Current Status of Cultured Human Limbal Epithelial Cell Transplantation

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Introduction :

The most important refracting surface of the eye is the cornea, amounting for more than 2/3 of the total diopteric power of the human eye. This is because of the huge difference between the refractive index of air (1.000) and that of the cornea (1.376). That's why the orchestrated role of the corneal surface epithelium, along with the preocular tear film, to maintain a stable refracting surface, can't be ignored. The corneal epithelium, which is exposed to the exterior, is in a constant process of self-renewal and its integrity is vital for visual outcome. The adult stem cells for regeneration of this epithelium are located at the corneoscleral junction, the limbus. Any damage to this component of stem cells leads to the so-called limbal stem cell deficiency (LSCD), with sight threatening consequences. Prior to the concept of limbal stem cell therapy, these cases were managed with standard penetrating keratoplasty (PKP), which invariably failed after a few months, when the transient amplifying cells (TAC) present at the central cornea (which are derived from limbal stem cells but have a limited replication potential) are exhausted. But with the advance of cell culture and the marriage of clinical medicine and basic science, this condition can now be successfully managed, another significant achievement in the field of regenerative medicine. In this review we will briefly discuss about LSCD, its presentation and the various modes of management of this condition.

Stem cell concepts

The most widely accepted stem cell definition is a cell with a unique capacity to produce unaltered daughter cells (self-renewal) and to generate specialized cell types (potency). Self-renewal can be achieved in two ways. Asymmetric cell division produces one daughter cell that is identical to the parental cell and one daughter cell that is different from the parental cell and is a progenitor or differentiated cell. Asymmetric cell division does not increase the number of stem cells, while symmetric cell division produces two identical daughter cells. However, stem cells are slow cycling in vivo. But in culture, many types of stem cells start multiplying rapidly, a feature helpful for the culture of these cells. The stem cells for different tissues are increasingly been discovered, and it is hypothesized that they are sustained at certain parts of the tissues in a specific microenvironment, the so-called "stem cell niche". These stem cells in tissues are called resident stem cells.

Different types of stem cells :

Stem cells can be classified according to their potency (totipotent, pluripotent, multipotent, oligopotent, unipotent) as well as stage (preimplantation, embryonic/ fetal, postnatal, adult) (Table 1). However, despite the great advances in molecular biology and regenerative medicine, the characteristics of the adult stem cells have remained an enigma.

Stem cells in corneal epithelium :

Coming back to cornea, the observation of centripetal pigmented lines in healed corneal injury led to the hypothesis of limbal localization of the epithelial progenitor cells.1 Thereafter, Thoft et al proposed the

x-y-z hypothesis of corneal epithelial maintenance.2 He suggested that the rate of cell loss from corneal surface (z-component) must equal the sum of the rate of proliferation of basal cells (x-component) and the rate of centripetal movement of peripheral cells from the limbus (y-component). Later, Schermer et al, noticed that keratin 3, a 64kDa basic keratin, was expressed in the whole of corneal epithelium but not in the basal layer of the epithelium of the limbus or the conjunctiva.3 This suggested the occurrence of the progenitor cells in that region. Now the problem was to isolate these cells from the pool of millions of other cells. For this purpose, some general methods as well as some specific methods were developed. General methods focused on the identification of the stem cells based on their slow cell cycle. Tritiated (3H) thymidine and 5-bromo-2-deoxyuridine (BrdU) labelling of these cells persist for a longer time than other rapidly multiplying cells. Another general method was the observation that the stem cells express an ATP-dependent cassette transporter protein, ABCG2, which pumps out a blue fluorescent dye, Hoechst 33342, readily from the cytoplasm.4, 5 This property is blocked by calcium channel blockers like Verapamil. 4 The cells that have this dye-exclusion property are termed as "side population" and they can be isolated by Fluorescence-assisted cell sorting (FACS). Specific methods to identify the stem cells depends on the expression of different lineage-specific stem cell markers, e.g. Keratin K19, Vimentin, P63, ABCG2 etc, and some differentiation markers like K3, K12, involucrin, nestin etc, by immunohistochemical methods.6-10

Ocular surface failure

The ocular surface physiology is maintained by many different factors acting in concert. A healthy preocular tear film (composed of basal mucinous layer secreted by conjunctival goblet cells, intermediate aqueous layer from the secretion of the lacrimal glands, and superficial lipid layer from the Meibomian glands at the lid margin), the corneal and conjunctival epithelium, and the eyelids - all are cardinal necessities for maintaining ocular surface homeostasis. Abnormal functioning of any of these elements (tear film: deficiency, unstable tear film; corneal and/or conjunctival epithelium: traumatic, inflammatory, allergic, infectious or congenital conditions; eyelids: malposition, infectious, inflammation) can lead to ocular surface failure. The other components of the ocular surface are also subsequently affected (e.g., primary tear film deficiency, by sicca syndrome or radiation, can lead to secondary changes in the corneal and conjunctival epithelial phenotype, giving rise to the clinical picture of dry eye syndrome). For this reason, identification of the primary component dysfunction is essential for the proper management of a case of ocular surface failure.

Limbal stem cell deficiency

Definition:

Limbal stem cell deficiency (LSCD) is a clinical picture caused by the destruction or dysfunction of the stem cell containing limbal epithelium, leading to failure of corneal epithelial regeneration, with resultant invasion of the cornea by conjunctival epithelial cells, which is associated with chronic inflammation, stromal scarring, neovascularisation and persistent epithelial defects (PEDs).

Causes of LSCD:

LSCD can be primary or secondary. Primary LSCD is characterized by absence of any identifiable external factors causing insufficient microenvironment or 'stem cell niche' to support the limbal stem cells. Causes include mainly congenital disorders like hereditary aniridia, congenital erythrokeratoderma and sclerocornea. Secondary LSCD occurs due to destruction of the limbal stem cells by external factors like trauma [chemical, thermal injury], radiation exposure, autoimmune diseases

[Steven Johnson Syndrome (SJS), ocular cicatricial pemphigoid (OCP), polyglandular autoimmune syndromes11], nutritional [Vitamin A deficiency], iatrogenic [multiple ocular surgeries, pterygium excision, cyclocryotherapy, antimetabolites (topical mitomycin C), systemic chemotherapy], contact les wear and some other ocular diseases [keratoconjunctivitis sicca, neurotrophic keratitis, atopic keratoconjunctivitis, postinfectious keratitis etc]. The causes are summarized in Table 2.

Clinical presentation of LSCD :

In a patient with suggestive history of chemical injury or other etiologies mentioned above, presenting with decreased vision, photophobia, tearing, blepharospasm and recurrent episodes of pain and redness, LSCD is clinically diagnosed by the triad of signs noted in the cornea, conjunctivalization, neovascularisation and chronic inflammation (Table 3). On a slit lamp microscopy, the corneal surface has a dull and irregular reflex due to variable thickness and loss of transparency of the cornea. There are tiny bud-like projections of normal corneal epithelium extending into the conjunctivalized area. Persistent and recurrent epithelial defects are better appreciated with fluorescein staining, characterized by a stippled pattern due to increased uptake of stain by the conjunctivalized corneal epithelium. There is associated stromal inflammation and sterile infiltrates. On higher magnification, loss of the palisades of Vogt at the limbus can be appreciated. In an advanced case, there is an ingrowth of thickened fibrovascular pannus, scarring and even calcification. There can be corneal ulceration leading on to keratectasia and perforation, which then becomes a surgical emergency. The clinical diagnosis can be supported by the demonstration of goblet cells by Periodic Acid Schiff staining for mucin of the corneal impression cytology specimen. Immunofluorescence microscopy of the sample for cytokeratin K3 and K19 can also be used to confirm a conjunctival phenotype.12

Limbal stem cell transplantation: different methods
Limbal autograft transplantation:

Clinical application of the basic research was also evolving. In 1989, Kenyon et al published the first successful case series of 26 cases of limbal autograft transplantation where the superficial conjunctivalized epithelium and fibrovascular pannus was removed surgically, and two free limbal autografts taken from the uninjured or less injured fellow eye was transplanted to the diseased eye for the treatment of severe ocular surface disorder. His case series consisted of both acute and chronic chemical injury (20 cases), thermal burns (2 cases), contact lens-induced keratopathy (3 cases), and ocular surface failure after multiple surgical procedures (1 case). After 6-18 months of follow-up of 21 patients he reported rapid re-epithelialization of the recipient cornea (90%), significantly improved visual acuity (81%), stable epithelial adhesion without recurrent erosion or persistent epithelial defect (95%) and arrest or regression of corneal neovascularisation (71%).10, 13 However the major problems were the potential for development of iatrogenic limbal stem cell deficiency in the donor eye, and the inapplicability in bilateral cases. Meallet et al, 2003, reported combined conjunctivo-limbal autograft (CLAU) with amniotic membrane transplantation for the management of total limbal stem cell deficiency (LSCD).14 In his case series of 5 eyes, after the removal of fibrovascular pannus from the corneal surface, two conjunctival limbal free grafts were harvested from the fellow eyes in all five patients with unilateral LSCD. Amniotic membrane, with the basement membrane side up, was grafted onto the defect created at the donor site and onto the recipient corneal and limbal sclera before placement of conjunctival limbal grafts. The amniotic membrane graft at the donor site promotes rapid wound healing. Amniotic membrane performs many functions for promotion of healing. The basement membrane contains

laminin, fibronectin, collagen type 1, keratan sulfate, chondroitin sulfate etc that promotes cell adhesion.15 In addition, it contains a number of growth factors like EGF (Epithelial growth factor), HGF (Hepatocyte growth factor), TGF-? (Transforming growth factor alpha), bFGF (Basic fibroblast growth factor) etc that promote cell proliferation. It also contains various anti-angiogenic and anti-inflammatory mediators16 and natural protease inhibitors17 that help in survival of the graft.

Cadaveric Kerato-limbal allograft (KLAL):

In bilateral cases of LSCD, Tsai et al, 1994, reported use of cadaveric donor corneas for allograft.18 In his case series of 16 cases [thermal or chemical burns (n = 5), Terrien's degeneration (n = 2), congenital sclerocornea (n = 1), Stevens-Johnson syndrome (n = 1), and chronic keratoconjunctivitis (n = 7)], cadaveric kerato-limbal grafts were applied after resection of fibrovascular pannus. It was followed by prolonged (2.9 +/- 1.3 months) immunosuppression with oral cyclosporine A. He reported improved visual acuity and rapid surface healing for most of the cases, while the initial angiogenic response at the limbus gradually regressed within 3 months after surgery. However, a later study by Ilari et al, showed that by KLAL procedure, over the time graft survival rate diminished dramatically (54.4% at 1 yr and 27.3% at 3 yrs follow-up).19 Based on these findings, some strategies were formulated to increase the long term success rate of this technique:20 (1) to restore ocular surface defense in cases of Stevens-Johnson Syndrome and related diseases prior to KLAL procedure; (2) to perform KLAL and penetrating keratoplasty as a staged procedure, with an interval of 3-6 months;21 (3) to restore limbal stromal microenvironment by amniotic membrane graft; and (4) to employ effective immunosuppressants in appropriate dosage, keeping in mind the potential complications of prolonged immunosuppression.

Living related conjunctivo-limbal allograft (lrCLAL):

HLA typing is not feasible from donor corneal tissue. That's why a living related HLA matched donor is sometimes preferred for selected cases, specially SJS and OCP patients.22, 23 This approach has significant success rate in the long term (84.6% maintaining a stable corneal surface after 1 yr and almost all of which maintaining it even after 4 years of follow-up, while having less dependence on systemic immunosuppression post-operatively).23

Cultivated autologous limbal epithelial cell transplantation (Fig. 1):

The idea came from the successful culture of human keratinocytes (epidermal cells from skin) onto a feeder layer of nonproliferative 3T3 fibroblasts (Rheinwald and Green, 1975).24 The 3T3 cells may secrete some growth factors that are conductive to the growth of the keratinocytes. This idea was incorporated in the culture of corneal epithelial cells; and the first report of this method was by Pellegrini et al, 1997,25 where 2 alkali burn patients were successfully transplanted with 3T3 fibroblast feeder-cultured autologous corneal epithelium, derived from mechanically and enzymatically disintegrated 1mm2 limbal biopsy of the other eye. The cultured layer was placed on the recipient eye, carried on a soft contact lens. The patients were followed up for more than 2 years and it was proved that long term maintenance of epithelial integrity is possible by this technique.

Since then there has been many modifications of this original technique. The major obstacles were to prepare a suitable support matrix for these cells to grow and later to transfer to donor eye. In late 1990-s, amniotic membrane was used as a carrier.26 Amniotic membrane has several advantages: it promotes growth of the stem

cells, retains their stemness for a longer period of time, and after transplantation, inhibits inflammatory reaction by interleukin-1 receptor antagonist,16 inhibits vascular invasion into the cornea by antiangiogenic effect of thrombospondin-1,16 and promotes rapid re-epithelialisation of the cornea. Also, it has easy availability and various storage methods have been studied in detail. Short term storage at temperature above 0oC has the advantage of retaining viability of the cells that secrete growth factors, while having disadvantage of more immunogenicity. Long term storage at sub-zero temperature does not retain cell viability, but has the advantages of less immunogenicity,27 and as it is used as a decellularized matrix for cell culture only, it is perhaps better to store by this technique.28 However there are certain drawbacks also, like the rare chance of transmission of viral diseases or prion diseases through this technique.

There were also various schools of thought regarding use of denuded or intact amniotic membrane. Grueterich M et al, 2002, reported that limbal epithelial stem cell cultures grown on denuded amniotic membrane (amniotic membrane whose epithelium has been stripped off) differentiate into a corneal phenotype, whereas those cultures grown on intact amniotic membrane (on the basal side) retain their stemness, by immunophenotypic studies.29 However, the clinical application of this finding is poorly understood.

Limbal epithelial culture method:

There are also various methods of culture of the limbal epithelial cells. One is explant culture,30 in which the limbal tissue is mechanically disintegrated into fine pieces and then transferred onto the amniotic membrane. Other is suspension culture,31 where the epithelium is first separated from the limbal specimen by the action of Dispase-IITM(neutral protease isolated from Bacillus polymyxa), then dispersed into a suspension by trypsin-EDTA treatment. The progenitor cells are preferentially attached to the amniotic membrane, and they form colonies upon culture in specialized culture medium [Dulbecco Modified Eagle's Medium (DMEM), with Ham's F12 serum, epidermal growth factor, autologous serum, insulin, and hydrocortisone].32 The epithelial cells grow to form sheet of cells which are monitored regularly. Comparison between these two methods of culture shows that suspension culture is superior to explant culture technique in terms of stem cell content, more desmosomal junctions and less intercellular spaces.31, 33

General principles for management of LSCD:

The cornea is an immunologically privileged site where there are no blood vessels, so the immune cells can not gain access to the penetrating keratoplasty allografts which are generally done without HLA matching. However, the situation is different in case of vascularised cornea due to LSCD. In these cases conventional PKP invariably fails due to two factors, one is due to immune attack from the blood; another is the lack of stem cells in the central cornea, which is transplanted. The central cornea, as mentioned earlier, contains transient amplifying cells that can support the epithelium for a few cell divisions only.

The plan of management depends on the state of the LSCD, whether it is unilateral or bilateral, partial or total. For unilateral partial cases there is scope for wait-and-watch or simpler procedures like mechanical debridement and amniotic membrane grafting. Ipsilateral translocation of limbus can be done to treat this condition if the earlier methods fail. In unilateral total LSCD, we have to take limbal tissue from the other healthy eye for either direct or cultured limbal epithelial transplant. Allografts (either living-related or cadaveric; direct or cultured epithelial transplant) are the treatment options

for bilateral LSCD cases. For allografts a long term immunosuppression regime is mandatory to prevent rejection. However, immunosuppression, being a double-edged sword, must be tailored to suit the individual patient's needs.

Future trends

Looking for tissue-engineered alternative substrate for culture:

Various researchers were also trying to develop different biomaterials on which the cells can be cultured. A gel scaffold comprising of fibronectin-fibrin cross linked with factor XIII was shown to be good enough for culturing and subsequent removal of the sheet of cells and handling to graft over the recipient.34 Nishida et al used another novel technique to produce carrier-free culture by using temperature-responsive culture plates.35 These culture plates have a polymer that converts to a hydrophobic to hydrophilic form when the temperature is lowered, allowing for hydration of the polymer and release of the cell sheet from the culture surface.36 However, difficulty of handling the cell sheet is an issue in this method.

Looking for alternate sources of epithelium:

For bilateral LSCD, for cases like Stevens-Johnson Syndrome, the need for prolonged immunosuppression for allogenic kerato-limbal grafts, and also their poor outcome, led to the search of alternate sources of epithelium. Buccal mucosa was long being used for reconstruction of conjunctival defects.37, 38 It was observed that it contains a similar type of keratin (Keratin-3) in the spinous layer, similar to the cornea.39 So it was viewed as a viable alternative. But the main problem of using buccal mucosa was the fact that it contained 20-30 layers of non-keratinised stratified squamous epithelium with a thick submucosa.40 After successful animal experiments using cultivated human oral mucosal epithelial (HOME) cells for transplant, the method was successfully utilised by two Japanese groups, Nishida et al35 and Nakamura et al.41 In contrast to cultivated limbal stem cell transplant, cultivated HOME cell transplant showed mild peripheral neovascularization, though the central region remained transparent.42 Continued research in this field is going on to develop a more useful alternative.

Questions that still need to be answered :

Despite the tremendous success of autologous stem cell transplant in curing ocular surface disorders, there are still many questions that come up after more studies. Still now there is a doubt about the long-term survival of the epithelial stem cells after transplantation to the recipient, as the central cornea theoretically cannot provide the niche required for the cells. A remarkable article was published recently that challenged the dogma of limbal localization of corneal epithelial stem cells.43 It was shown experimentally that oligopotent stem cells are distributed throughout the corneal surface and not only at the limbus; and the limbal stem cells only appear to contribute to corneal repair, not to steady state corneal renewal. The arguments put forward by the study included the questionable notion that the limbal stem cells migrate to the central cornea, a significant distance, which is in stark contrast to other squamous epithelia where each resident stem cell is in charge of only a limited area of epithelium. One recent article by Dua et al also supported this study, by a case series of 8 eyes from 5 human subjects with long standing clinically apparent 360o limbal stem cell deficiency with well preserved central corneal epithelium during a 5 year follow-up.44 These findings call for further introspection into this aspect and further testing of the dogma.

There is also need of further research into the development of synthetic biodegradable scaffolds for

limbal epithelial culture so as to obviate the need of human amniotic membrane, the use of which is a potential source of infection. Also there lies the need to identify the angiogenic signals that operate in case of cultivated HOME cells transplant. There is also need of development of some technique for easy storage and transportation of the cultured limbal epithelial cells to decentralize the service from centres of excellence to reach the farthest corners of the country.

With the continued thrust in research, this might not be a distant dream at all.

Table 1: Classification of stem cells

POTENCY	DEFINITION	STAGE			
		Preimplantation	Embryonic/ Fetal	Postnatal	Adult
Totipotent	Can form entire organism autonomously	Zygote			
Pluripotent	Can form almost all the body's cell lineages (endoderm, mesoderm, and ectoderm), including germ cells	Embryonic Stem (ES) cells	Embryonic Germ (EG) cells	Unrestricted somatic stem cells (USSC), Multipotent adult stem cells (MAPC)	Embryonic carcinoma cells (EC), Multipotent adult stem cells (MAPC)
Multipotent	Can form multiple cell lineages but cannot form all of the body's cell lineages			Embryonic carcinoma cells (EC), Multipotent adult stem cells (MAPC)	Mesenchymal stem cells (MS, MSC)
Oligopotent	Can form more than one cell lineage but are more restricted than multipotent cells; are sometimes called progenitor cells or precursor cells				Mesenchymal stem cells (MS, MSC) Neural stem cells (NS, NSC)
Unipotent	Can form a single differentiated cell lineage				
Terminally differentiated cells					

Table 2: Causes of Limbal stem cell deficiency

PRIMARY LSCD	SECONDARY LSCD
Hereditary aniridia Congenital erythrokeratoderma Congenital sclerocornea Epidermal dysplasia	**Trauma** - Chemical (Alkali, acid, other chemicals) - Thermal - Radiation (ultraviolet, ionizing radiations) - Iatrogenic (multiple surgical procedures, ptrygium or limbal tumor excision, cyclocryotherapy, topical mitomycin C, contact lens use) **Systemic causes** - Autoimmune (Steven Johnson Syndrome, ocular cicatricial pemphigoid, polyglandular autoimmune syndromes) - Nutritional (Vitamin A deficiency, protein energy malnutrition) **Other local causes** - Keratoconjunctivitis sicca (due to aqueous tear deficiency) - Neurotrophic (exposure) keratitis - Atopic keratoconjunctivitis - Post-infectious keratitis - Peripheral ulcerative keratitis (Mooren's ulcer)

Table 3: Clinical presentation of LSCD

CLINICAL FEATURES OF LIMBAL STEM CELL DEFICIENCY	
Presenting complaints	Decreased vision
	Photophobia and blepharospasm, reflex tearing
	Recurrent episodes of pain and redness
History	Trauma (chemical, thermal, radiation exposure, iatrogenic)
	History of ocular surgery or chemotherapy
	Contact lens use
	Neurological disorder (cerebrovascular accident, facial nerve paralysis)
Ocular examination	Thickened fibrovascular pannus
	Scarring of cornea
	Corneal ulceration
	Keratectasia and corneal perforation in advanced cases
Slit lamp examination	Dull and irregular reflex of cornea
	Variable thickness of cornea
	Tiny bud-like projections of normal corneal epithelium extending into the conjunctivalized area
	Neovascularisation of cornea extending from periphery
	Loss of palisades of Vogt at the limbus
	Stromal inflammation manifested by haze
	Sterile infiltrates in stroma
	Fluerescein staining: stippled pattern of the conjunctivalized epithelium
Investigations	Corneal impression cytology
	Periodic acid Schiff stain: Presence of goblet cells on corneal surface
	Immunofluorescence study by fluorescent labelled monoclonal antibody for cytokeratin K3 and K19: conjunctival phenotype of corneal epithelium

Table 4: Typical immunosuppression regime in limbal allografts (adapted from Espana EM et al. Keratolimbal allograft in corneal reconstruction. Eye 2004; 18(4): 406-417.)20

MEDICATION	DOSAGE	SCHEDULE	MONITORING
Prednisolone	0.5 mg/kg/day p.o.	Starts 3 days prior to surgery	Blood glucose level every month. Blood pressure/body weight
Cyclosporin A	2.5 mg/kg/day p.o.	Starts 3 days prior to surgery	Serum creatinine every 2 weeks, CsA and Mg2+ level in blood, Blood pressure (risk of hypertension)
Mycophenolate mofetil	2 g/day p.o.	Starts two to three weeks prior to surgery	CBC, platelets, and liver profile every month
Tacrolimus (FK506)	0.2 mg/kg/day p.o.	Used instead of CsA in patients with episodes of rejection	Blood glucose level every month, serum creatinine every 2 weeks

Acyclovir 200mg 5/day p.o. is used as a prophylactic measure against viral activation starting 3 days before surgery.

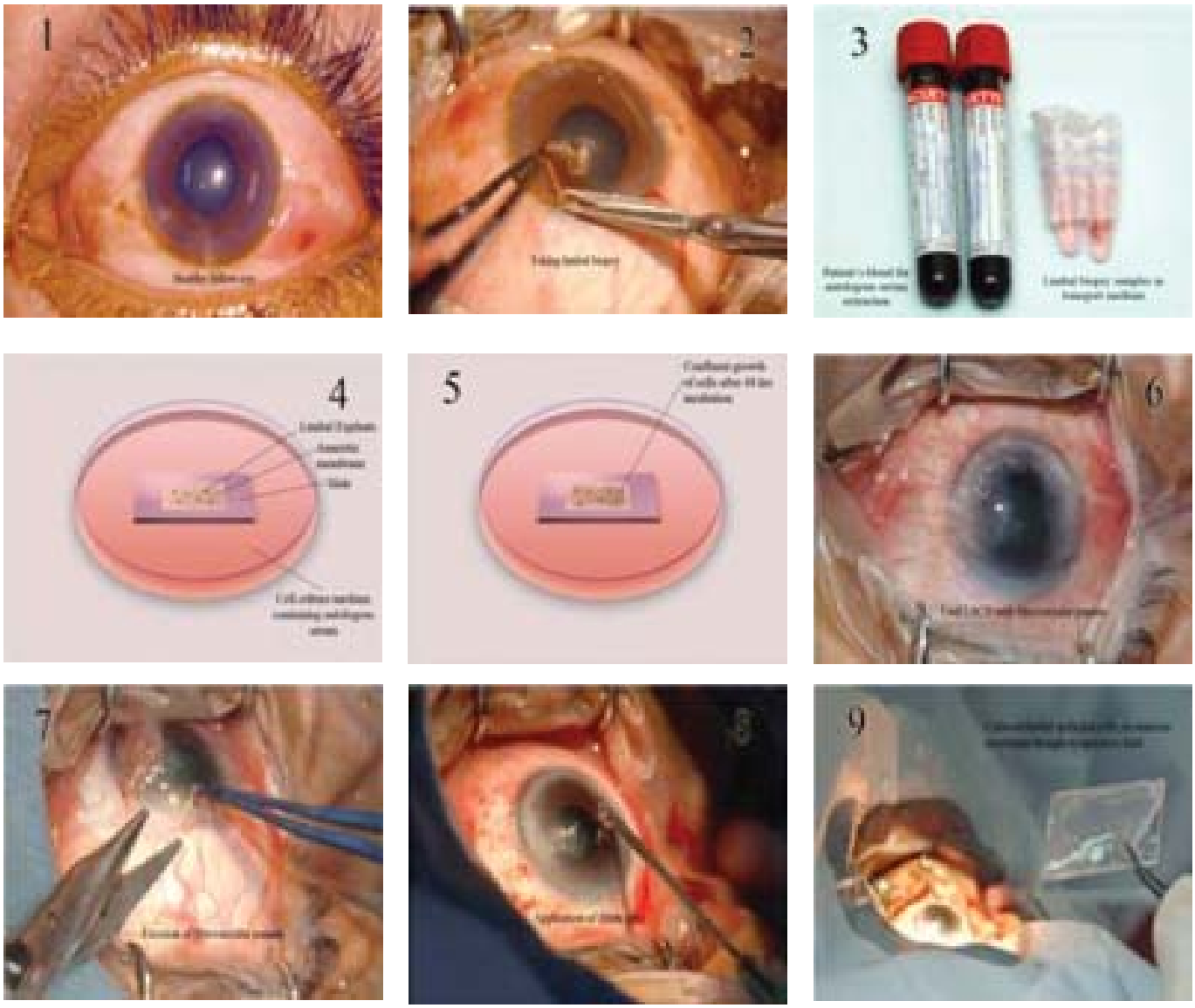

Fig. 1: Steps of cultured limbal epithelial transplant procedure.

1-2: Limbal biopsy taken from healthy fellow eye; 3: Limbal biopsy sample transported to cell culture facility in appropriate medium, patient's blood sample is also taken to extract autologous serum to prepare the cell culture medium; 4: Limbal biopsy sample is shredded into small pieces and explant-cultured on human amniotic membrane substrate; 5: Confluent growth observed after 48 hrs incubation at 37oC in 5% CO2 environment; 6: Photo showing the diseased eye with total limbal stem cell deficiency; 7: Fibrovascular pannus carefully excised; 8: Fibrin glue is put on to the cornea; 9: Confluent growth of epithelial cells on amniotic membrane is brought to the operative area.

10: Gently the amniotic membrane-cultured epithelium composite is spread over the whole cornea. Adhesion occurs by the fibrin glue.