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In vitro cultivation of porcine limbal transplants

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ABSTRACT

The stem cells that provide 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 corneal epithelium such sites are in the basal epithelial layer of the cornea residing in its limbal region, termed the palisade of Voight. The growing interest in allografts and xenografts implies a thorough study of the regenerative potential of these cells, as well as a clear description of their patterns in the *in vitro* tissue cultures to be grafted. The aim of this study was to evaluate the culturing patterns of porcine 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 porcine tissues. Porcine stem cell equivalents have potential in reparative/regenerative veterinary medicine for pets, horses and selected breeds of food animals, but also as xenografts for humans.

Key words: porcine, cornea, stem cells

Introduction

The anterior eye segment is covered with corneal, scleral and conjunctival epithelium. Conjunctival epithelium is well vascularised with goblet cells that produce mucous, and

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help 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 tars. The limbus is a thin border between the sclera and cornea, and besides epithelial cells, Langerhan's cells and melanocites are also present.

Stem cells, responsible for tissue regeneration across the body, are usually positioned in some well protected area (e.g. stem cells, responsible for intestinal epithelium regeneration are positioned in the depths of the intestinal crypts, those applied to the hematopoetical system are in the bone marrow (AKPED and FOSTER, 1999). Stem cells throughout the body are alike, and amongst their crucial characteristics there is very low mitotic activity, and a slow cell cycle, along with a huge ability to regenerate. Fenotipically, these are immature cells in whose cytoplasm there is a very small number of differentiated organelles. Owing to its structure and transparency, the cornea enables the passing and focusing of light onto the retina.

The multilayered squamous epithelium is an important part of the development of sight function. As in other epithelium types, during the desquamation process the outer layer of cells is constantly being 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 of the proliferating population. TAC cells migrate centripetally, from the limbus to the suprabazal, the outer layers of 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 to the palisades of Voigt. Various conditions can cause this pathology, from trauma, hereditary problems (aniridia, ectodermal dysplasia), radiotherapy, chryotherapy, to plain inflamation. Henceforth, the conjunctival epithelium grows along with its blood vessels into the cornea, ending in erosions, pain, inflammation and visual impairment. Limbal stem cell deficiency can be partial or total. With partial LSCD stem cells outside the limbal area are spared, but with total, 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: the limited transplant area originating from a living donor, and the possibility of tissue rejection in the case of 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.

Nowadays 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), to eventually obtain a transplant. However, the use of limbal epithelial cells in corneal surgery is very rare in veterinary medicine, in terms of procedures such as corneal transplantation, corneal conjunctivalisation therapy, or limbal insufficiency and keratopathy (O'SULLIVAN and CLYNES, 2007). The potential justification of their use in veterinary ophthalmology can lie in house pet or breed selection animal therapy. During current research, BRUNELLI et al. (2007) 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. The absence of complications in donor eyes is evidence of the advantages and efficiency of limbal autograft transplantation in managing stem cell deficiency after mechanical trauma or chemical injury.

Therefore, the purpose of this investigation was to apply *in vitro* growth technology of limbal stem cells to an animal model, aiming:

1. to adjust the conditions of *in vitro* cultivation of porcine limbal stem cells;
2. to prepare porcine limbal transplants from *in vitro* cultivated porcine limbal cells.

Materials and methods

Biological material. Eye bulb samples (2-4 cm width) were collected under the supervision of a veterinary inspector, from 4 pigs (German landras, male, 6 months old, weighting approximately 110 kg) within the "MM" slaughterhouse, Gornje Prekrižje, Krašić.

Eight eyes were transported and held for 24 hours at 4 °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 5 mm^2 samples were obtained using penetrating keratotomy from the cadaveric eye, at the premises of the Clinic for Surgery, Orthopaedics and Ophthalmology, Veterinary Faculty, University of Zagreb.

Procedure of porcine limbal cell extraction from the eye. In sterile conditions (Forma Scientific sterile chamber), limbal 5 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, and treated for 30 minutes in a 0.25% tripsin/1mM EDTA solution (Sigma), carefully stirred at 37 °C. In that 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, 1.5×10^6 cells were put into 6 well plates, onto a nutritive layer with γ-ray radiated 3T3 cells ($4\times10^4/cm^2$), 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), 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 at 37 °C. Every three days the medium was changed in sterile conditions.

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

a) in a concentration of $1\text{-}2\times10^6$ 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 plate bottom was overgrown. Every 3 days the medium was changed in sterile conditions. Secondary cultures on the nutritive 3T3 cell layer in the 100% confluention phase were treated with dyspasa solution (Invitrogen-Gibco) in DMEM of concentration 2.5 mg/mL, for 15-20 minutes at 37 °C, which caused elevation of the confluent limbal transplant from the bottom of the cultivation plate, after which the transplant was elevated onto a carrier made from silicon mash (Invitrogen-Gibco), sized about 1.5 cm^2.

b) in 3 mL SFM medium onto soft contact lenses (Focus Night & Day, Iotrafilcon A, CIBA Vision, Dublin), in a concentration of $2.5\times10^4/cm^2$ cells.

Preparation of nutritive mouse fibroblast plates (3T3 cells). Until their use, the 3T3 cells were kept at -70 °C in test tubes prepared for freezing, in which there was already approximately 1mL of cell suspension. Immediately before use, the 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 to 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 was added, and then ingrown in the concentration 2×10^6 per chamber of 75 cm^2 in 3 mL FM medium. Bottles, thus prepared were radiated with 56 Gy γ-rays for 37.5 minutes (Ruđer Bošković Institute, Zagreb), and incubated at 37 °C, 5% CO_2 for two days, before the limbal cells were added.

Cell colouring using May-Grünwald-Giemsa method. Primary culture cell samples were peeled with a scalpel blade off the cell cultivation chamber bottom (Tissue culture flask), and from those specimens, smears on slides were made. Specimens were fixated using air-drying, then dyed using the standard May-Grünwald-Giemsa method. The morphologic characteristics of those samples were analyzed using a light microscope.

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

The treatment of pigs and sampling were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC).

Results

Moderate cellularity was noted in all specimens. Most of the cells were larger groups of densely grouped cells in unilayered formations.

Fig. 1. The primary culture of porcine limbal cells cultivated on 3T3 cells 5 days after ingrowing (×10)

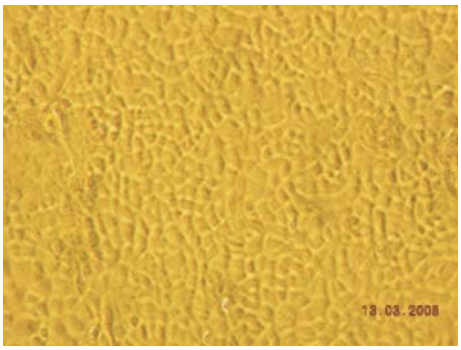

Fig. 2. The primary culture of porcine limbal cells cultivated in SFM medium *in explant* culture 7 days after ingrowing (×10)

Porcine limbal cells in primary culture, cultivated on 3T3 cells, already achieved 80% confluence after 5 days after ingrowing (Fig. 1).

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

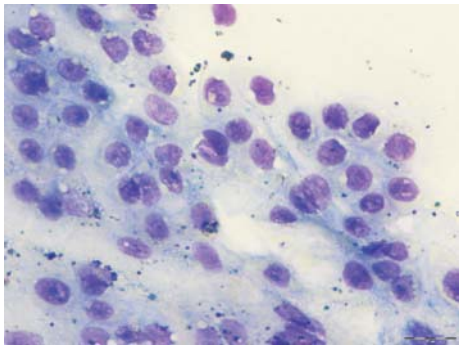

Fig. 3. The primary culture of porcine limbal cells cultivated in SFM medium *in explant* culture 7 days after ingrowing as visualized by May-Grünwald-Giemsa staining method (×20)

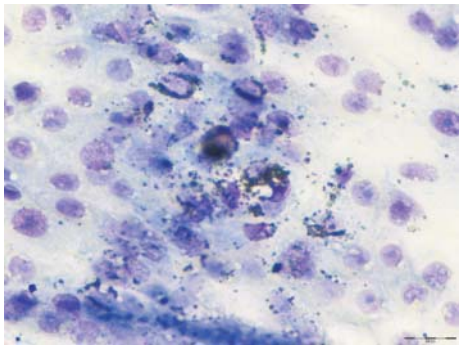

Fig. 4. The melanocites between limbal cells in the primary culture of porcine limbal cells cultivated in SFM medium *in explant* culture 7 days after ingrowing as visualized by May-Grünwald-Giemsa staining method (×20)

Fig. 5. Confluent limbal transplant, size 1.5 cm^2 on a silicon mesh, 19 days from the start of limbal cells isolation from limbal from cadaveric eye

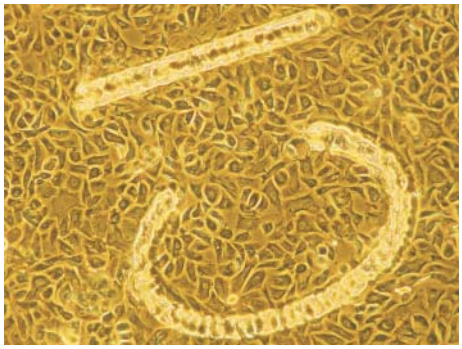

Fig. 6. The secondary *in explant* culture of porcine limbal cells in SFM medium 5 days after ingrowing on «Night & Day» lenses (×20).

In limbal cell samples peeled from primary cultures grown on 3T3 cells or from explant culture in SFM medium at the moment of 80% confluence, dyed by the standard May-Grünwald-Giemsa method, moderate cellularitiy was noted. Most of the cells within all specimens were in large groups of densely grouped unilayered cell formations (Fig. 3). Anisocytosis and anisokaryosis were noted moderately amongst the cells. Middle sized cells are oval to polygonal in shape with moderate to large amounts of light blueish

cytoplasm, in which a small number of vacuoles were noted in different places. Within some 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 contained one to two nuclei. Mitosis occurred rarely. According to their morphological characteristics, they were corneal epithelial cells.

Amongst the described cells, smaller groups of cells were seen, containing only few oval to polygonal cells with large cytoplasm with a greater number of melanin granule. Cell nuclei were oval, with poorly seen nucleoli. Thus described, these cells morphologically resemble the melanocites (Fig. 4) that are found between limbal epithelial cells.

Within secondary cultures, the cells were grown on a nutritive layer of γ-rays radiated 3T3 cells, which grew in the primary culture on 3T3 cells, achieved 100% confluence within 10 days from ingrowth. A confluent limbal transplant measuring approx. 1.5 cm^2, was elevated from the bottom of the cultivation plate, on a sterile silicone mesh, 15 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, achieved 100% confluence within 12 days from ingrowth. A confluent limbal transplant, size 1.5 cm^2, was elevated from the bottom of the cultivation plate, on a silicon mesh, 19 days from the start of limbal cell isolation from the limbal from the cadaveric eye (Fig. 5).

Cells within the secondary cultures were grown on a soft contact lens, in primary culture on 3T3 cells, and achieved 100% confluence 7 days from planting. Nevertheless, 5 days between ingrowths, grown on a 3T3 cell layer, 100% confluence was noted in cells from the *in explant* layer, in SFM medium (Fig. 6).

Discussion

The corneal epithelium plays a crucial role in the homeostasis and integrity of the eye. To maintain the integrity of the ocular surface, corneal epithelial cells must be balanced by stem cells, located in the limbus (CHEN et al., 2004). The limbus is the area between the cornea and sclera, 1 mm in width, and together with the conjunctival epithelium plays an important role in regenerating cornea after traumatic injuries (SANGWAN, 2001).

Limbal epithelial stem cells are the ultimate source of regeneration of the entire corneal epithelium in both normal and injured states (DANIELS et al., 2001). THORF and FRIED made a hypothesis in 1983, known as the XYZ hypothesis of limbal stem cells, as important cells for maintenance and regeneration of corneal epithelial cells (BRUNELLI et al., 2006). On the basis of a mathematical model of corneal epithelial cells kinetics, RHEINWALD and GREEN (1975) proved that the level of exfoliation of these

cells in precorneal tear film equals their production from limbal stem cells. Subsequently, in modern human and veterinary medicine, their use in corneal surgery became more and more frequent. It is already known that corneal epithelium has 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, recent research has 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 most frequent method used in medical research in limbal stem cell biology is penetrating keratotomy from the cadaveric eye. This method was also applied in this study to a porcine eye model. Furthermore, the most frequent "*ex vivo*" method of limbal cell sampling refers to penetrating the 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 self-renewing stem cells that are responsible for corneal epithelial reconstruction (CHEN et al., 2004).

Studies of the epithelial phenotype have shown that the limbal basal epithelium does not express corneal epithelial specific keratin 3 or keratin 12 (SCHLÖTZER-SCHREHARDT and KRUSE, 2005; O'SULLIVAN and CLYNES, 2007). Studies of cell cycle kinetics have shown that some portions of the the limbal basal epithelial cells are slow cycling and label retaining (KIM et al., 2008). Other studies have further confirmed that limbal epithelial cells have a greater growth potential *in explant* cultures and higher clonogenicity when co-cultured on 3T3 fibroblast feeder layers, and that their proliferative potential is resistant to tumour promoting phorbol esters (SHORTT et al., 2007).

The 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 its soluble factors and cell-cell contact aids growth and differentiation of epithelial cells in the co-culture (BORANIĆ et al., 1999; POPOVIĆ et al., 2009). Thus, in our research, applying this method to an animal model, porcine limbal stem cells cultivated *in vitro* on 3T3 cells, achieved 80% confluence within 5 days from ingrowth. However, although this method proved itself beneficial in our research or available references, it causes the problem of infection transmittance, whether human or animal. Furthermore, there is a possibility of immunological reactions development in molecules of animal origin. Current research proves that the nutritive layer of 3T3 cells could be replaced by a nutritive layer of human epithelial amniotic membrane cells. It seems that such a nutritive layer withdraws cells with less differentiation but with more stem cells (FIGUEIRA et al.,

2007). Limbal stem cells are identified on the basis of their morphological characteristics, e.g. small cells with high ratio of nucleus/cytoplasm area and certain markers, such as keratin K19, vimentin, p63 and ABCG2. During cultivation markers are lost, and cells express differentiation markers e.g. keratin K3/K12, involukrin, etc (SHORTT et al., 2007).

Furthermore, on the basis of theresults obtained in our research, cultivated limbal cells in explant culture in SFM medium, very quickly, in just two days, achieve 80% confluence, which is comparable with cells cultivated on a nutritive 3T3 cell layer. The advantage of this method in terms of 3T3 cell use would be that cells are not additionally treated with enzymes which eventually have a negative influence on cell cycle establishment and maintenance. Cytological analysis has shown that cells grown using both methods have the morphological characteristics of porcine limbal epithelium.

Current research emphasizes the definition and characterization of a suitable carrier for *in vitro* cultivation of limbal epithelium. The 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 the culture that with its soluble factors and cell-cell contact benefits the growth and differentiation of epithelial cells. A few things that are not beneficial in cultivation using mouse fibroblasts are the possibility of animal virus transmission and immunoreaction to the animal molecules. The amniotic membrane is frequently used as a carrier in limbus cultivation. Rich in growth factors and other molecules that enhance corneal epithelium regeneration in clinical use, it suppresses inflammation and fibrosis. Moreover, the multiplied cells are collected from the cultivation plate bottom along with the membrane, and therefore are easily applied to a wound. The difficulties in clinical use are the time and skill needed for membrane preparation (SHORTT et al., 2009). The amniotic membrane in conjunction with a feeder layer of 3T3 cells also appears to provide a good matrix for limbal cell 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 that diffusible factors or cytokines are responsible (CHEN et al., 2007).

Therefore, in our research we used sterile silicone mesh, with which is very easy to elevate a confluent limbal transplant size 1.5 cm^2, enough for clinical use on pigs.

Current research is showing that a nutritive 3T3 cell layer can be substituted by a nutritive layer of treated amniotic membrane human epithelial cells. It is likely that such a nutritive layer contains cells in a higher portion, in a less differentiated state, e.g. with more stem cell characteristics. Therapeutic "Night & Day" contact lenses with silicone hydrogel compounds have a major advantage in comparison with others, that is their

high oxygen permeability (as much as 6 times greater), which makes them suitable stem cell carriers (LOPEZ-ALMANY et al., 2002). This characteristic, along with their obvious simplicity in clinical use, makes them of great interest as a test model in research into a potential substrate for limbal epithelial cell growth. Results obtained in this research show that to obtain a confluent porcine limbal transplant, the method of choice is an *in vitro* explant culture in SFM medium, and then growth of these cells in secondary cultures on soft contact lenses. Cell growth in one layer in SFM medium with a low concentration of Ca_2^+ ions on a "Night & Day" contact lens resolves the problems related to the use of animal nutrition cells and foetal calve serum, and the cells are, because of the low Ca ions concentration, less differentiated.

In the Cell Laboratory of the Department for Biology, Faculty of Veterinary Medicine, University of Zagreb, from *in vitro* grown porcine limbal cells, 12 porcine corneal transplants, sized 1.5 cm^2 were obtained. Such results are encouraging, therefore the curative value of porcine corneal transplants should be evaluated within research conducted on patients of choice with corneal defects at the Clinic for Surgery, Orthopaedics and Ophthalmology, Faculty of Veterinary Medicine, University of Zagreb. The beneficial therapeutic effects could encourage the establishment of eye bank, divided according to species, and of interest for veterinary medicine.

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SAŽETAK

Zametne stanice, koje omogućuju regeneraciju tkiva, nalaze se u različitim organskim sustavima u tijelu i obično su smještene u dobro zaštićenim dijelovima organa i tkiva. Za zametne stanice rožnice takva su mjesta u bazalnom sloju epitela rožnice, njezinoga limbalnoga područja nazvanoga Voightove palisade. Rastuće zanimanje za alotransplantate i ksenotransplantate predmnijeva temeljita istraživanja regeneracijskog potencijala tih stanica, kao i definiranje njihovih osobitosti u *in vitro* kulturama za presađivanje. Cilj ovoga istraživanja bio je vrednovanje osobitosti uzgajanja svinjskih limbalnih zametnih stanica u kulturi i optimiziranje uvjeta njihovoga uzgoja radi uspostavljanja pouzdanoga biomedicinskoga modela pogodnoga za istraživanje potencijala alotransplantata i ksenotransplantata podrijetlom od svinjskih tkiva. Svinjske zametne stanice imaju potencijal u reparativnoj/regenerativnoj veterinarskoj medicini za kućne ljubimce, konje i visokoselekcionirane pasmine životinja namijenjenih ljudskoj prehrani, ali također i u ksenotransplantaciji za čovjeka.

Ključne riječi: svinja, rožnica, zametne stanice