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Regeneration of the Corneal Epithelium in the Animal Model of Limbal Stem Cell Deficiency (LSCD) Using the USSC Seeded on Amniotic Membrane

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Abstract

The loss of limbal stem cells (LSCs) results in the replacement of corneal epithelial cells with conjunctival epithelial cells, decreasing the usual clarity of the cornea and leading to impaired healing, scarring and vision loss. In this study, we evaluated the beneficial effects of Unrestricted somatic stem cells (USSC) from human cord blood (CB) seeded on the amniotic membrane in rabbits with limbal stem cell deficiency (LSCD) with corneal epithelium wounds. Accordingly, USSCs and LSCs were derived from human CB and limbus tissue. Flowcytometrymetry and karyotype are used to characterize USSCs andchromosomes'chromosomes and numbers. Then, such cells were seeded on the amniotic membrane (AM). The viability of USSCs and LSCs on AM was evaluated by MTT assay within 24, 48 and 72 hours of treatment.Further, the expression of epithelial markers, such as CK3, CK12 and P63, was evaluated using Real-time PCR and immunohistochemistry (IHC) to evaluate the differentiation of cells seeded on AM to corneal epithelium. Further, photographic images in rabbit models of LSCD monitored the healing of corneal epithelium wounds. Based on the analysis, AM could promote cell viability in all periods. Also, increased expression of CK3, CK12 and P63 genes and morphological analysis verified the efficient differentiation of USSCs to corneal epithelium cells. Also, photographic images of rabbit eyes verified the wound healing process in AM and USSCs seeded on AM compared with the control group. In conclusion, we found that transplantation of USSCs seeded on AM could be an effective, innovative therapy to alleviate corneal epithelium wounds in animal models of LSCD.

1. Introduction

Corneal limbal stem cells (LSCs) are located in the limbus, a specialized region separating the peripheral cornea from the nearby conjunctiva [1, 2]. LSCs are crucial for maintaining the balanced regeneration and clarity of the corneal epithelium, which consists of several layers. When injured, the dormant LSCs become active and help in the healing process of corneal epithelial wounds [3]. The degradation of vision caused by LSC loss or failure results from several issues, such as epithelial abnormalities, conjunctival ingrowth, neovascularization, and opacification of the corneal surface, which is typically transparent and lacks blood vessels. Limbal stem cell deficiency (LSCD), a medical ailment, may arise due to chemical or thermal burns and inflammatory or hereditary eye abnormalities [4, 5]. Although there have been many suggested approaches for treating LSCD, the transplantation of autologous or allogeneic LSCs is now the sole effective solution [6, 7]. The currently favoured method for treating unilateral LSCD is to transplant either a limbal autograft taken from the healthy opposite eye, known as simple epithelial transplantation (SLET), or to use ex vivo-produced autologous LSCs, referred to as cultured limbal epithelial transplantation (CLET) [8, 9]. The management of bilateral LSCD entails the grafting of Limbal Stem Cells (LSCs) acquired from a donor of the same species but with different genetic makeup. It is worth mentioning that the transplantation of a person's LSCs has been shown to have far greater success rates and lower occurrence of problems when compared to the transplantation of LSCs from another person (allograft transplantation) [10-12].

Mesenchymal stem cells (MSCs) have been widely researched for their regenerative properties and are recognized for their crucial role in tissue repair and maintenance [13, 14]. Animal studies have provided enough evidence that the many cytokines and growth factors present in the secreted factors (secretome) of MSCs may effectively decrease scarring, neovascularization (NV), and inflammation while also encouraging epithelialization after injuries [15, 16]. Nevertheless, there is a need for more research demonstrating the direct administration of extracted MSC into the cornea. This might be attributed to the absence of efficient delivery techniques, which affects the availability of MSCs and their biological function [17]. The distribution of soluble therapeutic components as an eye drop to the ocular surface is hindered by limited effectiveness and high costs, mainly due to fluid turnover and drainage, which lead to significant volume loss. Due to its anti-inflammatory and wound-healing capabilities, the amniotic membrane reduces scar formation and speeds recovery by promoting re-epithelization [18, 19]. The amniotic membrane is a protective barrier between the cornea and the top eyelid, facilitating quicker healing. In the present study, we administered human USSCs, as specific types of MSCs, that seeded on the amniotic membrane to investigate the healing of corneal wounds in animals with LSCs.

Material and Methods

Isolation of USCC

For the present study, umbilical cord blood under 180 cc was obtained by obtaining informed consent from the mother to isolate stem cells. The samples were checked for the presence of HTLV1,2, HIV, hepatitis B and C viruses, and cytomegalovirus, and the samples that were negative for all cases were selected. After collecting the samples, red blood cells were lysed by ammonium chloride (NH_4Cl), and then mononuclear cells (MNC) were isolated by Ficoll with a density of 1.077.

Characterizing of USCCs

The cells isolated in the previous step were cultured in low-glucose DMEM with 20% FBS and with mM dexamethasone, and then the non-adherent cells were removed from the medium. Adherent grown cells were collected from the plate and were tested by flow cytometry to check the surface markers. This test includes evaluation of markers CD44, CD73, CD90, CD105, CD166 , CD31, CD38, CD45, and CD34.

Karyotype was also used to check the chromosomal order and health of USSC cells' chromosomes. Both single USSC cells (in the third passage) and USSC cells cultured with the amniotic membrane (after ten days) were subjected to karyotyping and chromosomal status evaluation by counting 100 metaphase cells on the slide.

Preparation of human limbus tissue

Healthy human limbus tissue was obtained from the corneal surgery room of Shahid Labafinejad Hospital after registering the donor's information and confirming his health status with a cornea specialist. The ethical code of the study is “IR.SBMU.REC.1398.005”. In this light, it was tried to reduce possible tissue contamination, such as conjunctival cells or stroma. This tissue was transferred to the laboratory at a temperature of 4 degrees in a transfer medium (containing HBSS, amphotericin B, penicillin and streptomycin 4x) in the shortest possible time and was used for further processing.

Isolation and cultivation of corneal epithelium cells

The limbus tissue sample was processed to prepare a layer of epithelial cells that can be transplanted. Stem cells of the limbus were separated from the limbus tissue by enzymatic and mechanical processes in a sterile environment. First, the received tissue was cut into smaller pieces. Then, it is placed in 10 mM dispase enzyme at 37 degrees for 2 hours. After separating the epithelial layer, the limbus samples were incubated in collagenase enzyme for 20 minutes at room temperature and then exposed to cold trypsin for 10 minutes at 37 degrees. Finally, the samples were centrifuged with a force of 400 g at 4 degrees. The collected cells were suspended in a SHEM medium. SHEM and DMEM/F12 culture media are used in a ratio of 1:1 for the cultivation of limbus epithelial cells. This medium contains 10% human serum, 1.05 mM calcium, 5 µg/mL crystalline bovine insulin, 30 ng/mL cholera toxin, 2 ng/mL epidermal growth factor (EGF), 0.5% dimethyl sulfoxide (DMSO).), 0.5 µg/mL of hydrocortisone, 5 ng/mL of sodium selenite, and 5 µg/mL of apotransferrin. Also, the medium contains 100

units/mL of penicillin and 100 μg/mL of streptomycin. For the passage of LSCs, PBS with 0.02% EDTA 0.5 mM was used.

Cultivation of cells on the acellular surface of the amniotic membrane

USSC and LSC cells were cultured in a DMEM medium and morphometrically evaluated using an inverted phase contrast microscope. Moreover, when they reached the appropriate density, they were separated from the medium and cultured on the acellular amniotic membrane.

MTT assay

In order to investigate and compare the proliferation rate and viability of LSCs and USSC on the amniotic membrane, the MTT test was used at 24, 48 and 72 hours. Cells were separately cultured in 48 well ($5x10^4$/cells/well) plates with amniotic membrane. After 24 h, 48 h and 72h, MTT (Sigma, USA; 5 mg/ml in PBS) was added to each well and incubated for 3.5 h at 37°C. Then, dimethyl sulphoxide (Sigma, USA) was added to each well to dissolve the formazan crystals produced by the activity of live cells and the coloured supernatant was read at 570 nm using a plate reader (EL 800, Biotek, USA).

Real-Time PCR

The expression of P63, CK12 and CK3 genes, which are among the markers of differentiated epithelial cells in the cornea, was done using this technique. LSCs and USSCs cultured on amniotic membranes are tested using Real-Time PCR. First, total RNA is separated from the cells using the RNA extraction kit. In order to prevent genomic DNA contamination, DNase I treatment was used. The amount of RNA was measured by spectrophotometry. The specific primers were designed and synthesized using specialized Beacon Designer software. cDNA synthesis was done by kit. The positive control for this test was cDNA prepared from the limbus tissue itself. qRT-PCR was performed after 7 days of cell culture. In this way, it was possible to compare the results of these two cells in the in vitro phase. Considering that the expression of these genes indicates epithelial differentiation in the cornea, its observation in USSC cells indicates the power and ability of these cells to differentiate into the epithelial category. The primer pairs used in Real-time PCR has been provided in Table 1.

IHC staining

After culturing the corneal epithelium cells and USSC cells on the amniotic membrane and completing these steps (about two to three weeks), the prepared epithelial layer was immunologically examined to check the presence and differentiation of the corneal epithelium. At this stage, the cultured limbus, USSC cells, and the amniotic membrane tissue were directly fixed, paraffinized, cut, and finally immunohistochemically stained. Antibodies against CK3 and CK12 (corneal epithelium marker) and p63 (as epithelial stem cell marker) were used for this investigation.

Airlifting process

Airlifting is one of the most common methods of inducing epithelial differentiation in studies. After the cells cultured on the amniotic membrane reached the appropriate density, we exposed them to the atmosphere for 7 to 14 days. For this purpose, the supernatant culture medium of the cells was removed from the culture plate until the cells came out of the medium and were exposed to air. Considering that the culture medium is not completely emptied, the cells are safe from the risk of drying. On the other hand, the release of specific growth factors from the amniotic membrane itself acts as the primary stimulus for inducing differentiation towards the epithelial line, which can ultimately direct both cultured cell lines towards creating the epithelium layer. After performing the airlifting process, RT-PCR and IHC staining were performed again in the same way described to study the state of the cultured cells and compare the data with the results obtained before airlifting.

Animal model of LSCD

In order to prepare an animal model of LSCD disease, a chemical burn was used in the eyes of rabbits: using half molar NaOH, the surface of the corneal epithelium was damaged. In this way, nitrocellulose paper with a diameter of 0.5 cm dipped in half molar NaOH was placed for 30 seconds on the surface of the cornea of a New Zealand

white rabbit with an average weight of 2.5 to 3 kg, which was anaesthetized by injection of xylazine/ketamine (20/80). Then, the affected area was washed with saline for 5 minutes.

Transplantation

Tarsotrophy was also done to prevent possible injuries due to the impact of the animal's paw. After transplanting probiotic drops, Cyplex was instilled every 8 hours for 1 week, and Restasis drops were instilled every 60 days, one drop per day in each eye. Morphometric evaluations and examination of the healing rate of the injury area were also performed. Investigations were carried out within 60 days after transplantation, which included the evaluation of corneal epithelium regeneration using preparation of photographic images.

Statistical Analysis

The data were analyzed using the GraphPad Prism 7.01 program. The Student's t-test was used to compare two experimental groups as deemed suitable. A P-value less than 0.05 was determined statistically significant.

Results

1. USSC characterizing

We examined hUSSCs for the differentiation capacity and phenotypic markers. hUSSCs appeared spindle-shaped and adhered to the bottom of the culture flask (Fig. 1A). Flow cytometry analysis showed the positive markers for CD44, CD73, CD90, CD105, CD166 and negative for CD31, CD38, CD45, and CD34 (Fig.1B). Also, karyotype used to check the chromosomal order and the health of the chromosomes of USSC cells verified complete set of chromosomes, including their number, size, and shape (Fig. 1C).

2. Amniotic membrane improves cell viability

USSCs and LSCs cells were seeded on AM. The MTT assay was conducted in different groups 24, 48h and 72 h after the last treatment (Fig. 2). Based on the achieved results, The amniotic membrane increased cell viability of both USScs and LSCs within 24, 48 and 72 hours of treatment. This increase was more significant within 72 hours of treatment (Fig. 2)

3. LSCs and USSCs differentiation into corneal epithelium cell

The expression of two genes, P63, CK3 and CK12, which are among the markers of differentiated epithelial cells in the cornea, were evaluated in LSCs and USSCs cultured on the amniotic membrane by Real-Time PCR technique within days 7. The achieved results demonstrated an up-regulation in the expression of all genes in both LSCs and USSCs cultured on the amniotic membrane, verifying their efficient differentiation into corneal epithelium cells (Fig. 3A). Further, the IHC results verified the expression of P63, CK3 and CK12 in cultured USSCs cells on the amniotic membrane (Fig. 3B).

5. Photographic images on transplanted rabbit

Histology analysis was conducted on treated rabbits 60 days after transplantation of the amniotic membrane and USSCs-seed amniotic membrane. In these eyes with transplants, the ocular surfaces were smooth, which was more significant in the USSCs seeded amniotic membrane compared to the amniotic membrane (Fig. 4).

Discussion

Corneal inflammation, ulceration, neovascularization, conjunctivalization, limbal stem cell deficiency (LSCD), and stromal fibrosis may lead to blindness [20]. Chemical or thermal burns, mechanical damage, immunological or genetic problems, and severe corneal injury can cause these conditions. Standard treatment techniques include the injection of anti-inflammatory drugs, transplantation of limbal stem cells (LSC), and corneal transplantation [21]. However, these treatments are bound by certain clinical limitations. Anti-inflammatory medicines are unsuccessful in suppressing angiogenesis, conjunctivalization, and corneal fibrosis. LSC transplantation carries an elevated chance of immune rejection [22]. Corneal transplantation is the primary and most successful way to restore vision when a condition has affected the cornea's clarity. Although corneal transplantation is the most effective solid organ transplantation, immune rejection continues to be the significant reason for graft failure [23].

Several biomaterials have been used as biological dressings to enhance wound healing. Human amniotic membrane graft (AM) has been widely used in managing acute and chronic lesions for a significant period [24]. It is cost-effective and easily accessible, promotes the development of mesenchymal cells, improves the attachment and reproduction of epidermal cells, and has poor levels of toxicity and immunogenicity [25]. The amniotic membrane comprises a monolayer of epithelial cells, a compact basement membrane, and a stromal layer that lacks blood vessels. These three layers improve wound healing effectiveness, decrease the creation of scars, relieve pain, and promote faster re-epithelialization [26, 27]. One practical approach to improve the regeneration capabilities of tissue transplants is to augment them with stem cells. This animal research aimed to assess the safety, effectiveness, and practicality of tissue-engineered dressings made of amniotic membrane (AM) and USSCs in treating corneal epithelial lesions in rabbits with LSCD. This study used flow cytometry to examine the properties of USSC that were extracted from the human umbilical cord.

MSCs, such as USSCs, are becoming recognized as precursor cells for corneal tissue engineering to treat LSC deficit and rebuild ocular surfaces. MSCs can be easily isolated, grown, and expanded. Furthermore, they have the potential to maintain their ability to differentiate into many cell types. Gu et al. [28] examined the ability of MSCs to differentiate into corneal epithelial cells in vivo and ex-real life). Rabbit mesenchymal stem cells (Rb-MSCs) were transplanted into the surface of injured rabbit corneas in vivo using fibrin gels. The findings demonstrated that the Rb-MSCs exhibited the presence of the corneal epithelium-specific marker cytokeratin 3 (CK3) and promoted the recovery of the damaged corneal epithelium. Rb-MSCs were cultivated either in the conditioned media of Rb-LSCs or co-cultured in vitro with Rb-LSCs utilizing the Transwell culture technique. The Rb-MSCs rapidly changed their cell shape from fibroblast-like to resembling corneal epithelial cells, exhibiting the expression of CK-3. Similarly, it was suggested that the soluble substances released by Rb-LSCs had a notable impact on the transformation of Rb-MSCs. At the same time, the two kinds of cells did not interact directly. As part of a supplementary study, corneal stromal cells were used to prompt bone MSCs to undergo differentiation into cells resembling corneal epithelia and exhibit the expression of CK12 [29]. The corneal surface regeneration in rodents with corneal alkali damage is significantly improved by introducing MSCs in the amniotic membrane.

The treated groups with USCC and AM, either alone or in combination, showed a faster regeneration of the corneal epithelium compared to the control group. Additionally, the treatment group showed obvious benefits above the control group. Previous studies have shown that human umbilical cord blood (CB)-derived USSCs possess a wide range of differentiation abilities and provide regenerative benefits in animal models of several degenerative illnesses [30-32]. We have shown the feasibility of stimulating USSCs to exhibit gene expression patterns characteristic of epithelial development. After completing the specified procedures, we evaluated the existence and specialization of the corneal epithelium after the cultivation of USSC cells and LSCs on the amniotic membrane. According to the RT-PCR data, the cultured limbus and USSC cells and the amniotic membrane showed an upregulation in the expression of P63, cytokeratin 12, and cytokeratin 3 at this particular stage. The outcomes of the IHC staining corroborated the acquired findings. Cytokeratins (CKs) are proteins that make up the structural framework of epithelial cells, which line the surfaces and cavities of the body. The expression of cytokines (CKs) is particular to specific tissues, controlled throughout development, and relies on the differentiation process. Basic and acidic keratins are often expressed together in pairs. For example, the levels of CK12 and CK3 expression varied among various types of epithelium. CK3 is a superior indicator of conjunctival epithelial cells compared to CK19, and it is present in all levels of the epithelium. CK12 expression is restricted to the suprabasal and superficial cells from E15.5 to P10. It begins in the corneal peridermal epithelium during embryonic development.

The MTT test was used to examine the rate of cell growth and the viability of USSC cells and LSCs on the amniotic membrane. The findings demonstrated that AM can promote the growth of USSCs and LSCs. These findings were in line with previous studies. Hao et al. [33] conducted a study to examine the effectiveness of combining mesenchymal stem cells with a hydrogel containing an amniotic membrane extract (AME) enriched with RGD. AME upregulated the expression of the anti-apoptotic BCL2 and downregulated the expression of apoptosis-related genes Caspase-3 and Caspase-8 while also stimulating the development of MSCs in vitro. USCCS promote tissue regeneration by releasing a substance that controls angiogenesis and inflammation rather

than relying on their unique properties [34]. USSCs seearele to reduce the corneal inflammatory response and the growth of new blood vessels (neovascularization) in mice that have suffered a chemical injury. This effect is achieved via a paracrine mechanism, where the USSCs release signalling molecules that help relieve the inflammation and prevent the formation of new blood vessels.

5. Conclusion

The results of this study delivered the proof of the concept that USSCs delivery using amniotic membrane could be an effective and a rational strategy to accelerate wound healing process in LSCD rabbits.

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Conflict of interest

There is no conflict of interest.

Legends

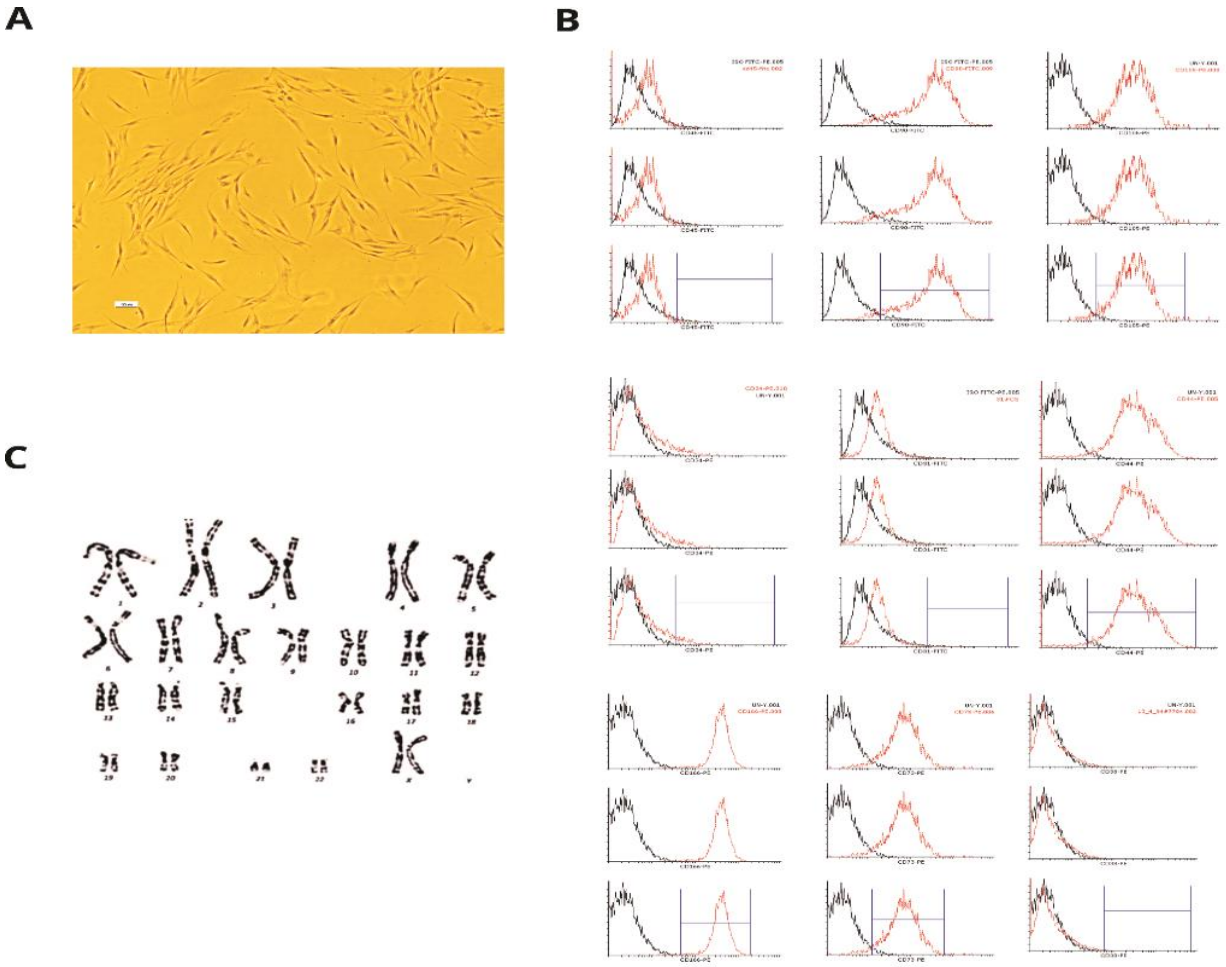

Fig 1. Characterizing of USSCs. (A) Morphology of USSC, (B) Flowcytometry analysis of a panel of CD markers for characterizing of USSCs, including CD44, CD73, CD90, CD105, CD166 , CD31, CD38, CD45, and CD34, and (C) Karyotype of USSCs.

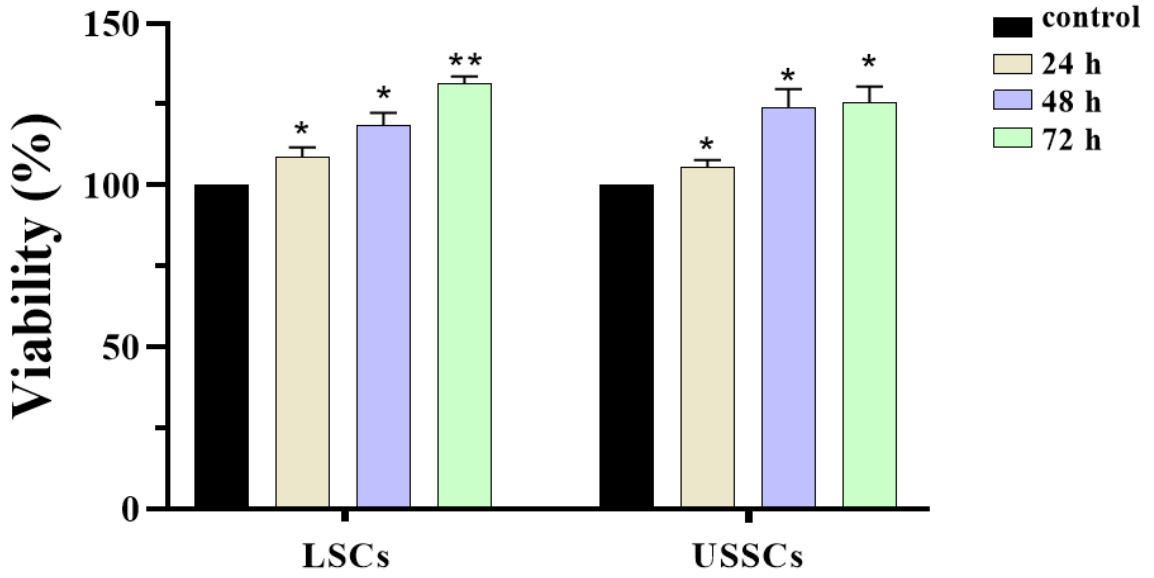

Fig 2. LSCs and USSCs viability seeded on amniotic membrane. The Student's t-test was used to compare two experimental groups as deemed suitable. A P-value less than 0.05 were considered statistically significant.

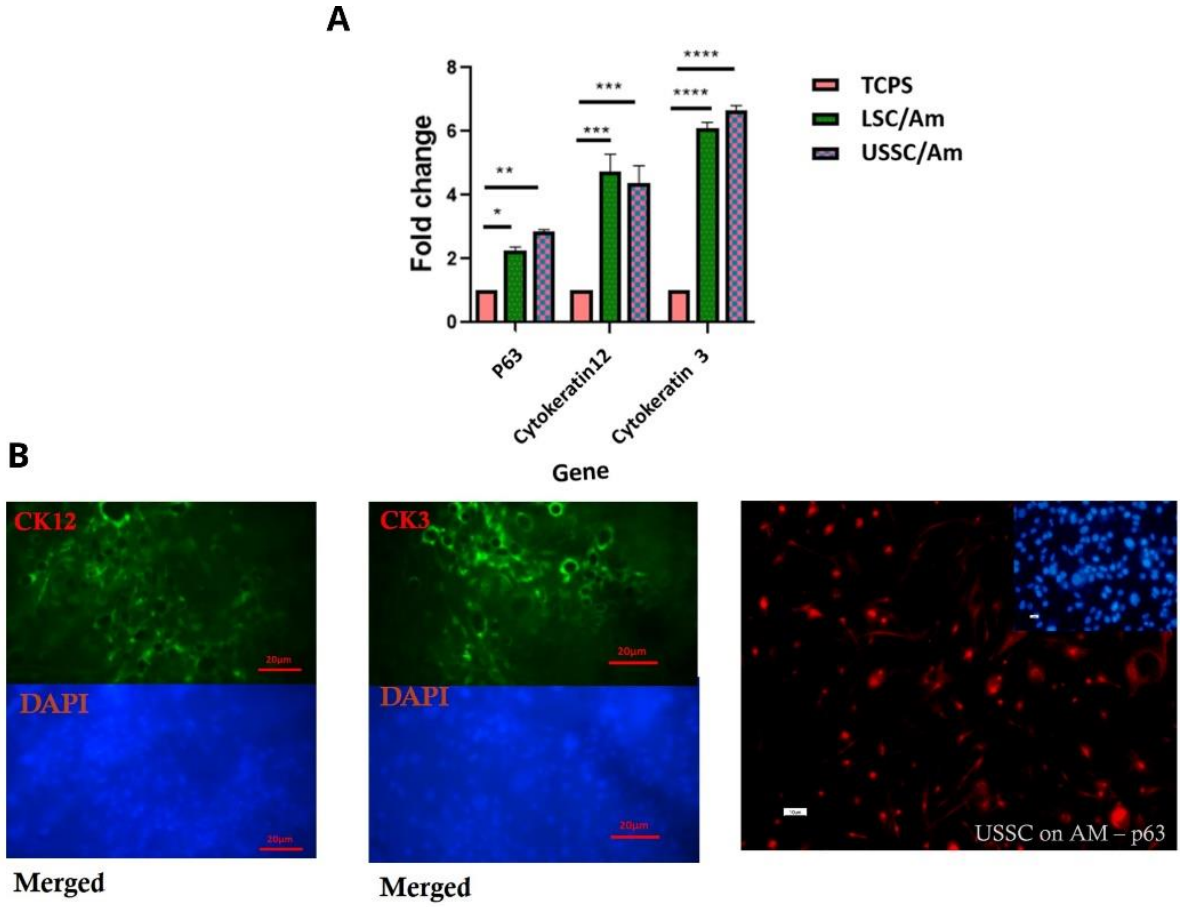

Fig 3. Efficient differentiation of USSCs and LSCs to corneal epitheliums. Within days 7 of the seeding of cells on amniotic membrane, Real Time PCR (A) and IHC analyses (B) verified the expression of CK3, CK12 and P63.

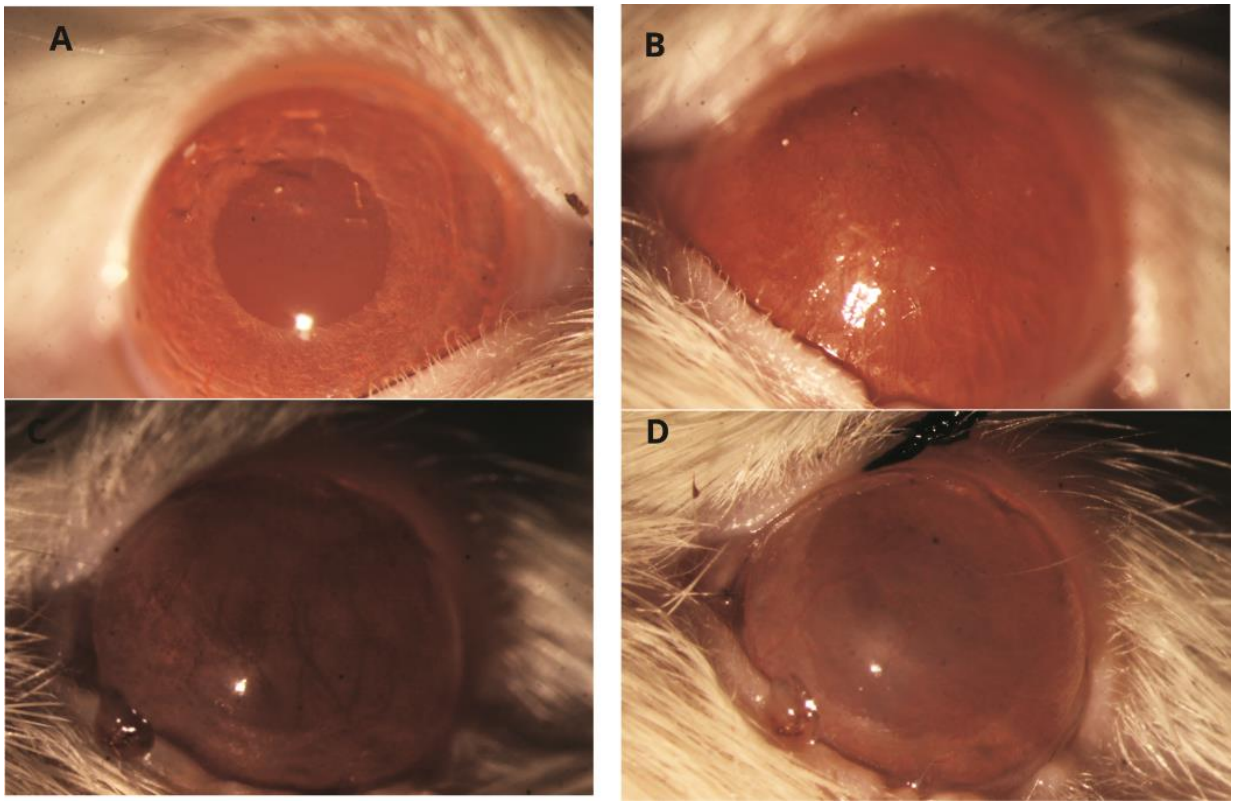

Fig 4. Photographic images of eyes of LSCD rabbits with corneal epithelium wounds.

Table

Table.1. Primers sequences used in qRT-PCR analysis

Gene	**Primer sequences 5'-3'**
CK3	F: AAGGACCCTCTACGACGCT R: CCAGGTCCAGGGAGCGAT
CK12	F: CTTCACAGAGCGATTACAGCA R: TCCTCAGCAGCTAGTCTCG
ABCG2	F: GCATTTACTGAAGGAGCTGTG R: GACCAGGTTTCATGATCCCA
P63	F: TGACCCTTACATCCAGCGTT R: CTGGAAAACCTCTGGACTGA