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IN VITRO CULTIVATION OF CANINE LIMBAL TRANSPLANT

VLAHOVIĆ KSENIJA*, PIRKIĆ B*, POPOVIĆ IVA**, BOROŠAK H*, HOHŠTETER M*, KIŠ IVANA*, MATIJATKO VESNA*, MULJAČIĆ A**, ŠPOLJARIĆ D* and POPOVIĆ MAJA*

**University of Zagreb, Faculty of Veterinary Medicine, Croatia*
***University of Zagreb, Medical faculty, University Clinic for Traumatology, Croatia*

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Limbal epithelial stem cells are the ultimate source of regeneration of the entire corneal epithelium under both normal and injured conditions. The corneal epithelium plays a crucial role in homeostasis and integrity of the eye. To maintain the integrity of the ocular surface, corneal epithelial cells must be balanced by stem cells, located at the limbus. The limbus is the crossing area between the cornea and sclera, 1 mm in width, and together with conjunctival epithelium plays an important role in regenerating the cornea after traumatic injuries. The aim of this study was to evaluate the culturing patterns of canine limbal stem cells and to optimize growing conditions of these cell cultures in order to develop a reliable biomedical model intended for studying the potentials of allografts/xenografts originated from canine tissues. Canine stem cell equivalents have potentials in reparative/regenerative veterinary medicine.

Key words: canine, cornea, stem cells

INTRODUCTION

Cell therapy is an emerging field whose technology is being investigated for the treatment of a variety of injuries and diseases affecting multiple tissue types. Many questions exist in the field of cell therapy, from understanding the basic mechanisms of action, to the optimal methods for preparing and administering cells to the patient. Answers to these questions will help to determine the best cell source, optimal protocols for *ex vivo* manipulation of cells, and the best methods for delivery of cells (Shi and Clegg, 2008).

An increasing number of embryonic-, foetal- and adult- derived stem cell preparations and lines are readily available today through various cell banks and commercial suppliers. Stem cells constitute a virtually inexhaustible supply of raw material from which to derive the specific differentiated cell types required for current biomedical research and future cell replacement therapy. Pluripotent stem cells are of the widest applicability and by their nature the most challenging stem cell types to differentiate reliably.

The stem cells, providing tissue regeneration are residents of different organ structures in the body and are usually located in well protected sites of organs and tissues. For stem cells of either skin or corneal epithelium such sites are in the deepest layer of epidermis or in the basal epithelial layer of the cornea residing its limbal region termed palisade of Voight (Sudha, 2007). A growing interest in allografts and xenografts implies a thorough study of regenerative potentials of these cells, as well as a clear description of their patterns in the *in vitro* tissue cultures to be grafted.

Limbal epithelial stem cells are the ultimate source of regeneration of the entire corneal epithelium under both normal and injured states (Daniels *et al*., 2001). The corneal epithelium plays a crucial role in homeostasis and integrity of the eye. To maintain the integrity of the ocular surface, corneal epithelial cells must be balanced by stem cells, located at the limbus (Chen *et al.*, 2004). Limbus is the crossing area between the cornea and sclera, 1 mm in width, and together with conjunctival epithelium plays an important role in regenerating the cornea after traumatic injuries (Sangwan, 2001). Thorf and Fried had in 1983 made a hypothesis, known as XYZ hypothesis of limbal stem cells, as important cells for the maintenance and regeneration of corneal epithelial cells (Brunelli *et al*., 2006). On the basis of mathematical model of corneal epithelial cells kinetics, Rheinwald and Green (1975) have proved that the level of exfoliation of these cells in the precorneal tear film equals their production from limbal stem cells. Subsequently, in modern human and veterinary medicine, their use in corneal surgery is more and more frequent. It's already known that the corneal epithelium has a good regeneration capacity, 7 to 14 days in most mammals, and that these cells can be found in the limbus, rich in nerves and blood vessels, unlike the cornea (Swift *et al.,* 1996). However, newer reserches proved that the epithelium itself has cells that are capable of regeneration, and the limbus is only a reservoir of such cells in conditions of severe damage (Pauklin *et al*., 2009).

The aim of this study was to evaluate the culturing patterns of canine limbal stem cells and to optimize growing conditions of these cell cultures in order to develop a reliable biomedical model intended for studying the potentials of allografts/xenografts originated from canine tissues/organs.

MATERIALS AND METHODS

Biological material: Eye bulb samples (2-4 cm width) were collected under the supervision of an ophthalmologist on the Clinic for Surgery, Orthopaedics and Ophthalmology, Veterinary Faculty University of Zagreb. Cats were in good condition, male, about 1 year old, domestic, euthanized with emmbutramide (T-61, Intervet®) by request of the owner. Eight eyes were transported and held for 24 hours at +4^{o}C in transportation medium: DMEM with 4.5 g/L glucose (Invitrogen–Gibco), 1% ABAM solution (penicilline-streptomycine-antimycotic) (Invitrogen – Gibco, cat. No. 15240-062).

Limbal 2 mm^2 samples were obtained using penetrating keratotomy from the cadaveric eye, within the premises of the Clinic for Surgery, Orthopaedics and Ophthalmology, Veterinary Faculty, University of Zagreb.

Procedure of canine limbal cell extraction from the eye: In sterile conditions (Forma Scientific sterile chamber), limbal 2 mm^2 samples were irrigated for 40 minutes with 5% ABAM in phosphate buffer (PBS, Invitrogen, Gibco). Specimens were then disinfected and fragmented into 1-2 mm^2 pieces, then treated during 30 minutes in 0.25 % tripsin/1 mM EDTA solution (Sigma), carefully stirred at +37°C. In such a cell suspension, the number of cells was measured in Neubauer's cell counting chamber, using trypan blue (Sigma, St. Louis, USA).

Cell cultivation in primary culture: For cell cultivation in primary culture, 1.5 x 10^6 cells were put into 6 well plates, onto nutritive layer with γ-rays radiated 3T3 cells (4 x $10^4/cm^2$), prepared in DMEM medium/Ham F12 (2:1), 10% FBS, epithelial growth factor (EGF, 10 ng/mL), insulin (5 μg/mL), adenine (24.3 μg/mL), cholera toxin (0.1 nM), L-glutamine (4 mM), hydrocortisone (0.4 μg/mL), triiodthyronine (1.36 ng/mL) and ABAM (Invitrogen-Gibco).

Cell cultivation in explant culture: In sterile conditions (Forma Scientific sterile chamber), 1-2 mm^2 fragmented limbal tissue was put into 6 wall cultivation plates in 3 mL of medium without cell growing serum (Serum Free Media SFM, Invitrogen-Gibco). Cells were incubated (Forma Scientific incubator) in the atmosphere with 5% CO_2 in +37°C. Every three days, the medium was changed under sterile conditions.

Cell cultivation in secondary culture: After cells from primary and explant culture have grown over 80% of the plate bottom, they were treated with 1 mL of 0.25% tripsine during 2-3 minutes, causing cell elevation from the cultivation plate bottom. Such cell suspension was transferred in sterile conditions into test tubes, centrifuged for 4 minutes at 1100 rpm. Cell sediment was resuspended in 1 mL SFM medium, and grown onto:

a) in concentration of 1-2 x 10^6 in 3 mL DMEM/Ham F12 medium on 3T3 cells in plastic plates. Cells were incubated in the atmosphere with 5% CO_2 at +37°C until 100% of the plate bottom was overgrown. Every 3 days a medium was changed in sterile conditions. Secondary cultures on nutritive 3T3 cell layer in the 100% confluention phase were treated with dyspasa solution (Invitrogen-Gibco) in DMEM at a concentration 2.5 mg/mL, during 15-20 minutes at +37°C, which caused elevation of confluent limbal transplant from the bottom of cultivation plate, after which the transplant was elevated on a carrier made from silicon mash (Invitrogen-Gibco), size about 1.0 cm^2.

b) in 3 mL SFM medium onto soft contact lenses (Focus Night & Day, Iotrafilcon A, CIBA Vision, Dublin), in concentration of 2.5 x $10^4/cm^2$ cells.

Preparation of nutritive mouse fibroblast plates (3T3 cells): Until their use, 3T3 cells were held at -70°C in test tubes prepared for freezing, in which is already put approx. 1 mL of cell suspension. Immediately before their use, cells were defrosted in a water bath at +37°C, to which was added 9 mL DMEM/Ham F12 medium or in FM medium (30 mL FBS, 3 mL L-glutaminate, 3 mL ABAM were added in 300 mL DMEM), then centrifuged for 4 minutes at 1100 rpm. After centrifuging in the cell containing residue, 1 mL DMEM/Ham F12 medium or FM medium is added, and then ingrown in a concentration of 2 x 10^6 per 75 cm^2 chamber in 3 mL FM medium. Bottles, thus prepared were radiated with 56 Gy γ-

rays during 37.5 minutes (Ruđer Bošković Institute, Zagreb), and incubated at +37°C, 5% CO_2 for two days, before limbal cells were added.

Cell colouring using May-Grunwald-Giemsa method: Primary culture cell samples were peeled with scalpel blade off the cell cultivation chamber bottom (tissue culture flask), and from those specimens, a smear on slides was made. Specimens were fixed using air-drying, than coloured using standard May-Grunwald-Giemsa method. Morphologic characteristics of those samples were analyzed using a light microscope.

Microscoping: Cells in primary/secondary cell culture were microscopically analyzed using Nikon TS 100 microscope with epifluorescency equipped camera and LCD control monitor.

Treatment and sampling were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). This study was supported by the grants nos. 053-0532265-2255, 053-0531863-1861, 053-0532264-2260 from the Ministry of Science, Education and Sport of Croatia.

RESULTS

Moderate cellularity was noted in all specimens. Most of cells were densely grouped in an unilayered formation.

Canine limbal cells in primary culture, cultivated on 3T3 cells, achieved 80% confluence already 4 days after ingrowing (Figure 1).

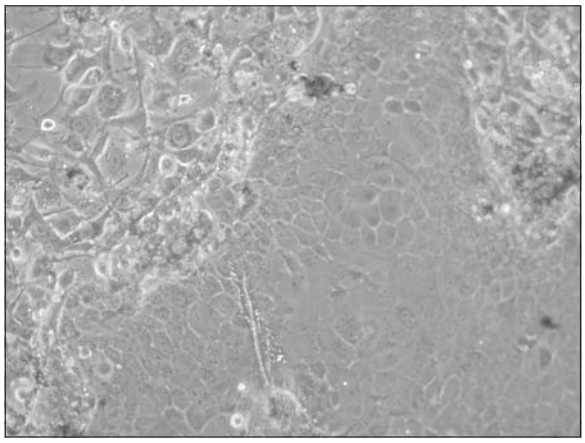

Figure 1. The primary culture of canine limbal cells cultivated on 3T3 cells 4 days after ingrowing (magnification x10)

Nevertheless, two days later, 80% confluence was achieved by cells cultivated solely in SFM medium *in explant* culture (Figure 2).

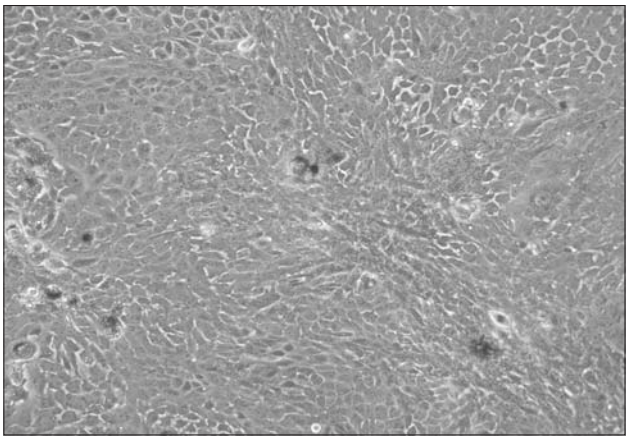

Figure 2. The primary culture of canine limbal cells cultivated in SFM medium *in explant* culture 6 days after ingrowing (magnification x10)

In limbal cell samples peeled from the primary cultures grown on 3T3 cells or from the explant culture in SFM medium at the moment of their 80% confluence, dyed with standard May-Grunwald-Giemsa method, a moderate cellularitiy was noted. Most of the cells within all specimens were in larger clusters of densely grouped unilayered cell formations. Anisocytosis and anisokaryosis were moderately present amongst the cells. Middle sized cells are oval to polygonal in shape with moderate to larger amounts of light blue cytoplasm in which a small number of vacuoles were noted in different places. Within some of the cells, a small number of brown circular granules were noted, looking like melanin granules. Cell nuclei were light violet, oval shaped with small grained chromatin and in some of them, a small vacuole was seen. Each cell contains one to two nuclei. Mitosis occurs rarely. According to morphological characteristics, these are corneal epithelial cells. Amongst the described cells, smaller groups of cells were recorded, containing only few oval to polygonal cells with larger cytoplasm with a greater number of melanin granules. Cell nuclei are oval, with poorly seen nucleoli. Thus described, these cells morphologically resemble to melanocites that are found between limbal epithelial cells (Figure 3).

Within secondary cultures, cells were grown on a nutritive layer of γ-rays radiated 3T3 cells, which have grown in primary culture on 3T3 cells, and have achieved 100% confluence within 10 days since ingrowth. Confluent limbal transplant measuring approx. 1.0 cm^2, was elevated from the bottom of the cultivation plate, on a sterile silicone mesh, 14 days from the beginning of limbal cell isolation from the cadaveric eye.

Cells in secondary cultures, cultivated on nutritive layer of γ-rays radiated 3T3 cells, grown *in explant* culture in SFM medium, have achieved 100% confluence within 12 days from ingrowth. Confluent limbal transplant, size

1.0 cm^2, was elevated from the bottom of the cultivation plate, on a silicon mesh, 18 days from the start of limbal cells isolation from the cadaveric eye.

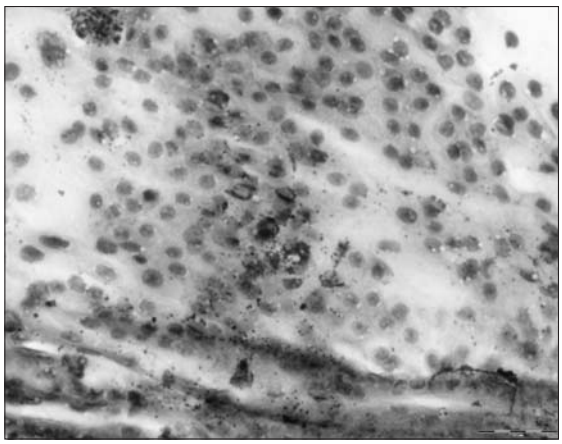

Figure 3. The primary culture of canine limbal cells cultivated in SFM medium *in explant* culture 6 days after ingrowing as visualized by May-Grunwald-Giemsa staining method (magnification x 20)

Cells within the secondary cultures were grown on the soft contact lens, in primary culture on 3T3 cells achieved 100% confluence 6 days since planting (Figure 4).

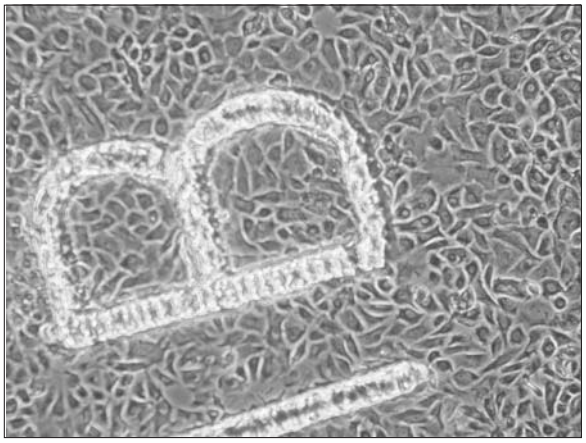

Figure 4. The secondary *in explant* culture of canine limbal cells in SFM medium 5 days after ingrowing on "Night & Day" lenses (magnification x20)

DISCUSSION

The anterior eye segment is covered with corneal, scleral and conjunctival epithelium. Conjunctival epithelium is well vascularised with goblet cells that produce mucous, and helps in keeping the corneal surface moist. Corneal tissue is transparent, avascular and innervated with numerous neural endings. Nutrients are obtained from the surrounding tissue, and from tears. Limbus is a thin border between the sclera and cornea, and beside epithelial cells, Langerhan's cells and melanocites are also present.

Stem cells, responsible for tissue regeneration across the body are positioned usually in a well protected area (e.g. stem cells responsible for intestinal epithelium regeneration are positioned in the depths of intestinal crypts, those applied to the hematopoetical system are located in the bone marrow etc. (Akped and Foster, 1999). Stem cells, all across the body are alike, and amongst the crucial characteristics there is a very low mitotic activity, a slow cell cycle along with a huge ability to regenerate. Fenotipically, these are immature cells in which cytoplasma small number of differentiated organelles is found. Owing to its structure and transparency the cornea ensures the passage and focus of light onto the retina.

Multilayered squamous epithelium is an important part in the development of sight. Like as in other epithelium types, during the desquamation process, the outher layer of cells is being constantly replaced. Regeneration starts in the limbal region, within the basal epithelial layer (palisades of Voigt), where the corneal stem cells are (Dua and Azuara-Blanco, 1999). Under certain conditions, stem cells change to *transit amplifiying cells* (TAC), which are the majority in the proliferating population. TAC cells migrate centripetally, from the limbus to suprabazal, outer layers of the cornea, where they gradually change to become postmitotically and terminally differentiated (Dua and Azuara-Blanco, 2000). Limbal stem cell deficiency (LSCD), as a disease, is a result of damage of palisades of Voigt. Various conditions can cause such a pathology; from trauma, hereditary problems (aniridia, ectodermal dysplasia), radiotherapy, chryotherapy, to plain inflammation. Henceforth, conjunctival epithelium along with its blood vessels grows into the cornea, ending in erosions, pain, inflammation and visual impairment. Limbal stem cell deficiency can be partial or total. Partial LSCD is characterized by some spared stem cells outside limbal area, and total stem cell deficiency where all stem cells are destroyed (Shortt *et al.,* 2007).

Severe clinical cases require limbal transplantation, either autograft or allograft (Bianco and Robey, 2001). Limbal allotransplantation is increasingly being used for ocular surface repair in animals with stem cell dysfunction. (Henry *et al*., 2004).

These therapeutic possibilities have their drawbacks; limited transplant area originating from a living donor and a possibility of tissue rejection if it's an allograft. However, it is uncertain whether donor cells survive long term on the ocular surface, and whether patients maintain the early benefits of the procedure.

Nowdays, in human medicine, we are witnessing the development of *in vitro* methods of multiplying limbal stem cells, isolated from very small biopsy samples

from the limbal region, further growing *in vitro* on a 3T3 cell nutritive layer (mouse fibroblasts), or on a suitable carrier (amniotic membrane, fibrin, collagen, contact lenses, etc.) (Shortt *et al.,* 2007; Kim *et al.,* 2008), in order to eventually obtain a transplant. However, the use of limbal epithelial cells in corneal surgery is very rare in veterinary medicine, and they concern procedures such as corneal transplantation, corneal conjunctivalisation therapy, or limbal insufficiency and keratopathy (O'Sullivan and Clynes, 2007). Potential justification of their use in veterinary ophthalmology can lie in house pet or breed selection animal therapy. During current researches, Brunelli *et al.* (2007) have postulated that the dog model of total limbal destruction is feasible and reliable in producing severe ocular surface wounds, resulting in loss of corneal clarity. Limbal autograft transplantation was effective in restoring corneal transparency with no ocular complications. Absence of complications in donor eyes is an evidence of the advantages and efficiency of limbal autograft transplantation in managing stem cell deficiency after mechanical trauma or chemical injury.

The most frequent method used within medical researches of limbal stem cell biology is penetrating keratotomy from the cadaver eye. The described method was very successfully applied on swine limbal cell cultivation by Pirkić *et al.* (2009). Furthermore, most frequent "*ex vivo*" method of limbal cell sampling refers to penetrating keratoplasty, where the transplantation of allograft grown on an amniotic membrane is in use, or even autotransplantation from a healthy eye (Bianco and Robey, 2001). Transplantation of the limbus for treating limbal stem cell dysfunction is based on the hypothesis that the transplanted donor graft will be able to provide the selfrenewing stem cells that are responsible for corneal epithelial reconstruction (Chen *et al*., 2004).

Rheinwald and Green (1975) method, used in this work includes *in vitro* cell cultivation on a nutritive layer of γ-rays radiated or mytomicin-C treated 3T3 cells. Treated 3T3 fibroblasts no longer multiply, but stay metabolically active within the culture, in a way that with it's soluble factors and cell-cell contact aids growth and differentiation of epithelial cells in co-culture (Boranić *et al*., 1999; Popović *et a*l., 2009a; Popović *et al*., 2009b). Thus, in our research, by applying this method on an animal model, canine limbal stem cells cultivated *in vitro* on 3T3 cells, have achieved 80% confluence a day earlier comparing to *in vitro* cultivation of swine limbal cells on 3T3 fibroblasts. However, although this method proved itself beneficial in our research or available references, it causes a problem of infection transmittance, whether it's human or animal. Furthermore, there is a possibility of development of immunological reactions against molecules of animal origin.

One of the biggest challenges of *in explant* cell therapy, articularly an autologous approach, is the transfer of cell-based products from *ex vivo* culture to the patient in a clinically and commercially viable way. While autologous cell therapy provides significant advantages including safety to the patient, it presents a unique manufacturing challenge. Within previous investigations Pirkić *et al.* (2009) described advantages of swine limbal cell cultivation in explants culture, when the cells thus cultivated achieved 80% confluence only two days after, comparing to cells cultivated on 3T3 culture. An approximately similar result was achieved in this investigation also, while cultivating explanted cat limbal stem cells

in SFM medium, for which was cytologically proved that they morphologically resemble swine corneal epithelium, which is in accordance with literature data (Sudha, 2007).

Current researches are pointed on the definition and characterization of suitable carriers for *in vitro* cultivation of limbal epithelium. Rheinwald and Green method (1975) includes growth on a nutritive layer of 3T3 cells treated with γ-rays, or mytomicin-C. Treated 3T3 fibroblasts no longer multiply, but stay metabolically active in culture in a way that with its soluble factors and cell-cell contact benefit the growth and differentiation of epithelial cells. To number a few things that aren't beneficial in cultivation using mouse fibroblasts are the possibility of animal virus transmission and immunoreactions on animal molecules. The amniotic membrane is frequently used as a carrier in limbus cultivation. Rich in growth factors and other molecules that enhance the corneal epithelium regeneration in clinical use, suppresses inflammation, fibrosis. Besides, multiplied cells are collected from the cultivation plate bottom along with the membrane, and therefore are easily applied on an injured wound. The setbacks for the clincal use of this proceure are the time and skill needed for the preparation of the membrane (Shortt *et al.*, 2009). Amniotic membrane in conjunction with a feeder layer of 3T3 cells also appears to provide a good matrix for limbal cells attachment and growth. Grueterich *et al.* (2003) showed that both amniotic membrane and 3T3 cells help maintain cells in a less differentiated phenotype at the monolayer stage. The beneficial effect is seen when 3T3 cells are not in direct contact with the expanded epithelium, suggesting diffusible factors or cytokines to be responsible (Chen *et al.*, 2007).

In earlier investigations, Pirkić *et al.* (2009) were using sterile silicone mesh, thus lifting very easily confluent limbal transplant sizing approx. 1.5 cm^2, which was sufficient for their further clinical application on swine. Similarly, in this investigation, for further clinical treatment of cats, a limbal transplant sizing 1 cm^2 was lifted using silicone mesh. Furthermore, in veterinary ophthalmology is of utmost interest the use of therapeutic contact lenses "Night & Day" with silicone hydrogel serving as a carrier for limbal cell growth. The here presented results point that the best method for confluent cat limbal cell transplant in *in vitro* conditions is explant culture in SFM media, thereon growing the obtained limbal cells in secondary cultures on soft contact lenses.

In the Cell laboratory of Department for Biology, Veterinary faculty, University of Zagreb, from *in vitro* grown canine limbal cells, 10 canine corneal transplants, size 1.0 cm^2 were obtained. Such results are encouraging, therefore curative value of canine corneal transplants should be evaluated within a research conducted on patients of choice with corneal defects on Clinic for Surgery, Orthopaedics and Ophthalmology, Veterinary faculty, University of Zagreb. Beneficial therapeutic effects could encourage an establishment of eye banks, divided according to species, and of interest for veterinary medicine.

Address for correspondence:
Prof. dr. sc. Maja Popović
Department of biology
Faculty of Veterinary Medicine
University of Zagreb
Heinzelova 55, Zagreb
Croatia
E-mail: mpopovic@vef.hr

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IN VITRO KULTIVACIJA LIMBALNOG TRANSPLANTATA MAČKE

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SADRŽAJ

Epitelne matične stanice limbusa osnovni su izvor regeneracije čitavog epitela rožnice, kako u zdravom stanju, tako i u slučaju traume. Epitel rožnice igra važnu ulogu u očuvanju homeostaze i integriteta oka. Kako bi očuvali cjelovitost površine oka, epitelne stanice rožnice održavane su djelovanjem matičnih stanica, smještenim na limbusu. Limbus je granično područje, širine 1 mm, između rožnice i bjeloočnice, a zajedno sa epitelom rožnice igra značajnu ulogu u regeneraciji rožnice nakon ozljeda. Cilj ovog istraživanja je bilo vrednovanje osobitosti uzgajanja mačjih limbalnih zametnih stanica u kulturi i optimiziranje uvjeta njihovog uzgajanja radi uspostavljanja pouzdanog biomedicinskog modela pogodnog za istraživanje potencijala alo- i kesnotransplantata podrijetlom od mačjeg tkiva. Mačji ekvivalenti zametnih stanica imaju potencijale u reparativnoj/regenerativnoj veterinarskoj medicini.