Decellularized human corneal stromal cell sheet as a novel matrix for ocular surface reconstruction

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Abstract
The shortage of donor corneas as well as the limitations of tissue substitutes leads to the necessity to develop alternative materials for ocular surface reconstruction. Corneal surface substitutes must fulfill specific requirements such as high transparency, low immunogenicity, and mechanical stability combined with elasticity. This in vitro study evaluates a decellularized matrix secreted from human corneal fibroblasts (HCF) as an alternative material for ocular surface reconstruction. HCF from human donors were cultivated with the supplementation of vitamin C to form a stable and thick matrix. Furthermore, due to enhanced cultivation time, a three-dimensional like multilayered construct which partly mimics the complex structure of the corneal stroma could be generated. The formed human cell-based matrices (so-called cell sheets [CS]) were subsequently decellularized. The complete cell removal, collagen content, ultrastructure, and cell toxicity of the decellularized CS (DCS) as well as biomechanical properties were analyzed. Surgical feasibility was tested on enucleated porcine eyes. After decellularization and sterilization, a transparent, thick, cell-free, and sterile tissue substitute resulted, which allowed expansion of limbal epithelial stem cells with no signs of cytotoxicity, and good surgical feasibility. DCS seem to be a promising new corneal tissue substitute derived from human cells without the limitation of donor material; however, future in vivo studies are necessary to further elucidate its potential for ocular surface reconstruction.

KEYWORDS
Cell sheet, cornea, corneal tissue engineering, keratocytes, ocular surface reconstruction

1 INTRODUCTION
Severe corneal diseases, for example, resulting from trauma or infection, often irreversibly reduce corneal transparency. According to the World Health Organization, this presents the fourth leading cause of blindness worldwide (WHO/NMH/PBD, 2010). Current surgical treatment is based on partial or full thickness corneal transplantation using donor corneas or, depending on the underlying disease, reconstruction of the corneal surface using other biomaterials such as amniotic membrane. Despite the slowly increasing donor-recipient rate for corneal transplants, there is a mismatch between the number of grafts required and those available for transplantation (EBAA, 2016). The total number of donors and also limitations in the quality of donor material as well as graft failure due to immunological responses to...
epithelial, keratocyte, or endothelial antigens are factors contributing to this gap. To date, the most frequently used tissue substitute for ocular surface reconstruction is amniotic membrane, which however displays some disadvantages. Human material needs to undergo time and cost-intensive quality controls including serological and microbiological testing of donor and transplant. Furthermore, the most important limitation of amniotic membrane is its poor transparency and its variable thickness and quality (Connon et al., 2010). Therefore, the requirement for new clinically utilisable corneal tissue substitutes is high.

The field of regenerative medicine and tissue engineering gained much interest in the past decade due to improved scientific and technical knowledge. Tissue engineered materials for ocular surface reconstruction however still face various challenges (Lynch & Ahearne, 2013). The scaffold needs to be transparent and suitably shaped to transmit and refract light. The material needs to be mechanically stable for surgical handling and suturing, as well as flexible because of the constant shear force due to the blink reflex.

The two main directions of tissue engineering evolving are the use of biopolymer-based scaffolds and the use of native tissue from different sources. To overcome difficulties concerning biocompatibility and immunoreaction of the host when using engineered tissue, one promising approach is the use of decellularized tissue. Decellularized corneal tissues as a scaffold came into the focus of recent research as this fulfills most of the biological, mechanical, and immunogenic requirements (He et al., 2016; Yam et al., 2016). As a potential source for decellularized tissue substitutes, human cadaveric donor corneas unsuitable for transplantation due to low endothelial cell density or other quality reasons have been used (Zhang et al., 2015). In addition, corneal stromal lenticules derived from small incision lenticule extraction (SMILE) are currently investigated as a corneal tissue source (Yam et al., 2016). Still, all these options require human donor material. Instead of using limited donor tissue, an alternative approach is to use human corneal cells to generate tissue substitutes for transplantation. Due to the ability to generate a high number of cells out of a small tissue biopsy, there is nearly no restriction on tissue availability for this approach.

The cell-based tissue engineering approach is a promising new method for the development of substitute tissue for ocular surface reconstruction. In contrast to combine biomaterial with living cells, these matrices are composed of densely growing cells which secrete their own extracellular matrix components, such as collagen. Once a confluent cell monolayer is generated through direct cell–cell contact, this so-called “cell sheet” also starts building its own 3D extracellular matrix, while simultaneously evolving into a cellular multilayer. Due to its natural human tissue-like composition, restrictions associated with scaffold degradation or immunologic reactions from the material can be avoided. Furthermore, as the cell sheets are harvest without the use of proteolytic enzymes, the structure of the matrix as well as cell interactions stay intact (Grobe & Reichl, 2013). A different approach of the cell sheet technology has already been successfully transferred to the clinic, where the cell sheets were used as cell-carriers but not as a thick matrix.

For example, an autologous myoblast sheet which was generated from skeletal muscle was used and transplanted to the lateral surface of the dilated heart of a patient suffering from dilated cardiomyopathy (DCM; Sawa et al., 2015). It was also used to reconstruct defects of the esophagus (Yamaguchi et al., 2017), middle cavity of the ear (Yamamoto et al., 2017), lung (Kanzaki, Takagi, Washio, Kokubo, & Yamato, 2017), periodontal tissue (Iwata et al., 2018), and the cornea (Nishida et al., 2004). For the cornea, this method has mostly been used for corneal epithelial cell and limbal epithelial stem cell transplantation in patients with unilateral stem cell deficiency (Umemoto, Yamato, Nishida, & Okano, 2013). Limitations of these cell sheets include the limited thickness and—if artificial polymers are used as carrier for cell sheet growth—that their surface can affect cell behavior (Chen, Yan, & Zheng, 2018) and therefore the quality of the cell sheet. It has already been shown that treatment of corneal and dermal fibroblasts with vitamin C leads to the formation of a cell sheet which is a suitable growth substrate for corneal epithelial and endothelial cells (Proulx et al., 2010). Additionally, a direct comparison of human primary corneal fibroblasts with human SV-40 immortalized corneal keratocytes for generating cell sheets revealed an enhanced collagen synthesis and improved biomechanical properties and higher transparency in matrices derived from primary cells (Grobe & Reichl, 2013).

Gouveia et al. (2017) developed a self-lifting auto-generated tissue equivalent (SLATE) as a possible corneal graft by seeding human corneal fibroblast onto peptide amphiphile-coated surfaces. This coating leads to an alignment of collagen fibers in the sheets, which showed improved mechanical properties and improved stability against enzymatic degradation compared to cell growing on noncoated surfaces. After 3 months of culture, their SLATES reached a thickness of 13.5 ± 3.3 μm. After stacking five sheets, they reached a thickness of 40.2 ± 11.7 μm. In their in vivo rabbit model, they could show that the SLATES, implanted into corneal pockets, caused no immunoreaction after 9 months of incubation. Due to the low thickness and therefore low stability of monolayer cell sheets, to date, most of the clinically used cell sheets are multilayered cell sheets. Miyahara et al. (2006) reached a thickness of a monolayer cell sheet of 6.2 ± 0.3 to 21.5 ± 0.8 μm after 4 days of cultivation. Grobe and Reichl (2013) generated human corneal fibroblast-based cell sheets with a thickness of 18 μm after 6 weeks in culture and 20–25 μm after 14 weeks. Triple-layering of cell sheets composed of endometrial-derived mesenchymal cells led to a thickness of 40 μm (Sekine, Haraguchi, Shimizu, Umezawa, & Okano, 2011). In their study, they found a limitation of thickness, as the stacking of more than three cell sheets leads to a decrease of oxygen concentration as well as to an increase of apoptosis in the cell sheets (Sekine et al., 2011).

In the current study, two main directions of tissue engineering were combined by using cell sheet technology and decellularization techniques to produce a human-cell-derived cell free matrix for ocular surface reconstruction. Based on previous work by Grobe & Reichl, 2013, primary human corneal fibroblasts were used for matrix production. In contrast to other studies, the cultivation time of the cell sheets was extended from 3 months up to 12 months to establish a three-dimensional multilayered construct which mimics the complex
structure of the corneal stroma as well as to reach a usable thickness and stability.

Additionally, the corneal cells from different patients for the sheet generation were not pooled, as it is normally done in other studies. This led to the generation of a patient matched cell sheet construct of enhanced thickness which could be used for corneal surface as well as for stromal reconstruction. Additionally, the constructs were decellularized, to reduce their immunogenicity for the use of the matrices as an allogenic transplant. The mechanical stability and the transparency of native and decellularized cell sheets as well as human amniotic membrane was compared in order to evaluate their suitability for the use in corneal surface reconstruction. Furthermore, their suitability as a growth substrate for the expansion of limbal epithelial stem cells and thus their biocompatibility in vitro was evaluated. Finally, the surgical handling of the sheets was evaluated by transplanting the constructs on ex vivo porcine corneas. The aim of this study was to develop an in vitro cultivated tissue substitute based on human corneal cells and characterize its suitability as an ocular surface reconstruction material.

2 | MATERIALS AND METHODS

2.1 | Cell culture and tissue

All cell culture medium components were purchased from Sigma-Aldrich (Schnelldorf, Germany) unless otherwise stated.

2.1.1 | Corneal stroma cell culture

Human donor corneas were obtained from the Lions Cornea Bank North Rhine-Westphalia (Department of Ophthalmology, University Hospital Düsseldorf, Heinrich-Heine-University, Germany) with the approval of the local ethics committee (number 4139, approved 2013) and under the tenets of the Declaration of Helsinki. The central part of human donor corneas was incubated in 1.2 U/ml dispase solution (Roche, Germany) over night at 4°C before epithelial cells and endothelial cells were removed using a blade. The remaining corneal stoma was cut into small pieces of about 1 mm³ and placed onto a 6-well dish. Tissue pieces and outgrowing cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.4 μg/ml hydrocortisone, 0.1 mM cholaer toxin, 0.075% sodium bicarbonate, 0.18 mM adenine, 2 nM T3, 5 μg/ml transferrin, 5 μg/ml insulin, 1% antibiotic and antimycotic) to harvest the cells. The cell suspension was transferred to a T75 culturing flask containing a cell feeder layer of 1.8 × 10⁶ mitomycin-C (MMS) treated (4 μg/ml for 2 h) 3T3 murine fibroblast cells. The feederlayer was renewed once a week with 6 × 10⁵ MMC-treated cells per flask. Medium was changed three times per week, and cells were cultured in a humidified incubator at 37°C and 5% CO₂.

2.1.3 | Human amniotic membrane

Human amniotic membrane (AM) was also obtained from the Lions Cornea Bank North Rhine-Westphalia with the approval of the local ethics committee (number 4722, approved 2014) and under the tenets of the Declaration of Helsinki. The AM was produced under the identical conditions as for the use in patients. Donor placentas after caesarean deliveries were washed intense with PBS, and the AM was separated from the chorion under sterile conditions. The AM was cut into pieces of 2–3 cm × 2–3 cm, spanned onto a nylon transfer membrane (Roti-Nylron 0.2 mm, Roth, Karlsruhe) and stored at −80°C in 1:1 (v/v) DMEM (Gibco, Life Technologies) and glycerol (Sigma-Aldrich, Schnelldorf, Germany). Prior to use, AM was thawed and washed three times using sterile PBS.

2.2 | Cell sheet generation

Primary corneal fibroblasts (1 × 10⁵ cells/cm²) were seeded on sterile glass slides in 24-well plates and supplemented with 50 μg/ml vitamin C 24 h after cell attachment. Medium was changed three times a week containing vitamin C, and cell sheets were kept in culture up to 12 months or until further use. Cell sheets were gently detached of the glass slide by grabbing the cell sheet with forceps on the rim in a PBS-floated petri dish and gently pull in one direction. No further enzymatic treatment was needed for lifting the cell sheets. For each donor, at least 48 wells were seeded. Three independent primary donor fibroblasts were used for cell sheet generation.

2.3 | Decellularization process and verification

2.3.1 | Decellularization process

For decellularization, CS were washed with 1 × PBS containing 5% penicillin-streptomycin (PBS-PS) for 2 h followed by incubation in 2% sodium deoxycholate monohydrate solution (NaDC, Sigma-Aldrich) for 3 h at 4°C on an orbital shaker. Subsequently, CS was incubated with fresh 2% NaDC for 24 h at 4°C followed by incubation with DNase solution (167 U/ml, Worthington, Lakewood, USA) for 3 h at
37°C. After DNase treatment, cell sheets were washed again in 1 × PBS-PS overnight. Between each decellularization step, three intense washing steps with 1 × PBS-PS were performed.

### 2.3.2 | DNA content measurement

For DNA extraction, CS and DCS were washed with 1 × PBS and transferred directly to RLT Buffer following manufactures instructions for total DNA extraction using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen). DNA was collected in 100 μl preheated EB Buffer and quantified using the Fluorescent DNA Quantitation Kit (BioRad, Hercules, USA) and the Viktor X multilabel plate reader (PerkinElmer, Waltham, MA, USA). A standard curve using calf thymus DNA was used to determine the amount of DNA per whole sheet (with 10 mm² size). N = 3 with at least two donor-different cell sheets per single experiment.

### 2.3.3 | Feulgen staining

Native and decellularized cell sheets were fixed with 4% paraformaldehyde for 1 h (Roti Histofix, Carl Roth), followed by paraffin embedding. For staining, CS and DCS were cut in 4 μm slides and deparaffinized. Feulgen staining was performed according to manufactures instructions (DNA staining kit according to Feulgen, Sigma Aldrich). N = 3 with two donor-different cell sheets per single experiment.

### 2.4 | Gamma sterilization of decellularized cell sheets

DCS were kept in deionized water and were sterilized by gamma-irradiation with 25 kGy (BBF Sterilization-Service GmbH, Rommelshausen, Germany). Sterilized DCS (γDCS) were used for all following experiments.

### 2.5 | Cell sheet properties

#### 2.5.1 | Determination of collagen content

For measurement of the total collagen content, the protein fraction of the CS and γDCS after processing with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) was used. Quantification of collagen was performed using a colorimetric assay (Sircol collagen assay, Biocolor, UK) according to the manufacturer's instructions. Samples were measured at 550 nm using a plate reader (Multiskan EX; Thermo Scientific), and collagen content was determined using a standard curve of collagen supplied by the Sircol assay. N = 3 with two donor-different cell sheets per single experiment.

#### 2.5.2 | Collagen staining

For immunohistological staining, deparaffinized CS and γDCS sections were washed, blocked with 5% Donkey serum for 60 min and incubated over night with anti-collagen I (Sigma C2456), III (Sigma C7805), and IV (Sigma C1926) antibodies (dilution 1:50 in 2% donkey serum). Secondary antibodies (Alexa Fluor 594 anti-mouse (Jackson Immunoresearch), dilution 1:400 and Alexa Fluor 488 anti-rabbit (Jackson Immunoresearch), dilution 1:400) were applied for 1 h at room temperature followed by three intense washing steps and samples were mounted with DAPI-Mowiol. Fluorescence was documented using a Zeiss microscope (Zeiss AxioVertA1). N = 3 with two donor-different cell sheets per single experiment.

### 2.5.3 | Histology

Paraffin embedded CS and γDCS slides (4 μm thickness) were deparaffinized, rehydrated, and subsequently stained using hematoxylin and eosin (H&E) staining following standard protocols (Spaniol et al., 2015).

### 2.5.4 | Thickness measurement

The thickness of CS and γDCS was evaluated using HE-stained paraffin-slides with different measurement points of 200 μm distance, beginning from the lateral side of the cell sheet. For each sheet, five HE-stained slides were evaluated, and the mean thickness over the whole sheet was calculated. N = 2 with two donor-different cell sheets per single experiment.

### 2.5.5 | Electron microscopy

The CS and γDCS with and without hLESCs were examined by transmission electron microscopy (TEM). The samples were fixed in 2.5% gluteraldehyde/4% PFA (v/v) in 0.1 M cacodylate buffer (pH 7.4) for 96 h at 4°C. Afterwards, samples were incubated in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. Following a dehydration step using acetone (50%, 70%, 90%, and 100%), a contrast agent with 1% phosphotungstic acid/0.5% uranylacetate (v/v) in 70% acetone was added. Embedding was done using SPURR embedding kit (Serva, Heidelberg, Germany) with a polymerization step overnight at 70°C. Samples were cut into 80 nm sections using an Ultratcut EM UC7 (Leica) and images were captured at 75 kV with an H600 TEM (Hitachi, Tokyo, Japan). N = 2 with two donor-different cell sheets per single experiment.

### 2.5.6 | Biomechanical properties

Biomechanical properties of CS and γDCS were examined by a stretch–stress test (Drechsler et al., 2015) using a Zwick/Roell Z 0.5 static material testing machine with a 10 N load cell. The CS and γDCS were spread onto a PBS soaked filter membrane, kept wet, and cut into 10 × 10 mm pieces. After the tissue pieces were placed between...
the splints of the testing machine, the membrane underneath was cut, leaving only the CS and γDCS samples in the middle of the splints. Afterwards, the samples were pulled vertically with a uniaxial stretching device until broken with a speed set to 25 mm/min for UTS measurement and with a speed set to 5 mm/min for E-modulus measurement. Mean thickness of all probes was measured by Image J with paraffin embedded probes and HE-staining. Ultimate tensile strength, elongation at break and elastic modulus (Emod) were analyzed using the testXpert® II software (Zwick, Ulm, Germany). Research consented AM with 10 × 10 mm tissue size was used as control. Thickness of amniotic membrane was evaluated using cryomicrotome sections and the software Image J. N = 6 with two donor different cell-sheets and n = 3 with two different AM.

2.5.7 | Transparency measurement

Transparency of CS and γDCS was assessed by photometric measurement of their optical density between 400 and 800 nm in 1 nm steps using a spectrophotometer (PowerWave XS). The CS and γDCS growing on glass slides in 24-well plates were carefully washed three times using 1 × PBS and placed into new 24-well plates with 500 μl 1 × PBS. A blank correction using a glass slide and 500 μl 1 × PBS was performed. At least three different CS and γDCS per donor cells were measured. AM was used as control. The recorded absorption spectrum was mathematically converted to the light transmission. N = 6 with three donor different cell-sheets (=18 different cell sheets in total for CS and γDCS).

2.6 | Seeding hLESCs onto matrices

Confluent hLESCs (P0) were trypsinized in a two-step procedure. First, cells were incubated with 1 ml 1x trypsin/EDTA (0.5 g porcine trypsin and 0.2 g EDTA × 4Na/L) until 3T3 cells detached. Cells were gently washed to remove 3T3 cells and 1 ml 10x trypsin/EDTA (5 g porcine trypsin and 2 g EDTA × 4Na/L) was applied for 5 min or until hLESC detachment was observed. Cells were counted and seeded in a density of 3 × 10^5 cells/cm² on top of the CS and γDCS and incubated for different time points (D3, D5, D7, and D14).

2.6.1 | WST assay

To assess cellular viability on CS and γDCS, a WST-1 assay was performed for measuring the enzymatic cleavage of the tetrazolium salt by mitochondrial dehydrogenase indicating metabolically active and vital cells. Thus, cells were seeded onto the CS and γDCS as well as directly on plastic in 24-well plates a density of 3 × 10^5 cells/cm². To eliminate possible variability in cell numbers due to seeding procedure, first measurement was done directly after cell attachment (4 h after seeding, D0); 24 h after attachment as well as at day 3 (D3), day 5 (D5), day 7 (D7), and day 14 (D14), cells were washed and incubated with WST-1 solution (100 μl/ml; Roche, Penzberg, Germany) for 1 h. Supernatant was transferred to a new 96-well plate. Measurement was performed using a plate reader (Multiskan EX; Thermo Scientific) at 450 nm. N = 3 with three donor different cell sheets per experiment and one hLESC donor per experiment.

2.6.2 | Immunostaining

For immunostaining on paraffin sections, dewaxed slides were washed three times with PBS followed a blocking step using 5% Donkey serum for 60 min. Anti-p63 (1:50, orb69390, Biorbyt), anti-CK15 (1:50, ab52816, abcam), anti-Ki67 (1:50, ab16667, abcam), and anti-caspase 3 (1:50, ab4051, abcam) were diluted in 2% donkey serum and incubated overnight at 4°C. After washing three times, Alexa Fluor 594 anti-mouse and Alexa Fluor 488 anti-rabbit (Jackson ImmunoResearch) secondary antibodies were applied for 1 h at room temperature followed by three washing steps with 1x PBS. Sections were mounted using DAPI-Mowiol. Fluorescence was documented using a Leica Microscope (Leica DM 4000B, Leica, Wetzlar, Germany). As negative controls, matching Ig antibodies were used. N = 3 with three donor-different cell sheets per experiment and one hLESC donor per experiment.

2.7 | Ex vivo evaluation of CS and DCS on porcine eye balls

Fresh cadaveric porcine eye balls (within 2–3 h after slaughtering) obtained from a slaughter house were used for ex vivo suture experiments. CS and γDCS were transferred onto a nylon transfer membrane (Roti-Nylon 0.2 mm, Roth, Karlsruhe) before placed onto the central part of the porcine cornea. CS and γDCS were sutured onto the cornea using eight single stitch sutures with Vicryl 8–0 (carried out by StS). Size of the matrices, suture time and number of tearings were measured. N = 3 with three donor-different cell sheets.

2.8 | Statistical analysis

All values are expressed as mean value ± standard deviation. Statistical analysis was performed using student’s t-test for the ex vivo experiments or ANOVA for the remaining experiments. The level of statistical significance was set at p < 0.05. All experiments were performed in independent triplicates using cell sheets generated by two to three different human donor cells in each independent experiment if not further indicated.

3 | RESULTS

3.1 | Generation of cell sheets

Native cell sheets, composed of corneal fibroblasts stimulated with vitamin C supplemented media to produce collagen fibers, were kept
in culture for 12 months to generate a stable collagen matrix (Figure 1). To generate a cell-free CS, a decellularization protocol using NaDC and DNase was used.

3.2 | Verification of successful decellularization and recellularization

3.2.1 | DNA content of CS and DCS before and after recellularization

Residual DNA content was measured using fluorescence DNA Quantification assay (Figure 2a). Native 12-month-old CS displayed a DNA content of 235.6 ± 44.7 ng DNA/CS, whereas decellularized 12-month-old CSs contained 31.3 ± 15.6 ng DNA/DCS residual DNA (p < 0.001). Cells seeded on top of the sheets generate an increase in DNA content, which was not significant between the different constructs. Comparing 12-month-old CS with hLESCs seeded on top for 14 days with CSs without cells, DNA amount was enhanced from 235.6 ng DNA/CS to 342.7 ng DNA/CS which is an increase of 107.07 ng DNA/CS caused by the cells seeded on top (n.s., p = 0.134). When cells were seeded on DCS for 14 days, the DNA amount increased from 31.3 ng/DCS to 82.3 ng/DCS, which is an increase of 51.0 ng DNA/CS which is an increase of 107.07 ng DNA/CS caused by the cells seeded on top (n.s., p = 0.268). Comparing directly the increase of DNA of CS with DCS caused by the hLESCs when seeded on top, a 209.95% increase of DNA amount is found in CS + hLESCs (107.07 ng DNA/CS) compared to DCS + hLESCs (51.00 ng DNA/DCS) set as 100%. Additionally, after the decellularization process, corneal fibroblasts were absent in all tested γDCSs as indicated by HE staining as well as the negative Feulgen staining (Figure 2c–f). Furthermore, TEM images revealed no remaining cells and only very few damaged cell components in the γDCS (Figure 2g–h).

3.3 | CS and DCS properties

3.3.1 | Conservation of collagen matrix

To evaluate if the decellularization process damaged the collagen matrix, collagen content was measured using a colorimetric assay as well as thickness measurements performed (Figure 2b). The native CS revealed a collagen content of 91.17 ± 4.5 μg collagen/CS in contrast to the γDCS with 44.96 ± 4.29 μg collagen/DCS (p ≤ 0.001). The decellularization process led to a loss of 50.6% of total collagen in the γDCS. Decellularized cell sheets with hLESCs seeded on top for 14 days exhibited 62.94 ± 8.53 μg collagen per sheet compared to native CS with hLESCs with 97.51 ± 0.81 μg collagen/cell sheet (p = 0.004). Here, we only found a difference of 35.45% when comparing CS with γDCS with hLESC. Comparing the collagen content with and without cells between CS and γDCS, the γDCS revealed a higher increase of collagen amount of 39.97% for γDCS compared to 6.95% for the CS when cells were grown on the matrices. As the proliferaations assay revealed no significant difference at day 14 between CS and γDCS, this is not based on a different quantity of cells on top of the sheets.

HE staining revealed similar collagen fiber arrangements in CS and γDCS (Figure 2c,d). Thickness measurements were performed using CS and γDCS with hLESCs seeded on top to additionally evaluate possible degradation effects caused by the cells (Figure 3a–c). For thickness evaluation, only the cell sheets themselves were measured without the cell layer on top. The thickness of the sheets revealed no significant differences comparing CS and DCS at all measured timepoints. The mean thickness of the CS was 126.62 μm ± 4.88 μm and 118.02 μm ± 6.38 μm for the γDCS. CS and γDCS thickness improved with culturing time of the cells, indicating no enzymatic degradation of the sheets by cells growth. Comparing the timepoints of γDCS among each other revealed significant differences between...
γDCS D1 and D3 (p = 0.016), D1 and D14 (p ≤ 0.001), D5 and D14 (p = 0.029), and D7 and D14 (p = 0.025). Comparing the timepoints of CS among each other showed significant changes between D1 and D3 (p ≤ 0.001), D1 and D5 (p = 0.016), D1 and D7 (p = 0.021), and D3 and D7 (p = 0.003; Figure 3a). Immunofluorescence staining of collagen revealed a distinct collagen I staining in CS and γDCS demonstrating the presence of collagen I in both cell sheets (Figure 3d–i). Furthermore, slight staining of collagen III and IV could be found in both matrices. In CS, a higher amount of positive staining for collagen IV was detected than in γDCS, both in close proximity of the cells (Figure 3f,i). This shows that collagen IV is not secreted to the same extent as collagen I by the cells due to the vitamin C treatment. By analyzing the ultrastructure of the cell sheets using transmission electron microscopy (TEM), again only slight differences could be observed between the two matrices (Figure 2g–j). The CS exhibited differentially arranged collagen fibers with orientation around the corneal fibroblasts and a few randomly arranged fibers (Figure 2g). After removal of the cells, the fiber arrangement was not strongly altered.

The difference seen in the TEM between CS and γDCS was based on the eliminated cells. There were hole-like structures visible where the cells were previously located (Figure 2h). The main collagen structure as well as the collagen fiber arrangement remained almost unaffected by the decellularization process. Only the compactness of the collagen seemed loosened (Figure 2i,j).

3.4 | Biomechanical properties

3.4.1 | Tensile testing

The ultimate strength (UTS) and the E-modulus (Emod) of both cell sheet types as well as amniotic membrane were evaluated using a material testing machine (Figure 4a–c). The elastic modulus described the elasticity of the tested material, whereas a higher Emod means a lower elasticity. No significant differences between AM, CS, or γDCS could be found for Emod measurement (118.56 ± 9.86 kPa AM,
89.61 ± 15.46 kPa CS, and 99.52 ± 17.79 kPa DCS), indicating comparable stability of all tested materials. The thickness of the used AM was 50–60 µm (Figure 4e). The UTS is the maximum force that a material can withstand while being torn. AM displayed significant higher UTS with 926.20 ± 150.25 kPa compared to CS (82.86 ± 25.24 kPa, p ≤ 0.001) and γDCS (86.56 ± 15.32 kPa, p ≤ 0.001). There was no significant difference between CS and γDCS in UTS (Figure 4f).
3.4.2 | Cell sheet transparency

The light transmission of native cell sheets ranged from 91.72 ± 1.44% at 800 nm to 69.04 ± 2.28% at 400 nm. The γDCS showed a slightly but not significantly enhanced transmission rate of 94.34 ± 0.12% at 800 nm and 77.41 ± 1.08% at 400 nm compared to CS. The amniotic membrane had the lowest transmission rate of 72.46% at 800 nm and 37.33% at 400 nm (Figure 4d), indicating an enhanced transparency of both cell sheets compared to amniotic membrane. Additionally, the decellularized cell sheet displayed the highest light transmission rate, which is equivalent with the highest transparency.

3.5 | Cell sheets as growth substrate

3.5.1 | Cytotoxicity

Human limbal epithelial stem cells were cultured directly on CS and γDCS for up to 14 days (D14) to evaluate a possible cytotoxicity of the decellularization process as well as to evaluate their eligibility as a substrate for corneal cell growth. Comparing the growth of hLESC on both matrices as well as the growth of hLESC on plastic, no significant difference in the WST-1 assay was observed between the three experimental conditions, indicating no cytotoxic effect of the decellularization process (Figure 5e). The cell viability increased from 2.878 ± 0.384 at day 1 (D1) γDCS with hLESC up to 4.032 ± 0.775 at day 14 (D14) for hLESC on γDCS, from 3.170 ± 0.041 D1 to 5.145 ± 0.336 at D14 CS with hLESC and from 3.246 ± 0.162 D1 to 4.556 ± 0.171 for hLESC on plastic (D0 measurements set as 1).

Growth on both matrices did not influence the cell viability significantly, indicating that CS as well as the γDCS are appropriate growth substrate for corneal cells. Furthermore, the decellularization process did not lead to cytotoxicity of the matrix.

3.5.2 | Stem cells

Beside the proliferation of the cells, it is of interest if the seeded human limbal epithelial stem cells keep their progenitor status or if they start to differentiate on the scaffolds. Therefore, immunohistological staining using corneal epithelial stem cell makers was performed. Staining for CK15 as well as p63α revealed a high number of positive cells on γDCS (Figure 5a–d) indicating that their progenitor status after 14 days of cultivation on the scaffolds was preserved. Furthermore, proliferative active cells could be detected by using Ki67 staining, but only a small number of apoptotic cells was found, indicated by a low number of positive caspase3 stained cells.

3.6 | CS and DCS ex vivo experiment

3.6.1 | Surgical applicability

The surgical applicability of CS and γDCS was evaluated using fresh cadaveric porcine eye balls (Figure 6). The scaffolds were placed on the central cornea and sutured by eight interrupted sutures using Vicryl 8-0. First, the size of each scaffold was evaluated. Both constructs exhibited an almost identical diameter with 10.83 ± 0.83 mm for CS.

![FIGURE 5](https://wileyonlinelibrary.com)  
Cell sheets as growth substrate tested by immunohistological staining (a–f) and proliferation assay (g). CK15 (a) as well as p63α (b) staining was found in a high number of cells cultivated on γDCS for 14 days. IgG control mouse staining revealed only slight background staining (c). Positive staining for the proliferation marker Ki67 (c) was found in a small number of cells. Apoptotic marker caspase 3 (d) was also detected in only a low number of cells. IgG control rabbit staining showed none unspecific staining (f). Proliferation of the LESC on CS and γDCS (g) showed no significant difference to growth of the LESC in 24-well plate measured from day 1, day 3, day 5, day 7, and day 14 [Colour figure can be viewed at wileyonlinelibrary.com]
and 10.67 ± 0.67 mm for γDCS. The time needed to suture the CS or γDCS onto the cornea did also not differ significantly. For CS, 18.52 ± 3.47 min was required for eight interrupted sutures and for γDCS 17.01 ± 3.07 min. During the suturing, only one suture at a CS scaffold resulted in a tear of the construct. The surgeon reported no objective difference concerning fragility or handling of the cell sheets compared to amniotic membrane. Both sheets could be handled with forceps without damaging them. Further manipulation to adjust the position on the cornea also resulted on no damage to the constructs.

4 | DISCUSSION

Severe cases of ocular surface diseases often result in the necessity of a corneal surface reconstruction or corneal transplantation. The number of required donor corneas has constantly increased during the last decades. On the one hand, this is due to improved early diagnosis, new therapeutics and improved surgical methods, but on the other hand also as a result of the rising number of patients with vision impairment provoked by the growing and ageing global population (Ackland, Resnikoff, & Bourne, 2017; Gain et al., 2016). Therefore, more than ever, there is a necessity to develop alternative corneal substitutes for clinical use. These substitutes need to fulfill some important optical and physical properties. One main desired feature is the transparency of the material. In the native cornea, the transparency is based on the parallel alignment of the collagen fibrils as well as on the distance of each fibril and the diameter. Beside the transparency, the directed arrangement of the collagen fibril in each layer of the corneal stroma is also important for the biomechanics of the tissue (Abass, Hayes, White, Sorensen, & Meek, 2015). Therefore, the choice of material for corneal tissue replacement is of great importance concerning the visual outcome and long-term stability.

Conventional tissue engineered materials such as for example artificial material based on synthetic polymers (Lee, Yoo, & Atala, 2014) or cell sheet technology for cell replacement (Green, Kehinde, & Thomas, 1979) have been successfully used for several different organ-systems in the past decades. But still, there are some important limitations to this method. Using biological material includes the risk of infections as well as rejection and can lead to tissue degradation due to the tissue-own proteins. Using artificial material also bears the risk of rejection and of toxic reactions against the artificial materials resulting in extrusion. To avoid these obstacles, one idea is to use patient-matched or patient-own cells and culture them on specific surfaces until they form a transplantable tissue sheet like structure. This technology has already been used successfully for a variety of organs (e.g., corneal endothelium, cartilage, heart, kidney, liver, endometrium, middle ear, periodontium, salivary gland, and esophageal living sheet transplants), where the matrices promote cell differentiation as well as tissue integrity (Baimakhanov et al., 2016; Kanai, Yamato, & Okano, 2014; Matsuura, Haraguchi, Shimizu, & Okano, 2013; Sato et al., 2008; Yu, Wang, Tai, & Cheng, 2018; Zhang et al., 2016). Furthermore, cell sheets are under investigation in clinical trials for the treatment of ischemic heart disease, esophageal stenosis, cholesteatoma, and cardiomyopathy (Sawa et al., 2015). Most of the studies regarding the use of cell sheet technology in the cornea—and in other tissues as well—are using the sheets as a carrier for cells but not as a tissue replacement due to the low thickness (Gomes et al., 2010; Nishida et al., 2004; Sumide et al., 2006). One limitation of this method is the availability of the cell source. For example, in patients with a bilateral limbal stem cell deficiency, the use of patient-own material for limbal reconstruction is not possible. In these cases, allogenic cell sheets have to be used, which involves the risk of immunologic reactions and rejections. Additionally, there are a few more obstacles to overcome, for instance, the preservation of the cell sheet
as the cells have to be kept alive until transplantation (Owaki, Shimizu, Yamato, & Okano, 2014).

The cell sheets generated in this study showed an enhanced thickness due to an extended cultivation time of 12 months and were additionally decellularized as well as gamma-irradiated. Furthermore, for generating the cell sheets, human corneal fibroblasts were used. Thus, in this study, two main directions of tissue engineering, (1) the use of human cells to generate biocompatible extracellular matrix and (2) the use of decellularization techniques, were combined.

Decellularization of biological material is an emerging technology in tissue engineering. The benefit of using decellularized material is that allogenic as well as xenogenic material can be used with a lower risk of rejection due to the decrease of immunogenicity. Furthermore, as the extracellular matrix (ECM) is important for normal cell growth and cell function, the use of decellularized material where the ECM stays intact seems a promising approach (Bryksin, Brown, Baksh, Finn, & Barker, 2014; Khan & Tanaka, 2017). As different cell types produce different types of ECM, the use of corneal fibroblast in this study provides an advantage over using for example skin fibroblasts to generate the cell sheets (Ebrahimi Sadrabadi, Baei, Hosseini, & Baghaban Eslaminejad, 2020). Using organ-matched cell types results in organ-specific ECM structures, which is important for biocompatibility. One main advantage of decellularized tissue is that the removal of all cellular components including the genetic material while simultaneously preserving the structure and biochemical and biomechanical components of the extracellular matrix leads to a nonimmunogenic but highly biocompatible construct due to the missing donor antigens in the tissue (Gilpin & Yang, 2017; Hillebrandt et al., 2019). Most of the research in this field has focused on the decellularization of whole tissue and organs. To date, nearly all occurring tissues of the human body have been partly or fully decellularized (see Porzionato et al., 2018), and some have already been used in the clinic, for example, for orthopedic or dental applications, showing good biocompatibility without any immunoreaction due to the decellularized tissue (Parnaksiz, Dogan, Odabas, Elicin, & Elicin, 2016). Decellularized corneas have also been investigated as scaffolds for corneal replacement or as a supportive matrix for ex vivo expansion of stem cells (Spaniol et al., 2018). However, even if the advantages of using humane decellularized cornea as a tissue substitute, human material is limited and therefore cannot be used as a reliable source.

Therefore, decellularized material, which is derived from growing cells, is a promising alternative. Only very few studies have described in vitro generated material for decellularization and most of them focused on periodontal tissue or blood vessel regeneration (Tavelli et al., 2019; Tondreau et al., 2015). Beside the material itself, the process of decellularization is also a critical step for decellularized tissue engineered material. There are several different methods for decellularization (see Gilpin & Yang, 2017) available including the use of SDS, Triton X-100, by freeze–thaw cycles or using sodium deoxycholate (NaDC), depending on the tissue composition and thickness (Fernandez-Perez & Ahearne, 2019). Xing et al (2015) studied three different methods (high and low SDS concentration and freeze–thaw cycles) for decellularization of cell sheets derived from human dermal fibroblasts and compared the impact of the methods on ECM ultrastructure, mechanical properties, in vitro inflammation response as well as comparing their effectiveness. They conclude that high concentration of SDS effectively removed DNA but also significantly reduced the mechanical stability. In our study, we used NaDC for decellularization (Witt et al., 2018) and did not find significant difference in mechanical properties, transparency, or repopulation of the decellularized cell sheet compared to the native cell sheet.

One main difference and advantage of the cell sheet produced in this study is the thickness. Recent approaches which used thicker cell sheets, generated them by stacking a number of single thin cell sheets. This method includes additional manipulation of the sheets, such as a gelatin layer (Williams, Xie, Yamato, Okano, & Wong, 2011) for connecting the single sheets or the use of nanofiber mesh (Kim et al., 2017). This stacking can lead to a to less stable alignment of the layers and also affect transparency. Furthermore, the thickness of stacked/layered cell sheets is also limited, as it was shown that stacking more than three sheets can lead to an enhance apoptosis and a loss of oxygen supply in the sheets (Sekine et al., 2011). Culturing monolayer human corneal fibroblast-based cell sheets for up to 14 weeks resulted in a thickness of 20–25 μm (Grobe & Reichl, 2013). An additional study who have cultured cell sheets for only 4 days reached a final thickness ranging between 6.2 ± 0.3 to 21.5 ± 0.8 μm (Miyahara et al., 2006). One study, using also corneal fibroblast to generate a substitute for ocular surface reconstruction, was able to generate a self-lifting cell sheet called SLATE. They used peptide-amphiphile-coated (PA) growth surfaces, which leads to an alignment of the collagen fibers in the construct. They prove that this alignment is beneficial concerning biomechanical stability and stability against enzymatic digesting, but no difference in transparency, cell viability of cells growing onto the constructs and in vivo biocompatibility was observed. Furthermore, they showed that the hLSECs seeded on top showed higher degree of differentiation into epithelial cells on their construct in comparison to sheets generated without the alignment-promoting coating (Gouveia et al., 2017). Gouveia et al (2017) reached a thickness of 13.5 ± 3.3 μm of their monolayer-construct, and even after stacking five sheets, they reached only a thickness of 40.2 ± 11.7 μm. Therefore, generating thicker cell sheets without stacking by only expanding the culturing time leads to a more biomechanically stable and more transparent construct.

After reaching a thickness of over 100 μm, the sheets in this study were decellularized, and the remaining DNA was quantified according to key criteria by Crapo, Gilbert, and Badylak (2011) for decellularized material used in patients. These criteria are (1) a significant reduction of DNA to <50 ng dsDNA per mg ECM dry weight and (2) a lack of visible nuclear material in tissue sections stained with HE or DAPI. The results showed that the DCS fulfill the criteria with a residual amount of DNA under 31.5 ng per whole construct and non-visible DNA/cell debris in TEM, HE-, and Feulgen staining.

When comparing the amount of collagen in the two cell sheets (CS and DCS) with cells growing on top, there is a higher amount of collagen in the DCS with cells compared to CS with cells. Proliferation assay revealed no difference in cell growth, which indicates that the
different amount of collagen produced by the cells may be based on the decellularized cell sheet itself and not on a different cell number. Regulation of collagen synthesis of the cells is normally achieved by the interaction of the cells with the surrounding extracellular matrix as well as by cytokines and different growth factors (Mauch, Hatamochi, Scharfetter, & Krieg, 1988). In this study, it seems that the cells inside of the native CS are inhibiting the collagen synthesis of the cells growing on top of the CS. This may be due to the fact that the response of cells inside of 3D gels on growth factors is reduced as well as the proliferation of these cells (Mio, Adachi, Romberger, Ertl, & Rennard, 1996; Rhee & Grinnell, 2007). This can lead to differences in cell signaling resulting in a lower collagen synthesis of the cells on top of the cell sheets. This has to be elucidated more in detail in further research to reveal the underlying mechanisms for this finding.

For clinical use, it is also necessary to sterilize the tissue to eliminate potential bacterial or viral DNA or endotoxins. Gamma irradiation is already used in a variety of tissue engineering approaches (Yoshida et al., 2015) and recent research also employed gamma-irradiation at higher doses for complete tissue decellularization (Ding, Ruan, Luk, He, & Wang, 2014). Consequently, the DCS were treated with 25 Gy gamma-irradiation before further use and mechanical and functional properties of the sheets were measured in the gamma-irradiated probes. Gamma-irradiation (GI) is already used as sterilization process of human donor corneas as well as of porcine corneas in clinical studies or Boston Keratoprosthesis (KPro) and has found to be a promising method to provide a long shelf life of transplants with the elimination of risk of infection (Daoud et al., 2011; Shi et al., 2017; Zhang et al., 2015). One disadvantage of using GI is that corneal samples can turned pale yellow and can fail to remain optical clear (Gonzalez-Andrades et al., 2018; Stevenson et al., 2012). Concerning biomechanical properties, using GI can be beneficial, as it was shown that GI enhanced the mechanical stability, tissue hydrophobicity, and resistance to enzymatic degradation in decellularized human cornea tissue (Islam et al., 2019). In our study, no significant difference between irradiated decellularized cells sheets and native cell sheets concerning transparency of mechanical properties was found. This may be due to the different thickness of our CS/DCS and the corneal tissue used in the other studies. Comparing the collagen content in the CS and γDCS, a significant reduction could be observed. Interestingly, this loss did not significantly alter the overall thickness of the γDCS in direct comparison to CS. The differences in collagen content can be partly explained by the loss of the cells that produce the collagen. Collagen staining revealed that the main component of the cell sheets is collagen I, which is also the main component of human corneal stroma. Additionally, a slight amount of collagen III and IV was found located around the fibroblasts. Collagen III is also part of the human corneal stroma, but collagen IV is mainly found in the Descemet membrane or corneal cells (Nakayasu, Tanaka, Konomi, & Hayashi, 1986), which corresponds to our findings. This indicates that using human corneal fibroblast for generating the cell sheets results in an in vivo-like composition of the matrix. TEM showed that the collagen fibrils in the sheets are not as regularly spaced and arranged in cross-oriented layers as in human corneal stroma (Komai & Ushiki, 1991). Nevertheless, the transparency of the material is high and significantly enhanced compared to amniotic membrane as well as higher in γDCS compared to CS. The different amounts of collagen as well as the fiber orientation in CS and γDCS did also not have a significant effect on the biomechanics of the cell sheets.

To assess the biocompatibility and eligibility of the cell sheets as growth substrate for corneal cells, proliferation and differentiation of isolated human limbal epithelial stem cells (hLESCs) seeded onto the constructs were quantified. Cell proliferation did not differ between cells on CS, γDCS, or cells grown on a plastic surface. Furthermore, the cells did express limbal stem cell markers after 14 days in culture, indicating the existence of stem cells after this culturing period. This supports the conclusion that CS as well as γDCS are nontoxic and biocompatible scaffolds. As the construct is supposed to be used in clinical application, it is also important that the material can be processed by surgeons. Transplantation of the CS and γDCS on corneas of porcine eye balls ex vivo by an ophthalmologic surgeon showed no difficulties in suturing or handling of the material. Furthermore, the surgeon did not notice any difference in the handling of the cell sheets compared to the handling of amniotic membrane, indicating that the sheets are not fragile and can be handled during normal surgical procedure without special precautions.

The aim of this study was to produce a material as a substitute for ocular surface reconstruction, which can be produced in high numbers in a standardized manner, has a high biocompatibility and is transparent as well as stable for handling during surgery. The cell sheets based on human corneal fibroblasts fulfilled these criteria due to the low immunogenicity based on the decellularization and the use of human cells, the enhanced thickness, stability, and the good transparency and might therefore be a promising tissue replacement for ocular surface reconstruction. However, the enhanced culturing time of 12 months is critical as this carries an enhanced risk of contamination of the cell sheets. Furthermore, it would be desirable for patient-matched cell sheet, to be able to deliver this in a short time period. Thus, current studies are performed investigating different medium substitutes such as growth factors to enhance cell sheet thickens in a shorter time period. Furthermore, more studies are needed to elucidate the biocompatibility in vivo.

5 | CONCLUSIONS

Our study showed that the tissue-engineered decellularized and gamma-irradiated cell sheet from human corneal fibroblasts may serve as a substitute for corneal surface reconstruction due to its high transparency, enhanced thickness, elasticity, suturing stability, and biocompatibility.

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CONFLICT OF INTEREST
The authors have declared that there is no conflict of interest.

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