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Keratin-chitosan membranes as scaffold for tissue engineering of human cornea

Natalia Vázquez[1,3,*], Manuel Chacón[1,3,*], Álvaro Meana[1,3,4], Yolanda Menéndez-Menéndez[2], Amaia Ferrero-Gutierrez[2], David Cereijo-Martín[5], Miguel Naveiras[1] and Jesús Merayo-Lloves[1,3]

[1]Eye Research Foundation - Instituto Oftalmológico Fernández-Vega, Oviedo, [2]Transplant and Cell Therapy Unit - Hospital Universitario Central de Asturias, Oviedo, [3]University of Oviedo, Oviedo, [4]U714 Centre for Biomedical Network Research on Rare Diseases (CIBERER), Oviedo and [5]Prodintec Foundation, Gijón, Spain

* These authors have contributed equally to this work

Summary. Purpose: To study the attachment and growth of human corneal cells on keratin-chitosan membranes. The end goal is to develop a bioengineered cornea based on this material.

Methods: Keratin-chitosan membranes were prepared as previously described by Tanabe et al., 2002. Briefly, 7.15 mg/cm^2 of keratin dialysate was mixed with 10wt% chitosan solution and 20 wt% glycerol. The solution was cast into a silicone mold and dried at 50°C for 36 hours.

Eyes were attained from a local eye bank after penetrant-keratoplastic surgery. Human epithelial, stromal and endothelial cells were obtained of the limbal, stromal and endothelial regions. Cells were cultured on keratin-chitosan membranes, as well as on plastic dishes as controls. When cultured cells reached confluence, they were fixed, incubated with primary antibodies (E-cadherin, cytokeratin high molecular weight (CK), vimentin and Na^+/K^+ ATPase) and visualized by indirect immunocytochemistry.

Results: Epithelial, stromal and endothelial cells were able to attach and grow on keratin-chitosan membranes. All the cells maintained their morphology and cellular markers, both in the membrane and on the culture plate.

Epithelial cells stained positively for CK and E-cadherin. A positive vimentin stain was observed in all stromal cells, while endothelial cells were positive for vimentin and Na^+/K^+ ATPase, but negative for E-cadherin.

Conclusions: Keratin-chitosan membranes have been shown to be a good scaffold for culturing epithelial, stromal and endothelial corneal cells; therefore, future applications of keratin-chitosan membranes may be developed for reconstruction of the cornea.

Key words: Keratin-chitosan scaffold, Human limbal epithelial cells, Human corneal stromal cells, Human corneal endothelial cells.

Offprint requests to: Natalia Vázquez, Eye Research Foundation, Instituto Oftalmológico Fernández-Vega, Avda. Dres. Fernández-Vega 34, 33012 Oviedo, Asturias, Spain. e-mail: natalia.vazquez@fio.as

Introduction

The human cornea is a transparent structure that acts as the external limit of the eye; it prevents its contamination and the loss of liquids and proteins. The cornea also works as a powerful refractive lens (42 diopters). When the cornea loses its transparency, the repair is usually performed with a corneal transplant from a cadaveric donor. To date, the most typical transplant was penetrant keratoplasty (Geerling and Seitz, 2005), in which the entire thickness of the cornea was replaced. Nowadays, surgical procedures increasingly tend to limit the repair to the damaged area of the cornea by employing anterior and posterior lamellar transplants (Zhang et al., 2013).

An alternative to the current methodology of corneal transplantation may be the production of tissue-engineered corneas. There already exist some artificial cornea models that contain the three human corneal cell

lineages (Germain et al., 1999). However, the tissue-engineered production of a complete cornea, endowed with a refractive component, still seems distant. The production of partial portions of the cornea (González-Andrades et al., 2009), which could be used as lamellar grafts, seems to be a more affordable goal with the current technology. The epithelia cultured on fibrin (Hirayama et al., 2012; San Martín et al., 2013) or amniotic membrane (Rendal-Vázquez et al., 2012), are widely used in the clinic for the treatment of limbal stem cell deficiency (Konomi et al., 2013). The endothelia developed *ex vivo* through culturing on different supports, have already been described in some experimental transplant models (Wang et al., 2012). Finally, the stromal keratocytes are cells that are easily grown on different scaffolds (Guan et al., 2013) that may be used for the repair of stromal scars. These tissue-engineered models could be incorporated into the new therapies as lamellar grafts.

Scaffolds in cornea tissue engineering should meet various criteria: biodegradable, optically transparent, non-toxic and surgically manageable. Keratin-chitosan membranes have already proven their ability to support fibroblast attachment and proliferation *in vitro*, being demonstrated as a good substrate for culturing mammalian cells (Tanabe et al., 2002). In the present study, we evaluate the ability of these membranes to support primary cultures of human epithelial, stromal and endothelial corneal cells and their potential use as a new carrier of human corneal cells for the selective reparation of the cornea.

Materials and methods

Materials and reagents

Materials for cell culture, including Dulbecco Modified Eagle's Medium (DMEM) culture medium, Ham's F12 culture medium, foetal bovine serum (FBS), penicillin-streptomycin, insulin, cholera toxin, adenine, triiodothyronine, hydrocortisone, non-essential amino acid solution, RPMI 1640 vitamin solution, ascorbic acid 2-phosphate, chondroitin sulfate, calcium chloride, collagenase I and trypsin/EDTA 0.25% solution, were purchased from Sigma-Aldrich (MO, USA). Epidermal growth factor was obtained from Austral Biologicals (CA, USA), Optimem I from Life Technologies (CA, USA) and FNC Coating Mix® was obtained from Athena Enviromental Sciences (MD, USA). All the reagents used for the purification and quantification of keratin and the preparation of the keratin-chitosan membranes were purchased from Sigma-Aldrich (USA).

Materials for the immunovisualization of the cell cultures including antibodies against cytokeratin high molecular weigth (CK) and vimentin were purchased from Dako (Glostrup, Denmark), Na^+/K^+ ATPase from Millipore (MA, USA), E-cadherin from Santa Cruz Technologies (CA, USA) and Vectastain ABC Elite kit and normal goat serum was purchased from Vector (CA, USA). All other reagents used were of reagent grade.

Preparation of the keratin-chitosan membranes

Keratins were prepared according to a previously reported method (Nakamura et al., 2002). Briefly, 20 g of human hair was immersed in 400 ml of extraction solution containing 25 mM Tris, 5 M Urea, 2.6 M Thiourea and 5% 2-mercaptoethanol. The mixture was shaken at 50°C for 5 days, and then was centrifuged using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) at 4500xg for 15 minutes and the supernatant was filtered through a 40 μm nylon mesh. The filtrate was dialyzed against 5 litres of distilled water using molecular porous membrane tubing (molecular cut-off 6-8kDa) for 3 days changing the water every day. After three days, the keratin dialysate was centrifuged at 10,000xg for 30 minutes discarding the precipitate formed. The protein concentration of dialysate was 30-50 mg/ml on average based on the Lowry method using protein assay kit. The obtained keratin solution was stored at 4°C before use.

As previously described (Tanabe et al., 2002) 7.15 mg/cm^2 of protein dialysate were diluted in 3 volumes of glacial acetic acid that was then mixed with 10 wt% chitosan solution (2.5 mg/ml in 75% acetic acid solution) and 20 wt% glycerol. The keratin-chitosan solution was cast onto a 2.1 cm diameter silicone mold and dried at 50°C for 36 hours. The film was stabilised for 24 hours at room temperature before its use.

Membrane manipulation

For cellular culture, all the keratin-chitosan membranes were cultured using a special device (Fig. 1) developed for our laboratory by the Prodintec Foundation (Prodintec, Gijon, Asturias) that facilitates the handling, cell culture and transport of the membranes. Briefly, this device is Teflon made and consists of two rings of 30mm external diameter and 15mm internal diameter with a pressure closure mechanism.

All the membranes were placed between the two rings, and once secured the device and the membrane were sterilized in 70% ethanol for 30 minutes and rinsed in a sterile solution of 0.1 M phosphate buffered saline pH 7.4. The device with the membrane was placed inside a 6-well culture plate filled with culture medium in order to stabilize the membrane before seeding the cells.

In order to simulate the diameter of the human cornea, membranes fastened in the handling device were punched using an 8.5 mm diameter trephine. Surgical handling was tested for its suitability for the selective reparation of the cornea by performing a simulated endothelial keratoplasty using enucleated rabbit eyes, treating keratin-chitosan membranes as a Descemet's membrane. Briefly, a 2.8 mm incision was made in a rabbit eye at the limit of the corneoscleral tissue and a

keratin-chitosan membrane was injected as a roll into the anterior chamber using an Endosaver® Corneal Endothelium Delivery System (Ocular Systems Inc., NO, USA), carefully unrolled, and floated up onto the back of the rabbit cornea on a gas bubble.

Cell isolation and culture

Human tissue was handled according to the Declaration of Helsinki. Corneoscleral rings from human donors eyes were obtained from our local Eye Bank (Centro Comunitario de Sangre y Tejidos, Oviedo, Asturias, Spain) and the Instituto Oftalmológico Fernández-Vega (Oviedo, Asturias, Spain). All of their central corneal buttons were used for corneal transplantation. The donor's ages were between 52-73 years. All tissues were maintained at 4°C in Eusol-C storage medium (Alchimia, Ponte S.Nicolò, Italy) for less than 10 days before study.

Culture of corneal epithelial cells

Human corneal epithelial cells were cultured in a 1:1 mixture of DMEM and Ham's F12 media supplemented with 10 v/v% FBS, 5μg/ml insulin, 8.33 ng/ml cholera toxin, 24 μg/ml adenine, 1.3 ng/ml triiodothyronine, 0.4 μg/ml hydrocortisone, 100 U/ml penicillin and 0.1 mg/ml streptomycin. This medium was used in the first three days of culture, after that, cells were cultured in the same medium with 10 ng/ml epidermal growth factor.

The limbal region was carefully dissected under a dissecting stereomicroscope according to its anatomical position and then cut into small pieces approximately 2-3 mm in diameter. The tissue pieces were placed as explants onto sterile keratin-chitosan membranes, previously placed on the handling device, and allowed to attach with only a small drop of culture medium. Other explants of the same ring were also plated on a culture dish as a control group. After 14-16 hours the membranes were submerged in culture medium.

Culture of corneal stromal keratocytes

Human corneal stromal keratocytes were cultured in a 1:1 mixture of DMEM and Ham's F12 supplemented with 10 v/v% FBS, 1 v/v% non-essential amino acid solution, 1 v/v% RPMI 1640 vitamin solution, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

The stromal region was dissected, cut into small pieces and digested with trypsin/EDTA 0.25% 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 0.4 rcf for 5 minutes and the supernatant was removed. Fresh medium was added and the cells were seeded on a culture plate. Once the culture was confluent, cells were trypsinized, centrifuged and reseeded, with a density of 10,000 cel/cm^2, on a sterile keratin-chitosan membrane fastened on a handling device and on a culture plate as a control group.

Culture of corneal endothelial cells

Human corneal endothelial cells were cultured in Optimem I supplemented with 8 v/v% FBS, 20 μg/ml ascorbic acid 2-phosphate, 200 mg/l calcium chloride, 0,08% condroitin sulfate, 5 ng/ml epidermal growth factor, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

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. The endothelium was digested using collagenase I (1 mg/ml) for 1 hour at 37°C followed by washing with Optimem I. Cells were centrifuged and seeded onto a sterile keratin-chitosan membrane held in a handling device as well as in a culture plate as a control group. Both, the membrane and the control plate were previously treated with FNC coating Mix®.

All of the cells were cultivated under the same conditions (humidified atmosphere at 37°C, 5% CO_2, medium changed 3 times per week).

Examination of cell cultures

Once the cultures were confluent, an 8.5 cm diameter punch was performed and used for contrast phase microscopy, scanning electron microscopy (SEM) and immunocytochemistry.

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

The surface morphology of keratin-chitosan membranes, and keratin-chitosan membranes with corneal cells attached, were observed with SEM. The membranes, previously fixed in 4% paraformaldehyde for 5 minutes, were dehydrated in a graded series of acetone (30%, 50%, 70%, 90% and 100%) for 10 minutes respectively and dried by the critical-point method. Afterwards, they were coated with gold under vacuum and observed with a JEOL 6610LV scanning microscope, (JEOL co., Tokyo, Japan) at 20 kV accelerated voltage.

Keratin-chitosan membranes were also fixed using 4% paraformaldehyde for 5 minutes for their immunocytochemistry analysis. Paraformaldehyde fixed cultures were rinsed with PBS solution twice for 10 minutes and permeabilized in a PBS solution containing 0.3% Triton-X100 for another 10 minutes. Next, the samples were incubated with 0.9% H_2O_2 for 30 minutes at room temperature, in order to inactivate endogenous peroxidase activity. Following this, the samples were incubated with primary antibody containing 10% normal goat serum at 4°C overnight. The E-cadherin (1:200), and CK (1:40) antibodies were used to identify corneal epithelial cells; vimentin monoclonal antibody (1:100)

was used as a mesenchymal marker to identify stromal keratocytes and endothelial cells. Finally, a Na^+/K^+ ATPase (1:100) and an E-cadherin (1:200) immunostain were performed on the corneal endothelial cells in order to confirm the phenotype of these cells.

Subsequently, the samples were incubated with a biotinylated secondary antibody (1:500) for 2 hours at room temperature. Between incubations cells were washed 3 times with PBS for 10 minutes. Immunolabeled cells were visualized using a Vectastain ABC Elite kit with diaminobenzidine (DAB) as the substrate. All the samples were critically examined in a Leica DMIL LED light microscope (Leica, Wetzlar, Germany).

Results

Handling the membrane

The device developed for the manipulation and transport of cultured keratin-chitosan membranes resulted in improvement of the performance of the cultures by avoiding breaks and scratches on the keratin-chitosan membranes while being held in a fixed position. Moreover, cell seeding and media changes could be performed without any risk of the membranes being damaged.

Furthermore, we also proved the ability of these membranes to be able to fold and unfold without breaking and resist the implantation inside an enucleated rabbit eye while performing a simulated endothelial keratoplasty (Fig. 2).

Microstructural characteristics

Figure 3 shows the SEM micrographs of the keratin-chitosan films. In figure 3A the surface of the keratin-chitosan film is visible. Figure 3B is a magnified field of micrograph 3A and shows the nano-particulate pattern of keratin-chitosan particles. The magnified view (3B) reveals that the keratin-chitosan film consists of spherical, tightly packed and well-connected nanostructures of keratin-chitosan.

Scanning electron micrographs of epithelial, stromal and endothelial corneal cells confirmed that these cells are capable of growing and expanding while attached to keratin-chitosan membranes maintaining their polygonal, fusiform or cobblestone morphology (Fig. 3).

In vitro evaluation

Corneal cells demonstrated their ability to attach and proliferate when cultured on keratin-chitosan membranes. Corneal cells maintained their morphology and cellular markers in the membrane yielding no differences compared to the control group.

After 4 days epithelial cells began to migrate from the limbal explants. Cells were flat and round in shape, forming a monolayer. The cultures were confluent at 10 days (Fig. 4). Epithelial cells showed positive staining

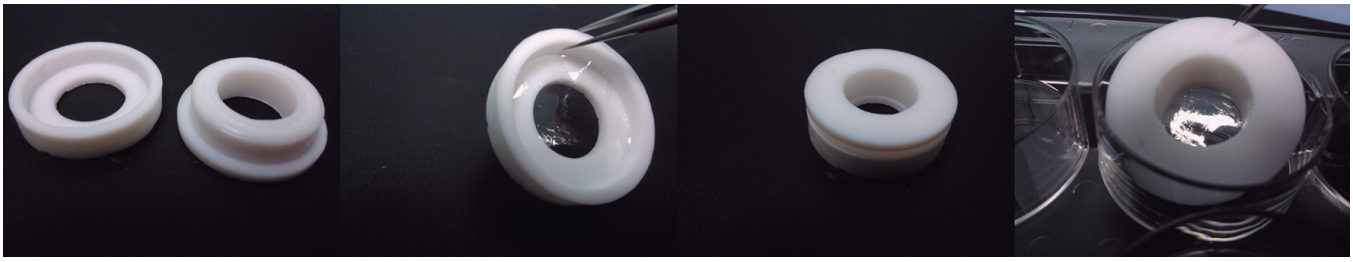

Fig. 1. Handling device with keratin-chitosan membrane.

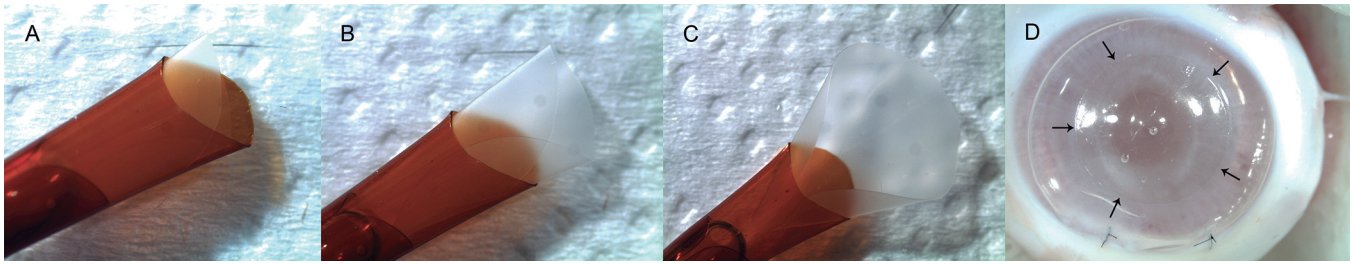

Fig. 2. Step by step unfolding of a keratin-chitosan membrane **(A, B, C)**. Keratin-chitosan membrane as an endothelial substitute in an enucleated rabbit eye. **D.** Arrows shows the edges of the membrane.

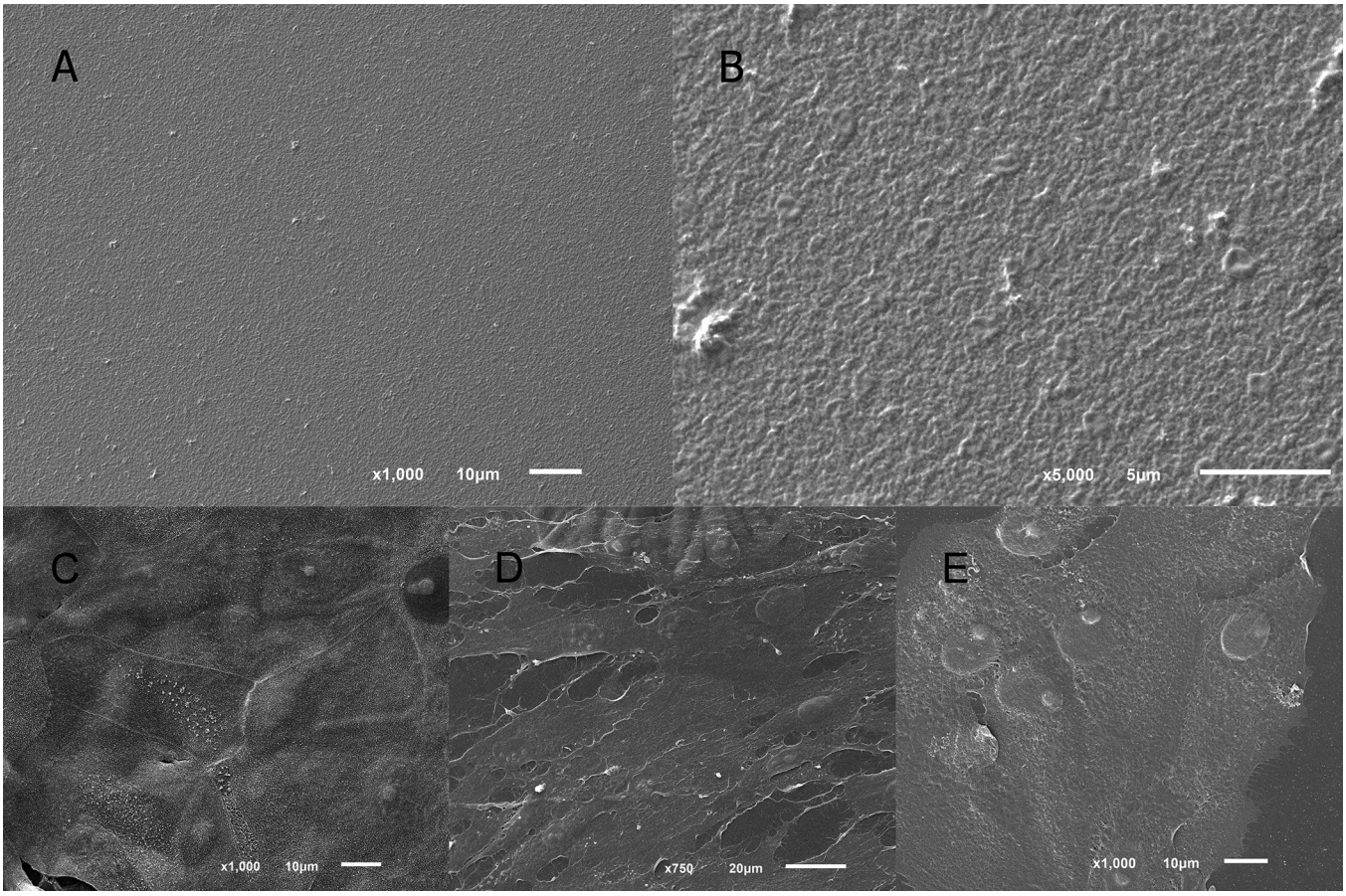

Fig. 3. SEM images of the surface of the membrane **(A, B)** and SEM images of primary culture of the three different corneal cell types: limbal cells **(C)**, stromal cells **(D)** and endothelial cells **(E)** on keratin-chitosan membranes.

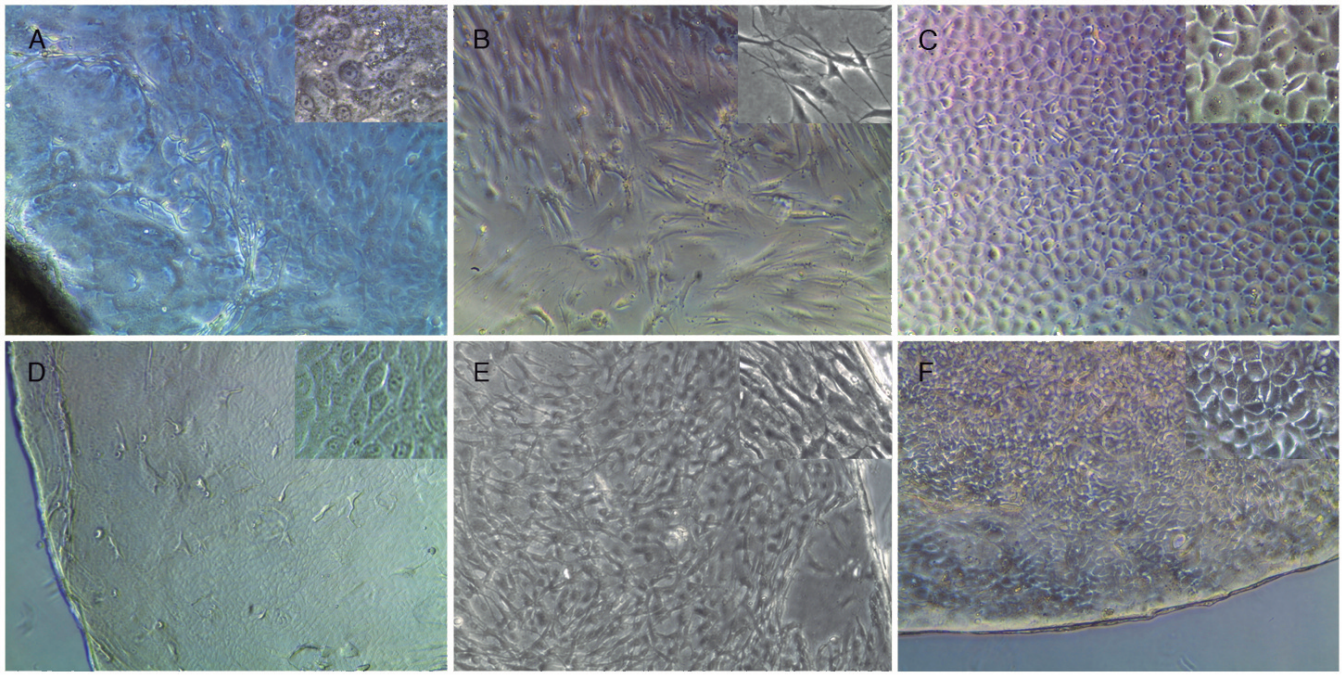

Fig. 4. Phase-contrast micrograph of primary culture of the three different corneal cell types (**A, D** limbal cells, **B, E** stromal cells and **C, F** endothelial cells) on keratin-chitosan membranes **(D-F)** and control group **(A-C)**. x 5, insert x 10

for CK and E-cadherin (Fig. 5).

Stromal cells were able to attach and proliferate on keratin-chitosan membranes and were confluent after 15 days showing a fusiform morphology (Fig. 4). A positive vimentin stain was observed in all stromal cells (Fig. 6).

After 2 days endothelial cell clusters were attached and endothelial cells began to proliferate. Cells showed cobblestone or polygonal morphology, and the culture was confluent at 12 days (Fig. 4). Immunocytochemistry showed positive staining for vimentin and Na^+/K^+ ATPase and was negative for E-cadherin (Fig. 7).

Discussion

The cornea is the most grafted human organ. Nowadays, corneal transplants are increasing in number due to them being the typical prognosis for visual impairment (Crawford et al., 2013). In the near future the total number of corneal surgeries will increase even more as the population grows. Therefore, more corneas will be required to meet clinical demands. However, there is a progressive decrease of keratoplasties that require the full thick cornea in favour of the selective interventions where only the damaged parts of the cornea are replaced (Young et al., 2012). Some of these clinical needs could be filled with the development of tissue engineered corneal layers (Ionescu et al., 2011).

We define a tissue engineered corneal layer as cultured cells on a scaffold. In the present study, we used a keratin-chitosan membrane to evaluate the potential use of this material as a carrier for human corneal cells. Our results shown that keratin-chitosan film could be used in an experimental graft and supports the growth of the three cell types of the cornea while maintaining the same morphology and cellular markers as in native cornea; even though endothelial cells required a fibronectin pre-treatment of both the culture plate and the keratin-chitosan membrane to improve the

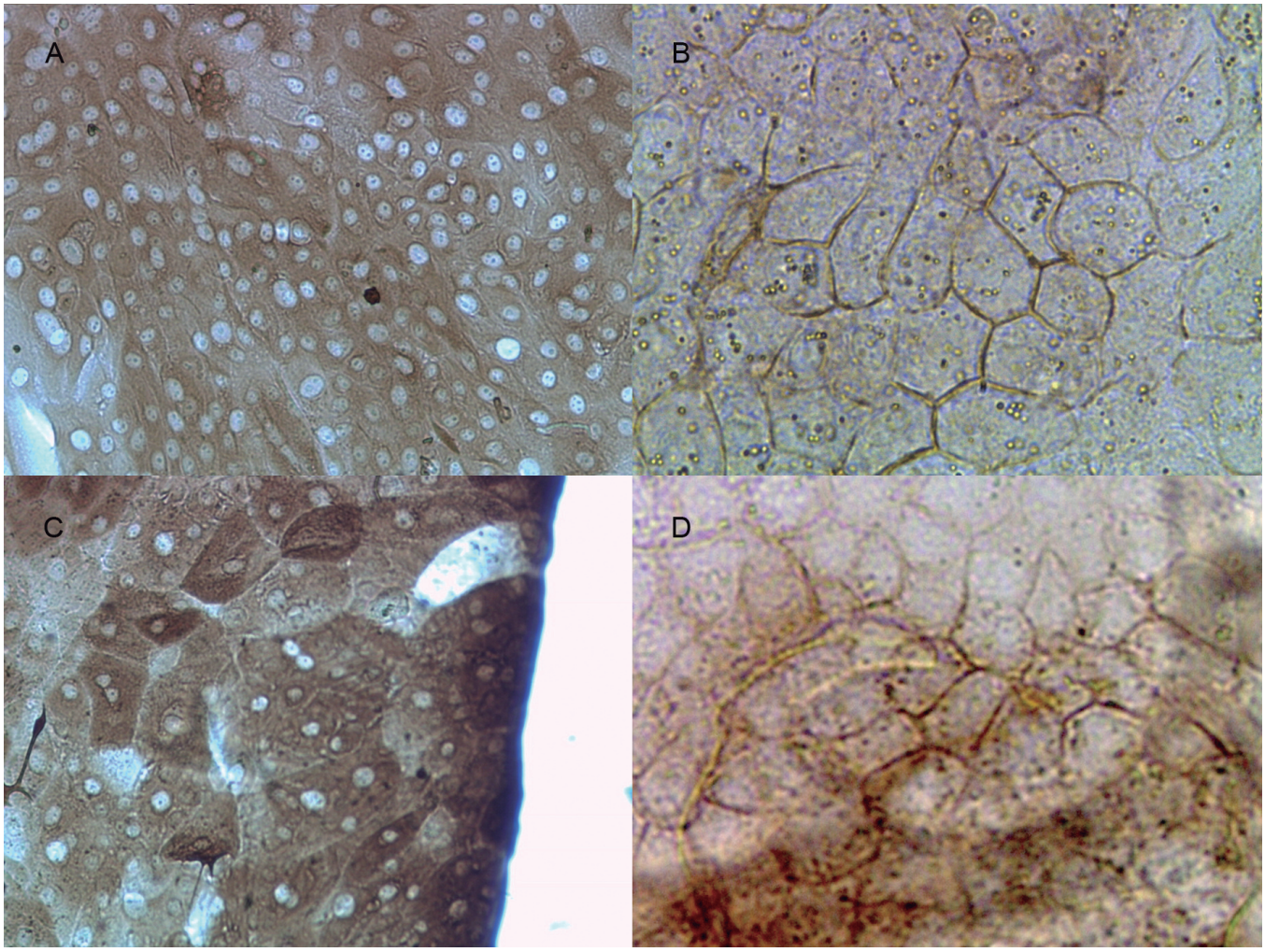

Fig. 5. Immunostain of limbal cells (**A, C**: CK, **B, D**: E-cadherin) on keratin-chitosan membranes **(C, D)** and control group **(A, B)**. A, x 10; B-D, x 20

attachment and proliferation ratio of these cells.

One of the biggest problems when facing the culture of cells on scaffolds is its manipulation without damage. In this study, we have shown a possible way to work with membrane type scaffolds preventing breaks and scratches by fastening them to a handling device. For this purpose, the membrane had to be quite strong and flexible to be able to fit and endure the culture process. Keratin-chitosan membranes were good enough to resist the whole procedure; moreover, this scaffold was able to resist the manipulation of a simulated experimental endothelial transplant without any visible damage.

In tissue engineering, it is of great importance that the cell culture models truly represent the organization and structure of the tissue being replaced (Guilak et al., 2014). In our case, by focusing on a corneal selective

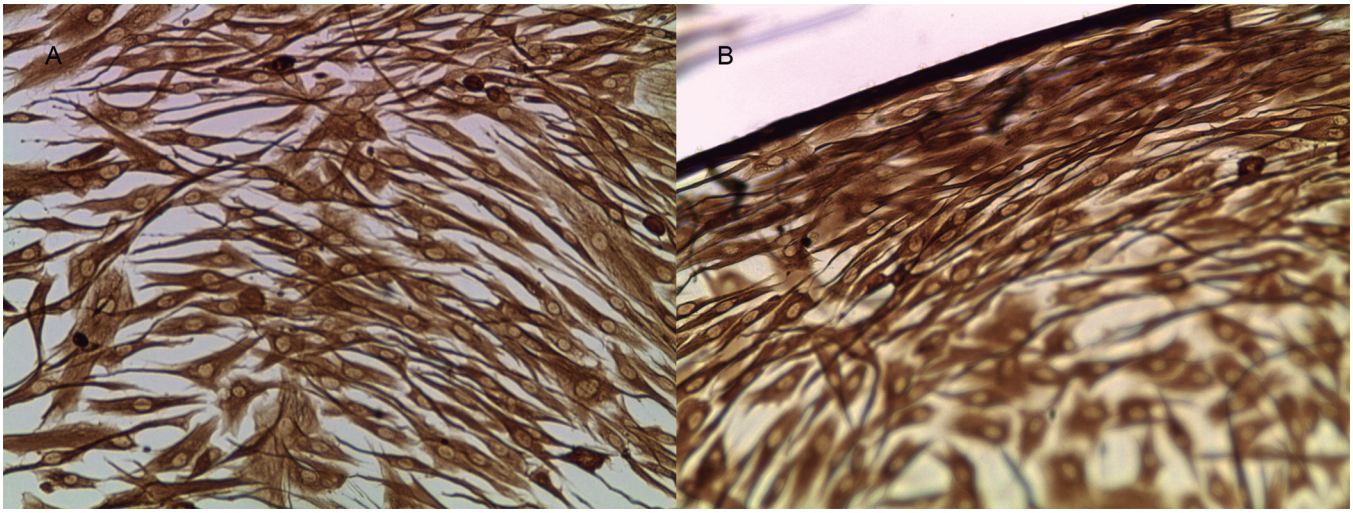

Fig. 6. Immunostain of stromal cells (vimentin) on keratin-chitosan membranes **(B)** and control group **(A)**. x 10

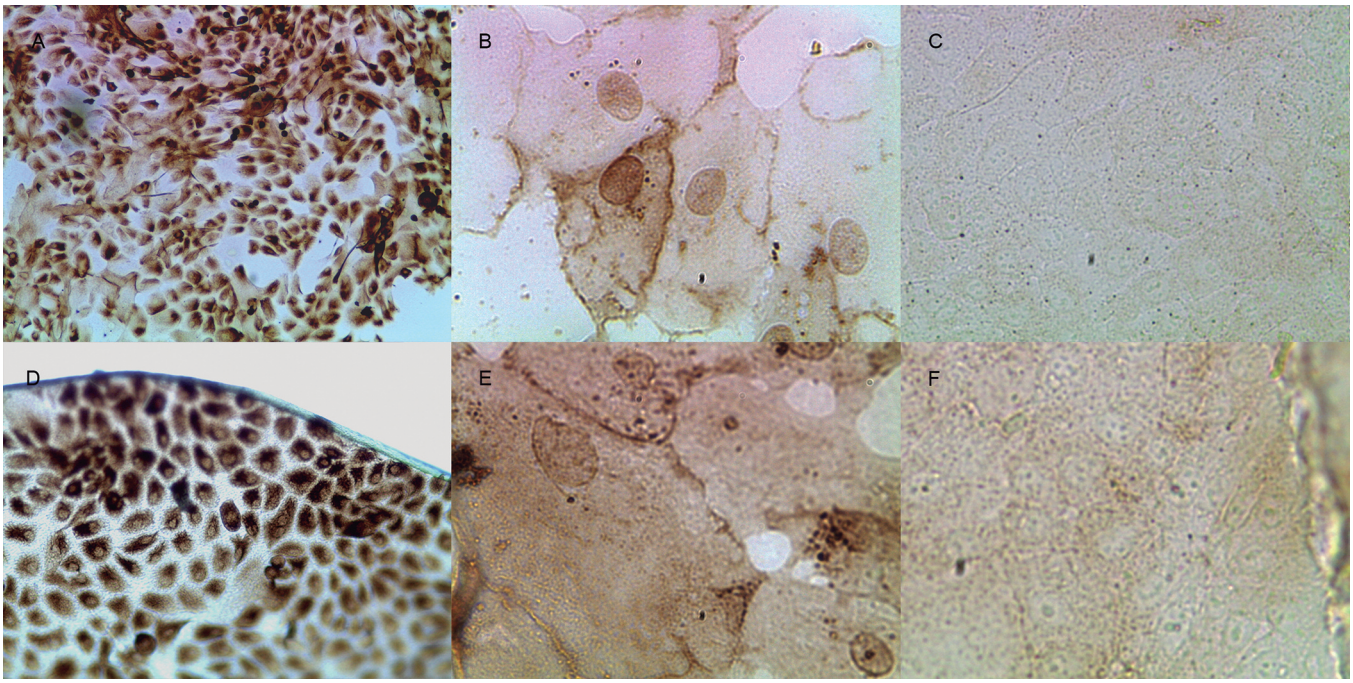

Fig. 7. Immunostain of endothelial cells (**A**, **D**: vimentin, **B**, **E**: Na^+/K^+ ATPase, **C**, **F**: E-cadherin) on keratin-chitosan membranes **(D-F)** and control group **(A-C)**. A, x 5; B, E, x 40; C, F, x 20; D, x 10

transplant methodology, each of the tissue-engineered layers must reproduce the natural structure of its correspondent corneal layer. We also proved that these cells, growing on keratin-chitosan membranes retained their phenotypic morphology as observed by SEM. Micrographs revealed a tightly joined cell structure with a characteristically polygonal morphology in the epithelial cultures. Stromal cells are specialized fibroblasts residing in the stroma and, as in native cornea, showed a fusiform pattern; finally, endothelial cells, a single layer of flattened cells, exhibited a predominantly hexagonal shape.

Although their morphology seemed to match their correspondent corneal layer, we also needed to prove that those cells also retained some of their specific cell markers by immunostains. As said, corneal epithelium is a multilayered structure with strong junctions between cells (Chen et al., 2004). We proved the presence of these bonds with an immunostain against E-cadherin; moreover, to ensure the corneal epithelial origin of these cells, an immunostain against CK was also performed. The positive stain of both of these markers along with the SEM results suggests that the culture of this type of cells on keratin-chitosan membranes truly represents the structure of corneal epithelium. Stromal keratocytes and corneal endothelial cells are known to originate from neural crest cells and then differentiate into mesenchymal cells as described by Nakamura (1982). In order to prove the correct origin of our cell cultures we performed an immunostain against vimentin (a type of intermediate filament protein that is expressed in mesenchymal cells) as evidence of the mesenchymal origin of these two cell types (Hayashi et al., 1986). Additionally, we needed to prove proper endothelial function on our cell cultures. Endothelial cells are known for transporting fluids and solutes across the cornea and maintaining the cornea slightly dehydrated with an active Na^+/K^+ pump activity (Peh et al., 2011). We proved a retained pump function, shown by a marked intercellular junction stain to Na^+/K^+ ATPase. These endothelial cells are bound together by strong intercellular bonds of the classical cadherin family (Vassilev et al., 2012), although a negative E-cadherin stain confirms the non-epithelial origin of these cells, suggesting other cadherins take the role of linking these cells together.

These results suggest the possibility that all the cell cultures on keratin-chitosan membranes, originated from their correspondent corneal layer, may reproduce their natural function. Therefore, it could be possible to use these membranes for selective tissue engineering of the cornea.

In conclusion, in this study, keratin-chitosan membranes have been shown to be a good scaffold for culturing human epithelial, stromal and endothelial corneal cells; therefore, future applications of keratin-chitosan membranes may be developed for the reconstruction of the cornea. However, animal experiments are necessary to assess the utility of this cell-carrier *in vivo* to demonstrate the potential use of these membranes as a biomaterial for tissue engineering.

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