Autologous Ex-Vivo Cultivated Limbal Transplantation for the Treatment of Unilateral Limbal Stem Cell Deficiency

Sayan Basu¹, Virender S Sangwan¹

Keywords: Ocular Burns, Limbus, Stem Cells and Therapy:

Ocular surface disease following chemical or thermal burns is a rare but severe form of corneal blindness. Initially, corneal surgeons believed that like other corneal diseases, corneal transplantation could restore corneal transparency and vision. In fact, the first successful corneal transplantation, by the Austrian surgeon Dr Eduard Zirm in 1905, was in the left eye of a farmer with bilateral chronic lime burns.¹,² However, with experience, corneal transplant surgeons realized that almost all corneal grafts performed for ocular burns failed within a year because of recurrence of epithelial defects and vascularization.³ In the late 1970s and early 1980s, Dr Richard Thoft showed in a small series of cases that autologous conjunctival transplantation as opposed to corneal transplantation was effective in stabilizing the corneal surface and moderately improving vision in eyes with ocular burns.⁴ He later proposed that the limbus and not the conjunctiva was the source of corneal epithelium hinting that adult corneal epithelial stem cells could be present at that location.⁵ Soon in 1986, Sun and associates actually demonstrated the presence of stem-cell like cells in the basal layers of the limbus which were slow-cycling, did not express cytological markers for either the conjunctiva or the cornea and were capable of proliferation in-vitro.⁶ This discovery led to a paradigm shift in the understanding of the patho-physiology of ocular burns suggesting that limbal stem cell deficiency (LSCD) was the reason behind corneal epithelial problems in ocular burns.⁷

The obvious implication of this new knowledge was whether limbal stem cell deficiency could be treated by performing limbal transplantation?⁷ Following successful pre-clinical animal trials,⁶ Kenyon and Tseng in 1989 provided the proof-of-principle by describing successful corneal regeneration in patients with unilateral acute and chronic chemical burns following limbal autograft transplantation.⁹ This technique involved removing as much as six clock hours of donor limbal tissue from healthy donor eyes and transplanting it on the recipient eyes after clearing the pathological pannus covering the cornea.⁹ Although this technique was extremely effective, other groups who tried to replicate the results reported rare incidents of iatrogenic LSCD in the donor eyes.¹⁰-¹² In 1997, Pellegrini and associates proposed a way around this problem by developing a technique of culturing the limbal cells ex-vivo in a laboratory to form a transplantable sheet of epithelium from less than one clock hour of donor limbal tissue.¹³ Following this, several large clinical trials have established the safety and efficacy of autologous ex-vivo cultivated limbal epithelial transplantation for the treatment of unilateral LSCD.¹⁴-¹⁷

Advantages of Ex-vivo Cultivated Limbal Epithelial Transplantation:

Although improving the safety of limbal transplantation was probably the driving force behind its development, this technique offers several other advantages as compared to conventional limbal transplantation.

1. **Minimal Donor Tissue:** Since first described in 1997, cultivated limbal transplantation has been performed in hundreds of patients with LSCD and till date there are no reports of donor site complications.¹⁴,¹⁵ The authors specifically looked at the donor eyes in 200 cases of unilateral LSCD which underwent autologous cultivated limbal epithelial transplantation and noted that the donor-site epithelized within two weeks without complications.¹⁷

2. **Repeatability:** One or two repeat limbal biopsies can be safely obtained from the same donor eye if the primary procedure fails, because more than 90% of the limbus is left untouched by a single biopsy.¹⁶,¹⁸ This is not possible in conventional limbal transplantation as the donor eye is not left with any limbal reserve.

3. **Early Corneal Epithelization:** Since a ready-made epithelial sheet is transplanted in cultivated limbal epithelial transplantation, corneal epithelization is
Conjunctivalization and consequently severe visual loss. Surface by the surrounding opaque conjunctival tissue or persistent epithelial defects, invasion of the corneal epithelium with 1 mm into clear corneal stromal tissue at the pigmented line (palisades of Vogt), and the limbal tissue is dissected; conjunctiva is excised just behind the limbus is epithelialized only by six weeks.

In conventional limbal transplantation, the entire cornea is epithelized only by six weeks. In conventional limbal transplantation as compared to conventional limbal transplantation.

Less Surface Inflammation: Post-operative ocular surface inflammation subsides faster after cultivated limbal epithelial transplantation as compared to conventional limbal transplantation.

Less Scarring: Cultivated limbal transplantation is associated with less scarring on the recipient corneal surface and probably better visual recovery as compared to the conventional technique.

Amplification in number of transplanted stem cells: Ex-vivo cultivation results in increase in the number of limbal stem cells obtained by biopsy and this in turn can lead to better long-term survival of the graft.

Indications for Autologous Limbal Transplantation:

Any traumatic or inflammatory damage to the limbus can cause permanent functional damage to the limbal stem cells. This leads to corneal epithelial instability, recurrent or persistent epithelial defects, invasion of the corneal surface by the surrounding opaque conjunctival tissue (conjunctivalization) and consequently severe visual loss.

The commonest indication of autologous limbal transplantation is unilateral ocular surface burns, due to chemical or thermal injury. Earlier this procedure was performed both during the acute and chronic stages, but it is presently indicated only in the chronic stage, after the acute event before performing limbal transplantation. Lid abnormalities like notches, improper closure, entropion and trichiasis also need to be looked for and addressed. Eyes with severe dry eye disease, with a Schirmer’s test 1 score of less than 10mm at 5 minutes are unsuitable for this procedure and punctual occlusion may be needed prior to surgery. In summary the ocular surface environment must be conducive for the limbal transplantation to succeed.

The extent of corneal stromal scarring is also difficult to assess pre-operatively and patients must be counseled that they may need additional surgery in the form of an anterior lamellar or penetrating keratoplasty (PK) for visual improvement despite a successful limbal transplantation. In the authors’ experience it is prudent to perform limbal and corneal transplantation as a two-stage rather than a single-stage procedure.

Technique of Limbal Biopsy: A biopsy is taken from a healthy part of the limbus; a 2x2 mm piece of conjunctival epithelium with 1 mm into clear corneal stromal tissue at the limbus is dissected; conjunctiva is excised just behind the pigmented line (palisades of Vogt), and the limbal tissue that contained epithelial cells and a part of the corneal stroma is obtained.

Technique of Limbal Culture: Broadly there are two techniques of limbal cultivation, a) the suspension culture where the a cell suspension of the biopsied limbal tissue is prepared and spread over a suitable substrate; and b) explant culture where the limbal tissue is sectioned into smaller pieces and directly placed on the substrate without separating the epithelial cells from the stroma. Additionally the constituents of the culture medium may or may not contain animal derived products or xenobiotic materials. Xenogenic constituents of a limbal culture system can be in the form of murine feeder-cells, bovine serum, or animal derived growth factors. To avoid the use of animal-derived products four groups, including our own, have independently developed completely xeno-free laboratory protocols of limbal culture.

In our technique, the tissue is transported to the laboratory in human corneal epithelium (HCE) medium. HCE is composed of modified Eagle’s medium/F12 medium (1:1) solution containing 10% (vol/vol) autologous serum.
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**Table 1. Techniques and outcomes of autologous cultivated limbal transplantation for unilateral limbal stem cell deficiency**

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Culture Technique</th>
<th>Substrate</th>
<th>Culture Time</th>
<th>Eyes</th>
<th>Clinical Success (%)</th>
<th>2-line visual gain (%)</th>
<th>Follow-up (years)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feeder-Free and Xeno-Free Cell Cultures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sangwan et al17</td>
<td>2011</td>
<td>Explant</td>
<td>hAM</td>
<td>10 to 14</td>
<td>200</td>
<td>71</td>
<td>60.5</td>
<td>3</td>
<td>1</td>
<td>1 to 7.6</td>
</tr>
<tr>
<td>Kolli et al26</td>
<td>2010</td>
<td>Explant</td>
<td>hAM</td>
<td>12 to 14</td>
<td>8</td>
<td>100</td>
<td>63</td>
<td>1.6</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Di Girolamo et al27</td>
<td>2009</td>
<td>Explant</td>
<td>CL</td>
<td>10</td>
<td>2</td>
<td>100</td>
<td>50</td>
<td>0.9</td>
<td>0.7</td>
<td>1 to 1.1</td>
</tr>
<tr>
<td><strong>Feeder-free but not Xeno-free Cell Cultures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Barand-Rafii et al29</td>
<td>2010</td>
<td>Explant</td>
<td>hAM</td>
<td>14</td>
<td>8</td>
<td>88</td>
<td>63</td>
<td>2.8</td>
<td>0.5</td>
<td>4</td>
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<tr>
<td>Pauklin et al30</td>
<td>2010</td>
<td>Explant</td>
<td>hAM</td>
<td>14</td>
<td>30</td>
<td>77</td>
<td>73</td>
<td>2.4</td>
<td>0.8</td>
<td>0.5 to 6</td>
</tr>
<tr>
<td>Shortt et al31</td>
<td>2008</td>
<td>Suspension</td>
<td>hAM</td>
<td>14 to 21</td>
<td>3</td>
<td>78</td>
<td>22</td>
<td>0.8</td>
<td>0.5</td>
<td>0.5 to 1.1</td>
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<td>Shimakazi et al32</td>
<td>2007</td>
<td>Explant</td>
<td>hAM</td>
<td>14.6</td>
<td>16</td>
<td>50</td>
<td>37.5</td>
<td>2.5</td>
<td>0.5</td>
<td>0.5 to 7.1</td>
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<td>Nakamura et al33</td>
<td>2006</td>
<td>Explant</td>
<td>hAM</td>
<td>15 to 16</td>
<td>2</td>
<td>100</td>
<td>67</td>
<td>1.2</td>
<td>0.5</td>
<td>0.5 to 1.6</td>
</tr>
<tr>
<td>Sangwan et al34</td>
<td>2006</td>
<td>Explant</td>
<td>hAM</td>
<td>11 to 15</td>
<td>88</td>
<td>73</td>
<td>37</td>
<td>1.5</td>
<td>0.3</td>
<td>0.3 to 3.3</td>
</tr>
<tr>
<td>Sangwan et al35</td>
<td>2003</td>
<td>Explant</td>
<td>hAM</td>
<td>10 to 14</td>
<td>2</td>
<td>100</td>
<td>50</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Grueterich et al36</td>
<td>2002</td>
<td>Explant</td>
<td>hAM</td>
<td>21</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>3.1</td>
<td>3.1</td>
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<tr>
<td>Tsai et al37</td>
<td>2000</td>
<td>Explant</td>
<td>hAM</td>
<td>14 to 21</td>
<td>3</td>
<td>100</td>
<td>50</td>
<td>2</td>
<td>0.3</td>
<td>0.3 to 10</td>
</tr>
<tr>
<td><strong>Neither Feeder-Free nor Xeno-Free Cell Cultures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Di Iorio et al38</td>
<td>2010</td>
<td>Suspension</td>
<td>Fibrin</td>
<td>NA</td>
<td>166</td>
<td>80</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
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<tr>
<td>Rama et al16</td>
<td>2010</td>
<td>Suspension</td>
<td>Fibrin</td>
<td>14 to 16</td>
<td>107</td>
<td>68</td>
<td>54</td>
<td>2.9</td>
<td>1</td>
<td>1 to 10</td>
</tr>
<tr>
<td>Gisoldi et al39</td>
<td>2010</td>
<td>Suspension</td>
<td>Fibrin</td>
<td>14 to 16</td>
<td>6</td>
<td>83</td>
<td>83</td>
<td>2</td>
<td>0.9</td>
<td>0.9 to 2.8</td>
</tr>
<tr>
<td>Kawashima et al40</td>
<td>2007</td>
<td>Explant</td>
<td>hAM</td>
<td>NA</td>
<td>2</td>
<td>100</td>
<td>67</td>
<td>2.7</td>
<td>1.7</td>
<td>1.7 to 3.7</td>
</tr>
<tr>
<td>Nakamura et al41</td>
<td>2004</td>
<td>Explant</td>
<td>hAM</td>
<td>23</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>1.6</td>
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<tr>
<td>Rama et al42</td>
<td>2001</td>
<td>Suspension</td>
<td>Fibrin</td>
<td>14 to 16</td>
<td>18</td>
<td>74</td>
<td>33</td>
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<td>1</td>
<td>1 to 2.2</td>
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<tr>
<td>Schawb et al43</td>
<td>2000</td>
<td>Suspension</td>
<td>hAM</td>
<td>21 to 28</td>
<td>10</td>
<td>60</td>
<td>36</td>
<td>1.1</td>
<td>0.5</td>
<td>0.5 to 1.6</td>
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<tr>
<td>Schawb et al44</td>
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<td>Suspension</td>
<td>hAM</td>
<td>28 to 35</td>
<td>17</td>
<td>76</td>
<td>16</td>
<td>0.9</td>
<td>0.2</td>
<td>0.2 to 2</td>
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<tr>
<td>Pellegrini et al33</td>
<td>1997</td>
<td>Suspension</td>
<td>3T3s</td>
<td>16 to 19</td>
<td>2</td>
<td>100</td>
<td>50</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

hAM= human amniotic membrane; CL=contact lens; NA=data not available

(AS), 2mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 10 ng/ml human recombinant epidermal growth factor, and 5 µg/ml human recombinant insulin. Under strict aseptic conditions, the donor limbal tissue is shredded into small pieces. Human amniotic membrane (hAM), prepared and preserved by our eye bank is used as a carrier. A 3x4cm hAM sheet is de-epithelized using 0.25% recombinant trypsin and EDTA solution for 15 minutes. The shredded bits of limbal tissue are explanted over the center of de-epithelized hAM with the basement membrane side-up. A similar parallel culture is also prepared as a backup. A submerged explant culture system without a feeder-cell layer is used. We used the HCE medium to nurture the culture. The culture is incubated at 37°C with 5% CO₂ and 95% air. The growth is monitored daily under an inverted phase contrast microscope and the medium is changed every other day. The culture is completed when a monolayer of the cells growing from the explants became confluent, typically in 10 to 14 days.

**Technique of Limbal Transplantation**: Any symblepharon which prevented adequate separation of the lids is released to permit the insertion of a wire speculum (no additional

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surgery to treat the symblepharon is performed). A peritomy is performed and the corneal fibrovascular pannus is excised. If an impending or frank corneal perforation is noted at this stage a PK is performed prior to placing the limbal graft.23 The hAM and monolayer of cultivated limbal epithelial cells is spread over the cornea, epithelial side up.17,18 The graft is then secured to the peripheral cornea by interrupted, circumferential 10-0 nylon sutures and to the surrounding conjunctival edge by interrupted 8-0 polyglactin sutures. Alternately, using a sutureless technique, the graft is secured to underlying ocular surface with fibrin glue (TISSEEL™ Kit from Baxter AG, Austria) and the margins of the graft are tucked under the surrounding conjunctival edge. Bandage contact lenses are not applied at the end of surgery.

Postoperative management: All patients receive 1% prednisolone acetate eye drops eight times a day tapered to once a day in 35-42 days and 0.3% ciprofloxacin hydrochloride eye drops four times a day for 1 week, in both the biopsied and transplanted eye. The latter are continued till the epithelial defect completely resolves. No systemic antibiotics or steroids are needed. Patients are examined on postoperative days 1, 7, 42 and at an interval of 90-180 days thereafter, as customized by the clinical appearance of the transplant. Each examination includes a complete history, visual acuity assessment with Snellen’s charts, intraocular pressure measurement and detailed ocular examination with slit-lamp bio-microscopy.

Clinical Outcomes of Cultivated Limbal Transplantation: The techniques and outcomes of autologous cultivated limbal transplantation described by various groups are summarized in Table 1.13,17,26-44 Success was defined clinically in most studies; a few studies additionally used impression cytology or symptom scoring. With our technique the hAM usually disappeared (it either disintegrates or is incorporated as a part of the corneal stroma) by 4 weeks and the recipient ocular surface stabilized by 6 weeks. The donor site completely epithelized without scarring within two weeks of limbal biopsy. Overall, the success rate of autologous cultivated limbal transplantation varied from 50 to 100% and a two-line improvement in visual acuity after cultivated limbal transplantation alone was seen in 22 to 100% cases (Table 1). More than 90% of failures occurred by the end of one year and more than half of these by six months after transplantation.15,16

Although on cursory review it appears that there is no clinical advantage that one culture technique holds over the other, comparing success rates among different culture techniques may be misleading as the indications for surgery, sample size, and follow-up duration are variable among different studies. Shimakazi et al32 and Nakamura et al33 compared the explant and suspension culture techniques, finding no significant difference in the outcomes. It is noteworthy in this context that with similar indications for surgery, clinical criteria for success, and follow-up Sangwan et al (explant culture, 71%, 200 eyes),17 Rama et al (suspension culture, 68%, 107 eyes)16 and Di Iorio et al (suspension culture, 80%, 166 eyes)38 reported similar and impressive success rates of cultivated limbal Table 2. Clinical Outcomes of Conjunctival Limbal Autografting or Conventional Limbal Transplantation for eyes with Unilateral Limbal Stem Cell Deficiency.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Eyes</th>
<th>Clinical Success (%)</th>
<th>2-line vision gain (%)</th>
<th>Complications</th>
<th>Follow-up (Months)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miri et al45</td>
<td>2011</td>
<td>25</td>
<td>NA</td>
<td>NA</td>
<td>Filamentary keratitis (4)</td>
<td>41</td>
<td>3</td>
<td>127</td>
</tr>
<tr>
<td>Miri et al46</td>
<td>2010</td>
<td>12</td>
<td>100</td>
<td>81.3</td>
<td>None</td>
<td>46</td>
<td>12</td>
<td>120</td>
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<tr>
<td>Santos et al47</td>
<td>2005</td>
<td>10</td>
<td>80</td>
<td>61</td>
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<td>33</td>
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<tr>
<td>Ozdemir et al48</td>
<td>2004</td>
<td>15</td>
<td>87</td>
<td>80</td>
<td>None</td>
<td>13.9</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>Dua et al49</td>
<td>2000</td>
<td>6</td>
<td>100</td>
<td>83</td>
<td>Filamentary keratitis (1)</td>
<td>18.8</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>Rao et al50</td>
<td>1999</td>
<td>16</td>
<td>94</td>
<td>82</td>
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<td>19.3</td>
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<td>45</td>
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<tr>
<td>Basti et al10</td>
<td>1999</td>
<td>3</td>
<td>100</td>
<td>100</td>
<td>LSCD in donor eye (1)</td>
<td>NA</td>
<td>9</td>
<td>15</td>
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<tr>
<td>Frucht-Pery et al11</td>
<td>1998</td>
<td>9</td>
<td>100</td>
<td>100</td>
<td>None</td>
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<td>60</td>
</tr>
<tr>
<td>Tan et al12</td>
<td>1996</td>
<td>9</td>
<td>77</td>
<td>77</td>
<td>LSCD in donor eye (1)</td>
<td>27.1</td>
<td>2.5</td>
<td>46</td>
</tr>
<tr>
<td>Morgan et al53</td>
<td>1996</td>
<td>6</td>
<td>83</td>
<td>83</td>
<td>Donor site micro-perforation (1)</td>
<td>3</td>
<td>2</td>
<td>45</td>
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<td>Kenyon et al9</td>
<td>1989</td>
<td>26</td>
<td>77</td>
<td>65</td>
<td>None</td>
<td>18</td>
<td>2</td>
<td>45</td>
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</table>

NA= data not available; LSCD: Limbal Stem Cell Deficiency
epithelial transplantation with mean follow-up ranging from 1.5 to almost 3 years and maximum follow-up of up to 8 years. It is noteworthy that of the various groups only Sangwan et al.,17 Kolli et al.,26 Di Girolamo et al.,27 and Zakaria et al.28 described completely xeno-free techniques of culturing limbal epithelium while others used at least one or more animal derived products for culture. None of the studies reported any donor-site complications.

Around 18 to 38% of all eyes treated with autologous cultivated limbal epithelial transplantation also needed a PK for visual improvement.14,15 The authors have found that adopting a staged approach of performing limbal transplantation first, followed at least six weeks later by PK resulted in better clinical outcomes as compared to a single-staged approach of combined limbal transplantation and PK.23 Therefore PK and limbal transplantation should not be combined unless PK is absolutely unavoidable as in the case of an impending or frank corneal perforation discovered after removal of the vascular pannus. The authors also found that a repeat limbal biopsy from the healthy eye followed by ex-vivo cultivation and transplantation of the cultured cells on the affected eye can successfully restore the ocular surface and improve vision in at least two thirds of cases with failure of therapy with primary autologous cultivated limbal epithelial transplantation.18 Therefore combining the efficacy of primary (71%) and repeat autologous cultivated limbal epithelial transplantation (67%) almost 90% of cases of unilateral LSCD can be treated successfully without any adverse impact on the healthy donor eye.17,18

Conclusions:

In terms of clinical efficacy there is hardly any difference between conventional and cultivated autologous limbal transplantation as treatment options for unilateral LSCD (Table 2).3-11,45-52 Proponents of ex-vivo cultivation cite the safety of the donor eye as the main advantage of their technique. However, ex-vivo cultivation requires specialized expertise and a licensed (by the Human Tissue Authority in the United Kingdom) laboratory. It can take up to 2 weeks to generate a sheet of desired dimensions, and it is expensive, costing approximately 10 300 Pounds Sterling or 12 000 Euros at current exchange rates for cultivation alone.46 Furthermore, many groups practicing this technique continue to use xeno-biotic materials for cell culture (Table 1). Not surprisingly, conventional limbal transplantations are still widely performed worldwide and cultivated limbal transplantation is restricted to few select centers around the globe.14,15 There are however certain clinical scenarios where cultivated limbal epithelial transplantation can be a superior alternative to conventional limbal transplantation. For example, in patients with bilateral but asymmetrical LSCD, even one clock hour of healthy limbal area in either eye can be utilized to restore the ocular surface and improve vision in both eyes.22

The only study that compared conventional and cultivated limbal transplantation reported slower epithelization rate, prolonged ocular surface inflammation and significantly more scarring with the conventional technique.53 However, for cultivated limbal epithelial transplantation to emerge as the more popular technique of treating unilateral LSCD the economic and logistic barriers of cell culture have to be overcome first. Moreover the actual mechanism by which limbal transplantation works is still debated. It is unclear whether this therapy replenishes the stem cell reserve16 or revives the surviving stem cells by improving the micro-environment.14 It is also widely accepted that the cause of failure of limbal transplantation is multi-factorial and poorly understood.16,17 In the large clinical trials Rama et al and Sangwan et al investigated the cause for failure and found that eyes with more severe injuries and post-operative complications were more likely to fail.16,17 Rama et al also found that the proportion of holoclone forming cells (actual stem cells) in culture needed to be more than 3% of the total cell population for the transplant to have higher chances of success.16

In summary, the last two decades have witnessed tremendous progress in the understanding of ocular surface disease due to ocular burns and this has in turn led to therapeutic innovations, at the cutting edge of which stands ex-vivo cultivated limbal epithelial transplantation. Stem-cell based therapy for LSCD has already benefitted hundreds of patients worldwide and continuous research and medical development in this field holds promise for an even more exciting future.

References:

6. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and


