Treatment of Total Limbal Stem Cell Deficiency With Autologous Ex Vivo Cultivated Limbal Epithelial Stem Cell Graft

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ABSTRACT:
Limbal epithelial stem cell (LSC) deficiency is severe disease of the anterior eye surface, causing corneal opacification and significant vision loss in the affected patients. Treatment of such patients by corneal graft is ineffective due to the fact that corneal donor tissue cannot survive in the eye without LSC. One of possible treatment options is to provide LSC from the other, unaffected eye, and to perform corneal graft later on, as a second procedure. In this paper, we have evaluated the visual outcome and clinical improvement in three eyes receiving ex vivo cultivated limbal epithelial stem cell graft to treat their limbal stem cell deficiency (LSCD) caused by corneal burn. We aimed at determining whether this treatment alone might provide sufficient visual improvement to avoid corneal grafting as a second surgery.

KEYWORDS: corneal burn, limbal stem cell deficiency, ex vivo cultivation of limbal stem cells, corneal transplantation

SAŽETAK:
Nedostatak limbalnih matičnih stanica rožnice je teška bolest prednje površine oka koja uzrokuje značajan pad vida radi zamučenja rožnicw. Liječenje takvih bolesnika transplantacijom rožnice nije moguće jer donorsko rožnično tkivo ne može preživjeti ukoliko nema limbalnih matičnih stanica rožnice koje su zadužene za obnovu epitela rožnice. Mogućnost liječenja takvih bolesnika sastoji se u tome da se prvo obnove limbalne matične stanice presadbom stanica sa kontralateralnog zdravog oka, a kasnije kao sekundarna kirurška procedura može se učiniti transplantacija rožnice. U ovom radu istraživali smo oporavak vida i kliničko poboljšanje u tri bolesnika sa opekotinama prednje površine oka koji su radi nedostatka limbalnih matičnih stanica rožnice primili presadak ex vivo uzgojenih limbalnih stanica koje su kultivirane iz uzorka dobivenog sa njihovog drugog, zdravog oka. Želja nam je bila ustvrditi dali sama presadba matičnih stanica može dovesti do dovoljnog oporavka vida i prednje površine oka kako drugi zahvat, transplantacija rožnice, nebi niti bio potreban.

KLIJUNČE RIJEČI: opekotina rožnice, nedostatak limbalnih matičnih stanica rožnice, ex vivo kultivacija limbalnih stanica, transplantacija rožnice
**Introduction**

According to the World Health Organization, corneal blindness is the fourth leading cause of blindness globally. The impact of corneal blindness is huge, since it tends to affect younger people. Corneal transplantation may restore vision in many corneal diseases, however in case of corneal burns in which limbal epithelial stem cells (LSC) are destroyed corneal transplantation is not feasible. Only renewal of the corneal epithelium, through replacement of the limbal epithelial stem cell population may bring long term visual improvement. LSCs are situated at the corneo-scleral junction in an area known as the limbus. They share common features with other adult somatic stem cells including small size, high nuclear to cytoplasmic ratio, high proliferative potential, clonogenicity, multipotency, expression of stem cell markers such as p63; but lacking expression of differentiation markers such as cytokeratin 3 and 12. The transcription factor p63 is required for formation of epidermis and has been proposed as a putative positive LSC marker. Limbal epithelial stem cell deficiency (LSCD) most often occurs due to the corneal burn caused by alkali or acid injury of the anterior eye surface. Dependent on the severity of injury, only partial or total LSCD may happen. Total LSCD is clinically characterized by growth of conjunctival tissue over the cornea, corneal neovascularization and opacification. For centuries, patients with total LSCD caused by corneal burns were untreated. Then a surgical approach was applied in which a grafting of limbal auto- or allo-graft was done combined with corneal grafting to supply a source of LSC. In such cases a larger amount of tissue had to be collected either from a contralateral healthy eye, thus carrying a risk of damage to healthy eye; or, if LSC graft was provided from allogenic tissue a risk from side effects of long-term immunosuppression was present. Two decades ago, first results of successful cultivation of smaller sample of LSC harvested from the contralateral healthy eye and amplified in number by ex vivo cultivation, followed by their transplantation in human eye were reported. Since that time different carriers for LSC cultivation have been tried in vitro, and their efficacy was tested in a clinical setting.

**Materials and methods**

Two patients with total, and one patient with partial LSCD were included in the study. Samples of LSC for ex vivo cultivation were obtained from a 1 mm² biopsy taken from the limbus of the contralateral healthy eye under topical anaesthesia.

**Cultivation of LSC**

All procedures regarding preparation of amniotic membrane as a carrier for LSC and cell cultures were made in aseptic conditions of clean room facility for cell cultures (class A and B of air cleanliness). Amniotic membranes were prepared from human placenta obtained from healthy woman during caesarean section, according to established procedure. After disinfection with 5% ABAM solution and DPBS, each sample was incubated in 0.05% enzyme trypsin/1mM EDTA solution. Human limbal cells are counted and seeded in 2:1 ratio to previously prepared mice fibroblast nutrient layer. Media is changed every third day till 80% confluence, when cells are counted. Cell cultures are regularly monitored by optical microscopy for their viability. Immunocytochemical method and confocal microscopy is used to test cells for the presence of stem-cell marker p63 and differentiation marker cytokeratin.

Clinical application of ex vivo expanded epithelial cells

After LSC cultivation, and once the appropriate number and percentage of p63 positive cells is achieved ex vivo, LSC on amniotic membrane as a carrier were transferred to corneal surgeon for clinical application. Surgeries were done under local anaesthesia. Limbal peritomy was performed and fibrovascular pannus was carefully dissected from the cornea in all 360°. The amniotic-membrane carrying cultivated LSC were then gently placed on the prepared corneal bed with LSC facing toward the cornea. Perilimbal region of 3-4 mm bare sclera is also covered with LSC sheet and the border of the amniotic membrane is sutured to the adjacent conjunctiva with resorptive 8.0 suture. The eyelids were kept closed for one week. Post-operatively, patients received 2-week systemic treatment with amoxicillin (500 mg tid) and 1-month systemic prednisone (24 mg) and ranitidine with gradual decrease of dosage. Topical steroid and antibiotic drops, and frequent artificial drops are started at day 14 after surgery, to prevent damage to the graft with lid manipulation. Throughout the postoperative follow-up, operated eyes were controlled for the regular epithelialization, decrease of corneal neovascularization and improvement of vision. Once the ocular surface becomes regular, improvement of vision was tested in order to determine whether patient is cured by LSC alone or second surgery – corneal transplantation (CT) is needed.

**Results**

The preoperative clinical appearance of the eyes treated for limbal stem cell deficiency is represented in Figure 1. From their contralateral eye LSC samples were taken for ex vivo cultivation. Limbal stem cells cultured ex vivo fully covered their carrier, the amniotic membrane, between 10 to 14 days after the beginning of cultivation process. Testing of their quantity, morphology and viability was performed by optical microscopy; the uniform layer of stem cells is represented in Figure 2. To check whether cultivated cells are indeed stem cells, the presence and quantity of LSC differentiation marker p63 was measured. In Figure 3, immunofluorescence analysis of p63 and cytokeratin 3 (C3K) markers on cells cultured on fibrin, intact amniotic membrane and denuded amniotic membrane are represented. Expression of stem cell marker p63, and lacking expression of differentiation marker CK3 proved that the cultured cells were LSC and such cells were issued to surgeon for clinical application.

Patients with LSCD received LSC auto-graft cultivated ex vivo
and the clinical outcome regarding regeneration of their anterior surface and visual improvement was tested. As shown in Figure 4, patient with total LSCD caused by tear gas burn reached postoperative visual acuity of 60% at 6-months after surgery, increasing to 100% vision at 1 postoperative year. His cornea became completely transparent and epithelialized with tiny vessels at the limbus of the cornea and remained stable for 2 postoperative years. Patient with partial LSCD caused by alkali burn obtained postoperative vision of 55% at 6 and 85% at 1 year, with normal appearance of the anterior eye segment (Figure 5). Only in one case with total LSCD caused by explosive wound, grafted limbal stem cells did not provide significant visual recovery (from light perception preoperatively to 10% postoperatively at 1 postoperative year); and clearance of the cornea was not fully achieved due to the fact that the patient had completely abnormal lid function causing constant inflammation and abnormal tear film at the anterior eye segment (Figure 6).

**DISCUSSION**

In case of LSC deficiency normal corneal epithelial homeostasis is disrupted and this may occur as a result of primary or acquired insults. Deficiency of the LSC may be partial or total. The leading cause of total LSC deficiency are corneal burns with chemical or thermal substances and corneal diseases such as aniridia and Stevens Johnson syndrome. As a result of LSCD, conjunctivalisation, neovascularisation, chronic inflammation, recurrent erosions, ulceration and stromal scarring can occur causing painful vision loss. Conventional corneal transplantation has been unsuccessfully tried in such cases, since donor cornea cannot survive in a “milieu” where no LSC are present. First successful attempt to grow ocular surface epithelial cells in a culture for consequent use as grafts, was made in a mice model. Twenty years ago, Pellegrini et al. have reported first successful epithelialization of a burned human cornea treated with a novel approach – transplantation of ex vivo expanded LSC. Normal corneal differentiation in vitro was tested with a specific biochemical marker, and then the cells were grafted onto the burned eye. Successful re-epithelialization of the corneal surface was achieved in a 2-year follow-up. These results provided a new perspective on the treatment of ocular disorders characterised by stem-cell deficiency. Cultured LSC therapy has been further developed by several investigators using different protocols and carriers - mostly amniotic membrane or fibrin, in the presence or absence of growth arrested 3T3 fibroblast feeder layers. After those first findings many groups around the globe have continued to treat patients with LSC deficiency with ex vivo expanded limbal stem cells on different carriers such as amniotic membrane, fibrin or contact lens. The most widely used ones in...
clinical work today are fibrin and amniotic membrane, although other potential carriers are also tested. Same methodology is theoretically applicable for patients with bilateral corneal burns, when LSC allografts obtained from a relative or unrelated donor are used; however, with much lower success rate and a negative impact of aggressive systemic immunosuppressive treatment. The effort is also made to grow LSC in a media completely free of any animal-derived products, and successful clinical results of such technique, using only autologous human serum as the only growth supplement are already reported. Later on, oral mucosal epithelial cell and mesenchymal stem cell grafts have been tried, which is of special interest in cases of bilateral corneal burns where autologous grafts from the other healthy eye can’t be obtained. The transplantation of bioengineered oral mucosal epithelium is also a good strategy in bilateral LSC deficiency; however further studies are required to optimize the culture conditions and visual outcome.

In our clinical study on cases with unilateral total and partial LSCD we have investigated whether transplantation of limbal stem cells alone (without subsequent corneal graft) may restore normal anterior surface and adequately improve vision. On this small number of eyes, we have seen that in case of normal lid morphology and function patients had total visual recovery and normal anterior surface and did not need another surgery – corneal graft. In one case of serious lid abnormality caused by explosive wound significant improvement in both anterior surface and visual acuity was achieved after one year, however another surgery in form of corneal graft may be needed for full visual recovery. If the disease is bilateral, other autologous stem cells, such as oral mucosal cells, orbital fat cells and mesenchymal stem cells, might be an option. Recent data from literature, analysing overall published results on LSCD treatment have shown that an “older” approach of grafting limbal stem cells from a healthy eye without their amplification ex vivo may be equally...
good, or even superior option of treatment\(^1\). In our opinion, since a larger stem cell graft has to be excised from a healthy eye with this approach, it is still questionable whether this may have consequences in a long run.

**Conclusion:**
In case of limbal stem cell deficiency (LSCD) best source of LSC is from the other uninjured eye with their multiplication by ex vivo cultivation. In eyes with normal lid function vision can be significantly improved by LSC graft alone, without any further need for subsequent corneal graft, which is not the case if serious lid abnormality is present.

**Literature:**

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