

Original Article

Evaluation of short-term *ex vivo* culture with air/liquid system of porcine cornea

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Regarding therapies for treatment of corneal wounds, *ex vivo* corneal culture is the most effective for minimizing expensive animal studies. Eighteen porcine enucleated eyes were soaked in 0.2% povidone iodine solution for disinfection prior to cornea excision. Subsequently, corneas were excised from whole eyes and filled with an agar/medium mixture. Corneas were transferred into culture dishes, after which culture medium was added until the limbus was covered. Cultures were then placed onto a plate rocker to mimic blinking action, followed by incubation at 37°C and 5% CO₂. Corneas were harvested on Days 0, 3, and 7 after incubation, and optical coherence tomography (OCT) was performed on Day 7. Two eyes from each group were fixed in 2% glutaraldehyde/4% paraformaldehyde for low-vacuum scanning electron microscopy (LV-SEM), and four eyes from each group were fixed in 10% neutral-buffered formalin for histological analysis. OCT results showed that central corneal thickness significantly increased by Day 7 compared to Day 0 ($P < 0.05$). Using LV-SEM, gaps between endothelial cells were detected on Day 7 of *ex vivo* culture. In the histological evaluation, four to five stratified squamous cell layers, wing cells, and basal cells in the epithelium as well as flat-shaped keratocytes in the stroma were found on Day 0. By Day 7, stratified squamous cells and basal cells had decreased in number, and slightly round-shaped keratocytes were observed; however, the number of keratocytes was similar to that on Day 0. In this short-term *ex vivo* culture, epithelium and endothelium were sensitive to culture, whereas stroma and keratocytes were well maintained. An additional deswelling method will be needed to obtain more successful results in porcine corneal *ex vivo* culture.

Key words: cornea, *ex vivo* culture, air/liquid system, short-term, pig

Introduction

Ex vivo culture of corneas, first used in the 1980s for

corneal transplantation, is a method of keeping the cornea alive under culture conditions. This culture method is used to preserve the human cornea up to the time of transplantation [1] as well as to study the corneal response to drugs, various injuries, and transplants in animal eyes. Moreover, this method facilitates preclinical studies preceding animal studies in order to avoid unnecessary animal sacrifice [2, 3]. In corneal *ex vivo* culture, bovine, porcine, and rabbit corneas are most commonly used. Several studies have used human corneas in *ex vivo* culture; however, these are difficult to obtain. In comparison, bovine and porcine corneas can be easily obtained. The biology of the porcine eye is similar to that of the human eye and is therefore commonly used in *ex vivo* culture [4]. Whereas porcine corneal epithelium is slightly thicker, Bowman's layer and the stroma are similar to those in the human eye [5].

In nature, the cornea is provided with nutrients and growth factors primarily from the aqueous humor. However, in *ex vivo* culture, an additional nutritional supply is needed, and culture media are used to maintain the excised cornea. In humans, Optisol[®], which contains tissue culture medium 199, Earle's balanced salt solution, minimum essential medium, HEPES buffer, chondroitin sulfate, dextran, vitamins, precursors of adenosine triphosphate, gentamycin, and streptomycin, is widely used to preserve donor corneas, which can be stored in medium for up to 14 days [6]. However, Optisol[®] is expensive, and the required storage temperature for corneas is 4°C [7]. Therefore, long-term preservation is difficult due to low cultivation temperature [8]. Thus, commercial organ culture media are used for this type of *ex vivo* culture, and various media supplements and compositions have been studied [9, 10].

In this study, to mimic blinking action, an air-liquid system was used with a rocker. In previous studies, most corneal organ culture models used static culture methods

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[11, 12]. However, in an *in vivo* situation, corneas are kept intermittently moist by blinking motion and are not submerged. Further, Richard *et al.* reported that static, submerged organ cultured corneas present epithelial, stromal, and endothelial edemas [13].

In the present study, we evaluated normal changes during short-term porcine corneal *ex vivo* culture with optical coherence tomography (OCT), low vacuum scanning electron microscopy (LV-SEM), and histological analysis.

Materials and Methods

Ex vivo culture of excised porcine corneas

Eight pig heads were obtained from a local slaughterhouse (Samho Livestock Co., Gwangju, Korea), and eyes were enucleated within 4 hr of death. Enucleated eyes were soaked for 2 min in 0.2% povidone iodine solution for disinfection and were irrigated with sterile saline. For *ex vivo* culture, corneas were excised from the eye globe, leaving a 3~5 mm circular rim of sclera (Fig. 1A). After excision, corneas were filled with agar mixed with culture medium and transferred into culture dishes. Subsequently, culture medium was added until it covered the limbus (Fig. 1B). Culture medium consisted of Dulbecco's modified Eagle's medium (Gibco, USA)/F12 (Gibco, USA) supplemented with an insulin-transferrin-selenium mixture (BD Biosciences, USA), penicillin/streptomycin, and amphotericin B. Cultures were placed on a plate rocker and incubated at 37°C and 5% CO₂ (Fig. 1C). Corneas were harvested on Days 0, 3, and 7 days after incubation commencement.

Optical coherence tomography (OCT)

A custom OCT system was used in this study. A swept-wavelength light source (SSOCT-1310, AXSUN Technologies) was used with a center wavelength of 1310 nm, bandwidth of 107 nm, sweeping speed of 50 kHz, and imaging depth range of 6 mm in air. Light from the source was split with 95:5 ratios: 95% of light went into the interferometry setup, and the remaining 5% was directed to a fiber Bragg grating to generate an external trigger signal

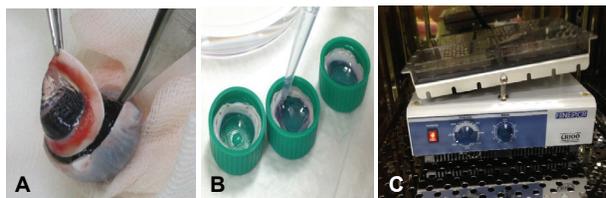


Fig. 1. The procedure of porcine corneal *ex vivo* culture. (A) Excision of sclera with #15 blade and elevation of the corneal button from the porcine globe, (B) Mold formation beneath the corneal button, (C) Rocking motion with rocker for the air-liquid system.

for data acquisition. For interferometry, light was split into the sample and reference arms by using an 80:20 coupler. In the sample arm, light was collimated to 1.8 mm in diameter with a fiber collimator (HPUCO-13A-1300/1550-S-11AS, OZ optics), passed through a 2D galvano mirror scanner (GVS012, Thorlabs) and an objective lens (LSM03, Thorlabs), and then focused at the sample. Light in the reference arm passed through a delay line in order to match optical path lengths in both arms. Reflected light from both the sample and reference arms was combined and collected by two balanced photodetectors (PDB410C, Thorlabs) in the polarization diverse detection setup. Signals from detectors were digitized by a two-channel data acquisition board (Alazartech) after passing through low-pass filters (81 MHz cut-off frequency, Mini-circuits). Stored data were processed to obtain OCT images. Dispersion difference between the reference and sample arms was compensated numerically by using precalibration data with a mirror sample. OCT images were obtained in 3D by acquiring multiple cross-sectional images in the x-z plane with step-wise increment in the y direction. Imaging field was typically 5 mm × 5 mm in the x and z directions, consisting of 500 pixels × 640 pixels. The signal-to-noise ratio of the system was 103 dB, and the frame rate was 100 frames/s. Central corneal thickness (CCT) was reported as the mean ± S.D. To compare OCT results between Days 0 and 7, an independent t-test was used. A *P*-value of <0.05 was considered to be statistically significant.

Low vacuum scanning electron microscopy (LV-SEM)

After OCT measurement, two corneas from each group were fixed in 2% glutaraldehyde/4% paraformaldehyde overnight at 4°C and dehydrated through an increasing series of ethanol solutions. Thereafter, specimens were submitted to critical point drying using liquid carbon dioxide and air-dried. Endothelial surfaces were photographed by LV-SEM (JEOL-JSM IT300, Tokyo, Japan).

Histological evaluation

After OCT measurement, four corneas from each group were fixed in 10% neutral-buffered formalin, dehydrated in an ascending series of alcohol rinses, and embedded in paraplast (Sherwood Medical Industries, USA). Embedded specimens were sectioned to a thickness of 5 μm with a microtome (Cambridge Instruments, Germany). Slides

Table 1. Central corneal thickness assessment by optical coherence tomography (OCT) on Days 0 and 7 of *ex vivo* culture

Technique	Central corneal thickness (μm)	
	Day 0 (n=6)	Day 7 (n=6)
OCT	868.53 ± 107.28	2925.00 ± 413.60*

Quantitative data are expressed as the mean ± S.D.
**P*<0.05 when compared with the Day 0 group.

were stained with hematoxylin and eosin and evaluated for histological changes by microscopy.

Results

Evaluation by OCT

In OCT, mean CCTs on Days 0 and 7 were $868.53 \pm 107.28 \mu\text{m}$ and $2925.00 \pm 413.60 \mu\text{m}$, respectively (Table 1). On Day 7, mean CCT had increased about 3.36-fold more than that on Day 0.

Evaluation by LV-SEM

LV-SEM for corneal endothelium was performed on Days 0 and 7. On Day 0, a regular polygonal pattern was observed, and a round nucleus was detected in the center of the endothelial cell (Figs. 2A and 2C). On Day 7, a regular polygonal pattern of endothelial cells was also detected, although, gaps between endothelial cells in partial areas were found (Figs. 2B and 2D).

Histological evaluation

On Day 0, typically four to five layered, stratified squamous cells as well as wing cells and basal cells were found in the epithelium (Fig. 3A). On Day 3, overall epithelial thickness had decreased, and the stratified squamous cell layer and basement membrane were thinner than those on Day 0 (Fig. 3B). On Day 7, overall epithelial thickness was similar to that on Day 3. However, the stratified squamous cell layer decreased, along with numbers of wing cells and basal cells (Fig. 3C). In the stroma, parallel-arranged collagen lamellae and flat-shaped keratocytes were observed on Day 0 (Fig. 3A).

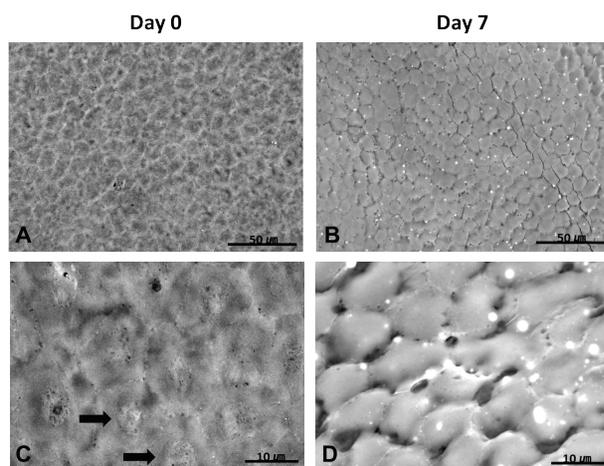


Fig. 2. Low vacuum scanning electron micrographs of porcine corneal endothelium on Days 0 and 7 after *ex vivo* culture. (A) Porcine corneal endothelium on Day 0 ($\times 500$), (B) Porcine corneal endothelium on Day 7 ($\times 500$), (C) Porcine corneal endothelium on Day 0 ($\times 2,000$), (D) Porcine corneal endothelium on Day 7 ($\times 2,000$). Arrow: Nucleus in endothelial cell.

On Day 7, slightly round-shaped keratocytes were found, although the overall population of keratocytes was not reduced (Fig. 3C).

Discussion

Ex vivo culture of intact corneas has many advantages over culture of small pieces of explanted corneas or corneal cells. First, cultured intact corneas are very similar to *in vivo* tissue [3]. Intact cornea has an intact epithelium, stroma, and endothelium, which maintains corneal hydration and cell signaling; the latter is very important for the wound healing process. Second, the limbus at the corneal margin has many stem cells and thus can provide a source of proliferative cells [14, 15]. Third, in wound studies, healing of wounded corneas in *ex vivo* culture is a similar wound healing process as that *in vivo*.

Corneas can be cultured *ex vivo* for up to 48 days [16]. However, corneal re-epithelialization after chemical burns or photorefractive keratectomy requires a shorter culture period (4–7 days) [17, 18].

To investigate normal changes in short-term corneal culture, *ex vivo* culture was performed for 7 days with porcine eyes. Using OCT, we evaluated CCT and swelling of tissue. Using LV-SEM, corneal endothelium was evaluated for cell-cell interdigitation and morphology to observe the general status of the endothelium with histology. Using histological evaluation, corneal epithelium and stroma were identified. In OCT, the mean CCT on Day 7 increased by about 3.36-fold compared to Day 0. In LV-SEM, on Day 7, a regular polygonal pattern of endothelial cells was detected; however, several cells were separated at tight junctions. Additionally, in the histological findings, the stratified squamous cell layer was reduced, numbers of wing and basal cells had decreased in the epithelium, and slightly round-shaped keratocytes and stromal swelling were observed. However, the overall population of keratocytes had not decreased in the stroma on Day 7. These results suggest that corneal swelling was present on Day 7 possibly due to breaking-up of endothelial cell-cell junctions. The corneal endothelium forms a barrier between the aqueous humor and

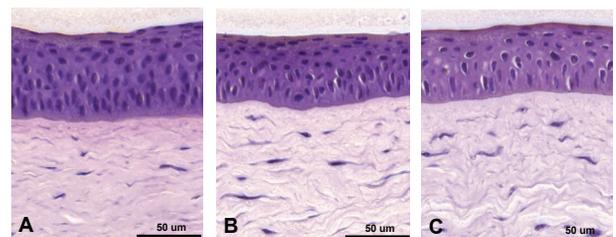


Fig. 3. Histological findings of porcine corneal epithelium and stroma on Days 0, 3, and 7 of *ex vivo* culture. (A) Day 0 of culture, (B) Day 3 of culture, (C) Day 7 of culture (hematoxylin and eosin staining, scale bar=50 μm).

stroma *in vivo*. Tight junctions and metabolic-pump sites in the epithelium maintain corneal deturgescence, and destruction of the endothelium results in corneal edema and swelling [19]. Although the cause of corneal swelling in *ex vivo* culture is unknown, one of the causative factors is endothelial-cell disruption, along with other factors such as metabolic changes in the culture medium, nutrient deprivation, mechanical stress, and loss of survival factors [20].

According to a similar study by Smith *et al.* [21], extensive wing cell death and epithelial detachment from the stroma occurred on Day 5 of *ex vivo* culture in Optisol®. However, there was no severe increase in corneal thickness. In contrast, epithelial thickness decreased, whereas epithelial detachment was not observed in any samples. Further, in histological evaluation, reduced keratocyte population was not detected. To confirm these results, further investigation such as keratocyte density assay is needed.

In conclusion, corneal *ex vivo* culture with organ culture medium is an effective culture method. However, to prevent corneal swelling, addition of growth factors or specified culture media that protect the endothelium or a deswelling agent should be considered in future studies.

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