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Short review of the Ph.D. thesis

Regenerative Medicine in Ocular Surface Reconstruction: Advancing Cell-Based Therapies for Limbal Stem Cell Deficiency

Joao Victor de Sousa Cabral, MD

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Předseda oborové rady: doc. MUDr. Tomáš Kučera, Ph.D.

Školící pracoviště: Laboratoř biologie a patologie oka, Ústav biologie a lékařské genetiky, Albertov 4, 128 00 Praha 2

Školitel: prof. Mgr. Kateřina Jirsová, Ph.D.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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Abstract

Limbal stem cell deficiency is a disease caused by the impairment of limbal epithelial stem cells (LESCs), leading to the replacement of the corneal surface with nontransparent conjunctiva, and its treatment is not standardly available worldwide. This work focuses on preparing cells for treating both uni- and bilateral forms of the disease using advanced cell-based therapy. It investigates LESCs in various scaffolds (fibrin, nanofibers) and explores non-limbal cell sources like oral mucosal epithelial cells (OMECs) under standard complex and xenobiotic-free culture conditions. The study uses immunofluorescence and gene expression to detect stem cell markers, proliferation, and differentiation capacity of cultured cells. Moreover, long-term OMECs storage with different media and cryoprotective agents and the healing properties of the amniotic membrane (AM) were also examined.

We found that adding interleukin-13 to the culture media enhanced LESCs' stemness. LESCs on fibrin gels showed higher expression of stemness markers, while those on polymers expressed more mesenchymal ones. Using standard and xenobiotic-free media, we successfully prepared OMEC-containing cell sheets on fibrin gel substrates. The cultured cells exhibited high expression of stemness genes (*ΔNp63α*, *NGFR*, *KLF4*) and decreased levels of differentiation (lower *KRT13* expression). Keratins related to basal layer and progenitor cells (*KRT14*, *KRT15*, *KRT17*, *KRT19*) were highly expressed in both conditions. The cells in complex media had a higher proliferation rate, evidenced by the upregulation of *MKI67*, with an earlier onset of the growth and reaching confluence sooner than xeno-free cultures. OMECs formed a confluent cell sheet even after storage in liquid nitrogen. Better outcomes (confluence, viability) were observed for OMECs stored in complex media alone or with 5% glycerol, compared to complex media with 10% glycerol or 10% dimethyl sulfoxide, particularly when stored after the first passage instead of using primary cells. Lastly, we showed that cryopreserved AM is a safe and effective treatment for non-healing wounds, with consistent interplacental quality among AM grafts and a strong analgesic effect.

In conclusion, we have prepared and finalized protocols for cultivating limbal and oral mucosa cells. The cell culture can now be transferred to the cleanroom conditions of the tissue bank for verification, and the protocols can be forwarded to the State Institute for Drug Control for approval for clinical use.

Keywords: limbal stem cell deficiency, stem cells, ocular surface, oral mucosa, cell culture, amniotic membrane, transplantation.

Abstrakt

Deficience limbálních kmenových buněk je onemocnění způsobené poškozením limbálních epitelových kmenových buněk (LESC), které vede k přerůstání průhledné rohovky netransparentní spojivkou a ke ztrátě zraku. Léčba není standardně dostupná. Tato práce se věnuje přípravě buněk pro léčbu jedno- i oboustranné formy deficience pomocí moderní buněčné terapie. Zkoumá růst LESC na různých substrátech (fibrin, nanovlákna), dále buňky epitelu bukální sliznice (OMEC), kultivované ve standardním komplexním médiu i v podmínkách bez xenobiotik. K detekci markerů kmenových buněk, stanovení proliferační a diferenciační kapacity kultivovaných buněk byla použita imunocytochemie a genová exprese. Kromě toho byl hodnocen vliv dlouhodobého skladování OMEC v médiích s různými kryoprotektivními látkami. Součástí teze bylo i hodnocení hojivých vlastností amniové membrány (AM).

Zjistili jsme, že přidání interleukinu-13 do kultivačního média zvýšilo kmenovost LESC. LESC pěstované na fibrinu více exprimovaly márkry kmenovosti, buňky na nanovlákenných polymerech exprimovaly více márkrů mezenchymálních. Na fibrinu se nám kultivací ve standardním komplexním médiu, ale i v prostředí bez cizorodých látek (xeno-free) podařilo připravit kultury OMEC. Buňky vykazovaly vysokou expresi kmenových márkrů (*ΔNp63α*, *NGFR*, *KLF4*) a sníženou diferenciaci do fenotypu epitelu bukální sliznice (nižší exprese *KRT13*). Keratiny typické pro bazální vrstvou a progenitorové buňky (*KRT14*, *KRT15*, *KRT17*, *KRT19*), byly exprimovány v obou podmínkách. Buňky v komplexním médiu rostly rychleji (upregulace *MKI67*). Tyto buňky také dosáhly ve srovnání s buňkami kultivovanými v xeno-free médiu rychleji 100% konfluenci. OMEC vytvořily souvislou vrstvu buněk i po kryokonzervaci. Lepší výsledky (konfluence, viability) byly pozorovány u OMEC skladovaných v komplexním médiu bez kryoprotektiv, nebo v komplexním médiu s 5% glycerolem ve srovnání s komplexním médiem s 10% glycerolem nebo 10% dimethylsulfoxidem, zejména pokud byly mrazeny po první pasáži. Prokázali jsme, že kryokonzervovaná AM je bezpečná a účinná v léčbě dlouhodobě se nehojících ran, že má silný analgetický účinek, a že mezi AM štěpy není patrný rozdíl v intenzitě hojení.

Závěrem: připravili jsme postupy pro kultivaci limbálních buněk a epitelových buněk ústní sliznice. Kultivace lze nyní přenést do podmínek čistých prostor tkáňové banky k ověření, a protokoly předat Státnímu ústavu pro kontrolu léčiv ke schválení klinického hodnocení.

Klíčová slova: deficience limbálních kmenových buněk, kmenové buňky, povrch oka, bukální sliznice, kultivace buněk, amniová membrána, transplantace.

Abbreviations

AA	Antibiotic-Antimycotic Solution
ABCB5	ATP-Binding Cassette, Sub-Family B, Member 5
ABCG2	ATP-Binding Cassette, Subfamily G, Member 2
ACTA2	Actin alpha 2, smooth muscle
ALDH3A1	Aldehyde dehydrogenase 3 family member A1
ALS	Alkali Labile Sites
AM	Amniotic Membrane
CFA	Colony Forming Assay
COM	Complex Medium
COMET	Cultivated Oral Mucosal Epithelial Transplant
CPA	Cryoprotective Agent
dAM	Deepithelized Amniotic Membrane
DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
FBLN1	Fibulin 1
FBS	Fetal Bovine Serum
FPG	Formamidopyrimidine DNA Glycosylase
HS	Human Serum
IL13	Interleukin-13
ITS	Insulin-Transferrin-Selenium
K	Keratin
KLF4	Krüppel like factor 4
KRT3	Keratin 3 (gene)
KRT7	Keratin 7 (gene)
KRT8	Keratin 8 (gene)
KRT12	Keratin 12 (gene)
KRT13	Keratin 13 (gene)
KRT14	Keratin 14 (gene)
KRT15	Keratin 15 (gene)
KRT17	Keratin 17 (gene)
KRT19	Keratin 19 (gene)
LECs	Limbal Epithelial Cells
LESCs	Limbal Epithelial Stem Cells
LSCD	Limbal Stem Cell Deficiency
LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1

MKI67	Marker of proliferation Ki-67
MSCs	Mesenchymal Stem Cells
NANOG	Nanog homeobox
NGFR	Nerve growth factor receptor
NHW	Non Healing Wounds
OCT4	POU class 5 homeobox 1
OMECs	Oral Mucosa Epithelial Cells
P	Passage
PAX6	Paired box 6
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDLLA	Poly(L-lactide-*co*-DL-lactide)
RT-qPCR	Reverse Transcription Quantitative Real-time Polymerase Chain Reaction
SBs	Single-Strand Breaks
SOX2	SRY-box transcription factor 2
TACs	Transient Amplifying Cells
THY1	Thy-1 cell surface antigen
XF	Xenobiotic-free
ΔNp63a	ΔN p63 Transcription Factor Alpha Isoform

1. Introduction

1.1. Limbal Stem Cells Deficiency

Limbal stem cell deficiency (LSCD) is an ocular surface disease caused by the destruction or dysfunction of limbal epithelial stem cells (LESCs), leading to a loss of the corneal epithelium's barrier function (Ahmad, 2012). It can result from injuries, burns, inflammation, or inherited diseases, causing conjunctivalization, vascularization, and opacification of the cornea (Bonnet, Roberts, & Deng, 2021). Treatment options depend on factors like the extent (partial, total, unilateral, or bilateral) of LSCD. Research has explored alternative non-limbal cell sources like oral mucosa epithelial cells (OMECs) (Nakamura et al., 2004) and mesenchymal stem cells (MSCs) (Calonge et al., 2021), due to the scarcity of allogeneic limbal tissue (in case of bilateral disease) and the difficulties brought on by an immunological rejection of allografts. Stem cell-based therapies like Holoclar® (LESCs) (Pellegrini et al., 2018) and Ocural® (OMECs) (Toshida et al., 2023) have been approved for commercial use. Amniotic membrane transplantation has been utilized in various ways to treat LSCD due to its beneficial properties (Grueterich, Espana, & Tseng, 2003). The present study aims to enhance the understanding and treatment of LSCD by optimizing LESCs culture, exploring OMECs as an alternative cell source, and evaluating AM's healing properties for chronic non-healing wounds. The following chapters will delve into the anatomy and physiology of relevant structures to provide a comprehensive understanding of the topic.

1.2. Cornea and Limbus

1.2.1. Cornea

The cornea, the eye's transparent and avascular outermost layer, plays a crucial role in allowing light to enter and focus on the retina (DelMonte & Kim, 2011). Composed of six layers (Nishida, Saika, & Morishige, 2021) – corneal epithelium, epithelial basement membrane, Bowman's layer, the stroma, Descemet membrane, and endothelium –, the cornea provides two-thirds of the eye's refractive power and acts as a barrier to prevent fluid loss (Gonzalez-Andrades, Argüeso, & Gipson, 2019). The cornea lacks blood vessels, ensuring transparency and resistance to neovascularization (Ghafar, Jalil, & Kamarudin, 2021). The corneal epithelium, which renews every 7 to 14 days (West, Dorà, & Collinson, 2015), originates from LESCs located in the limbus, a transition zone between the cornea and sclera (Schlötzer-Schrehardt & Kruse, 2005). The corneal stroma's uniform fibril distribution reduces light scattering (Hassell & Birk, 2010), and the endothelium regulates fluid movement, maintaining corneal transparency (Hassell & Birk, 2010), both contributing to the cornea's transparency.

1.2.2. Limbus

The limbus, a 1.5 – 2.0 mm-wide transition zone between the cornea and sclera (Bonnet et al., 2021), consists of an epithelium and a stroma containing various cell types, including MSCs and melanocytes (Schlötzer-Schrehardt & Kruse, 2005). Research suggests that the basal layer of the limbus is the prime location for LESCs, making up less than 10% of basal limbal epithelial cells (Lavker, Tseng, & Sun, 2004). The limbal subepithelial connective tissue contains papilla-like structures called the palisades of Vogt, which house the LESCs (Bizheva et al., 2017).

1.2.2.1. LESCs and Cornea Regeneration

LESCs are crucial in maintaining corneal transparency and vision as they continuously renew the corneal epithelium (Ebrahimi, Taghi-Abadi, & Baharvand, 2009). These cells are typically inactive in mitosis and have distinct characteristics, such as cuboidal shape and heterochromatin-rich nuclei with a high nucleus-to-cytoplasm ratio (Barbaro et al., 2007). The "X, Y, Z" hypothesis proposes that corneal homeostasis is achieved through equal rates of cell gain and loss (Thoft & Friend, 1983). LESCs, located in the basal layer of limbal epithelial crypts (Lavker & Sun, 2000), have high proliferative potential and can divide symmetrically or asymmetrically, producing transient amplifying cells (TACs) (Kaplan et al., 2019). Early TACs multiply and move towards the corneal periphery, gradually losing their regenerative capacity as more mature TACs take their place (Lehrer, Sun, & Lavker, 1998). Finally, the terminally differentiated cells migrate outward to form the superficial corneal layers, eventually sloughing off from the ocular surface. The regulation of LESCs' proliferation and phenotype is of paramount importance, given their role in corneal epithelium restoration under normal and pathological conditions (Masood et al., 2022).

1.2.2.2. Characterization of LESCs

Various molecules have been proposed as potential biomarkers to identify LESCs and early TACs in the limbal epithelium (Takács et al., 2009). Notably, the nuclear p63 transcription factor (ΔNp63α isoform) has been detected in limbal basal cells, differentiating LESCs from TACs (Pellegrini et al., 2001). Additionally, the ATP-binding cassette subfamily G member 2 (ABCG2) has been suggested as a putative biomarker for LESCs (de Paiva et al., 2005). Other markers, such as ATP-binding cassette, sub-family B, member 5 (ABCB50 (Ksander et al., 2014), Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) (Gur et al., 2004), and Krüppel-like factor 4 (KLF4) (Cieślar-Pobuda et al., 2016), have also been linked to stem cell behavior in the limbal epithelium. Proliferation markers like Proliferating Cell Nuclear Antigen (PCNA) and Ki-67 have been used to assess cell proliferation in the limbal epithelium (Moll, Divo, & Langbein, 2008; Merjava et al., 2011). Keratins (K) 3/K12 are specific for corneal epithelium, while K5/K14 and K15 are proposed as potential LESCs biomarkers. However, identifying LESCs remains challenging due to the lack of LESC-specific biomarkers.

1.2.2.3. LESCs and Interleukin-13

Based on previous work from our group (Stadnikova et al., 2019), interleukin-13 (IL13), one of the anti-inflammatory interleukins and a T helper 2-type cytokine (Junttila, 2018), has a favorable effect on the proliferation and expression of the *ΔNp63a* gene in the conjunctival epithelium produced from limbal explants. We then hypothesized that IL13 would have a similar effect on the expansion of LESCs from limbal explants, preserving their stemness. In general, data on how IL13 affects LESCs or stem cells are limited. It makes IL13 a promising stem cell research target when combined with our finding of the function of IL13 in the stemness of conjunctival epithelial cells.

1.3. Oral Mucosa

The oral buccal mucosa is a structured and avascular tissue composed of squamous stratified epithelium (Brizuela & Winters, 2022). It consists of two layers: the *stratum spinosum*, composed of elliptical-shaped cells, and the underlying *stratum basal* (Squier & Kremer, 2001). These layers are connected to the lamina propria, a supportive connective tissue containing salivary glands, fibers, blood vessels, fibroblasts, and other cell types (Squier & Kremer, 2001). The interface between the epithelium and the lamina propria is formed by *rete ridges* and dermal papillae (Brizuela & Winters, 2022). Epithelial stem cells

in the basal layer play a crucial role in tissue renewal (Iglesias-Bartolome, Callejas-Valera, & Gutkind, 2013), with cell division mainly occurring in this region. Committed cells undergo differentiation, express structural keratin proteins, and eventually slough off the surface (Oda & Watson, 1990). The turnover time of the oral epithelium is around 14 – 24 days for a stem cell to divide and its progeny to traverse the entire thickness of the epithelium (Richard & Pillai, 2010).

1.3.1. OMECs for Treating LSCD

The oral buccal mucosa shares structural similarities with other stratified epithelia and has characteristics that make it suitable for ocular surface reconstruction (Ramachandran et al., 2014). It contains epithelial stem cells with similar features to LESCs and can be transdifferentiated into cells resembling corneal epithelial cells (Nakamura, Endo, & Kinoshita, 2007; Hancox et al., 2020). However, finding a specific marker to distinguish oral mucosal tissue from ocular surface epithelia remains a challenge (Kolli et al., 2014).

Oral keratinocyte stem/progenitor cell phenotypes could be characterized by their expression of p75NTR, a low-affinity neurotrophin receptor (Nakamura, Endo, & Kinoshita, 2007). The putative stem cell marker p63 is observed in oral mucosal tissue's basal to suprabasal cell layers (Kasai et al., 2016). The characterization of the oral mucosa involves the expression of specific keratins, such as K4/K13 (Kasai et al., 2016) in the suprabasal to the upper cell layers and K14, K15, and K19 in basal cells (Presland & Jurevic, 2002; Squier & Brogden, 2011; Kasai et al., 2016). K8 exhibits intense staining in the basal and parabasal layers (Moll et al., 2008), while K5 and K7 are expressed from the basal layer to the upper layer (Kasai et al., 2016).

1.4. Culture of LESCs and OMECs

1.4.1. Culture Media for *in vitro* Expansion of LESCs and OMECs

Complex medium (COM) is commonly used for cultivating LESCs (Pellegrini et al., 2018), containing fetal bovine serum (FBS, or fetal calf serum), antibiotic-antimycotic solution (AA), epidermal growth factor (EGF), hydrocortisone, insulin-transferrin-selenium (ITS), cholera toxin, and adenine (Brejchova et al., 2018). FBS promotes cell proliferation (Kruse & Tseng, 1992), but it is possible to culture LESCs without it. AA prevents biological contamination (Weiskirchen et al., 2023), while EGF enhances clonogenic potential (Meyer-Blazejewska et al., 2010). Hydrocortisone aids in keratinocyte proliferation (Rheinwald & Green, 1975), and ITS reduces the need for animal serum (Mainzer et al., 2014). Cholera toxin and adenine promote colony growth (Okada, Kitano, & Ichihara, 1982; González, Chen, & Deng, 2017).

For OMECs culture, a similar medium, including triiodothyronine, is used to reduce the need for FBS (Hayashi, Larner, & Sato, 1978). While FBS is effective for cell growth, it carries risks of disease transmission and immunologic rejection (Shortt et al., 2007). To minimize the use of animal-derived products, human serum (HS) can be an alternative to FBS (Utheim et al., 2015), being less likely to cause adverse effects. The present study aims to culture OMECs in xenobiotic-free (XF) media (containing HS) to produce a viable cell sheet, avoiding FBS and other animal-derived components, which is preferred for advanced stem cell therapies to avoid contamination issues carried by the graft.

1.4.2. Substrates for *in vitro* Expansion of LESCs and OMECs

In clinical trials for treating LSCD, various substrates have been used, with AM being the most common choice for cultivating both LESCs and OMECs (Nguyen et al., 2018). Intact AM preserves native amniotic epithelia, promoting stem cell maintenance, but it may

cause immunological reactions (Li et al., 2006). Deepithelialized AM (dAM) is preferred as it eliminates the risk of immunological reactions but requires skilled preparation and exhibits variable properties (Utheim et al., 2016).

Another substrate used is fibrin gel (Rama et al., 2001), which is effective and easy to handle. It offers advantages like biocompatibility, self-assembly, and inhibition of fibrosis and inflammation (Weisel, 2005). However, it stimulates angiogenesis, which is undesirable for corneal grafts (Gonzalez-Andrades, Argüeso, & Gipson, 2019), though this effect diminishes over time (Radosevich, Goubran, & Burnouf, 1997).

Synthetic scaffolds (Nguyen et al., 2018), particularly electrospun membranes made from biodegradable polymers (Zdraveva et al., 2023), offer potential advantages, including high porosity, large pore size, and low thickness, supporting cell growth and differentiation (Zdraveva et al., 2023).

1.4.3. Feeder Layer

In cell culture research for growing epithelial cells like limbal and oral mucosa, 3T3 feeder cells (mouse embryonic fibroblasts cell line) are commonly used as a feeder layer (Sharma et al., 2012). This technique has been shown to enhance stem cell properties during cultivation (Pellegrini et al., 1999). However, a major challenge is that the epithelial sheets cocultured with 3T3 feeder cells are xenogeneic, limiting their clinical application (Martin et al., 2005). To address this limitation, the study aims to develop a feeder layer-free culture system for culturing limbal and oral mucosa cells.

1.5. Genotoxicity

In ocular surface transplantation, analyzing DNA damage in cultured cells is crucial to ensure the procedure's safety and success (Lorenzo et al., 2018). The comet assay is a widely used technique to detect DNA single-strand breaks (SBs) and alkali-labile sites (ALS) in cells before transplantation (Rojas et al., 2014). It helps identify potential genotoxicity and ensures that the cells used for transplantation do not carry significant DNA damage that could lead to adverse effects (Rojas et al., 2014). The standard alkaline comet assay can only detect SBs and ALS (Muruzabal et al., 2021), but modifications, such as incorporating a digestion step with DNA glycosylases, have been made to identify additional types of DNA damage caused by various substances. One common modification involves using the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) to assess the level of oxidized purines (Magdolenova et al., 2012).

1.6. Cryopreservation of Stem Cells for the Long-term Storage

Cryopreservation is vital for the long-term storage of stem cells and other biological materials (Hunt, 2011). It enables the maintenance of cell viability and structure at cryogenic temperatures, supporting various applications like stem cell therapy and tissue engineering (Sambu, 2015). For ocular surface transplantation, cryopreservation of limbal suspension cultures has been advantageous as it eliminates the need for additional biopsies in case of graft failure and allows for consecutive surgeries (Kaufman et al., 2014). To preserve cultured tissue's stem cell content and integrity, standardized freezing and thawing protocols with cryoprotective agents (CPAs) like DMSO and glycerol are commonly used (Martín-López et al., 2023). However, these agents have associated adverse effects and risks (Yang et al., 2020), leading to a search for CPA-free or low-CPA solutions to optimize cryopreservation techniques (Jang et al., 2017). In the present study, the effectiveness of a CPA-free solution is compared to CPA-based solutions with different concentrations of DMSO and glycerol for advanced therapy medicinal products, aiming to minimize potential

adverse effects and the use of xenogeneic materials.

1.7. Amniotic Membrane in Regenerative Medicine

AM's unique biological and mechanical properties make it highly suitable for various clinical applications (Pogozhykh et al., 2018). Its transparency, lack of immunogenicity, and antimicrobial properties facilitate wound healing and reduce inflammation and pain (Svobodova et al., 2022). AM has been proven valuable in the treatment of corneal and conjunctival defects, either as a scaffold or a bandage, aiding in ocular surface reconstruction (Dua et al., 2004). Additionally, AM has the potential for skin regeneration and prevents infection due to its strong adhesion to wound surfaces (Deihim, Yazdanpanah, & Niknejad, 2016). In the context of LSCD, AM transplantation is commonly combined with cultured limbal or oral mucosal epithelial cell transplantation (Le & Deng, 2019). Although research on other materials has been conducted, AM remains widely used as a substrate and carrier for these cell-based therapies (Tsai, Li, & Chen, 2000). For the thesis work, two approaches are taken: 1) OMECs will mainly be cultured in fibrin gels to overcome AM's limitations as a scaffold, and 2) cryopreserved AM will be grafted for the treatment of chronic wounds to study its properties in tissue engineering and healing dynamics, providing insights into its variability for wound treatment, including ocular surface wounds.

2. Hypotheses and Aims

2.1. Hypothesis 1: Increasing the Stemness of LECs Cultures and Alternative Substrates for Cell Culture

Our previous study (Stadnikova et al., 2019) showed that IL13 enhanced proliferative activity and *ΔNp63α* gene expression in conjunctival epithelium from limbal explants. This suggests IL13's potential role in maintaining stemness, useful for developing new culture media for LESCs from limbal explants. To explore this, we plan on conducting experiments with LECs cultures supplemented with IL13, comparing them with controls.

Building on our earlier work (Brejchova et al., 2018), LECs cultured on fibrin gel exhibited high growth rates with minimal fibroblast-like contamination. Exploring alternative synthetic materials might yield more benefits. Thus, we plan to culture LECs on fibrin gel and electrospun poly(L-lactide-*co*-DL-lactide) (PDLLA) nanofibrous scaffolds.

Aims:

- To determine the effect of IL13 on the stemness, differentiation, proliferation, clonogenicity, and morphology of cultured LESCs;
- To compare LECs growth and cell behavior under two different culture substrates: electrospun PDLLA nanofibrous scaffolds coated with human plasma fibronectin and fibrin gel; to analyze the differences in the gene expression of specific markers, including stem cell, proliferation, keratins, and fibroblast genes.

2.2. Hypothesis 2: Preparation of OMECs on Fibrin Gel for Grafting

Oral mucosa holds stem/progenitor-like cells in its basal layer, allowing graft preparation with OMECs. Bilateral LSCD is also treated with OMEC-sheet grafting. Cells are usually cultured with xenogeneic materials, raising infection and immunological risks. We aimed to enhance safety by removing animal components and improving cell sheet quality for bilateral LSCD treatment. Additionally, we explored enhancing the cell culture

technique by combining OMEC culture in XF culture media on fibrin gel, potentially boosting stem cell percentage in the resulting cell sheet.

Aims:

- To culture oral mucosal epithelial cells on fibrin gel and AM and compare standard complex medium and xenobiotic-free culture systems, both supplemented with pooled human serum;
- To compare the kinetics of growth, stemness maintenance, differentiation, and DNA damage between the groups;
- To prepare a stable cell sheet on fibrin gel containing viable stem cells while preserving genome stability. Additionally, to develop a protocol that can be readily transferred to the State Institute for Drug Control to undergo clinical approval in the Czech Republic.

2.3. Hypothesis 3: Long-term Storage of OMECs in Liquid Nitrogen

Cryopreservation, vital for long-term storage in ultra-low temperatures, utilizes CPAs to counteract ice damage. This process is pivotal across domains like stem cell research, regenerative medicine, and tissue engineering, including LECs and OMECs for treating LSCD patients. Our study hypothesizes that CPAs impact stem cell count and subsequent culture proliferation. We will compare the effects of CPAs (5% glycerol, 10% glycerol, 50% glycerol, or 10% DMSO) against cryoprotectant-free media on stem cell preservation and culture proliferation. We aim to explore whether cryoprotectant-free media can retain stem cells and their proliferation status post-thawing, potentially aiding cell sheet preparation.

Aims:

- To compare different CPAs (glycerol and DMSO), including not using a CPA, for long-term storage of oral mucosal epithelial cells in suspension;
- Analyze gene expression of cultured thawed cells, including a comparison with the gene expression of samples before storage (control);
- To find the best condition for long-term storage of OMECs, which could be used for transplantation purposes.

2.4. Hypothesis 4: (A) Cryopreserved AM for the Treatment of Non-healing Wounds and (B) Inter-placental Variability in the Healing Efficiency of AM

Chronic non-healing wounds pose a significant economic burden. AM acts as an ideal biological wound dressing, aiding granulation, epithelialization, exudate reduction, and pain relief. We hypothesize that cryopreserved AM allografts can induce wound healing, shorten closure time, and alleviate pain compared to the standard of care. Typically, multiple AM sheets from a placenta lack specific sub-region tracking, complicating intra-placental variation assessment in clinics. However, individual placental AM tracking adheres to legal regulations. Our hypothesis suggests that inter-placental differences do not hinder AM's effectiveness in healing chronic non-healing wounds.

Aims:

- To evaluate the effect of cryopreserved AM from different donors on wound healing efficiency (wound closure) and to determine whether the dynamics of the wound closure can be used as a predictor for the efficacy of the AM treatment of nonhealing wounds;
- To assess the effectiveness of using cryopreserved AM grafts for treating chronic wounds and for determining if the healing process differs depending on the origin of the AM grafts (inter-placental variability);
- To determine the average percentage of wound closure achieved per application of AM.

3. Materials and Methods

3.1. Preparation of LESCs Culture and Analysis (H1)

Study on the effect of IL13 on LECs:

- Culture of limbal explants and comparison of media supplemented with and without IL13.
- The cells were also subjected to colony forming assays (CFA), immunofluorescence, and RT-qPCR to evaluate their characteristics and expression of specific genes, and the WST-1 assay determined the proliferation activity of living cells.
- Data analysis and statistical analysis.
- Preparation and publication of paper containing the results (Trosan et al., 2022).

Study on the culture of limbal explants on PDLLA membrane and fibrin gels:

- Limbal explants were cultured in PDLLA nanofibrous membranes and fibrin gels. The cells were also subjected to RT-qPCR to evaluate their characteristics and expression of specific genes (stem cell, proliferation, keratins, and fibroblast marker genes).

3.2. Preparation of OMECs Culture and Analysis of Stem/ Progenitor Cells Specific Markers (H2)

- Preparation of a review paper containing relevant clinical data on the use of OMECs for the treatment of LSCD (Cabral et al., 2020).
- Preparation of *in vitro* oral mucosal epithelial cell cultures from retrieved cadaverous tissue and monitoring of the growth of the cells – microscopy;
- Preparation of oral mucosa for immunofluorescence analysis involved cryosectioning of the oral mucosa epithelium (whole tissue) and *in vitro* cultures using cytospin;
- Gene expression analysis of cells before culture and after confluence for both tested media conditions (COM and XF);
- DNA damage analysis by comet assay;
- Data analysis and statistical analysis.

3.3. Long-term Storage of OMECs in Liquid Nitrogen (H3)

- Preparation of cell suspension of oral mucosal epithelial cells from retrieved cadaverous oral mucosa tissue and monitoring of the growth of the cells – microscopy;
- Primary cells and cultured cells after confluence (first passage cells) were stored resuspended in storage media (with or without CPAs) and stored in liquid nitrogen;
- Stored cells were thawed and seeded for culture;
- Gene expression analysis of cells before culture and after confluence for both tested media conditions.
- Data analysis and statistical analysis.

3.4. Amniotic Membrane Grafts for the Treatment of Non-healing Wounds (H4)

- Preparation of AM grafts from the placenta and cryopreservation of AM;
- Preparation of papers (Svobodova et al., 2022; Horvath et al., 2023) containing the results on grafting of cryopreserved AM for treating non-healing wounds and analysis of the healing dynamics and pain relief (as subjectively reported by the patients).

4. Results

4.1. Influence of Interleukin-13 in LESCs (H1)

4.1.1. Limbal Epithelial Cell Growth and Morphology

The study determined that LECs cultures in the passage (P) 0 exhibited growth from limbal explants around the fifth day, reaching 90 – 100% confluence after 14 days, regardless of IL13 presence. Interestingly, P1 and P2 cultures achieved confluence earlier than P0, with no IL13-related differences. The cultures showed decreasing success rates from P0 to P1 and P2, notably dropping to 9.7% confluence for IL13- in P2 compared to 42% in IL13+. Morphologically, IL13- P2 cells had flattened/fibroblast-like morphology, unlike typical cuboidal LECs IL13+ P2 cells. Notably, fibroblast-like cell contamination was lower with IL13 presence. Colony forming assay results revealed higher growth potential in IL13+ P1 (13.79%) and P2 (8.63%) compared to IL13- P1 (4.78%) and P2 (1.19%). IL13 significantly impacted colony numbers, with a more pronounced decrease in IL13- cultures than in the IL13+ cultures ($p < 0.001$ vs. $p < 0.05$).

ΔNp63α gene expression was higher in IL13+ cultures. Expression decreased consistently during culture, and there was a significant difference between P0 – P1 and P1 – P2 cultures in both IL13+ and IL13- groups ($p < 0.01$). *KRT14* gene expression was higher in IL13+ P0 ($p < 0.05$) and P1 ($p < 0.001$) cultures. The *KRT17* gene expression was significantly higher in P0 samples with IL13 ($p < 0.05$) compared to samples without IL13. All cultures exhibited p63+ cells. IL13's absence led to significantly decreased p63 expression during culture. IL13+ cultures maintained stable p63 expression. P2 IL13+ had significantly more p63+ cells than P2 IL13- (90.69% and 75.55%, respectively, $p < 0.05$).

MKI67 gene expression did not differ significantly among groups. P2 IL13+ showed higher proliferation by WST-1 assay. Proliferation decreased significantly between P1 and P2 in IL13- cultures ($p < 0.01$). *KRT3* and *KRT12* gene expression remained consistent across groups. Conjunctival *KRT7* gene expression was significantly higher in IL13+ cultures, notably in P0 and P1 (both $p < 0.05$). *MUC4* gene expression decreased between passages P0 – P2 and P1 – P2 in IL13- conditions ($p < 0.05$).

4.2. Culture of Limbal Explants on PDLLA membranes compared to Fibrin Gel (H1)

4.2.1. Growth Dynamics, Cell Morphology and Gene Expression

Cell growth on fibrin gels started at 3 – 5 days post-seeding, with small epithelial cuboidal cells expanding around explants. By 7 days, growth reached around 70%, maintaining cuboidal morphology even at 90 – 100% confluency (9 – 14 days). Spindle cells were rarely observed. On PDLLA nanofibrous membranes, growth began at 5 – 7 days with cuboidal and round cells, unlike fibrin cultures. From 7 – 10 days, both epithelial and fibroblast-like cells appeared, migrating and forming complex structures (multicellular cell structures, whirling patterns, and self-organizing structures). Confluence on PDDLA was reached at 16 – 21 days with mostly fibroblast-like cells. Comparing fibrin and PDLLA, *NGFR*, *OCT4*, *ΔNp63α*, *KLF4*, *KRT12*, and *KRT14* were significantly upregulated in fibrin. Conversely, PDLLA led to higher expression of fibroblast markers *ACTA2*, *FBLN1*, and *THY1*.

4.3. Oral Mucosal Epithelial Cells (H2)

Oral buccal mucosa samples from four donors underwent hematoxylin-eosin staining for preliminary imaging and showed that the enzymatic treatment effectively separated the epithelium from the submucosa, which is a crucial step to proceed to the preparation of a cell suspension for cell culture.

4.3.1. Viability and Cell size

Primary cell suspensions showed 77.7% ± 18.3% viability, and about 68.7% of the live cells were ≤ 11 μm. The cell size of confluent cultures in COM and XF was also measured by the automated cell counter. In COM cultures, 45.8% of the live cells were ≤ 11 μm, and in XF, 59% of the live cells were ≤ 11 μm.

Stemness markers p63α and p40 were found in the basal layer and on *rete ridge* tips. Another stemness marker, p75NTR, was uniformly present in the basal layer. Ki-67 was expressed in parabasal and scarcely in suprabasal layers. Differentiation markers had varying presence: K3 was absent to minimal in the suprabasal layer; K8 was primarily found in the basal layer; K13 was detected homogeneously throughout the entire suprabasal layer, and K19 was heterogeneously present in the basal layer.

4.3.2. Oral Mucosa Cell Growth Dynamics and Cell Morphology

Cells on COM showed earlier proliferation than XF (4.7 ± 1.1 days and 5.4 ± 1.0 days, respectively. Viability was similar between COM and XF groups (on average 89.8% and 86.3%, respectively). The COM group reached confluence about 11.8 days, on average, while the XF group took 12.7 days.

Attempts were made to culture OMECs on dAM, with challenges in cell amount and harvesting: it requires a much higher cell density to obtain cell attachment, which is further challenged by the lack of a flat and stable surface.

4.3.3. Immunofluorescence

4.3.3.1. Cultured Cells

Stemness markers (p63α, p40, p63α + p40, p75NTR) were detected in all samples. p63α, p40, and p63α + p40 were similarly present in primary cell suspension, COM, and XF, with about 60% for each marker in each condition. p75NTR showed similar presence patterns. Ki-67, a proliferation marker, was more present in cultured cells compared to primary cells, with no significant difference. Differentiation markers K3 showed a minor presence (less than 15% in cultured cells), K8 was similar in all three conditions (around 50%), and K13 was significantly lower in cultured cells compared to the primary cells.

In COM, there were 4.3% of small cells (≤ 11 μm) positive for the stemness marker p63α, whereas in XF, there were 1.9%.

4.3.4. Reverse Transcription Quantitative Real-time PCR (RT-qPCR)

Significant differences were observed for *SOX2* and *ABCG2*. *ABCG2* was upregulated in XF ($p < 0.05$), while *SOX2* was downregulated in both media conditions (COM $p < 0.05$, XF $p < 0.01$), compared to primary cells.

PCNA was upregulated in XF ($p < 0.05$), *MKI67* was upregulated in COM ($p < 0.01$), and *ALDH3A1* was downregulated in COM ($p < 0.05$), both compared to the primary cells.

KRT3 (COM, $p < 0.01$, XF, $p < 0.05$) and *KRT13* (COM, $p < 0.0001$, and XF, $p < 0.05$) were downregulated in both conditions cultured conditions compared to the primary cells, whereas *KRT7* (COM and XF, $p < 0.0001$) and *KRT17* (COM and XF, $p < 0.01$) were

upregulated in the cultured cells.

4.3.5. Genotoxicity Assay

Genotoxicity assay was performed on cells cultured in COM and XF. Low levels of DNA SBs and ALS were observed. Net FPG-sensitive sites were lower in XF. There was no significant difference in genotoxicity between COM and XF.

4.4. Long-term Storage of OMECs in Liquid Nitrogen (H3)

Primary cells stored without CPAs (COM or XF) were not able to proliferate after thawing. Primary cells stored with 5% glycerol or 10% DMSO (for both cases, diluted in COM and XF) achieved confluence on an average of 21.71 days. In contrast, COM + 10% glycerol required more days to achieve confluence (27 days), and there was no growth in XF + 10% glycerol. Interestingly, both groups in XF (5% glycerol and 10% DMSO) and COM + 5% glycerol had higher viability than other COM groups (10% glycerol, 10% glycerol). First passage (P1) cells stored in COM + 5% glycerol, COM + 10% glycerol, or COM + 10% DMSO were able to achieve confluence, with an average of 11 days and 14.6 days in the COM group (no CPA), the highest viability after confluence was in COM, 87.37%, and the lowest in COM + 5% glycerol, 71.31%.

4.4.1. Reverse Transcription Quantitative Real-time PCR (RT-qPCR)

Regarding gene expression, we observed a consistent pattern of expression for stemness, proliferation, and differentiation, similar to that detailed earlier (**4.3.4 Reverse Transcription Quantitative Real-time PCR (RT-qPCR)**, with one exception, *SOX2*, where a visible pattern in expression is observed between the cells before storage and after storage, with lower expression in the latter.

4.5. Treatment of Non-healing Wounds with Cryopreserved AM (H4, A)

Twenty-six non-healing wounds from 18 patients were treated. Three wound response groups emerged: healed wounds (62% of cases), partially healed wounds (19%), and unhealed wounds (19%). These groups showed different wound area reduction patterns, serving as indicators for predicting the healing outcome when using cryopreserved AM. Patients experienced decreased pain levels during treatment, irrespective of full wound closure. Pain scores decreased from 3.25 to 0.47 out of 10 after ten weeks of AM therapy.

4.6. Inter-placental Variability in the Healing Efficiency of AM When Used for Treating Chronic Non-healing Wounds (H4, B)

Cryopreserved AM application led to complete wound closure by the end of treatment. The study focused on wound healing's proliferative phase. The average wound closure rate varied among individual cases, ranging from 5.7% to 20.99% seven days after AM application. The median wound closure rate was 17.75%, significantly higher than the average of 12.17 ± 20.12%. This suggests the effectiveness of cryopreserved AM in treating non-healing wounds.

5. Discussion

5.1. Limbal Epithelial Cell Culture

We have shown that IL13 can potentially improve the properties of cultured LECs in terms of their stemness and their use for treating LSCD. Gene expression analysis revealed that adding IL13 to LECs culture boosted the expression of the stem cell genes *KRT14*, *KRT17*, and, most significantly, *ΔNp63α*, often used to denote stemness in LESC cultures. Adding IL13 to culture media significantly increased the clonogenic capacity (evaluated by CFA assay) of LECs cultures after both passages, correlating with the upregulation of *p63α*'s expression. We demonstrated that IL13 promoted a more significant differentiation into the conjunctival phenotype, as evidenced by the increased K7 gene expression in all cells (passage 0 – passage 2), whereas K7 is regarded as a conjunctival epithelial marker (Jirsova et al., 2011). Based on our results, the increased gene expression of putative stem cell markers in limbal cultures supplemented with IL13 could be beneficial for preparing cell sheet grafts containing a higher amount of stem cells.

Moving to scaffold materials, we examined electrospun PDLLA membranes as an alternative to fibrin gels for LEC culture. PDLLA membranes support cell culture, although different morphologies (fibroblast-like) are observed compared to fibrin gel. Gene expression analysis highlights differences between the two scaffolds, with fibrin gel supporting the higher expression of stem cell markers such as *ΔNp63α*, *OCT4*, and *NGFR*.

We also observed that cells cultured on fibrin gels expressed higher levels of the cornea-specific gene *KRT12*, reflecting uniform cobblestone morphology akin to corneal epithelial cells. In contrast, cells on PDLLA membranes exhibited a significant upregulation of genes associated with mesenchymal and fibroblast cells, including *ACTA2*, *THY1*, and *FBLN1*. The higher expression of *ACTA2* on PDLLA is linked to endothelial-to-mesenchymal transition (Roy et al., 2015). The presence of fibronectin on PDLLA membranes could influence this transition toward a fibroblast phenotype.

To conclude, our results indicate that fibrin gel maintains a higher proportion of stem cell markers in cultured limbal cells compared to PDLLA membranes. Cells cultured on PDLLA membranes demonstrate potential for fibroblast transition, while those on fibrin gels display enhanced stemness and differentiation toward corneal epithelial phenotype.

5.2. OMECs for Limbal Stem Cell Deficiency

5.2.1. Whole Tissue Characterization

In our study, we started with the characterization of the whole tissue to analyze the expression of stemness, proliferation, and differentiation markers in solid tissue, i.e., before cell suspension for culture was prepared, aiming to choose the best markers for cell characterization.

Our findings are consistent with previous research (Ilmarinen et al., 2013), where stemness markers were primarily concentrated in the basal layer of the epithelium. Both p63α and p40 markers were predominantly localized in the basal layer, with significant co-localization between them. Similarly, the p75NTR marker's uniform presence in the basal layer aligned with previous studies' observations (Chen et al., 2009). The proliferation marker Ki-67 was unevenly expressed in the parabasal and suprabasal layers, in agreement with other studies (Nakamura et al., 2007; Ilmarinen et al., 2013). On the other hand, Tra et al. (Tra et al., 2012) observed Ki-67 in the basal layer only.

Differentiation markers were also examined, with K3, a corneal epithelium marker, found in a minority of suprabasal cells. Attico et al. (Attico et al., 2022) have also

demonstrated that K3 is present in the oral mucosa epithelium. K8 was mainly present in the basal layer, echoing its role as a keratin for simple epithelial cells and its potential significance in stemness. It indicates that K8 can also be a positive marker in terms of stemness and not yet completely differentiated cells (Moll et al., 2008). K19 exhibited a heterogeneous distribution, mainly in the basal layer. A similar result was observed by multiple groups (Tra et al., 2012; Gaddipati et al., 2014; Sheth et al., 2015), as all of them identified K19 on the basal layer only, but different from the study by Kolli et al. (Kolli et al., 2014), in which K19 was not expressed in the oral epithelium. K13 was mainly located in the suprabasal and superficial layers, aligned with its role as an indicator of mucosal keratinocyte differentiation, which is in agreement with other studies as well (Tra et al., 2012; Ilmarinen et al., 2013).

Overall, p63, p40, p75NTR, Ki-67, K8, K13, and K19 were identified as valuable markers for characterizing cultured OMECs in terms of stemness, proliferation, and differentiation. Additionally, K3 was noted for its importance as a corneal-specific marker despite its relatively low presence in the whole tissue.

5.2.2. Culture Substrates

The standard practice involves culturing OMECs on AM due to its beneficial properties for tissue engineering, including anti-inflammatory, antimicrobial, and mechanical characteristics (Baradaran-Rafii et al., 2017). However, using AM presents challenges, such as variations in product quality and differences between AM samples from different placental locations (Massie et al., 2015). To overcome these, fibrin gel was introduced as an alternative substrate and cell carrier. Fibrin gel has a well-standardized use for LECs and is also applicable for OMECs culture and grafting (Hirayama et al., 2012). When attempting OMECs culture on deepithelized AM (dAM) as a comparison to fibrin gels, we encountered challenges with cell attachment on dAM. The irregularities in the dAM surface caused by its heterogeneity and fixation on the culture insert led to difficulties in achieving a uniform cell layer. In contrast, OMECs cultured on fibrin gels displayed successful proliferation and confluence. Notably, the transparent fibrin gel allowed unobstructed cell growth with no initial cell attachment issues. We cultured the cells in a 24-well plate, which has a diameter of 14.0 mm, in comparison to the cornea, which is about 11.7 mm in diameter. Thus, the resulting cell sheet would be large enough to cover the entire cornea.

5.2.3. Cell Morphology, Culture Media and Culture Growth

We introduced a novel approach, not explored previously, involving xenobiotic-free culture without feeder layers and FBS replacement with HS on a fibrin gel substrate. OMECs exhibited characteristic cobblestone-like morphology with high nucleus-to-cytoplasm ratios in both media. The transparent fibrin gel used for support did not interfere with cell growth, and contamination by fibroblast-like cells was minimal to none. The epithelial-to-mesenchymal transition was not observed. Growth kinetics differed between COM and XF, with COM showing faster growth onset (4.7 days in COM and 5.4 days in XF) and earlier 80 – 90% confluence. Cell viability was higher in both conditions (89.8% ± 5.1% in COM and 86.3% ± 7.9% in XF, mean ± SD) than in previous studies (70.5%) (Hyun et al., 2017).

Based on our results, the earlier onset of growth and shorter time to reach confluence in the COM group suggest that the COM may provide a more favorable environment for cell proliferation.

5.2.4. Cell Size, Stemness

The size of cultured cells holds significance in LSCD therapies, particularly when stem cells are involved in cell sheet grafts. Research indicates that evaluating p63 levels in

epithelial cell sheets is crucial for assessing sheet quality (Baba et al., 2020). Studies suggest that smaller LESCs express stem cell markers while larger LECs express differentiation markers (Kim et al., 2004). Despite conflicting data on defining cell size thresholds, small cells are often associated with stem cells. For instance, studies categorized small cells as ≤11 μm (De Paiva, Pflugfelder, & Li, 2006), or also 6 – 10 μm (Di Iorio et al., 2006).

The data from other studies led us to consider the 11 μm cell size as a small cell, similar to Priya et al. (Priya et al., 2011), data correlated with the putative stem cell marker (p63α and p40). We obtained about 4.9% of cells ≤ 11 μm in COM and 3.1% in XF; among these, 84.5% were p63α+ in COM, and 73.4% were p63α+ in XF, resulting in 4.5% small cells-p63α+ in COM and 1.9% in XF to the total amount of cells in each condition.

CFA was considered to assess growth potential under different conditions. However, the feeder layer (mitomycin C-inactivated 3T3 mouse fibroblasts) would not survive in culture when HS replaced FBS; thus, the proper analysis of the growth potential of OMECs under both conditions, including xeno-free, would not be adequately assessed. Other studies also encountered this issue (Lužnik et al., 2017). We have then used the analysis of the cell size in combination with the presence of stemness markers, which will be discussed further, as a surrogate method to identify and characterize stem cells (Di Iorio et al., 2006).

Immunofluorescence analysis indicated co-localization of stem cell markers p63α and p40. The lack of significant differences in *ΔNp63α* gene expression suggests stemness was maintained across both conditions; other groups obtained similar results (Krishnan, Iyer, & Krishnakumar, 2010; Gopakumar et al., 2019). The p75NTR protein, indicative of stem/ progenitor cells, was present at higher levels in this study than in previous work. Gene expression supported this trend, suggesting that p75NTR-positive cells were maintained in cells that proliferated from the primary cell suspension.

ABCG2, a marker for LESCs (de Paiva et al., 2005), showed significant upregulation in XF culture. The significant upregulation of *ABCG2* in XF indicates this media condition allows the maintenance of a side population of stem cells, as other studies have shown the presence of ABCG2 in OMECs confirmed the presence of a stem cell population (Krishnan, Iyer, & Krishnakumar, 2010; Dhamodaran et al., 2016). The expression of other markers like *OCT4*, *LRIG1*, and *KLF4* remained stable. *SOX2*, a stem cell marker, was downregulated in culture, in line with differentiation processes. Low expression of *PAX6*, *NANOG*, and *NESTIN* indicated the absence of these stemness markers.

In summary, both COM and XF conditions maintained stemness markers in OMECs. Immunofluorescence and gene expression analyses confirmed the presence of stem cell markers. COM favored smaller cell sizes, often indicative of stem cells. Reduced expression of *KRT13*, a mucosa epithelium marker, in both media conditions indicated a lower degree of differentiation, beneficial for ocular regeneration grafts. Combined with p63α and cell size correlations, COM-cultured OMECs offer a promising outcome.

5.2.5. Proliferation Markers

Ki-67, a proliferation marker, was higher in cultured cells (COM: 35.9%, XF: 24.2%) than primary suspension (12.0%), but not significantly due to limited samples. Gene expression showed upregulation of *MKI67* in both COM and XF, significantly different in COM. This reflects the higher proliferation of cultured cells, as Ki-67 indicates active cell division. *PCNA*, another proliferation marker (Bologna-Molina et al., 2013), was significantly upregulated in XF, while *ALDH3A1*, a proliferation-suppressive marker, was downregulated in both conditions but only significantly in COM. COM's combined upregulation of *MKI67* and downregulation of *ALDH3A1* may enhance proliferation. COM-cultured cells reached confluence earlier (11.8 days) than XF (12.7 days), supporting COM's superior proliferation rate.

5.2.6. Differentiation Markers

K13, an oral mucosa marker, decreased significantly in cultured cells (COM and XF) versus primary suspension, confirmed by gene expression. This indicates less differentiation in cultured cells. *KRT7* was significantly upregulated in cultured cells (COM and XF) compared to primary cells. This could be explained by the fact that K7 is a secondary keratin of simple epithelia (Pekny & Lane, 2007), meaning that K7 is produced in addition to or instead of primary keratins (Bragulla & Homberger, 2009). *KRT3* and *KRT12*, cornea-specific markers, showed low expression in cultured cells, consistent with limited corneal differentiation. K8 presence from immunofluorescence did not match gene expression (*KRT8*), indicating possible post-transcription modifications. *KRT14*, *KRT15*, *KRT17*, and *KRT19*, associated with basal stem cells, were highly expressed in cultured cells, reflecting a high content of stem cells and progenitors.

Both cultured conditions had similar gene expression patterns and marker presence. Stemness markers were prevalent, while cornea-specific and other differentiation markers were less expressed. The cell sheet's goal for ocular surface transplantation favors high stemness markers and low differentiation markers, as cultured OMECs can acquire corneal phenotype after grafting.

5.2.7. Genotoxicity

DNA damage assessment in cultured cells is critical for ocular surface transplantation's safety and efficacy. Epithelium renewal depends on functional stem cells, but ex vivo systems can alter cellular function via oxidative reactions and stressors (Pathak et al., 2016). This can damage cellular molecules and DNA integrity. Factors like medium composition and culture duration affect genome stability and oxidative damage. DNA SBs and oxidative damage to purine bases indicate culture quality.

In our study, no significant difference existed in SBs + ALS or net FPG-sensitive sites between COM and XF groups. Net FPG-sensitive sites were mostly lower in XF, suggesting varying DNA stability effects. Our results aligned with Baričević et al. (Baričević et al., 2012), who found similar DNA damage levels in cultured and non-cultured cells. Our results contrasted with Lorenzo et al. (Lorenzo et al., 2018), where LECs showed substantial SBs.

The findings confirm OMECs' effective cultivation in both COM and XF media without significant DNA damage. XF media's preference in clinical settings stems from lower contamination and animal-derived pathogen transmission risks.

5.3. Long-term Storage of OMECs

In cell-based therapy, LSCD patients often require regrafting due to the 70% success rate of COMET (Cabral et al., 2020). Cryopreservation offers a way to store stem cells long-term, ensuring availability for future treatments and reducing donor burden (Jaiswal & Vagga, 2022). Two cryopreservation approaches were employed: direct storage from primary cell suspension and storage after first culturing to confluence. COM and XF media were compared, also with the addition of CPAs (glycerol, 5%, 10%, 50%, and 10% DMSO) to storage media. Results showed cultured cells stored after confluence (first passage cells) had better growth rates and viability than those stored directly from the primary cell suspension. Only primary cells in COM or XF with 5% glycerol or 10% DMSO proliferated with good viability after storage. Regarding other gene expressions, we observed a consistent pattern of expression for stemness, proliferation, and differentiation, as detailed earlier (**5.2.4 Cell Size, Stemness**, **5.2.5 Proliferation Markers**, and **5.2.6 Differentiation Markers**), with one exception, *SOX2*, as stored cells showed a decreased expression of *SOX2* compared to cells before storage. Some studies have shown that CPAs can influence gene expression (Sumida

et al., 2011; Cordeiro et al., 2015); particularly, it was shown that DMSO could affect the expression of pluripotency genes in human embryonic stem cells, leading to a decrease in stem cell markers (Czysz, Minger, & Thomas, 2015).

Based on our results, storing cells after the first passage without CPAs is suggested to improve viability and growth potential. For grafting, a recommended approach involves first culturing OMECs in COM and parallelly storing first passage cells in COM or COM + 5% glycerol, considering stemness and viability preservation.

5.4. Cryopreserved AM for the Treatment of Non-healing Wounds and Inter-placental Variability in the Healing Efficiency of AM

Our study explored the healing rate of cryopreserved AM in treating NHW. A healing rate of 62% was observed, consistent with similar studies. Lavery et al. (Lavery et al., 2014) achieved a 62% success rate, while Farivar et al. (Farivar et al., 2019) reported 53% healing in a limited trial. A multicenter trial using viable cryopreserved placental membranes reported a 48.4% success rate (Ananian et al., 2018). Healing efficiency was linked to baseline wound size, although our study did not statistically confirm this due to larger baseline sizes in the unhealed group. The average wound size before AM therapy was larger than in other studies. Treatment frequency varied in studies, and our approach was adapted based on the wound's response.

We identified three categories of wound response to AM treatment: healed, partially healed, and unhealed. The healed group showed steady progress, with values like 70% closure after 10 weeks and 50% after 5 weeks. This could serve as a predictors of successful treatment. AM not only promoted healing but also had an analgesic effect. Pain relief was reported after the first AM application, attributed to lipid molecules like palmitoylethanolamide, oleoylethanolamide, and anandamide found in placental tissues (Svobodova et al., 2023; Vrkoslav et al., 2023). The study did not find significant variability in healing efficiency between different AM placentas, suggesting other factors, such as individual health status, might influence outcomes (Horvath et al., 2023).

6. Conclusion and Future Perspectives

6.1. Conclusion 1: Increasing the Stemness of Limbal Epithelial Cell Cultures and Alternative Substrates for Cell Culture

- IL13 functions as a culture supplement to enhance LEC stemness by increasing clonogenicity and stem cell marker expression while maintaining morphology.
- IL13's positive impact on stemness comes with a downside: it raises the expression of conjunctival markers in LECs. This is problematic due to LSCD, where conjunctival cell growth on the cornea is a major issue; thus, adding IL13 to culture media might lead to the undesirable outcome of conjunctival cell proliferation. Another drawback is that IL13 is a xenobiotic additive, unsuitable for advanced cell-based therapy.
- LECs cultured on PDLLA showed noticeable transdifferentiation towards mesenchymal traits in morphology and gene expression.
- PDLLA membrane culture is suboptimal due to the deficiency of epithelial cells in LSCD, whereas fibrin gels sustain normal corneal morphology and high stemness marker expression. Fibrin gels are a superior alternative for LEC grafting, intended for transfer to clinical practice.

- Future focus: Replace or remove fibronectin from PDLLA coatings to prevent pro-mesenchymal effects.

6.2. Conclusion 2: Preparation of Oral Mucosal Epithelial Cells on Fibrin Gel for Grafting

This study aimed to optimize the culture technique for OMECs by comparing the use of COM and XF on fibrin gel. Our findings provide significant advancements in ocular surface reconstruction and cell-based therapies.

- OMECs can be cultured on fibrin gel without xenogeneic additives, maintaining an undifferentiated state and sufficient stem cell pool;
- COM seems to support a better cell sheet containing OMECs;
- Crucial stemness genes (*ΔNp63a*, *NGFR*, *ABCG2*) are expressed in both conditions.
- COM has more stem cells due to small cell size and p63α presence.
- Low DNA damage in both conditions and even lower in XF OMEC cultures, enhancing safety.

Based on the results obtained in this study, several future perspectives and directions for further research can be suggested:

- Optimization of culture protocols: Although this study successfully cultivated OMECs in both COM and XF media, further optimization of culture protocols is necessary. Even though this work did not initially focus on the comparison involving cholera toxin, it serves as a surrogate possibility and can be considered an interim solution. Animal components (e.g., cholera toxin) can be removed from the media and compared to the original complex media containing such additives.
- Comparative studies with other cell sources: It would be valuable to compare the characteristics and functionality of OMECs with other cell sources used to treat LSCD, such as LESCs and hair follicle bulge stem cells.

In summary, the results of this study provide a solid foundation for future research on OMECs for treating LSCD. We are already preparing the documentation for the State Institute for Drug Control in the Czech Republic to obtain approval for the commencement of clinical studies using the OMECs prepared as described in this work and later introducing this OMEC-based therapy in the Czech Republic.

6.3. Conclusion 3: Long-term Storage of Oral Mucosal Epithelial Cells in Liquid Nitrogen

In conclusion, this study demonstrates the importance of CPAs in the cryogenic preservation of OMECs. The choice of storage media and the presence of CPAs significantly affect the viability, proliferation capacity, and cell size of OMECs after cryopreservation.

- Primary cell suspension needs suitable CPAs like 5% glycerol or 10% DMSO for successful post-thaw outcomes;
- Storing cells after the first passage enhances viability and proliferation potential. First-passage cells can reach confluence in COM without CPAs;

Suggestions for future studies:

- To investigate the long-term effects of cryopreservation on the functionality and genetic stability of OMECs.
- To explore different thawing protocols to optimize cell viability and functionality after cryopreservation.

In conclusion, it can be summarized that after repeating the experiments and confirming the results, the protocol will be optimized, according to which it will be possible to store OMEC for treating LSCD in the long term.

6.4. Conclusion 4: (A) Cryopreserved Amniotic Membrane for the Treatment of Non-healing Wounds and (B) Inter-placental Variability in the Healing Efficiency of Amniotic Membrane

My work on this project resulted in preparing hundreds of AM grafts for wound treatment. It was essential to meticulously prepare high-quality AM for clinical studies. Additionally, these AM grafts also served as a cell culture substrate.

The study found that cryopreserved amniotic membrane as a treatment for NHWs was safe and effective, with 62% of wounds completely healed. The study also identified three distinct groups of NHWs with different healing characteristics, which can be used to predict treatment outcomes. Moreover, no significant difference in healing capacity was observed between the individual placentas.

AM is a very effective biomaterial not only for its healing effects but also as a suitable substrate for cell cultivation. The properties of AM need to be further investigated and used in clinical practice.

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8. List of Publications Related to the Thesis

1. Trosan, P., **Cabral[1], J. V.**, Smeringaiova, I., Studeny, P., & Jirsova, K. (2022). Interleukin-13 increases the stemness of limbal epithelial stem cells cultures. PLoS One, 17(8), e0272081. https://doi.org/10.1371/journal.pone.0272081 (Impact Factor: 3.75);
2. **Cabral, J. V.**, Jackson, C. J., Utheim, T. P., & Jirsova, K. (2020). Ex vivo cultivated oral mucosal epithelial cell transplantation for limbal stem cell deficiency: a review. Stem Cell Res Ther, 11(1), 301. https://doi.org/10.1186/s13287-020-01783-8 (Impact Factor: 7.5);
3. Svobodova, A., Horvath, V., Smeringaiova, I., **Cabral, J. V**., Zemlickova, M., Fiala, R., Burkert, J., Nemetova, D., Stadler, P., Lindner, J., Bednar, J., & Jirsova, K. (2022). The healing dynamics of non-healing wounds using cryo-preserved amniotic membrane. Int Wound J, 19(5), 1243-1252. https://doi.org/10.1111/iwj.13719 (Impact Factor: 2.38);
4. Horvath, V., Svobodova, A., **Cabral, J. V.**, Fiala, R., Burkert, J., Stadler, P., Lindner, J., Bednar, J., Zemlickova, M., & Jirsova, K. (2023). Inter-placental variability is not a major factor affecting the healing efficiency of amniotic membrane when used for treating chronic non-healing wounds. Cell Tissue Bank. https://doi.org/10.1007/s10561-023-10096-y (Impact Factor: 1.75);
5. Smirnov, E., Trosan, P., **Cabral, J. V.**, Studeny, P., Kereïche, S., Jirsova, K., & Cmarko, D. (2020). Discontinuous transcription of ribosomal DNA in human cells. PLoS One, 15(3), e0223030. https://doi.org/10.1371/journal.pone.0223030 (Impact Factor: 3.75).

[1] Equivalent to the first author.