Silk Fibroin Films for Corneal Endothelial Regeneration: Transplant in a Rabbit Descemet Membrane Endothelial Keratoplasty

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Abstract: We have developed SF films with biological properties that supported the growth of rabbit and human CECs, which showed normal morphology and characteristic markers; and with mechanical properties that allowed its use in a DMEK surgery, proving its in vivo functionality in a rabbit model of endothelial dysfunction.

Keywords: silk fibroin films, biomaterials, corneal endothelial cells, DMEK

Descemet membrane endothelial keratoplasty (DMEK) and Descemet stripping automated endothelial keratoplasty (DSEAK) are two of the most used methods in the treatment of corneal endothelial dysfunction.1 In these techniques, Descemet’s membrane and the endothelial monolayer, or Descemet’s membrane with its endothelium and a thin layer of stroma, are transplanted. These procedures have inherent advantages1,2 in terms of visual recovery, final visual acuity, and rejection risk compared with penetrant keratoplasty (PK), which involves the replacement of the full thickness of the cornea. Moreover, these types of surgeries are minimally invasive compared with the current PK technique, in which the anterior chamber is exposed to the environment, avoiding the risk of ocular surface complications,3–5 suture-related infections,6,7 and devastating expulsive haemorrhage intraoperatively or postoperatively.8,9

Despite that DMEK surgery is more complex in graft preparation and handling than other posterior lamellar keratoplasties,10 it is the most in-demand surgical technique because it does not have the disadvantages of transferring another cell type and offers faster and more complete visual rehabilitation than DSAEK.11–17 However, as in other surgical techniques, it still requires of human donors.

The treatment of corneal pathologies using artificial endothelial grafts18 or isolated endothelial cells, associated19,20 or not21 with the use of ROCK kinase, represents a promising future in which there would be less need of human donors when performing surgical interventions. However, cell therapy with injected corneal endothelial cells (CECs) into the anterior chamber may lead to systemic cell dispersion via aqueous flow20 or could lead to secondary glaucoma if the cells obstruct the trabecular meshwork.22 This inherent risk could be avoided by grafting CECs already attached to an artificial Descemet membrane.

In recent years, tissue engineering techniques have been aimed at developing new artificial endothelial grafts, which could supplant the natural endothelium, through optimization of CEC culture techniques23–25 and the development of adequate scaffolds that support their growth.18,20–40 These scaffolds not only have to offer an adequate environment for CEC growth, moreover, they must be able to be used in the...
current minimally invasive surgical techniques while maintain-
ing endothelial function when they are grafted.

Silk fibroin (SF), the structural protein obtained from the
cocon of the silk worm Bombyx mori, has been used in tissue
engineering due to optical properties, nonimmunogenic
response, controllable degradation rates, and tuneable and
robust mechanical properties.41–48 Several studies have report-
ed SF films as an optimal scaffold for different types of cell
cultures.49,50 On the ocular surface, SF films also have been
shown to be an optimal substrate for the culture of corneal
endothelial cells.42,51,52 All these facts make SF films a good candidate for
their use in current surgical techniques.

In the present study, we used SF films for the development
of artificial endothelial grafts. These artificial endothelial grafts
were transplanted into rabbit corneas using a minimally
invasive surgical technique. The tissue-engineered SF endothe-
lial graft restored endothelial function, leading to complete
recovery of the corneal transparency and thickness at 6 weeks.
Finally, we show that human CECs also can be cultured on SF
films, so that SF films may be an alternative for the
development of corneal endothelial artificial grafts for its use
in a human DMEK surgery.

**Materials and Methods**

**Preparation of the SF Films**

*Bombyx mori* cocoons were obtained from silkworms reared in
the sericulture facilities of the IMIDA (Murcia, Spain). Cocoons
were chopped into 4 or 5 pieces and boiled in 0.02M Na2CO3
for 30 minutes to remove the glue-like sericin proteins. Then
raw SF was rinsed thoroughly with water and dried at room
temperature for 3 days. The extracted SF was dissolved in 9.3M
LiBr (Acros Organics, Geel, Belgium) for 3 hours at 60
temperature for 3 days. The resultant 5% wt/vol SF dissolution was
recovered, filtered, and stored at 4°C for no longer than 30 days.
SF films were obtained by casting 580 µL 5% wt/vol SF aqueous
dissolution on a plastic Petri dish, 5.8 cm in diameter, to give a
10-µm-thick film. Once dried at room temperature, the water-
annealing was performed by placing the SF films in a water-filled
desiccator in vacuum conditions for 24 hours.

SF films were sterilized in 70% ethanol for 15 minutes and
rinsed in a sterile solution of PBS pH 7.4 before cell seeding.

**Characterization of Mechanical and Optical
Properties of the SF Films**

Tensile tests were performed using a universal test frame machine
(Qtest; MTS Systems, Eden Prairie, MN, USA). The mechanical
properties of specimens (10 × 30 mm) were recorded with a
crosshead speed of 0.1 mm/s and a load cell of 5N, under ambient
conditions. The thickness of each piece of film was determined
with an electronic digital micrometer, with an accuracy of 1 µm.
Young’s modulus (MPa), ultimate strength (MPa), and elongation
at rupture (%) were determined using the stress-strain curves.
This studio was performed using either dry films or prewet films
(in PBS 1X, for 24 hours at room temperature). Each test was
performed at least three times per condition.

Light transmission measurements were made using a
narrow spectral region between 400 and 700 nm using a
SPELEC Spectroelectrochemical Instrument (Dropsens, Astu-
rias, Spain) equipped with a Deuterium 215 to 400 nm and
Tungsten Halogen 360 to 2500 nm light source and a linear
silicon charge-coupled device array with a detection range 200
to 900 nm detector. The entire tests were carried out in
duplicate at 20°C ± 0.1°C, using prewet (1X PBS for 24 hours
at room temperature) 10-mm-diameter SF films.

For the statistical analyses, IBM SPSS Statistics v.22 software
(IBM Corp., New York, NY, USA) was used. Data followed the
normality and homogeneity of variance requirements and they
were compared by means of the parametric ANOVA test (*P* <
0.05).

**Cell Isolation and Culture of CECs on SF Films**

CECs were cultured in Optimem I (Life Technologies, Carlsbad,
CA, USA) supplemented with 8% vol/vol fetal bovine serum,

**Table. Mechanical Properties of Dry and Prewet SF Films**

<table>
<thead>
<tr>
<th></th>
<th>Dry Films</th>
<th>Prewet Films</th>
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<tr>
<td>Young’s modulus, MPa</td>
<td>34.6 ± 3.3</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Elongation, %</td>
<td>8.3 ± 1.2</td>
<td>23.5 ± 9.1</td>
</tr>
<tr>
<td>Ultimate strength, MPa</td>
<td>21.5 ± 3.3</td>
<td>2.1 ± 1.0</td>
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Values are shown as mean ± SD.
0.3 mM ascorbic acid 2-phosphate, 200 mg/L calcium chloride, 0.04% chondroitin sulfate, 10 U/mL penicillin, 10 μg/mL streptomycin, 20 ng/mL nerve growth factor (Sigma-Aldrich Corp., St. Louis, MO, USA), and 5 ng/mL epidermal growth factor (Austral Biologicals, San Ramon, CA, USA).

Culture of Human CECs. Human tissue was handled according to the Declaration of Helsinki. Corneoscleral rings from corneas previously used for PK were obtained from the local Eye Bank (Centro Comunitario de Sangre y Tejidos, Oviedo, Asturias, Spain) and the Instituto Oftalmológico Fernández-Vega (Oviedo, Asturias, Spain). All tissues were maintained at 4°C in Eusol-C storage medium (Alchimia, Ponte S. Nicolò, Italy) for fewer than 10 days before use. Corneoscleral rings were placed endothelial side up in a Petri dish containing endothelial culture medium. Descemet’s membrane, along with endothelial cells, was carefully dissected under a dissecting stereomicroscope following the Schwalbe line and the peripheral endothelial ring was maintained overnight at 37°C in a culture plate (2 cm²), previously treated with FNC Coating Mix (Athena Environmental Sciences, Baltimore, MD, USA), with 1 mL culture medium. The following day, the excess medium was removed to a volume of 150 μL and then, the peripheral endothelial ring was placed as an explant in the culture plate.

Culture of Rabbit CECs. Rabbit CECs were isolated from healthy male New Zealand white rabbits (2 months of age and body weight of 2.0–2.5 kg) obtained from the Animal Housing Facility of the University of Oviedo (Oviedo, Asturias, Spain). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved (PROAE 14/2014) by the Committee on the Ethics of Animal Experiments of the University of Oviedo and the Animal Production and Health Service of Asturias. Rabbits were kept under a 12/12 day/night light cycle with food and water ad libitum and were monitored on a daily basis.

Descemet’s membrane along with endothelial cells were carefully peeled and digested with trypsin/EDTA 0.25% (Sigma-Aldrich Corp.) for 30 minutes at 37°C. After that, the trypsin was neutralized with culture medium. The loosened cells were centrifuged using an Eppendorf 5702R centrifuge (Eppendorf, Hamburg, Germany) at 0.4g for 10 minutes, and the supernatant was removed. Fresh medium was added and the cells were seeded on a culture plate (10 cm²) previously treated with FNC Coating Mix.

Culture of CECs on SF Films. When human or rabbit cultures were confluent, cells were digested with Accutase (Sigma-Aldrich). The loosened cells were centrifuged using an Eppendorf 5702R centrifuge at 0.4g for 10 minutes and the supernatant was removed. Fresh medium was added and the cells were seeded (100,000 cells/cm²; passage 1) on one of the sides of a SF film, previously treated with FNC Coating Mix, being cultured for a week in an 11-mm-diameter culture device.

All cells were cultured under the same conditions (humidified atmosphere at 37°C, 5% CO₂, medium changed three times per week).

Examination of Cell Cultures

Cellular growth was assessed using a Leica DMIL LED phase-contrast microscope (Leica, Wentzler, Germany); photos were taken with an attached EC3 camera (Leica).

Confluent cultures on SF films were fixed using ice-cold methanol for 10 minutes, an 8.5-mm-diameter punch was performed and used for phase-contrast microscopy, immuno-cytochemistry, and scanning electron microscopy (SEM).

Methanol fixed cultures were rinsed with PBS solution twice for 10 minutes and permeabilized in a PBS solution containing 0.3% Triton-X 100 for another 10 minutes. Next, the samples were incubated with primary antibody containing 10% normal goat serum (Abcam, Cambridge, UK) at 4°C overnight. Mouse Na⁺/K⁺ ATPase (Millipore, Billerica, MA, USA) (1:100)
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RESULTS

Preparation of the SF Films

SF films were obtained by evaporating the SF dissolution extracted from cocoons of *Bombyx mori* after removing the glue-like sericin proteins. Fabricated films had an average area of 3.46 cm² and thickness of 10 μm showing a smooth surface when observed under SEM (Fig. 2).

Characterization of Mechanical and Optical Properties of the SF Films

Average values of Young’s modulus, the elongation at break, and the ultimate strength were calculated using stress-strain curves generated from tensile tests and are shown in the Table. All were found to be statistically different depending on the hydration of the film (ANOVA, *P* < 0.05).

As can be observed in the Table, the average value of Young’s modulus was much higher in dry films (346 MPa) than in prewet films (22 MPa), with the latter being less rigid. The elongation was improved with the hydration of the films, with average values of 8.3% and 23.5% in dry and prewet films, respectively (Fig. 3). Otherwise, the estimated ultimate
strength decreased from 21.5 MPa to 2.1 MPa after prewetting the films.

Optical testing revealed (Fig. 4) that SF films were optically transparent at all wavelengths of the visible light electromagnetic spectrum with a mean value of light transmission of 89.447% ± 0.84%.

**Cell Isolation, Culture, and Examination of CECs on SF Films**

Human CECs were observed to have migrated from the explant onto the plate after 3 to 4 days in culture, and by day 30 a monolayer of compact cells had formed on the plate immediately adjacent to the explant. These cells displayed their typical hexagonal endothelial morphology. After 30 days, confluent (80,000 cell/cm²) hexagonal human CECs were detached by Accutase digestion, and subcultured for a week on SF films previously treated with FNC Coating Mix. Without this treatment, human CECs showed signs of endothelial-mesenchymal transition with an elongated and fibroblast-like abnormal phenotype (Fig. 5).

In the same way, rabbit CECs were able to attach and proliferate until confluence (150,000 cells/cm²) when isolated by trypsin/EDTA digestion. In this case, when rabbit CECs were subcultured for a week on SF films, treatment with FNC improved morphology of rabbit CECs only slightly (Fig. 6).

These results were confirmed under SEM, whereby human and rabbit CECs displayed their polygonal morphology when they were cultured on SF films previously treated with FNC Coating Mix (Fig. 7).

Immunohistochemical analysis revealed positive staining for ZO-1 and Na⁺/K⁺ ATPase, a tight junction-associated protein and an integral membrane protein, respectively, characteristic markers of CECs and responsible for endothelial barrier and pump functions (Fig. 8).

**FIGURE 9.** Photographs of the exterior appearance of transplanted rabbit eyes during the follow-up period. From top to bottom: rabbits transplanted with or without rabbit CECs cultured on SF films and rabbits with only peeled Descemet’s membrane.

**FIGURE 10.** AS-OCT of control, descemetorhexis, and transplanted rabbit corneas displaying corneal thickness at 6 weeks.
Transplantation of SF Films in a Rabbit Model

Corneal edema and white turbidity appeared a few days after surgery in the three groups: rabbits transplanted with or without rabbit CECs cultured on SF films and rabbits with only peeled Descemet’s membrane. Corneas transplanted with cultured rabbit CECs on SF films began to become transparent by day 15, and corneal transparency was maintained up to 6 weeks. However, the eyes in the group without rabbit CECs cultured on SF films and the eyes in the group of rabbits with peeled off Descemet’s membrane did not restore corneal transparency and retained the corneal edema during the follow-up period. No obvious signs of immune rejection were found in any group (Fig. 9).

Results of AS-OCT revealed SF films with cultured rabbit CECs as a fully integrated component in the corneal tissue, displaying a similar corneal thickness when compared with its healthy contralateral cornea. SF films without cultured rabbit CECs and rabbits with peeled off Descemet’s membrane revealed only an enhancement in corneal thickness reflecting a loss of corneal endothelial functionality (Fig. 10).

Topography mapping of transplanted corneas revealed irregular values of peripheral corneal thickness (392–477 μm), whereas central cornea appeared homogeneous (366 μm) in corneas with cultured rabbit CECs, displaying a similar distribution when compared with its healthy contralateral cornea (359 μm) (Fig. 11). Moreover, the CECs count in eyes transplanted with cultured rabbit CECs on SF films had a number of cells with a size and shape similar to the ones found in the contralateral control eye (Fig. 11).

The histologic analysis showed a slightly marked fibrotic tissue in the posterior segment of the stroma in rabbits transplanted with SF films without cultured rabbit CECs and an apparent corneal edema and fibrotic tissue in the rabbits with peeled off Descemet’s membrane. SF films, in rabbits transplanted with cultured cells, was attached tightly to the corneal stroma, and rabbit CECs formed a continuous monolayer with the same morphology and phenotypical markers, ZO-1 and Na⁺/K⁺ ATPase, as a healthy control eye (Fig. 12). No sign of endothelia remains were found in any of the transplanted corneas.

DISCUSSION

Currently, corneal transplantation from cadaveric donors is the main alternative for the treatment of endothelial dysfunctions. However, this procedure is limited by the shortage of high-quality donor corneas. Due to recent advances in the field of biomaterials and the culture of CECs, the development of artificial endothelial grafts could be an alternative to traditional surgeries.

In this study, we have isolated SF from cocoons of Bombyx mori and manufactured it into 10-μm-thick transparent films that support human and rabbit CEC growth. Madden et al. were the first to prepare 5-μm thick SF transparent films, modified with coatings of collagen IV, FNC Coating Mix, and
chondroitin sulfate laminin mixture to assess cell attachment and proliferation of the B4G12 cell line and primary culture of human CECs. The group achieved successful cell confluence and proliferation with the collagen coating, concluding that SF films could be a potential substratum for the transplantation of artificial endothelial grafts for keratoplasty. In our case, the treatment of SF films with FNC Coating Mix allowed the growth of both rabbit and human CECs, maintaining the hexagonal morphology and the expression of its characteristic markers ZO-1 and Na⁺/K⁺ ATPase.

The second outcome of this study was that SF films have mechanical properties that permit their use in a disposable inserting set for Descemet lenticle. Our SF prewet films showed a Young's modulus value of 22 MPa and an ultimate strength of 2.1 MPa. These values are similar to the values of posterior corneal stroma described in the literature and allowed that SF films were easily handled for transplantation into rabbit eyes in a DMEK model. Our SF artificial graft was able to fold in the interior of a disposable inserting set for Descemet lenticle, and was easily introduced and unfolded in the anterior chamber in a DMEK surgical procedure, indicating that it could be used in this type of surgery. This point is important in corneal regenerative medicine because a tissue-engineered endothelium should be able to adapt to the current surgical procedures, avoiding the need for the development of new grafting techniques, and, therefore, making it easier to implement in the clinical practice.

In our study, we established a 6-week follow-up period. Throughout this follow-up period, it was observed that transplanted corneas with cultured rabbit CECs on SF films began to become transparent by day 15 and corneal transparency was maintained up to 6 weeks. Results of AS-OCT revealed SF films with rabbit CECs as a fully integrated component in the corneal tissue, displaying a similar average corneal thickness when compared with their healthy contralateral cornea. Although CEC density was found to be lower in the rabbits transplanted with SF films without rabbit CECs, the density of CECs seems adequate because it restored corneal transparency.

It is known that rabbit CECs have the ability to proliferate both in vivo and in vitro. However, in rabbits transplanted with SF films without rabbit CECs and in rabbits with peeled off Descemet’s membrane only, the cornea remained edematous during the entire follow-up period and the corneal thickness was greater than that of the healthy contralateral cornea. These findings indicate that corneal transparency, at
the 6-week follow-up period, is due to SF artificial endothelial graft and not due to spontaneous regeneration of autologous rabbit endothelium.

Histologic staining showed that the SF artificial endothelial graft was attached and integrated in the corneal stroma without a significant inflammatory reaction, and rabbit CECs consisted of a monolayer that showed their characteristic markers ZO-1 and Na+/K+ ATPase, suggesting proper intercellular junctions and cellular pump function. These results confirmed that the SF artificial endothelial graft performs as expected on in vivo implantation and contributes to the maintenance of corneal clarity.

Several groups, including our own, have already developed different artificial endothelial grafts that allow the recovery of endothelial functionality in different in vivo models.18,27,29,57,58 From our point of view, SF films present several advantages, such as having better mechanical properties than other biomaterials, being a biomaterial that has been used for centuries in medicine and that could be easily accessible and have a lower cost of production than other biomaterials in some countries. However, future studies with more animals and a longer follow-up period will be necessary to elucidate the ideal biomaterial for this purpose.

Our current results still have some issues to resolve. On the one hand, the number of animals was limited, and on the other hand the differences in the size of anterior chamber and the IOP between rabbits and humans make rabbit DMEK surgery even a more complex procedure, limiting the correct placing of the graft. In our case, pachymetry and AS-OCT revealed some irregular values of corneal thickness in the peripheral cornea. Several studies have demonstrated that the presence of anomalies, such as folds or wrinkles in the graft, secondary to posterior keratoplasties, could compromise visual acuity. However, this limitation could be overcome with an appropriate handling of the graft.

In conclusion, we developed SF films with biological properties that supported the growth of rabbit CECs, which expressed its normal morphology and characteristic markers; and with mechanical properties that allowed its use in a DMEK surgery, proving its in vivo functionality in a rabbit model of endothelial dysfunction. Furthermore, clinical translation to human therapies might be feasible because, as shown, human CECs also can be cultured on SF films.

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