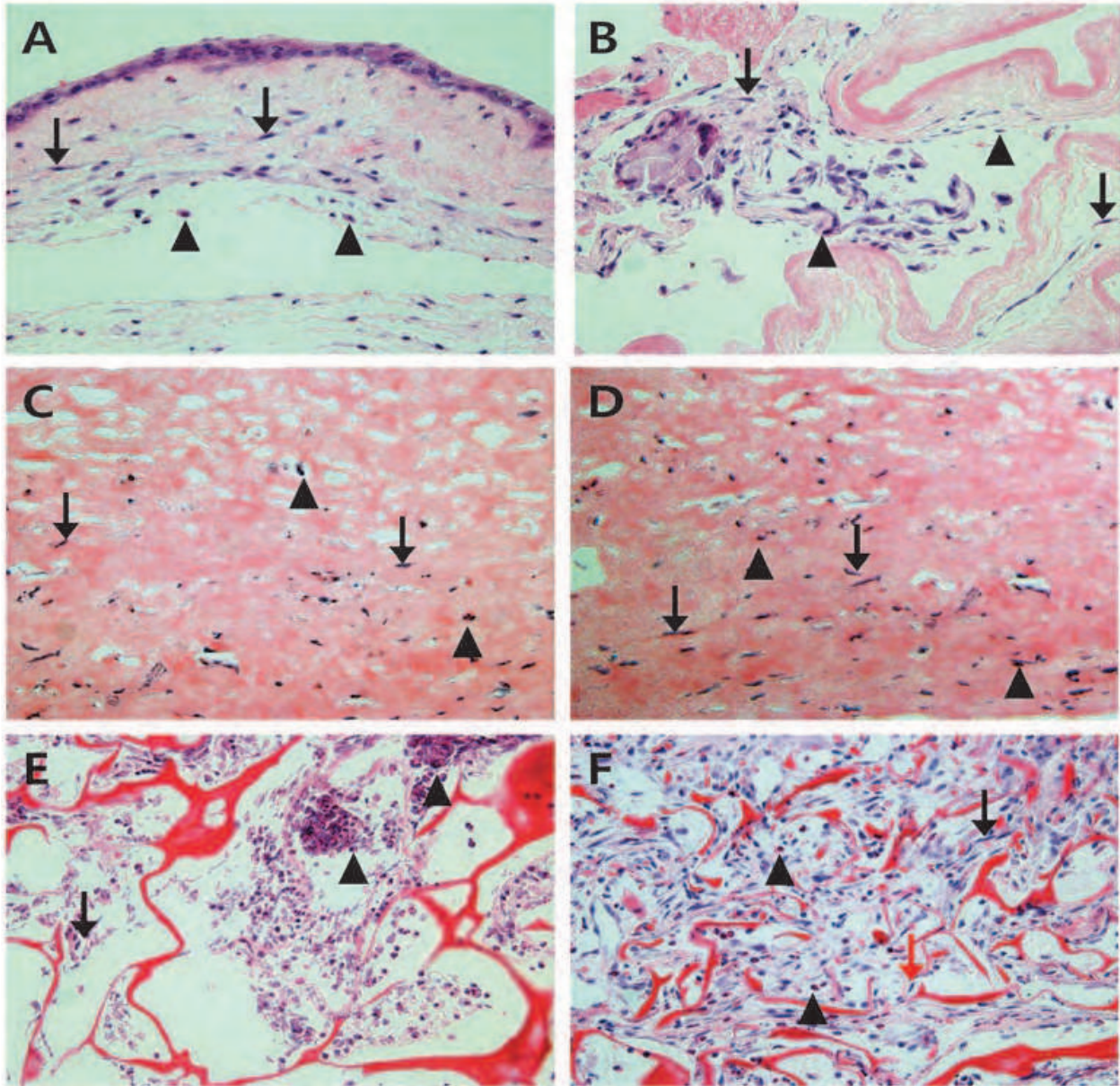


Fig. 2. Hematoxylin-eosin staining of amniotic membranes (A, B), lyophilized acellular porcine corneas (C, D), and collagen sheets (E, F) (B, D, F soaked in substance-P). Amniotic membranes and lyophilized acellular porcine corneas had more keratocytes (black arrow) than collagen sheets. Lyophilized acellular porcine corneas were shown to have less inflammatory cells (black arrowhead) than amniotic membranes and collagen sheets. Keratocytes were more visible in implants treated with substance-P. Original magnification: x400.

3.1.3 Expression of protein markers

The expression of protein markers was examined on implants that were kept for 7 and 14 days after surgery using RT-PCR (Fig. 3). The overall expression of protein markers was more remarkable in implants that were kept for 7 days than 14 days post-operatively, which is in agreement with the histologic results of stroma-like cells 7days after surgery.

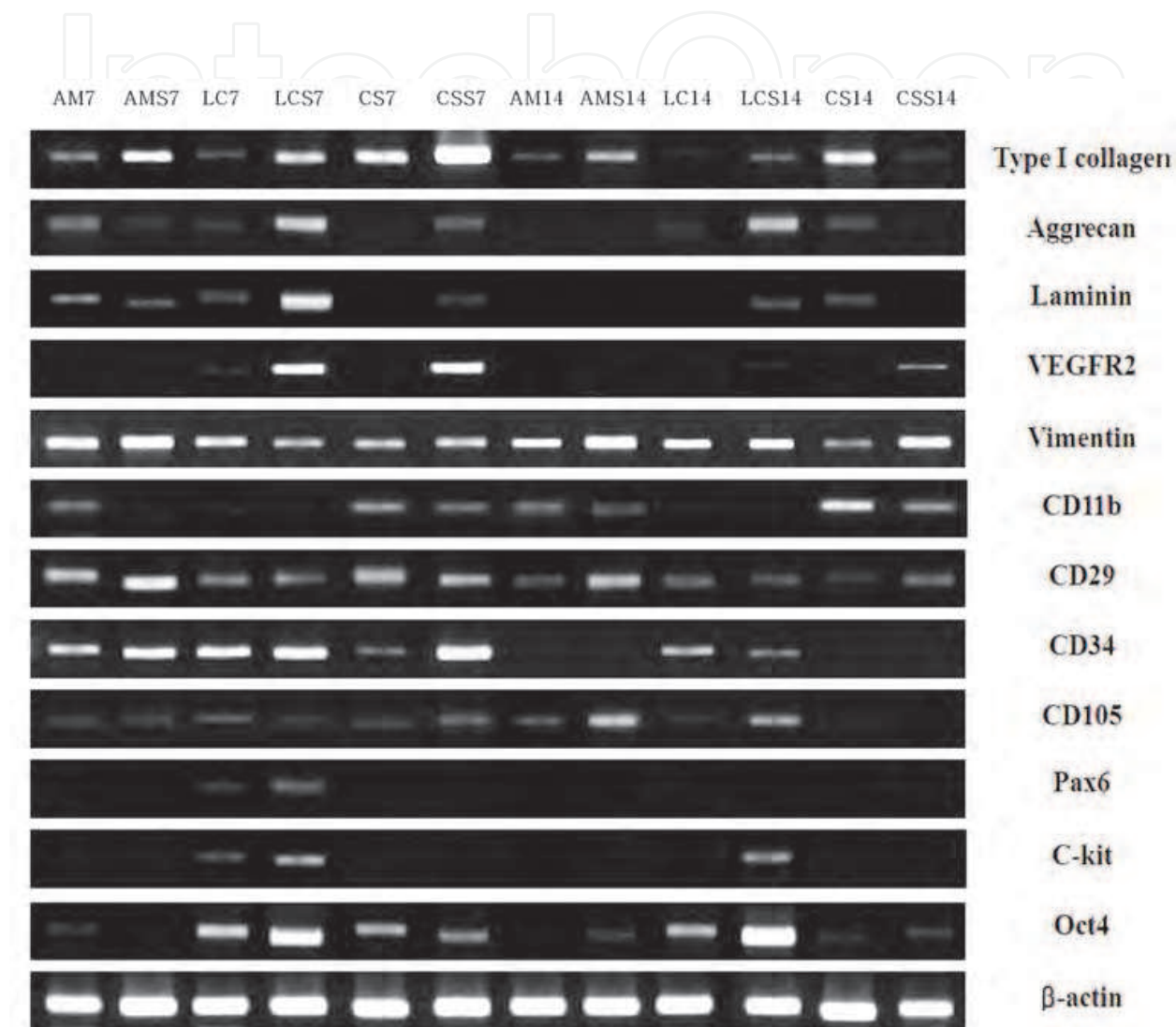


Fig. 3. Expression of various markers in subconjunctival-inserted implants. Most markers were more highly expressed in the implants 7 days after insertion than implants 14 days after insertion. Also, stem cell markers (c-kit and VEGFR2) and protein markers (aggrecan and laminin) were more expressed in implants treated with substance-P. AM : Amniotic membrane, AMS : Amniotic membrane soaked in substance-P, LS : Lyophilized acellular porcine cornea, LSS : Lyophilized acellular porcine cornea soaked in substance-P, CS : Collagen sheet, CSS : Collagen sheet soaked in substance-P, 7 : Implant which was kept for 7days after subconjunctival insertion, 14 : Implant which was kept for 14days after subconjunctival insertion

The corneal protein markers, aggrecan and laminin, had significantly increased expression in lyophilized APCs, especially APCs treated with substance-P. Among the stem cell markers, CD34, CD29, and CD105 had a similar level of expression between each implant, but c-kit was only expressed in lyophilized APCs. The expression of c-kit and VEGFR2 was more prominent in the lyophilized APCs treated with substance-p. Increased expression of the transcription and differentiation genes (Oct4 and Pax6, respectively) was also observed in lyophilized APCs in comparison with AMs and CSs. The expression of Oct4 and Pax6 was also more distinct in lyophilized APCs treated with substance-P. The expression of type I collagen was higher in CSs compared with AMs or lyophilized APCs.

3.2 Biocompatibility of lyophilized APCs treated with substance-P used in lamellar keratoplasty

Although corneal haziness was initially observed and aggravated around the graft in rabbits by 2 weeks, none of the lyophilized APCs showed signs of rejection or severe inflammation. Corneal opacity and neovascularization began to improve 3~4 weeks after surgery, and completely cleared after 6 weeks (Table 1, Fig. 4). The epithelium over the graft was beginning to heal 1 week after surgery, and was usually completed 6 weeks after surgery.

	Opacity scores	Neovascularization scores
1week	1.5±0.8†‡	1.4±0.5†‡
2weeks	2.8±1.0†‡	2.1±0.9†
3weeks	2.4±0.7†‡	2.8±0.7†‡
4weeks	1.4±0.7†‡	1.6±0.5†‡
5weeks	0.4±0.5†	0.8±0.5†‡
6weeks	0.0±0.0	0.4±0.5†
7weeks	0.0±0.0	0.0±0.0
8weeks	0.0±0.0	0.0±0.0

† p<0.05 comparison with grade 0 (Mann-Whitney U test)

‡ p<0.05 comparison with previous finding (Mann-Whitney U test)

Table 1. Comparison of corneal opacity and neovascularization in acellular porcine cornea group after surgery.

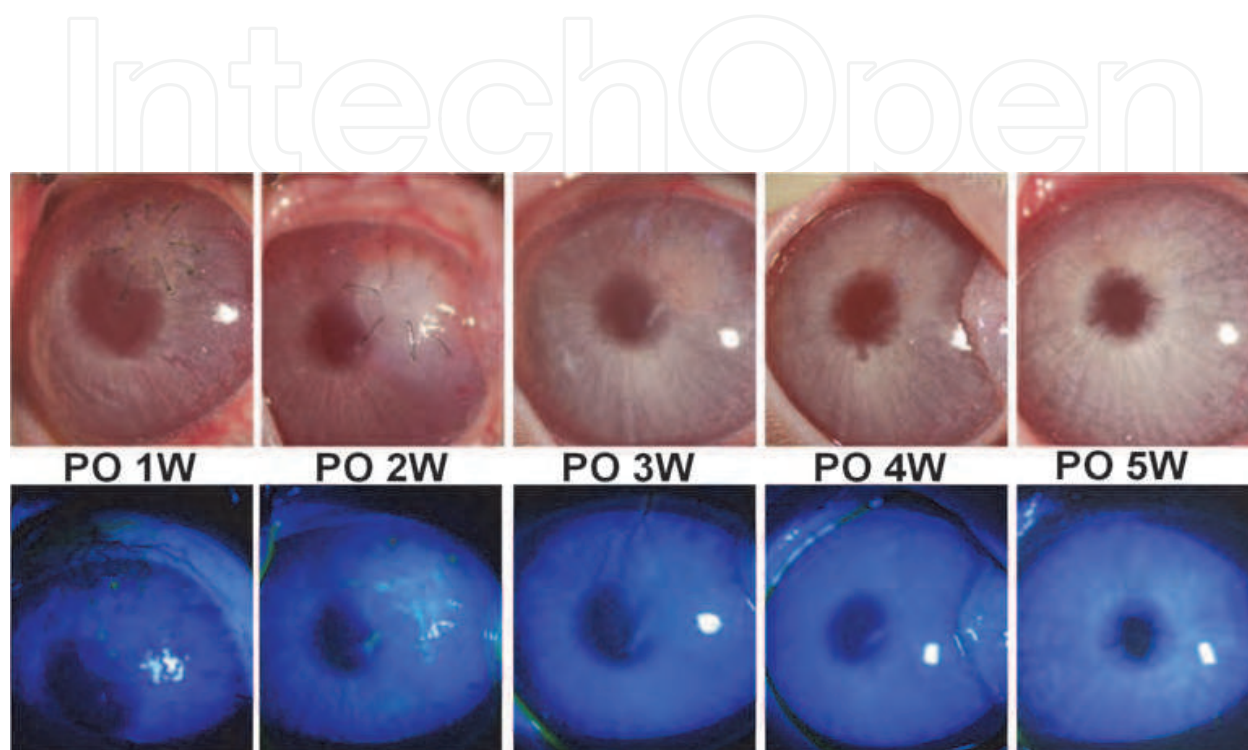


Fig. 4. Results of lamellar keratoplasty using lyophilized acellular porcine corneas. Corneal opacities and neovascularization appeared 1 week post-operatively, which became more severe 2 weeks after surgery. Opacity and neovascularization decreased 3~4 weeks post-operatively, and corneas recovered transparency 5 weeks after surgery.

Based on H&E staining, corneal stromal cells were observed in lyophilized APCs with rare infiltration of inflammatory cells 1 month after surgery. The lyophilized APCs were well-integrated into the host tissues, showing indistinct borders with normal rabbit corneal stroma on histology examination. These findings were more prominent 8 weeks after surgery. Vimentin staining showed viable stromal cells in grafted lyophilized APCs and recipient rabbit corneas, and a similar histologic pattern of grafted lyophilized APCs with recipient rabbit corneas (Fig. 5).

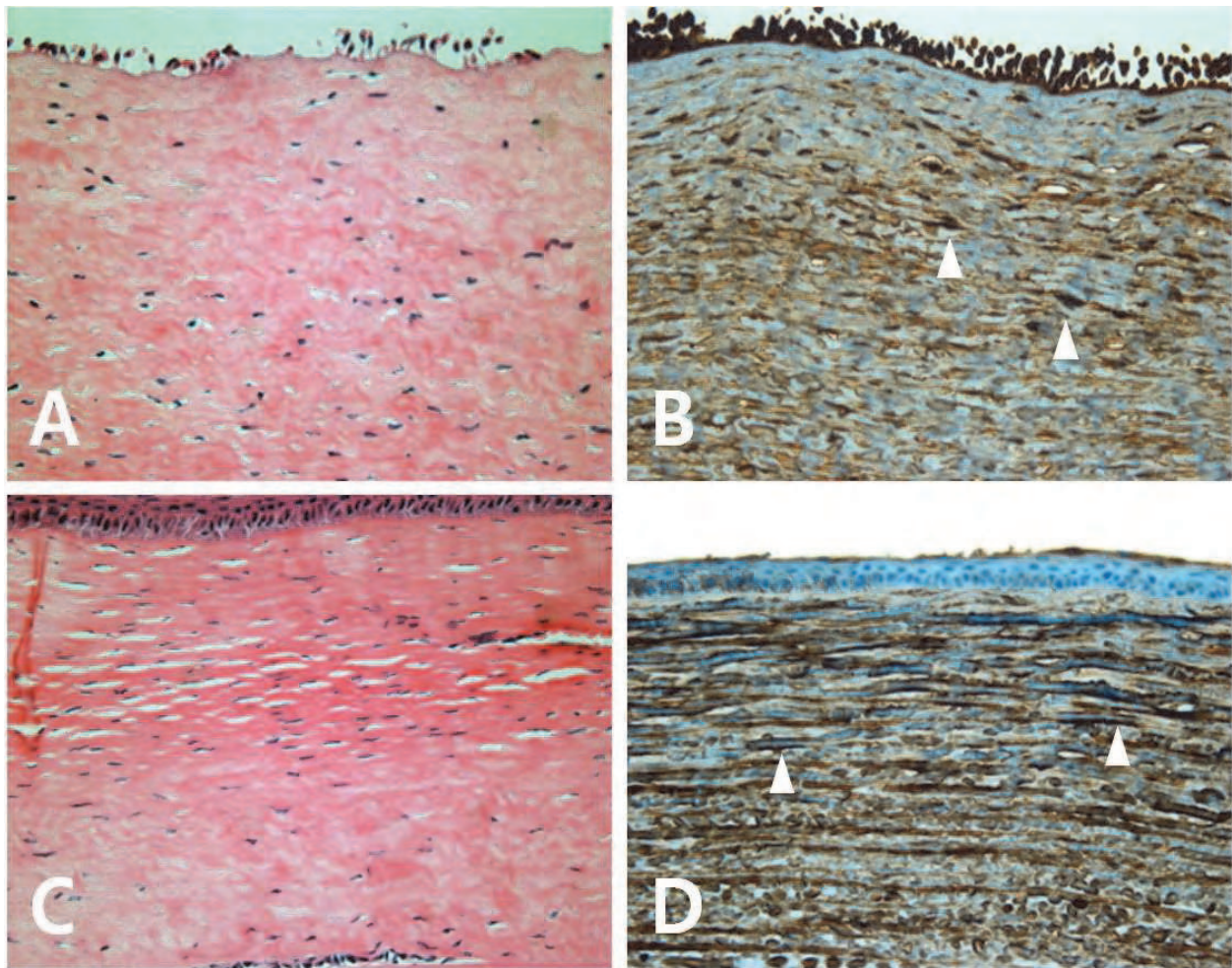


Fig. 5. Hematoxylin-eosin staining (A, C) and vimentin immune-histochemical staining (B, D) of grafted lyophilized acellular porcine cornea.

Corneal stromal cells presented in lyophilized APCs with rare infiltration of inflammatory cells 4 weeks after surgery (A). The lyophilized APCs were well-integrated into rabbit corneas with indistinct borders, and incomplete epithelization was shown. At 8 weeks, the number of corneal stromal cells increased and complete epithelization was observed (C). The staining aspects of vimentin in porcine corneas were very similar to the host cornea (B, D). (original magnification: x400)

4. Discussion

Porcine corneas have a well-organized structure similar to human corneas, and have now been extensively studied as a supplement to corneal collagen (Kampmeier et al., 2000; Xu et al., 2008). With respect to the use of porcine corneas, the most significant limitation is hyperacute or acute rejection, mainly due to xenogenic antigen (Li et al., 1992; Tseng et al., 2005). Several decellularization methods have been reported to decrease the antigenicity by removing stromal cells, but the ideal method for graft survival has not been determined

(Gilbert et al., 2006; Grauss et al, 2005; López-García et al, 2007). In the current study, we used physical methods to decellularize the porcine cornea by a freeze-thaw technique in combination with a centrifuge. In comparison with chemical methods destroying cells and collagen microstructures, this freeze-thaw technique has the advantage in eliminating stromal cells, while minimizing damage to the extracellular matrix (Gulati, 1988; Jackson et al., 1991). Lyophilization makes tissues less immunogenic by removing antigen-expressing cells and keeps the tissue sterilized for a longer period of time (Coombes et al., 2001; Pepose et al., 1991; Rostron et al., 1988; Zavala et al., 1985). We have previously reported the clinical and histological importance of lyophilization in producing less antigenicity and longer survival after xenotransplantation in rabbits (Lee et al., 2010).

Although decellularization is an essential process to minimize immune rejection, but transplantation of acellular lyophilized tissue alone might impede the healing process and increase the risk of infection, especially in immune-privileged tissues, such as the cornea. Efficient tissue regeneration usually needs repopulation of biologic decellularized scaffolds with interstitial cells (Lichtenberg et al., 2006). In the cornea, the role of corneal cells in optical transparency is already known. Accordingly, co-transplantation of acellular scaffolds with viable corneal cells appears to be important for graft survival and optical transparency in the cornea. In this study, we removed the stromal cells, and attempted *in vivo* cultivation rather than direct seeding of cells to increase graft survival and rapid acceptance. *In vivo* cultivation has been used for reconstruction of the ocular surface, and has advantage of maintaining corneal cell characteristics due to cell mitosis *in vivo* (Kim et al., 2008). Direct seeding of cells usually requires *ex vivo* cultivation, which is limited by facilities and equipment, and has a higher risk of infection.

In the present study, we showed that lyophilized APCs recellularized for 1 week had better optical transparency than APCs recellularized for different periods of time. The implants that were kept > 2 weeks had severe fibrosis adherent to the recipient tissue. This result indicates that implants in the limbus appear to serve as a medium for cultivation, and lacking the immune privilege of the eye, were ultimately rejected by the immune response. Nevertheless, it is remarkable that originally opaque APCs had better transparency after recellularization process for 1 week. Histologic examination demonstrated an abundance of stroma-like cells spreading into lyophilized APCs, which appear to be responsible for implants transparency. The expression of aggrecan and laminin in RT-PCR of lyophilized APCs implanted in the limbus of rabbits showed that cells grown in lyophilized APCs had the specific activity of keratocytes. Aggrecan is main glucoprotein of the sclera, and laminin is the main protein of the basement membrane, and thus has an important role in cell proliferation, migration, and adhesion (Doege et al, 1991; Dunlevy & Rada, 2004; Filenius et al., 2001; Yurchenko & Batton, 2009). These findings provide a plausible explanation as to why recellularized APCs in rabbit eyes showed better transparency, and supported the effectiveness of *in vivo* recellularization over the implants. Compared with lyophilized APCs, excessive infiltration of cells (primarily inflammatory cells) were noted on the bovine AMs and CSs. Multiple micropores on bovine AMs and CSs in comparison with the small surface area of lyophilized APC could be responsible for over-infiltration of cells.

Substance-P is known to stimulate migration of bone marrow stem cells, and accelerate the wound healing process (Hong et al., 2009). In this study, we attempted to evaluate the effect of substance-P treatment on implants, and found that expression of mesenchymal stem cell factors (CD 29 and CD105) was not affected by substance-P treatment. However, endothelial

stem cell factors (VEGFR2 and c-kit, but not CD34) were highly expressed in lyophilized APCs treated with substance-P. In addition, the increased expression of transcription and differentiation genes (Oct4 and Pax6) and ocular specific proteins (aggrecan and laminin) was also noted in lyophilized APCs treated with substance-P. The transcription factor Oct4 is critical for self-renewal and maintenance of embryonic stem cells, which has a role in controlling cellular phenotype (Zhou et al., 2010). Pax6, located on the short arm of chromosome 11, is known to produce a protein which is very important in ocular development (Ton et al., 1991; Glaser et al., 1992). It is difficult to validate the specific role of mesenchymal and endothelial stem cells during the recellularization process in this study. It is possible that the expression of stem cell factors might contribute to rejection in coordination with higher cellularity and enhanced angiogenesis. Still, higher expression of genes imperative for corneal development in lyophilized APCs treated with substance-P implicated the substantiality and validity of substance-P treatment in xenografts.

To identify the usefulness of our methods in preparing APCs, we transplanted lyophilized APCs with cells grown *in vivo* in rabbit corneas using lamellar keratoplasty. Previous studies using APCs reported at least 8 weeks to accept porcine collagen, suggesting that rejection could be inhibited by removing antigenicity, but failed to decrease the inflammatory reaction caused by the graft itself (Xu et al., 2008; Lin et al., 2008). Our study showed that corneal opacity improved within 4 weeks after the grafting, indicating that corneal stromal cells surviving in APCs function immediately after transplantation. Also, the histologic examination showed well-integrated implants with indistinct borders, and viable corneal stroma-like cells in vimentin staining. It is interesting that corneal haziness becomes aggravated immediately after transplantation, then improved 3~4 weeks after transplantation. The improvement in corneal haziness was accompanied with epithelialization over the implants, indicating the importance of tight junctions in the epithelium that controls the flow of fluid into the cornea.

This study suggests that lyophilized APCs repopulated with recipient allograft cells might be a physiologically functional tissue substitute in xenotransplantation. The rabbit cornea recipients stayed clean after receiving a lyophilized APC. Although, additional experiments are required to clarify the role of substance-P and the recellularization process, we believe that our results might provide a valuable clinical input to tissue-engineered corneal scaffolds using porcine corneas to facilitate the rapid restoration of the ocular surface.

5. Conclusion

The increase of ocular surface disease and shortage of cornea donors need the tissue-engineered corneal equivalent. The lyophilized acellular pig corneal stroma, which is devoid of α -gal epitope, is less antigenic than fresh pig corneal stroma, and might be a useful alternative to corneal tissue. We previously demonstrated that lyophilized APCs survived longer than fresh porcine corneas in pig-to-rat model, but the delayed healing and the risk of infection caused by an acellular substrate graft in immune-privileged corneal tissue require another resolution. In the present study, we investigated the effectiveness of lyophilized APCs with cells grown *in vivo* and stimulated with substance-P. The results showed that lyophilized APCs repopulated with cells grown *in vivo* for 1 week had better optical transparency compared with controls. More infiltrated corneal stromal-like cells observed in lyophilized APC in comparison with controls in histology might explain the better optical transparency, and higher expression of stem cell markers (c-kit and VEGFR)

and corneal protein markers (aggrecan and laminin) in lyophilized APCs repopulated with cells grown *in vivo* for 1 week support the importance of recellularization of graft before transplantation. These findings were more remarkable in lyophilized APC treated with substance-P, which implicated the possible role of substance-P in stromal cell maturation in cornea. Lamellar keratoplasty using lyophilized APC containing cells grown *in vivo* and stimulated with substance-P had good graft survival without rejection for 8 weeks. These results might provide a valuable clinical input to xenotransplantation using porcine cornea, and lyophilized APC with cells grown *in vivo* might be useful for ocular surface reconstruction.

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Unit 405, Office Block, Hotel Equatorial Shanghai
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Phone: +86-21-62489820
Fax: +86-21-62489821

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