

# Early Results of Autologous Cultivated Limbal Stem Cell Transplantation in Total Limbal Stem Cell Deficiency

Mohammad Ali Javadi<sup>1</sup>, MD; Bahram Einollahi<sup>1</sup>, MD; Alireza Baradaran Rafiei<sup>1</sup>, MD; Hossein Baharvand<sup>2</sup>, PhD; Marzieh Ebrahimi<sup>2</sup>, PhD; Nasrin Rafati<sup>1</sup>, MD; Mojgan Rezaei Kanavi<sup>1</sup>, MD

<sup>1</sup>Shaheed Beheshti Medical University, Tehran, Iran; <sup>2</sup>Rooyan Research Center, Tehran, Iran

**Purpose:** To report the early results of transplantation of autologous limbal stem cells cultivated on amniotic membrane (AM) in patients with total unilateral limbal stem cell deficiency (LSCD).

**Methods:** Four eyes of 4 patients with total unilateral LSCD confirmed with impression cytology underwent transplantation of autologous limbal stem cell cultivated on AM. At each follow up visit, a complete eye examination with special attention to recurrence or regression of vascularization, corneal opacification, and epithelial defect healing was performed. Digital imaging was performed at each follow up visit. Impression cytology was repeated in all cases after surgery.

**Results:** The patients were followed for 5-13 months. Visual acuity improved in all cases. Decrease in corneal opacification and vascularization was obvious in 3 cases with coverage of the cornea with corneal epithelium. Sectoral conjunctivalization was evident in these 3 cases, however the corneas were ready for transplantation. The procedure failed in one case with total corneal conjunctivalization.

**Conclusion:** Transplantation of autologous stem cells cultivated on AM seems to be an effective way for total LSCD. More definite judgment needs longer follow up together with long-term results of corneal transplantation in these patients.

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**Correspondence to:** Mohammad Ali Javadi, MD. Professor of Ophthalmology; Ophthalmic Research Center, Labbafinejad Medical Center, Boostan 9 St., Pasdaran Ave., Tehran 16666, Iran; Tel: +98 21 22585952, Fax: +98 21 22590607, e-mail: ma\_javadi@yahoo.com

## INTRODUCTION

Limbal stem cells are responsible for corneal epithelial cell replacement and tissue regeneration. These cells are poorly differentiated slow cycling cells with great capacity for clonogenic expansion and error-free division and have long life span. The immediate progeny of stem cells have the potential to proliferate rapidly and provide the capacity for rapid expansion of

the cells. These cells are referred to transient amplifying cells (TAC) which pass different stages of differentiation and eventually convert to terminally differentiated cells. Furthermore, limbal stem cells act as a barrier against corneal vascularization and conjunctivalization.<sup>1,2</sup> Limbal stem cells are dependent on the limbal stroma (stem cell niche), normal tear production and normal conjunctival vasculature for tissue regeneration.

Limbal stem cell deficiency (LSCD) is characterized by conjunctivalization of the corneal surface, recurrent and persistent epithelial defects, chronic inflammation, scarring and ulceration of the cornea. LSCD has been detected in a number of corneal disorders which can be divided into two major categories. Category I disorders destroy the epithelial stem cell population and include chemical or thermal injuries, Stevens-Johnson syndrome, long-term contact lens wear, advanced ocular cicatricial pemphigus (OCP) or multiple previous operations. Category II disorders lead to dysfunction in limbal stem cell stromal microenvironment and include diverse causes such as aniridia and keratitis associated with multiple endocrine deficiency.<sup>1,2</sup>

LSCD may be focal or diffuse depending on the extent of limbal involvement and the underlying disease process. Patients with this group of disorders often suffer from photophobia, decreased vision, epiphora, blepharospasm and recurrent episodes of pain. LSCD can best be confirmed histologically using impression cytology (IC) which can detect goblet cell containing conjunctival epithelium on the corneal surface.<sup>3</sup>

Treatment of LSCD is stepwise starting from simple measures such as administration of artificial tears and punctal occlusion. Correction of palpebral lesions, blepharorrhaphy or tarsorrhaphy and superficial keratectomy in combination with amniotic membrane transplantation (AMT) should be performed if needed. Many cases may heal by simple measures, for instance radiation injury is temporary and could be managed medically. Partial LSCD, especially in the presence of adequate number of TACs, may be cured with AMT alone. In these situations, improper interventions may damage the remaining cells and should be avoided. In presence of LSCD, corneal transplantation will fail, because only short life-span TACs will be transferred during this procedure.<sup>4</sup> Autologous limbal transplantation is useful for patients with unilateral LSCD but the rate of success is low in severe cases, furthermore it may damage the healthy eye.<sup>1</sup>

Regarding the recent success in cultivating corneal stem cells on amniotic membrane (AM), transplantation of autologous limbal stem cells cultivated on AM seems to be a promising option in unilateral LSCD. Advantages of this technique compared to previous techniques include less need for limbal tissue and that the AM acts as a bed for proliferation of stem cells and transformation of them into corneal epithelium. Stem cell transplantation could be considered for cases of total LSCD with lack or deficiency of TACs.<sup>4,9</sup> In this study we describe the surgical technique for transplantation of autologous limbal stem cell cultivated on AM and the early outcome of a series of patients with total unilateral LSCD.

## METHODS

Four patients with total unilateral LSCD and complete vascularization of the corneal surface due to chemical or thermal burn underwent autologous limbal transplantation. Inclusion criteria were basal tear secretion at least 5 mm on the 5-minute Schirmer test and visual acuity of at least light perception (LP) in the affected eye. Total LSCD and conjunctivalization of the cornea were verified by IC.

All eyes were subject to comprehensive ophthalmologic examination including visual acuity, biomicroscopy, tonometry and funduscopy. In cases of inadequate media clarity, B-scan ultrasonography was performed to rule out gross posterior segment pathology. Informed consent was obtained from all subjects.

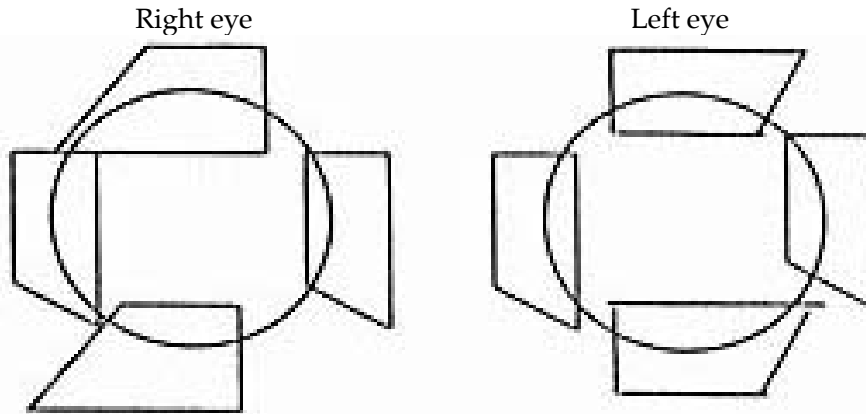
All patients had negative history for transmittable disease and also negative serologic tests for hepatitis B and C and HIV and a negative VDRL.

## Impression Cytology

After instilling a drop of tetracaine 0.5% into the conjunctival sac of the affected eye and clearing excess moisture in the conjunctival sac, 4 Millipore papers were put on the superior, inferior, nasal and temporal quadrants of the limbus so that one half of the paper covered the

cornea and the other half covered the conjunctiva (Fig.1). The papers were kept on the surface for approximately 3 to 5 seconds and were then removed with a peeling motion. Papers were sent to pathology laboratory of the Eye Bank of I.R. Iran in a labeled 24-well containing fixator

(alcohol 70% and acetic acid 5%). Slides of the specimens and the controls were stained with periodic acid Schiff and Papanicolaou's stains. Presence of goblet cells on the corneal half of the specimen was considered as evidence for corneal conjunctivalization and LSCD.



**Figure 1** Schematic diagram showing the placement of Millipore papers for sampling limbal cells

### Limbal Biopsy in Healthy Eye

Under local anesthesia (tetracaine 0.5%), a small biopsy (1×1 mm, 30% deep) was harvested from the superior limbus of the healthy eye which consisted of corneal-limbal-conjunctival tissue. The biopsy was preserved in Optisol solution and sent to Rooyan Research Center for cultivating limbal stem cells.

### Cell Culture

The limbal biopsy was irrigated 3 times in Dulbecco's modified Eagle's medium and F12 (DMEM/F12) containing amphotricin B (1.25 µg/ml) and gentamicin (50 µg/ml). Excess tissue including corneal endothelium, sclera and conjunctiva were removed from the biopsy under a stereomicroscope. The remaining tissue was irrigated in the above-mentioned solution and then incubated in dispase II (1.2 U/ml) in Hanxs buffer salt solution (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) for 10-15 minutes at 37°C and 5% CO<sub>2</sub>. The tissue was then irrigated by DMEM/F12 medium containing embryonic stem cell

qualified-fetal bovine serum (ES-FBS) 10%, amphotricin B (1.25 µg/ml), and gentamicin (50 µg/ml) to neutralize the effect of dispase.<sup>10,11</sup>

Human AM was obtained under sterile conditions after elective caesarian section from healthy, HIV and HBV negative donors. After washing with phosphate buffered saline (PBS) containing penicillin (1000 u/ml), streptomycin (50 µg/ml) and ofloxacin (0.3%), the inner AM was separated from the rest of the chorion. The membrane was then flattened onto a nitrocellulose paper with the epithelium/basement membrane side up. The membrane with the paper was cut into pieces and placed in a sterile vial containing dimethylsulfoxide (1.5 M) and preserved at -80°C until use.

The membrane was defrosted immediately before use and was rinsed in PBS and then incubated in ethylene diamine tetraacetic acid (EDTA) 1% at 37°C for 15 minutes. Then the medium was evacuated and the epithelial cells of the membrane were separated using a glass bar. Each membrane piece, after ensuring it was bared, was flattened onto a 6-well plate with the epithelial surface up. Thereafter the

prepared limbal tissue was placed on the center of the membrane and was cultivated on DMEM/F12 medium containing ES-FBS (10%), dimethyl sulfoxide (DMSO) (0.5%), epidermal growth factor (27 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 µm/ml), hydrocortisone (0.5 µg/ml), cholera toxin A (30 µg/ml), amphotricin B (1.2 µg/ml), gentamicin (50 µg/ml) and L-glutamine (4 mM) at 37°C, 5% CO<sub>2</sub> and 95% O<sub>2</sub>.

After 13-15 days, the limbal tissue was removed from the plate and cultivation was continued for 7 more days in the absence of limbal tissue. During the cultivation period, the rate of stem cell migration was evaluated by phase-contrast microscopy and inverted microscopy. The cellular phenotype of the cultivated cells was evaluated by hematoxylin-eosin staining. At last, the cultivated limbal stem cells were ready for transplantation in an expansion of 2×2 cm.

### Transplantation Surgery

Under general anesthesia a 360° limbal peritomy was performed. Subconjunctival scar tissue was dissected and excised with scissors and forceps. Next, the fibrovascular scar tissue (corneal pannus) was stripped off. The AM with overlying cultivated stem cells was placed on the bared surface of the cornea and adjacent sclera with epithelial side up and sutured onto the recipient conjunctiva and episclera with 10-0 nylon tangentially with long separate bites. A viscoelastic material was applied on the external surface of the transplanted AM to prevent damage to cultivated stem cells. Finally the ocular surface was covered with a layer of AM with epithelial side down to protect the transplant. At the end of the operation, the puncta were cauterized and lateral tarsorrhaphy was performed.

Postoperative medical treatment included topical steroid, antibiotic and autologous serum eye drops 20% or preservative-free artificial tears. Autologous serum eye drop was used for two weeks and an antibiotic eye drop was used until complete epithelialization. Topical steroid

was tapered gradually based on severity of ocular surface inflammation and discontinued in 1.5 to 2 months.

Follow up visits were scheduled on days 1, 2 and 5, weekly up to one month, every two weeks up to 3 months and monthly up to one year. Thereafter, the patients were visited every 3 months. The patients were examined with special attention to corneal transparency at each follow up visit. Digital photos were taken at each visit. IC was repeated at final follow up. Herein we present the early results of the procedure, while the follow up is maintained.

## RESULTS

### Case 1

A 40-year-old man who had sustained severe thermal burn with molten plastic to his left eye 15 years ago, presented with opacity and extensive corneal vascularization. He had undergone corneal transplantation together with cataract extraction and intraocular lens implantation 4 years after the injury. Corneal graft developed vascularization and opacification after a short time.

The patient was referred for graft failure. Ophthalmologic examination in the right eye was normal with visual acuity of 20/20. Examination of the left eye revealed extensive corneal vascularization and opacity with mild symblepharon in the temporal upper fornix (Fig. 2A), open puncta in both lids and reduced tear meniscus, visual acuity was hand motions (HM). IC of the limbal area in the left eye verified LSCD (Fig. 2B). The patient underwent transplantation of autologous limbal stem cell cultivated on AM in the left eye (Fig. 2 C, D, E). Visual acuity improved to counting fingers at one meter and corneal opacity and vascularization decreased after 2 months. IC of the limbal area was repeated 3 months after the procedure and revealed few goblet cells only in the cornea with expansion toward the center. There was no conjunctivalization on the center and other quadrants of the cornea (Fig. 2F).

**Case 2**

An 18-year-old man was referred for thermal burn to his left eye due to fireworks 4 years ago. The eye suffered extensive corneal opacity and vascularization. His right eye was completely normal with visual acuity of 20/20. The left eye had complete corneal conjunctivalization and vascularization on biomicroscopy (Fig. 3A) with visual acuity of counting fingers at 2.5 meters. IC of the limbal area of the left eye showed moderate decrease in limbal stem cell population (Fig. 3B). Schirmer tests I and II revealed mild dryness of the left eye.

The patient underwent autologous cultivated limbal stem cell transplantation in his left eye (Fig. 3C). After 2 months, visual acuity was counting fingers at 4 meters and considerable decrease in corneal opacity and vascularization was noted (Fig. 3D). Schirmer tests I and II were found also to be significantly improved. At the 4 month visit, visual acuity remained the same, corneal transparency improved despite the presence of deep stromal vessels. IC at the one month visit showed few goblet cells in the lower and nasal quadrants but not in other parts of the cornea (Fig. 3E).

**Case 3**

A 22-year-old man presented with acute alkali burns to both eyes. Visual acuity was 2/10 in the right eye and LP in left eye. There was an epithelial defect in the lower one-fifth of the cornea in the right eye with limbal ischemia at this area. The left eye demonstrated diffuse corneal opacity along with severe and extensive limbal ischemia and scattered points of conjunctival and episcleral necrosis. The eyes were initially treated medically.

Three months later, visual acuity was 10/10 in the right eye and counting fingers at 20 centimeters in the left eye. The left eye had extensive corneal vascularization and opacity (Fig. 4A). Five months after the injury, the patient underwent left upper lid laser epilation due to trichiasis.

One year after the initial injury, he was di-

agnosed with unilateral LSCD in the left eye based on examination and IC and underwent transplantation of cultivated limbal stem cell. Two months later entropion repair in the left upper lid was performed. At the 8 month visit, visual acuity in the left eye improved to 20/800 along with mild symblepharon of the upper fornix and remarkable decrease in corneal opacity and vascularization. At the 9 month visit, visual acuity was counting fingers at 1.5 meter with recurrence of corneal vascularization and failure of transplantation based on IC of the cornea (Fig. 4B). He underwent reconstruction of the left eye fornices, followed by keratolimbal allograft surgery and finally penetrating keratoplasty.

**Case 4**

A 20-year-old man was referred for extensive alkali burn to his right eye 8 month ago. The left eye was normal with visual acuity of 20/20. The right eye had visual acuity of HM, upper temporal symblepharon extending to the corneal surface and severe corneal opacity and vascularization. Ten months later he underwent symblepharon release.

About 5 year after the initial injury, he had profound visual loss due to severe corneal opacity and dry eye in his right eye. Right eye conjunctivalization was verified by IC (Fig. 5A) and transplantation of limbal stem cell cultivated on AM was performed. At the first month visit, the AM was completely resolved, visual acuity improved to 20/400, and corneal opacity and vascularization was decreased. Schirmer tests 1 and 2 revealed considerable improvement in tear production. IC 7 months after transplantation revealed only a few goblet cells in the temporal quadrant of the cornea and the other quadrants were covered with normal corneal epithelium (Fig. 5B).

**DISCUSSION**

Management of LSCD depends on ocular conditions such as type (partial or total), laterality (uni- or bilateral) of the condition, severity of

ocular surface inflammation, tear production status, presence of symblepharon or ocular surface keratinization in addition to systemic factors such as age and general health condition of the patient.<sup>5-8</sup> Preliminary measures such as punctal occlusion, correction of trichiasis and lid lesions and tarsorrhaphy should be considered before performing stem cell transplantation.<sup>3</sup> Early management of LSCD especially in reversible processes, such as early damage in radiotherapy, includes use of emollients and transient or permanent punctal occlusion. These measures can provide symptomatic improvement in mild cases, especially in the presence of adequate TACs in the center of the cornea. Undue surgery or administration of eye drops containing preservatives should be avoided in these cases.<sup>1</sup>

In cases of partial LSCD, AMT alone may suffice<sup>1</sup>, but in total LSCD, the main strategy includes replacement of limbal stem cells. Kenyon and Tseng<sup>12</sup> have shown that corneal epithelial cells could be regenerated by limbal stem cell transplantation. Donor tissue for limbal transplantation can be obtained from the healthy fellow eye (limbal autograft) in case of unilateral deficiency or from a living related or a cadaver donor (limbal allograft) when both eyes are affected. Cadaver donors have a large supply of stem cells and have the potential to be repeated but the main problem is the high rate of immune reaction and need for immunosuppression. Living related donors usually provide better tissue matching compared to cadaver donors but there are limitations in finding an HLA-matched donor and it is less repeatable. Furthermore, the risk of inducing partial LSCD in the donor eye is another drawback.<sup>5</sup> In spite of the above-mentioned disadvantages, limbal autograft has revealed promising results in recent years.<sup>13-21</sup> Histopathologic examination of postoperative corneas has revealed stratified squamous epithelium similar to normal corneal epithelium with K3 positive staining in one study.<sup>22</sup>

Limbal autograft has some advantages over limbal allograft. This technique overcomes the problem of immunologic rejection but may

only be used for patients with unilateral mild to moderate LSCD. The drawback is that due to removal of fairly large segments of limbal tissue, the donor eye is at risk of surgically induced LSCD.<sup>13,19</sup> There is no definite agreement on the maximum safe size for harvesting limbal tissue but it is prudent not to remove more than 120°. The use of autologous cultivated limbal stem cell transplantation has been suggested to overcome this limitation. Cultivating a small amount of limbal tissue provides adequate limbal stem cells for treatment of total LSCD.

Use of AM as a carrier medium for cultivated limbal stem cells carries the following advantages: 1) AM stromal matrix is similar to conjunctival basement membrane and is a suitable medium for growth of limbal stem cell;<sup>14</sup> 2) It is antigen-free and resorbed gradually;<sup>23</sup> 3) AMT facilitates ocular surface epithelialization by releasing growth factors and suppressing fibrovascular proliferation;<sup>13,24</sup> 4) It down-regulates inflammatory cytokines such as interleukin 1 $\alpha$  and 1 $\beta$  in culture media;<sup>25</sup> 5) It facilitates epithelial cell differentiation<sup>2</sup> and 6) The AM may be used for ocular surface reconstruction<sup>24</sup>.

Nakamura et al<sup>26</sup> have reported histopathologic features after autologous limbal stem cell transplantation. Postoperative sampling revealed that 4-5 layers of well differentiated epithelial cells were formed on the surface of the cornea and the ocular surface remained stable up to 19 months with no epithelial defect. Grueterich et al<sup>27</sup> performed corneal transplantation in a patient 5.5 months after cultivated limbal stem cell transplantation. Postoperatively corneal sampling revealed a 5-6 cellular layer attached to the basement membrane side of the AM. These cells were similar to normal corneal epithelial cell as they contained laminin-5, integrin  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$ ; however they were different from corneal epithelium and similar to limbal stem cells because of lacking keratin-3 and connexin-43. They concluded that AM provides an ideal substrate for supporting the growth of epithelial progenitor cells.

The growth rate of different corneal cells

on AM used as a culture substrate is as follow: 0% for central corneal cells, 8.3% for peripheral corneal cells and 96.2% for corneal limbal cells. Cultivated stem cells retained their in vivo properties of undifferentiation, slow-cycling and label-retention.<sup>10</sup> The presence of cell markers P-63 and cytokeratins 14 and 19 on the surface of cultivated limbal stem cells indicate retention of these properties.<sup>28</sup>

There have been a number of reports on ex vivo expanded autologous limbal stem cell transplantation. Pellegrini et al<sup>22</sup> have reported two cases of unilateral LSCD who underwent autologous cultivated limbal stem cell transplantation resulting in stable ocular surface. Tsai et al<sup>29</sup> performed autologous transplantation of ex vivo expanded stem cell on 6 eyes of 6 patients including 5 cases of partial LSCD and one case of total LSCD and found that the epithelial defect healed in 4 days in all cases and the ocular surface remained stable up to 15 months. Schwab et al<sup>13</sup> have reported 10 cases of autologous cultivated limbal epithelial transplantation resulting in stable ocular surface after 6-9 months of follow up.

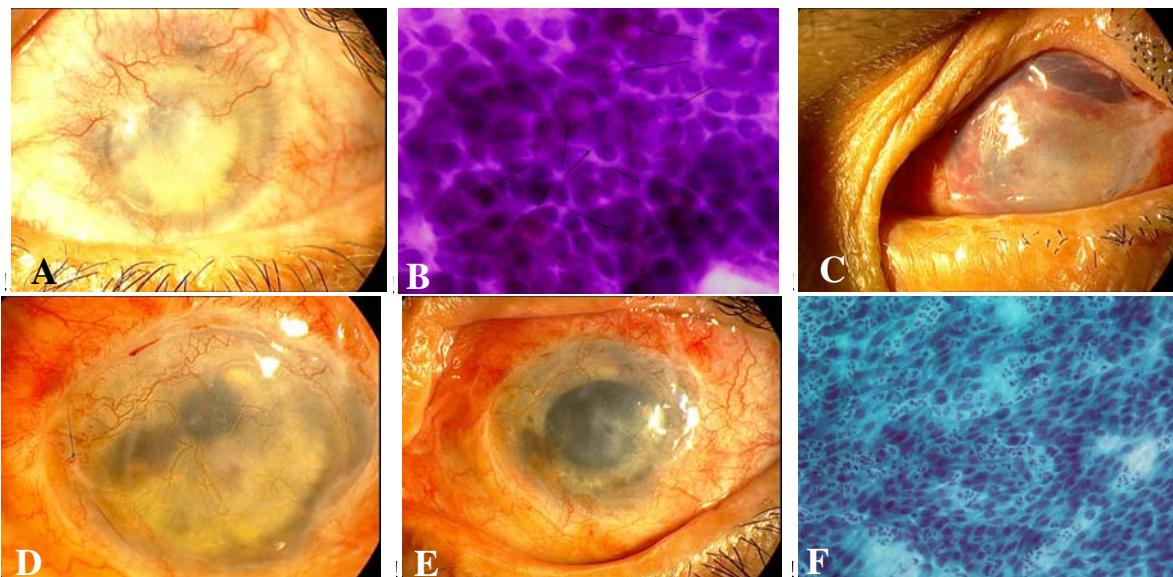
Shimazaki et al<sup>14</sup> performed cultivated stem cell transplantation on 13 cases of total

LSCD. The etiology of LSCD was Stevens-Johnson syndrome in 8 cases, OCP in 3 cases and chemical burn in 2 cases. Donor tissue was obtained from cadavers in 7 cases and from a living related donor in 6 cases. Complete epithelialization occurred only in 61% of the cases.

Herein we have reported the early results of autologous cultivated limbal epithelium transplantation in 4 cases of unilateral total LSCD due to chemical or thermal burns. The anatomic and visual outcomes of our series after a follow up of 5 to 13 months were favorable. IC performed at the last visit revealed reversal of LSCD in 3 cases. More extensive follow up is essential for evaluating the long-term results. The ultimate goal of this procedure is to replenish limbal stem cells and maintain a stable ocular surface in order to increase the success rate of simultaneous or subsequent penetrating or lamellar keratoplasty.

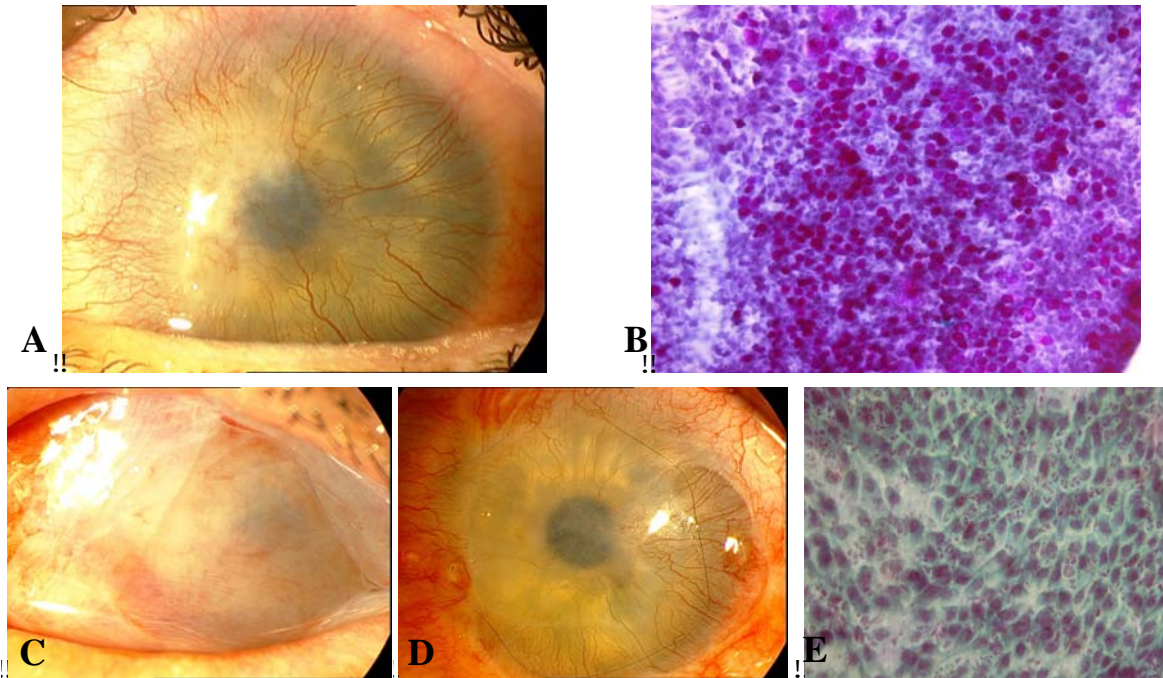
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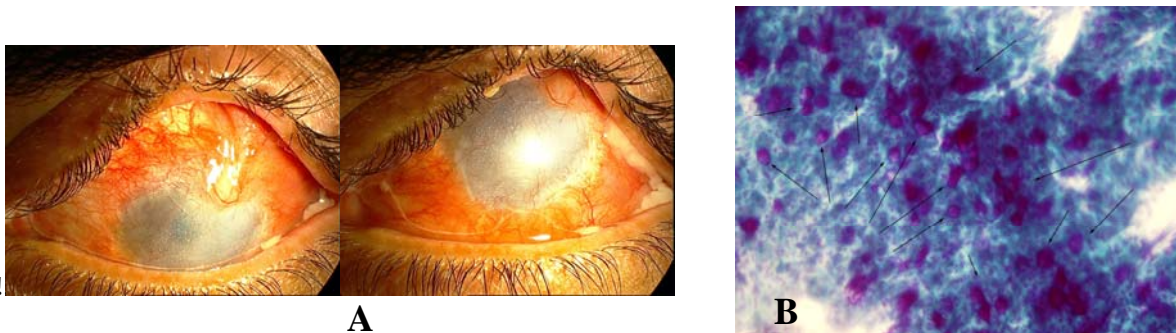


**Figure 2** Clinical photographs of the left eye of case 1: (A) Preoperative view showing extensive corneal vascularization, (B) Preoperative impression cytology displaying the presence of goblet cells (arrows) in the cornea (periodic acid Schiff and Papanicolaou's staining,  $\times 450$ ), (C) One week postoperatively, (D) 2 weeks postoperatively, (E) 2 months postoperatively, (F) Impression cytology 3 months postoperatively revealed corneal epithelial cells and scattered multinuclear cells without any goblet cell (periodic acid Schiff and Papanicolaou's staining,  $\times 100$ ).!

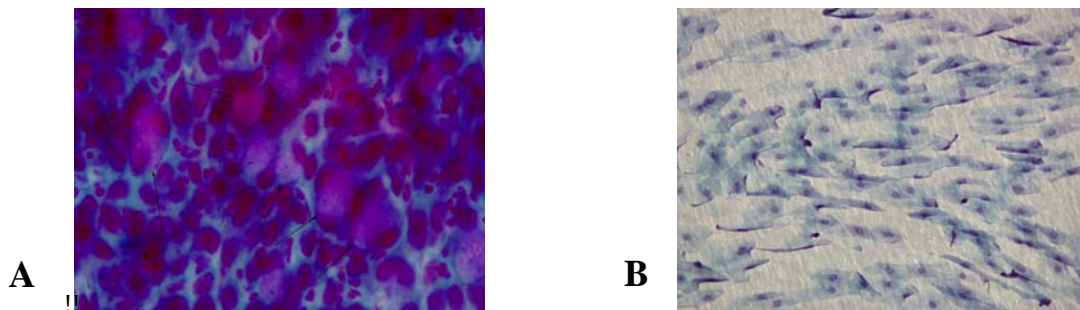




**Figure 3** Clinical photographs of the left eye of case 2: (A) Preoperative view displaying corneal conjunctivalization and vascularization, (B) Preoperative impression cytology showing a large number of goblet cells among corneal epithelial cells (Periodic acid Schiff and Papanicolaou's staining,  $\times 100$ ), (C) postoperative day one, (D) 2 months postoperatively, (E) Impression cytology one month postoperatively revealed corneal epithelial cells and scattered multinuclear cells without any goblet cell (Periodic acid Schiff and Papanicolaou's staining,  $\times 100$ ).!



**Figure 4** Clinical photographs of the left eye of case 3: (A) preoperative views revealed corneal vascularization and severe opacity, (B) Impression cytology 9 months postoperatively displaying the presence of goblet cells (arrows) among corneal epithelial cells (periodic acid Schiff and Papanicolaou's staining,  $\times 100$ ).



**Figure 5** Impression cytology of the right eye of case 4: (A) Preoperative view displaying the presence of remarkable goblet cells in the cornea (periodic acid Schiff and Papanicolaou's staining,  $\times 250$ ). (B) 7 months postoperative view revealed the absence of goblet cells in the nasal quadrant of the cornea (periodic acid Schiff and Papanicolaou's staining,  $\times 100$ ).



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