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CORNEA AND EXTERNAL DISEASES

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Ocular Gvhd in Allogeneic Hematopoietic Stem Cell Transplantation – Indian Scenario

Dr. Rehan Khan, Dr. Murugesan Vanathi, Dr. Seema Kashyap, Dr. Pravas Mishra, Dr. Tulika Seth, Dr. Anita Panda

Though allogeneic Hematopoietic stem cell transplantation (allo HSCT) [peripheral blood stem cells transplantation (PBSCT), bone marrow transplantation (BMT), and cord blood cells transplantation (CBT)] has been described as an accepted standard treatment in the management of several hematological and non hematological malignancies, ocular Graft versus host disease (OGVHD) remains a common manifestation of allogeneic hematopoietic stem cell transplantation. Ocular Graft versus host disease (oGVHD) occurs when immunologically competent donor T lymphocytes recognize recipient tissues alloantigens like lacrimal gland, conjunctiva, meibomian glands resulting in inflammation and fibrosis. Ocular GVHD has been reported to occur in 40-60% of post allo HSCT patients.1-2 Dry eye associated with chronic GVHD is one of the major complications with 50% of patients having been known to develop dry eye or progression of pre-existing dry eye after allo HSCT.3 No clinical study has been done on oGVHD in Indian population. Hence this study was carried out to evaluate the ocular surface changes after allogeneic HSCT in a tertiary care centre in India.

MATERIALS AND METHODS

Cross-sectional hospital based observational study carried out at the outpatient department in hematological patients undergoing allogeneic hematopoietic stem cell transplantation between study periods November 2009 to November 2011. Criteria for recruitment into the study were followed. Institutional Ethics Committee approval was sought and obtained. Informed consent was taken from all patients participating in the study. Systemic evaluation of all patients was done by the referring hematologist. Clinical data recorded including demographic detail, diagnosis, type of transplant, and GVHD detail. Comprehensive ophthalmic evaluation including visual acuity assessment, symptom score, ocular surface disease index score (OSDI)4, slit lamp biomicroscopy, ocular surface evaluation tests [tear meniscus height (TMH), fluorescein tear break-up time (FTBUT), Schirmer’s test I without anaesthesia, Meibomian gland dysfunction, ocular surface staining (with lissamine green)] and conjunctival impression cytology (CIC) 5 were done. Diagnosis of keratoconjunctivitis sicca was made based on FTBUT < 5 seconds, Schirmer’s I test < 5 mm in 5 min, ocular staining score ≥ 3. Dry eye severity classification was done in accordance to the DEWS recommendations.6
RESULTS

Demographic Data

40 allo-HSCT patients [mean age 25.7 ± 11.03 years (median 23.5 years, range: 8 -48 years), (males: 30 (mean age 24.2 ± 10.65), females: 10 (mean age 30.2 ± 11.78)] were studied of which 38 patients had undergone PBSCT and two had BMT (HLA matched, sibling allogenic transplant). Mean time of recruitment of the patients into the study after allo-HSCT was 10.12 ± 14.13 months (range: 1.1–78.7 months). The most common indication for allo-HSCT was aplastic anaemia (n = 20, 50%). Chronic systemic GVHD occurred in 13 patients (33%). Chronic oGVHD was noted in 30% of the allo-HSCT patients (n=12) with mean time of occurrence being 17.41 ± 21.52 months after the allo-HSCT (range: 1.3 months to 78.7 months).

Of the 80 eyes of 40 patients examined, 68.5% (55 eyes) had a Snellen’s visual acuity of 6/6. The most common cause for decreased visual acuity was refractive error (19 eyes).

Dry eye disease due to chronic oGVHD was seen in 24 eyes (30 %) with dry eye severity of level 3 in 17.5 % (14 eyes), level 2 in 2.5 % (2 eyes), level 1 in 10% (8 eyes).

Ocular surface evaluation data

Analysis of ocular surface evaluation data between eyes with oGVHD and eyes without oGVHD was done.

Symptom score noted in eyes with chronic oGVHD was grade 0 in 8.33%, grade 1 in 41.67%, grade 2 in 33.33%, grade 3 in 16.67% while eyes without oGVHD had symptoms score of grade 0 in 82.14%, grade 1 in 17.86% (p < 0.001).

OSDI was noted to be mild in 16.67%, moderate in 45.83% and severe in 20.83% oGVHD eyes; while it was seen to be mild in 94.64%, moderate in 5.36% of eyes without oGVHD eyes (p < 0.001).

Schirmer’s I test score was ≤ 5 mm in 58.33% of eyes with oGVHD while 96.43% eyes without oGVHD had a score of > 5 mm (p < 0.001).

FTBUT was ≤5 seconds in 45.83% and > 5 seconds in 54.17% of eyes with chronic oGVHD, while 96.43% eyes without ocular GVHD had FTBUT of > 5 seconds (p < 0.001).

MGD in eyes with ocular GVHD was of grade 0 in 29.17%, grade 1 in 50%, grade 2 in 8.33%, grade 3 in 12.50%, while eyes without ocular GVHD had grade 0 MGD in 92.86%, grade 1 in 7.14% (p < 0.001).

50 % of the eyes with chronic oGVHD had TMH ≤ 0.2mm, while in eyes without oGVHD, 3.57% had TMH ≤ 0.2mm and 96.43% had TMH > 0.2mm (p < 0.001).
Corneal findings: Eyes with oGVHD had no corneal findings in 33.33%, grade 1 in 25%, grade 2 in 8.33%, grade 3 in 33.33% while all eyes without oGVHD had no corneal finding (p < 0.001).

 Conjunctival staining score of < 3 in 25%, ≥ 3 was noted in 75% of oGVHD eyes, while it was < 3 in 96.43%, ≥ 3 in 3.57% of non - oGVHD eyes (p < 0.002).

 Corneal staining score of < 3 in 79.17 %, ≥ 3 in 20.83% was seen in oGVHD eyes, while no corneal staining was observed in non - oGVHD eyes (p<0.002).

 CIC Grade 0 and grade 1, conjunctival cytology changes are considered as normal while grade 2 and grade 3 changes considered as abnormal. CIC of each eye was classified as normal if grade 0/grade 1 changes were seen in >2 quadrants and abnormal if grade 2/grade 3 changes were seen in > 2 quadrants. CIC was abnormal in 60 eyes (75%) with altered morphology seen in 22 eyes with oGVHD (91.7%, 22/24) and 38 eyes without oGVHD (67.9%, 38/56) (p=0.024). Normal CIC was seen in 20 eyes (oGVHD – 2 eyes and non-o GVHD – 18 eyes).

 Comparison of ocular surface evaluating parameters in eyes with ocular GVHD or without ocular GVHD showed statistical significance in symptoms (P <0.001), meibomian gland disease (P <0.001) corneal finding (P <0.001), tear meniscus height (P <0.001), tear breakup time (P <0.001), cornea staining score (P <0.002), conjunctival staining score (P <0.002) and Ocular surface disease index score (P <0.001).

 Overall statistical analysis of association of CIC of eyes of post allo-HSCT patients with the various ocular surface evaluation parameters showed statistical significant association of CIC with corneal findings (p = 0.021), Schirmer’s 1 test score (p = 0.008) and OSDI (p = 0.007). However statistical analysis of association of CIC with ocular surface evaluation parameters in eyes with and without oGVHD did not show any significant association.

**DISCUSSION**

The severity of the ocular surface disease in chronic oGVHD depends upon the clinical extent of tissue involvement of the ocular surface tissues (lacrimal glands, lids, conjunctiva and cornea) and tear film. Ocular surface morbidity in chronic GVHD is the result of T-cell–related immune inflammatory processes, apoptosis and fibrosis, along with TH1- associated chemokines which have been identified in the conjunctiva of patients with ocular cGVHD. These result in conjunctival inflammation and cicatrization; decreased goblet cell density causing mucus layer disturbance in the tear film; lacrimal gland dysfunction due to inflammation and fibrosis of the lacrimal gland thereby altering the aqueous layer integrity of the tear film; meibomian gland dysfunction causes lipid layer instability of the tear film. Microstructural changes in terms of
both altered number and morphology of conjunctival mucosal microvilli have been observed in cGVHD patients.

Dry eye is the most common clinical finding occurring in about 90% of patients. A recent prospective study on the ocular complications after allogenic PBSCT in 101 hematological patients reported development of oGvHD in 54% of patients.

In our study, of the 40 patients of allo-HSCT evaluated, chronic systemic GVHD was noted in 13 patients (33%). Chronic oGVHD (dry eye disease) was seen in 12 patients (30%) in our study with mean time of occurrence of GVHD after allo-HSCT being 17.41 ± 21.52 months (range 1.3 months–78.7 months). Chronic oGVHD was observed to be present in 92.3% of chronic systemic GVHD cases (12 out of the 13 patients of chronic systemic GVHD) in allogenic hematopoetic stem cell transplantation patients in our study. This corroborates with earlier studies, which showed a strong association of chronic oGVHD with chronic systemic GVHD. Several studies have also mentioned ocular involvements occurring in GVHD in allo-HSCT patients. The risk factors for the development of dry eye in GVHD patients were older age group (> 27 years), PBSCT, chronic GVHD, and chronic/ acute myeloid leukemia, with dry mouth and Schirmer’s test value of <5 mm being strong predictive factors for dry eye. There seems to be a strong association between the development of systemic GVHD and the development of oGVHD (dry eye disease) in allo-HSCT patients with oral or dermatological involvements in the systemic GVHD showing an increased risk for developing oGVHD.

The common manifestations of oGVHD are dry eyes, conjunctivitis, blepharitis and uveitis. Our study (predominantly PBSCT), found keratoconjunctivitis sicca as the clinical feature in oGVHD patients. The ocular surface baseline characteristics evaluations revealed conjunctival and corneal involvement, meibomian gland disease in our patients with oGVHD. Alterations in conjunctival epithelial morphology were also seen in oGVHD patients as a result of the dry eye disease. CIC of eyes of post allo-HSCT patients showed significant association with corneal findings, Schirmer’s 1 test score and OSDI score in our study. However, further statistical analysis did not find a significant association of CIC with ocular surface evaluation parameters, when the evaluation parameters were grouped into eyes with oGVHD and eyes without oGVHD.

One of the most recent studies on oGVHD by Wang et. al. (2010), which was published during the course of our study period, also elaborates the baseline profiles of ocular surface and tear dynamics of oGVHD and non-oGVHD related dry eye disease in allo-HSCT patients. They evaluated fifty eyes of 25 post allo-HSCT patients and 28 controls to study meibomian gland
obstruction, tear evaporation rate, corneal sensitivity, Schirmer test-I, TBUT, ocular surface vital staining, CIC and brush cytology. They described altered conjunctival epithelial morphology decreased conjunctival goblet cell density, and increased inflammation in chronic GVHD-related dry eyes. They also noted comprehensive ocular surface alterations in post allo-HSCT patients, irrespective of the presence of chronic GVHD-related dry eye or not. Our study also places us in agreement with their conclusions that the extent of inflammatory process has an essential role in the outcome of the chronic GVHD-related dry eye.

The search for clarity in the immunopathogenic processes that contribute to ocular surface morbidity in chronic GVHD continues in order to find effective therapeutic options for its treatment and progress prevention. Corticosteroids, calcineurin inhibitors (cyclosporine and tacrolimus), and recently, antifibrotic agents such as tranilast are available as topical treatment options. Anti-inflammatory treatments that target T-cell suppression seem to be of use in managing oGVHD. Topical cyclosporine 0.05% has been found to be effective in the treatment for dry eye in GVHD patients. It causes improvement in the ocular surface and tears functions by decreasing inflammation, increasing goblet cell density and MUC5AC mRNA expression in these eyes.

In summary, our study shows clinical involvement of the ocular surface in chronic oGVHD eyes and conjunctival cytological changes in the eyes of chronic GVHD with and without manifestation of dry eye disease. Corneal involvement, Schirmer’s 1 test score and OSDI score can be considered to be important parameters of dry eye disease in oGVHD patients. Ocular surface alterations are seen in post allo-HSCT patients with chronic GVHD with dry eye disease being seen in 30% of the patients evaluated. Our study helps to establish baseline data on clinical characteristics of oGVHD. Further research into other advanced parameters of dry eye disease may help to understand the immunopathogenesis involved in GVHD related dry eye disease.

REFERENCES


Ocular Surface Changes in Patients of Pseudoexfoliation (PEX) Syndrome Undergoing Cataract Surgery

Dr. Indu Govindaraj, Dr. Subashini Kaliaperumal, Dr. Vasudev Anand Rao

John G. Lindberg, in 1914, was the first person to describe PEX (PEX). He found that this phenomenon was more common in cataractous eyes and in patients with glaucoma. Since its discovery PEX syndrome has undergone extensive research for its unique structure and its effects on the eye. Patients ‘with’ a PEX can develop corneal endotheliopathy, sphincter atrophy of the iris, poor mydriasis, iris neovascularization, transillumination defects, flaky material on the lens capsule, zonular dialysis and spontaneous dislocation of lens. They are also more predisposed to goblet cell loss and dry eye. Cataract surgery and glaucoma medications can induce the risk of dry eye in such patients.

Our study aimed to assess the conjunctival morphological changes and tear film abnormalities with PEX syndrome and compare the findings with eyes without PEX.

MATERIALS AND METHODS

In this prospective non-randomised study, group 1 consisted of 30 eyes of 15 normal subjects and group 2 consisted of 30 patients with PEX syndrome at least in one eye. The study was approved by institute ethics committee. Patients who presented for cataract surgery and diagnosed to have PEX syndrome in the lens or iris were included in Group 2 of the study. Patients with ocular surface disorder, PEX glaucoma, previous ocular surgeries and adnexal abnormalities were excluded. The demographic profile, slit lamp examination for zones of pseudoexfoliative material, laterality of PEX and grading of cataract were documented. To detect tear film changes Schirmer’s 2 test, tear film break up time and conjunctival impression cytology of both the eyes were performed. Conjunctival biopsy was also taken at the time of cataract surgery.

Conjunctival impression cytology

Cellulose acetate membrane filter paper (AXIVA Lab filters, India) was used to take impression cytology from the conjunctiva. Diameter of each filter paper was 13mm and the thickness was 0.2µm. Each filter paper was cut into two halves and one side of each half was cut.

A large cut was placed for the right eye and small cut for the left eye. The eye was anesthetised with 0.5% proparacaine eye drops. The excess drops outside the eye were wiped off. After 30s the filter paper was placed in the lateral bulbar conjunctiva by holding it with an untoothed forceps. The impression
was taken in such a way that when the paper is placed with base down and the cut end to the right the impression is present on the superior surface.

The filter paper was then placed in a Teflon plate and 70% ethyl alcohol was added as fixative. The Teflon plate was then covered and the paper stained with Periodic acid Schiff within 24 hours or stored at 4 degrees until staining. The conjunctival impression cytology was studied under light microscopy. Nelson grading was used to grade the slides.

**Grade 1**
The epithelial cells are small with eosinophilic staining cytoplasm. The nuclei are large and basophilic with nucleocytoplasmic ratio of 1:2. The goblet cells are abundant, plump, and oval and have intensely PAS positive cytoplasm.

**Grade 2**
The epithelial cells are larger and polygonal and occasionally multinucleated with variably staining cytoplasm. The nuclei are small with nucleocytoplasmic ratio of 1:4 to 1:5. The goblet cells are markedly decreased, less intensely PAS positive with poorly defined cellular borders.

**Grade 3**
The epithelial cells are large and polygonal with basophilic staining cytoplasm. The nuclei are small and pyknotic and in many cells completely absent. The nucleocytoplasmic ratio is more than 1:6. Goblet cells are completely absent.

**Conjunctival biopsy**
Conjunctival biopsy was taken from the eye undergoing cataract surgery. 2mm x 2mm strip of conjunctiva was taken from the superior conjunctiva during peritomy. The tissue was stored in formalin and sent to histopathological examination for PEX material. The specimen was stained with Periodic acid Schiff and analysed under light microscopy for PAS positive PEX material.

**Statistical analysis**
Statistical analysis in this study was done using GraphPad Instat 3. The results were compared using the unpaired ‘t’ test and the chi-square test. Schirmer’s test and TBUT were analyzed by unpaired ‘t’ test. The chi-square test was used to compare impression cytology grades between the different groups.

**RESULTS**
The study included 30 eyes of 15 normal subjects (Group 1) and 43 eyes of 30 patients with PEX (Group 2). Seventeen (56.6%) of the thirty patients in the study group had unilateral PEX and the rest 13 (43.4%) had bilateral presentation. The mean age of the patients in Group 1 and Group 2 was 59.4±6.1 years (Range 51 to 72) and 66.27± 6.7 years (Range 55 to 80) respectively.
Among the eyes with PEX, 67.4% of cases had PEX material in both zone 1 and zone 3, 20.9% of cases had only in zone 1, and 13.9% had only in zone 3. Hence 81.3% of case had PEX material in zone 3. The average mydriasis in eyes with PEX syndrome was 4.9mm and in eyes without PEX syndrome was 5.5mm.

Average Schirmer’s and TBUT in Group 1 were 22.05±4.4mm and 14.75±2.5s respectively whereas in Group 2 the values were 10.6±7mm and 5.6±2.8s and the differences were statistically significant (P value- <0.0001). Within group 2, among the 17 unilateral PEX syndrome the average Schirmer’s and TBUT in eyes with PEX material was 11.7±6.4mm and 6.4s respectively whereas in fellow uninvolved eyes the values were 12.9±5.9mm and 6.6s. This difference was not clinically significant (P=0.065).

In group 1 CIC score was stage 1- 66.7%, stage 2- 33.3%, Stage 3- 0% which was significantly lower when compared to Group 2. In eyes with PEX, 60% had stage 3 cytology with total loss of goblet cells, 37.2 % had stage 2 and 2.3% had stage 1.

Twenty five biopsies were taken from 25 eyes with PEX from Group 2 at the time of cataract surgery. Only seven biopsies revealed suspicious PAS positive material on the surface of the epithelium. One biopsy showed uniform PAS staining on the surface of the epithelium suggestive of keratinisation.

To conclude this study reveals that PEX syndrome causes tear film abnormalities and a decrease in goblet cells which can even precede the appearance of PEX in lens or iris. The changes produced can lead to symptoms of dry eye which become significant especially following cataract surgery in these PEX patients and in those on antiglaucoma medications for PEX glaucoma.

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**Clinicohistopathological Analysis and Outcome of Management in Cases of Suspected Ocular Surface Neoplasia: Retrospective Analysis of 109 Cases**

Dr. Roopasree B V, Dr. Sean Socrates D'SILVA, Dr. Prajna N V, Shanti R

Ocular surface tumours refer mainly to three types of malignant and premalignant neoplasias arising from conjunctiva and cornea *i.e.* ocular surface squamous neoplasia (OSSN), ocular surface melanocytic tumours and lymphoid tumours of the conjunctiva. Although rare entities, they have a significant impact on patient’s morbidity and mortality. Conjunctival squamous cell carcinoma is the most common of all the ocular surface neoplasias while conjunctival melanoma is the most dangerous malignancy.
Potential of conjunctival malignancies increases when the epithelium is breached and the stroma gets involved. These malignant neoplasms are both sight and life threatening with potential to invade locally the ocular tissues and to metastasize to distant organs.\textsuperscript{3,4,5} Conjunctival melanoma has a higher rate of metastasis of 26\% by 10 years\textsuperscript{7} compared to squamous cell carcinoma which is 1\%.

In most cases of surface neoplasias presumptive diagnosis can be made clinically with high index of suspicion which is confirmed by histopathology, the gold standard for diagnosis. The treatment options for ocular surface neoplasias includes tumour excision with or without cryotherapy, radiotherapy, immunotherapy, topical chemotherapy, enucleation and exenteration. The reported incidence of recurrence after initial treatment is quite variable from 12\% to 21\%. Positive pathological margins and higher grade of neoplasia have been shown to increase the risk of recurrence.

\section*{MATERIALS AND METHODS}

The study is a retrospective case review of 109 eyes of 109 consecutive patients with suspected ocular surface neoplasias between January 2010 and May 2011. Patient selection required access to patient’s medical records, pathology reports and color photographs. Collected data included patient age, gender, visual acuity, laterality, clinical appearance, tumour size, extension, invasion (cornea, sclera, uvea, orbit, lymph node and distant metastasis) and treatment modality. Analysis was also done on histopathological diagnosis, duration of follow-up and recurrence.

At presentation, all patients underwent slit-lamp examination of conjunctival surface (including eversion of the upper eyelid and tarsus) and cornea. Lymph nodes (preauricular and submandibular) were examined for enlargement. Slit-lamp photographs of the lesion were documented. Tumor size was divided into small, medium, and large. Small tumors includes those less than 3 clock hours (for limbal tumors) or less than 5 mm in largest diameter. Medium tumors were defined as those between 3 to 6 clock hours (for limbal tumors) or 5 to 10 mm in largest diameter and large were those with more than 6 clock hours (for limbal tumors) or more than 10 mm in largest diameter.

Small and medium sized tumours were treated by excision biopsy with 2 mm of healthy margin with reverse cryotherapy. Preoperative MMC was given in large tumours for chemoreduction and postoperative MMC was given in higher grades of neoplasias.

\section*{RESULTS}

Patient’s age ranged from 8 yrs to 82 yrs. Maximum number of patients were in the age group of 40-50 yrs and 60-70 yrs, each group containing 22 patients.
66 were males and 43 were females. Right eye was involved in 54 patients and left eye in 55 patients. Visual acuity ranged from hand movements to 6/6. In 13 patients defective vision was due to corneal lesion extending to pupillary area and in rest it was due to cataract, glaucoma and diabetic retinopathy changes. Growth in eye, irritation and foreign body sensation were the most common presenting complaints.

Most common site of involvement was limbus in 73% cases followed by cornea in 15%, bulbar conjunctiva in 10% and fornix being the least common in 2% cases. 53% of tumours were small in size, 28% were medium and 20% were large.

The lesions were associated with pterygium in 11%, pinguecula in 3% of cases. Leukoplakic appearance was the most common in 88 cases and pigmented lesion, papillomatous lesions, keratinized lesions were seen in 7 cases each. 38% of malignant and 25% of benign lesions had feeder vessels.

Preoperatively Mitomycin C (MMC) was given in 2 cycles for 5 patients. Mitomycin C was given as 0.02% eyedrops twice a day for 2 wks and repeated after 2-4 weeks. Preoperative MMC was given maximum for 2 cycles only. Histopathologically 45% were benign lesions with dyskeratosis being the most common (27%) followed by granulomas (9%). 55% of lesions are malignant. Amongst the malignant lesions, CIN was the most common (62%) followed by squamous cell carcinoma (33%), malignant melanoma (3.33%) and Kaposi sarcoma (1.6%). Local spread with scleral involvement was seen in 3 cases (5%), intraocular in 1 case (1.8%), and orbital involvement in 3 cases (5.4%). Preauricular lymphnode enlargement was seen in 2 cases of invasive

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squamous cell carcinoma. Distant metastasis to fronto-temporal lobe of brain and lung was seen in 1 case who had xeroderma pigmentosa with squamous cell carcinoma. Follow up period ranged from 12-18 months. Post excision 9 patients received two cycles of MMC 0.02% and 2 patients received 3 cycles. Recurrence was seen in 15 cases (13%), more in 60-70 yr of age group (33%), with medium and large size lesions involving limbus with extension to cornea and conjunctiva (46%), more in squamous cell carcinoma with invasion (46%). HIV positivity was seen in 7 cases (6%), among them 2 cases had extensive actinic dyskeratosis, 2 had CIN, 2 had invasive squamous cell carcinoma and 1 had Kaposi sarcoma. Primary excision biopsy for 5 amongst 15 recurrent cases was done elsewhere. Rate of recurrence for CIN was 33%. Earliest recurrence was seen after 1 month in squamous cell papilloma with malignant transformation. Late recurrence was seen in a patient with xeroderma pigmentosa after 4 yrs of excision biopsy which was again a squamous cell carcinoma. Excision with cryo(double freeze slow thaw) with (absolute alcohol application for corneal involvement) amniotic membrane transplantation with post-op MMC was the most common treatment for recurrence. Partial sclerectomy was done in 1 case with scleral involvement. Enucleation was done in 1 case with intraocular extension and exenteration was done in 3 cases (5.4%) who had intraorbital extension. Radiotherapy was done in 1 case with moderately differentiated invasive squamous cell carcinoma after exenteration.

**DISCUSSION**

In our case series, Squamous cell neoplasia accounted for the maximum number of cases which is similar to other studies done earlier. Our clinical suspicion correlated with histopathological diagnosis in most of the cases. Predictive indicators for recurrence in our series were age more than 40 yrs, lesions involving limbus with extension to cornea and conjunctiva, medium and large sized lesions and invasive tumours. Recurrence was not correlated significantly to gender, laterality and clinical appearance. As reported previously, our study also had slight male preponderance (61%). Involvement of intraocular structures suggests invasion which has been reported in 2-8% of cases, we had in 4% cases. Orbital invasion has been seen in 2-16% in various studies, our study had 5% of such cases. Metastasis is rare, but we had 1 patient with lymph node involvement and 1 patient had distant metastasis. When adjunctive cryotherapy is used with excision of lesion, recurrence rate can be reduced between 7% and 22% as reported earlier. This was 15% in our study. Recurrence in cases who underwent excision with cryotherapy with postoperative MMC was 40%. Recurrence is more in higher pathological grade of tumour, which was also observed in our study.
Logistic regression analysis was used to assess the risk factors of Ocular surface neoplasia. We included age, sex, size and site. Of these variables Age>40, Large and medium size of the lesion, and Site was statistically significant risk factors for occurrence of malignant lesions.

Leukoplakia is not always benign. Histopathology is mandatory in all suspected cases of ocular surface neoplasias. Excision biopsy with cryotherapy to margins results in good tumour control. Old age, medium to large invasive lesions, lesions involving limbus extending to cornea and conjunctiva are at higher risk of recurrence. Excision with cryotherapy with MMC gives better outcome in locally recurrent tumours.

REFERENCES
5. Shields et. al, Conjunctival melanoma risk factors for recurrence, exenteration, metastasis and death in 150 consecutive patients, 2000;118:1497-1507.

Clinical Risk Factors for Failure of Autologous Limbal Stem Cell Transplantation

Dr. Anupam Bagdi, Dr. Sayan Basu, Dr. Md Hasnat Ali, Dr. Virender Sangwan

The eye is a highly specialized organ of photoreception. It involves convergence of light by the transparent media of the eye i.e. cornea, aqueous humor, lens and vitreous onto the retina. The maintenance of transparency of the cornea plays a vital role in the process as it forms a part of the Ocular Surface which is exposed to the environment. The epithelium of the cornea is composed of stratified squamous cells and makes up about 5-10% of the total corneal thickness. The epithelium is in contact with external
environment and is subjected to regular wear and tear. These epithelial cells are replaced through proliferation of a distinct sub-population of cells known as Stem Cells. There is now enough clinical and laboratory evidence to prove that these are located in the basal layers of the limbus in specialized structures called Pallisades of Vogt.

Severe Ocular trauma or inflammatory disease may damage the limbus leading to a functional loss of these stem cells. This induces Corneal epithelial instability which is clinically termed as LIMBAL STEM CELL DEFICIENCY (LSCD). It is characterized by loss of epithelial clarity, persistent epithelial defects, vascularization and conjunctivalization of corneal surface leading to loss of vision associated with pain, redness and photophobia.

With better understanding of Stem Cells and Ocular Surface it was proven that transplantation of stem cells from healthy limbus could be used for restoring the damaged ocular surface. Limbal transplantation has now become the standard of care for eyes with LSCD. In conventional Limbal Transplantation donor tissue obtained either from unaffected eye (unilateral disease) or from live related/cadaveric donor (bilateral disease) is transplanted directly onto the affected eye. The concerns regarding donor site complications lead to the development of cultivated Limbal Stem Cell Transplantation (LSCT) which involves expansion of cells ex-vivo on a suitable substrate before transplantation.

However, studies have shown that success rate of these procedures is around 50–70% and a substantial proportion of patients suffer from recurrent disease. It has also been proposed that causes of failure in such cases are multifactorial. It is therefore important to study the various factors that might be associated with failure of transplantation as it will help us in better understanding of the disease, customizing the prognosis and to better communicate to the patients about the expected outcome.

The previous studies suffered from lacunae such as insufficient sample size, heterogenous population, multiple surgeons, multiple techniques and limited follow-up. Therefore in this study we aim at elucidating the risk factors using a homogenous group of cases operated by a single surgeon with an adequate follow-up.

**Purpose**

Customizing the prognosis of limbal transplantation for an individual patient is difficult because the risk factors predisposing to failure of surgery are not clearly known. To address this issue, this study aimed to identify the clinical risk factors associated with failure of autologous stem cell transplantation for the treatment of limbal stem cell deficiency (LSCD).
**MATERIALS AND METHODS**

This is a retrospective study which included 526 eyes of patients with unilateral LSCD following ocular surface burns who were treated with ex-vivo autologous cultivated limbal epithelial transplantation between 2001 and 2011.

A standard procedure for LSCT was followed for all the patients which is described as follows:

A 2 mm × 2mm piece of conjunctival epithelium extending 1 mm into clear corneal stromal tissue at the limbus is dissected and limbal tissue that contains the epithelial cells and a part of the corneal stroma is obtained. The tissue is transported to the laboratory in human corneal epithelium (HCE) medium. HCE is composed of modified Eagle’s medium/F12 medium (1:1) solution containing 10%(vol/vol) autologous serum, 2 mL-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 10 ng/ml human recombinant epidermal growth factor and 5 μg/ml human recombinant insulin. Under strict aseptic conditions, the donor limbal tissue is shredded into small pieces. De-epithelialised Human Amniotic Membrane (hAM) is used as a carrier. The shredded bits of limbal tissue are explanted over the centre of de-epithelialised hAM with the basement membrane side up. A similar parallel culture is also prepared as a backup. A submerged explant culture system without a feeder-cell layer is used. HCE medium was used to nurture the culture.

The culture is incubated at 37°C with 5% CO₂ and 95% air. Growth is monitored daily under an inverted phase contrast microscope and the medium is changed every other day. The culture is completed when a monolayer of the cells growing from the explants become confluent, typically in 10–14 days. On the day of transplantation the cultivated limbal cells on the hAM substrate are transported back to the operating room. The affected eye is prepared surgically by performing a peritomy all around the limbus. The corneal fibrovascular pannus is excised. The hAM and monolayer of cultivated limbal epithelial cells is spread over the cornea. The graft is then secured to the limbal side by interrupted circumferential 10-0 nylon sutures and to the surrounding conjunctival edge by interrupted 8-0 polyglactin sutures or with fibrin glue (TISSEEL™ Kit, Baxter AG, Austria).

Data collection included entry of various parameters from each case folder which included pre-operative factors like demographics, age at injury, previous ocular interventions etc.; intra-operative factors like combined with keratoplasty or only CLET etc.; and post-operative factors like success/failure, time to fail, complications etc. Failure was defined as any recurrence of conjunctivalization or superficial vascularization.
A multivariate analysis was performed using multiple logistic regression to elucidate the strength of association between the pre-operative, intra-operative and post-operative clinical factors and recurrence of LSCD.

**RESULTS**

Successful restoration of the ocular surface was seen in 292 (55.5%) of the 526 eyes at a mean follow-up of 1.4 years. Among all the pre-, intra and post-operative clinical factors that were assessed: pediatric age group (OR=1.4, P=0.04), previous ocular surgeries (OR=1.3, P=0.02), presence of symblepharon (OR=1.2, P<0.001) and simultaneous keratoplasty (OR=3.2, P=0.02) were found to be associated with greater chances of failure of autologous cultivated limbal epithelial transplantation.

In conclusion children, patients previously having undergone multiple ocular reconstructive procedures, patients with extensive symblepharon and patients requiring keratoplasty along with autologous limbal transplantation are at higher risk of recurrence of LSCD and need to be adequately counseled about this possibility before surgery.

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**Microbiological Profile in Infective Keratitis and its Correlation with the Conjunctival Flora of the other Normal Eye: A Cross-Sectional Study**

Dr. Joginder Chugh, Dr. Ankush Mutreja, Dr. Rajendra Singh Chauhan

To identify the (i) Microbial pathogens (bacterial, fungal and parasitic) of infectious keratitis, (ii) To compare the profile of microbial pathogens of infective keratitis with the normal flora of the uninvolved other eye. (iii) To study the pattern of antibiotic susceptibility in bacterial isolates

**MATERIALS AND METHODS**

- Prospective cross-sectional, single visit, unmasked study
- **Patient Selection**
  - All the patients included in this study underwent thorough slit lamp biomicroscopic examination.
  - Non-infectious corneal ulcers and viral ulcers were excluded.
Data of patients regarding demographic features, predisposing factors, history of corneal trauma, associated ocular conditions, systemic diseases, therapy received prior to presentation, visual acuity at the time of clinical presentation and clinical course was collected.

History of any medication taken for keratitis was recorded.

Corneal features were noted and drawings made.

**Inclusion criterion**
- Patients of any age, any race and either sex.
- Clinical diagnosis of infective keratitis in one eye only.

**Exclusion criterion**
- Non-infectious corneal ulceration such as Mooren’s ulcer, sterile neurotropic ulcers, marginal keratitis and ulcers associated with autoimmune disorders
- Viral corneal ulcers
- Patients with diagnosis of blepharitis and/or infective conjunctivitis and/or dacryocystitis
- Single eyed patients
- Bilateral infective keratitis

Corneal scrapping of 100 patients with clinically suspected bacterial, fungal or parasitic keratitis was performed and conjunctival swab of both the eyes were taken.

These scrapings were subjected to Gram stain, KOH mount and culture on blood and Sabauraud’s Dextrose Agar. The conjunctival swab was subjected to culture only.

Those patients who had a history of prior antimicrobial drug usage were admitted and all antimicrobial medications were stopped for 24 hours. The affected eye was patched overnight.

**RESULTS**

<table>
<thead>
<tr>
<th>Table 1: Sex distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males: 64</td>
</tr>
<tr>
<td>Females: 36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2: Age distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 30 Years : 21%</td>
</tr>
<tr>
<td>31 – 60 Years : 52%</td>
</tr>
<tr>
<td>61- 80 Years : 27%</td>
</tr>
</tbody>
</table>
Table 3: Distribution of patients according to local risk factors present

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trauma</td>
<td>64</td>
</tr>
<tr>
<td>OSD</td>
<td>13</td>
</tr>
<tr>
<td>Dacryocystitis</td>
<td>2</td>
</tr>
<tr>
<td>Contact lens wear</td>
<td>4</td>
</tr>
<tr>
<td>Exposure keratopathy</td>
<td>4</td>
</tr>
<tr>
<td>Unknown</td>
<td>13</td>
</tr>
</tbody>
</table>

Microbiological Investigations

Table 4: Detection of bacterial and fungal ulcers based on microbiological investigations

<table>
<thead>
<tr>
<th>Method of detection used</th>
<th>Bacteria/Fungi detected</th>
<th>NO Micro Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
</tr>
<tr>
<td>Staining/microscopy</td>
<td>39 (39%)</td>
<td>23 (23%)</td>
</tr>
<tr>
<td>(Grams / KOH Mount)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>29 (29%)</td>
<td>18 (18%)</td>
</tr>
</tbody>
</table>

Table 5: Distribution of various bacteria in culture positive cases

<table>
<thead>
<tr>
<th>Bacterium Isolated</th>
<th>Number of Cases</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. Aureus</td>
<td>12</td>
<td>41.38</td>
</tr>
<tr>
<td>Staph. epidermidis</td>
<td>9</td>
<td>31.03</td>
</tr>
<tr>
<td>Strep. pneumoniae</td>
<td>3</td>
<td>10.34</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>2</td>
<td>6.89</td>
</tr>
<tr>
<td>Enterococcus sp.</td>
<td>2</td>
<td>6.89</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>1</td>
<td>3.45</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 6: Incidence of various fungal species (in culture positive cases)

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of Cases</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium sp.</td>
<td>9</td>
<td>50</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>8</td>
<td>44.44</td>
</tr>
<tr>
<td>Curvularia sp.</td>
<td>1</td>
<td>5.56</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 7: Conjunctival flora of the affected eye

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus epidermidis</td>
<td>46</td>
<td>67.64</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>14</td>
<td>20.59</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>10</td>
<td>14.71</td>
</tr>
<tr>
<td>Neisseria catarrhalis</td>
<td>4</td>
<td>5.88</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>2</td>
<td>2.94</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>2</td>
<td>2.94</td>
</tr>
</tbody>
</table>

### Table 8: Polymicrobial flora of the affected eye

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. of eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus epidermidis and Staphylococcus aureus</td>
<td>6</td>
</tr>
<tr>
<td>Staphylococcus epidermidis and Corynebacterium</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus epidermidis and Neisseria catarrhalis</td>
<td>1</td>
</tr>
<tr>
<td>Corynebacterium and Staphylococcus aureus</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table 9: Correlation between corneal scrappings and the conjunctival flora of the affected eye

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of cases</th>
<th>Statistical significance (p value, chi square value, statistical significance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>3</td>
<td>0.698, 0.151, p&gt;0.05, NS</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>2</td>
<td>0.912, 0.012, p&gt;0.05, NS</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>1</td>
<td>0.436, 0.606, p &gt;0.05, NS</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

### Table 10: Conjunctival flora of the normal eye

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus epidermidis</td>
<td>66</td>
<td>73.33</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>15</td>
<td>16.67</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>11</td>
<td>12.22</td>
</tr>
<tr>
<td>Neisseria catarrhalis</td>
<td>8</td>
<td>8.88</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>3</td>
<td>3.33</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>3</td>
<td>3.33</td>
</tr>
</tbody>
</table>

### Table 11: Polymicrobial flora of the normal eye

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus epidermidis and Staphylococcus aureus</td>
<td>9</td>
<td>56.25</td>
</tr>
<tr>
<td>Staphylococcus epidermidis and Corynebacterium</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Staphylococcus epidermidis and Neisseria catarrhalis</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>Corynebacterium and Staphylococcus aureus</td>
<td>1</td>
<td>6.25</td>
</tr>
</tbody>
</table>
Table 12: Correlation between corneal scrappings and flora of other eye

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number of cases</th>
<th>Percentage of bacterial positive cases</th>
<th>Statistical significance (p value, chi square value, statistical significance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>2</td>
<td>6.89</td>
<td>0.611, 0.874, p&gt;0.05, NS</td>
</tr>
</tbody>
</table>

**In conclusion**

1. Infective keratitis mostly affects people of middle age (31-60 years). In our study, the youngest patient was 5 years old while oldest was 80 years old. Maximum incidence was in the age group of 31-60 years while minimum incidence was seen in extremes of age.

2. Males are affected more commonly than females.

3. Males in the economically most productive age group are more vulnerable to infectious keratitis than females.

4. Infectious keratitis is more prevalent in rural agricultural population. 68% of our cases were from a rural background and 36% were farmers.

5. Incidence of ulceration was maximum in the warm, humid months of April to September.

6. Trauma, frequently with vegetative matter is an important cause in the initiation of infectious keratitis. 43.75% of trauma cases had a positive history of injury with vegetative matter.

7. Based on microbiological investigations, 62% cases were smear positive, out of which 23% cases were diagnosed as fungal corneal ulcer and 39% as bacterial corneal ulcers.

8. Staphylococcal sp. was most commonly isolated (21, 72.4%) followed by Streptococcus Pneumoniae (9, 10.34%). 5 cultures were positive for gram negative bacteria, viz. Pseudomonas (2, 6.89%), Enterococcus (2, 6.89%) and Acinetobacter (1, 3.45%).

9. Fusarium sp. was the commonest fungus identified (50%) followed by Aspergillus (44.44%). 1 case was positive for Curvularia.

10. For bacterial infections, ampicillin/penicillin covered most of gram positive isolates. Most gram negative isolates were maximally sensitive to gentamycin and ciprofloxacin (3, 100%). Two isolates of Pseudomonas were observed which were found to be maximally sensitive to Ceftizoxime and Ciprofloxacin (2,100%).

11. Polymicrobial flora was cultured from 10 cases from the affected eye and in 16 cases from the conjunctiva of the normal eye as shown in Table 8 and 11 respectively.

12. In only 6 culture proven cases, the organism isolated from the conjunctiva of the affected eye was same as that from the corneal scrappings, which is not statistically significant.

13. In only 2 cases, the same organism was cultured from conjunctiva of both the eyes and corneal scrappings.

14. There is no correlation between the conjunctival flora and the causative organism in infective keratitis.

15. Microbiological evaluation should be performed in all the cases before starting any treatment.
Partial Limbal Stem Cell Deficiency (LSCD): Do We Need to Disturb the Fellow Eye?

Dr. Jayesh Vaziran, Dr. Sayan Basu, Dr. Virender Sangwan

Limbal stem cell deficiency (LSCD) clinically manifests with recurrent or persistent epithelial defects, vascularization, pannus formation, conjunctivalization of the corneal surface and loss of normal corneal clarity. Cell based therapy for restoration of the ocular surface in limbal stem cell deficiency has been utilized with good results at various centers across the world. The conventional paradigm of cultivated limbal stem cell transplantation (CLET) in unilateral LSCD rests on harvesting a limbal biopsy from the contralateral eye, cultivating it in a laboratory and transplanting the resultant epithelial sheet on to the affected eye. Though small, there is a definite potential for donor-site complications in the eye from which the limbal biopsy is harvested. In cases with partial LSCD, there are clinically normal appearing areas of the limbus in the affected eye. We hypothesized that it may be possible to harvest limbal stem cells from the healthy part of the limbus in eyes with partial LSCD and cultivate them ex-vivo for transplantation on to the same eye. To the best of our knowledge, no study thus far has explored such a possibility or compared outcomes of such a procedure with conventional CLET.

To report the outcomes of autologous CLET utilizing the healthy part of the affected eye or the fellow eye as a source of limbal stem cells in patients with partial LSCD.

MATERIALS AND METHODS

This retrospective study included 70 eyes of 70 patients with unilateral partial LSCD who underwent autologous CLET between 2001 and 2011. The study was approved by the Institutional Review Board of L V Prasad Eye Institute, Hyderabad, India, and was conducted in strict adherence to the tenets of the Declaration of Helsinki. Prior written informed consent was obtained from all patients or guardians, as appropriate.

A retrospective chart review of all patients who underwent cultivated limbal epithelial transplantation for the treatment of LSCD between 2001 and 2011. The inclusion criteria for this study were as follows: (A) patients with a documented history of chemical or thermal burns; (B) patients with age at injury of more than 8 years; (C) patients who underwent autologous limbal transplantation for unilateral (defined as no clinical signs of ocular surface disease in the other eye) and partial LSCD (defined as less than 360 degree superficial corneal vascularisation, diffuse fluorescein staining of the corneal surface with or without persistent epithelial defects, conjunctivalisation of
the corneal surface and absence of limbal palisades of Vogt. The following exclusion criteria were applied: (A) patients who had bilateral LSCD or had allogeneic limbal transplantation; (B) patients who had limbal transplantation for total LSCD or LSCD due to causes other than ocular surface burns; (C) patients with dry eye disease (Schirmer’s test without anaesthesia of <10 mm in 5 min; (D) patients with no visual potential as determined by clinical examination and electrophysiological testing; (E) patients with untreated concurrent problems, such as glaucoma and infection.

The limbal biopsy was taken either from the healthy part of the limbus of the same eye or from the healthy fellow eye. Cells were cultivated in the laboratory to form a confluent monolayer, which was subsequently transplanted after dissecting the pannus. The surgical technique has been described in detail in our earlier publications.3 Primary outcome measures were visual acuity and graft survival rate. Success was defined clinically as a completely epithelised, avascular and clinically stable corneal surface. Failure was defined as the occurrence of superficial corneal vascularisation or persistent epithelial defects. Survival time was calculated in months from the date of limbal transplantation to the date of failure or the date of last follow-up depending on the clinical outcome.

RESULTS

The mean follow up was 17.5 ± 7 months. The mean age of patients was 24 ± 12.5 years. Male:female ratio was 4:1. In 36 eyes the limbal biopsy was taken from the contralateral eye and in the remaining 34 eyes from the ipsilateral eye. The mean pre-operative visual acuity was 1.1 ± 0.6 logMAR, which improved to 0.8 ± 0.6 logMAR(p=0.04). The one year graft survival was 85±7 percent in the contralateral group and 71±8 percent in the ipsilateral group(p=0.74). Failure of limbal transplantation was seen in 9 eyes in the contralateral group and 10 eyes in the ipsilateral group.

DISCUSSION

Our results using autologous CLET for unilateral partial LSCD show comparable outcomes in both groups. The biological plausibility of harvesting stem cells from the relatively healthy part of the limbus in an eye with partial LSCD, cultivating them ex vivo and transplanting the resultant layer of cells back on to the affected eye to restore the ocular surface is therefore confirmed. In our experience, taking a small limbal biopsy from the contralateral normal eye usually does not result in clinically significant complications. Nonetheless, patients with unilateral problems are often hesitant to consent for a procedure with the potential risk of damage to the only normal eye. The results of this study provide proof that even this small theoretical risk of iatrogenic LSCD in the contralateral eye can be completely eliminated by utilizing the eye with
partial LSCD as the source of stem cells. This may encourage more patients to undergo the procedure. The findings have particular importance in cases where one eye has total LSCD and the other eye has partial LSCD. Conventional procedures would not allow for allogenic CLET to be carried out in such a situation. Extrapolating our results, it would be possible to harvest stem cells from the eye with partial LSCD for CLET. Indeed, our results with such cases have been extremely gratifying. Recently, our group has also published the technique of Simple Limbal Epithelial Transplantation (SLET), which combined the advantages of conjunctivo-limbal autografting and CLET.5 Though we only used CLET in the current study, similar results may conceivably obtained using a single-stage, single-eye procedure by performing SLET in unilateral partial LSCD, using the same eye for harvesting limbal tissue.

In conclusion ocular surface restoration in partial LSCD is possible with cell-based therapy. Outcomes are similar irrespective of whether the limbal biopsy is taken from the healthy part of the ipsilateral eye or the contralateral eye.

REFERENCES

Corneal Biomechanics Changes following Collagen Crosslinking Treatment in Keratoconus in Indian Eyes

Dr. Ravi Bypareddy, Dr. Anita Panda, Dr. Murugesan Vanathi, Dr. Tushar Agarwal, Dr. Tanuj Dada, Dr. Sudarshan Khokhar

Keratoconus is the most common primary progressive ectasia of the cornea resulting from non-inflammatory thinning of the corneal stroma.1 Keratoconus usually becomes apparent during 2nd decade of life, around
puberty and may progress until 4th decade, when it usually stabilizes. Abnormal corneal protrusion causes high myopia and irregular astigmatism, which exerts a negative impact on quality of life, causing a significant visual acuity reduction due to the progressive changes of the disease, and hence remains one of the common indications for corneal grafting.

The biomechanical properties of the cornea depend on the characteristics of collagen fibers, interfibrillar bonds, and their spatial-structural disposition. Early experiments of Cannon and Foster implicated the role of degraded normal collagen or synthesis of abnormal collagen in the pathogenesis of keratoconus. There is increased expression of lysosomal and proteolytic enzymes and decreased concentration of protease inhibitors in keratoconus which results in corneal thinning and altered configuration of corneal collagen lamellae. These changes are thought to reduce the biomechanical stability of the corneal stroma by 50% with consequent changes in both the cornea’s anatomical and topographic architecture.

There are multiple treatment options for management of keratoconus ranging from spectacle correction to deep lamellar anterior keratoplasty (DALK). However, none addresses the inherent pathology of increase in weakening of the corneal stroma. In the last decade advancements in ophthalmology have found an efficient technique for arresting progression of disease by Collagen Cross Linking using UV-A/Riboflavin 0.1%

Since the cornea is a viscoelastic structure, when stress is applied, an immediate elastic response of the cornea followed by a prolonged, time-dependent, viscoelastic response is seen. Early studies measured a decrease in elasticity in corneas with keratoconus. UV A/riboflavin–mediated collagen cross-linking (CXL) for the treatment of keratoconus is thought to increase the biomechanical strength of the cornea. Wollensak et al reported that immediate stress measurements increased by 71.9% and 328.9% in porcine and human corneas, respectively, after CXL. Despite laboratory and clinical findings, however, to date it has been difficult to quantify the actual biomechanical changes effected by CXL in vivo. But with the development of Ocular Response Analyzer (ORA) now it is possible to measure the biomechanical properties of cornea in vivo.

Corneal hysteresis (CH) characterizes the viscoelastic property of the cornea which is expressed as a measure of its stiffness or rigidity. It is an indication of the viscous damping in the cornea and reflects the capacity of the tissue to absorb and dissipate energy. The electro-optical collimation detector system of the ocular response analyzer (ORA) monitors the corneal curvature in the central 3.0 mm diameter throughout the 20 millisecond applanation measurement of a precisely delivered metered air pulse. This air pulse effects an inward corneal movement (first applanation event) causing a corneal
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When the air pulse is shut down, the pressure applied to the eye decreases, effecting a return of the cornea from concavity (second applanation event) to its normal convex curvature. The information obtained from these applanating events is depicted in graphic form with the two well-defined peaks of pressure corresponding to inward and outward applanation events (one while the cornea is moving inward (P1), and the other as the cornea returns from its concavity, it passes through a another state of applanation called P2). Corneal hysteresis is the difference between these two pressures peaks. The ORA also measures the Goldmann-correlated IOP (IOPg), corneal-compensated IOP (IOPcc) and corneal resistance factor (CRF). IOPg is the average of the P1 and P2 applanation pressures, while IOPcc represents the IOP that has been compensated for the corneal biomechanical properties. Corneal resistance factor (CRF) is more strongly associated with central corneal thickness (CCT) than corneal hysteresis and is derived as follows: CRF= P1 – k P2, (k is an empirically determined constant).

In this study, in an effort to evaluate corneal biomechanical changes in vivo after CXL, ORA measurements of CH and CRF were analyzed over a 6 months period after the CXL procedure and also were correlated with visual acuity and topographic outcomes after CXL.

MATERIALS AND METHODS

Single centre, prospective, longitudinal study of ORA recordings of 35 progressive keratoconic eyes that had undergone collagen cross linking using riboflavin 0.1% and UV A light were done. Informed consent was obtained from all patients. Progressive keratoconus was defined as one or more of the following changes over a period of 12 months: an increase of ≥1 diopter (D) in the steepest keratometry, an increase of ≥1 D in manifest cylinder, or an increase of ≥0.5 D in manifest refractive spherical equivalent. We excluded the patients with corneal scarring, severe dryness of eyes or any corneal infection, history of any autoimmune disease, history of any prior corneal surgery or intra corneal ring segments (ICRS), history of delayed wound healing, patient with history of allergy to riboflavin, pregnant women or patients who are breast feeding and finally patients with central corneal thickness of less than 400 microns because of risk of damage to endothelium with de-epithelial technique of cross-linking. Prior to CXL, we recorded the UCVA, BSCVA, SE, RC, average and maximum keratometry, ORA readings and Goldmann intraocular pressure(IOP GAT). CXL was performed according the methodology described by Wollensak et. al. Initially, a topical anesthetic was administered and the central epithelium was removed. After topical 0.1% riboflavin administration (0.1% in 20% dextran T-500 solution, Medio-Cross; Peschke Meditrade, GmbH, Zurich, Switzerland) every 2 minutes for a total
of 30 minutes. The cornea was exposed to UVA 365 nm light for 30 minutes at an irradiance of 3.0 mW/cm² (UV-X System; IROC, Zurich, Switzerland), and riboflavin administration was continued every 2 minutes for the duration of the treatment.

ORA (Reichert Ophthalmic Instruments, Buffalo, N.Y.) recordings were performed by a single observer (RB) according to a standard protocol. Recordings were done to obtain four consecutive readings in each eye, with only the good quality measurement with two distinct peaks, being included. The mean of the four measurements was used for statistical analysis. CH, CRF, IOPg and IOPcc were noted. CCT was measured with ORA attached hand held ultrasonic pachymeter.

Data was entered into Excel spreadsheet and all statistical analyses were performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). “Paired student t” test was used to analyse the post CXL visual acuity, keratometry and ORA parameter changes over time and compared with baseline. A p value of <0.05 was used as statistically significance level.

RESULTS

A total of 35 eyes of 27 patients underwent CXL and were followed for 6 months. The mean (SD) age of keratoconus patients was 20.74 ± 7.48 years [range: 12 years to 30 years, males 18(67%), females 09 (33%)].

Visual acuity

The results have been shown in Table 1 below.

UCVA: The visual acuity changed from baseline (pre-operative) value of 0.6786±0.1 log MAR to 0.6583 ± 0.27, 0.6346 ± 0.27 and 0.5246 ± 0.23 logMAR at 1st, 3rd and 6th month respectively after cross-linking. The change was not statistically significant at first month, however the change was significant statistically at 3rd month (p=0.050) and 6th month (p=0.000) following cross-linking. (Figure. 1).

BCVA: Preoperatively the mean BSCVA was 0.306 ± 0.21 logMAR (range from 6/36 to 6/5). Mean post operative visual acuity at months 1, 3 and 6 was 0.289± 0.19, 0.237 ± 0.20 and 0.156 ± 0.18 logMAR respectively. However the change was statistically significant only at month 6 (p=0.000). (Figure.1)

Refraction

The mean baseline refractive spherical equivalent (RSE) was -6.0 ± 2.3 D (range from -1.5 D to -10 D). Mean spherical equivalent improved after cross linking with values being -5.71D ± 2.2 at month 1, -5.52D±1.8D at month 3 and -5.20D ± 1.7D at month 6 following CXL. Thus, there was significant change at months 3 (p=0.03) and 6 (p=0.000). (Figure.2)
Keratometry

Average Keratometry: (Avg Km)
Mean preoperative Avg Km preoperative was 50.59±4.73D. Postoperative keratometry improved at different time periods of follow-up. Mean Avg km at month 1, 3 and 6 after crosslinking was 50.31±4.69D, 50.12±4.62D and 49.61±4.51D respectively. Thus, there was significant improvement in km at month 6 (p=0.000).

Maximum Keratometry
Following crosslinking the Kmax values decreased from 56.29±5.24D at baseline to 56.31±5.57D at month 1, 55.61±5.21D at month 3 and 55.12±5.06D at month 6. Thus, there was significant improvement in Kmax. There was a decrease in average Kmax by 1.19±0.23D at post op 6 months compared to pre CXL (p=0.012). (Figure 3)

CH and CRF
Preoperative CH was 7.18±1.33 mmHg and at 6 months postoperatively changed to 8.34±1.14 mmHg (P=0.000). Preoperative CRF was 6.20±1.29 mmHg and at 6 months was 6.84±1.49 (P=0.024).

Postoperative Time Course of CH and CRF
The postoperative changes in CH were 7.33±1.28, 7.78±1.27 and 8.34±1.14 mmHg between baseline and 1 month, 1 and 3 months, 3 and 6 months respectively. The changes showed statistical significance level at 6 months (p=0.000) (Figure 4 and 5).

Similarly CRF changed from 6.20±1.29 at baseline to 6.52±2.45 at 1 month, 6.65±1.50 at 3 months and 6.84±1.49 mmHg at 6 months following CXL, with the changes being significant at 6 months (p=0.024).

DISCUSSION
Keratoconus is progressive ectasia of the cornea due to non-inflammatory thinning of the stroma leading to diminishment of visual acuity due to induced myopia and astigmatism. Majority of these patients can maintain good vision with the help of spectacles and contact lenses. However 20% of patients progress to advanced keratoconus, where contact lenses fail to maintain vision of 6/18 or better; stressing the need for penetrating or lamellar procedures.

Collagen cross linking is a promising new treatment for the stabilization and strengthening of the cornea in keratoconus. Reduced biomechanical strength of the cornea has been shown to be a main characteristic of keratoconus. Corneal collagen crosslinking using a combination of riboflavin and ultraviolet A is a new treatment modality for increasing corneal biomechanical resistance by adding additional polymer
The effect of this treatment has been previously assessed only using clinical parameters such as corneal topography and subjective refraction, whereas we sought to demonstrate physical corneal changes. Previous in vitro studies described physical changes in the cornea after cross-linking. Wollensak et al. used stress–strain measurements to evaluate the effect of riboflavin–UV-A CXL on corneal rigidity in human and porcine corneas. Using microcomputer-controlled biomaterial tester, they showed significant increase in rigidity in both human and porcine corneas. The experimental stress–strain investigations ex vivo revealed an increase of the tangential bands between collagen fibers.
value for Young's modulus (strain/stress) after Riboflavin/UV A collagen cross linking. Therefore a change in biomechanical properties was also expected in vivo.

Because cross linking of cornea supposed to be halting the progression of keratoconus by increasing the stiffness, we expected to observe changes in CH and CRF after CXL. Theoretically, therapeutically inducing crosslinking of collagen might act similarly to processes that retard keratoconus progression during aging and prolonged uncontrolled hyperglycemia.

In our study, we observed significant changes in biomechanical properties of the cornea after CXL in patients with progressive keratoconus as measured in vivo using ocular response analyzer. These factors estimate the cornea’s biomechanical parameters, which can be measured in vivo by biomechanical wave form analysis. Shah et al. found decreased biomechanical properties in eyes with keratoconus using the same biomechanical wave form analyzer we used. Our data give further evidence of this decline. However, the baseline values of CH and CRF in our study were lower than those reported on previous studies of keratoconic eyes. Albe, Goldich et al. and Sedaghat et al. studies revealed no change in CH after CXL in three different studies. Recently, Steven A et al. also confirmed no change in biomechanical properties at 1 year after CXL. However, our study did show statistically significant increase in corneal hysteresis and corneal resistance factor following CXL at 3 and 6 months. There are studies documenting that keratoconus may be more prevalent, have earlier onset, and have greater disease progression in certain

Table 1: Showing the changes in UCVA, BCVA, Keratometry and ORA parameters pre and post CXL

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-operative</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCVA (logMar)</td>
<td>0.6786±0.30</td>
<td>0.6583±0.27</td>
<td>0.6346±0.27</td>
<td>0.5246±0.23</td>
<td>0.000</td>
</tr>
<tr>
<td>BCVA (logMar)</td>
<td>0.306±0.21</td>
<td>0.289±0.19</td>
<td>0.237±0.20</td>
<td>0.156±0.18</td>
<td>0.000</td>
</tr>
<tr>
<td>RE (D)</td>
<td>6.0±2.3</td>
<td>5.71±2.2</td>
<td>5.52±1.8</td>
<td>5.20±1.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td>5.40±2.37</td>
<td>5.25±2.25</td>
<td>4.91±2.22</td>
<td>4.70±1.99</td>
<td>0.000</td>
</tr>
<tr>
<td>Kmax (D)</td>
<td>56.29±5.29</td>
<td>56.31±5.57</td>
<td>55.61±5.21</td>
<td>55.1±5.06</td>
<td>0.012</td>
</tr>
<tr>
<td>Avg K (D)</td>
<td>50.59±4.73</td>
<td>50.31±4.69</td>
<td>50.1±4.62</td>
<td>49.61±4.51</td>
<td>0.021</td>
</tr>
<tr>
<td>K2 (D)</td>
<td>53.99±5.07</td>
<td>53.8±5.10</td>
<td>53.71±5.17</td>
<td>53.1±5.01</td>
<td>0.032</td>
</tr>
<tr>
<td>K1 (D)</td>
<td>46.1±4.93</td>
<td>45.9±4.28</td>
<td>45.5±4.34</td>
<td>45.2±4.58</td>
<td>0.031</td>
</tr>
<tr>
<td>CH (mmHg)</td>
<td>7.18±1.33</td>
<td>7.33±1.28</td>
<td>7.78±1.27</td>
<td>8.34±1.14</td>
<td>0.000</td>
</tr>
<tr>
<td>CRF (mmHg)</td>
<td>6.20±1.29</td>
<td>6.52±2.45</td>
<td>6.65±1.50</td>
<td>6.84±1.49</td>
<td>0.024</td>
</tr>
<tr>
<td>IOPg (mmHg)</td>
<td>13.76±3.34</td>
<td>14.68±6.05</td>
<td>13.78±3.21</td>
<td>13.78±2.18</td>
<td>1.000</td>
</tr>
<tr>
<td>IOPcc (mmHg)</td>
<td>9.26±3.81</td>
<td>10.04±3.31</td>
<td>10.06±3.31</td>
<td>9.36±3.38</td>
<td>1.000</td>
</tr>
<tr>
<td>CCT</td>
<td>454.3±22.90</td>
<td>452.6±20.13</td>
<td>453.0±24.06</td>
<td>455.1±24.03</td>
<td>1.000</td>
</tr>
</tbody>
</table>
Asian and non-Asian ethnicities, particularly Indians, Pakistanis, Middle Easterners, and Polynesians, compared with white populations. Studies have also proved that efficacy of CXL is more pronounced in adolescence and progressive keratoconus. Since 33% of study group were of less than 16 years of age, one would definitely expect pronounced and rapid changes in biomechanics in contrary to previous studies.

In the Goldich et. al. and Sadaghat et. al., the ORA software used was 1.06 but the ORA software which we have used in 2.06. The old software system might have been successful in measuring the subtle changes in biomechanics and moreover, the surface optical irregularity of these ectatic corneas may introduce error and variability into the ORA signal that may prevent meaningful quantitative comparison of preoperative and postoperative CH and CRF in the older version of the ORA software.

In conclusion, in the present study, we did observe significant change in corneal biomechanical properties, as measured with the ORA parameters CH and CRF, after CXL in keratoconus. The changes in keratometry, UCVA, BSCVA and refractive spherical equivalent were similar to previously published studies.

REFERENCES


Keratoconus is a disorder characterized by a conical ectasia of the cornea in the absence of clinical signs of corneal inflammation. Typically, the pathology is characterized by a central or inferior corneal thinning with increased curvature at the apex of the cone. The etiology of this disease is unknown, but it has been classically described as being associated with certain systemic diseases, such as atopy and connective tissue disorders. In most cases, keratoconus occurs bilaterally but asymmetrically. It generally affects young adults and has an incidence of about 1:2000 of the general population. Many cases progress slowly and gradually in severity, but the rate of progression and the length of time that keratoconus remains actively progressive vary considerably. The factors governing the progression and stabilization of keratoconus are currently unknown. In recent years, extensive studies of the biochemical and pathologic changes that occur at the structural and cellular levels of the cornea have been carried out. Nevertheless, the specific mechanisms underlying the development of keratoconus and its relationship to heredity or environment are still not fully understood. Although there may be a relationship between keratoconus and several conditions of allergic etiology the influence of these conditions on the pathogenesis and natural history of keratoconus remains unclear. Tissue degradation in thinning disorders, such as keratoconus, involves the expression of inflammatory mediators, such as proinflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases. With a view to contributing to our understanding of the factors that govern the etiology and development of keratoconus, we evaluated the levels of matrix metalloproteinase 9 (MMP9) in tears of Indian patients with keratoconus.

The purpose of this study was to evaluate levels of the inflammatory biomarker MMP9 (Matrix Metalloproteinase 9) in patients with keratoconus of Indian origin. SECONDARY OBJECTIVE- We also attempted to study the difference in the level of MMP9 between allergic and non allergic individuals.

**MATERIALS AND METHODS**

The subjects included in the study had the following criteria:

**Inclusion Criteria**

Patients of keratoconus, of Indian origin, with/without any association with allergy *i.e. ocular or systemic*, who reported to our tertiary care centre in South India were included.
Exclusion Criteria
Patients using contact lenses, any type of topical ocular medications, any systemic medications (e.g. anti-allergic, anti-inflammatory drugs) or who had undergone any intervention (e.g. Penetrating keratoplasty/ Corneal Collagen Cross Linking, etc.) for either of the eyes were excluded from the study.

Demographics
64 eyes of patients with keratoconus were included in the study. The Male to female ratio was 39:25. The age group of the study population ranged from 10 years to 53 years, with a mean of 25 years of age. 18 patients had allergies, 4 of which reported systemic allergies like eczema, and the rest 14 had ocular allergies. All patients underwent corneal topography using the Pentacam, Oculus Inc.

The study was approved by the Institutional ethics committee. Tear samples were collected under aseptic precaution using capillary micropipette tubes. These can contain up to 15-20 microlitres of fluid. The tears were collected from the exterior 1/3rd of the lower fornix, taking care not to touch the conjunctiva or produce any reflex tearing.

The micropipette tubes were placed into dry Eppendorf tubes and freeze stored at -20 degrees Celsius, until analysis. An ELISA enzyme assay, Biotrak Ltd., was used to analyse the samples and obtain values of the MMP9.

**RESULTS**

<table>
<thead>
<tr>
<th>N=64</th>
<th>STEEP K (in Diopters)</th>
<th>MMP 9 (relative units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MINIMUM</td>
<td>41.4</td>
<td>5</td>
</tr>
<tr>
<td>MAXIMUM</td>
<td>68.5</td>
<td>60</td>
</tr>
<tr>
<td>MEAN</td>
<td>49.3</td>
<td>43.47</td>
</tr>
</tbody>
</table>

The mean level of MMP 9 in the study group was 42.45. We classified the subjects into 3 groups based on the value of the steeper meridian obtained on the Pentacam. They were divided into grade 1: <48 Diopters, grade 2: 48.25-51 Diopters, grade 3: 51.25-56 Diopters, grade 4: >56 Diopters. Mean MMP9 levels for each individual group was as follows: Grade 1: 42.45, Grade 2: 40.06, grade 3: 46.07, grade 4: 46.55.

We further divided the subjects into 2 groups based on the co-existence of allergy (ocular/systemic). There were 18 eyes with allergic involvement, who had a mean MMP9 level of 41.1 as opposed to the remainder patients whose mean level was 44.39. There was no statistically significant difference in the MMP9 levels of these groups (p=0.361)

**DISCUSSION**

Numerous clinical studies in recent years of keratoconus support the idea
that its pathogenesis has an inflammatory component. Various changes in the ocular surface of patients with keratoconus have been found, like increased fluorescein and rose bengal staining scores, reduced corneal sensitivity, and abnormal impression Cytology. The tear may be a vehicle of some of the pathogenic protagonists of keratoconus, such as IL-6, TNF or MMP-9. Increased levels of these molecules may be sporadic, but sufficient to provoke slowly progressive ectasia.\(^{15}\)

Our results indicate that the concentration of inflammatory molecules in tears is associated with the intensity of keratoconus in Indian eyes; however, a similar association with the progression of ectasia was not determined.

Increased MMP 9 levels were found in patients with keratoconus as compared to normals. However, these values were lower than those of patients with other origin e.g. Caucasians, etc. The possibility that Indian eyes are more susceptible to lower levels of inflammatory markers exists. The level of MMP 9 correlates with increasing grades of the condition. Though many studies exclude patients with allergies, we included such subjects and found that there was no significant difference in the MMP9 values of the 2 groups. This may indicate that MMP9 is a direct marker for keratoconus, irrespective of allergy. However, the role of other inflammatory markers needs to be evaluated before such a statement holds true.

The sample size in our study may not be representative of all patients. Other inflammatory biomarkers need to be evaluated to know the interplay of these in the pathogenesis of the condition.

REFERENCES


**Epithelium-on and Epithelium-off Riboflavin Uva-Induced Corneal Collagen Cross-Linking: A Comparative Study in Pediatric Keratoconus**

**Dr. Archita Agrawal, Dr. Ashish Nagpal, Dr. Sunita Mohan**

Paediatric age at the time of diagnosis represents a negative prognostic factor for keratoconus progression, with increased probability of corneal transplant. Moreover, pediatric keratoplasty has a higher chance of rejection and a worse visual prognosis than adults. According to international results, corneal collagen cross-linking (CXL) should be the primary choice in young patient with progressive keratoconus.

In the classic CXL procedure, removal of the corneal epithelium not only causes discomfort and postoperative pain to patients, it can also increase the risk of corneal infections, ulcers, haze, scarring, infiltrates, and longer recovery time. These problems have led surgeons to explore trans-epithelial CXL (TE-CXL), and the initial results are promising, though variable. Looking at the advantages of TE-CXL, specially in pediatric age-group, we started performing trans-epithelial CXL in our institute in 2010.

To evaluate the efficacy and safety of corneal collagen cross-linking (CXL) with epithelium-on and epithelium-off in progressive pediatric keratoconus.

**MATERIALS AND METHODS**

In this retrospective study, we analyzed 12 eyes of 11 pediatric patients with keratoconus undergoing standard epithelium-off or trans-epithelial CXL.
Consecutive patients were enrolled using the following inclusion criteria: Age younger than 18 years at the time of procedure, and progressive keratoconus (defined as an increase of at least 1D in astigmatic refraction or maximum curvature on corneal topography within past 1 year). 6 eyes underwent epithelium-on/trans-epithelial CXL (group-1), and 6 eyes underwent standard epithelium-off CXL (group-2).

Patients were examined preoperatively and at 1 day, 1 week, 1, 3, 6, 12, 18 and 24 months postoperatively. Each examination included slit-lamp evaluation, uncorrected and best-corrected visual-acuity (UCVA and BCVA respectively), refraction, corneal topography (pentacam, oculus inc.), optical pachymetry and specular microscopy.

**Surgical procedure**

The surgical procedure of corneal cross-linking with Riboflavin and UVA was performed using modified Dresden protocol using the IROC X-linker. The treatment was conducted under topical anaesthesia (0.5% proparacaine drops). Under sterile conditions, eyelid speculum was applied and a 9 mm diameter circular alcohol swab was put over central cornea for 10 seconds. Alcohol was thoroughly flushed-off with normal saline, and epithelium was removed using a blunt forceps. After epithelial scraping, a disposable solution of Riboflavin 0.1% and Dextrane 20% was instilled every 1-2 minutes for 15-30 minutes before starting UVA irradiation, and then every 1-5 minutes for 30-45 minutes during UVA exposure (3 mW/cm2). Treated eyes were dressed with an antibiotic ointment and patched for a day. The postoperative medications given included antibiotics (moxifloxacin drops 4 times/day) and lubricants (1% HPMC 4 times/day) starting on first post-op day, and fluorometholone 0.2% drops (4 times/day) started after epithelium had healed and continued for a month.

In the transepithelial group, all steps were same except that no alcohol was used and epithelium was not debrided.

In patients in whom the corneal thickness was less than 400 microns, distilled water was used for 5-15 minutes before putting riboflavin drops.

**RESULTS**

The age of the patients ranged from 12-18 years (mean 15.4 yrs), and the follow-up ranged from 6-21 months (mean 11.7 months). In group-1, the mean age was 15 yrs, and the mean follow-up was 11.5 months. In group-2, the mean age was 15.8 yrs, and mean follow-up was 11.8 months. We compared pre-op data with data at the last follow up, and the results found are shown in tables 1 and 2. Statistical analyses were done using paired t-test and 2-samples t-test, and p-values<0.05 were considered significant.
Immediate postoperative period: All patients in group-2 complained of pain, watering and foreign body sensation and showed signs of conjunctival hyperemia and epithelial defect, which took 3-4 days to resolve. In contrast, only 1 patient in group-1 had conjunctival hyperemia and watering, which resolved in 2 days. No patient in this group had pain or foreign body sensation.

Visual and topographical parameters: The visual and topographical parameters in all patients in both the groups stabilized or improved after the crosslinking procedure. The results are shown in tables 1 and 2. On statistical analysis using SPSS software, both the groups had statistically similar improvements in UCVA, BCVA, K-steep, manifest refraction and surface-asymmetry index.

### Table 1: Group-1 Preoperative and postoperative mean values for the measured parameters, compared with standard t-test. p-values are demonstrated

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean Pre-op</th>
<th>Mean Post-op</th>
<th>Difference of means (post-pre)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCVA</td>
<td>0.2266</td>
<td>0.2533</td>
<td>0.0267</td>
<td>0.363</td>
</tr>
<tr>
<td>BCVA</td>
<td>0.5283</td>
<td>0.6117</td>
<td>0.0834</td>
<td>0.201</td>
</tr>
<tr>
<td>K-STEEP</td>
<td>55.37</td>
<td>54.4</td>
<td>-0.97</td>
<td>0.443</td>
</tr>
<tr>
<td>K-FLAT</td>
<td>47.37</td>
<td>47.95</td>
<td>0.58</td>
<td>0.285</td>
</tr>
<tr>
<td>Refraction (Spherical Equivalent)</td>
<td>-6.08</td>
<td>-5.33</td>
<td>-0.75</td>
<td>0.06</td>
</tr>
<tr>
<td>Surface Asymmetry Index</td>
<td>1.97</td>
<td>1.97</td>
<td>0</td>
<td>0.98</td>
</tr>
<tr>
<td>Endothelial Cell Count</td>
<td>2980</td>
<td>2765</td>
<td>-215</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Group-2 Preoperative and postoperative mean values for the measured parameters, compared with standard t-test. p-values are demonstrated

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean Pre-op</th>
<th>Mean Post-op</th>
<th>Difference of means (post-pre)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCVA</td>
<td>0.1133</td>
<td>0.1367</td>
<td>0.0234</td>
<td>0.520</td>
</tr>
<tr>
<td>BCVA</td>
<td>0.4433</td>
<td>0.5</td>
<td>0.0567</td>
<td>0.465</td>
</tr>
<tr>
<td>K-STEEP</td>
<td>56.31</td>
<td>55.31</td>
<td>-1.0</td>
<td>0.460</td>
</tr>
<tr>
<td>K-FLAT</td>
<td>48.69</td>
<td>48.26</td>
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Corneal transparency: 2 corneas in group-1 developed clinically visible haze (grade 1+), while all 6 corneas in group-2 developed clinically visible haze (grade 1+ to 3+).

Pachymetry: The mean pre-op and post-op corneal thickness (thinnest point at topography map) was 409 and 390 respectively in group-1, and 430 and 419 in group-2.

Endothelial cell count: Both the groups had insignificant decrease in endothelial cell count following the CXL procedure.

Complications
In group-1, no other complication was noted except deep stromal scarring and persistent photophobia in 1 eye. In group-2, 2 patients developed transient superficial punctate keratopathy during follow-up, and 1 patient complained of persistent photophobia.

DISCUSSION
Although this study was nonrandomized and the number of patients small, the results of transepithelial CXL are encouraging. Not only did the TE-CXL treatment appear to halt the progression of keratoconus in all treated eyes, it also resulted in improvement (though statistically non-significant) in all visual and topographic outcome measures. Also, the improvements in outcome measures were statistically similar in both groups (p>0.05). Further long-term observation of these patients is required to determine for how long disease progression is arrested.

The protocol used by us was variable and not fixed as no protocol was considered standard at that time. But the two groups were comparable in this regard, as both had similar number of patients undergoing a particular protocol.

The majority of patients in group-1 were comfortable and symptom-free in the immediate post-op period, while the patients in group-2 had severe pain and watering. Intra-operatively also, the patients in group-1 were much more cooperative and comfortable.

One patient in group-1 developed deep stromal scarring and photophobia. This patient had a pre-op corneal thickness of 382 microns and distilled water was not used before riboflavin. This could account for the deep stromal scarring, emphasizing the importance of a minimal pre-op corneal thickness of 400 microns, and intraoperative pachymetry (not done in our study).

Few earlier studies in which TE-CXL was performed using riboflavin with epithelial permeability enhancers showed TE-CXL to be having limited but favourable results in arresting keratoconus progression, but at the same time...
less effective than standard CXL. Another study\textsuperscript{14} in which in-vivo confocal analysis was performed on eyes undergoing TE-CXL, showed a limited apoptotic effect (around 1/3rd of standard CXL) and penetration (upto 140 microns depth). In contrast, a bilateral study by fillipello \textit{et. al.}\textsuperscript{11} in which fellow eye was left untreated and used as control eye showed TE-CXL to be effective in halting CXL progression and improving visual and topographical parameters. Our study shows that trans-epithelial CXL is efficacious and comparable with classic CXL. It not only arrests progression of keratoconus and improves visual and topographical parameters, its results are comparable to the standard CXL. The procedure is pain free, can be performed on uncooperative patients like children, does not need a sterile environment, can be performed on relatively thinner corneas, there is minimal or no postoperative discomfort, and the risk of complications associated with epithelial debridement is negated.

In conclusion, transepithelial CXL treatment appeared to halt keratoconus progression with improvement in measured visual and topographic parameters which were comparable to classic CXL procedure. The treatments were safe and well tolerated. Its noninvasive nature makes it a potentially useful treatment in cases in which epithelial debridement is ideally avoided, such as pediatric cases, uncooperative patients, and in eyes with thin corneas (thicknesses nearing 380 μm). A larger, prospective study comparing transepithelial and standard CXL is required, and, should transepithelial CXL prove to be as efficacious as classic CXL, its noninvasive nature and simplicity would make it the procedure of choice for corneal collagen CXL.

REFERENCES


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**Emergence of Multiple Drug Resistance in Pseudomonas Aeruginosa Keratitis**

**Dr. Shikha Gupta**, Dr. Sudeep Mittal, Dr. Niranjan Nayak, Dr. Gita Satpathy, Dr. Tushar Agarwal, Dr. Anita Panda

To report the antibiotic sensitivity pattern of *Pseudomonas aeruginosa* isolates cultured from corneal scraping of patients with infectious keratitis presenting to the outpatient department of a tertiary eye care centre.

**MATERIALS AND METHODS**

Retrospective chart review of in vitro antibiotic susceptibility of *P. aeruginosa* cultured from corneal scrape samples of patients who presented with infectious keratitis to our outpatient department over the last 6 months (December 2011-May 2012) was done. Minimum Inhibitory Concentration (MIC) (in milligrams per liter) was determined for the following antimicrobials: polymyxin B, ciprofloxacin, tobramycin, tetracycline, gentamycin, gatifloxacin, moxifloxacin, cefazolin, ceftazidime and cefuroxime for each case. The isolate was labelled as resistant if the MIC was greater than the systemic breakpoint MIC as defined by BSAC. Multidrug resistance (MDR) was defined as resistance to > 5 drugs against which susceptibility was tested. Pan drug resistance (PDR) was defined as resistance to all the tested antibiotics except one. Multi and pan drug resistance were grouped together as poly drug resistance.
RESULTS

Of the total number of infectious keratitis presenting between December 2011- May 2012, isolates from 32 corneal scraping isolates tested positive for Pseudomonas Aeruginosa. Of these 14 isolates (43.75%) showed poly drug resistance. Additionally, ten of these cases of multiple antibiotic resistant P. aeruginosa infectious keratitis were resistant to commonly used antibiotics including fourth generation fluoroquinolones (gatifloxacin and moxifloxacin) currently available in the Indian market (table). 7 of these cases showed susceptibility to only polymyxin B amongst the tested array of drugs. Further, Pseudomonas aeruginosa isolated from the corneal scraping demonstrated in vitro susceptibility to polymyxin B in all these cases of poly drug resistance.

DISCUSSION

P. Aeruginosa is the most frequently isolated gram negative pathogen in bacterial keratitis in India.4 Though scattered reports of antibiotic resistance in Paeruginosa corneal infections were available5,6,7 but finding MDR in approximately half of our patients is definitely alarming. The protocol for treating bacterial infective keratitis in our institute is to start with fortified antibiotics (cefazolin and tobramycin) in cases of moderate (2-5 mm) and large (>5 mm) ulcers or ulcers associated with hypopyon empirically. For small ulcers (<2 mm), we start with fourth generation fluoroquinolone monotherapy (gatifloxacin/ moxifloxacin) till the culture sensitivity report is established. Unfortunately, P. Aeruginosa isolates were resistant to all these front line drugs in 10 of our cases. Remarkably in our setting, susceptibility to gatifloxacin was more frequently encountered compared to moxifloxacin which should be thus preferred in bacterial keratitis. Falagas et. al. reported that the increasing use of fluoroquinolones against Pseudomonal strains contribute to the upsurge of MDR species.8

Multidrug resistance amongst Pseudomonal isolates is frequently reported in systemic infections.1,2 Most often, different mechanisms of resistance are present simultaneously, thereby conferring multi-resistant phenotypes.3 The proposed mechanisms include the intrinsic resistance of the organisms, a low permeability of the outer membrane to administered antibiotics, expression of chromosomal AmpC cephalosporinase; production of plasmid mediated b-lactamases against different molecular classes; diminished outer membrane permeability; enhanced expression of active efflux systems for wide variety of substrates; synthesis of aminoglycoside modifying enzymes (phosphoryltransferases, acetyltransferases and adenyllyltransferases); synthesis of an antibiotic-resistant biofilm (consisting of bacterial communities embedded in an exo-polysaccharide matrix) and structural alterations of topoisomerases II and IV preventing efficacy of fluoroquinolones.
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Table 1 shows culture sensitivity profile of Pseudomonas isolates against the tested antibiotics. (R: resistant, S: sensitive, PS: partially sensitive, Pol B: polymyxin B, Gat: gatifloxacin 0.3%, Mox: moxifloxacin 0.5%, Cip: ciprofloxacin 0.5%, Cefa: cefazolin, Cefur: cefuroxime, Tetra: tetracycline, Tob: tobramycin, Amika: amikacin, Genta: gentamycin).

*P. aeruginosa* is intrinsically susceptible to polymyxin B, an old class of cationic, cyclic polypeptide antibiotic. Positively charged polymyxins interact electrostatically with the negatively charged lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria and are subsequently taken up via the ‘self-promoted uptake’ pathway leading to cell death. Acquired resistance to polymyxins in MDR Gram-negative bacilli is not common currently, probably due to the infrequent usage of these agents over the last 50 years. In another study, isolates from bacterial keratitis were found to be most susceptible to chloramphenicol and fusidic acid; followed by
ciprofloxacin which favours a trend towards use of older antibiotics.\textsuperscript{10}

The limitations of our study include that the sensitivity profile of cultured isolates was not tested against newer antibiotics like carbapenems. The correlation of resistance patterns of Pseudomonas organisms with the clinical presentation and outcomes would be helpful in understanding the virulence of these poly antibiotic resistant strains.

In conclusion there is an emergence of multidrug resistance in Pseudomonas aeruginosa strains isolated from corneal scraping samples, to most of the currently available topical antibiotics including fourth generation fluoroquinolones. However, these have been found susceptible to polymyxin B and hence it may be included as a front line drug for treating Pseudomonas keratitis in North India.

REFERENCES


Healing Response of the Donor Cornea following Harvesting for Cultivated Limbal Stem Transplantation

Dr. Srilathaa Gunasekaran, Dr. Himi Singh, Dr. Shweta Sharma, Dr. Anil Kumar, Dr. Sujata Mohanty, Dr. Radhika Tandon

To evaluate the healing response of the donor cornea harvested for cultivated limbal stem cell transplantation (LSCT) in patients with unilateral limbal stem cell deficiency.

Stem cells research in ophthalmology has been in much focus in the last decade, particularly the limbal stem cells. These cells rest in the stem cell niche present in the palisades of Vogt. Loss of these cells disrupts the normal homeostatic milieu in the maintenance of healthy corneal epithelium which in turn gets replaced by conjunctival epithelium associated with superficial vascularisation, the clinical hallmark of limbal stem cell deficiency (LSCD). It is more often due to secondary causes than primary. Chemical injury, Steven Johnson syndrome, ocular cicatricial pemphigoid, vernal keratoconjunctivitis are some of the most common causes for LSCD. Congenital LSCD occurs as a result of hereditary aplasia of limbal stem cells as in aniridia.1

Limbal Stem Cell Transplantation (LSCT) is the established technique for ocular surface reconstruction in total LSCD.2 In unilateral cases, an autolimbal graft from the contralateral healthy eye is preferred. Ex vivo expansion of the harvested limbal graft on a substrate requires harvesting of only a small limbal biopsy and has become very popular wherever the laboratory facilities exist.3 The wide acceptance of this technique is due to the better safety profile to the donor cornea. In the past, Miri et. al.4 have described the donor site complications in 50 donor sites of autolimbal and living related allolimbal transplantation where 2 clock hours of tissue was biopsied from superior and inferior limbus. They observed that it was a safe procedure with donor eyes demonstrating stable vision and an intact corneal epithelium during mean follow up period of 41 ± 38 months. Basu et. al.5 have reported from their series of 50 eyes that repeat autologous cultivated limbal epithelial transplantation successfully restores ocular surface without adversely affecting donor eyes. There are no specific reports of healing response of donor eye following primary harvesting and hence we purposed to do this study.

MATERIALS AND METHODS

All cases of unilateral LSCD who underwent autologous cultivated LSCT from January 2006 to December 2011 with atleast one year of completed follow up were included in the study. Independent review of serial photographs of the donor cornea was done at the end of one year of harvesting. The parameters
evaluated were 1) continuity of palisades of vöggt 2) presence of any corneal scar 3) persistent epithelial defect (PED) 4) superficial neovascularisation.

**RESULTS**

32 patients who had completed one year of follow up were included in this study. 19 were males and 13 females. The most common indication for autologous cultivated LSCT in these patients was alkali injury. Complete healing of donor site in terms of restoration of continuity of palisades of vöggt in the absence of corneal scar/PED/neovascularisation was seen in 25 patients (78.1%) at the end of one year of follow up. Partial healing in terms of discontinuous palisades of vöggt and corneal scar was noted in 7 (21.9%) patients. The mean extent of the discontinuity of palisades of vöggt was 1.5±0.5 clock hours (max: 3) and the mean extent of corneal scar was 1.5±0.5 clock hours (max: 3). No patients had signs of focal limbal stem cell deficiency manifested by PED and/or superficial neovascularisation of the cornea. (Figure 1).

In conclusion the donor site following ex vivo cultured LSCT shows a complete healing response in 78% of the patients at the end of one year. No patients had any signs of focal limbal stem cell deficiency suggesting that harvesting from the healthy contralateral cornea in patients with unilateral LSCD is a very safe procedure.

**REFERENCES**

Irregular astigmatism doesn’t have gradual changes in refraction between meridians. It is characterized by an irregular change of refractive power in different meridia. There are multiple meridia which admit no geometrical analysis. Because of the inability of the eye to properly refract light, host of problems are faced. There are a variety of conditions that we see in our daily practice which amount to widely ranging amounts of irregular astigmatism. Keratoconus, pellucid marginal degeneration, post keratorefractive surgery, post corneal transplantation being the most common. The irregular astigmatism created by these conditions of the cornea is not sufficiently corrected by spectacles. The application of rigid contact lenses is required to obtain acceptable vision in all but the mildest cases of the disease.\(^1\)\(^,\)\(^2\) Today, most of the contact lens designs for these conditions are rigid gas-permeable contact lenses. A recent trend in rigid lens design for keratoconus is the development of proprietary rigid gas-permeable contact lens designs. The “Rose K Lens for Keratoconus” is a proprietary design that has gained popularity since its introduction in the United States in 1995. There are a number of key design features in the Rose K2 system. These include: an extensive range of back optic zone radii (BOZR - 5.1-7.6mm), smaller back optic zone diameters (BOZD controlled) as the radius steepens, simplified edge lift control to facilitate ordering and significantly increased lens power as the radius decreases when fitting advanced cases. These unique design features guarantee a high success rate when fitting the potentially difficult keratoconic patient. The Rose K2 set includes:

1. Rose K2 set – for most keratoconic cones
2. Rose K2 NC – for small nipple cones
3. Rose K2 IC – for irregular cornea like PMD
4. Rose K2 post graft – for corneas post corneal transplant surgery

This study represents a retrospective review of patients assembled to evaluate the clinical performance of the Rose K2 Contact Lenses. The purpose of this study is to determine the safety and efficacy of Rose K2 contact lenses for optical management of irregular corneas.

**MATERIALS AND METHODS**

A retrospective non randomised review was performed on the medical records of patients with irregular corneas who underwent Rose K2 lens trial at Shroff Eye Hospital, Mumbai. 52 eyes of 36 patients who were successfully fitted and
dispensed with Rose K2 lenses with a minimum follow up of 3 months were included in the study.

**Clinical Evaluation**

The following parameters were noted prior to contact lens fitting in all patients: demographics including age at time of fitting, refraction with the best high contrast spectacle-corrected visual acuity (BSCVA) and ocular diagnoses. Corneal topography (Oculyzer, WaveLight) using Scheimpflug imaging was performed on all patients. All contact lens fitting parameters were noted for all patients. The best high contrast contact lens-corrected visual acuity (BCLCVA) was recorded at the time of dispensing. Slit lamp complications were recorded on every follow up and the average wearing time (which was calculated as the mean time for which lenses had been worn over the past three days) was assessed in the latest follow up.

**Rose K2 Contact Lens Fitting**

A diagnostic CL-fitting method was used to finalize the CL parameters. The fitting procedure was determined by the Rose K fitting guide. The BC of the initial trial lens was selected as 0.2 mm steeper than the mean keratometry value. After giving an adaptation period of 30 min, the dynamic and static fit was assessed. In dynamic fit assessment, the lens fit was considered to be acceptable when the lens was centered adequately on the cornea with good postblink movement, with good stability on different gaze movements, and provide comfortable wearing period. In static fit, the goal was to achieve a “light feather touch” in the centre with mid peripheral bearing and peripheral clearance. The trial was repeated until we achieved an acceptable dynamic and static fit. After finding the optimal lens fit, the final power was calculated after performing a spherical over refraction over the trial lens.

**Outcome Measures**

**Primary**
- Visual acuity – Best contact lens corrected visual acuity

**Secondary**
- Average contact lens daily wearing times
- Complications

Statistical analysis was done by the paired ‘t’ test using the SPSS software. An alpha level of p<0.05 was considered significant.

**RESULTS**

**Demographic Data**

Fifty two eyes of 36 patients were included in this retrospective review. The mean age of the patients was 28.17 +/-9.18 (range: 14–48) years, and 28 were male
patients. Ocular diagnoses of the Rose K CL fitted eyes included – Keratoconus (34 eyes), Pellucid marginal corneal degeneration (4 eyes), Corneal tear repair (2 eyes), post radial keratotomy (2 eyes) and post corneal transplantation (10 eyes). Topometric keratometry values ranged from 27.9D to 78.5 D (mean = 52.304 D +/- 11.133). Astigmatism ranged from 2D to 15.9D (mean 6.212D +/- 3.773).

Range of CL Fitting Parameters
The mean base curve of the final Rose K2 lens was 6.64 +/- 1.11 (range: 4.8 – 8.7), diameter was 9.53 +/- 1.04 (range: 8.2 – 11.4) and power was -9.5 +/- 10.3 D (range: -29.5 to +14.0 D). Good centration, movement and comfort were achieved in all eyes. The contact lens sets used were classified as – K2 (22 eyes), Nipple cone (6 eyes), Irregular cornea (8 eyes) and Post graft (16 eyes).

Visual Acuity
- Mean prefitting BSCVA was 0.505 +/- 0.391 (range 6/6 to CF)
- Mean BCLCV A was 0.079 +/- 0.17 (range 6/5 to 6/36)
- Extremely statistically significant difference between BSCVA and BCLCV A (p < 0.0001) was noted

Average daily wearing time was between 6-8 hours.

No significant biomicroscopic complications were observed.

**DISCUSSION**

For many practitioners, fitting a patient with irregular astigmatism is a time consuming and frustrating undertaking. The irregular corneal topography, a wide range of severity and a variable rate of progression present the practitioner with unique fitting requirements in each case. A poorly fitting lens can cause considerable damage to the cornea and may result in significant scarring and loss of visual acuity. An optimum fitting minimises the risk of lens related tissue damage. Successful RGP lens fitting for such corneae requires an extensive range of trial lenses that are suited to a wide variety of keratometry values and altered corneal topographies. A golden rule for such eyes is that the best fitting trial lens must be as close as possible in design and power to the ordered lens. The Rose K2 system is designed to achieve a high percentage of first time successful fittings at any stage in the progression of the disease. Our review is one of the first reports highlighting the results of fitting Rose K2 contact lenses.

52 eyes of 36 patients who underwent Rose K2 contactlens fitting were retrospectively analysed. A wide range of ocular pathologies inducing irregular astigmatism were successfully visually rehabilitated with the use
of the Rose K2 set of contact lenses. Special sets for keratoconus, nipple cones, irregular corneas and post graft eyes cover the whole gamut of ocular disorders causing irregular astigmatism. Also, based on topography analysis, choosing of the contact lens set for a particular patient becomes very easy. Extremely flat as well as steep corneas (keratometry range: 27.9D to 78.5 D) were satisfactorily fitted with these contact lenses. Varying amounts of irregularity were covered in our patients (astigmatism range: 2D to 15.9D).

Range of contact lens fitting parameters were as follows - base curve 4.8 to 8.7 (mean = 6.64 +/- 1.11), diameter 8.2 to 11.4 (mean = 9.53 +/- 1.04) and power -29.5 to +14.0 (mean = -9.5 +/- 10.3). This shows the wide range of contact lenses available for fitting in the Rose K2 set. This enables the practitioner to fit a trial lens which is as close as possible to the final lens. Inspite of such a large range of fitting parameters, good centration, movement and comfort were achieved in 100% eyes.

The vision was improved from 0.505 +/- 0.391 (mean prefitting best spectacle-corrected LogMAR visual acuity) to 0.079 +/- 0.17 with the final Rose K lens in LogMAR visual acuity (paired t-test, P < 0.0001). Extremely statistically significant change in visual acuity was achieved.

Excellent quality of vision was attained in all patients. Disabling visual phenomenon like glare, haloes, star bursts and reduced contrast visual acuity associated with irregular astigmatism were eliminated or significantly reduced in all patients.

Also we are able to achieve acceptable wearing times with no complications of contact lens wear.

Our study is probably the first reported series of fitting Rose K contact lenses for a wide variety of corneal conditions (other than keratoconus) with an extremely high success rate, good visual acuities, acceptable wearing times and no biomicroscopic complications related to contact lens wear. However, studies with a larger sample size and longer follow up would substantiate these results further. Comparative studies with other proprietary contact lenses would also help reduce the confusion in the mind of the practitioner fitting the lenses.

In conclusion fitting of specialty contact lenses for irregular corneas is quite challenging. Rose K2 family of CL’s are successful in visually rehabilitating a wide variety of irregular corneas.
Femtosecond Laser-Assisted Sutureless Anterior Lamellar Keratoplasty (FALK) for Anterior Stromal Corneal Pathologies - Long Term Results

Dr. Mukesh Taneja, Dr. Jayesh Vazirani, Dr. Varsha Rathi, Dr. Pravin Vaddavalli, Dr. Somasheila Murthy

Anterior stromal corneal pathologies include corneal degenerations, corneal dystrophies and corneal scars, amongst others. Prominent modalities for management of these conditions include phototherapeutic keratectomy (PTK) as well as anterior lamellar keratoplasty (ALK).

Limitations of PTK include post operative shifts in refraction, which can be unpredictable in both degree and direction, residual haze, as well as recurrence of the original condition.

Another approach to treatment of anterior corneal pathologies is ALK, which involves targeted lamellar replacement of corneal tissue, while retaining normal cornea. Advantages of this technique over penetrating keratoplasty include superior wound strength, faster visual rehabilitation as well as reduced intra and postoperative complications (graft rejection).

Types of ALK include manual ALK, microkeratome assisted ALK and Femtosecond laser assisted ALK (FALK). Compared to earlier techniques, FALK offers advantages such as a highly reproducible fit between donor and recipient lenticules, precise pre-programmed corneal dissections at a variety of depths and orientations as well as the possibility of sutureless surgery.

Outcomes of suture-less FALK for anterior stromal corneal pathologies have been reported. The successful outcome in these cases indicates that FALK is a promising technique for dealing with anterior corneal pathologies.

MATERIALS AND METHODS

Eleven eyes of 11 patients with varying pathologies in the anterior 250 microns of corneal stroma underwent FALK procedure using femtosecond laser (VisuMax, Carl Zeiss Meditec Inc., Germany). Parameters noted included indication for surgery, logMAR visual acuity, pachymetry, duration of follow up, refraction at last follow up as well as complications were noted.

FALK surgical procedure: Surgical technique:

All procedures were performed under strict asepsis in LASIK OR.

This was done in two steps. First, a donor button was fashioned and then the recipient bed.
Procedure of Donor graft preparation
Corneo scleral donor rim was mounted on the Moria artificial chamber. Pressure was built up inside. Care was taken that epithelium remained intact when the free cap was created with Visumax 500 kHz femtosecond laser. Preoperative OCT was performed in all cases to see the level of the opacities in the cornea. The thickness of donor lenticule was based on preoperative level of the opacity and the thickness of the cornea.

In all the cases the graft thickness was taken 10–20% more than the recipient because of preexisting oedema in the eyebank eyes.

Preparation of the recipient bed
All procedures were performed under strict asepsis. The lids were separated with wire speculum. Patient was asked to look at the green fixation light in the laser machine. The suction was built up and then the laser was fired. The recipients lenticule was removed with the flap lifter. All these surgeries were done using Visumax femtosecond laser (Carl Zeiss) with a pre-planned donor.

Table 1: Surgical Indications

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>f</td>
<td>os</td>
<td>Reis Buckler dystrophy recurrence in graft</td>
</tr>
<tr>
<td>73</td>
<td>m</td>
<td>od</td>
<td>Reis Buckler dystrophy recurrence in graft</td>
</tr>
<tr>
<td>37</td>
<td>m</td>
<td>os</td>
<td>Reis Buckler dystrophy recurrence in graft</td>
</tr>
<tr>
<td>35</td>
<td>m</td>
<td>od</td>
<td>Anterior corneal scar</td>
</tr>
<tr>
<td>61</td>
<td>m</td>
<td>os</td>
<td>Spheroidal degeneration</td>
</tr>
<tr>
<td>54</td>
<td>f</td>
<td>od</td>
<td>Granular dystrophy</td>
</tr>
<tr>
<td>62</td>
<td>m</td>
<td>os</td>
<td>Granular dystrophy</td>
</tr>
<tr>
<td>65</td>
<td>f</td>
<td>os</td>
<td>Spheroidal degeneration</td>
</tr>
<tr>
<td>42</td>
<td>m</td>
<td>od</td>
<td>Anterior corneal scar</td>
</tr>
<tr>
<td>73</td>
<td>f</td>
<td>od</td>
<td>Granular dystrophy recurrence</td>
</tr>
<tr>
<td>34</td>
<td>m</td>
<td>os</td>
<td>Corneal scar, intrastromal foreign body</td>
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</tbody>
</table>

Table 2: Visual Acuity

<table>
<thead>
<tr>
<th></th>
<th>Pre-operative</th>
<th>Last follow-up</th>
<th>p</th>
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<tbody>
<tr>
<td>Mean UCVA</td>
<td>1.92</td>
<td>0.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Mean BCVA</td>
<td>1.51</td>
<td>0.57</td>
<td>0.05</td>
</tr>
<tr>
<td>Spherical equivalent</td>
<td>-0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cylinder</td>
<td>-1.96</td>
<td></td>
<td></td>
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</table>
lenticule thickness and diameter, Spiral-out fashion with 2.5 microns spot distance and track distance for the bed and 2.5 microns spot distance and track distance for side cut with an angle of 90°.

Thereafter the donor lenticule was placed in prepared host bed and ironed to remove any folds and kept preseed for two minutes and then a bandage lens was placed over the lamellar graft. No sutures were placed.

RESULTS
Indications for FALK included granular corneal dystrophy (two eyes), Reisbuckler’s dystrophy (three eyes), spheroidal degeneration (two eyes) and anterior stromal corneal scar with intrastromal glass foreign body (one eye), corneal scars (three eyes) (Table 1) Anterior lamellar donor corneal tissue was successfully integrated into the host bed without need for sutures in all 11 eyes. Mean duration of follow up was 11.2 months (range: 8 - 20 months). Uncorrected logMAR visual acuity improved significantly from a mean of 1.92 (range: 0.9 to 3) to 0.9 (range: 0.3 to 1.6) following FALK (p<0.05) (table 2). No significant intraoperative or postoperative complications were noted in any of the cases other than partial displacement of corneal lenticle in one case which was subsequently replaced successfully.

Complications
We did not see any major complications in our series of eleven cases. One patient had an irregular side cut on the donor lenticule and the lenticule had to be prepared again. One patient had partially displaced lenticule on first post-op day, however this could be easily centered. In few patients centration with the suction cup was found to be difficult due to poor view of fixation light to the patients because of anterior corneal pathologies.

FALK appears to be a safe and effective procedure for anterior stromal corneal pathologies, providing excellent improvement in visual acuity without the need for sutures or tissue ablation.

REFERENCES
Comparison of Corneal Topography in Keratoconus Patients Treated by Standard (CXL) Versus Accelerated (Avedro KXL) Corneal Collagen Cross Linking

Dr. Supriya Bhagat, Dr. Mala B, Sathish Prabhu, Dr. Deepthi Bhat, Dr. Sri Ganesh

Keratoconus is considered a slowly progressive, non-inflammatory corneal dystrophy characterized by changes in corneal collagen structure and organization. Decreased mechanical corneal stability plays an important role in the progressive protrusion of the keratoconic cornea, resulting in mild to marked impairment of visual acuity owing to irregular astigmatism, progressive myopia, corneal thinning, and central corneal scarring. Corneal collagen cross-linking using riboflavin and UV-A irradiation has become a norm to arrest the progression of keratoconus. The basic principle of corneal collagen cross linking is the induction of cross-links in the corneal stroma, producing a stiffening effect and increasing corneal strength and stability. The standard corneal collagen cross-linking (CXL) involves 30 minutes exposure to UV-A. Newer accelerated corneal collagen cross-linking (KXL) using the Avedro’s KXL system reduces this exposure time to 3 minutes.

The aim of this prospective study was to compare changes in corneal topography in patients with progressive keratoconus treated with CXL versus KXL.

MATERIALS AND METHODS

This prospective comparative study done between Feb 2012 to June 2012 included 30 eyes of 20 patients with progressive early to moderate keratoconus (grade I to III according to the Amsler-Krumeich classification) who were classified into two groups according to the treatment they underwent: standard CXL group 1 (15 eyes, 11 patients-4 Female and 7 Male) with a mean age of 25.82±4.39 years and accelerated KXL group 2 (15 eyes, 9 patients-4 Female and 5 Male) with a mean age 23.47±4.47 years. The two groups were matched for age, preoperative uncorrected distance visual acuity (UDVA) and best spectacle corrected distance visual acuity (BSDVA), refractive error (cylinder, and spherical equivalent), steep topography keratometry (K) value, flat K value, and average K value by independent sample t test for equality of means. All operations were performed by one surgeon.

Inclusion criteria were a documented keratoconus progression in the previous 6 months, corneal thickness of >400 μm at the thinnest point, and being aged between 18–40 years. Preoperative keratoconus progression was confirmed by serial differential corneal topographies in all eyes included in the study.
Exclusion criteria included corneal thickness <400 μm at the thinnest point, a history of herpetic keratitis, concurrent corneal infections, concomitant autoimmune diseases, and any previous ocular surgery. Also excluded were pregnant or nursing women, patients with central or paracentral opacities, patients with poor compliance and patients wearing rigid gas permeable lenses for at least four weeks before baseline examination.

At baseline and at 3 months all patients underwent UDVA and BSDVA assessment; slit-lamp biomicroscopy; corneal topography by pentacam.

\textbf{C³R Procedures}

- The treatment is carried out in sterile conditions, preferably in the operating theatre. After topical anaesthesia, the epithelium of the central 8-9 mm of cornea is removed.

- Standard CXL: The surface is then treated by the application of riboflavin (vitamin B2) 0.1% solution (10 mg riboflavin-5-phosphate in 10 ml dextran 20% solution) every 3 minutes for 30 minutes. Cornea is exposed to a UV source emanating from a solid-state device (UV-X System, Switzerland), which emits light at a wavelength of 370 nm and an irradiance of 3 mW/cm² or 5.4 J/cm². Exposure lasted for 30 minutes, during which time riboflavin solution was again applied, this time once every 10 minutes.

- Accelerated KXL: Riboflavin dye 0.1% - every 2 minute for 10 minutes. Cornea is exposed to a UV source emanating from Avedro’s KXL system which emits light at a wavelength of 365 nm and an irradiance of 30 mW/cm² or 5.4 J/cm². Exposure lasted for 3 minutes, during which time riboflavin solution is applied once before starting the radiations.

- Antibiotic drops are instilled as prophylaxis and a bandage contact lens is inserted, which is then removed at the follow-up visit once epithelial healing is complete.

\textbf{RESULTS}

\textbf{Visual acuity}

No significant differences in changes in UDVA and BSDVA between the two procedures.

\textbf{Refractive}

No significant differences in changes in Cylinder and Spherical Equivalent between the two procedures.

\textbf{Corneal Topography}

No significant differences in changes in K1, K2 and mean K between the two procedures. Reduction in thinnest Pachymetry was significantly different (statistically \( P < 0.05 \)) – lesser in KXL versus CXL.
DISCUSSION

There were no significant differences found in the two groups in terms of improvement in UDVA and BSDVA, change in manifest refraction spherical equivalent and cylinder and keratometric values. Similar results were found by Minoru Tomita. However, we found a significant difference in reduction in thinnest pachymetry in the two groups, reduction was more in standard CXL (5%) compared to KXL(2.8%). One of the pathologic characters of keratoconus is the progressive corneal thinning, thus we think the corneal thickness may be an important efficacy parameters of stabilization of the keratoconus. In our study, we found a decrease in thinnest pachymetry in the initial 3 months in both the groups which is comparable with the study done by Doors et. al and Vinciguerra and associates. A study by Seiler and Hafezi implied that the refractive index of the cornea changed, especially at the site of the demarcation line, because of structure modifications after corneal crosslinking. So it is partly refractive index and partly other factors that introduce these pachymetry measurement differences. Another reason of corneal thinning is associated with the keratocytes. According to Gang Li et. al at the initial stage, there are many keratocytes apoptosis which may reduce the synthesizing of cornea collagen. During the follow-up months, there are more and more activated keratocytes and there are also more new collagen synthesized. Transmission and scanning electron microscopy show clearly that the collagen fiber is thicker by 13% in the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Standard C.R.</th>
<th>Accelerated C.R.</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-op 3 months</td>
<td>Difference</td>
<td></td>
</tr>
<tr>
<td>UDV A (logMAR)</td>
<td>0.84±0.20</td>
<td>0.76±0.19</td>
<td>0.06</td>
</tr>
<tr>
<td>BSDVA (logMAR)</td>
<td>0.27±0.12</td>
<td>0.22±0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td>-2.83±0.91</td>
<td>-2.65±0.72</td>
<td>0.18</td>
</tr>
<tr>
<td>Spherical Equivalent (D)</td>
<td>-3.33±1.78</td>
<td>-3.21±1.76</td>
<td>0.42</td>
</tr>
<tr>
<td>K1</td>
<td>45.8±2.1</td>
<td>45.4±2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>K2</td>
<td>49.2±2.4</td>
<td>48.6±2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Mean K</td>
<td>47.5±2.1</td>
<td>47.0±1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Thinnest Pachymetry</td>
<td>476.4±23.3</td>
<td>474.8±22.3</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Corvis ST or Ocular Response Analyzer: Which is better to Evaluate Corneal Biomechanics?

Dr. Gurjeet Maggo, Dr. Rohit Shetty, Dr. Himanshu Matalia, Dr. Sharon Dsouza, Dr. Harsha Nagaraja

Corneal biomechanics plays an important role in screening of refractive surgery patients and diagnosis of patients with potential risk of ectasia. Ocular Response Analyzer (ORA, Reichert) was the 1st instrument to evaluate corneal biomechanical properties in vivo (Figure 1).

Newer Corneal Visualization with Scheimpflug Technology” (CorVis ST, Oculus Inc) (Figure 2,3) provides real time imaging of the corneal deformation based on ultra-high speed Scheimpflug imaging, 4330 frame/sec. Both the machines based on similar principle of air puff applanation /distortion of the cornea but both the machine provide different variable,which are directly not comparable.

To study the correlation between two different methods of studying corneal biomechanics in normal and keratoconic eyes.

MATERIALS AND METHODS

Study design- It was a prospective non-randomized observational case series. We studied normal eyes and eyes with keratoconic eyes using ORA and CorVis ST. Both the machines were studied for biomechanics. Parameters Studied in ORA were Corneal hysteresis (CH), Corneal Resistant Factor (CRF), Corneal crosslinked eye which explains it eventually returns to its original thickness according to Gang Li et. al.

Also Prof. John Marshall found that endothelial cells showed similar or better cell viability when treated at higher irradiance for shorter time (3 min) compared to the cells treated at lower irradiance for a longer time (30 min). Keratocytes showed similarly good cell viability when treated at higher or lower irradiance which can be attributed in our study as the cause for the lesser reduction in thinnest pachymetry in KXL.

Accelerated corneal collagen cross-linking (KXL) is as effective as standard corneal collagen cross-linking (CXL) in stabilizing the corneal topographic changes in progressive keratoconus but KXL causes lesser reduction in the thinnest pachymetry as compared to CXL.
compensated intraocular pressure (IOPcc), Gold Standard calibrated intraocular pressure (IOPg). In CorVis ST parameters like Deformation amplitude (Def amp), CorVis ST IOP were studied. After confirming the normal distribution of data, using Pearman’s correlation we tried to compare both these machines.

RESULTS
We studied 62 normal and 56 keratoconic eyes. In both keratoconic and normal eyes, CorVis ST (Def amp) showed no correlation with ORA (CH) and ORA (CRF). Comparison of Def amp in normal (1.041) and keratoconus (1.146) showed statistically significant difference (p<0.0001). CH between normal(9.66) and keratoconus (8.75) showed statistically significant difference (p=0.0098). CRF between normal (9.5) and keratoconus (8.33) showed statistically significant difference (p=0.0020). CorVis IOP and Def amp showed fair negative correlation (R2=0.53). Keratoconic eye did not show any correlation between CorVis ST-IOP and ORA(IOPcc and IOPg). Normal eyes showed poor correlation between CorVis ST IOP and ORA IOPcc (r=-0.25) and fair correlation between CorVis ST IOP and ORA IOPg (r=0.51).

DISCUSSION
This study was carried out to study the correlation between two different methods of studying corneal biomechanics in normal and keratoconic eyes. We found that both of them cannot be used interchangeably for various measurements of corneal biomechanics and intraocular pressure as no good correlation has been found.
CorVis ST and ORA do not any show good correlation in terms of measurement of corneal biomechanics and intraocular pressure. Hence, these machines cannot be used interchangeably for various measurements of corneal biomechanics and intraocular pressure. Follow-up on the same machine is mandatory.

**Intraoperative Applicability of Hand held high Resolution Spectral Domain OCT in Various Refractive Procedures**

**Dr. Gurjeet Maggo, Dr. Rohit Shetty, Dr. Sharon Dsouza, Dr. Kareeshma Wadia**

Bioptigen is an anterior and posterior segment OCT. Depth independent axial resolution of 3-4.5 µ. Hand held probe-intraoperative measurements are possible.

**Study design:** Prospective, interventional, comparative, case series.

**MATERIALS AND METHODS**

High resolution Spectral domain OCT (Bioptigen) was used to study three refractive procedures. Procedure 1: Group 1-Femtosecond laser (FS 200 Wavelight) with intended thickness of 100 micron thick flaps and Group 2-Microkeratome with intended thickness 120 microns was measured in 50 eyes of 25 patients. Imaging was done preoperatively and then intraoperatively by measuring flap thickness at centre and near periphery prior to lifting flap for laser ablation and postoperative day 1. Procedure 2: 20 eyes (divided in 2 groups) undergoing collagen crosslinking of cornea imaged intraoperatively using Bioptigen. Inclusion criteria: Patients with progressive keratoconus, minimal thinnest pachymetry ≥ 400µ on pentacam. Exclusion criteria: advanced keratoconus, apical scarring. Study population was divided into two groups: In Group 1(10 eyes) epithelium was removed completely in central 8 mm zone and in Group 2(10 eyes) epithelium was removed in a grid pattern. Depth of maximal penetration of riboflavin (seen as band of increased reflectivity measured) imaging was done. Imaging was done -prior to scraping the epithelium, then after 30 minutes of riboflavin 0.1% instillation and after 30 minutes of exposure to UVA light. Depth of hyper reflective band seen in anterior corneal stroma, measured perpendicular to anterior corneal surface by two independent observers Procedure 3: Depth predictability of Intacs
(Addition Technology, Inc.) placed at preplanned depth in tracts created by FS laser was measured in 20 eyes. Using bioptigen, the shortest distance from the epithelium to the segment was measured at proximal and distal to the incision.

**RESULTS**

In FS flap procedure mean central flap thickness was 111 ± 2 microns while at the periphery it was mean 113 ±5 microns. In microkeratome flap procedure mean central flap thickness was 127 ± 10 microns while at the periphery it was mean 130± 25 microns (Figure 2).

The average difference in flap thickness is about 2 microns in the center and about 5 microns in the periphery with very low standard deviation for femto flaps whether thin 90 micron flaps or regular thickness flaps. However the microkeratome flaps tend to be about 10 microns thicker in the center and nearly 25 microns thicker in the periphery with a wide variation in the standard deviation indicative of the unpredictability of the flap thickness

In collagen crosslinking Group 1, a homogenous hyper reflective band seen (mean depth –MD  53 microns) which increased in thickness (MD 73 microns) after exposure to UVA light. In Group 2 greater depth of band was seen in ‘epi off’ areas(MD 78 microns) while it was minimal under areas of intact epithelium (MD 24 microns).

The mean pre planned depth of the 20 segments was 376±6 µm. The mean depth measured of the placed rings was 358±29 µm, no significant difference was found (p=.07). Distal and proximal point measurements of all INTACS were 354±27 and 363±30 µm respectively. No part of the segments tended to be more superficial than others (p=0.98).
DISCUSSION

Femtosecond laser shows more predictability and very homogeneous flap thickness as well as even stromal bed in comparison to microkeratome seen on intraoperative measurement. Our results confirms earlier studies performed with regional subtraction pachymetry and histology. Corneal epithelium blocks adequate penetration of riboflavin into the stroma as directly imaged intraoperatively. This may lead to a nonhomogenous crosslinking effect. Our suggestion is thus to completely remove the epithelium for an adequate and homogenous effect. We found out that there was no statistically difference between the depth of placed INTACS ring and the preplanned depth establishing the precision of channel depth made by femtosecond laser and method of measurement by Bioptigen. However some studies reported shallow placement of rings then expected.

Bioptigen can prove as a useful intraoperative tool to confirm the depth of treatment in various refractive procedures.

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2. Comparison of the depth predictability of intra corneal ring segment implantation by mechanical versus femtosecond laser-assisted techniques using optical coherence tomography. Optical Coherence. Kouassi FX, Buestel C, Raman B.

Dynamics of Stem Cell Replication in Limbal Epithelium ‘in niche’ During Extended Explant Culture

Dr. J Mario Wolosin, Dr. Ozlem Barut Selver

Limbal epithelium and/or limbal zone loss, due to disease, trauma, or congenital deficiencies, results in visual decrease or vision loss. Reintroduction of epithelial cells derived from a contralateral eye pre-expanded in vitro can reestablish a fully functional lineage, provided the engrafted cell population incorporates cells retaining the SC/progenitor phenotype. One indicator of the stem/progenitor cell phenotype is the expression of the multidrug resistance protein ABCG2/BCRP1, a xenobiotic transporter expressed at high levels within subpopulations of adult stem cells. Cells with the highest levels of functional ABCG2 exclude the dyes. This feature has allowed the isolation of viable stem and progenitor cells.
by flow cytometry from multiple lineages and organs, including cells from the limbal and conjunctival epithelia. One common approach to generate therapeutic epithelial cell populations for ocular surface reconstruction is based on the explant method, where cells outgrow into a suitable biological or synthetic substratum from a small tissue segment. It is not clear what the contribution of bonafide SCs to the outgrowth is, vis-à-vis the contribution of other proliferative limbal epithelial cells. In particular, the unique niche localization of SCs also raises questions about the permanence or survival of the SC phenotype within the explant proper, which may or may not be transferred with the expanded cell populations used for corneal regeneration. To start addressing these questions we have now investigated the ability of limbal explants to generate outgrowths over prolonged time periods. In these studies we have replaced Hoechst 33342, a DNA binding dye that displays substantial toxicity, with JC1, a new ABCG2 substratum that binds to mitochondria instead of nuclei and shows minimal toxicity.

MATERIALS AND METHODS
Donor human corneas from unidentifiable cadavers were obtained through the National Disease Research Interchange (Philadelphia, PA, USA). Rabbit corneas were excised from the eyes of 2–2.5 kg rabbit cadavers. Limbal strips (0.5 mm x 2 mm) were deposited, epithelial side up, on six-well plate 0.4-μm-pore polyethylene terephthalate (PET) membrane inserts. After 72 hours of culture at the air–liquid interface, after confirming the presence of an encircling island of outgrowing cells, and every 48 to 72 hours thereafter, the culture solution refreshed. Human cells were cultured in supplemented human epithelial medium (SHEM) containing (per liter) 950 mL D/F-12; 50 mL FBS complemented with 5 μg each of insulin, transferrin, and selenium; 10 ng cholera toxin; 5 ng epidermal growth factor; and 28 mg phosphoethanolamine. Rabbit cells were cultured in 84% D/F-12 + 16% FBS. For continued cell production from the explant, after 11 to 14 days for human or 8 to 10 days for rabbit (at which point outgrowths were 3–4 cm wide but had not yet reached the edges of the insert), were incubated overnight in calcium-free D/F-12 + 3% FBS to loosen cell–cell attachments. The were cut away, and repositioned, epithelial-side-up in a new insert, to extend the explant culture beyond the initial round for additional 9-12
days. Fresh tissue (FT) cells were obtained by direct trypsinization of the Dispase released epithelium Figure 1),

FT and explant outgrowth cells were trypsin harvested and replated for overnight culture. After overnight incubation, the medium was complemented with 0.25 μg/mL JC1 (Axxora, San Diego, CA) for 45 minutes. The attached cells were washed twice with PBS, released by a 2- to 3-minute trypsinization, spun down, resuspended in ice-cold Hanks’ balanced salt solution + 4% FBS + 1 μg/mL propidium iodide (PI; Invitrogen) solution and subjected to flow cytometry for analysis of JC1 exclusion and/or sorting. Fluorescent emissions from the 488-nm laser were collected at 531 nm (green) and 572 nm (red) for JC1 and at 610 nm for PI. Aggregated (elevated forward scatter width parameter) and dead (PIhigh) cells were excluded from the analysis. The ABC transporter responsible for the dye exclusion was identified through the effect of the ABCG2-specific inhibitors: FTC and Ko1-43, the ABCC1/MRP1 inhibitor MK57132 and the ABCB1/MDR1 inhibitor Ivermectin. The entire process of explant transfer and outgrown cell collection and overnight reculture was then sequentially repeated, thereby generating in total first second, third and four cycles of explant culture outgrowth. To correlate these results with in situ cell behavior corneas maintained in organ culture were
subjected to single or repeated limbus to limbus corneal debridements and JC1low content was assessed as described above.

**RESULTS**

JC1, an argon laser (488 nm)–excitable dye, accumulates in the inner mitochondrial membrane, where it displays a concentration- or aggregation-dependent bathochromic shift in emission. When limbal human or rabbit epithelial cells are stained with this dye at selected concentrations and incubation times and subjected to analysis by flow cytometry, green versus red (531 vs. 572 nm) emission plots include a cell small cohort displaying reduced staining (JC1low) and a higher green/red ratio than that of the general cell population (Figure 2 A and C). The size of JC1low is markedly reduced by two different ABCG2 inhibitors, Ko1-43 (Figure 2B), and FTC (Figure 2D), demonstrating that the phenotype reflects the pumping activity of the stem cell–identifying ABCG2 transporter (*i.e.*, JC1 low is equivalent to ABCG2+). Neither MK571 nor Ivermectin affected JC1low (not shown).

Representative images of JC1 staining images for multiple cycles of outgrowths from individual biopsies are shown in Figure 3 A and B. JC1 low percentiles in the first cycle of explant outgrowth in older adult specimen (Figure 3A) tended to be only marginally larger than that previously reported for freshly harvested (*i.e.*, not cultured) basal limbal epithelial cells. The second cycle of explant resulted in some increase in the percentile but by the third cycle, corresponding to about 35 days of continuous proliferation within the source explant, the increase was dramatic. This time dependent increase in ABCG2+ cells occurred more rapidly and was more pronounced in specimens from younger human donors or in the 3-month old rabbit (Figure 3B). Using 6 explants from a single donor at the end of the second cycle of outgrowth we also examined the ratio of JC1low content between the cells over the explant biopsy and those in the outgrowth zone (Figure 3C). The population recovered from the explant zone contained a statistically higher percentile of JC1 low cells (Figure 3C). Figure 3D provides average JC1 low percentiles for cell obtained from fresh tissue or in the outgrowth zone of the first and second cycle of explant culture.

The organ culture experiments, as schematically depicted in Figure 4, represent equivalent experiments to the explant experiments, except that a) the outgrowth occurs over the corneal surface/Bowman membrane and b) unlike the open ended continuous rapid growth in the explant condition, once the corneal surface fully repopulates and re-stratify, rapid proliferation is replaced by normal slow growth at steady state. In these conditions, undebrided corneas (not shown) or corneas debrided once on day 0 and let rest in organ culture for 30 days showed very little JC1 low (Figure 4A). Corneas
scraped one or two extra times during the culture period showed a marked increase in JC1 low content. The content over the outgrowth zone (i.e., corneal zone) was very similar to the increase within the cell origination (limbal) zone. Overall our results show that; a) limbal explants can continuously give rise to outgrowths for at least 45 days; b) the JC1 low (= ABCG2+) phenotype is as much as 10-50 times more frequent in the limbal epithelium and its outgrowths than in the limbal cell population isolated from fresh tissue. Since earlier experiments showed that the clonogenic capacity of the expanded cell population resides nearly exclusively in the JC1 low cells we conclude that in ex-vivo, in situ culture, as the limited proliferative capacity non stem cells (also known as transient amplifying cells) are lost to terminal differentiation, the normally slow cycling or quiescent, limbal epithelial stem cells become activated to undergo enhanced conservative self renewal. As a result, over time, the basal cell layer of both the originating biopsy explant and its zone of outgrowth become over-populated by stem/precursor-like cells which may have a much higher regenerative capacity than the initial outgrowth, in particular when the donor tissue derive from an old adult. The organ culture results indicate that this is a reversible process. Once steady state is re-established, the bona fide niche stem cells return to their slow cycling phenotype and the low stem cell to transient amplifying cell ratio is reestablished.

REFERENCES


