Limbal stem cell deficiency (LSCD) can result from a variety of corneal disorders, including chemical and thermal burns, infections, and autoimmune diseases. The symptoms of LSCD may include irritation, epiphora, blepharospasms, photophobia, pain, and decreased vision. There are a number of treatment options, ranging from nonsurgical treatments for mild LSCD to various forms of surgery that involve different cell types cultured on various substrates. Ex vivo expansion of limbal epithelial cells (LEC) involves the culture of LEC harvested either from the patient, a living relative, or a cadaver on a substrate in the laboratory. Following the transfer of the cultured cell sheet onto the cornea of patients suffering from LSCD, a successful outcome can be expected in approximately three out of four patients. The phenotype of the cultured cells has proven to be a key predictor of success. The choice of culture substrate is known to affect the phenotype. Several studies have shown that amniotic membrane (AM) can be used as a substrate for expansion of LEC for subsequent transplantation in the treatment of LSCD. There is currently a debate over whether AM should be denuded (i.e., de-epithelialized) prior to LEC culture, or whether this substrate should remain intact. In addition, crosslinking of the AM has been used to increase the thermal and mechanical stability, optical transparency, and resistance to collagenase digestion of AM. In the present review, we discuss the rationale for using altered versus unaltered AM as a culture substrate for LEC.

INTRODUCTION

In the early 1900s, Davies was the first to report the therapeutic use of human amniotic membrane (AM) in skin transplantation to treat burned and ulcerated skin surfaces [1]. A considerable decrease in pain and improved rate of skin-surface healing was reported. Subsequently, there was a lag period of more than 2 decades before any additional use of AM was reported in the literature. In the 1930s, AM was applied in surgical reconstruction of vaginas [2]. Thereafter, AM has been used following head injury to prevent meningocerebral adhesions [3], in repair of abdominal herniation [4], in closure of pericardium [5], for treatment of nonhealing wounds in diabetic patients [6], to aid head and neck surgery [7], as a biological dressing in correction of abdominal birth defects [8], for surgical repair of refractory labial adhesions [9], in wounds as a biologic dressing [10], and after total removal of the tongue [11].

In the 1940s, several authors reported the beneficial role of AM in treating a variety of ocular surface disorders [12–15]. It was first used as a substitute for rabbit peritoneum in the management of chemical burns of the eye. Successful outcomes were reported with dried amniotic tissue,
termed “amnioplastin” [12, 13]. Following these initial procedures, there was no report on the use of AM in ophthalmology until the early 1990s, when AM experienced a renaissance with reference to treatment of ocular surface disorders. In 1993, Batle and Perdomo introduced AM preserved in 95% ethyl alcohol as a substitute for conjunctival membranes in fornix reconstruction and in the treatment of recurrent pterygia and alkali burns [16]. Two years later, Tseng and Kim performed AM transplantation in rabbits for ocular surface reconstruction [17]. Subsequently, various authors have reported the beneficial effects of human AM transplantation in ever-expanding ocular indications [18].

Numerous studies have demonstrated that AM also can be used as a substrate for expanding limbal epithelial stem cells (LEC) for subsequent transplantation in the treatment of limbal stem cell deficiency (LSCD) [19]. Tsai et al. were the first to report the use of AM to culture LEC [20]. The choice of culture substrate for LEC is of key importance for growth characteristics and phenotype preservation. However, so far there is no standardized culture method for LEC on the AM. Various culture techniques are used are used to culture LEC on AM. They differ regarding the composition of AM (e.g., AM with or without the epithelium), air-lifting prior to transplantation, and the use of an additional 3T3 feeder layer. Furthermore, there are challenges with human AM that still are undetermined, for example, the thinness of membrane affecting the suture strength, crushing while transplanting, early detachment, and considerable dissolution of the membrane after transplantation [21]. In order to improve these characteristics, researchers have focused on different methods to alter the AM and increase the mechanical and thermal stability, optical transparency, and resistance to collagenases. It has been proposed that the devitalized epithelium on preserved AM may be of significant importance to promote expanded human LEC maintain a less differentiated phenotype compared with the limbal basal epithelium in vivo [22]. On the other hand, studies have shown that the intact AM (with the amniotic epithelium) exhibits higher levels of growth factors compared with epithelially denuded AM [23]. The growth factors are implicated in epithelium–stroma interactions of the human ocular surface [24]; therefore, the amniotic epithelium may have a substantial role in the microenvironment niche of limbal progenitor cells. More research is warranted to explore this potential mechanism of action in order to control LEC behavior. Additionally, further research on alteration of AM may improve its properties and thereby increase the therapeutic efficacies.

The present review is also timely as AM has recently been used as a culture substrate for simple limbal epithelial transplantation (SLET) [25]. This is a new clinical procedure for the treatment of unilateral LSCD. In SLET, a small piece of limbal tissue (e.g., 2 × 2 mm) is divided into smaller pieces and distributed over an AM placed on the cornea. Although long-term results are not available, the results so far are promising. The influence of AM preparation method on short- and long-term clinical outcome following SLET is unknown, but laboratory and clinical data based on LEC cultured on altered and unaltered AM ex vivo are clearly relevant to consider when designing future SLET studies where the culture is performed in vivo instead of ex vivo.

**Mechanical Properties and Possible Mechanisms of Action**

The AM is the innermost layer of the fetal membranes, and is normally 0.02–0.5 mm in thickness [26, 27]. The AM consists of five layers, from the innermost outward: (a) epithelium, (b) basement membrane, (c) compact layer, (d) fibroblast layer, and (e) spongy layer (Fig. 1) [26]. The monolayer of cells in the epithelial layer varies from columnar over the placenta to cuboidal or flat away from the placenta [26]. The basement membrane is a thin layer composed of reticular fibers. It adheres closely to the amniotic epithelium from which multiple processes interdigitate into it. The remaining three layers are collectively termed the stroma. The compact layer is a dense layer almost totally devoid of cells and consists mainly of a complex reticular network. The fibroblastic layer is the thickest layer of the AM and consists of fibroblasts embedded in a loose network of reticulum. The outermost spongy layer forms the interface between the AM and chorion and consists of wavy bundles of reticulum covered with mucin [27]. The AM supports the homeostasis of amniotic fluid [28]; however, its precise function is still elusive. During pregnancy, the amniotic epithelium is metabolically active [28, 29]. It lacks a blood supply of its own; oxygen and nutrients are derived from the amniotic fluid, surrounding choriocoele fluid, and fetal surface blood vessels. It is suggested that energy is derived through an anaerobic glycolytic process due to limited oxygen supply [30].

The AM exhibits several properties that makes it suitable for use in tissue engineering [31]. Cells in the epithelial layer of the AM have significant similarities to stem cells. They express pluripotent markers of stem cells, have the ability to be differentiated into all three germ layers, and have no need for a feeder layer throughout their cultivation [31]. Other important characterizations of AM crucial for use in tissue engineering are its antitumorigenic, anti-fibrotic, anti-inflammatory, antimicrobial, antiscarring, low immunogenicity, and useful mechanical properties [31].

There are, however, some challenges with the use of AM in tissue engineering. The AM has a thin structure and exhibits technical limitations when suturing. It has been suggested that the use of glue as a substitute for suturing may be promising [32]. Furthermore, the AM shows a viscoelastic mechanical response [31]. In a majority of tissues, viscoelasticity is crucial for scaffolding, for example, stiff scaffolds of the arteries that may encourage hyperplasia and occlusion [33]. It has been demonstrated that preterm AM exhibits greater mechanical integrity compared with term AM. However, the stiffness of term AM is more applicable for a majority of protocols in tissue engineering [34]. It has been suggested that this may be related to the collagen content, although there are contradictory studies showing that the content of amnion collagen decreases with gestational age [35]. Moreover, it is...
also proposed that elastin, which is detected in the fetal amnion, provides the molecular basis for elasticity in the AM [36].

There are differences concerning AM location, that is, samples of AM taken from locations distal and proximal to the placental disc. It has been demonstrated that proximal human samples of AM are thicker and stronger, however, with poorer optical properties compared with distal samples [37]. Furthermore, AM may be used in surgical procedures either fresh or modified through different preservation methods such as cryopreservation, freezing, or lyophilization [38]. Cryopreservation, compared to freezing, seeks to reach very low temperatures without causing additional damage by the formation of ice during freezing. It has been reported that cryopreservation better preserves growth factors compared to freezing [38]. When comparing cryopreserved and fresh AM, it is shown that epithelial cells do not survive cryopreservation and they exhibit poor proliferative capacity. No morphological differences were detected between fresh and cryopreserved AM [39]. Recently, studies have shown that the combination of AM preservation and sterilization by gamma-irradiation, paracetamol, and/or trehalose reduces the risk of infections that may be transmitted by AM [38].

The AM secretes several growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), basic fibroblast growth factor, platelet-derived growth factor (PDGF), and transforming growth factor β (TGFβ) [40, 41]. EGF is a powerful mitogen for the growth of epithelial cells and its high level of expression following transplantation may explain improved wound healing of the ocular surface [23]. It has been shown that EGF is mainly found in the amniotic epithelium [38]. PDGF participates in cellular responses including proliferation, migration, survival, and the deposition of extracellular matrix and tissue remodeling factors [42]. Koizumi et al. reported that the amniotic epithelium secretes HGF and keratinocyte growth factor (KGF), which are also produced by mesenchymal cells such as fibroblasts in corneal stroma [23]. Growth factors transferred in the epithelium of AM may affect wound healing of cornea through paracrine action [43, 44]. It may therefore be suggested that ocular surface re-epithelialization may be accelerated by HGF and KGF secreted by the amniotic epithelium following transplantation of AM.

Studies have also shown an anti-inflammatory effect associated with AM [19, 45, 46]. Expression of IL-1α and IL-1β by human LEC was significantly suppressed when cultured on the stromal matrix of the AM, even when challenged by application of bacterial derived lipopolysaccharides [46]. In a study in which the corneas of rabbits were covered by human AM after phototherapeutic keratotomy, acute inflammatory reaction was significantly reduced by apoptosis of polymorphonuclear neutrophils [47]. This finding was also supported in patients with acute burns where CD20+ lymphocytes were trapped by the AM and exhibited cell death [48]. Upon inoculation of rat corneas with herpes simplex virus type 1 to induce necrotizing keratitis, inflammation decreased when the cornea was covered with preserved human AM [49]. Chronic inflammation in the limbal region can cause LSCD. Furthermore, inflammation can negatively affect integration of transplanted conjunctival-limbal auto-grafts in the treatment of LSCD [50]. Thus, the anti-inflammatory property of AM may be a considerable benefit. Furthermore, numerous factors participate in the antifibrotic effect of the AM [24, 51]. Tseng et al. have shown that it induces a downregulation of TGFβ signaling, which is responsible for activation of fibroblasts in wound healing [51].

**Culture Techniques and Use of Intact and Denuded AM**

Currently, there is no standardized method for ex vivo expansion of LEC. Culture of LEC can follow the explant or cell suspension method. In the explant method, cells grow out from a small biopsy attached to the base of the culture dish. Cell suspension means that cells are first enzymatically released from the tissue. Once attached to the base of a culture dish the single cells divide and grow to form a confluent layer. Some culture methods use air-lifting to encourage differentiation of the superficial layer. This is achieved via lowering the medium until it is just at the level of the superficial cell layer. The use of irradiated or Myotomicin C treated mouse embryonic fibroblasts was originally developed to enable culture of skin epidermal cells [52]. It is now a culture technique often used for culture of all types of epithelial cells to supply cytokines and growth factors that promote proliferation.

**In Vitro Experiments with Intact and Denuded AM**

The precise role of the devitalized amniotic epithelium is not yet fully understood. It is suggested that the devitalized epithelium covering the amniotic basement membrane may be important to help expanded human LEC assume a less differentiated epithelial phenotype [22]. Native intact AM has been found to comprise higher levels of growth factors compared to denuded AM [23], suggesting that they are primarily present in the amniotic epithelium. These growth factors are believed to be involved in epithelium–stroma interactions of the human ocular surface [24].

Several studies have shown that LEC cultured on an intact AM maintain a more stem cell-like phenotype compared with LEC cultured on a denuded AM [22, 53, 54]. Expression of slow cycling and label-retaining cells that do not express the differentiation-associated markers K3, K12 [22, 55], or Cx43 [22] has been demonstrated in limbal epithelial sheets cultured on intact AM. Krishnan et al. compared the expression of ΔNp63α, a marker for nondifferentiated cells, in LEC cultured on intact human AM with AM denuded human AM [56]. Interestingly, only LEC cultured on intact AM gave rise to ΔNp63α expression [56]. The expression of ΔNp63α isoforms ΔNp63α and ΔNp63α has been confirmed in other studies in which LEC has been cultured on intact AM.

The nerve growth factor signaling pathway, which is known to be involved in stem cell survival, was preserved in the intact AM culture system [22]. Furthermore, cultured LEC on intact human AM has been found to maintain high proliferative potential when compared to denuded human AM [56]. However, contrary results have also been demonstrated [22, 59]. Koizumi et al. showed that LEC cultured on a denuded AM formed a more stratified and differentiated epithelium and exhibited a higher number of desmosomes and hemi-desmosomes compared to culture on intact AM [59, 60]. The authors concluded that for purposes of transplantation of differentiated epithelial sheets, denuded AM is probably the more suitable carrier for human LEC cultures when using the cell-suspension culture system. However, denuded AM did not improve the structural integrity of cultured human LEC following 1 week of eye bank storage [61]. Moreover, the highest levels of K3 and Cx43 were observed when denuded AM was used without an additional 3T3 feeder layer (fibroblasts synthesizing the extracellular layer and collagen) [22]. Addition of a 3T3 feeder layer to denuded AM increased the level of Cx43 but decreased that of Cx50, reflecting a less differentiated phenotype compared with denuded AM without 3T3 fibroblasts.
Clinical Studies Using Intact and Denuded AM

Only seven clinical studies (substudies excluded) involving transplantation of ex vivo cultured LEC have applied intact AM [20, 62–68] (Table 1) as a culture substrate, whereas 29 clinical studies used denuded AM to culture LEC [69–97] (Table 2).

In 2000, Tsai et al. were the first to report the use of intact AM to culture LEC to treat patients with unilateral partial or total LSCD [20]. The authors used autologous limbal tissue obtained from a biopsy of the contralateral eye for explant cultures on cryopreserved intact AM. The results showed a success rate of 83% with reference to visual acuity and a 100% success rate regarding reconstruction of a stable ocular surface. During the follow-up time of 15 months, no conjunctivalization was observed in the treated eyes (Table 1). The remaining six studies all performed transplantation of ex vivo cultured limbal epithelium on intact AM without the use of a 3T3 fibroblast feeder layer or air-lifting (Table 1). With a mean follow-up time of 22 months (range: 14 [67] to 48 [68] months), visual acuity improved, ranging from 56% [68] to 83% [20, 67]. Immunosuppression was used in four studies [62, 64, 65, 68] and conjunctivalization was reported in one study [68].

The first clinical trial using denuded AM as a culture substrate for LEC in treating LSCD was published in 2000 by Schwab et al. [90]. LEC were expanded on an inactivated 3T3 fibroblast feeder layer and subsequently seeded onto denuded AM. Ten of fourteen patients with allogeneic and 6 of 10 patients with autologous transplants maintained a stable corneal surface after a follow-up period of between 6 and 19 months. A year later, two cases of acute Stevens–Johnson syndrome with large persistent epithelial defects were treated with the same technique [79]. The authors expanded allogeneic limbal tissue from donor corneal buttons on denuded AM, taking advantage of an inactivated 3T3 fibroblast feeder layer. The renewed epithelium was stable and without defects after a follow-up time of 6 months. Koizumi et al. thereafter used the same approach to treat 13 patients with total LSCD. Ten of thirteen eyes exhibited visual improvement and a stable ocular surface without epithelial breakdown after a mean follow-up period of 11.2 months [78].

In 2002, Shimazaki et al., using denuded AM, reported on the transplantation of ex vivo expanded LEC from allogeneic (n = 7) and living related (n = 7) donors to 13 eyes with total LSCD [93]. They showed that corneal epithelial restoration was achieved in 46.2% of cases. One eye did not show epithelialization at all, five eyes failed with recurrent conjunctivalization, and one eye failed with dermal epithelialization. Following transplantation of cultivated allogeneic LEC on AM, improved visual acuity was observed in 77% of patients.

The remaining studies using denuded AM as a culture substrate for LEC used both allogeneic [69, 74, 77, 80, 82, 86, 91, 92, 95–97] and autologous [70–73, 75–77, 80, 81, 83–87, 89–92, 94–97] explants, with and without the use of a 3T3 fibroblast feeder layer or air-lifting (Table 2). Immunosuppression was used in all studies using allogeneic limbal explants except for one [88], and in some studies using autologous explants (Table 2). The reported follow-up period was up to 66 months. Following transplantation of cultured LEC on denuded AM, visual acuity ranged from 53% to 100%. Moreover, 100% clinical success was reported in 7 of 29 studies (Table 2).

Table 1. Clinical studies using ex vivo expansion of LEC on intact amniotic membrane

| Author, year | Culture system | Air-lifting | 3T3 | Immunosuppression | Follow-up time (months) | Clinical success |
|--------------|----------------|-------------|-----|-------------------|------------------------|-----------------|
| Tsai et al. (2000) | Autologous explant | No | No | No | Mean: 15 (12–18) | Stable ocular surface: 100% |
|               |                |             |     |                   | Visual acuity: Improved in 83% |
|               |                |             |     |                   | Conjunctivalization: No |
| Grueterich et al. (2002) | Autologous explant | No | No | No | 21 | Stable ocular surface: 100% |
|               |                |             |     |                   | Visual acuity: Improved |
|               |                |             |     |                   | Conjunctivalization: No |
| Tseng et al. (2002) | Autologous and allogeneic explant | No | No | No | 14 | Stable ocular surface: 100% |
|               |                |             |     |                   | Visual acuity: Improved in 83% |
|               |                |             |     |                   | Conjunctivalization: No |
| Fatima et al. (2007) | Autologous explant | No | No | Topical steroids | 37 | Stable ocular surface: 100% |
|               |                |             |     |                   | Visual acuity: Improved |
|               |                |             |     |                   | Conjunctivalization: No |
| Kolli et al. (2010) | Autologous explant | No | No | Topical steroids | Mean: 19 (12–30) | Stable ocular surface: 100% |
|               |                |             |     |                   | Visual acuity: Improved in 62% |
|               |                |             |     |                   | Conjunctivalization: No |
| Pauklin et al. (2010) | Allogeneic explant | No | No | Cyclosporin A | Mean: 28.5 ± 14.9 | Stable ocular surface: 68% |
|               |                |             |     |                   | Visual acuity: Improved in 73% |
|               |                |             |     |                   | Conjunctivalization: No |
| Pathak et al. (2013) | Autologous explant | No | No | Topical steroids | 11–48 | Stable ocular surface: 56% |
|               |                |             |     |                   | Visual acuity: Improved in 22% |
|               |                |             |     |                   | Conjunctivalization: 440025; |

Abbreviation: LEC, limbal epithelial cells.
| Author, year                  | Culture system                                      | Air-lifting | 3T3 | Immunosuppression                           | Follow-up time (months) | Clinical success                  |
|-----------------------------|-----------------------------------------------------|-------------|-----|--------------------------------------------|-------------------------|----------------------------------|
| Schwab et al. (2000)        | Autologous and allogeneic cell suspension           | Yes         | Yes | Topical and systemic steroids, Cyclosporin A | Median: 11 (6–19)       | Stable ocular surface: 71%       |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 100%  |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: 29%        |
| Koizumi et al. (2001)       | Allogeneic explant                                  | Yes         | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | Mean: 11.2 ± 1.3       | Stable ocular surface: 77%       |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 100%  |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: 23%        |
| Koizumi et al. (2001)       | Allogeneic explant                                  | Yes         | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | 6                       | Stable ocular surface: 100%      |
| Shimazaki et al. (2002)     | Allogeneic explant                                  | No          | No  | Topical and systemic steroids, Cyclosporin A | Short-term              | Stable ocular surface: 46%       |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 77%   |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: 38%        |
| Nakamura et al. (2003)      | Allogeneic explant                                  | Yes         | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | 14                     | Stable ocular surface: 100%      |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Markedly improved|
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: No         |
| Sangwan et al. (2003)       | Allogeneic explant                                  | No          | No  | No                                         | 5                       | Stable ocular surface: 100%      |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 100%  |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: No         |
| Harkin et al. (2004)        | Autologous cell suspension                          | Yes         | Yes | No                                         | 15                     | Stable ocular surface: 100%      |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 100%  |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: Not reported|
| Nakamura et al. (2004)      | Autologous explant                                  | Yes         | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | 19                     | Stable ocular surface: 100%      |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 100%  |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: No         |
| Sangwan et al. (2005)       | Autologous and allogeneic explant                   | No          | No  | Systemic steroids, Cyclosporin A           | Mean: 8.3 ± 5.0         | Stable ocular surface: 93%       |
|                             |                                                     |             |     |                                            |                         | Visual acuity: Improved in 53%   |
|                             |                                                     |             |     |                                            |                         | Conjunctivalization: No         |
| Author, year | Culture system | Air-lifting | 3T3 | Immunosuppression | Follow-up time (months) | Clinical success |
|-------------|----------------|-------------|-----|-------------------|------------------------|-----------------|
| Sangwan et al. (2005) | Autologous and allogeneic explant | No | No | Systemic steroids, Cyclosporin A | 25–34 | Stable ocular surface: 100% Visual acuity: Improved in 100% Conjunctivalization: No |
| Fatima et al. (2006) | Autologous explant | No | No | No | 6 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: No |
| Nakamura et al. (2006) | Autologous and allogeneic cell suspension | Yes | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | Mean: 14.6 ± 4.4 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: No |
| Sangwan et al. (2006) | Autologous explant | No | No | No | Mean: 18.3 | Stable ocular surface: 73% Visual acuity: Improved Conjunctivalization: No |
| Ang et al. (2007) | Allogeneic cell suspension | Yes | Yes | Systemic steroids, Cyclosporin A, Cyclophosphamide | 48 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: No |
| Kawashima et al. (2007) | Autologous and allogeneic explant | Yes | Yes | Systemic steroids, Cyclosporin A | Mean: 25.1 ± 13.2 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: No |
| Shimazaki et al. (2007) | Autologous cell suspension | No | No | Systemic steroids, Cyclosporin A | 31 | Stable ocular surface: 59% Visual acuity: Improved Conjunctivalization: No |
| Shortt et al. (2008) | Autologous and allogeneic cell suspension | No | No | Systemic steroids, Cyclosporin A | 13 | Stable ocular surface: 100% Visual acuity: Improved in 70% Conjunctivalization: No |
| Gomes et al. (2009) | Allogeneic explant | Yes | Yes | Systemic steroids | 12 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: Yes |
| Sahu et al. (2009) | Autologous explant | No | No | Topical steroids | 10 | Stable ocular surface: 100% Visual acuity: Improved Conjunctivalization: No |
| Author, year          | Culture system                          | Air-lifting | 3T3 | Immunosuppression                     | Follow-up time (months) | Clinical success                                      |
|-----------------------|-----------------------------------------|-------------|-----|---------------------------------------|-------------------------|------------------------------------------------------|
| Satake et al. (2009)  | Autologous explant                      | No          | No  | No                                    | 43                      | Stable ocular surface: 100%                           |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: No                               |
| Baradaran-Rafi et al. (2010) | Autologous explant                   | No          | No  | Topical and systemic steroids         | Mean: 34.0 ± 13.5       | Stable ocular surface: 100%                           |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: 12%                              |
| Dobrowolski et al. (2011) | Autologous explant                       | Yes         | Yes | No                                    | Mean: 4.9 ± 1.1         | Stable ocular surface: 72%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: 60%                              |
| Sangwan et al. (2011) | Autologous explant                      | Yes         | Yes | Systemic steroids                     | Mean: 36.0 ± 19.2       | Stable ocular surface: 71%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved in 60%                        |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: Yes                              |
| Sharma et al. (2011)  | Autologous and allogeneic explant       | No          | No  | Systemic steroids, Cyclosporin A      | Mean: 11.0 ± 8.0        | Stable ocular surface: 68%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved in 74%                        |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: Yes                              |
| Basu et al. (2012)    | Autologous explant                      | No          | No  | Topical steroids                      | Mean: 27.6 ± 16.8       | Stable ocular surface: 100%                           |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: 34%                              |
| Shigeyasu et al. (2012) | Autologous and allogeneic explant     | Yes         | Yes | Topical steroids                      | 48–66                   | Stable ocular surface: 100%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: No                               |
| Vazirani et al. (2014) | Autologous and allogeneic explant       | No          | No  | Topical steroids                      | 12                     | Stable ocular surface: 63%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved in 100%                        |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: 37%                              |
| Guarneri et al. (2014) | Autologous explant                      | No          | No  | Topical steroids                      | Mean: 17.5 ± 7          | Stable ocular surface: 59%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved in 63%                        |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: 27%                              |
| Zakaria et al. (2014) | Autologous and allogeneic explant       | No          | No  | Systemic steroids, Cyclosporin A      | Mean: 22 (4–43)         | Stable ocular surface: 67%                            |
|                       |                                         |             |     |                                       |                         | Visual acuity: Improved                               |
|                       |                                         |             |     |                                       |                         | Conjunctivalization: Yes                              |

Abbreviation: LEC, limbal epithelial cells.
CROSSLINKING OF AM

The topography of the underlying substrate affects the cells, and it has been shown that physical cues control cell morphology, migration, and embryonic development [98]. Studies using photolithography showed that surfaces with single 5-μm-tall steps was sufficient to selectively slow the migration rate of baby hamster kidney and fibroblast cell types, but not of neutrophils [99]. Microarray analysis of cells seeded onto substrates with hexagonal pits compared with flat surfaces demonstrated significant changes in expression of hundreds of genes that were associated with extracellular matrix protein production and regulation of cell-cycle modifications that are important for its structural and mechanical properties, and the interruption of some of these processes leads to severe dysfunction of the cells. The final steps in the formation of collagen include the cleavage of the N and C pro-peptides, self-assembly of the resulting collagen molecules into fibrils, and formation of covalent crosslinks [102]. Optimal crosslinking of collagen is essential for collagen binding to its receptors; however, it is also important for regulation of the availability of growth factors and for the mechanical characteristics of the extracellular matrix [103]. Previous studies have shown that the inhibition of collagen crosslinking in the mouse preosteoblast cell line weakens the osteogenic program [104]. Furthermore, impairing the crosslinking of collagen is associated with exposure of cryptic nucleation sites, resulting in enhanced mineralization [105]. Insufficient collagen crosslinking makes the collagen more prone to proteolytic degradation [106].

Collagen nanofibers, an essential structural component of the AM, exhibit significant degradation after being exposed to endogenous collagenases in vivo. The collagenase activity is enhanced in many diseases affecting the cornea and may therefore lead to accelerated degradation of AM transplants [107]. Spoerl et al. demonstrated that insufficient biological stability of an AM graft may be a significant cause of early AM detachment during corneal wound healing [108]. As enzymatic degradation of the AM matrix is considered a major cause of failure after surgical transplantation, the development of strategies for improvement of the molecular biostability of AM is warranted. Since it is desirable that the collagen in the AM serves as a limbal stem cell niche, several researchers have tried to modify it to a crosslinked molecular biopolymer chain network. Different crosslinking strategies have been used in order to increase the stability of AM for culture of LEC, including glutaraldehyde [108–111], carbodiimide [112–117], radiation- [111], photo- [118], and Al2(SO4)3- [21] crosslinking (Table 3).

Glutaraldehyde Crosslinking

Glutaraldehyde is a widely used, highly effective, chemical crosslinking substrate used for the stabilization of collagenous biomaterials. Fujisato et al. have demonstrated that glutaraldehyde crosslinked AM is more resistant to degradation from collagenases [111]. It has also been demonstrated that the effect of glutaraldehyde crosslinking on the nanostructure of AM material is critical to maintenance of LEC stemness [109]. Furthermore, glutaraldehyde crosslinking of collagenous materials affects corneal epithelial characteristics of stem cell culture [109]. After modification with glutaraldehyde using a variable crosslinking activation time, the AM samples were investigated by determining the degree of crosslinking, nanofibrous structure, in vitro degradability, cytocompatibility, anti-inflammatory activity, and stemness gene expression. After a 6-hour reaction time, the crosslinking degree and in vitro degradability of glutaraldehyde treated samples were much lower than those of the carbodiimide crosslinked counterparts. Furthermore, the increased biostability of collagen within crosslinked AM was positively correlated with the amount of crosslinker in the reaction system. Nevertheless, a method involving chemical modification of AM with glutaraldehyde likely reduces the level of safety, especially when the extent of crosslinking reaches high levels [119]. Various studies have reported that using glutaraldehyde as a crosslinking agent is not advisable due to its toxic nature [120, 121].

Carbodiimide Crosslinking

The modification of AM with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) does not introduce foreign structures into the biomaterial network and is therefore considered a more biocompatible technique [122]. The EDC/NHS carbodiimide method of crosslinking has been previously used for the development of chemically crosslinked AM materials [117]. However, with carbodiimide treatment for a longer duration (i.e., 4 hours), the AM samples showed significant weight loss after 4 weeks of incubation with matrix metalloproteinases, suggesting low crosslinking efficiency of biological tissues [115]. With an optimum concentration of 0.05 mmol EDC/NHS per mg AM, chemical crosslinking can significantly enhance mechanical stability and retard enzymatic degradation [117]. It is expected that the increased stability introduced by crosslinking could be useful in an inflammatory wound. However, in vitro cell culture studies demonstrate that EDC crosslinked AM can support human LEC proliferation and preserve epithelial progenitor cells in vivo and in vitro [117]. Enhanced expression of p63 and ABCG2 and increased LEC growth were also significantly associated with the greater crosslinking degree of AM samples [115]. The expression of K3 and ABCG2 suggests that both differentiated and progenitor phenotype can be preserved by crosslinking AM.

Radiation and Photo Crosslinking

In a study by Lai, it was demonstrated that UV radiation physically crosslinks AM [118]. Results of crosslinking density measurements and in vitro degradation tests showed that the biostability of these biological tissues strongly depended on the number of crosslinked structures, which was affected by the duration of exposure to UV radiation. The number of crosslinks per unit mass of photo-crosslinked AM played an important role in determination of matrix permeability. In vitro biocompatibility studies, including cell viability and pro-inflammatory gene expression analyses, demonstrated that the physically crosslinked biological materials did not cause harm to the corneal epithelial cells, irrespective of UV radiation time. It was found that undifferentiated precursor cell phenotype was significantly improved with an increase in crosslinking density [123]. Therefore, both duration of UV radiation and riboflavin may be important for the generation of AM matrices for cultivation of LEC.
## Table 3. Different methods and effects of crosslinking of amniotic membrane

| Author, year | Crosslinking type | Methods | Results |
|--------------|-------------------|---------|---------|
| Fujisato et al. (1999) | GA and radiation crosslinked amniotic membrane | Measurement of water content, membrane permeability, and mechanical properties; In vitro degradation test of membranes | Radiation decreased the tensile strength; Protein permeation not influenced by the GA concentration; GA crosslinking increased the biodegradation properties of the membranes; Radiation crosslinking is less effective than GA crosslinking |
| Spoerl et al. (2004) | GA crosslinked amniotic membrane | Measurement of biomechanical force-elongation and resistance to enzymatic digestion; Transplantation to patients with ocular surface defects | Significantly increased biomechanical strength of GA crosslinked membranes; Membranes were completely resistant to enzymatic digestion; The membranes did not dissolve for months after transplantation |
| Ma et al. (2010) | Carbodiimide (EDC/NHS) crosslinked amniotic membrane | ATR-FTIR; DSC; EM; Mechanical degradation tests; Membrane permeability | The optimal concentration was 0.05 mmol EDC/mg amniotic membrane; Increased mechanical and thermal stability, optical transparency, and resistance to collagenase digestion; EDC/NHS crosslinked membranes supported LEC proliferation and preserved epithelial progenitor cells in vitro and in vivo |
| Kitagawa et al. (2011) | Hyperdried GA crosslinked amniotic membrane | Transplantation with hyperdried GA crosslinked amniotic membrane; In vitro degradation tests | Hyperdried GA crosslinked amniotic membrane did not dissolve until 48 hours; Corneal perforation repaired after transplantation; No recurrence during follow-up period 3–6 months |
| Tanaka et al. (2012) | Carbodiimide crosslinked amniotic membrane | Measurement of light transmittance and tensile strength | Significantly increased tensile strength after crosslinking; Greater light transmittance in crosslinked membranes under wet conditions |
| Lai et al. (2013) | Carbodiimide crosslinked amniotic membrane | Varying crosslinking durations (1–4 hours); Measurement of light transmittance, water content; In vitro degradation tests | Increased water content, light transmittance, and resistance to enzymatic degradation; Enhanced LEC growth and increased expression of p63 and ABCG2 on membranes with greater crosslinking degree |
| Lai et al. (2013) | GA crosslinked amniotic membrane | In vitro degradation tests; Ninhydrin assays; TEM | Significant collagen molecular aggregation; Crosslinking with GA for 6 hours resulted in lowest in vitro degradability of the membranes |
| Sekar et al. (2013) | AlgSO_4 crosslinked amniotic membrane | Measurement of mechanical properties, percentage of swelling in water, and stability; In vitro static culture system; Infrared spectroscopy; SEM | 125% increase in the tensile strength in the crosslinked membranes; Membrane was sterile up to 1 year; Confluent sheets of epithelial cell at the end of 14th day resembled the morphology of limbal epithelium |
| Lai (2014) | Photo-crosslinked amniotic membrane | Measurement of crosslinking structure, degradability, and nutrient permeation ability; Expression of ABCG2 | The number of crosslinks per unit mass of membrane increased with increasing illumination; Biological stability and matrix permeability dependent of the crosslinking density; Increased expression of ABCG2; LEC exhibited the undifferentiated precursor cell phenotype |
| Lai et al. (2014) | L-lysine Carbodiimide crosslinked amniotic membrane | Measurement of crosslinking structure, water content, light transmittance, and cell viability; In vitro degradation tests; Expression of p63 and ABCG2 | Increase in L-lysine concentration increased crosslinking density and decreased water content; Decreased thermal denaturation and enzymatic degradation in accordance with the number of crosslinks; High levels of L-lysine decreased light transmittance and biocompatibility; Increased expression of p63 and ABCG2 was dependent on improved crosslinking formation |
| Lai (2015) | Carbodiimide crosslinked amniotic membrane (treated with glycine, lysine, or glutamic acid) | Measurement of crosslinking structure, diffusion permeability, biocompatibility, and zeta potential; In vitro degradation tests | Addition of lysine to crosslinked membranes improved formation of covalent crosslinkages and increased tensile strength; Crosslinked membrane implants exhibited biocompatibility; Glycine molecules were insufficient to increase the resistance to thermal denaturation and enzymatic degradation |
| Ma et al. (2015) | Carbodiimide (EDC/NHS) crosslinked amniotic membrane | Culture of LEC; Colony formation efficiency; Immunoconfocal microscopy; Real-time qRT-PCR; Western blot | LEC cultured on EDC/NHS crosslinked membranes exhibited great colony formation efficiency and expressed high levels of p63, ABCG2, integrin B1, and integrin-linked kinase; Membranes exhibited high rigidity and rough ultrastructure, and preserved progenitor LEC in vitro |

Abbreviations: ATR-FTIR, attenuated total reflection-Fourier transform infrared spectroscopy; EDC, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride; DSC, differential scanning calorimetry; EM, electron microscopy; GA, glutaraldehyde; LEC, limbal epithelial cells; NHS, N-hydroxysuccinimide; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, scanning electron microscopy; TEM, transmission electron microscopy.
Aluminum Sulfate Crosslinking

A recent study showed that aluminum sulfate (Al₂(SO₄)₃) may be used as a crosslinking agent to improve the mechanical properties of AM. Crosslinking with Al₂(SO₄)₃ supported improved attachment and proliferation of corneal LEC [21]. Using infrared spectroscopy to confirm the crosslinking of AM with Al₂(SO₄)₃, it has been demonstrated that there is an approximate 125% increase in tensile strength in the crosslinked AM. Importantly, the crosslinked AM was found to be sterile for up to 1 year and the morphology of confluent sheets of epithelial cells resembled in vivo morphological features of LEC. Based on these results, the Al₂(SO₄)₃ crosslinked AM should be further investigated as a candidate substrate for ocular surface reconstruction.

Crosslinking and the Limbal Stem Cell Niche

Stability and biocompatibility are both important factors that need to be taken into consideration when studying biomaterial crosslinking and its applications. Using l-lysine as an additional amino acid bridge the stabilization of an EDC/NHS crosslinked AM collagen matrix for potential use as a limb stem cell niche was investigated [114]. The results showed that the number of positively charged amino acid residues incorporated into the tissue collagen nanofibers was highly correlated with the l-lysine-pretreatment concentration, thereby influencing the crosslinked structure and hydrophilicity of the resulting scaffold. The variation in thermal and biological stability was correlated with the number of crosslinks per unit mass of AM. It is noteworthy that the samples prepared using a relatively high l-lysine-pretreated concentration (i.e., 30 mM) appeared to have decreased light transmittance and cell viability. This was likely due to the effects of an increase in nanofiber size and subsequent higher charge density. However, in the 1–30 mM range of l-lysine pretreatment, expression of p63 and ABCG2 in LEC were upregulated. This corresponded with an increased number of amino acid bridges in the chemically crosslinked AM scaffolds. Therefore, mild to moderate l-lysine pretreatment appears to be a useful strategy to assist in the construction of a stable LEC niche using EDC/NHS crosslinked AM.

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