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RESEARCH ARTICLE

# Limbal Stem Cells in Dogs and Cats Their Identification Culture and Differentiation into Keratinocytes

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## Abstract

Limbal epithelial stem cells are the source of regeneration of the corneal epithelium in normal and diseased conditions and it is proven that they are located at the area known as limbus. Maintaining avascularity and clearity of the cornea is a crucial role in homeostasis and integrity of the eye. This study focuses on cats and dogs limbal derived mesenchymal stem cells (LMSCs) differentiation potentials into keratinocytes for developing a culture model for corneal diseases. Limbus-derived mesenchymal stem cells were obtained from the limbus of terminated fetuses of the pregnant dogs (45 d) and cats (35 d). Explant cultures were allowed to grow in the culture medium untill 3rd passage. LMSCs differentiated into keratinocytes and allowed to grow into the decelularized amniotic membrane (AM) until three layer formation is maintained. Characterization was carried out by transition of cells from all three germ layers (osteogenic, chondrogenic and adipogenic). Histology and immunohistochemistry was carried out with cytokeratin 19 (CK 19) and pancytokeratin detected in the cornea. Three lineage differentiation and keratinocyte transformation was succesfully maintained. Immunohistochemically LMSCs were confirmed for the cell surface markers and cells were positive for CD90, CD44 and CD49 and negative for CD45 and CD11b\c. LMSCs are potentially the native cells of the cornea and reconstruction of the cornea with these cells will have a better outcome.

***Keywords:*** *Cat, Dog, Cornea, Keratinocytes, Limbal mesencymal stem cell (LMSC)*

# Kedi ve Köpeklerde Limbal Kök Hücrelerin Kültürü ve Keratinositlere Farklılaştırılması

## Özet

Limbal epitel kök hücrelerin limbus olarak bilinen bölgede bulundukları kanıtlanmış olup normal ve hastalık durumlarında kornea epitelinin yenilenmesinden sorumludurlar. Korneanın damarsızlığının ve saydamlığının devamı, gözün homeostazisinin ve bütünlüğünün korunmasında önemli role sahiptir. Bu çalışmada kedi ve köpeklerin korneal hastalıklarının tedavisinde kulanılmak üzere keratinositlere farklılaştırılan limbal kaynaklı mezenkimal kök hücrelerin (LMSCs) farklılaşma potansiyelleri ve kültür özellikleri üzerinde durulmaktadır. Limbus kaynaklı mezenkimal kök hücreler gebe köpek (45 gün) ve kedilerin (35 gün) istenmeyen gebeliklerinin sonlandırılması ile elde edilen fötuslardan elde edilmiştir. Eksplant kültürler, kültür ortamında üçüncü pasaja kadar büyümeye bırakılmıştır. Keratinositlere farklılaştırılmış LMSCs desellülerize amniyotik membran (AM) üzerinde üç katman oluşumu sağlanana kadar üremeye bırakılmıştır. Hücrelerin karakterizasyonu her üç germ tabakasına (osteojenik, kondrojenik ve adipojenik) hücrelerin geçişi ile sağlanmıştır. Histoloji ve immonohistokimyasal analizler gerçekleştirilmiş ve korneada sitokeratin 19 (CK 19) ile pansitokeratinin pozitifliği gösterilmiştir. Keratinositlere ve her üç germ tabakasına dönüşüm başarı ile gerçekleştirilmiştir. Immunohistokimyasal olarak LMSC'ler hücre yüzey işaretleyicileri tespit edilmiş ve CD90, CD44 ve CD49 için pozitiflik, CD45 ve CD11b\c için ise negatiflikleri belirlenmiştir. LMSC'ler kornea dokusunun kendi hücreleri olması sebebiyle, ileriki çalışmalarda bu hücreler kullanılarak kornea onarımından daha iyi sonuçlar alınacağı düşünülmektedir.

***Anahtar sözcükler:*** *Kedi, Köpek, Keratinosit, Kornea, Limbal mezenkimal kök hücreler (LMSC)*

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## INTRODUCTION

Many eye diseases such as abnormal growth or disorders of the corneal epithelium are observed in veterinary ophthalmology. Among them, the most common are corneal ulcers, neurotrophic ulcers, feline corneal necrosis (FKN), keratoconjunctivitis sicca (KCS), Uber-Reiters Syndrome, Feline herpesvirus type-1 (FHV-1) [1].

The use of stem cells in veterinary medicine is limited. Limbal stem cells found within the basal layer of the limbal epithelium are responsible for continious renewing the entire corneal epithelium and ensures the integrity of the eye [2-4]. The XYZ hypothesis clearly explains the epithelial cell renewal in the eye. X (proliferation of basal cells) + Y (proliferation and centripedal migration of limbal cells) = Z (epithelial cell loss from the surface). Under normal conditions the cells that are lost from the corneal epithelium are renewed by epithelial cells derived from the limbal stem cells. When limbus area is damaged, limbal stem cell deficiency related disorders can be seen. These are as follows; corneal ingrowth of conjunctival epithelium, neovascularization, scarring, chronic inflammation, pain and reduced vision [3,5,6]. In cases of unilateral limbal stem cell deficiency limbal autograft can be harvested from the healthy eye. when limbal stem cell deficiency is bilateral a limbal allograft from a living relative or cadaver donor can be applied. Unfortunately corneal allografts may couse some problems like patient rejection of the graft or avalibility of the graft in the time it is needed. As an alternative to limbal grafting corneal stem cell theraphy or exvivo expanded limbal epithelial cells can be used and these cells can preserve the structural and functional integrity of the cornea. The use of limbal stem cells in corneal defects are very effective, although not yet available [6]. The use of limbal stem cells in the treatment of diseases by *in vitro* cultivation is considered.

Compared to bone marrow derived stem cell limbal mesenchymal stem cells are the native cells of the eye and will ensure a better corneal healing when used in cornea. Limbal mesenchymal stem cells differentiation into keratocytes and use of these cells in the corneal diseases is believed to show a better outcome.

The aim of this study is to focus limbal derived mesenchymal stem cell culture characteristics and optimization of the cell culture conditions and to show their differentiation capacity into keratocytes in cats and dogs. In this way, we are intending to treat primarily corneal diseases and then stem cell deficiency related diseases. In future therapeutic use of limbal-derived mesenchymal cells is aimed to clinically diagnosed corneal ulcer, Uber-Reiters syndrome, FKN, KCS and FHV-1 patients.

## MATERIAL and METHODS

Fetus samples were collected from the bitches of both cats and dogs while termination of the unwanted pregnancies during the ovariohysterectomy operation. Samples were used with the permission of the Board of Ethics in Animal Experiments of Ankara University (20.01.2014/53184147-50.04.04/3800). Samples taken from fetuses were transferred to the laboratory as fresh. Transferred newborn dogs and cats eyes were placed into the Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Belgium).

Limbus region was determined and divided into small pieces. The removed tissue was divided into segments. The explants were performed by the method of cultivation, and then seeded in T25 flasks and incubated at 37°C in a humidified atmosphere 5% $CO_2$.77% DMEM (Lonza, Belgium), 20% fetal bovine serum (Lonza, Belgium) 2% L-Glutamine (Lonza, Belgium) 1% Penicillin, streptomycin, amphotericin (Biological Industries, Israel) composed medium was added. Medium was replaced every 2-3 days and nonadherent cells were discarded. At aproximately 70% confluence, adherent LSCs were passaged at split ratio of 1:2 using 0.25% trypsin in PBS. The cells were grown untill the 3$^{rd}$ passage.

All placental samples were obtained under sterile conditions after elective caesarean delivery from the same bitches. To separate the amniotic membrane from the whole placenta, the amniotic membrane was peeled off from the chorionic membrane mechanically. Under sterile conditions, the collected amniotic membranes were rinsed with normal saline (0.9%) several times. Generally, amniotic membrane (AM) composed of epithelial monolayer and an avascular stroma. Under a laminar flow hood, the placenta was first washed free of blood clots with sterile saline and later in 4%, 8%, and 10% dimethylsulphoxide (DMSO) and phosphate buffered saline (PBS) for 5 min each. The membrane was then flattened onto a nitrocellulose paper, with the epithelium/basement membrane surface up. The amniotic membrane was then cut into 5-5 cm pieces. Each of them was placed in a sterile vial containing 10% DMSO medium. The vials were frozen at -70°C. The membrane was defrosted immediately before use by warming the container to room temperature for 10 minutes, and rinsed three times in saline. Limbal derived MSCs (grown into 3$^{rd}$ passage) growth medium was was replaced by keratinocyte differention medium. Limbal derived MSCs differentiation into keratinocytes were as follows: medium change was carried out 3 days intervals and the differentiation was maintained in 14 days the differentiation was ended in total 21 days. Before seeding onto the amniotic membrane the cells were stained with Kodak invivo fluorescent stain to allow the folllow of the cells in vivo by Kodak equipment. Initially the AM was prapared then the cells were seeded and allowed to

differentiate into keratinocytes. Keratinocyte seeding onto the AM was carried out in six well plate and $5x10^4$ cells were seeded in total. The second layer of cells were seeded onto the first layer and allowed to grow for 4-6 days with 2 days intervals medium change. The third layer of cells were seeded onto the second layer and allowed to grow for 4-6 days with 2 days interval medium change. This three layer tissue formation maintained in 30 days and at the end of 30 days the medium is replaced with formol and fixed. Three layer of cells on the both side of the membrane could be visualised and this sandwich structure is embedded into parafine. The tissue block is stained with hematoxylin-eosin and immunohistochemically surface markers are visualised.

The LMSCs were seeded at a density of $5x10^4$ cells\cm$^2$ in six-well plates at P3 and differentiated into adipogenic, chondrogenic or osteogenic differentiation medium for 21 days as previously described [7,8]. The differentiation of cells into the adipogenic, osteogenic, and chondrogenic lineages was determined using different histological staining techniques. Von Kossa staining demonstrated deposition of minerals in osteogenic cultures. Adipogenic differentiation was evaluated using Oil red O to reveal lipid droplet accumulation in the cell cytoplasm and Alcian blue (pH 2.5) staining was used to reveal chondrogenic differentiation based on the production of a ground substance matrix. Each passage was photographed and in each passage in days 1, 3, and 6 photography was documented as well. In all these staining methods, the nuclei were counterstained with Mayer's hematoxylin and the stained cells were examined under a light microscope.

For cytokeratin 19 (CK19) and pancytokeratin staining, frozen section tissue slides were fixed in cold methanol for 30 min, followed by two PBS washes. After being blocked in 5% skim milk at room temperature for 1 h, slides were incubated with a mouse antibody against human/mouse CK19 (1:500, Millipore, Billerica, MA, USA), followed by incubation with DyLight 488-conjugated goat anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA), or DyLight 594-conjugated goat anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 30 min. Nuclei were then stained with hematoxylene eosin and samples were assessed under a fluorescence microscope (Leica Microsystem).

## RESULTS

The third passage cat and dog LSCs were grown in T25 culture flasks and showed fibroblast-like morphology. At the beginning of the culture period, dog cells showed better proliferation than cat LSCs. But overall, throughout the culture period, the confluence of the cells was similar (10-12 days). By the end of the first week, both the cat and dog LSCs displayed interconnection and monolayer confluence was observed.

The cultured LMSCs typically showed colony forming unit fıbroblast CFU-U morphology both in cat and dog cultures *(Fig. 1)*. LMSCs were cultured in normal medium and the expression of a panel of cell surface markers were studied. Analysis of LMSCs cultured in normal medium confirmed the cell surface markers for mesenchymal stem cells. At P3 95% of cells were positive for CD90, CD44 and CD49 and negative for CD45 and CD11b\c.

The ability of LMCSs to differentiate into adipogenic, osteogenic and chondrogenic lineages were assesed. It is observed that LMSCs differentiated into all three lineages when cultured in a normal medium. In adipogenic cultures the Oil red O stained fat vacuoles were observed throughout the culture *(Fig. 2)*. Chondrogenesis was observed with Alcian blue staining of glycosaminoglycans. Deep staining

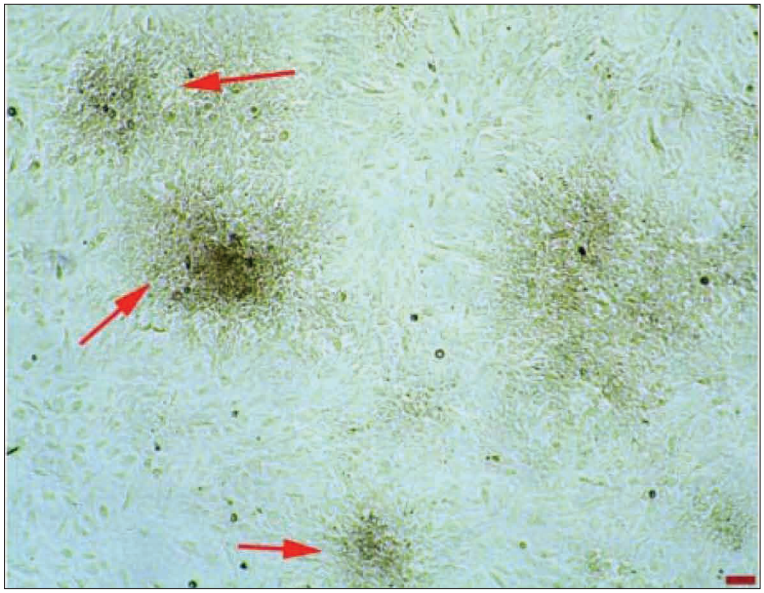


**Fig 1.** The cultured LMSCs showing colony forming unit fıbroblast (CFU-U) morphology both in cat and dog cultures

**Şekil 1.** Kedi ve köpeklerde kültüre edilmiş LMSC'lerde koloni forming fibroblast (CFU-F) yapılarının gözlenmesi

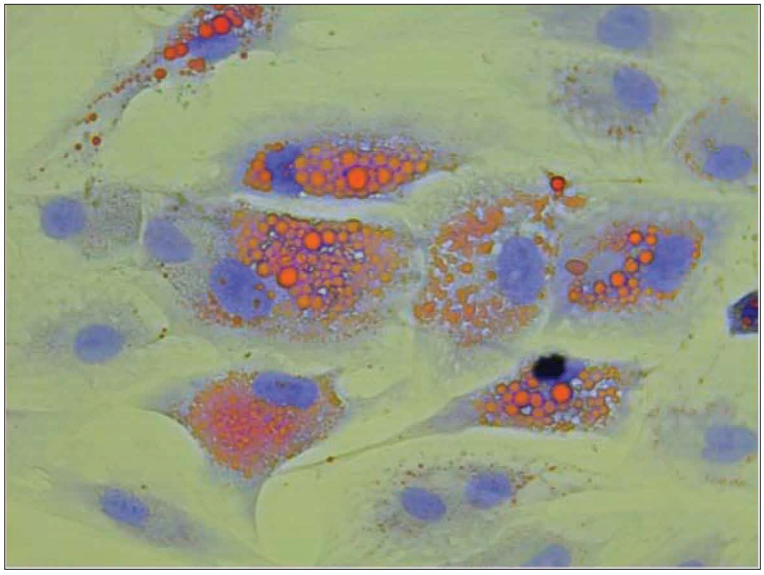
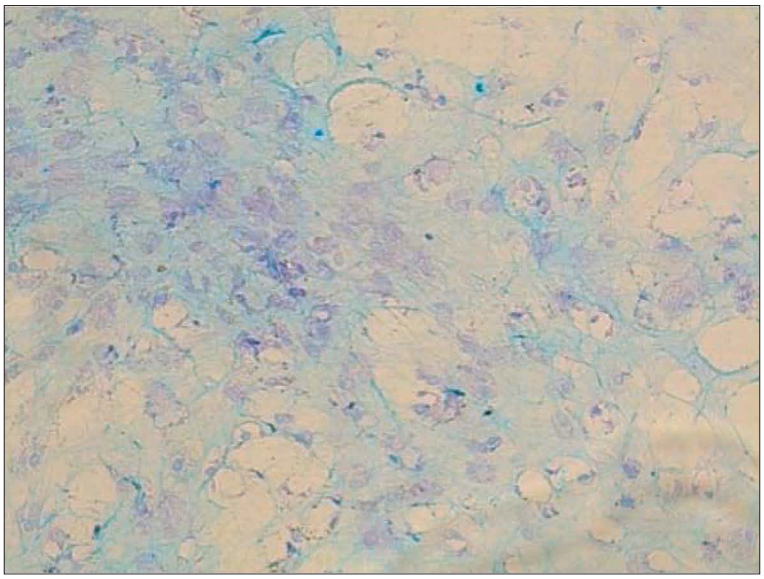


**Fig 2.** Adipogenic differentiation with Oil red O stain showing fat vacuoles throughout the culture

**Şekil 2.** Adipogenezin göstergesi olan Oil red O bayama ile yağ damlacıklarının kültürde yaygın olarak gözlenmesi

**Fig 3.** Chondrogenic differentiation with Alcian Blue showing glycosaminoglycans

**Şekil 3.** Kondrojenik farklılaşmada glikozaminoglikanların Alcian Blue ile boyanması



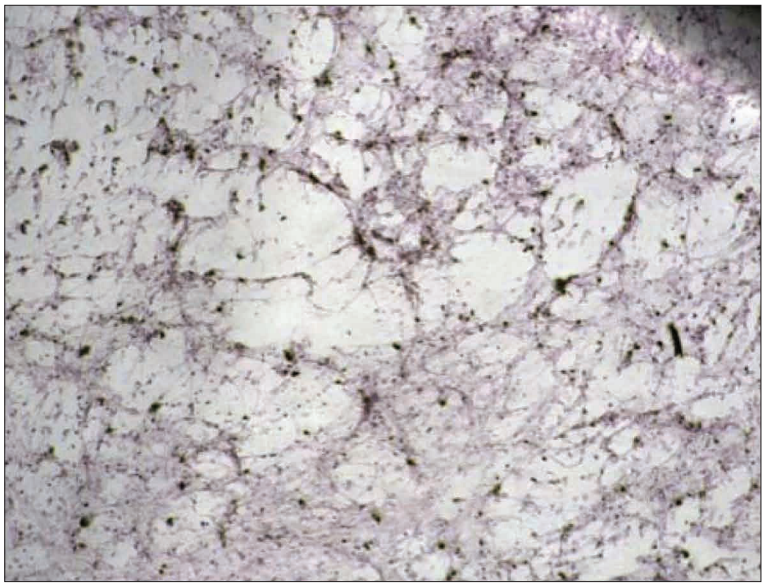


**Fig 4.** Osteogenic differentiation with Von kossa showing calcium deposition in cultures

**Şekil 4.** Osteojenik farklılaşmada kalsiyum birikimlerinin Von Kossa ile boyanması

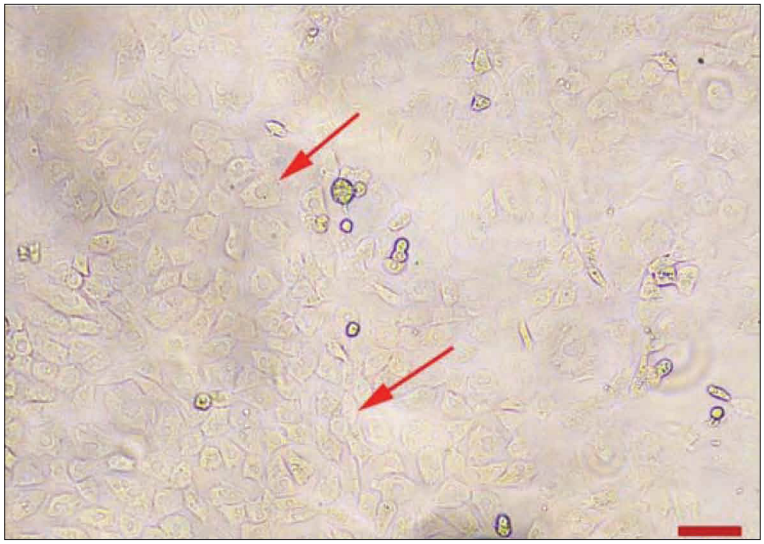


**Fig 5.** The LMSCs differentiation into keratinocytes

**Şekil 5.** Limbal kaynaklı mezenkimal kök hücrelerin keratinositlere dönüşümü

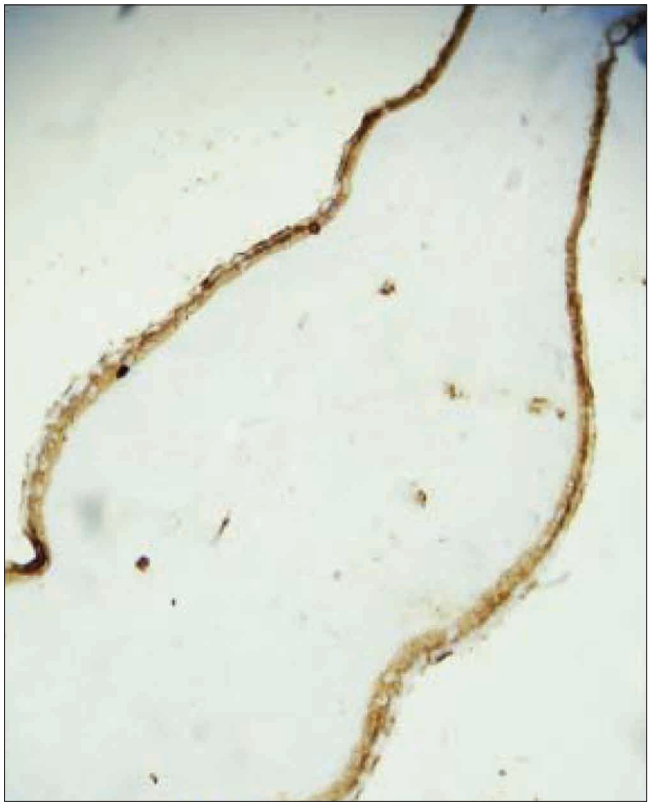


**Fig 6.** Positivity of CK19 and pancytokeratin bilaterally on amniotic membrane

**Şekil 6.** CK 19 ve pansitokeratinin amniyotik membran üzerinde çift taraflı pozitifliği

of chondrogenic nodules can be seen in cultures *(Fig. 3)*. Osteogenic differentiation was shown by Von Kossa staining of calcium deposition in cultures. Also individual bone nodules can be seen in the cultures *(Fig. 4)*.

The LMSCs were put through the procedure for keratinocyte differentiation *(Fig. 5)* and subsequently analysed by using flow cytometry for CK19 and pancytokeratin markers of progenitor and diffrentiated corneal epithelium respectively. The high positivity of CK19 and pancytokeratin was observed in both sides of the amniotic membrane with a multilayer manner *(Fig. 6)*.

## DISCUSSION

The placenta is comprised three-layer structure of the amnion, chorion and decidua. The roles of the placenta are to provide nutrients and oxygen, which are essential for fetal survival and development. The amnion is a thin, nonvascular membrane, which has a two-layer structure: an epithelial monolayer and a stromal layer [9]. In particular, the amnion is the sac that binds the fetus and constructs the environment. The amniotic membrane has clinical applications in covering wounds and burn lesions and ocular surface reconstruction [10]. Recently, canine stem cells have been studied for use in cell therapy [11,12]. However, they have been confined to limited stem cell sources. Most of the cells were isolated from canine adipose-derived tissue, umbilical cord blood or bone marrow [13-15]. Therefore, limbal mesenchymal stem cells provided from the fotal tissue seems to be a new cell source for corneal reconstruction. Beyond this, these cells are the native cells of the eye itself and it is thought that with the use of these cells a better corneal healing and more transparent cornea could be obtained.

Systemic transplantation of a high dose of MSCs reduces induced inflammatory damage to the cornea by secretion of TNF-α-stimulated gene/protein 6 with minimal engraftment [16], which shows that systemic administration is not an efficient way to deliver stem cells to the cornea, a physiologically avascular tissue. However, the abundant paracrine effect of MSCs contributes to inhibition of inflammation. It has been reported that BM-MSCs expanded on an amniotic membrane can reconstruct cornea by inhibiting inflammation and angiogenesis [17]. Alternatively, application of stem cells with the help of

the amniotic membrane provides stem cells with direct contact with the corneal epithelium and stroma on a damaged cornea.

Wound healing is essential for tissue regeneration. Corneal transparency determines visual acuity. Corneal tissues are physiologically composed of the corneal epithelium, stroma and endothelium. Clarity of the cornea depends on an intact corneal epithelium, tight organisation of epithelial cells, constant water content, and regular arrangement of keratocytes and keratocyte produced extracellular matrix in the stroma [18-20]. For the future applications, when LMSCs differentiated into keratinocytes are applied to the corneal wound, it is believed that it will reduce the time needed for healing of the corneal opacity.

In our previous study, we found that cell-cell interactions between stem cells were crucial for cell signalling [21]. Recently, application of autologous MSCs to the bottom of a corneal ulcer for a persistent sterile corneal epithelial defect has been reported [22]. The corneal stroma is connective tissue maintained by keratocytes [23]. Administration of stem cells to the cornea with an epithelial defect provides a diffuse distribution of cells in the stroma, and which favors direct stem cells-keratocyte interactions. Furthermore application of keratocytes with the help of amniotic membrane will result with a better corneal healing and transparent cornea. At the same time it will reduce the time of these cells to turn into keratinocytes.

Initially it is believed that anti inflammatory effect of stem cells will inhibit the cellular response and at a late stage promotion of the differentiation of stem cells into keratinocytes will have a high impact corneal transparency. Disorders like corneal ulcers, neurotrophic ulcers, feline corneal necrosis (FKN), keratoconjunctivitis sicca (KCS), Uber-Reiters Syndrome, Feline herpesvirus type-1 (FHV-1) associated disorders can be cured with cultured limbal epithelial stem cells. Furthermore the fetal limbal stem cell cultivation and differentiation into keratinocytes is a simple and a reliable method of delivering stem cells to cornea for corneal tissue regeneration. Once it is produced it can safely and effectively be used in the same species and also be stored for subsequent applications.

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