Cornea and Ocular Surface Treatment

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Abstract: In addition to being a protective shield, the cornea represents two thirds of the eye's refractive power. Corneal pathology can affect one or all of the corneal layers, producing corneal opacity. Although full corneal thickness keratoplasty has been the standard procedure, the ideal strategy would be to replace only the damaged layer. Current difficulties in corneal transplantation, mainly immune rejection and shortage of organ supply, place more emphasis on the development of artificial corneas. Bioengineered corneas range from prosthetic devices that solely address the replacement of the corneal function, to tissue-engineered hydrogels that allow regeneration of the tissue.

Recently, major advances in the biology of corneal stem cells have been achieved. However, the therapeutic use of these stem cell types has the disadvantage of needing an intact stem cell compartment, which is usually damaged. In addition, long *ex vivo* culture is needed to generate enough cell numbers for transplantation.

In the near future, combination of advanced biomaterials with cells from abundant outer sources will allow advances in the field. For the former, magnetically aligned collagen is one of the most promising ones. For the latter, different cell types will be optimal: 1) for epithelial replacement: oral mucosal epithelium, ear epidermis, or bone marrow- mesenchymal stem cells, 2) for stromal regeneration: adipose-derived stem cells and 3) for endothelial replacement, the possibility of *in vitro* directed differentiation of adipose-derived stem cells towards endothelial cells provides an exciting new approach.

Keywords: Cell therapy, ophthalmology, cornea, stem cells, ocular surface.

1. DISEASES AND CLINICAL PROBLEMS OF THE CORNEA AND OCULAR SURFACE

The anterior part of the eye is formed by the sclera, the "white" part of the eye, and the cornea, the central, transparent one (Figs. **1A** and **1B**). The ocular surface corresponds to the part that is exposed anteriorly, whose function is to protect the eye from external insult and allow visual function. It includes the conjunctiva and the corneal epithelium. Both the conjunctiva and the cornea are always covered, in a healthy eye, by the tear film. The tear film allows for the adequate trophism of the corneal epithelium, the antibacterial protection of the ocular surface, and the refractive properties of the eye [1].

Histologically, the conjunctiva shows a non-keratinized stratified epithelium with goblet cells producing mucine, an essential part of the tear film, and a loose and richly vascularized stroma, with numerous immune cells. In a healthy eye, the conjunctival epithelium does not cover the surface of the cornea.

In addition to being a protective shield, the cornea represents two thirds of the eye's refractive power. The cornea is composed mainly of three layers (Fig. **2**). 1. The non-keratinized stratified epithelium, with no goblet cells. 2. The avascular and transparent corneal stroma that represents 90% of the corneal thickness, which is formed mainly of ordered collagen fibrils and proteoglycans and the cells that secrete them, the keratocytes. And the endothelium, a thin cell layer with extraordinary pumping functions in contact with the aqueous humor of the anterior chamber of the eye. The acellular basement membrane separating stroma and endothelium is known as Descemet's membrane (Fig. **2**).

The corneoscleral border is termed the limbus (Fig. **1A** and **1B**). Most studies locate the stem cells responsible for the turnover of the specialized corneal epithelial cells in the limbus, because corneal epithelial repopulation is both centripetal and apical, as in other epithelia [2-5].

Chemical or thermal burns, autoimmune diseases, infections, prolonged contact lens wear, iatrogenia (chronic topical treatments, multiple ocular surgeries) may damage the conjunctiva and the corneal limbal stem cells. Some hereditary deficiencies (especially the disease called aniridia) specifically show a dysfunction of the limbal stem cells. In response to the external insult, the conjunctiva scars, loses its goblet cells, becomes rough and dry and can no longer perform its function of protection and lubrication of the ocular surface. The loss of corneal limbal stem cells causes inability to maintain the normal specialized corneal epithelium. In order to compensate for the lacking corneal epithelium, the conjunctiva grows into the cornea. However, since the characteristics of the conjunctival epithelium make it inadequate to provide the smooth and transparent surface that the cornea needs to allow good vision, the corneal surface becomes irregular, with frequent epithelial erosions, vascularization and tendency towards stromal scarring. The limbal stem cell deficiency may be partial or complete, depending on the degree of loss of stem cells in the affected area, and total or partial, depending on the number of clock hours the limbus has been affected. These characteristics, and its uni- or bilaterality, will decide the therapeutic options, which will be based on the replacement of the normal corneal epithelium through the potentiation of the residual healthy stem cells or through a stem cell transplant [6]. However, the high expression of HLA antigens in the limbus increases the risk of rejection when allogenic corneo-limbal-scleral transplants are performed.

When the corneal stroma is also harmed, the degree of the lesion determines how the corneal wound heals: the keratocytes may produce new extracellular matrix and new collagen fibrils, and regenerate the stromal tissue while maintaining its transparency. However, greater damage causes keratocyte loss, infiltration by inflammatory cells, tissue destruction, and leads to more profound repairing mechanisms: the extracellular matrix becomes denser and

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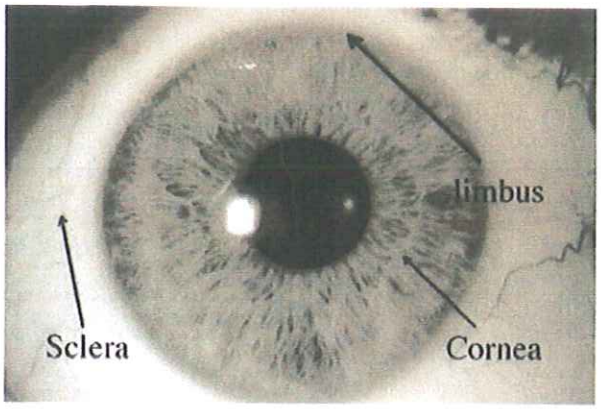

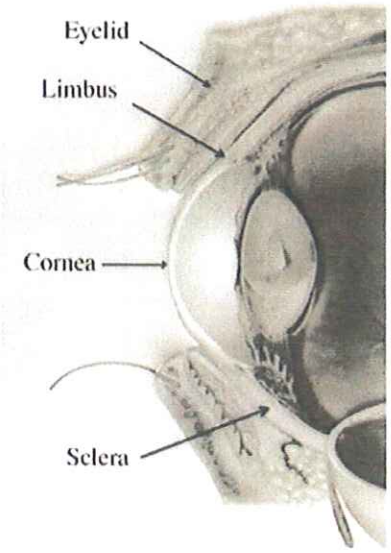

Fig. (1). Anatomical parts of the eye. A: Photograph of a human eye. 10x. B: Schematic representation of an eye sagittal section.

the collagen fibrils more disorganized, the stroma is filled with fibroblasts and blood vessels, and therefore the cornea loses its transparency. The way to restore it is to replace the cornea with a new, transparent one: a corneal graft. Due to the moderately high expression of HLA antigens in the cornea, the main problem in the long-term is the risk of rejection. To overcome this problem, several keratoprosthesis have been used with different results, but all of them encounter other complications such as infections and dehiscence that prevent them from constituting the definitive solution for corneal replacement [7-8]. The possibility of stromal repopulation and regeneration with cells from the host could decrease the need for allogenic corneal grafts.

The corneal endothelium covers the internal face of the cornea, in contact with the aqueous humor. Its main function is to keep the cornea in its optimal state of hydration and therefore transparent. The endothelial cells are the corneal cells that show the lowest mitotic activity [9-11]. Therefore, when after surgeries, traumas, etc., or just spontaneously, the number of endothelial cells decreases below a certain threshold, the cornea becomes edematous and loses its transparency. Currently, the only way to restore the endothelial function is to perform an allogenic graft, with the secondary risk of rejection. The possibility of growing autologous endothelial cells in the laboratory would improve the long-term outcomes in these corneas.

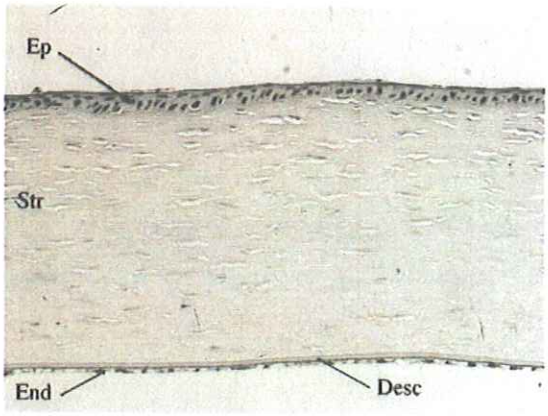

Fig. (2). Histological layers of the cornea. H&E stained rabbit's cornea. Ep: epithelium. Str: Stroma. End: Endothelium. Des: Descemet membrane. 200x.

2. ACELLULAR TREATMENT FOR THE CORNEA AND OCULAR SURFACE

Current difficulties in corneal transplantation, which are mainly primary immune rejection and shortage of organ supply, place more emphasis on the development of artificial corneas. Such replacements must fulfill the most important functions of a normal cornea, mainly refraction, transparency and protection. Bioengineered corneas are designed to replace part of the full thickness of a damaged or diseased cornea. They range from prosthetic devices that solely address replacement of the corneal function to tissue-engineered hydrogels that allow some regeneration of the host tissue.

The idea of an artificial cornea originated already in 1771 when Guillaume Pellier de Quengsy suggested replacing an opaque cornea with a silver-rimmed glass window, (reviewed in [12]), but it was not put into practice until 1853 when Nussbaum placed a quartz crystal implant in a rabbit's eye [13].

The ideal keratoprosthesis is defined as an epithelialized artificial button that could be implanted in a similar fashion to a penetrating keratoplasty [14]. Most of the recent designs employ a flexible, optically functional core intimately bonded to a surrounding microporous skirt that permits fibroblast ingrowth and extracellular matrix deposition, thereby providing anchorage and complete integration. The posterior surface should prevent attachment and proliferation of cellular component, therefore avoiding the formation of retroprosthetic membrane formation. It also has to allow for endothelial growth to prevent edema. Furthermore, in order to maintain any cellular component of the device, the optic must be permeable to oxygen and nutrients.

Clinically available keratoprostheses still have significant limitations such as retroprosthetic membrane inflammation and development of glaucoma, and are only used to treat the most high-risk patients. Some of the more commonly used keratoprosthesis are

briefly described in Table **1** [15-25]. An example of a keratoprosthesis made of polymethyl-methacrylate is shown in Fig. (**3**).

Newer keratoprostheses, like Aachen (silicone) used for short time surgery aid or SupraDescemetic (HEMA-MMA34) non penetrating keratoprosthesis, which avoids penetration of the prosthesis into the anterior chamber, and maintained Descemet's membrane and endothelium preservation have shown promising results [25], although further studies are still needed to solve crucial limitations.

3. TISSUE TRANSPLANTATION FOR THE CORNEA AND OCULAR SURFACE

Corneal pathology can be limited to one of the corneal layers or affect the full thickness, producing corneal opacity and therefore diminished visual acuity. Although full corneal thickness or penetrating keratoplasty (PKP) (Fig. **4A**) has been the standard procedure for most of the diseases, the ideal strategy would be replacing only the damaged layer [26].

3.1. Corneal Epithelium

Several surgical interventions use donor corneal tissue to treat ocular surface diseases with limbal stem cell deficiency. Amniotic membrane (AM) transplantation is often associated with limbal grafting to increase success rates. Moreover, corneal transparency is compromised on many occasions, and reconstruction of ocular surface is followed by penetrating or lamellar keratoplasty [27-32].

In contrast to standard corneal grafting, systemic immunosupression with cyclosporine A and/or mycophenolate mofetil maintains graft survival following limbal stem cell transplantation [32, 33].

3.1.1. Conjunctival Limbal Autograft (CLAU)

CLAU [34] is useful in unilateral limbal stem cell deficiency, such as in chemical or thermal burn of the cornea [33]. A conjunctival-limbal graft from the unaffected contralateral fellow eye is dissected and sutured into the recipient eye. The maximum amount of tissue that can be taken from the donor eye are two grafts of no more than 3 clock hours each taken from 12 and 6 clock hours. High reported success rates in most of the series have been reported, with long term survivals of up to 61% after 6 years [35] and improvement on visual acuity in around 2/3 of the eyes [28].

3.1.2. Living-Related Conjunctival Limbal Allograft (lrCLAL)

LrCLAL uses a conjunctival-limbal graft obtained from a patient's living relative to provide some degree of immune histocompatibility. Systemic immunosupression, although not always used, was associated with a further increase in survival rates [30]. Stable surface was achieved in 80-100% of patients after 17-24 months as reported by different authors [30, 36, 37], but long term results are lacking.

3.1.3. Keratolimbal Allograft (KLAL)

KLAL [34, 38] is a technique in which allogenic cadaveric limbal stem cells are transplanted to a recipient eye using the peripheral rim of cornea-scleral tissue (including limbus) as a carrier [39, 40]. It is mainly used to treat severe bilateral ocular surface disorders and unilateral disease if there are concerns about damaging the healthy fellow eye. This procedure produces best results in diseases with minimal conjunctival involvement such as aniridia or iatrogenic limbal stem cell deficiency [32]. Severe Stevens Johnson syndrome (SJS), ocular cicatrizial pemphigoid or recent chemical injuries, have poorer prognosis in the long term. In a series of 39 patients by Solomon *et al.* [41], the overall survival of ambulatory vision was 53.6% at 3 years and 44.6% at 5 years, significantly worse in SJS. In another case series by Ilari *et al.* [31] that included 23 eyes, graft survival rate was 54.4% at 1 year, 33.3% at 2 years, and 27.3% at 3 years.

3.1.4. Homologous Penetrating Central Technique of Sundmacher's Limbo-Keratoplasty or Eccentric Keratolimbal Allograft

First described as a penetrating procedure [42], it can also be performed as lamellar surgery [43]. It consists of an eccentrically trephined donor cornea, which includes limbus in 1/3 of the circumference. The donor button is placed centrally in the recipient corneal bed, so limbal tissue can be identified within the paracentral clear cornea. After 5 years, 65% of HLA matched grafts but only 14% of the untyped grafts were centrally clear [44], despite the use of systemic immunosuppressant.

In summary, autologous tissue transplantation is a reasonable option for unilateral ocular surface disease, especially when there is no total limbal stem cell failure. On the contrary, allogenic tissue transplantation has an only moderate-mild degree of success in the long term.

3.2. Corneal Stroma

Partial thickness or lamellar surgery has gained momentum recently, despite its higher technical complexity than PKP. Superficial (ALK) or deep anterior lamellar keratoplasty (DALK), replaces only the stromal tissue that is abnormal or damaged (Fig. **4B**), spar-

Table 1. Common Used Keratoprosthesis and their Most Common Complications

Keratoprosthesis Type	Background	Core/Skirt	Skirt/Haptic	Survival Reports	Most Common Complications
Osteo-Odonto Keratoprosthesis	1963[15], best proven results [16-18]	Polymethyl-methacrylate (PMMA) optical cylinder	Patient tooth	85% at 18 years [18]	Retroprosthetic membrane (RPM), inflammation, support tissue resorption, glaucoma.
Dohlman-Doane	Introduced in the 1990s [19]	PMMA	PMMA	75% at 10 months [20]	RPM, glaucoma.
Seoul-Type	2002 [21]	PMMA and fluorinated silicone	Polyurethane or polypropylene	66% at 62 months [22]	RPM, retinal detachment, glaucoma and endophthalmitis.
BIOKOP	1993 initial results good, recent studies disappointing maybe due to patients characteristics	PMMA/silicone	Porous expanded polytetrafluoroethylene	36% at 25 months [23]	Corneal melting, endophthalmitis, extrusion, RPM, dislocation.
AlphaCor	1997 [24]	Flexible poly2-hydroxyethyl-methacrylate (pHEMA)	Opaque porous sponge pHEMA	80% at 12 months [25]	Melting, retroprosthetic membrane, calcium deposition.

ing the healthy Descemet membrane and endothelium [45]. This is the procedure of choice in purely stromal diseases such as stromal dystrophies, corneal scarring or keratoconus. Visual outcome is comparable to standard penetrating keratoplasty (PK) when exposure of the Descemet membrane is achieved in deep anterior lamellar keratoplasty [46].

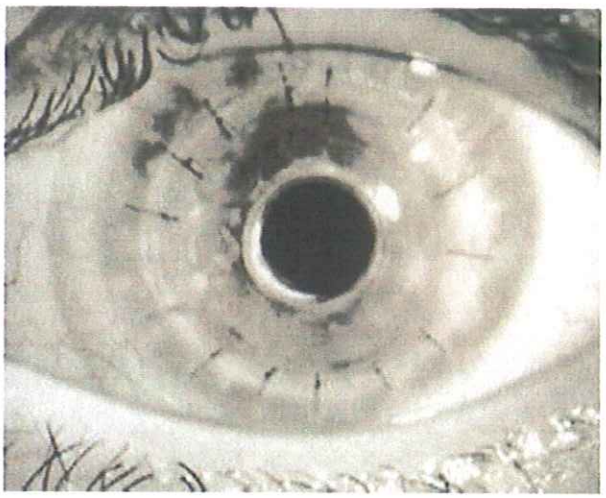

Fig. (3). Keratoprosthesis made of polymethyl-methacrylate in a human eye. 10x.

3.3. Corneal Endothelium

Endothelial keratoplasty, such as Descemet stripping automated endothelial keratoplasty (DSAEK), which is the most popular technique of its kind, replaces diseased host Descemet membrane and endothelium with a healthy donor posterior corneal button which includes some posterior stroma (50-100 μm), Descemet and endothelium [47]. The results of DSAEK surgery compared to conventional penetrating keratoplasty have shown more predictable results, quicker and with fewer postoperative complications. For example, a case series showed that 97% of the 74 eyes had a vision of 20/40 or better at 6 months and 14% obtained 20/20 or better [48]. Transplantation of the pure Descemet-endothelium complex has been described in the Descemet Membrane Endothelial keratoplasty (DMEK), but it is technically more difficult, has higher rates of failure, and still needs to show superiority over DSAEK [49].

4. CELL THERAPY FOR THE CORNEA AND OCULAR SURFACE

One of the most recent approaches to overcoming corneal damage is the use of Cell Therapy. Different types of cells have been used in this strategy.

4.1. Cells from the Eye

4.1.1. Corneal Epithelium

The limbus is the anatomical junction between the cornea and the conjunctiva (Fig. **1A &B**). The theory of the limbal zone serving as a source of corneal epithelial cells was first suggested in 1971 by Davanger and Evensen [50]. In 1986, Schermer *et al.* [3] created a model of corneal epithelial maturation *in vitro* and, using a monoclonal antibody against a 64kD keratin (a marker of mature epithelium), were able to characterize two cell populations at different stages of maturation – the suprabasal and the basal one. The latter was the youngest, and thus the progenitor. They also demonstrated that 64kD keratin was suprabasally located in the limbus. However, it was present throughout the central corneal epithelium, basal cells included. They concluded that the limbus was the niche of corneal epithelial stem cells, as confirmed later by other researchers [51]. Several reviews have been published about this issue [52-55]. Recent studies have suggested that the limbus is not the only niche for corneal stem cells [56]. They demonstrated that the entire ocular surface of the pig, including the cornea, contains stem cells with the ability to generate individual colonies of corneal and conjunctival cells.

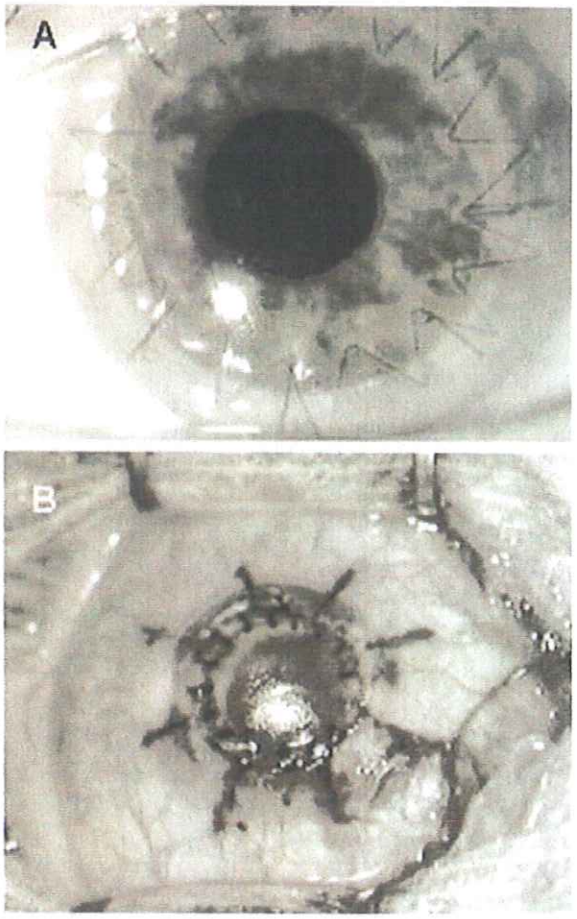

Fig. (4). Human corneal transplants. A: Penetrating keratoplasty. B: Lamellar keratoplasty. 20x.

Transplantation of *ex vivo* expanded limbal epithelial stem cells (LESCs) to reconstruct the ocular surface has been advocated as an alternative to direct grafting of the limbus, as detailed in section 3. It has several advantages over tissue transplantation, such as the possibility of autografting with cells obtained from minimal biopsy, and harvesting higher number of cells from a single donor without transplanting the immunogenic limbal tissue. On the other hand, as we will see, stem cells lose their original niche, and it is unclear if this, in the end, exhausts the stem cell capacity of the cells.

There are several clinical situations in which the limbus may be partially damaged. In these cases transplantation of AM alone may be sufficient to restore the corneal surface as it promotes the expansion of the residual LESCs [57-61]. However, if extensive damage is present, cell therapy is needed.

Several techniques have been used in order to cultivate LESCs. Firstly, Lindberg *et al.* [62] used a feeder layer system consisting of irradiated 3T3 fibroblasts which enable cell growth from limbal biopsy specimens as small as 1 mm^2. Pellegrini *et al.* [63] were the first to describe and successfully transplant autologous cultured

corneal sheets obtained by this method in two patients. Cell sheets were mounted either on a petrolatum gauze or on a soft contact lens and placed on the injured eye after removing the conjunctival epithelium. Secondly, the suitability of different carriers for cultivating LESCs has been tested, AM being the most appropriate one. Schwab, [64] was the first to successfully transplant autologous *ex vivo* expanded epithelial stem cells on denuded AM in patients. Several groups have later demonstrated the efficiency of this method [65-70]. The technique consists of obtaining expanded LESCs from a 1-3 mm^2 limbal biopsy from a healthy eye cultivated on 3T3 cells. Then AM is processed in order to remove its epithelium leaving the basement membrane intact. This structure is placed at the bottom of culture dishes and the donor epithelium is then harvested and cultured on the basement membrane side of the AM. After confluency is reached, everything is transplanted onto the denuded stromal corneal surface. Regarding these studies, other authors have compared the usefulness of intact and denuded AM as substrate [71, 72]. The epithelial outgrowth was quicker when cells were cultured onto denuded AM compared to intact AM. Likewise, the stratified appearance of this culture was similar to that of normal corneal epithelium, whereas intact AM culture didn't show a normal corneal epithelial morphology [71]. Grueterich *et al.* [72] used corneal differentiation markers, like keratin 3 (K3) and connexin 43 (Cx43), which are expressed on the basal layer of corneal epithelium but not on that of limbal epithelium, to address the process that was taking place in these two different cultures. In this work, the strongest expression of K3 and Cx43 on the basal layer was found in cultures grown on denuded AM with a 3T3 feeder layer. After transplantation, the full-thickness of the stratified epithelium cultivated on denuded AM was positively stained, whereas only suprabasal layers were positive for these markers in cultures grown on intact AM. They concluded that denuded AM with 3T3 feeders was the best option for cultivating, differentiating and transplanting corneal epithelial cells, whereas intact AM was more suitable for maintaining LESCs in culture. In bilateral total disease, if obtaining autologous healthy limbus proves impossible, fresh cadaveric eyes [67] or living related donors [73] as a source of donor cells may be used. Scientists have focused on improving techniques to make these cell sheets more suitable and easier to manipulate. This was the case of Nishida *et al.* [74] who expanded *ex vivo* human and rabbit LESCs co-cultured with mitomycin C treated 3T3 feeder layers on culture surfaces covalently coated with a temperature-responsive polymer. Sheets were harvested by reducing the temperature. These kinds of sheets did not need suturing to attach the stroma. Now what is of interest is to establish culture techniques that do not require fetal bovine serum, feeders or any unknown growth factor, as achieved in the studies of Nakamura *et al.* [75] and Yokoo *et al.* [76]. Several reviews have been published with further information [77-79].

The outcome measures used to define successful treatment and the follow up time are very heterogeneous and poorly described in most studies (reviewed in [78]). Clinical parameters are the most common measure of success, such as the establishment of a complete transparent corneal epithelium and resolution of conjunctivalization, vascularization and epithelial defects. Together, the studies achieve 76% successful treatment. Visual acuity is another outcome data some studies provide. In combination, the studies performed reach 79% of success. The most controversial question that arises from these studies is donor cell survival. As reviewed in [78], donor cells may persist for 7 to 9 months but thereafter are replaced by host cells [69, 78], suggesting that LESCs might promote the regeneration of the epithelium by the proper host stem cell population.

4.1.2. Conjunctival Epithelium

Cells of conjunctival origin are known to cover the corneal surface after epithelial damage. The goblet cells from the conjunctiva migrate to the injured area and transdifferentiate into corneal epithelial cells in a few days [80-82]. The existence of conjunctival epithelial stem cells located in the fornical epithelium has been reported [83]. Although conjunctival limbal graft associated with AM has been performed [84, 85], cultivated conjunctival stem cells have not yet been used in epithelial reconstruction.

4.1.3. Corneal Stroma

The presence of progenitor cells capable of differentiation into functional corneal keratocytes was first described in 2005 [86]. These progenitor cells expressed stem cell genes and early neural crest and ocular development genes such as PAX6. The same authors also reported the presence of pluripotent cells with keratocyte progenitor potential in the limbal corneal stroma [87]. When keratocytes are expanded *in vitro* in a serum-containing medium they lose their *in vivo* quiescent phenotype and acquire a fibroblastic phenotype with abnormal physiological properties [88]. However, in serum-free cultures they maintain their dendritic morphology and the production of keratan sulphate proteoglycans [89, 90]. In 2005 Yoshida *et al.* [91] reported a new method for subculturing mouse keratocytes in large quantities in a serum free medium aiming at maintaining their secretion of the cornea-specific proteoglycan keratocan, and the aldehyde dehydrogenase enzyme. Those cells might be used to restore corneal stroma when damaged. More recently, Mimura *et al.* [92] have constructed a substitute for corneal stroma using corneal fibroblast precursors or corneal fibroblasts together with porous gelatin hydrogels *in vitro* and transplantation into a rabbit model. In order to obtain the precursor cells, they dissociated the stroma into single cells which were cultured in a sphere-forming assay, and the primary spheres or the fibroblasts were then applied to the hydrogel and cultured. The whole structure was then transplanted into the rabbit ocular surface. They concluded that transplantation of fibroblast precursors combined with gelatin hydrogels is another possible treatment for corneal stromal regeneration. However, rabbits were sacrificed one and four weeks after transplantation, which makes it impossible to determine the stability of the graft. In our opinion, gelatin hydrogels might not be strong enough to resist eventual mechanical damage to the cornea. In fact, there are several papers in which the authors state that their scaffolds were "weak" or "unstable" [93-95, reviewed in 96]. Further development of stronger stroma-like scaffolds needs to be developed (see later, Conclusions and Future Perspectives).

4.1.4. Corneal Endothelium

Whikehart *et al.* in 2005 [97] demonstrated the existence of endothelial precursors in the human corneal endothelium for the first time. They reported the presence of these cells adjacent to the endothelial periphery at the limbal zone. In 2007 this group demonstrated the existence of stem cell markers (nestin, alkaline phosphatase, telomerase, Oct-3/4 and Wnt-1) in the same limbal cells [98]. The last 2 markers appeared after wounding and so did the differentiation markers Pax-6 and Sox-2. Similarly, Mimura *et al.* [99] have carried out a comparison of the central and peripheral cornea, finding that although both zones contain endothelial precursors, the peripheral one is enriched in these precursors.

The first studies using corneal endothelial cells for therapy did not take advantage of these endothelial stem cells. In 1998 several laboratories reported the isolation and long-term culture of human corneal endothelial cells *in vitro* [100, 101] and the optimization of their culture technique [102]. Some of them already used these cells to carry out transplantation onto human corneas [100, 103]. In the last 10 years several studies have reported the use of corneal endothelial cells to restore injured corneal endothelium in many different ways. Firstly, recipient corneas were stripped of their own endothelium by different methods (mechanical, chemical or physical), the last procedure proving to be the more efficient one. Cultured corneal endothelial cells were then seeded onto recipient corneas followed by centrifugation. They were then cultured and entirely transplanted into the recipient eye and attached with sutures [104-

106]. Functional studies of reconstructed corneas have shown that they achieve a maximum of 75% of the pump function of normal corneas [106, 107].

The most recent strategies use corneal endothelial precursors and new techniques in order to improve the suitability of transplantation. Using the sphere-forming assay, Mimura *et al.* [108] isolated endothelial progenitor cells and created a new method for transplantation. They injected the primary spheres with the human precursor endothelial cells into the anterior chamber of corneal endothelium deficient rabbits and showed that the graft reached almost 100% functionality [108-110]. The same group has performed a study using collagen sheet as a carrier. The Descemet membrane of the recipient was removed, and cultured human corneal endothelial cells were seeded on it. The sheet was then attached to the posterior stroma [111]. This technique has been shown to be less functional. All these experiments have been performed in rabbits and need a very large amount of corneal endothelial cells. An alternative source of endothelial precursor cells has to be searched for and developed.

4.2. Cells from Extraocular Sources

4.2.1 Epithelium

Although LESC allograft is a feasible treatment option for bilateral total limbal epithelial stem cell deficiency, there are risks associated with it, such as graft rejection despite oral immunosuppressive treatment. The use of autologous cells from extraocular origin could overcome some of these drawbacks. Nakamura *et al.* [112] carried out an experiment using autologous oral mucosal epithelial cells co-cultured with 3T3 feeders treated with mitomycin C and AM as a carrier. After 48 hours the entire corneal surface was free of epithelial defects and remained stable for about 13 months. By slit lamp examination using fluorescein staining they confirmed the survival of oral epithelium manifesting the characteristic staining pattern different from both the cornea and the conjunctival epithelium. Yano and colleagues performed similar experiments, but using the previously reported technique of temperature-responsive cell culture without AM as a carrier, both in rabbits [113] and human beings [114]. After transplantation, reconstructed ocular surfaces resembled native corneas morphologically, and corneal surfaces were completely protected from fluorescein penetration. As reviewed by Inatomi *et al.* [115], one of the most remarkable benefits of this approach is the swift epithelization, apart from also being an autologous source of cells that avoid rejection. A potential area of concern is the relatively high rate of peripheral corneal neovascularization [78, 112, 114, 115] although most of those vessels did not cause any postoperative complications. Again, further research is necessary.

Another approach to solving this problem has recently been proposed by Yang *et al.* [116, 117]. They developed a method for isolating and characterizing the epidermal adult stem cells (Epi-ASC) from ear of a goat. They cultured EpiASCs on denuded human AM and transplanted the autologous sheet onto the ocular surface of a goat model of limbal stem cell deficiency. Although the corneal surface was smooth and the epithelium was integral without goblet cells, clearness was not achieved in all transplanted eyes. Thirty percent of the treated eyes had whole transparent cornea, however part of the limbus was opaque; 50% of the treated eyes regained three quadrants of clear cornea, but the limbus of those eyes was also opaque. The remaining 20% failed. The authors compared a series of markers of corneal epithelium in normal and reconstructed corneas. Cytokeratins 3 and 12 and PAX-6 showed a similar expression profile in normal and reconstructed corneas.

Very recent studies have suggested that bone marrow-derived cells might be implicated in promoting corneal wound healing *in vivo* [118]. This finding might be supported by a proven strategy to repair corneal epithelial damage using human bone marrow-derived mesenchymal stem cells (BM-MSCs), as reported for the first time in 2006 by Ma *et al.* [119]. Briefly, cultured human BM-MSCs on AM were transplanted into chemically burned rat corneas achieving the same results in corneal epithelization and vision acuity as achieved by the same procedure using LESCs. Inhibition of both inflammation and inflammation-related angiogenesis as the main therapeutic effect was suggested, since human MSCs were detected in the rat epithelium but did not express corneal epithelial markers like CK3. These findings demonstrate the lack of differentiation of MSCs into corneal epithelial cells. However, Gu *et al.* [120] have found CK3 positive cells after transplanting BM-MSCs into a damaged rabbit cornea, although at a low percentage.

4.2.2. Stroma

The only published study using cells from an extraocular source in order to regenerate a corneal layer different from the epithelium is the one carried out by our laboratory [121]. We succeeded in repairing damaged corneal stroma in a rabbit model using MSCs from processed human lipoaspirate. We created a flap in the stroma and a 50 μm thick ablation was made. Adipose derived mesenchymal stem cells (ASC) were then delivered to the stromal pocket. Rabbits were sacrificed 12 weeks after transplantation. ASCs were able to differentiate into functional keratocytes when transplanted into the stroma, expressing keratocan and Aldh as normal keratocytes do (Fig. **5**). They also produced collagens type I and VI (the

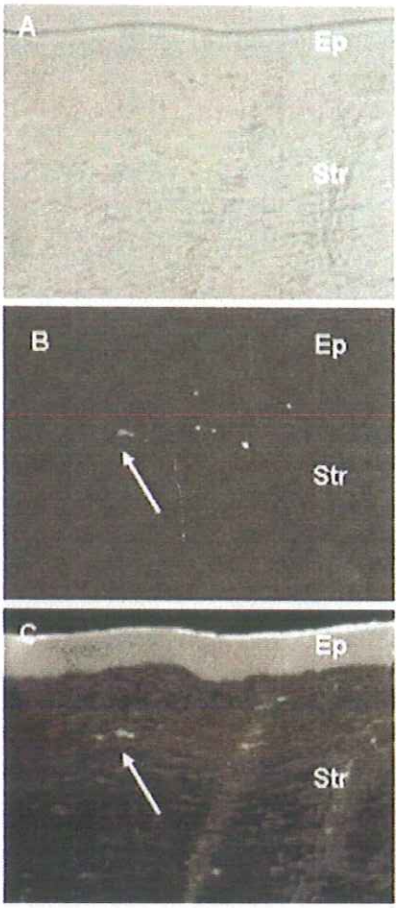

Fig. (5). ASC regeneration of the corneal stroma. Human ASC were introduced into partially-ablated stromal rabbit corneas and histologically sectioned after 12 weeks. Human keratocyte-specific keratocan was immunodetected, demonstrating ASC differentiation into functionally human keratocytes *in vivo*. A: Phase contrast microphotograph. B: The same section showing CM-DiI labeled human cells. C: Same section showing human keratocan in some of the transplanted cells (arrow). Ep: epithelium. Str: Stroma. 400x.

main components of corneal extracellular matrix). Furthermore, none of the rabbits showed immunological rejection. Our results are now being further tested for long-term restoration and stability of the transplanted cells in the stroma.

4.2.3. Endothelium

No studies have been performed using extraocular cell sources for corneal endothelial reconstruction. Interestingly, ASCs can be induced to differentiate into endothelial cells *in vitro* ([122, 123], and our own unpublished observations, Fig. 6), opening a whole new strategy for endothelial cell failure treatment. Our group is currently performing studies to test this approach for endothelial repair *in vivo*.

Fig. (6). CD31 positive endothelial cells induced *in vitro* from human ASC. 100x.

5. CONCLUSION AND FUTURE PERSPECTIVES

In the past few years, great advances in ocular surface and corneal stem cell identification and characterization have been described. In particular, major advances in the biology of corneal epithelial limbal stem cells [51, 124,125], corneal stromal stem cells [86, 87], corneal endothelial stem cells [90, 126], neural crest-derived corneal stem cells [91], limbal stromal mesenchymal cells [127, 128] and endothelial precursors [97] have been achieved (for review see [129]). However, despite these advances, the therapeutic use of these stem cell types presents the challenge of a need an intact stem cell compartment – constituting the main cause of the need of a transplant in the first place – or, in cases where it is possible, the use of the stem cells of the healthy contralateral eye, as autografts are more likely to succeed than allografts and do not need immune suppression [69, 78]. Even for patients with only one eye affected, the risk of damaging the stem cell compartment of the healthy eye is high [130]. Also, the number of stem cells in each compartment is very low, and long *ex vivo* culture is needed to generate sufficiently high numbers for successful transplantation.

As well as cell therapeutic approaches, new and improved biomaterials compatible with human cornea have been developed leading to advanced scaffolds that can be used to engineer an artificial cornea (keratoprostheses), such as poly-hydroxyethyl methacrylate hydrogels [12, 131], collagen-chondroitin sulphate hydrogels [93, 132], polyvinylpyrrolidone-coated silicon rubber [133, 134], polyurethanes [135] and perfluoroethers [136], together with a myriad of chemical modifications to promote epithelization (for review see [137]). Most of these studies aim to develop the corneal stroma, due to the high contribution of the stroma to the corneal thickness, together with its low cellularity (for an engineering-point-of-view review see [96]).

The combination of these scaffolds with cells is the future of corneal tissue engineering (the so-called tissue-engineered corneal equivalents). Some studies have already been published that use mainly corneal cell lines (for an elegant review see [137]), including corneal epithelium [138] and corneal stromal keratocytes [92, 139], or even the three corneal layers cells [93, 140].

In the near future, combination of advanced biomaterials with autologous cells from outer sources where they are abundant will allow further advances in the field. For the former, magnetically aligned collagen [141], which provides both strength and transparency, is one of the most promising. For the latter, different cell types will be the optimal sources for the different corneal layers: 1) for epithelial replacement: oral mucosal epithelium, ear epidermis, or BM-MSC [114, 117, 120], 2) for stromal regeneration: ASCs [121] and 3) for endothelial replacement: although still in development, the possibility of *in vitro* directed differentiation of ASC into endothelial cells [122, 123], provides an exciting new approach.

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