Original Article

Diagnosis of limbal stem cell deficiency based on corneal epithelial thickness measured on anterior segment optical coherence tomography

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Purpose: The purpose of this study is to investigate the epithelial thickness in the cornea and limbus in limbal stem cell deficiency (LSCD) using anterior segment optical coherence tomography (AS-OCT). **Methods:** This was a cross-sectional, comparative study. OCT images of 30 eyes of 19 patients with LSCD collected by AS-OCT were scanned. Corneal epithelial thickness was recorded at the central cornea and the superior, nasal, inferior, and temporal limbus. Measurment of the same region of 30 normal eyes served as control. Epithelial thickness in all locations was measured by 2 independent observers. **Results:** The mean epithelial layer thickness was 61.3 ± 2.9 μ in the central cornea and 62.7 ± 4.3 μ in the limbus in the control. The epithelial thickness in LSCD patients was found to be 41.33 ± 2.8 μ. An average reduction of 22.2% in the central cornea and 32.15% in the limbus was found in patients with LSCD ($P < 0.05$). Epithelial thinning correlated with the severity of LSCD in both cornea and limbus. In eyes with sectoral LSCD, a similar degree of epithelial thinning was also detected in the clinically unaffected limbal regions. **Conclusion:** Both corneal and limbal epithelia become progressively thinner in LSCD. Epithelial thickness assessment using AS-OCT as a noninvasive tool could be used as a diagnostic measure of LSCD.

Key words: Anterior segment optical coherence tomography, corneal epithelium thickness, limbal stem cell deficiency

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The junction of the cornea with sclera, known as limbus, is anatomically very significant part of the eye as it helps to regenerate the corneal epithelium. Regular shedding of the surface epithelium and transparency is maintained by the limbal stem cells. Limbal stem cells (LSC's) reside in the palisades of Vogt (POV) and in the deepest limbal epithelial cell layer. The basal cells of the limbus may consist LSC's, trans amplifying cells, and niche cells.[1-4] Limbal stem cell deficiency (LSCD) occurs with the destruction of LSCs through either injury or congenital abnormality. Common causes of LSCD include chemical injuries, severe dry eyes, contact lens wear, Stevens–Johnson syndrome (SJS), multiple surgeries, and severe infectious keratitis.[5] The hallmark of clinical picture of LSCD is an invasion of conjunctival epithelium onto the cornea. Common symptoms and clinical findings include pain, superficial neovascularization, and recurrent or persistent epithelial defects.[6-8]

The diagnosis of LSCD is largely based on careful clinical examination using fluorescein staining to detect the abnormal conjunctival epithelium on the cornea. Stippling staining in a vortex pattern along with epithelial thinning, irregularity, and opacity are often present. Goblet cells are normally found in the conjunctival epithelium, and their presence on the cornea indicates invasion of the conjunctival epithelium onto the corneal surface. Detection of goblet cells on the cornea by impression cytology confirms the diagnosis.[9] However, goblet cell deficiency can be concurrent with LSCD and may lead to a false negative result. In addition, in the early stage of LSCD, goblet cells might not be present in the cornea. Use of conjunctival biomarkers is a recent development and still needs to be confirmed in larger clinical studies.[10-12] Pannus or corneal neovascularization is seen in LSCD, but their presence does not necessarily indicate LSCD. In addition, the degree of LSCD cannot be reliably evaluated by impression cytology because of sampling error. To date, no classification system for LSCD has been established, partly because of the lack of a specific diagnostic marker of LSCD, and the lack of a quantitative method to measure corneal epithelial cells on the corneal surface.

Accurate diagnosis of LSCD is important, as a corneal transplant will not survive in LSCD. Replenishment of the LSCs is the only appropriate treatment.[4,5,13,14] Impression cytology was considered to be the most sensitive investigation for the diagnosis of LSCD, but practically it is difficult to perform, special stains are needed, and special training is needed for the same. Laser scanning confocal microscopy (LSCM) has been used to analyze the corneal epithelium and associated structures in normal controls and patients with LSCD *in vivo* earlier.[15-23] Central corneal basal cell density and sub-basal nerve density are criteria that have been investigated as new indices to help in the diagnosis and potentially, to quantify

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the degree of LSCD. LSCM has various drawbacks as its lens touches the cornea, hence traumatic, expensive, and special training is required.[15]

OCT is a practicable and reliable instrument for noninvasive measuring corneal epithelial thickness (CET) and detects various abnormalities in corneal stroma *in vivo* in cases of LSCD.[11] Anterior segment-OCT (AS-OCT) imaging is versatile, nontraumatic, nontouch technique, easy to use, without any stain, and subsequent images may be compared easily.[16]

Methods

This was a screening and diagnostic prospective, cross-sectional study conducted after getting approval from ethical committee of the institute. All the patients attending the cornea clinic with the history of chemical burn, SJS, congenital coloboma, recurrent corneal epithelial defects, chronic CL bearer, and conjunctival encroachment on cornea were included in the study, and cases with comorbid condition were excluded from the study. The patients with acute chemical injury and associated stem cell deficiency were excluded from the study. The study group consisted of 30 eyes from 19 patients of 20–50 years of age of both sexes. The control group had 30 eyes of 15 age-match individuals selected from patients reporting for refractive error correction. After recording symptoms and history, each patient underwent a comprehensive eye examination and slit-lamp microscopy, fluorescein staining followed by AS-OCT scanning. LSCD was diagnosed clinically with specific complaints, history, and slit-lamp examination of the patients. Early stage of LSCD was characterized by stippling or late fluorescein staining. The intermediate stage of LSCD was characterized by persistent late fluorescein staining in a vortex pattern. The advance stage of LSCD was characterized by the same vortex staining and a history of recurrent cornea epithelial defect or persistent epithelial defect. Affected limbal and corneal areas were identified by the location of fluorescein staining on slit-lamp examination. Affected areas were then stratified into superior, nasal, inferior, and temporal limbal sections. Unaffected areas were determined as the limbal sections outside of the affected sections. Each patient's chart was also reviewed to determine any underlying etiology and predisposing factors that led to LSCD. Patients with acute chemical injuries with LSCD were excluded from the study. A total of 30 eyes with normal presentation on slit-lamp examination and no previous history of ocular disease were selected as the control group.

Optical coherence tomography

Scan images were taken by high-definition cirrus optical coherence tomography (Cirrus HD OCT with software package for Anterior Segment Imaging [Ver 5.1], Carl Ziess, Meditech, Dublin). Scans of the central cornea and the superior, nasal, inferior and temporal limbus were collected. The five-line raster protocol was used. It scans through 5 parallel lines of equal length. This scan can be used to view high-resolution images of the cornea. The line length is fixed at 3 mm, but the rotation and spacing are adjustable. Each line is composed of 4096 A-scans. By default, the lines are horizontal and separated by 250 µm (0.25 mm), so that the 5 lines together cover 1-mm width. Radial scan covers from the periphery to center. This five-line raster may be rotated from radial to horizontal or vertical to cover the point of interest. Scleral spur and root of iris were used as reference marks.

In normal controlled eyes, superficial conjunctival epithelium layer is thin consisting only 2 cells layer followed by loose connective tissue as compared to 5–6 cells layer thick corneal epithelium followed by basal cells layer and Bowman's layer interface and well-arranged stroma, hence give well-demarcated layers on AS-OCT [Fig. 1]. Irregularity of outer surface epithelium, fibrosis of subepithelial layers

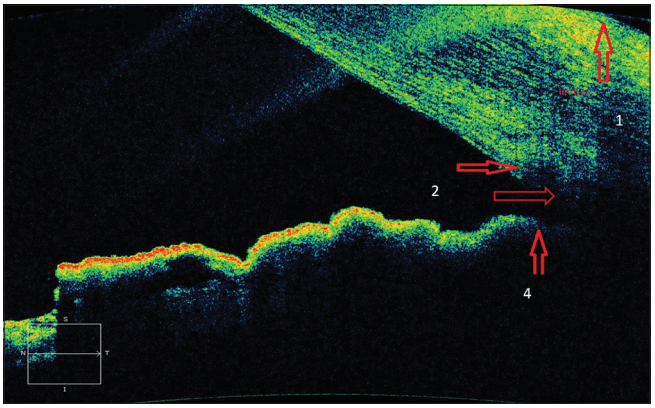


Figure 1: Anterior segment-optical coherence tomography image showing anatomical landmarks around limbus, (1) limbus, (2) Scwalbe's line, (3) scleral spur, (4) root of iris

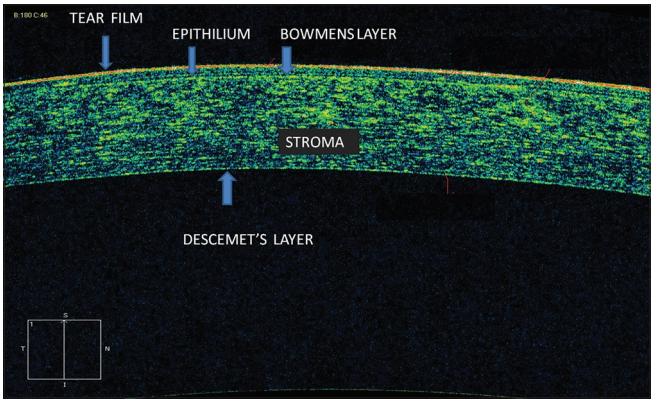


Figure 2: Anterior segment-optical coherence tomography image showing various layers of cornea

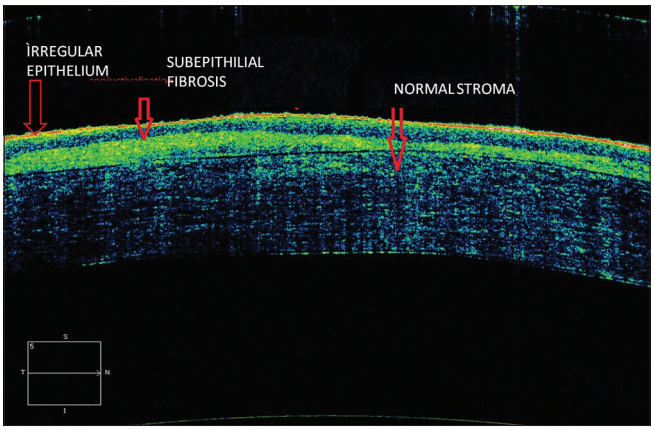


Figure 3: Anterior segment-optical coherence tomography image showing prominent subepithelial fibrosis in patients with limbal stem cell deficiency LSCD stage-1

and scarring of stroma can be easily identified as compared to normal corneal AS-OCT scan [Figs. 2 and 3].

Epithelial thickness was obtained by manually counting the focus positions of the initial image of the superficial epithelium to the final image of the basal cell layer as well as total corneal thickness also. Five-line raster scale was used to analyze 2.0-mm breadth of the limbus. Other specific findings of cornea and limbus were also recorded and correlated with the clinical picture.

Statistical analysis

Statistical analyses were performed with SAS software (SAS Institute, Cary, North Carolina, USA). Intraclass correlation coefficients were used to assess the reliability of thickness measurements obtained by 2 independent observers. Kruskal–Wallis tests were used to compare the difference in thickness measurements among control group and different stages of LSCD. Any $P < 0.05$ indicated statistical significance.

Results

We recorded the symptoms and found watering, followed by foreign body sensation, and diminution of vision were significantly associated with LSCD. Table 1 shows symptoms of LSCD.

Five-line raster imaging and recording technique were used for horizontal, vertical, and radial scan (3-mm length × 1-mm width) of limbus as described earlier.

Epithelium thickness varied in different limbal regions

In control group, mean epithelium thickness was thickest in the inferior limbus, that is, 65.4 μ (standard deviation [SD] =4.96) and was thinnest in the nasal limbus, that is, 59.47 μ (SD = 7.70) with temporal and superior being 62.8 μ and 64.23 μ, respectively. In LSCD group, thinnest mean epithelium thickness was seen in inferior limbus, that is, 41.9 μ (SD = 11.14).

The mean epithelial thickness including central corneal epithelium of the ocular surface in the control group was 62.64 μ compared to LSCD groups 42.767 μ. The difference in mean epithelial thickness of two groups was found to be statistically significant ($P < 0.001$). In the control group, the mean central corneal epithelium thickness was 61.3 μ and 41.33 μ in the cases of LSCD. The difference between two groups was statistically significant ($P < 0.001$). In the control group, the mean limbal corneal epithelium thickness was 62.975 μ and 43.188 μ in the cases of LSCD. The difference between two groups was statistically significant ($P < 0.001$). Graph 1 shows thickness of epithelium in each quadrant in control and case group. Graph 2 shows mean epithelium thickness in central, peripheral, and total in control and case group.

Table 1: Symptoms of limbal stem cell deficiency

Symptoms	Number of eyes (%)
Watering	30 (100)
Foreign body sensation	30 (100)
Diminution of vision	24 (80)
Pain	12 (40)
Discharge	10 (33)
Stickiness of the eye	10 (33)
Redness	21 (70)

We tried to evaluate the correlation between average epithelial thinning in LSCD patients with various etiologies. In our study, we found that contact lens induced epithelial thinning was the most common etiology.

In addition to corneal epithelial thinning following, OCT-based microstructural changes were also noticed.

1. Irregular limbal epithelium – It is represented in the OCT by the discontinuation of the surface epithelium or stippling of the epithelium. Histologically, it is the epithelium defect that is not associated with the underlying inflammation in the surrounding epithelium. It is the most sensitive feature of LSCD ($P < 0.05\%$)
2. Subepithelial fibrosis – It is represented in the OCT as the hyper-reflective layer just beneath the epithelium. Histopathologically, subepithelial fibrosis is the chronic inflammatory response to the continuous insult of epithelium associated with the fibroblastic cell proliferation [Fig. 4]
3. Stromal scarring – It is represented as the hyper-reflective layer in the mid-stromal region on OCT. Stromal scarring is the deposition of the fibroblast replacing the normal collagen and cells. It is the most specific finding of LSCD [Fig. 4]
4. Conjunctivalization – On OCT, it is represented as the hyper-reflective layer with decreased ray penetration limiting the visibility of underlying tissue. It is the encroachment of the conjunctiva over the cornea associated with the vascularization [Fig. 5].

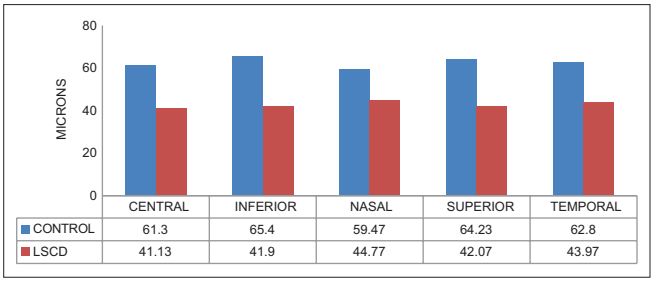
Graph 3 shows the OCT-based microstructural changes in diagnosed cases of LSCD.

Discussion

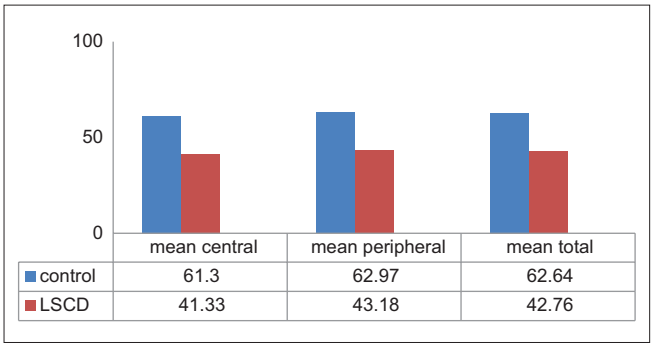
LSCs maintain the normal homeostasis and ensure normal wound repair of the corneal epithelium. Epithelial stratification and self-renewal of corneal epithelial cells require sufficient function of stem/progenitor cells. The basal corneal epithelial cell density and subbasal nerve density are significantly reduced in LSCD;[15] therefore, it is not surprising that the epithelium thickness is also significantly reduced secondary to LSC dysfunction or deficiency.

In our study, a total of 30 eyes of 19 patients with LSCD were studied, with 11 patients having bilateral involvement at the time of presentation. The mean age of the study population was 37.06 ± 12.58 years; the mean age of the control group was 35.33 ± 13.55 years. There was no significant difference in age ($P = 0.2905$) between the control and LSCD groups.

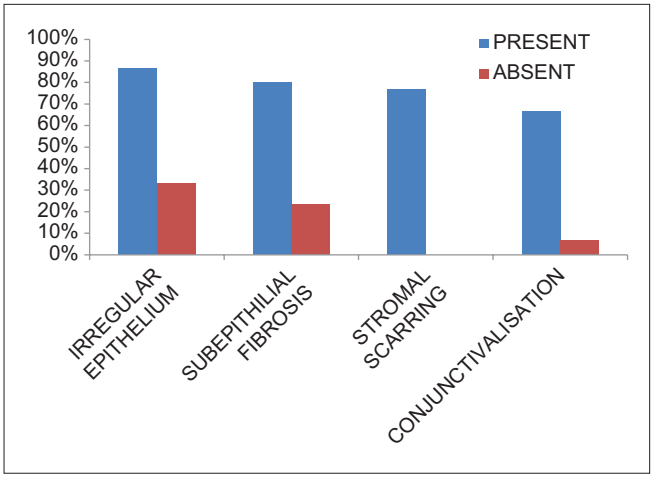
Etiological factors for LSCD found in various studies were congenital causes, namely, aniridia and Peter's Anomaly. Other causes include inflammatory insults such as those seen in SJS, ocular cicatricial pemphigoid, and graft versus host disease. Chronic ocular allergy such as vernal keratoconjunctivitis is another reported cause. Neurotrophic keratopathy, whether neuronal or ischemic, can lead to this disease as well, as can bullous keratopathy. Any infections of the corneal surface such as herpes, keratitis, and trachoma can predispose to this condition. Acquired causes also include trauma from chemical or thermal burns, and patients who have undergone prior



Graph 1: Corneal epithelium thickness in each quadrant in control and limbal stem cell deficiency group



Graph 2: Mean epithelium thickness in central, peripheral, and total in control and limbal stem cell deficiency group



Graph 3: The optical coherence tomography-based finding in diagnosed cases of limbal stem cell deficiency

ocular surgeries or cryotherapies at the limbus may be more susceptible. Radiation and chemotherapy are other potential causes, and systemic as well as topical chemotherapeutic medications may be sufficient to cause deficiency. LSCD has also been seen with benzalkonium chloride toxicity with glaucoma medications. Inappropriate contact lens use with consequent hypoxia and ocular irritation with destruction of the limbus may also contribute to both focal and total LSCD.[17]

In our study, the most common cause of LSCD was contact lens wear (n = 10, 33%), followed by pterygium (n = 8, 27%). Other etiological factors for LSCD were dry eye (7%), SJS (13%), and chemical injury (13%). In a study done by Chen *et al*., various etiologies found were SJS, multiple surgeries, contact lens, dry eye syndrome, chemical injury, drug toxicity, chronic keratoconjunctivitis, and infectious keratitis, they found contact lens wear to be the most common cause of LSCD.

Corneal epithelial thickness

We analyzed the percentage decrease in the average CET between the contact lens users and nonusers, and we found significant difference in the average CET of the two study groups, in contrast to the study done by Chen *et al*. who did not find any significant difference. In our comparative analysis, the percentage decrease in the average corneal thickness in patients with LSCD using contact lens was 0.253 ± 0.15. This can be explained by the fact that the thinning is dependent on the chronicity of the use of contact lenses.[18]

All patients of LSCD had gritty sensation and dryness. Although most of the patients complained of watering, this was most probably reflex watering due to foreign body sensation. Two eyes of SJS had TBUT <2 s and one eye TBUT was < 1 s. Deficiency of tears led to collection of desquamated epithelium of conjunctiva and cornea in the form of debris leading on to stickiness of eye. Frequent rubbing and unhygienic environment were responsible for redness and discharge. Inadequate replacement of epithelium resulted into erosion, ulceration, and pain.

On analyzing of signs of the LSCD patients, the signs we took into consideration were the presence of epithelial defect, scarring, ulceration, neovascularization, and corneal sensations. The most common sign was scarring which was due to nonreplacement of denuded epithelium in LSCD patients. The nonreplacement of the epithelium also led to epithelial defect resulting in superadded infection and ulceration. The neovascularization and ischemia led on to scarring.

On comparing the mean epithelial thickness in central and four limbal regions by AS-OCT, our study suggested a significant difference between study and control groups. Similar results were found in the study done by Chen *et al*. Both studies suggested a significant decrease in the mean epithelial thickness in the patients with LSCD. Such an epithelial thinning in our patients is likely due to the dysfunction or deficiency of LSCs, which are required for adequate homeostasis of corneal epithelium.[16]

On comparing of the average limbal thickness in the four quadrants by AS-OCT in normal patients, we found that the thickest portion of the limbus is the inferior quadrant. There is a significant decrease in the average limbal thickness in the LSCD group. Similar results were found in the study done by Chen *et al*.[16] Another important finding in our study was a positive correlation between the average decrease in the thickness of the epithelium of the superior and inferior limbus. We found that the average decrease in the epithelial thickness of the cornea is significantly related to the insult of the superior and inferior limbus. Similar results were found in the study done by others.[16]

This finding suggests an underlying susceptibility to LSCD in the superior and inferior limbus and the development of disease occurring at different rates. It has been suggested that there is a variation in the distribution of LSCs at the limbus. The superior and inferior limbus, in particular, the POV or the

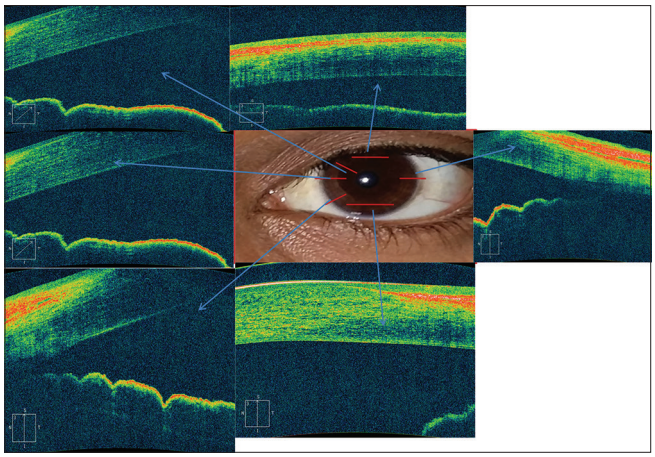


Figure 4: Anterior segment-optical coherence tomography image showing a mosaic raster scans at various places in a normal cornea

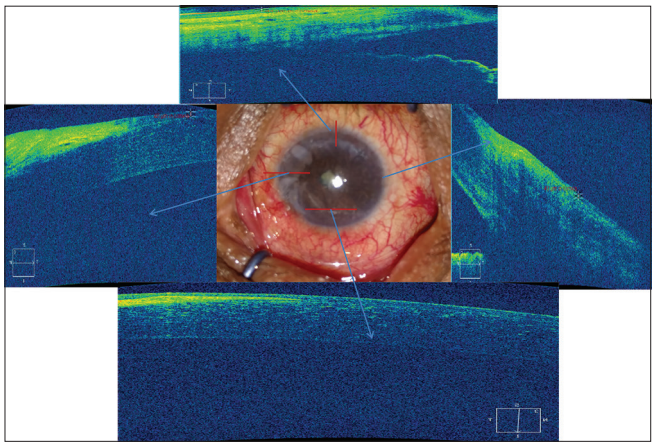


Figure 5: Anterior segment-optical coherence tomography image showing a mosaic raster scans at various places in a patient with limbal stem cell deficiency stage-2

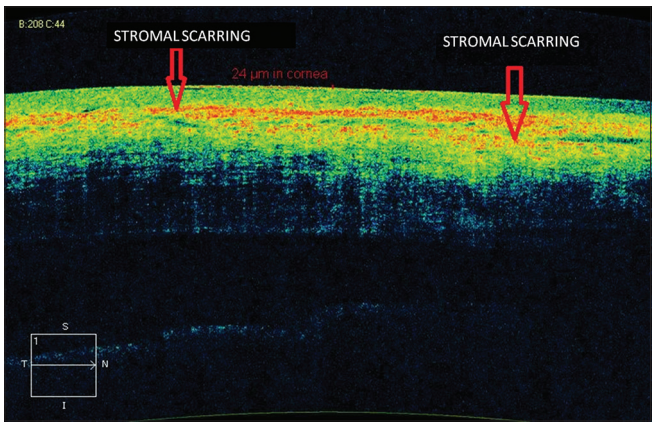


Figure 6: Anterior segment optical coherence tomography image showing anterior and mid stromal scarring in a patient with Limbal stem cell deficiency stage-3

limbal crypts, harbor a higher density of LSCs.[16] Damage to the superior limbus has a greater impact on corneal epithelial maintenance, and thus could result in an earlier clinical presentation of disease. Espana and associates found in their study of 7 LSCD eyes that only the superior limbus was involved. It was hypothesized that blinking of the upper eyelid may cause more frequent microabrasions, leading to a greater loss of epithelial cells. Thus, the superior limbus and inferior limbus would require a constant supply of LSCs to regenerate epithelial cells in that region. Given the repair stress in that area, sectoral LSCD is more likely to develop in the superior limbus and inferior limbus.

One interesting finding in our study was that the limbal epithelium is affected mostly in all cases of LSCD with the sectoral presentation. Despite a normal clinical presentation of the unaffected limbal regions at slit-lamp examination, the LSCs and their niche are abnormal at the micro structural level, as evident by the significantly thinner limbal epithelium and the loss of the normal stromal structure. This observation suggests that insults affect the LSCs globally at the cellular level. Therefore, the onset of LSCD occurs sooner in the more susceptible region, such as the superior and inferior quadrant. This observation also suggests that LSCD is a spectrum of disease and that subclinical damage to LSCs and their niche precede the clinical presentation. Invasion of conjunctival epithelial cells including goblet cells might be a rather late phenomenon of LSCD [Fig. 5]. It would be beneficial to detect subclinical damage and determine the threshold for irreversible damage.

Interestingly in our study, we found some of the microstructural abnormalities in the early and late cases of LSCD. We tried to summarize them under the following headings:

Irregular limbal epithelium.

In our study, the irregular epithelium was found in 26 out of 30 eyes and was found to be statistically significant ($P < 0.001$). The normal corneal epithelium has the presence of tight junctions between subepithelial cells which make it optically smooth. Normal corneal epithelium is five layers thick which develop from basal layer to stratified epithelia regularly. In LSCD, the normal epithelial thickness is reduced as the replacement of the normal epithelial layer is hampered, and hence, we found irregular epithelial surface [Fig. 6].

Stromal scarring

We found 24 out of 30 eyes in which stromal scarring was present and the value was found to be statistically significant ($P < 0.001$). The regular arrangement of collagen fibrils in stroma is responsible for the transparent cornea, and hence, we get the optically clear stromal area in AS-OCT. But in LSCD, the regular arrangement of stromal fibrils is disturbed which is the cause of stromal scarring [Fig. 5].

Subepithelial fibrosis

Another significant finding on AS-OCT was subepithelial fibrosis in 23 out of 30 eyes with LSCDs and 20 out of 30 eyes with conjunctivalization. Both the values were statistically significant ($P < 0.001$). Normally, the limbal epithelial stem cells act as a barrier between the conjunctiva and the corneal epithelium but in case of LSCD, this barrier is lost, and hence, the presentation of subepithelial scarring takes place.

Conjunctivalization

Twenty out of 30 patients with LSCD was associated with conjunctivalization. It is the encroachment of the conjunctiva

Table 2: Proposed classification of limbal stem cell deficiency based on optical coherence tomography parameters

Stage	Symptoms	Slit-lamp findings	OCT findings
Stage 1	–,+	Present	Present
Stage 2	+	Peripheral corneal opacity leaving central cornea not affected	Subepithelial fibrosis with stromal scarring
Stage 3	+	Corneal opacity or ulceration affecting the vision >6/60	Dense stromal scarring, breach in the epithelium, or stroma
Stage 4	+	Advanced signs, blindness affecting the vision <6/60	Total conjunctivalization with keratinization or ulceration of the cornea. OCT picture details not recordable [Fig. 7]

OCT: Optical coherence tomography, – Vague symptoms, + Present

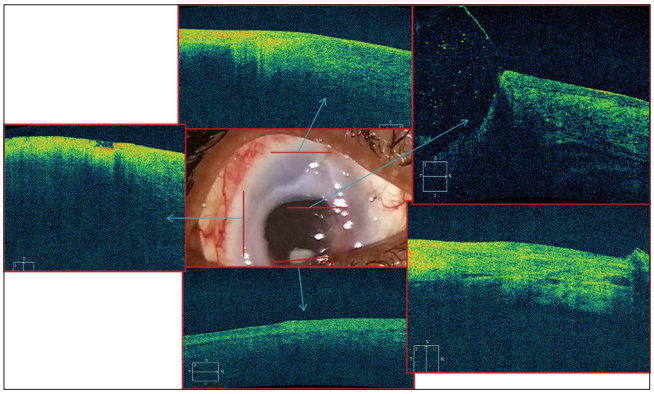


Figure 7: Anterior segment-optical coherence tomography scans of 60 years male with corneal melt and desmetoceole

over the cornea due to disruption of the normal limbal barrier. Hence, we propose a classification of LSCD based on symptoms and OCT findings.

Table 2 shows the proposed LSCD classification based on AS-OCT.

Conclusion

LSCD relatively a new entity has been able to explain various corneal entities. There have been various studies to evaluate the symptoms, signs, and diagnosis of LSCD with the help of impression cytology and confocal microscopy. None of the aforementioned techniques elucidate the exact pathophysiology of LSCs and their niche and they are associated with high false positive and negative results. In our comparative analysis, AS-OCT was found to be an important tool in the diagnosis of clinical and subclinical cases of LSCD. It provides better insights *in vivo* evaluation of limbus and provides a better visualization of various clinical signs of LSCD patients.

Individually comparing impression cytology, confocal microscopy, and AS-OCT, impression cytology has been the gold standard technique of confidently diagnosing LSCD, but this procedure is time-consuming, requires specialized equipments, is invasive and with variable results. It is difficult to perform impression cytology and sometimes even in cases of LSCD we do not get a positive result. On the other hand, confocal microscopy has a poor resolution as compared to AS-OCT in the diagnosis of LSCD.

We evaluated the role of AS-OCT in diagnosis and evaluation of patients with LSCD and since we found that there was a significant decrease in the mean epithelial thickness in patients with mild-to-moderate LSCD. We conclude that AS-OCT being a noninvasive modality of evaluation, having higher magnification, repeatability of results, hence has an imperative role in the clinical evaluation of patients with LSCD.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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