Original Article

Transplantation of Autologous *Ex Vivo* Expanded Human Conjunctival Epithelial Cells for Treatment of Pterygia: A Prospective Open-label Single Arm Multicentric Clinical Trial

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Abstract

Purpose: To establish the efficacy and safety of *ex vivo* cultured autologous human conjunctival epithelial cell (hCjEC) transplantation for treatment of pterygia.

Methods: Twenty-five patients with pterygia were recruited at different centers across the country. Autologous hCjEC grafts were prepared from conjunctival biopsy specimens excised from the healthy eye and cultured *ex vivo* on human amniotic membrane mounted on inserts using a unique mounting device. The hCjEC grafts were then transported in an in-house designed transport container for transplantation. Post-surgery, the patients were followed up on days 1, 7, 14, 30, 90, and 180 as per the approved study protocol. Clinical outcomes were assessed by slit lamp examination, visual acuity, imprint cytology, fluorescein/rose bengal staining, Schirmer's test, and photographic evaluation three and 6 months post-transplantation.

Results: Two patients were lost to follow-up and final analysis included 23 cases. No recurrence of pterygium was observed in 18 (78.3%) patients; all of these eyes showed a smooth conjunctival surface without epithelial defects. Recurrence was observed in 5 (21.7%) patients at 3 months post-treatment. No conjunctival inflammation, secondary infections or other complications were reported. Adequate goblet cells were present in 19 (82.6%) patients at the site of transplantation.

Conclusion: We have, for the 1st time, standardized a protocol for preparing autologous hCjEC grafts that can be safely transported to multiple centers across the country for transplantation. The clinical outcome was satisfactory for treating pterygia.

Keywords: Autologous; Human Amniotic Membrane; Conjunctival Epithelial Cells; Goblet Cells; Pterygium; Multicenter Study

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INTRODUCTION

A pterygium is a triangular or wing-shaped fibrovascular overgrowth of abnormal conjunctiva onto the cornea. The etiology of pterygia remains largely unknown;[1] but it is believed to be caused by exposure to ultraviolet radiation, dust, and dry climates.[2] In severe cases, visual loss

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may arise from induced irregular corneal astigmatism, corneal stromal scarring and obscuration of the visual axis.[3,4] Several surgical methods have been employed for management of pterygium. Simple excision using the bare sclera technique has been shown to be associated with a recurrence rate of more than 70%.[5-8] This high recurrence rate has led to the search for adjunctive treatment options such as chemotherapy or radiotherapy. Different surgical techniques combined with adjuvants such as mitomycin-C,[9] β–irradiation,[10] thiothepa,[11] anti-VEGF (vascular endothelial growth factor) agents,[12] and more recently, alcohol[13] have been tried in various combinations for the treatment of pterygia.

The use of amniotic membrane grafts (AMG) has been shown to be efficacious in treating primary pterygia.[14] AMG serves as an alternative to conjunctival tissue in eyes with large conjunctival defects and inadequate tissue to cover the bare sclera, commonly seen in recurrent pterygia.[15] However, in terms of preventing recurrence, most studies have shown this treatment modality to be less effective than conjunctival autografting.[16] The conjunctival autografting technique was introduced by Kenyon et al in 1985 and has become a popular treatment for recurrent and advanced pterygia.[17] Although more time consuming, conjunctival autografting is a much safer and effective approach than chemotherapy or radiotherapy. This technique, targeted to reduce recurrence, has resulted in varying degrees of success.[18] Recurrence rates as high as 39% after conjunctival autografting has been reported.[19] Limbal-conjunctival autografts after pterygia excision have also shown promise for treatment of recurrent pterygia.[20,21]

There is wide variation in the extent of surgical excision of pterygia and subconjunctival fibrovascular tissue by various investigators.[22,23] Excision of larger conjunctival grafts from the bulbar conjunctiva may help reduce the recurrence rate of pterygia but may result in complications such as scarring, fibrosis and inflammation at the donor site.[24] Since the preferred site for autograft excision is the superior bulbar conjunctiva, patients requiring subsequent glaucoma surgery could face further problems.

In 1997, Pellegrini et al reported the first successful use of *ex vivo* expansion for autologous transplantation without inducing iatrogenic injury normally associated with autograft excision.[25] Since then, this technique is being used extensively for treating various ocular disorders with long-term positive clinical outcome.[26,27] The use of cultivated and *ex vivo* expanded conjunctival epithelial cell sheets for treatment of pterygia has been in practice for a few years now. The advantages of using cultivated conjunctival epithelium include reduction in inflammation and early epithelialization leading to faster recovery.[28-29]

We have standardized a method for *ex vivo* culture of autologous conjunctival epithelial cells which would benefit patients who are geographically distant from the cell culture facility. During the development stage, the major challenge was preparation of human conjunctival epithelial cell (hCjEC) grafts which could be transported to hospitals across the country. To overcome this issue, we developed a novel device for mounting human amniotic membrane (HAM) which would serve as a substrate for culturing the cells.[30] Further, we also designed a transport container which would ensure graft integrity during shipment.[31] To the best of our knowledge, this is the first multicentric clinical study to assess the safety and efficacy of autologous hCjEC grafts transported across the country and used for treatment of pterygia.

METHODS

This study adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committees of the study sites. Informed consent was obtained from all participants. The study was conducted at four sites across the country from January 2008 to December 2009. Twenty-five patients were enrolled in the study as per the inclusion criteria [Table 1].

Human Amniotic Membrane Processing

Placentas were obtained, after due consenting process, from mothers undergoing Caesarean section and were used to prepare HAM. Screening tests for infectious disease were done for human immunodeficiency viruses 1 and 2 (HIV-1, HIV-2), hepatitis B and C viruses

Table 1. Criteria for selection of patients for the study

Inclusion criteria
Patients with unilateral conjunctival disorders
Male or female patients of 18 years and above
Patient willing to comply with protocol described procedures
Written informed consent from patient/patient's legal representative
Exclusion criteria
Previous conjunctival transplant surgery
Pregnant or lactating women
Patient with documented HIV infection/AIDS
History of significant hematological, hepatic, renal, cardiovascular, respiratory, neurological, endocrinal or allergic disease
Patients with history of diabetes
Coexisting conditions limiting the successful outcome of the transplant surgery such as dry eye (Schirmer's <6 mm), lid margin disorder or actively inflamed eye
Any debilitating disease/disorder or psychiatric condition that in the judgment of the investigator would interfere with the adherence to the study protocol or ability to give informed consent

HIV, human immunodeficiency viruses; AIDS, acquired immune deficiency syndrome

(HBV, HCV) by polymerase chain reaction (PCR) and for cytomegalovirus (CMV-IgM, CMV-IgG), and Syphilis IgM/IgG by serology. Amniotic membrane was processed according to the method proposed by Kim et al.[32] Briefly, the placenta was cleaned under aseptic conditions and the amnion was separated from the chorion by blunt dissection. The membrane was cut into pieces admeasuring 4 cm × 4 cm and placed on separate pieces of nitrocellulose paper. Each membrane was placed in the sterile specimen cryogenic vial (Thermo Fisher Scientific-Nunc, DK-4000 Roskilde, Denmark) containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA, USA) and glycerol (Sigma Aldrich, St. Louis, MO, USA) [1:1] and cryopreserved at – 80°C. Sterility checks and endotoxin tests were performed before releasing the membranes for clinical use.

Mounting on Human Amniotic Membrane

On the day of biopsy processing, two membranes were thawed and washed thrice with sterile Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen-Gibco, Carlsbad, CA, USA) for 5 min each time. The basement membrane side of HAM was then treated with trypsin-EDTA (Invitrogen-Gibco, Carlsbad, CA, USA) for 15 min at 37°C. The amniotic epithelium was gently removed using a cell scrapper and washed thrice for 5 min in DPBS (1X) to remove cellular debris.

The HAM was oriented correctly, as described by Zakaria et al, with its basement membrane facing upwards.[33] The nitrocellulose membrane on the millicell insert (Millipore Corporation, Billerica, MA, USA) was gently peeled off. The insert was then placed on top of the membrane and the edges of the membrane were pulled over the rim of the insert. This is now referred to as "HAM construct". The HAM construct was then flipped over and placed on the base of the mounting device [Figure 1a]. A silicone ring was slipped onto the HAM construct using the plunger of the mounting device in order to provide a wrinkle-free substrate [Figure 1b]. The HAM construct was then flipped back and placed in the 6-well plate (Thermo Fisher Scientific-Nunc, DK-4000 Roskilde, Denmark) containing supplemental hormonal epithelial medium (SHEM).

Preparation of Human Conjunctival Epithelial Cell Grafts

Conjunctival biopsies, approximately 2 mm × 4 mm in size, with underlying stroma were obtained under strict aseptic conditions from the superior fornix of the contralateral healthy eye of patients. Biopsies collected at the respective sites by trained investigators were sent to the current Good Manufacturing Practices (cGMP) facility for processing. They were transported at 2°C–8°C in SHEM [Table 2]. The biopsy was cut into pieces and seeded on two HAM constructs in SHEM. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Growth of cells from the explants was monitored with an inverted phase-contrast microscope (Olympus Corporation, Tokyo, Japan) for a period of 14–21 days. The culture medium was replenished on alternate days.

Uniqueness of the Transport Container

The transport container was designed to hold the graft assembly in place and protect the graft from

Table 2. Composition of supplemental hormonal epithelial medium

Medium components	Final concentration	Manufactured by
DMEM/F-12 (1:1)	1X	Invitrogen-Gibco, Carlsbad, CA, USA
Fetal bovine serum (ES cell tested)	10%	HyClone Laboratories Inc., Logan, Utah, USA
Dimethyl sulphoxide	0.1%	Sigma-Aldrich, St. Louis, MO, USA
Epidermal growth factor	10 ng/ml	Sigma-Aldrich, St. Louis, MO, USA
Insulin-transferrin -selenium	1X	Invitrogen-Gibco, Carlsbad, CA, USA
Hydrocortisone	0.5 µg/ml	Sigma-Aldrich, St. Louis, MO, USA
Basic fibroblast growth factor	4 ng/ml	R&D systems, Minneapolis, MN, USA
Penicillin-Streptomycin	1X	Invitrogen-Gibco, Carlsbad, CA, USA
Gentamicin	50 µg/ml	Sigma-Aldrich, St. Louis, MO, USA

DMEM, dulbecco's modified eagle's medium; ES, embryonic stem

Figure 1. (a and b) Indigenously designed and patented mounting device for mounting human amniotic membrane onto a millicell insert, (c) In-house designed and patented stainless steel transport container for transportation of the graft to hospital site.

damage during transport. The transport container is cylindrical in shape, made of SS316 L and has a screw cap lid [Figure 1c]. A specially designed silicone gasket holds the graft firmly in place and prevents leakage of medium during transport.

Packaging of the Graft

Upon attaining confluence (approximately 14–21 days), the grafts were individually packaged in the in-house designed transport containers. The graft assembly was placed gently inside the container and the medium was added slowly along the sides. The silicone gasket was placed on top and the container was closed with the lid. The prepared grafts were transported to the hospital site for transplantation within 24 h of packaging. Only one graft was needed for the transplantation. The second graft was sent as a standby in the unlikely event of damage to the first graft.

In-Process Testing

During graft preparation, spent medium was checked for sterility by direct inoculation method as prescribed in the current Indian Pharmacopoeia and endotoxin test using the Limulus Amebocyte Lysate (LAL) gel-clot method (Wako Chemicals, Richmond, VA, USA) at different stages during the process [Table 3]. Mycoplasma testing of the spent medium was carried out as per the manufacturer's instructions (Minerva Biolabs GmbH, Berlin, Germany).

Characterization of the Human Conjunctival Epithelial Cell Graft

After successful transplantation, the second graft was sent back to the facility. The expanded cells were checked for viability and expression of key markers by semi-quantitative RT-PCR.

Gene Expression Profiling by Semi-Quantitative Reverse Transcription-polymerase Chain Reaction

RNA extraction was performed using RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. One microgram of RNA was converted into complementary DNA (cDNA) utilizing the first strand synthesis kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. PCR was performed as reported previously.[34] The primer details are as given in Table 4.

Cellular Characterization

During the standardization process for preparation of hCjEC grafts, immunofluorescence staining was performed to confirm the identity of conjunctival epithelial cells. Formalin-fixed paraffin-embedded sections of hCjEC graft were deparaffinized with xylene and graded alcohol treatment. The sections were dipped in 10 mM sodium citrate buffer, pH 6.0 for antigen retrieval. The sections were heated in a microwave oven for 30 s and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). The non-specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 60 minutes at 4°C. Sections were incubated with mouse anti-cytokeratin epithelial clone AE1 (Millipore, Billerica, MA, USA), mouse anti-cytokeratin epithelial clone AE3 (Millipore, Billerica, MA, USA), goat anti-mucin MUC4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-mucin MUC5AC (Millipore, Billerica, MA, USA) overnight at 4°C followed by incubation with their respective FITC-conjugated secondary antibodies for 30 minutes at 4°C. Excess antibodies were washed off and samples were mounted in immunofluorescence mounting medium (Sigma-Aldrich, St. Louis, MO, USA) and observed under a fluorescence microscope (Nikon E600) for the presence of immunoreactive cells.

Surgical Procedure

The surgical procedure was carried out as described previously.[18] Under local anesthesia with 2% lignocaine, the head of the pterygium was completely removed by blunt dissection. The hCjEC graft was washed 4 times with DPBS (1X) containing D-glucose and sodium pyruvate (Invitrogen-Gibco, Carlsbad, CA, USA) and placed over the diseased conjunctival surface with the epithelial cells facing upwards. The graft was trimmed to fit the entire conjunctival defect, including the bulbar surface of the fornix and the deeper portion of the palpebral aspect of the fornix, if required. It was then secured to the recessed conjunctival edge with a few interrupted or running 8.0 vicryl sutures so that its margin remained placed under the conjunctival margins. Sutures were tied carefully ensuring that the trailing

Table 3. Schedule for in-process testing

Stage	In-process quality checks		
	Sterility testing	Endotoxin testing	Mycoplasma detection
Conjunctival culture medium	√*	√	-†
Spent conjunctival biopsy collection medium	√	√	-
Spent conjunctival culture medium (day 10)	√	√	-
Spent conjunctival culture medium (day 12)	-	-	√
Spent conjunctival culture medium (at the time of packaging)	√	√	-

*Test was done; †Test was not done

Table 4. Primers used for semi-quantitative RT-PCR

Gene	Primer sequence	Annealing temperature (°C)	PCR product (bp)	Gene bank accession number
p63	F - AGCAGCAAGTTTCGGACAGT R - GCTGCTGAGGGTTGATAAGC	60	378	NM_001114978
Oct4	F - AGTGAGAGGCAACCTGGAGA R - CAAAAACCCTGGCACAAACT	60	447	NM_002701
CK4	F - CCAGGAGCTCATGAGTGTGA R - CCAAACTCCAAGAGGCAGAG	60	491	NM_002272
CK7	F - CAGGAACTCATGAGCGTGAA R - GGGTGGGAATCTTCTTGTGA	60	346	NM_005556
CK12	F - AGGACTGGGTGCTGGTTATG R - CAGGGCCAGTTCATTCTCAT	58	436	NM_000223
CK19	F - AGCAGGTCCGAGGTTACTGA R - CCTCCAAAGGACAGCAGAAG	60	367	NM_002276
ABCG2	F - TTATCCGTGGTGTGTCTGGA R - CCTGCTTGGAAGGCTCTATG	58	429	NM_004827
Integrin β1	F - AATGAAGGGCGTGTTGGTAG R - CCTCGTTGTTCCCATTCACT	63	664	NM_002211
Connexin 43	F - GGACATGCACTTGAAGCAGA R - GGTCGCTCTTTCCCTTAACC	60	368	NM_000165
MUC4	F - AAAACAGCCCACTGATGTCC R - CCAGCCTTCACGAAACTCTC	60	353	NM_004532
GAPDH	F - TTCACCACCATGGAGAAGG R - CATGTGGGCCATGAGGTC	60	690	NM_002046

RT-PCR, reverse transcription-polymerase chain reaction; CK, cytokeratin; MUC, mucin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

suture did not drag along the surface of the membrane. If clinically required (based on investigator's discretion), the conjunctival graft was then tucked to the deep fornix by a muscle hook anchored to the palpebral side of the fornix by passing two or three double-armed 6.0 vicryl sutures through the full thickness of the lid and secured to the skin with silicone bolsters. The rest of the conjunctival graft was then secured and flattened to the bulbar aspect by interrupted 10.0 nylon sutures with superficial scleral bites.

A topical broad-spectrum antibiotic was used and the eye was patched. The patch was not disturbed for the next 24 h. Post-operatively, prednisolone eye drops were instilled 4 times a day for 4 weeks, followed by topical fluorometholone and tobramycin 4 times a day for the next 4 weeks. Lubricating eye drops were administered at 2-4 h intervals for the first 1-month and then tapered down to 3 times a day for the next 2 months.

Post-operative Evaluation of Clinical Outcomes

Throughout the study, operated eye was observed for signs of inflammation and infection. Patients were monitored on at post-operative days 1, 7, 14, 30, 90, and 180 for graft edema, lacrimation, graft retraction/necrosis and irritation.

Physiological and anatomical assessment of conjunctival epithelium was done on day 90 and day 180 using local and slit lamp examination, imprint cytology, fluorescein/rose-bengal staining, Schirmer's test, and visual acuity. The graft success was assessed based on the smoothness, vascularity, epithelial integrity, adequate hydration, tear formation and the presence of goblet cells on the conjunctival surface. Any evidence of inflammation of the conjunctival surface, necrosis at the site of transplant, dry cornea with blurred vision and mucopurulent discharge from the recipient eye within 1-week of transplantation were considered as signs of graft failure.

Statistical Analysis

Data processing, tabulation of descriptive statistics, and calculation of inferential statistics was performed primarily using SAS software (release 9.0 or higher) for Windows. Statistical analysis was performed using 95% confidence interval along with counts and proportions.

RESULTS

The demographic and clinical details are summarized in Table 5. A total of 25 patients from four different centers were recruited. These patients underwent pterygium excision followed by transplantation of autologous human conjunctival epithelial cell graft. The patients included 12 (48.0%) male and 13 (52.0%) female subjects with mean age of 43.9 (range, 25–67) years. All patients were followed for 6 months. Two (8.0%) patients were lost to follow-up.

All conjunctival biopsies even from the farthest site (approximately 2,000 km away) were received at the facility within 24 h of excision. They were well transported at 2°C–8°C in a validated shipper. The biopsies were cut into small pieces and seeded as explants on two different HAM in parallel. Initial migration of the conjunctival epithelial cells from the explants was seen by day 2 [Figure 2a]. The cells started expanding in a concentric manner and showed typical cobble-stone morphology [Figure 2b]. On day 12, mycoplasma testing by PCR was done on the spent medium. All the cultures (n = 24) were found to be negative [Figure 3]. A confluent sheet of epithelial cells was obtained within 14-21 days. These hCjEC grafts were then packaged and transported in specially designed stainless steel transport containers that maintained graft integrity during transit.

All the cultivated conjunctival grafts, at process development stage, showed high expression of cytokeratin epithelial clones AE1 and AE3; and goblet-cell rich mucins, MUC4 and MUC5AC by immunofluorescence staining, thus confirming that they were conjunctival cells [Figure 4].

Much as it would have been desirable to characterize the cultivated conjunctival grafts prior to transplantation, the size of the excised tissue from the donor eye did not provide adequate cells for characterization. Hence, it was decided to confirm the identity of the cultured cells post-operatively, from the second graft sent back by the investigator.

The second hCjEC graft was transported back at 2°C–8°C and received at the cGMP facility within 48 h of transplantation. Upon receipt, these unused grafts were assessed for viability and molecular characterization. The viability of the cells as determined by trypan blue dye exclusion assay was between 78% and 90% [Figure 5a] in all specimens. Semi-quantitative RT-PCR analysis was done on conjunctival cells. High levels of mRNA expression of epithelial stem cell marker, p63; conjunctival epithelial cell markers, CK4 and CK7; epithelial basal marker, CK19; integrin β1 (CD29); connexin 43 (CX43); and goblet-cell marker, MUC4 were observed in these cells. We also observed that these cells weakly expressed CK12 and ABCG2. These results clearly indicate that the cells retain their normal characteristics [Figure 5b].

The focus of the study was also to determine the clinical acceptance and success of the graft and the rate of pterygium recurrence. Figure 6a-f shows a case with pterygium extending up to the central cornea from the nasal region in the right eye prior to and after hCjEC grafting on days 1, 14, 30, 90, and 180.

One of the evaluated parameters was reduction in ocular surface inflammation. We observed that conjunctival inflammation subsided to normal levels in 16 (69.6%) patients within 7 days and in 18 (78.3%)

Table 5. Demographic and clinical details of the study

Number of patients	25 (100.0)
Left eye affected	15 (60.0)
Right eye affected	10 (40.0)
Mean age (years) ±SD	43.9±11.09
Range of age (years)	25-67
Gender	12 (48.0) male 13 (52.0) female
Race	Indian/Asian
Treatment modality	hCjEC graft transplantation
Graft rejection	None
Pterygium recurrence	5.0 (21.7)
Patients lost to follow-up	2.0 (8.0)

SD, standard deviation; hCjEC, human conjunctival epithelial cell

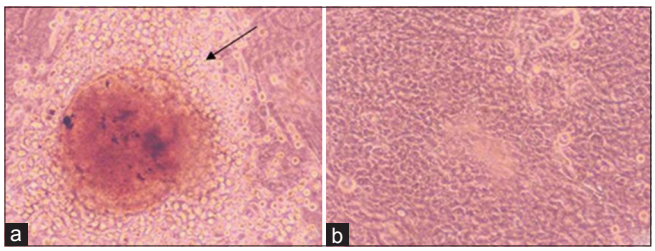

Figure 2. Phase-contrast microscopy of *ex vivo* expanded hCjECs from conjunctival biopsy. (a) Primary explant culture from conjunctival explants seeded on HAM show budding of cells by day 2. (b) Confluent monolayer of cells showing cuboidal morphology typically of epithelial cells by day 14. Total magnification of the phase-contrast image: ×100. Arrow shows budding of cells from the explants. hCjECs, human conjunctival epithelial cells; HAM, human amniotic membrane.

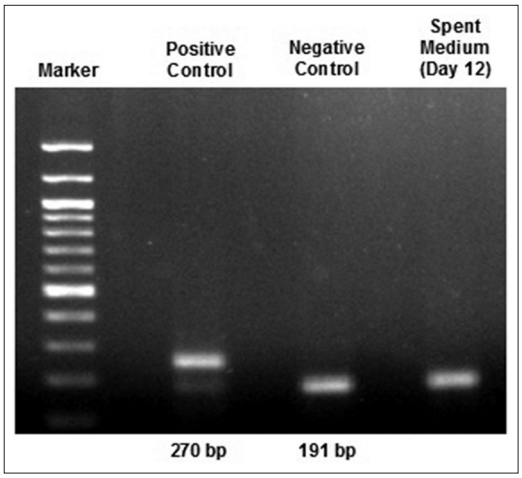

Figure 3. Detection of Mycoplasma DNA. Spent medium (day 12) was tested by polymerase chain reaction (PCR) using primer sets specific to the highly conserved 16S rRNA coding region in the mycoplasma genome. The expected product sizes for positive and negative controls are 270 and 191 base pairs, respectively. Spent medium (day 12) was found negative. A 100 bp DNA ladder was used as the molecular size marker. PCR data presented here are representative of 24 independent clinical samples.

patients within 14 days of transplantation. On day 90, no conjunctival inflammation was seen in the operated eye of 22 (95.7%) patients but in 1 (4.3%) patient it persisted until the end of study period. Other parameters such as overall luster, discharge, and bleeding were also monitored in the treated eye in all patients. Luster and discharge from the recipient eye was normal from day 14 onwards until the end of the study. Minor bleeding was observed in 2 patients on day 7 but there was no conjunctival bleeding in any patient from day 14 up to the end of the study as shown in Table 6. There were no secondary infections or other complications observed with respect to the transplant.

Visual acuity of the recipient eye was checked on day 1 and post-operative days 90 and 180. The number of patients with visual acuity of 6/6 increased from 7 (28%) pre-operatively to 12 (48%) at final visit. No worsening of

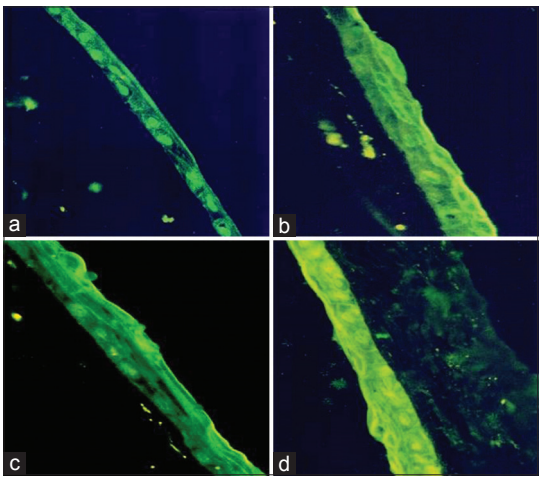

Figure 4. Cellular characterization of hCjEC graft. Representative images of immunofluorescence staining for (a) acidic (type I) cytokeratin (AE1), (b) basic (type II) cytokeratin (AE3), and goblet-cell rich mucins, (c) MUC4 and (d) MUC5AC on paraffin sections of the conjunctival graft. hCjEC, human conjunctival epithelial cell.

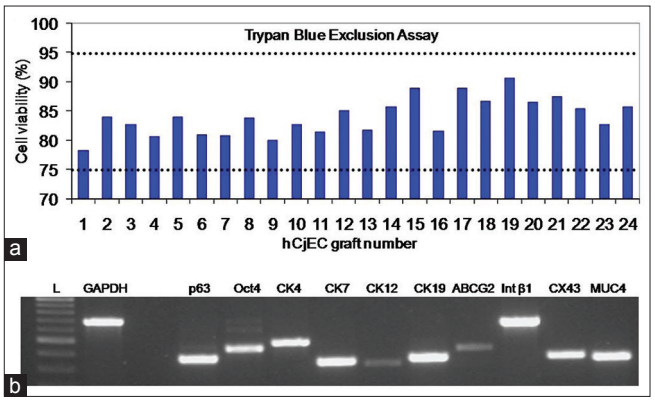

Figure 5. Viability and gene expression analysis of returned hCjEC grafts. (a) Cell viability was determined by trypan blue dye-exclusion assay on conjunctival cells recovered from the returned grafts. (b) RNA was isolated from the graft and expression of p63, Oct4, CK4, CK7, CK12, CK19, ABCG2, integrin β1 (Int β1), connexin 43 (CX43), and MUC4 were analyzed by semi-quantitative RT-PCR. GAPDH was used as an internal standard. The expected product sizes for GAPDH, p63, Oct4, CK4, CK7, CK12, CK19, ABCG2, integrin β1 (Int β1), connexin 43 (CX43), and MUC4 are 690, 378, 447, 491, 346, 436, 367, 429, 664, 368, and 353 base pairs, respectively. A 100 bp DNA ladder was used as the molecular size marker. Data presented here are representative of 24 independent clinical samples. RT-PCR, reverse transcriptase-polymerase chain reaction.

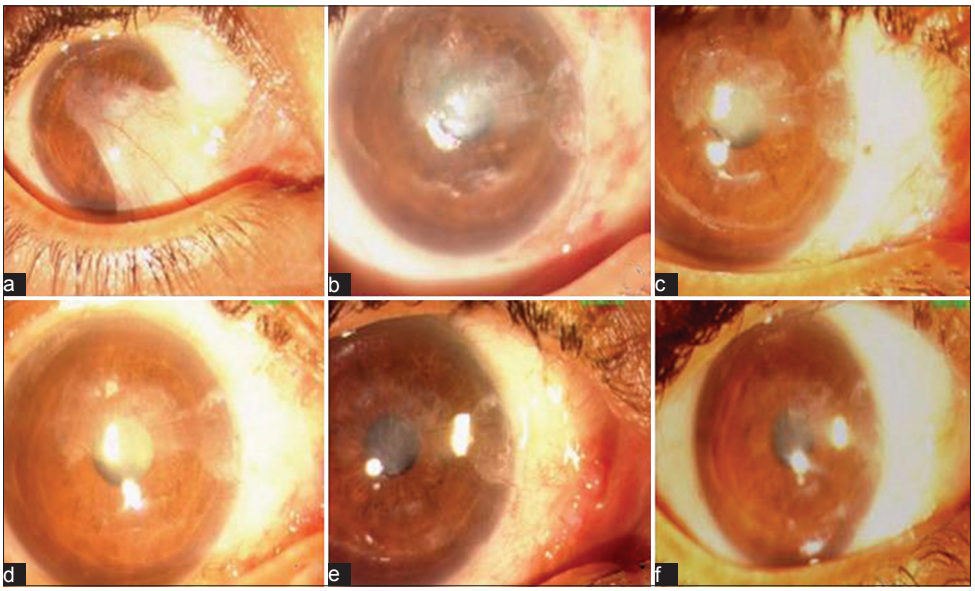

Figure 6. (a) Pre-operative photograph of the eye of a patient with recurrent pterygium extending from the nasal region. Post-operative photographs, after hCjEC graft transplantation, taken on (b) day 1, (c) day 14, (d) day 30, (e) day 90, and (f) day 180 respectively, shows no recurrence of pterygium. hCjEC, human conjunctival epithelial cell.

vision was observed in any patient during the follow-up period.

Imprint cytology showed that goblet cells at the site of transplantation were seen in 17 (73.9%) patients on day 30 and in 19 (82.6%) patients by day 180. No goblet cell was seen in 3 (13%) patients at the end of the study. In addition, Schirmer's test confirmed adequate hydration with presence of tears in all 23 (100%) patients at days 90 and 180.

No recurrence of pterygium was observed in 18 (78.3%) patients until day 180 and conjunctival surface appeared clinically normal in these patients from 3 months post-operatively. In the remaining 5 (21.7%) patients, pterygium recurrence was observed within 6 months.

DISCUSSION

Pterygia are complex fibrovascular conjunctival overgrowths on the cornea and surgical excision is the most prevalent treatment option. Recurrence is common and therefore, newer approaches to treat pterygia are being contemplated and tried by researchers and ophthalmic surgeons.[15] More recently, autologous conjunctival epithelial cell grafting has been reported for reconstructing conjunctival defects.[29] The results of our clinical trial to assess the safety and efficacy of such autologous grafts to stabilize the conjunctival surface have been promising. This clinical study was conducted with formal approval from the Drug Controller General of India, the highest approving authority, in the Indian context. All procedures for graft preparation including cell characterization and transport conditions were established prior to commencement of the study.

Conjunctival epithelial cell transplantation requires a carrier tissue, as it is not possible to transfer these cells alone. A natural basement membrane, HAM is already in clinical use and is believed to minimize ocular inflammation, reduce pain and aid in epithelialization.[35] HAM has been used as carrier tissue for conjunctival stem cells.[29] In our study, we used HAM as the substratum for culturing conjunctival epithelial cells.

The hCjEC grafts were prepared from the ipsilateral conjunctival tissue. Regardless of age, the biopsy samples were all 2 mm × 4 mm in size and all of them were cultured on HAM as per the procedure described in the methods section. The quality of the prepared graft is directly related to the conjunctival biopsy tissue excised from the donor eye. Despite this inherent variation, our results showed consistency in the quality of the grafts prepared using this standardized method.

Transporting the grafts to distant sites across the country was a challenge. We did validation runs to establish the logistics and transport conditions. The in-house designed transport container maintained proper orientation and integrity of the graft during transit. Our validation study showed that the graft can be transported at 2°C–8°C without affecting its viability and integrity [Table 7]. No instance of graft damage was noted during transport. Also, cell viability of all returned grafts was found to be greater than 80% when assessed 48 h after surgery. At the hospital site, the doctors and the paramedical staff were trained to handle the graft to ensure that there is no damage during transplantation.

Most of our patients had primary pterygia while five cases had recurrent lesions. The primary objective of the study was to assess feasibility of the graft through manifestations such as inflammation, graft melting, mucopurulent discharge, necrosis, and redness on the recipient eye. The entire study was uneventful with no cases having post-operative infections or redness. Minor adverse events like eye pain and local irritation subsided with medications.

Table 6. Parameters monitored in the recipient eye post-transplantation

Parameter	Follow-up (days)	Number of patients (*n*=23) (%)	
		Normal	Abnormal
Inflammation	7	16 (69.6)	7 (30.4)
	14	18 (78.3)	5 (21.7)
	30	18 (78.3)	5 (21.7)
	90	22 (95.7)	1 (4.3)
	180	22 (95.7)	1 (4.3)
Luster	7	23 (100)	0 (0)
	14	23 (100)	0 (0)
	30	23 (100)	0 (0)
	90	23 (100)	0 (0)
	180	23 (100)	0 (0)
Discharge	7	22 (95.7)	1 (4.3)
	14	23 (100)	0 (0)
	30	23 (100)	0 (0)
	90	23 (100)	0 (0)
	180	23 (100)	0 (0)
Bleeding	7	21 (91.3)	2 (8.7)
	14	23 (100)	0 (0)
	30	23 (100)	0 (0)
	90	23 (100)	0 (0)
	180	23 (100)	0 (0)

Table 7. Stability study of the hCjEC* grafts (*n*=10)

Parameter analyzed	Storage time at 2°C-8°C			
	6 h	12 h	24 h	48 h
Attachment of cells	Good	Good	Good	Good
Morphology of cells	Cuboidal	Cuboidal	Cuboidal	Cuboidal
Cell viability %	95±2	90±2	88±3	82±3
pH of the transport medium	7.2	7.3	7.5	8.3

*hCjEC, human conjunctival epithelial cell

Surgical excision remains the conventional treatment for pterygium and recurrence is the most common undesirable outcome. The two important concerns in pterygium surgery are to reduce pterygium recurrence and minimize complications arising from surgery. There is no standard definition of recurrence but when a fibrovascular outgrowth is observed at the site of previously excised pterygium, it is generally accepted as recurrence.[15] It has been reported that at least 97% of all recurrences manifest within the 1st year after excision.[36] Much as it would have been desirable to extend follow-up to 1-year, our approved protocol was limited to 6 months. However, we did follow the patients for up to 1-year through telephone interactions with the investigator, purely out of interest. In our study, 18 (78.3%) patients had no recurrence of the pterygia at 6 months. Informal communication confirmed that there was no recurrence in these patients even after 1-year.

Ocular surface inflammation plays a significant role in increasing the risk of pterygium recurrence. Hence, control of inflammation before and after surgery is essential for reducing recurrence rate. In our study, conjunctival inflammation subsided in 18 (78.3%) patients within 14 days post-operatively and no conjunctival inflammation was present in 22 (95.7%) patients by day 90. This observation continued until the end of the study. No other serious complications were reported during the study indicating that hCjEC graft was well tolerated at the site of transplantation.

Integrity of the conjunctiva is a very important end point. The conjunctival surface became smooth gradually by the end of 3 months and continued up to the end of the study. Further, at the end of 1-year, we had informal communication that there was no change in the smoothness at the transplant site.

Presence of adequate goblet cells is a critical parameter that reflects the overall health of the ocular surface.[37] At least 19 (82.6%) patients showed sufficient goblet cells at day 180. This is another important indicator of normal conjunctival regeneration post-transplant.

In other studies, transplantation of an autologous cultivated conjunctival epithelial sheet facilitated early post-operative epithelialization and recovery. However, true recurrence was observed in 22.7% cases.[18] In our clinical study, 5 (21.7%) patients showed signs of recurrent pterygia 3 months post-transplant which is consistent with other reports.

In conclusion, we demonstrated for the 1st time, the feasibility of transporting *ex vivo* expanded autologous human conjunctival epithelial cells (hCjECs) to distant locations without compromising viability and integrity of the graft. This method of transporting cultured grafts in the present study seems satisfactory. Larger studies with longer follow-up will be needed before one can consider this method as the preferred alternative for treatment of pterygia.

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