**Application of Limbal stem cells for regenerative therapy in corneal diseases**

**Running title:** limbal stem cells and ocular surface regeneration

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**Abstract**

Transparency of the cornea as a clear window on the front surface of the eye is an important issue in visual system that a number of ocular surface diseases affect this. The human ocular surface is constructed by two various types of epithelia: the corneal epithelium in center and the conjunctival epithelium that surrounds this. These two epithelia are separated by limbal epithelium which is known as a major source of stem cells. The limbal stem cells (LSCs) provide the source of corneal epithelial renewal and also provide a promising approach to treat chronic epithelial defects on the corneal surface. Therefore, this review summarizes the current knowledge regarding to cornea and ocular surface diseases with special focusses on stem-cell-based therapeutic methods to improve and restore sight in affected patients.

***Keywords*:** Limbal stem cell, Ocular surface disease, corneal homeostasis

**Introduction:**

The cornea is an important transparent layer at the front of the eyes. This layer carries out two vital purposes in visual system [1](#_ENREF_1). First, cornea is necessary for exact concentration of the light on the retina which improves created images and second, it plays a significant role in protection of eyeball from external hazards [2](#_ENREF_2). The cornea is consisting of five main parts including, the corneal epithelium, Bowman’s layer, the corneal stroma, Descemet’s membrane and the corneal endothelium. The subtle changes in structure of each corneal layer have converted it to a potential tool for visual accuracy and eyeball protection [3](#_ENREF_3). The corneal epithelium is the outermost layer of cornea and the nearby layer to outer environment. This layer consists of a non-keratinizing stratified squamous epithelium which covers the entire surface of the avascular cornea up to the limbus [4](#_ENREF_4). The limbal epithelium is also a non-keratinizing stratified squamous surface at the outer edge of the cornea that separates the corneal epithelium and conjunctival epithelium [5](#_ENREF_5). Recent investigations demonstrated that limbal epithelium is a major source of stem cells (SCs) for corneal epithelium renewal and also prevents from conjunctival epithelium invading onto corneal surface [6](#_ENREF_6), [7](#_ENREF_7).

**Corneal epithelium homeostasis: limbal stem cell theory**

Continuous clinical observations and basic science researches identified the primary evidence for the limbal location of corneal epithelial stem cells almost 40 years ago. Reconstruction of the corneal epithelial through limbal pigment migration during wound healing process in guinea pig eyes demonstrated a significant role for the limbus regarding to the corneal epithelium renewal [8](#_ENREF_8). These results were confirmed clinically, when it was observed that the corneal epithelium restored by the peripheral aspects of the cornea in patients with corneal epithelial disorders [9](#_ENREF_9). These clinical findings led to the formation X, Y, Z hypothesis of corneal epithelial homeostasis. This theory for corneal homeostasis was firstly proposed by Thoft et al. in 1983 which stated that the limbus serves as a reservoir of ocular stem cells. X and Y refers to movement of cells from the basal layers of the corneal epithelium and from the periphery of the cornea, subsequently and Z shows those cells that are lost from the corneal surface during natural shedding. According to this hypothesis, to maintain the mass of cornea, the sum of X and Y must be equal Z [10](#_ENREF_10). Since then, various studies have localized the limbal stem cells to the basal layer of the limbal epithelium. Obtained results from DNA labeling in mouse models have indicated that a subset of epithelial cells in the basal layer of the limbal epithelium migrate slowly during the cell cycle which have potential to replace the lost cells [11](#_ENREF_11), [12](#_ENREF_12). These stem cells produces a stem-like daughter cell through asymmetric division which remains within the limbus and a number of transient-amplifying cell (TAC) which migrates centripetally and anteriorly [13](#_ENREF_13). Asymmetric division of limbal stem cells and maintaining of their stem cell state depends on their cellular microenvironment [14](#_ENREF_14). This is known as the stem cell niche. This niche might control SC behavior via intercellular contacts and signals. Limbal stem cells are no different from other adult stem cells in this respect [15](#_ENREF_15). Many findings suggest that the adult LSC niche exists in the basal epithelium of the limbus. The basal layer of the limbus provides the stem cells closeness to stromal vasculature, which brings important growth factors to the stem cells [16](#_ENREF_16). To date, four anatomic structures have been proposed as the corneal stem cell niche including Palisades of Vogt, limbal epithelial crypts, limbal crypts and focal stromal projections. The basement membrane of the limbus corrugates to increase LSCs contact with their microenvironment which can be seen on the surface of the limbus [17](#_ENREF_17). These visible undulations are known as the palisades of Vogt. The palisades of Vogt are located at the superior and inferior limbus where provide essential protection for limbal stem cells by the upper and lower eyelids [18](#_ENREF_18). More recently, there are a large body of evidence that deeper epithelial ingrowths are located at the interpalisade epithelial rete ridges which have been termed limbal epithelial crypts. It is suggested that the true limbal stem cells actually reside in the depth of these crypts [19](#_ENREF_19). Moreover, Shortt et al suggested two additional niches in vivo confocal microscopy; limbal crypts and focal stromal projections [20](#_ENREF_20). Limbal crypts are projections of limbal epithelium into the limbal stroma, whereas, focal stromal projections are finger-like projections of limbal stroma into the epithelium. These mentioned structures have a protective role for resided cells from injuries and other external hazards, and also provide a large surface area. Recently, it was shown that in patients with limbal stem cell deficiency (LSCD), these four proposed niche structures are absent [20](#_ENREF_20), [21](#_ENREF_21).

**Cornea and ocular surface: diseases and clinical problems**

As the outermost layer of the eye, the ocular surface is exposed to external environment protecting eyes from external insults and also help to better visual function. The ocular surface comprises the conjunctiva and the corneal epithelium. In normal eyes, the ocular surface are always covered by tear film, which can act as a protective barrier against infectious agents and also leads to an adequate trophism of corneal epithelium [1](#_ENREF_1). Ocular surface might be hurt because of chemical or thermal burns, autoimmune diseases, infection, wearing of contact lens, iatrogenia and also secondary to other systemic diseases [22](#_ENREF_22). Some hereditary defects in limbal stem cells such as aniridia may also lead to remarkable damages in conjunctiva and the corneal epithelium [23](#_ENREF_23). As a result of external hazards, the conjunctiva loses its ability of protection and lubrication, leading to rigidity and dryness of ocular surface [24](#_ENREF_24). In several diseases, limbal SC failure results in major problems in the corneal epithelium turn over. This results in growing of conjunctiva into the cornea to compensate the lacking corneal epithelium. Because many conjunctiva epithelium properties such as transparency are not similar to cornea, the visual power decreases and ultimately leads to blinding [25](#_ENREF_25), [26](#_ENREF_26).

**Limbal stem cell deficiency**

Limbal stem cell deficiency (LSCD) is a complex corneal disorder in which the stem cell functions of the limbus are destroyed [27](#_ENREF_27). Failure of epithelial regeneration as a result of either loss or dysfunction of LSCs leads to a different ranges of signs and symptoms including conjunctivalisation, corneal vascularisation, pain, tear, redness, oedema, poor vision and blindness [28](#_ENREF_28), [29](#_ENREF_29). Several investigations indicated that severity of pathology and symptoms is dependent to degree of limbal tissue loss [30](#_ENREF_30). Over the past decade, many causes of limbal stem cell deficiency have been identified. These causes classify in two groups; hereditary or genetic and acquired causes [29](#_ENREF_29), [31](#_ENREF_31). Aniridia is a hereditary developmental dysgenesis of the anterior segment of the eye, including the limbus [32](#_ENREF_32). Acquired causes include both chemical and thermal burns, inflammatory diseases such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, and chronic limbitis [22](#_ENREF_22). Some studies demonstrated that contact lens wearing is associated with more limbal stem cell deficiency [33](#_ENREF_33). It seems that contact lens solution toxicity and mechanical irritation of contact lens leads to inflammatory response in limbal stem cell area. In addition, radiation, cryotherapy, multiple surgeries, infection or drug use can lead to limbal stem cell defects [22](#_ENREF_22).

**The clinical applications of limbal stem cell in management of limbal stem cell deficiency**

Corneal grafting is classically known as a historical treatment option in total LSCD which can be failed over a short period of time. Clinical observations have indicated that LSCD can be successfully treated with transplantation of healthy limbal tissue [34](#_ENREF_34). The major sources of limbal cells are usually limbal autograft for unilateral LSCD and allogenic limbal graft from living related donors [35](#_ENREF_35). The main disadvantage of these two procedures is the risk of creating LSCD in the donor eye if a large amount of tissue is required [36](#_ENREF_36). Ex vivo expanded limbal cells on transplantable substrate is another important source of limbal cells. In this technique a small biopsy or explant is taken from the patient’s other eye (autoexplant) or from the healthy eye of a living or cadaveric donor (alloexplant). Then, the explant is used for culture and limbal epithelial cells are digested from the explant to produce a pure limbal stem cell suspension. The suspension is then cultured on inactivated 3T3 mouse fibroblasts or on human amniotic membrane for a period of time. The obtained cultured limbal epithelial sheet is then transfer to the eye with limbal stem cell deficiency after the conjunctival tissue is removed from the corneal surface .[37](#_ENREF_37)Whole limbal tissue transplantation and ex vivo expanded limbal epithelial cell transplantation are the beneficial approaches to corneal epithelial homeostasis be normalized. The overall success rate of limbal cell transplant varies between studies and it is approximately estimated at 76% [38](#_ENREF_38). The success rates are different in each study based on ex vivo expansion protocol, length of follow-up and etiology of LSCD [23](#_ENREF_23), [38](#_ENREF_38).

**Amniotic membrane** **as a** **gold standard substrate for ocular surface reconstruction**

There are three important factors for development of ocular surface reconstruction (OSR) using tissue engineering including: 1) the stem cell source, 2) a suitable substrate and 3) essential growth factors [39](#_ENREF_39). Recent research has shown promising results in generation of suitable substrate to improve tissue functions [40](#_ENREF_40). The quality of generated substrates is one of the significant factors in production of tissue engineered sheet for OSR. A suitable substrate for OSR must be biocompatible, non-immunogenic and non-inflammatory character, and also can maintain transparency of cornea and its mechanical stability [41](#_ENREF_41). Many cell substrates, such as biological scaffolds, biosynthetic scaffolds and synthetic scaffolds have been suggested for OSR [42](#_ENREF_42), [43](#_ENREF_43). Among them, amniotic membrane (AM) is the most extensively-used and the current gold standard substrate for OSR [44](#_ENREF_44). Human AM is the innermost layer of the fetal sac. It is composed of some layers such as amniotic epithelial cells, basement membrane and a subjacent avascular stroma. Previously AM has been utilized in epidermal field for surgical materials and biological dressing [45](#_ENREF_45). Several studies have shown that AM has strong potential to be used for conjunctival reconstruction in some ocular surface diseases [46](#_ENREF_46). AM have some characteristics including growth factors and cytokines inducing epithelialization and wound healing, anti-scarring and anti-inflammatory properties which make it proper in OSR [47](#_ENREF_47), [48](#_ENREF_48). AM can also resembles the basement membrane of the corneal epithelium [49](#_ENREF_49). From this mentioned evidence, AM is known as an appropriate substrate in OSR usages. Although clinical results using AM as a substrate are promising, some problems such as AM preparation standard protocol, donor-dependent variability and regional variations are still remained. These problems lead to development of alternative substrates for OSR. As mentioned above, AM has some unique characteristics which make it a good choice for conjunctival reconstruction in some ocular surface diseases [50](#_ENREF_50). Because of some biological problems, many ophthalmologists suggest AM cryopreserved under sterile conditions. However complete sterilization cannot be carried out by existing procedures, but AM must be as sterile as possible for clinical usages. In addition, AM also should be easily to obtain and preserve at room temperature. Cryopreservation of AM is an expensive method and bulky deep freezer is required [51](#_ENREF_51), [52](#_ENREF_52). Recently, Nakamura et al., have successfully produced sterilized, freeze-dried AM under vacuum conditions and vacuum-packed at room temperature with gamma-irradiation which has been used as a good substrate in OSR [52](#_ENREF_52). They also used this biomaterial for OSR in patients with pterygium [53](#_ENREF_53). Moreover, the physical properties of freeze-dried AMs could be improved by use of trehalose during the freezing process [54](#_ENREF_54). Therefore, suitable sterilization of both native and dried AM is a vital step in AM preparation for conjunctival reconstruction.

**Alternative sources for corneal epithelium reconstruction**

Because a very small amount of healthy limbal tissue is only required in the ex vivo expansion of LSCs, the risk of LSCD in the donor eye is insignificant during transplantation for the treatment of LSCD. In patients with bilateral total LSCD, ex vivo expansion of allogeneic limbal tissue taken from a living relative or cadaveric donor is suggested as a possible option [55](#_ENREF_55). Several investigation indicated that cadaveric donor tissue has been used to produce cultured limbal epithelial sheets that have improved vision in some cases of bilateral LSCD [56](#_ENREF_56), [57](#_ENREF_57). Because the tissue would be allogeneic, these methods could still be associated with some problems such as tissue rejection and use of immunosuppression drug with its serious side effects[7](#_ENREF_7) .

**Adult epithelial stem cells**

Recent studies indicated that using alternative autologous epithelial cells could introduce new ways to overcome the problems in the transplantation of allogeneic tissue. Oral mucosal, conjunctival, nasal, esophageal, vaginal and rectal epithelia are the potential sources of non-keratinizing stratified squamous epithelium in the adult human. Among these, the oral mucosal epithelia have some properties including lack of advanced differentiation, high proliferative potential, cytokeratin K3 expression, easy access and rapid healing which intrigue ophthalmologists to use it in resurface of ocular surface. The use of oral epithelium in reconstruction of ocular surface was associated with a very thick epithelium, leading to a poor vision and also discomfort in visual system. Ex vivo expansion of oral epithelium can lead to production of an epithelium similar to corneal epithelium in appearance and function. Some investigation demonstrated that expansion of rabbit oral mucosa epithelial SCs on amniotic membrane and subsequently transplantation of the cells onto the ocular surface of rabbits with total LSCD resulted in the successful re-epithelialization of the corneal surface [58](#_ENREF_58). In addition, promising early and longer term results was observed when this method has been used to humans [59](#_ENREF_59), [60](#_ENREF_60). These results introduce new approach which provide an opportunity for treatment of patients with blindness due to bilateral total LSCD.

**Embryonic stem cells**

hESCs are pluripotent derived from blastocysts through in vitro fertilization. ESCs could be differentiated theoretically to a corneal epithelial lineage using replication of the LSC niche. For the first time, this approach was used to generate corneal epithelium from mouse ESCs [61](#_ENREF_61). Recent researches showed that hESCs culture on an extracellular matrix of collagen-IV and fed with medium conditioned by limbal fibroblasts leads to corneal epithelial lineage [62](#_ENREF_62). Nevertheless, theuse of corneal epithelial lineage as a therapeutic approach for humans need to more investigation to elucidate associated problems regarding to immune rejection and ethical concerns.

**Induced pluripotent stem cells**

Induced pluripotent stem cells (also known as iPS cells or iPSCs) are a type of pluripotent stem cell that can be generated directly from adult cells. The iPSC technology was first pioneered by Takahashi and Yamanaka [63](#_ENREF_63). The induction of pluripotency in adult cells might decrease the need for blastocyst-derived hESC as a source for pluripotent cells. Although the generation of human iPS cells has been achieved in several laboratories, still there are many challenging problems that require resolution before it can be used as a clinical approach [64](#_ENREF_64). Some of these limitations are purifying a specific population, formation of tumors, potential immune rejection and some problems in appropriate experimental model for future studies [65](#_ENREF_65).

**Conclusion and future directions**

Reconstruction of ocular surface and development of cell therapies for corneal diseases are the main issues that need tenacity in recent years. This main issue needs further knowledge about potential cell sources, scaffold material, trophic factors and fabrication technologies. There are still many challenges and unanswered questions that need to be addressed before translation of stem cell therapy approaches to the clinical stages. One major challenge is providing an appropriate biocompatible tissue equivalent. Unfortunately, current use of animal products and allogeneic human cells and tissue corneal stem cell therapy has resulted in the development of serious ocular complications including graft-versus-host disease, cataract, dry eye, glaucoma and squamous cell carcinomas of the conjunctiva. Despite these practical problems, there are promising results by advancement of in vitro cell culture and expansion techniques and by identifying new appropriate biocompatible scaffold/nanoparticles in the near future for stem cell–based therapies of corneal blindness. These promising results would lead to the identification of possible newer therapeutic approaches for cellular transplantation to address corneal diseases. In the near future, with the combination of stem cell applications, novel ocular drug delivery systems would be capable of improving the therapeutic effects of current medication in corneal regenerative medicine.

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**Conflicts of interest statement**

The authors indicate no potential conflicts of interest.

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**Figure legends**

**Figure 1.** The cornea structure. The cornea contains five main parts including, the corneal epithelium, Bowman’s layer, the corneal stroma, Descemet’s membrane and the corneal endothelium.

**Figure 2.** Limbal epithelium as a major source of stem cells (SCs) for corneal epithelium renewal**.**





**Figure 1.** The cornea structure. The cornea contains five main parts including, the corneal epithelium, Bowman’s layer, the corneal stroma, Descemet’s membrane and the corneal endothelium.

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**Figure 2.** Limbal epithelium as a major source of stem cells (SCs) for corneal epithelium renewal**.**