In Vivo-Like Culture Conditions in a Bioreactor Facilitate Improved Tissue Quality in Corneal Storage

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The cornea is the most-transplanted tissue worldwide. However, the availability and quality of grafts are limited due to the current methods of corneal storage. In this study, a dynamic bioreactor system is employed to enable the control of intraocular pressure and the culture at the air-liquid interface. Thereby, in vivo-like storage conditions are achieved. Different media combinations for endothelium and epithelium are tested in standard and dynamic conditions to enhance the viability of the tissue. In contrast to culture conditions used in eye banks, the combination of the bioreactor and biochrom medium 1 allows to preserve the corneal endothelium and the epithelium. Assessment of transparency, swelling, and the trans-epithelial-electrical-resistance (TEER) strengthens the impact of the in vivo-like tissue culture. For example, compared to corneas stored under static conditions, significantly lower optical densities and significantly higher TEER values were measured (p-value <0.05). Furthermore, healing of epithelial defects is enabled in the bioreactor, characterized by re-epithelialization and initiated stromal regeneration. Based on the obtained results, an easy-to-use 3D-printed bioreactor composed of only two parts was derived to translate the technology from the laboratory to the eye banks. This optimized bioreactor facilitates noninvasive microscopic monitoring. The improved storage conditions ameliorate the quality of corneal grafts and the storage time in the eye banks to increase availability and reduce re-grafting.

1. Introduction

The cornea represents the window of our eye, allowing light to reach the sensory cells and us to see. In humans, it is 0.5 mm thick, 10–12 mm in diameter, spherically shaped, and has the main refractive power of the eye with 43 dioptres. Due to corneal damage or degenerative, infectious, dystrophic, and inflammatory disorders, numerous corneal diseases lead to opacity and blindness. Currently, 1.9 million people worldwide are affected and 5.2 million people are visually impaired.[1] The main method of visual rehabilitation after corneal opaqueness is the transplantation of a donor tissue, and corneas are among the most successful-transplanted tissues.[2] However, a lack of a sufficient number of donor tissues results in long waiting lists, especially in developing countries. Moreover, the number of donors is declining due to demographic changes and an increasing frequency of corneal refractive surgery.[3] To tackle this need, more donors should be recruited and the storage of corneal tissues should be improved.[2] In addition to availability, corneal grafts of high quality might increase the transplantation success rate, thereby reducing the need of re-grafting and preventing associated negative impacts on patients. Currently, there are two main preservation storage methods: hypothermia and organ culture. Hypothermic storage of corneas at 2–8 °C with Optisol-GS is the most-used system in the US; allowing to maintain corneas for up to 14 days.[4] In hypothermic storage, the epithelium is lost, and thus 7 days of storage are usually not exceeded. In Europe, the organ culture of corneas at 28–37 °C in biochrom medium 1 is applied in the majority of

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eye banks.[2] Thereby, corneas are cultured for up to 4 weeks. However, high quality standards lead to a high rate up to 41% of discarded corneas, depending on donor age and storage duration.[3]

The main criterion, which is associated with the transparency of the transplant, is the state of the endothelial cells covering the inner corneal surface. An impaired integrity of the epithelium and swelling are further indicators for tissue degradation. Current corneal storage conditions allow the preservation of endothelial cells for sufficient clinical outcomes. These conditions, however, lack the ability to sufficiently preserve the epithelium, and can thereby entail postoperative complications.[5] Although the donor epithelium is replaced by host cells following the transplantation of a cornea, epithelial defects can heal slowly and an intact donor epithelium can be essential for graft survival.[6] Especially for patients that suffer ocular surface diseases such as Stevens–Johnson syndrome or severe dry eyes, an insufficient integrity of the donor epithelium increases the risk of infection, scarring, stromal thinning, and perforation.[5,7]

In addition to clinical applications, the in vitro culture of corneas is also interesting for basic research, e.g. in drug development or for the mechanistic understanding of wound healing and target-driven therapies. Thus, for clinical and basic research applications, the identification of methods that allow to preserve the endothelium and the epithelium has gained importance. To ameliorate the state of the endothelial and epithelial layer during corneal storage, different media combinations were tested in our study. Moreover, to provide in vivo-like stimuli, a bioreactor system was developed and compared to the standard conditions employed in eye banks.

2. Experimental Section

2.1. Design of Bioreactor and Cell Crowns

The bioreactors and the cell crowns were designed in SolidWorks (Dassault Systèmes SolidWorks Corp, Waltham, USA). The cell crowns were milled from stainless steel. The bioreactors were either milled from polysulfone or 3D-printed, using the digital light processing technology. The material for 3D printing was a biocompatible acrylate-based polymer (Technical University Munich, Germany).

2.2. Biological Material

Porcine eyes from 5-month-old pigs were obtained from the local slaughter house immediately after slaughter. Eyes were directly transferred in DMEM (Invitrogen, Darmstadt, Germany) + 10 % fetal calf serum (Lonza, Cologne, Germany), and 1% Penicillin/Streptomycin (PAA, Coelbe, Germany). At the same day, corneas were removed together with a 3 mm scleral ring and washed eight times in PBS (Invitrogen) under sterile conditions.

2.3. Culture Conditions

Different media combinations were tested in static and dynamic culture:

(I) Native cornea as reference.
(ii) Standard culture with biochrom medium 1 (Merck Biochrom GmbH, Berlin, Germany) as reference.
(iii) Biochrom medium 1 either static (cell crown) or dynamic (bioreactor) to identify the impact of the two culture systems.
(iv) 14.4 mM $CaCl_2$ solution (Sigma Aldrich, Munich, Germany) on the epithelial side and VascuLife® with EnGS-Mv Microvascular LifeFactor Kit with 5% fetal bovine serum (LifeLine® CellTechnology, Maryland, USA) on the endothelial side. According to a patent for eye drops (US 8648057 B2), an elevated calcium concentration has a positive effect on epithelial growth. Thus, we tested this condition in static and dynamic cornea culture.
(v) Epilife™ Basal medium with defined growth supplements (Cascade Biologics, Portland, USA) and 1.44 mM $CaCl_2$ solution on the epithelial side and VascuLife® with EnGS-Mv Microvascular LifeFactor Kit with 5% fetal bovine serum on the endothelial side. This medium combination provides optimal medium conditions for the epithelium and endothelium.
(vi) Epilife™ Basal medium with 20% of the recommended growth supplements and 1.44 mM $CaCl_2$ solution on epithelial side and VascuLife® with EnGS-Mv Microvascular LifeFactor Kit with 1% fetal bovine serum on the endothelial side. This condition allows to identify the impact of the growth factors on tissue characteristics.

Except for the biochrom medium 1, all media were supplemented with 1% Gentamicin (Genaxxon bioscience GmbH, Ulm, Germany) and 1% Penicillin/Streptomycin as well as 1% Amphotericin B (Gibco™, Thermo Fisher Scientific, Waltham, USA). Corneal tissues were stored in standard submerse culture and in cell crowns[8] at 37 °C, 5% CO_2 and 95% humidity for 7 days. For the dynamic culture, the bioreactor was operated at 37 °C and 5% CO_2. The corneas were mounted in the bioreactor by a fixator between the main part and the lid (**Figure 2**A). A supporting membrane was not required due to the intraocular pressure and the stability of the tissue. Pressure was monitored by a pressure transducer (PI in the schematic drawing in Figure 2A, HJK Sensoren + Systeme GmbH & Co. KG, Merching, Germany). Based on the measured pressure value, the speed of the pump (P1 in the schematic drawing in Figure 2A, MCP-E MS/CA 4-12, ISMATEC Laboratoriumstechnik GmbH, Wertheim, Germany) was adjusted to reach the required intraocular pressure of 7–15 mmHg. Therefore, a microcontroller (ET200S, Siemens AG, Munich, Germany) was used. Flow rates were in the range of 2.9 mL min^{-1} on the endothelial side and 1.9 mL min^{-1} (one drop each 5 s) on the epithelial side. Due to the modified geometry, the 3D-printed bioreactor was operated with a flow rate in the range of 9 mL min^{-1} on the endothelial side and an alternating air-liquid phase with 37.5 mL min^{-1} influx and efflux flow rate on the epithelial side. All tailored culture systems were adapted to the size and the shape of native porcine corneas.

2.4. Corneal Wounding

A standardized wound with a deepness of 300 µm and a wound-diameter of 1 mm was induced with an Excimer Laser (Schwind Amaris® 750S, Kleinostheim, Germany). Following, corneas were cultured in the bioreactor as well as in the cell crowns for 7 days using medium condition V.

2.5. Fluorescein Eye Stain Test

Fluorescein staining was performed by applying one drop Fluoreszein SE Thilo® (Alcon, Hünenberg, Switzerland). Tissues were immediately washed with PBS and pictures were taken under blue-filtered light.

2.6. Absorption Scan

The absorption spectrum of the corneas was measured with the plate reader infinite® M200 (Tecan, Crailsheim, Germany) within the visual field (400–800 nm) and a step size of 5 nm. Therefore, the corneas were placed in a 6-well plate. For statistical comparison, the absorption curve was integrated using the Simpson's rule.

2.7. Impedance Measurement

After culture, impedance spectroscopy was performed as endpoint measurement to calculate the TEER. Therefore, the corneas were harvested and transferred into a measuring chamber. In the
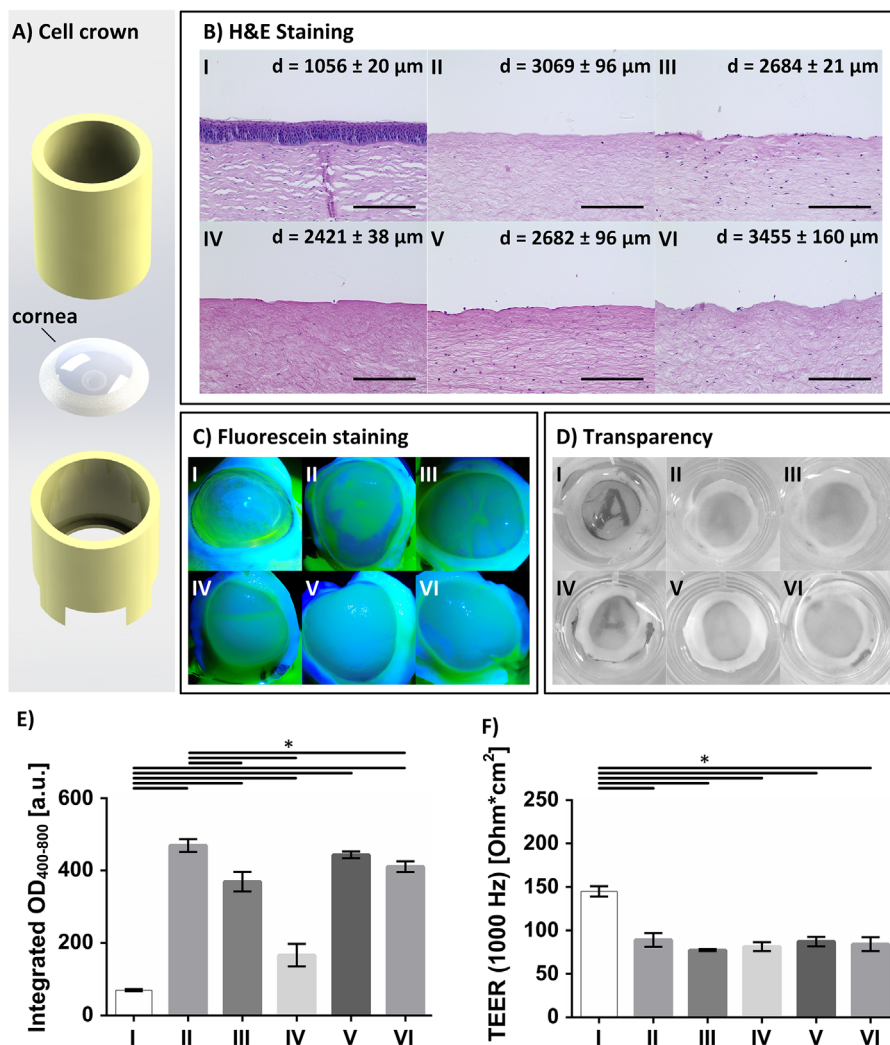
Figure 1. Static culture of native cornea. A) A cornea was fixed between two rings of a cell crown and cultured for 7 days in different media combinations (III, IV, V, and VI). As control, native tissue (I) and standard eye bank conditions (II) were used. B) (H&E) staining, C) fluorescein staining, D) transparency, E) absorption, and F) electrical resistance measurements were performed to assess the tissue following culture. Scale bar depicts 200 µm; $n = 3$; star indicates significant differences ($p < 0.05$); d in B) denotes the tissue thickness; I: native porcine cornea; II: standard culture in eye banks (biochrom medium 1 without compartmentalization); III: biochrom medium 1 on both sides; IV: $CaCl_2$ in the upper (epithelial side) and VascuLife® in the lower (endothelial side) compartment; V: EpiLife™ in the upper and VascuLife® in the lower compartment; 6: EpiLife™ in the upper and VascuLife® with a reduced amount of growth factors in the lower compartment.

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chamber, electrodes are incorporated to measure the complex electrical resistance in a frequency range from 1 to 100 kHz through the tissue. From the measured spectrum, the impedance at 1000 Hz was selected and multiplied with the tissue surface to characterize the epithelial barrier. The measuring chamber and the technical approach is described in Groeber et al.[9]

2.8. Histological Analysis

For histological analysis, corneas were immersion-fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Samples were deparaffinised in xylene and rehydrated in a graded series of ethanol. Hematoxylin and eosin staining (H&E) for general morphological assessment was performed.

2.9. Statistical Analysis

Normal distribution was confirmed using the Shapiro–Wilk test. Significant differences were identified by the one-way ANOVA with Tukey's multiple comparison method. Values are shown as mean ± SD. A p-value <0.05 was considered as significant.

3. Results

3.1. Static Culture with Different Media Combinations Reveals a Low Impact on Tissue Quality

In eye banks, the cornea is fixed in a cage and the epithelium as well as the endothelium are exposed to the same medium, although it is known that most cells require specific nutrients and growths factors. To investigate the impact of cell-specific culture media combinations, porcine corneal tissues were fixed in a cell crown culture system (**Figure 1**A); resulting in an apical and basolateral compartment. VascuLife®, a medium designed to promote endothelial culture conditions was chosen for the basolateral compartment. For the apical compartment, media variations based on the basal medium Epilife™, which was supplemented with epidermal growth factor or/and calcium stimuli, were evaluated considering their capacity to support cell growth and differentiation. Biochrom medium 1, the standard culture medium in eye banks, served as control. H&E staining was performed to evaluate the tissue architecture (Figure 1B). Directly after explantation, native corneas showed a multilayered epithelium (Figure 1B I), whereas no epithelial cells could be seen (otherwise two times observed) in the standard conditions in biochrom medium 1 (Figure 1B II) and in all other media combinations after 7 days in culture (Figure 1B III–VI). Moreover, tissue swelling was observed. In contrast to the fresh native porcine cornea with a thickness of 1056 ± 20 μm, static culture resulted in a thickness up to 3455 ± 160 μm (Figure 1B, d denotes tissue thickness). The absence of the epithelium was confirmed by fluorescein staining (Figure 1C). In this clinical standard test, a patterned yellow staining visualizes areas, where the epithelial cell layers are disrupted. The yellow-stained areas at the border of the native cornea are associated with pooling effects of the fluorescein (Figure 1C I). The standard culture

(Figure 1C II) revealed a disrupted epithelium at the center of the tissue. For all the other conditions, a complete loss of the epithelial layer was detected (Figure 1C III–VI). To note, due to the severe swelling of the stroma (Figure 1B) and the complete degeneration of the epithelium, the staining, however, resulted in an unnatural homogeneous blue color, e.g., Figure 1C V. Additionally, corneal tissue cultured in medium conditions II, III, V, and VI showed a reduced transparency compared to condition IV and native corneal tissue (Figure 1D). This result could be confirmed by the measurement of the optical density, whereby condition IV had the lowest integrated OD with 166 ± 31 a.u. (OD, Figure 1E). Furthermore, the epithelial barrier of the corneas was assessed by TEER measurements (Figure 1F). Compared to the native cornea with a TEER value of 145 ± 6 Ω*cm², all other corneas revealed significantly decreased TEER values down to 77 ± 1 Ω*cm²; demonstrating an impaired barrier function. These results emphasize that it is not possible to maintain a functional epithelial layer of porcine corneas for at least 1 week in static culture conditions.

3.2. Dynamic Culture in a Bioreactor Improves Tissue Quality

To improve the culture conditions, a bioreactor was designed to facilitate an in vivo-like organ culture (Figure 2A). The bioreactor harnessed two fluid circuits; one to mimic the intraocular pressure (circuit 1) and a second one to simulate tear drops (circuit 2). During culture, the intraocular pressure was continuously monitored and controlled by a μ-controller-operated pump. Both circuits hosted different media. In contrast to the static cultures (Figure 2B II), H&E staining of the dynamic cultures showed that the epithelial cell layers were preserved for all media combinations (Figure 2B III–VI). Moreover, swelling was reduced. The maximum measured corneal thickness following dynamic culture was 2248 ± 74 μm in condition IV whereas condition 3 resulted in a minimal increase of approximately 500 μm (Figure 2B, d denotes tissue thickness). These findings were confirmed by fluorescein staining (Figure 2C). Considering the transparency, conditions III and VI resulted in a slight opacity, whereas in conditions II, IV, and V, the transparency was strongly impaired (Figure 2D and E). Most striking, between the native control (OD = 69 ± 2 a.u.) and conditions III (OD = 100 ± 35 a.u.) and VI (OD = 114 ± 38 a.u.) no significant differences were found in OD. TEER values of 145 ± 6 Ω*cm², measured for native corneas, are a further indicator that strengthens the positive effect of the bioreactor culture on the epithelial barrier (Figure 2F). The dynamic culture of a cornea also allowed the preservation of the endothelium, addressing the currently accepted quality criteria for transplantation (exemplarily shown for condition V, **Figure 3**A).

3.3. 3D-Printed Bioreactor for Noninvasive Monitoring and Improved Handling

To examine the impact of the dynamic culture system on tissue regeneration, corneas were wounded by an Excimer-Laser. Following, tissues were cultured either under static conditions in
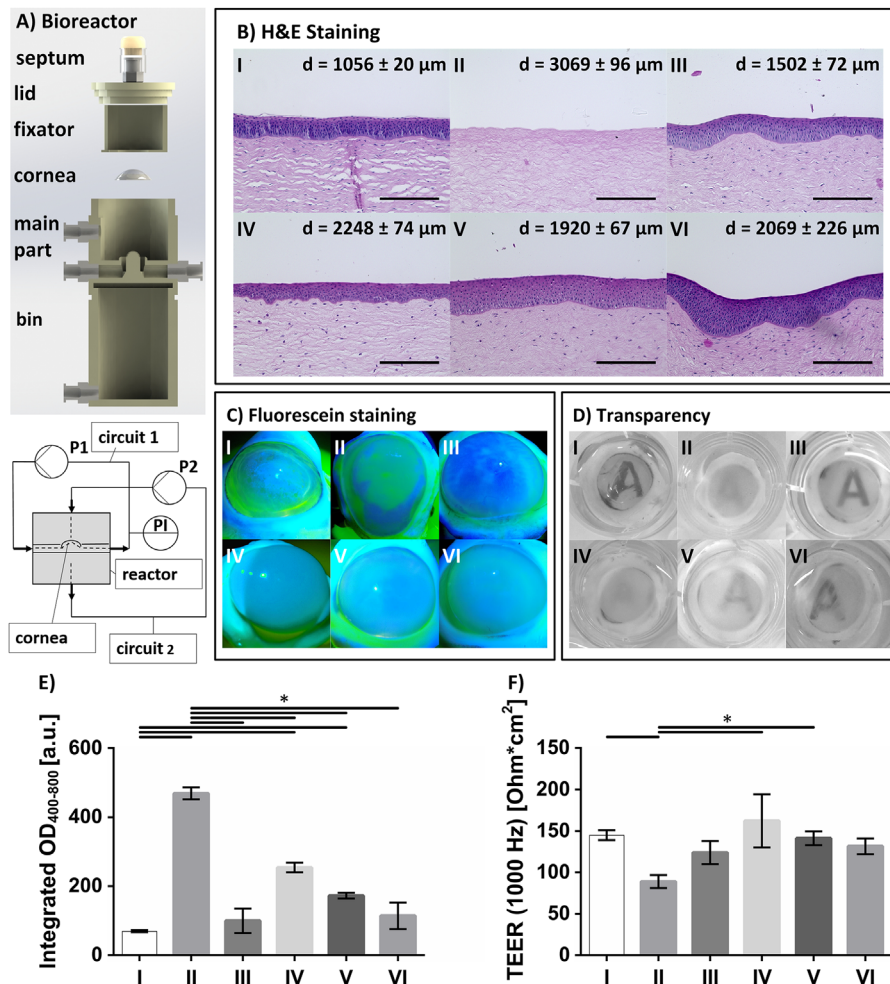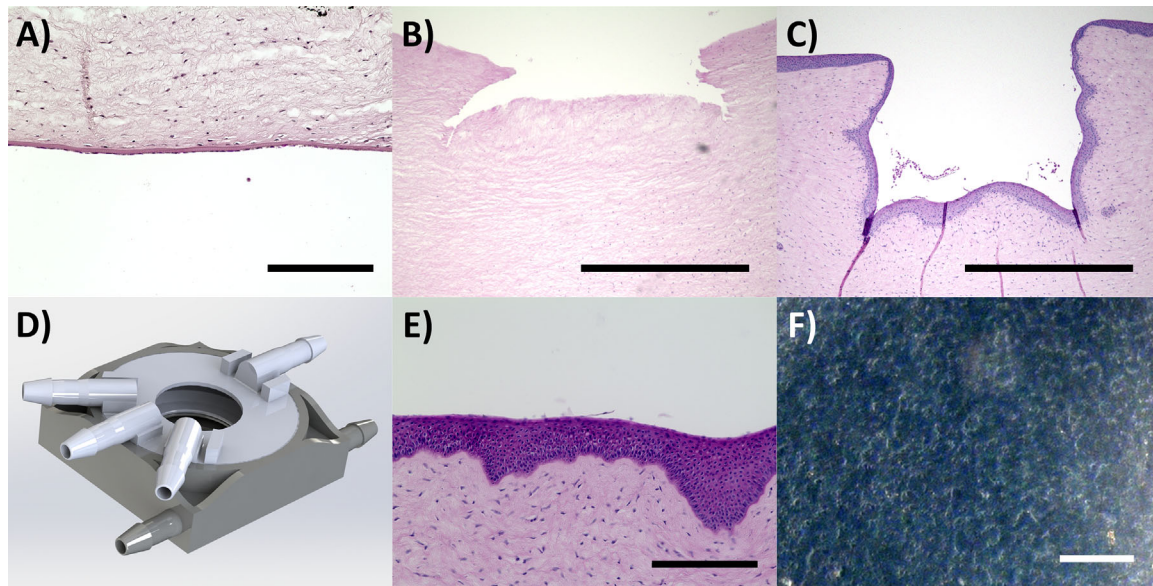
Figure 2. Dynamic culture of native cornea. A) A cornea was fixed in a bioreactor using a custom-made fixator. The bioreactor system consisted of two separate circuits. Circuit 1 with the pump P1 provided medium to the endothelial side of the cornea; in circuit 2 the pump P2 delivered medium to the epithelial side of the cornea to mimic tear fluid. The sensor PI measured the intraocular pressure. After 7 days of culture in different media combinations (III, IV, V, and VI), the corneal tissue was investigated by (B) (H&E) staining, and C) fluorescein staining. D) The assessment of transparency, E) the integrated optical density, and F) the electrical resistance allowed the comparison to (I) native corneas and (II) standard submerse culture used in eye banks. Scale depicts 200 μm; $n = 3$; star indicates significant differences ($p < 0.05$); d in B) denotes the tissue thickness; I: native porcine cornea; II: standard culture in eye banks (biochrom medium 1 without compartmentalization); III: biochrom medium 1 on both sides; IV: $CaCl_2$ in the upper (epithelial side) and VascuLife® in the lower (endothelial side) compartment; V: EpiLife™ in the upper and VascuLife® in the lower compartment; 6: EpiLife™ in the upper and VascuLife® with a reduced amount of growth factors in the lower compartment.

a cell crown or in dynamic culture conditions in the bioreactor system. H&E staining showed a closed epithelial layer in the bioreactor culture after 7 days (Figure 3B) and a beginning stromal healing process, whereas the corneal tissue stored under static culture conditions lost its epithelium (Figure 3C). Despite the advantages of the bioreactor system, the in vivo-like culture conditions entailed a higher complexity regarding the culture process compared to the methods harnessed in eye banks. To support the handling and the use of the bioreactor for cornea storage, a 3D-printed bioreactor, consisting of only two parts, was developed (Figure 3D). This disposable bioreactor facilitated an epithelial multi-layer comparable to the initial bioreactor setup (Figure 3E). The major advantage of the new bioreactor was the possibility to monitor the cornea noninvasively; allowing to assess the integrity of the endothelial cell layer (Figure 3F).

4. Discussion

In eye banks that perform organ culture, corneal tissue is stored under submersed conditions in biochrom medium 1, which represented the reference in our study. Thereby, the epithelium and the endothelium are exposed to the same nutrients, amino acids, and growth factors. To supply endothelial and epithelial cells with different media, a cell crown system was employed in our study. In a cell crown, the cornea tissue is stored between two separated medium reservoirs, which allowed to apply different cell culture media combinations for the apical and basolateral tissue surface. Despite the adapted biochemical storage conditions, only minor improvements regarding the histology and the functionality of the epithelium were achieved in comparison to standard submerse culture in biochrom medium 1. For example, the typical

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Figure 3. Application domain of the bioreactor device. (H&E) of the A) endothelial mono layer of a porcine cornea cultured for 7 days in the bioreactor using condition V. H&E staining of a wounded corneal tissue cultured under condition V for 7 days in B) static submerse conditions in a cell crown or C) cultured in the bioreactor. D) Optimized 3D-printed microscopable bioreactor. E) H&E staining of a porcine cornea after a 1 week culture in the 3D-printed bioreactor. F) Light microscopic picture of the endothelial cell layer of a porcine cornea fixed in the bioreactor. Scale bar depicts 200 µm in A; E, 1500 µm in B; C and 100 µm in F; $n = 3$.

loss of the epithelium within 1 week of culture was detected.[10] These results are in accordance to the outcome of previous studies that were comparing the influence of the storage medium on the properties of corneal tissue.[7] In contrast to these previous investigations that harnessed submerse cornea culture, even the adapted culture conditions for each cornea side, which were never tested before, revealed no positive impact. Interestingly, also the use of cell culture medium that is optimized for epithelial cells, e.g., EpiLife™ medium, resulted in the degeneration of the outer cornea surface. The limited benefit of tissue-specific media combinations indicated that in addition to a suitable medium also the physical culture conditions determine the success of cornea storage. Similar findings were observed for other tissues. For example, the culture at the air-liquid-interface is an essential stimulus for the formation of an epidermal barrier in tissue engineering of skin.[11] In analogy, the exposure of the tissue to the air has an impact on key enzymes of the lipid synthesis and induces changes in gene expression of structural proteins.[12] To achieve an in vivo-like tissue culture, a cornea bioreactor was developed. In analogy to the cell crowns, the bioreactor allowed to apply epithelium- and endothelium-specific media combinations. Moreover, the control of the intraocular pressure and the culture at the air–liquid interface was possible. To mimic the in vivo situation, a periodic moisturizing of the eye surface was performed. Compared to the standard culture system, all assessed tissue characteristics were improved for the tested media combinations. Especially the biochrom medium 1 facilitated tissue characteristics comparable to the properties of native cornea tissues. This indicates that the biochrom medium 1, currently used in eye banks, in combination with the bioreactor supports a physiological storage of native corneas without degradation of the epithelium. Even healing of an epithelial defect was detected in the

bioreactor, whereas a persistent wound was found in the statically cultured tissues. The preserved barrier function of the epithelium, the presence of the endothelium, and the ability to heal indicate that in vivo-like culture conditions also bear potential for in vitro toxicity and irritation testing. Although this technology has impact on corneal grafting and basic research, in comparison to the standard submerse culture, the bioreactor-based technology is more complex, entails an impaired handling, and requires more lab space. These disadvantages hamper the translation of the technology into a cornea storage system used in an eye bank. Thus, the design of the bioreactor was optimized based on the first classically manufactured bioreactor. Modern 3D-printing technologies allowed to reduce the number of bioreactor components to a lid and a bioreactor base. The redesigned disposable bioreactor can be placed in a rack system to operate several bioreactors in parallel and supports microscopic tissue characterization via an optical access without manipulating the cornea. Thereby, the presence, the density, and the morphology of the endothelium – the current readout factor for the assessment of the quality of cultured cornea tissue – can be assessed noninvasively.

Although the literature partially describes the development of bioreactors for corneal storage, current studies are focusing on the technical aspects of such a system. For example, it has been shown that poly(etheretherketone) and titanium–6Al–4V are suitable materials for corneal storage, whereas poly(oxymethylene) copolymer and poly(tetra-fluoro-ethylene) result in an impaired proliferation and viability of corneal fibroblasts.[13] Our results strengthen that polysulfone can be added to the group of materials available for the engineering of a corneal storage system. In addition to the material properties, also the impact of the mechanical conditions on the quality of the cultured tissues has been investigated.[14] In contrast to the bioreactor presented

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here, the system previously described by Leonard et al. requires a more complex setup and quality criteria for corneal storage that are used in eye banks are not addressed. Moreover, no information on the anatomy and physiology of the endothelium and epithelium of cultured corneas are provided. In a further application, bioreactors are used in ophthalmology to generate cell-free scaffolds for tissue regeneration.[15] Such scaffolds can serve as a substrate for corneal keratocytes and endothelial cells. To our knowledge, no bioreactor system is currently used in a clinical application. Thus, the developed bioreactor can be the first step towards a full-automated eye bank, which facilitates reduced labor intensiveness, good economic efficiency, and continuous monitoring for high quality grafts.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

bioreactor, corneal endothelium, corneal epithelium, corneal storage, tissue culture

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