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Use of corneal endothelium and amniotic membrane for transplantation purposes

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S disertací je možno se seznámit na děkanátu 1. lékařské fakulty Univerzity Karlovy.

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Abstrakt

Část I: Endotelové buňky tvoří zadní vrstvu rohovky a jsou nezbytné pro udržení její průhlednosti. Dysfunkční endotel lze obnovit pouze transplantací. Globální nedostatek rohovek dárců vyžaduje hledání alternativních způsobů léčby. Přípravu štěpu metodami tkáňového inženýrství komplikuje nízká proliferační kapacita endotelu a jeho rychlá transformace v buňky s markery epitelu či fibroblastů. Dosud nebyl definován marker, jenž by byl exprimován pouze endotelem a nebyla potvrzena existence kmenových buněk pro endotel.

Připravili a zavedli jsme protokol pro kultivaci endotelových buněk z tkáně určené k výzkumu, tj. korneosklerálních rimů získaných po transplantaci a rohovek vyřazených z transplantačního procesu. Sledovali jsme lokalizaci vybraných proteinů, včetně markerů kmenových buněk v nativní tkáni a v primárních buněčných kulturách. Z jedné hypotermicky uskladněné tkáně jsme připravili až 6,4 cm^2 buněk endotelu, které měli buněčné rysy nativního endotelu. Tímto přístupem lze získat endotel pro výzkumné i transplantační účely. Pomocí nepřímé imunohistochemie jsme prokázali, že žádný z dříve navrhovaných molekulárních markerů endotelu není pro tyto buňky specifický a detekovali jsme expresi markerů kmenových buněk v celé vrstvě endotelu. Na modelu prasečí rohovky jsme po navození centrálního poškození endotelu sledovali jeho reparační kapacitu. Zjistili jsme, že reparaci signifikantně neovlivňuje přítomnost periferního endotelu, ale především délka kultivace a vyšší koncentrace séra v médiu.

Část II: Lidská amniová membrána (AM) je membrána placenty, která má značný potenciál v léčbě dlouhodobě nehojících se ran různých etiologií. Mechanismem je akcelerace a urychlení granulace a epitelizace defektu. V České republice byla dosud terapie pomocí AM standardizována pro léčbu povrchu oka. Podstatnou součástí přípravy AM je účinná, ale netoxická dekontaminace. V klinické praxi se k transplantaci používá především intaktní AM, deepitelizovaný AM se preklinicky využívá jako nosič pro kultivaci buněk k transplantačním účelům.

Připravili jsme laboratorní dekontaminační roztok s vlastnostmi (vysoká mikrobiologická účinnost, nízká toxicita) obdobnými komerčnímu roztoku. Vyvinuli jsme protokol pro deepitelizaci AM, pomocí kterého jsme získali jednak AM s intaktní bazální membránou a vitální epitelové buňky. Oba produkty se využít v tkáňovém inženýrství. Na základě předběžných výsledků ukazujeme pozitivní účinek AM na hojení nehojících se ran u pacientů zařazených do multicentrické studie.

Klíčová slova: rohovka, endotel, amniová membrána, kmenové buňky, kultivace, imunohistochemie, transplantace

Abstract

Part I: Endothelial cells form the posterior layer of the cornea are important for maintaining its transparency. Dysfunctional endothelium can only be restored by transplantation (Tx). The global shortage of donor corneas requires the search for alternative treatments. The preparation of the graft by tissue engineering methods is complicated due to low proliferative capacity of the endothelium. To date, no endothelium-specific marker has been found and the existence of endothelial stem cells has not been confirmed yet.

We have prepared a protocol for culturing the endothelial cells from research-grade tissue, i.e. corneoscleral rims obtained after Tx and from complete corneas excluded from the transplant process. We monitored the localization of selected proteins, including stem cell markers, in native tissue and in primary cell cultures. We prepared up to 6.4 cm^2 of endothelium from one cornea/rim, which had cellular features comparable to the native endothelium. This approach can increase the amount of endothelium for research or Tx purposes. Using indirect immunohistochemistry, we showed that none of the previously proposed endothelial molecular markers is specific for these cells. We detected the expression of stem cell markers in endothelial monolayer. In the porcine cornea model, we monitored its repair capacity after inducing central damage to the endothelium. We found that the repair is not significantly affected by the presence of peripheral endothelium, but mainly by the length of culture and by concentration of serum in the medium.

Part II: The human amniotic membrane (AM) is a placental membrane that has a great potential in the treatment of non-healing defects of various etiologies. The mechanism is the acceleration of granulation and epithelialization of the wound. In the Czech Republic, AM therapy has so far been standardized for the treatment of the ocular surface only. A crucial part of AM preparation is effective but non-toxic decontamination. In clinical practice, intact AM is mainly used for Tx, but de-epithelialized AM is preclinically used as a carrier for culturing cells for Tx purposes.

We prepared own laboratory decontamination solution with properties (high microbiological efficiency, low toxicity) similar to a commercial solution. We developed protocol for AM de-epithelialization, with which AM with an intact basement membrane, and vital epithelial cells are obtained simultaneously for their further use in tissue engineering. Based on preliminary results, we show a positive effect of AM on the healing of chronic non-healing wounds in patients enrolled in a multicenter study.

Key words: cornea, endothelium, amniotic membrane, stem cells, cultivation, imunohistochemistry, transplantation

Abbreviations

5HT1D/HTR1D	5-Hydroxytryptamine Receptor 1D
AECs	amniotic epithelial cells
AESCs	amniotic epithelial stem cells
AM	amniotic membrane
AMCs	amniotic mesenchymal cells/stromal keratocytes
AMSCs	amniotic mesenchymal stem cells
BM	basement membrane
CD166/ALCAM	Activated Leukocyte Cell Adhesion Molecule
CE	corneal endothelium
CECs	corneal endothelial cell(s)
CM	chorionic membrane/chorion
COL8A2	collagen Type VIII Alpha 2 Chain
DM	Descemet membrane
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
EGF	epidermal growth factor
EnMT	endothelial-to-mesenchymal transition/transformation
EP	corneal epithelium
EPC	corneal epithelial cells
FBS	fetal bovine serum
FGF-2/b-FGF	basic-fibroblast growth factor
FNC	a mixture of fibronectin, collagen I and bovine serum albumin
GPC4	glypican 4
HT	hypothermia/hypothermic
KC	keratocytes
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
MRGX3/MRGPRX3	Mas-Related G-Protein Coupled Receptor Member X3
OC	organ culture(-ed)
PCR	polymerase chain reaction
PPCD	posterior polymorphous corneal dystrophy
PRDX6/Prdx6	peroxiredoxin 6
ROCK	rho-associated protein kinase inhibitor
SOX2	SRY (Sex Determining Region Y)-Box 2
TGF-β	transforming growth factor -ß
TM	Trabecular meshwork
Tx	transplantation
TZ	transition zone
ZO1/TJP1	Zona Occludens Protein 1
ZP4	Zona Pellucida Glycoprotein 4
α-SMA	alpha smooth muscle Actin

Introduction

Cornea

The human cornea is the outermost transparent part of the eye, which allows light to enter the eye. This avascular and lymph vessel devoid tissue is the strongest lens of the eye, having about +43 diopters. It consists of corneal epithelium with its basement membrane, the acellular Bowman's layer, stroma, Descemet's membrane and endothelium.

Corneal endothelium

The corneal endothelium (CE) is the innermost 4-6 μm thick monolayer of cornea, which preserves corneal transparency. The corneal endothelial cells (CECs) lies on their basement membrane, called Descemet's membrane (DM). The CECs maintain the stable corneal hydration by "pump-leak" mechanism, where the CE pump rate equals the inward passive leak (Bonnano, 2012). The CECs are derived from the neural crest (Bahn et al., 1986) and mesoderm (Gage et al., 2005) and share features of epithelial and mesothelial cells. The CECs are polarized and have mostly hexagonal (cobblestone-like) shape. The endothelial cell density (ECD) declines with age and ECD below 500 cells/mm^2 leads to cornea opacification (Bahn et al., 1986; Lass et al., 2010).

Adult human CECs do not proliferate, as they are arrested in G1-phase of the cell cycle, due to various inhibitory mechanisms (Joyce, 2012). The corneal periphery, including the transition zone (TZ) and trabecular meshwork (TM) is thought to contain stem/progenitor cells, because cells in this region express stem markers, such as Sox-2, Oct-3/4, or Lgr5 (McGowan et al., 2007; Yam et al., 2019). Physiologically the CE is the repaired only by cell spreading and migration to recover barrier/pump function of CE (Srinivas, 2010). In older corneas, repair/regenerative capacity of CE is worsened (Joyce, 2012), and *in vitro* expansion of CECs from old donors is more difficult compared to young tissue (Peh et al., 2011; Joyce, 2012; Bartakova et al., 2018).

The wound healing (Miyamoto et al., 2010) or *ex vivo* propagation (Wu et al., 2017) of the CECs may lead to unwanted phenotypic switch towards fibroblastic appearance, called the endothelial-to-mesenchymal transition (EnMT) (Roy et al., 2015), which may disrupt the function of CE and is related to pathological fibrosis and various endothelial dystrophies (Bayyoud et al., 2019). The underlying mechanisms of EnMT in CE is still unknown.

In vitro cultivation of CE is negatively influenced by naturally low proliferative capacity of CE and the rapid onset of EnMT after the start of cultivation (Okumura and Koizumi, 2020). The transcriptome and proteome of cultured CECs' vary according to donor and culture conditions (Frausto et al., 2016). Current protocols standardly include a peel-and-digest method, which includes manual peeling of CE on DM lamella from cornea and the enzymatic digestion of DM as a second step (Parekh et al., 2017). The most used culture approach is the dual-media method (Peh et al., 2015), which use two different culture media, the proliferation medium (PM), supporting proliferation of CECs, and stabilization medium (SM), allowing maintenance of CECs and preserving CEC phenotype before next passage.

Amniotic membrane

A full-term human placenta is composed of the placental disc, placental membranes and the umbilical cord (UC). The placental membranes include the amniotic membrane (AM) and the chorionic membrane (CM). AM is a 35–60 μm thin innermost layer of the placenta that surrounds the embryo/fetus and protects it from unwanted materials during intrauterine development (Benirschke et al., 2012).

AM is composed of five layers: the innermost monolayer of epithelial cells (AECs), basement membrane (BM), compact layer, fibroblast layer and spongy layer that contain mesenchymal cells (AMCs). AM does not contain blood or lymph vessels. Due to incomplete fusion of AM and CM during development, AM can be separated from underlying CM by blunt dissection.

The AECs forms a monolayer of metabolically active cuboidal epithelial cells that are derived from the embryonic ectoderm (Enders and King, 1988). AECs contain populations of stem/pluripotency-like cells (AESCs) that express molecular markers such as stage specific embryonic antigen (SSEA)-4, tumor rejection antigen (TRA) 1-60 and TRA1-81, Oct-4, nanog, Sox2 or Kruppel-like factor 4 (Klf-4) (García-López et al., 2019). These AECSs can be differentiated towards multiple cell lineages for clinical use (Miki, 2018).

The amniotic stroma contains stem/pluripotent cells, the amniotic mesenchymal stem cells (AMSCs) expressing stem cell/pluripotency markers and can be used clinically, as they have multilineage differentiation, superior proliferation ability and anti-inflammatory potential (Ryan et al., 2013).

Biological and mechanical properties (the presence of various growth factors and cytokines) directly predispose AM for clinical use (Pogozhykh et al., 2018). AM promotes granularization, re-epithelialization, reduces fibrosis, angiogenesis, pain, inflammation and it has antimicrobial and anti-viral features (reviewed in Jirsova and Jones, 2017a). Since its first use in ophthalmology (de Rotth, 1940), the AM has been used for treatment of various pathologies of ocular surface, where the AM was used as a scaffold or as a bandage (Dua et al., 2004). In dermatology, the cryopreserved, air-dried or lyophilized (freeze-dried) AM is used for treatment of skin burns and chronic non-healing wounds (diabetic foot ulcers, venous leg ulcers, etc.) (Haugh et al., 2017). Numerous clinical studies have demonstrated that AM increase frequency and probability of wound closure, compared to application of standard of care (SOC) (Haugh et al., 2017; Serena et al., 2020). The AM can be also utilized as a scaffold for expansion of cell types intended for grafting or tissue-engineering.

Hypotheses and aims of work

Corneal endothelium (Hypothesis 1, H1):

Corneal endothelium contains stem/progenitor-like cells in the endothelial periphery, which allows a preparation of endothelial graft from discarded corneas with preserved endothelial periphery; the endothelial cell phenotype can be confirmed with novel markers referred to be specific to corneal endothelium.

Based on available research data, we assumed that we would find a positive expression of a selected stem/pluripotency markers in the periphery of CE (transition zone) in both normal and wounded corneas. This would allow us to prepare confluent CE cultures from corneoscleral rims discarded after ocular surgery. This approach could increase a pool of available donor CE for research or grafting. Due to requirement of proper characterization of CE phenotype prior they clinical use, we assumed that novel endothelial-specific markers (CD166, GPC4, PRDX6, etc.) will be expressed exclusively in the CE of native tissue, and not in other corneal cells.

Aims:

- To detect stem/progenitor markers in the periphery of CE and TZ of corneas excluded from transplant program to confirm their presence in the corneal periphery.
- To explore the possibility of culturing CECs, isolated from discarded corneoscleral rims and corneas to find the best ratio between proliferation and CE phenotype.
- To characterize an expression of reported CE-specific markers in native corneas and in endothelial cultures for their potential use for identification of CE phenotype.

Corneal endothelium (Hypothesis 2, H2):

Wounded corneal endothelium of corneas with preserved endothelial periphery containing stem/progenitor-like cells, will be repaired/regenerated faster than the endothelium of corneas lacking the endothelial periphery (Paper 1).

We presumed that centrally injured porcine CE of corneas with preserved TZ, possibly containing stem/progenitor CECs, would be repaired/regenerated faster than the corneas lacking the TZ, and the organ culture would allow more efficient wound healing than hypothermia.

Aims:

- To introduce mechanically damaged pig corneas as a mammalian model for the assessment of CE wound healing.
- To determine whether the pig CE, with the preserved periphery (TZ), will repair faster and more efficiently after central injury than CE lacking the periphery.
- To find the best wound healing conditions in terms of serum concentration and organ culture period leading to the most extensive and fastest reparation of porcine CE.

Amniotic membrane (Hypothesis 3, H3):

In house-made decontamination solution have similar effect on amniotic cell viability, as well as antimicrobial efficiency and stability than the commercial decontamination solution (Paper 2 and 3).

We standardly prepare cryopreserved AM for grafting, which we sterilize with the only certified decontamination solution in Europe, which is not always available. We supposed that this solution can be substituted by our laboratory decontamination solution (LDS), with a similar antimicrobial efficiency and cytotoxicity to the commercial solution.

Aims:

- To prepare tissue decontamination solution (LDS), alternative to commercial product, for its future approval by national authority and clinical use.
- To test LDS for its cytotoxicity, antimicrobial efficiency and stability and compare the results to commercial solution **(Paper 2**, **Paper 3)**.
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Amniotic membrane (Hypothesis 4, H4):

Using a gentle method for de-epithelialization of amniotic membrane, it is possible to obtain both, viable epithelium and intact denuded membrane simultaneously, for their further clinical/experimental use (Paper 4).

Intact AM is primarily used in clinical practice, but the de-epithelialized AM with well-preserved basement membrane seems to be more suitable as a substrate for cultivation of various cells. The AECs that are released from AM have a great clinical potential. We hypothesized that it is possible to develop a gentle enzymatic decellularization protocol where both, intact cells and AM matrix can be obtained simultaneously.

Aims:

- To develop an efficient, gentle and safe de-cellularization protocol to obtain both living epithelial cells and denuded amnion with intact structure.

Amniotic membrane (Hypothesis 5, H5):

Amnion grafts, prepared according to the protocol we use, stimulate and facilitates wound healing of non-healing skin wounds.

AM is considered as an ideal biological wound dressing because it promotes granulation and epithelialization of wound and pain relief. We hypothesized that AM will induce wound healing of chronic wounds, shorten the time of wound closure and reduce pain more than standard-of-care methods.

Aims:

- To prepare standard protocol for amnion graft application for healing of chronic wounds in Czech Republic.
- To assess the benefits of AM application in diverse groups of frequent non-healing wounds (venous leg ulcers and diabetic foot ulcers).

Material and methods

To achieve the aims of our work, a wide spectrum of methods was used. Individual material and methods are described in the appended publications or within a following paragraphs. Author managed following steps:

- Preparation and monitoring the repair of porcine endothelium - histological staining, microscopy, evaluation of endothelial parameters (cell density, hexagonality, coefficient of variation) from taken photographs of the samples;
- Processing of the placenta and preparation of AM, monitoring of the cell vitality (live/dead assay, trypan blue staining) in tissue before and after decontamination – histological staining, microscopy;
- Immunohistochemical/immunofluorescence analysis of porcine and human tissue;
- Preparation of decontamination solutions and analysis of stability (pH changes, osmolality) of cold-stored solutions; analysis of antimicrobial efficiency data;
- Preservation of denuded amnion; histological and immunohistochemical staining;
- Preparation of *in vitro* corneal endothelial cell cultures from discarded cadaverous tissue and monitoring the growth of the corneal endothelial cells – microscopy;
- Immunolocalization of proteins in cornea – cryosections, flat-mounts, cell imprints;
- Preparation of amnion grafts from human placentas in clean room facility;
- Evaluation of wound healing from taken photographs by software analysis;
- Preparation of amnion/chorion samples for biochemical and histological analysis.
- Statistical analysis.

Material and methods in experimental part regarding H1 (corneal endothelium):

The donated research-grade donor tissue (n=59), either whole corneas (n=19) that were excluded from Tx process or corneoscleral rims (n=40), discarded after ocular surgery, was stored under hypothermic conditions (4-8 °C) in Eusol-C storage medium prior their use. Donors' age ranged from 23 to 78 years. The harvested DM-EC sheets were incubated overnight at 37 °C in stabilization medium (SM1), i.e. human endothelial serum free medium (h-SFM) with 15% (v/v) FBS prior digestion with collagenase. In seven cases, the lamella was cultured without digestion, i.e. explant culture. For propagation of CECs on FNC coated dishes, two types of proliferation media (PM) were used, either Opti-MEM I, or M199/F12 (1:1), varying in supplements (FBS, growth factors, lipids, vitamins etc.), following published protocols for expansion of CECs from high quality corneas (Zhu and Joyce, 2004; Peh et al. 2013). PM was exchanged every other day and the cell culture progress was observed by phase contrast microscopy. After the CEC reached confluency (4 weeks on average), cells were incubated in SM2 (h-SFM with 5% FBS), until their hexagonal morphology was restored (5-7 days on average). The cell density was measured from photo-documentation with NIS Analysis Software.

Control corneas were stained by either enzyme immunohistochemistry (by Mouse and Rabbit Specific HRP/AEC (ABC) detection kit) or immunofluorescence, following standard staining protocols. The examined tissue included cryosections (7-µm thin), whole mounted corneas, *in vitro* cultured CECs and cell imprints. The primary antibodies used were: 5HT1D

Receptor, Alpha smooth muscle actin (α-SMA), CD44/HCAM, Anti-CD166, Keratin 7, Keratin 19, Glypican 4, LGR5, MRGX3, Nanog, Oct4, Prdx6, Sox2, ZO-1, ZP4, Ki67, Na/K-ATPase-α1. The staining pattern was examined using NIS Analysis Software.

Material and methods in experimental part regarding H5 (amnion):

Human AM grafts for Tx were prepared by a protocol approved by the State Institute for Drug Control from placentas obtained at caesarean section delivery. The cryopreserved AM (DMEM/glycerol, -80°C) and air-dried AM were prepared within 24 hours after birth. AM grafts approved for Tx were applied by collaborating surgeons at the clinic to patients selected according to predefined project criteria. The change of AM was done once a week. The change of wound size/area/volume, granulation and epithelization progress (%), curve of closure progress (wound area/volume change per week), and the time to complete healing were observed on a regular basis and analyzed from taken photographs by NIS software.

Results

In the following text, the most important published results (Paper 1 - 4) and yet unpublished data (Hypothesis 1, 5) are summarized. For more details, please see the appended publications.

Part I: Corneal endothelium

Preparation of an *in vitro* cultured corneal endothelium (H1)

We prepared 38.98% (23/59) of CEC monolayers, i.e. 3.2–6.4 cm^2 of confluent CE monolayers, with cobblestone-like shaped cells, from one corneoscleral rim or cornea. From all successful cultures (n=23), the 47.82% (11/23) of successful cultures were derived from whole corneas (n=19) and 52.17% (12/23) of the cultures were derived from rims (n=40). The unsuccessful cultures (61.02%) were characterized as those with low cellular attachment, no mitotic cells, presence of apoptosis and senescence, or irreversible fibroblastic changes of majority of CECs.

Explant method was less successful (28.5%, 2/7) than peel-and-digest method (40.38%, 21/52). Considering the cell isolation method (peel-and-digest), we prepared 57.89% (11/19) successful cultures from whole corneas and only 30.30% (10/33) from rims. The cell densities varied (848.5 ± 443.5 cells/mm^2) and were higher in cultures derived from younger donor. Prolonged HT and higher donor age negatively influenced the success of our CEC cultures. Successful CE cultures were derived from donor tissue with average donor age of 56.82 ± 13.67 (28 – 77) years and average HT storage length of 14.68 ± 5.19 days (6-23). The average age of donors in case of unsuccessful cultures was 59.33 ± 12.19 (23-78) and years average HT storage length of 16.67 ± 7.99 (4-41) in case of unsuccessful cultures. Slightly less successful cultures were derived from older donors (38.2%, 13/34, 60-77 years old) than from younger donors (40%, 10/25, 28-59 years old). The success of prepared cultures was improved by overnight stabilization of manually peeled CE-DM lamellae in SM1, Viscoat® application and culturing CECs at confluency for 5-7 days in SM2, which improved the morphology of CECs and reduced (up to 10%) the portion of fibroblast-like CECs that occurred at 7.56 ± 2.25 days of cell culture. Slightly more successful cultures were obtained with Opti-MEM I medium (60%) than with F99 (58.33%), both supplemented with BPE, EGF, FGF and ROCK inhibitor Y-27632. Slightly more mitotic cells were detected within CECs grown in Opti-MEM I (14.17%) than in F99 (11.76%). None of the media could prevent EnMT or senescence. The successfully cultured CECs expressed endothelial markers, Na+/K+-ATPase, ZO-1 (at cell membrane), CD166 and Prdx6 (in cytoplasm).

Molecular markers found in native and in vitro cultured endothelial cells (H1)

None of the selected molecular markers was found to be specific for native CE and its close vicinity. The following markers were examined (regions of their expression are summarized in brackets): CD166 (endothelium, TZ, TM, stroma, limbus), Prdx6 (epithelium, limbus), GPC4 (all three main corneal cell types, predominantly endothelium and epithelium), MRGX3, 5HT1D, ZP4 (all corneal cells), CD44 (epithelium, stroma), α-SMA (TM, stroma, limbus), K7 (endothelium, epithelium, limbus) and K19 (endothelium, epithelium, limbus). Positive staining for stem/progenitor markers Sox2 and Lgr5 was located predominantly at the periphery of CE, but also in some CECs of the central CE. The Ki-67 positive CECs were found predominantly in the periphery of CE but also in the central CE, especially near the wounded CE or denuded DM. More Ki-67 positive CECs were detected in organ-cultured corneas than in corneas stored under hypothermic conditions. The expression of Nanog or Oct4 was not confirmed due to a weak/unclear staining signal.

Repair and regeneration of corneal endothelium – a porcine model (H2, Paper 1)

The reparative capacity of the CE was assessed by a computer-aided measurement of the common CEC parameters: endothelial cell density of live cells (LECD), percentage of dead cells (%DC), percentage of hexagonal cells (6A) and coefficient of variation (CV) in the central (C) and paracentral (PC) CE, surrounding the central lesion. The parameters were assessed in fresh discs (n = 42) and cultured discs (n = 124), either intact or injured, i.e. with 6.8 mm^2 central lesion. The CE imprints were immunoassayed for the Ki-67 and fibrillarin.

In fresh corneas, the LECD/mm^2 (mean ± standard deviation) were 3998.0 ± 215.4 (C) and 3888.2 ± 363.1 (PC). Cultured (intact) controls had stable LECD in C. Most of the CEC parameters worsened in injured cultured discs – lower values of LECD and 6A and higher %DC and CV, compared to control discs. The mean values of LECD of large injured discs were numerically higher than in small injured discs, but the difference was not statistically significant. Prolonged storage (9d) considerably improved CEC parameters of injured discs than shorter storage (5d). After 9 days, complete reparation was observed in 99% of discs and Ki-67 positive cells were found in the central cornea. Higher serum concentration (10%) and prolonged OC (9d) facilitated the wound closure.

Part II: Amniotic membrane

Effect of decontamination on vitality of amniotic membrane cells (H3, Paper 2)

The cytotoxic effect of BASE128 and LDS, with analogous composition of antibiotics (AA), was measured as the percentage of dead AECs (%DEC) in fresh (control) and decontaminated AM. The %DEC was assessed by trypan blue staining of untreated (fresh) and decontaminated AM samples under three protocols: 1) 6 h, 37 °C, 2) 24 h, at room temperature (RT), 3) 24 h, 4 °C. The samples were analyzed for apoptotic cells via terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) method.

In fresh tissue, the %DEC varied between 4.8–28.1%. In general, prolonged storage (24h) and low temperature led to the highest %DEC independent of the type of decontamination solution. The lowest %DEC was found after the treatment of AM with BASE128 for 6 h at 37 °C and the highest %DEC after storage of AM in LDS for 24 h at 4 °C. The mean percentage of apoptotic epithelial cells was less than 1% (at all conditions) and varied between 16.1-59.7 % (42.0 ± 18.5) in case of apoptotic mesenchymal cells (AMCs).

Efficiency of decontamination solutions against selected pathogens (H3, Paper 3)

The two solutions used for decontamination of AM, the BASE128 and LDS, were compared in their antimicrobial efficiency against five human pathogens (*S. aureus, P. aeruginosa, P. mirabilis, E. coli* and *E. faecali*s) via agar well diffusion method and their stability (pH, osmolarity) in time (fresh, 1, 3 and 6 months at 4°C) was determined.

The difference in the inhibition of bacterial growth was mostly not statistically significant. Both solutions effectively eliminated *P. mirabilis*; the lowest antimicrobial activity was measured against *S. aureus*, which slightly decreased with cold storage. The BASE128 had lower antimicrobial efficiency against *E. coli* and *E. faecalis* than LDS. The pH of LDS was slightly higher (7.72 ± 0.19) than pH of BASE128 (7.58 ± 0.07) after storage for 6 months. The osmolarity values for the LDS ranged between 277.50 units (T3) to 392.00 units (T0), while the values of BASE128 were mostly below the range of the osmometer.

De-epithelization of human amniotic membrane for experimental use (H4, Paper 4)

The AM was successfully de-epithelialized using all three types of enzymatic methods, i.e. TrypLE Express, trypsin/EDTA, and thermolysin that had comparable efficiency. Unlike other two enzymes, only trypsin/EDTA treatment led to the best preservation of BM and stroma, and the highest vitality of AECs (60%). The AECs isolated from AM after trypsin/EDTA treatment could be successfully cultured for several passages. With increasing passages, the proliferation activity raised, and expression of stem cells markers Sox2 and Nanog decreased.

Treatment of chronic wounds with human amniotic membrane (H5)

Ten patients (80 % of them polymorbid) with 17 chronic, non-healing wounds were treated by cryopreserved AM (n=9) and dried AM (n=1) allografts. In general, after application of AM onto wound, the wound healing was initiated, and pain reduced in all the treated wounds. The 47% (8/17) wounds were completely healed up to 9 months since first AM application, while 29% (5/17) wounds were healed from more than 80 % up to now. The average length of wound healing period using SOC prior AM application was 77.1 ± 74.9 weeks (18-260), and wound healing period using AM was 20.5±13.4 (6-54) weeks. In all cases there was a significant decrease in pain after five to ten weeks of AM application. The treatment of unhealed wounds continues.

Discussion

Part I: Corneal endothelium

Corneal endothelium (H1): *In vitro* culture of corneal endothelium

Due to the global shortage of donor corneas for endothelial keratoplasties, alternative therapies are being developed including tissue engineering construct and cell injection therapy (Chang et al., 2018). The current trend is to use a cadaveric CECs for graft preparation. In response to the lack of donor corneas, we focused on exploring the possibility of in vitro propagation of CECs from corneas deemed unsuitable for Tx or corneoscleral rims (surplus tissue after CE keratoplasty) to increase the supply of donor CE for research and potentially for grafting. Since our results have not been published yet, they are discussed in more detail in this section.

We prepared 38.98% of successful monolayers of CECs from donor corneas or rims. These results suggest that CE cultures can be prepared not only from whole corneas, but also from rims that do not contain central CE but have preserved periphery, where stem/proliferating cells could reside. Considering only successful cultures, slightly more

successful cultures were prepared from rims (52.17%) than from whole corneas (47.82%) by both cell isolation methods. Peel-and-digest method led to more successful cultures derived from corneas (57.89%) than from rims (30.30%), showing the whole corneas as better source of CECs, as expected. Peel-and-digest method was better method for cell expansion than explant culture method. These results are like that achieved by Choi et al. (2014), who derived the 31.97% (86/269) of successful cultures, with 47.06% (8/17) of cultures derived from whole cornea and 27.83% (64/230) from the rims.

Our results could be affected not only by donor-to-donor characteristics, but also by characteristics and sizes of tissue. Unlike the whole corneas, the rims contained less CECs due to lacking central CE, as well as they often contained areas of impaired/dead cells as a result of tissue processing and storage (Jirsova et al. 2017b). On the other hand, this could be balanced by a shorter storage period of rims (6-18 days), compared to whole corneas (11-23 days), which could minimize the negative effects of hypothermia on CE quality. Moreover, the presence of corneal periphery in rims, where the higher ECD than in central CE was observed, could compensate the loss of central CE in rims (He et al., 2012). Other factors that could possibly influence the outcomes of this study is cause of death (traumatic vs. non-traumatic) (Ventura et al., 1997), cancer or diabetes of the donor (Briggs et al., 2016), race or previous ocular surgery (cataract, refractive), duration from death to enucleation and the time from preservation to establishment of culture affect quality of CE (Kwon et al., 2016; Parekh et al., 2019a). We did not analyze other factors than the age of donor and the length of storage on quality of CEC cultures and it will be examined in the future studies.

Prolonged storage of corneas in hypothermic conditions and higher donor age negatively affected the success of our CEC cultures, similarly to other studies (Corwin et al. 2013; Parekh et al., 2019a). The average age of donors in case of successful cultures was slightly lower (56.82 years) than the average donor age of unsuccessful cultures (59.33 years). Slightly less successful cultures were derived from older donors (38.2%) than from younger donors (40%). Choi et al. (2014) prepared more successful cultures from younger donors (42.9%, 64/149, <60 years old) than from older donors (18.3%, 22/120, >60 years old). This shows the possibility to successfully expand CECs even from donors over 60 years old. Moreover, it was easier to peel CE lamella from older corneas than from younger ones. Similar observation was previously reported (Bennett et al., 2015). Due to lower thickness of DM at younger age, the CE-DM harvested from young corneas have also a tendency to form tighter scroll than lamella obtained from older donor, which may represent an additional stress to CECs (Bennett et al., 2015).

The average storage length of successful cultures was slightly lower (14.68 days) than in case of unsuccessful cultures (16.67 days). It has been shown that hypothermia (for 7 days at 4 °C) negatively affects the growth capacity of CE, and thus success of CEC cultures, compared to organ-culture storage at 31°C (Parkeh et al., 2019a). Due to prolonged hypothermia, whole corneas often contained DM folds and largely sloughed off epithelium. Considering the already published positive effects of organ cultures on CE (Nejepinska et al., 2010; Spinozzi et al., 2018), donor tissue in our experiments was pre-incubated in medium with 15% FBS (overnight), and this increased the success of the cultures, compared to those derived from non-incubated tissue. In such stabilized corneas we detected more Ki-67 positive cells, in both, peripheral and central CE, than in HT corneas. Other factors that were found to positively influence the establishment of CEC cultures that scored as a success were the use of PM (preferably the Opti-MEM I), supplemented with FBS, BPE, EGF, bFGF and ROCK inhibitor, SM2 for 5-7 days, the use of FNC coating as a culture surface and Viscoat®, which forced CECs to attach to surface (Parekh et al. 2019b). We did not analyze

other factors than the age of donor and the length of hypothermic storage on quality of CEC cultures. The evaluation of other parameters will be one of the goals of the following studies.

We were able to prepare up to 3.2 and 6.4 cm^2 of confluent CE from one corneoscleral rim or cornea, respectively. Cell reached confluency after one month and had features of native CECs, i.e. they expressed endothelial markers Na+/K+-ATPase, ZO-1, CD166 and Prdx6. Despite all effort, the total CECs yields were small (LECD: 848.5 ± 443.5 cells/mm^2), which is still unsatisfactory for possible clinical use. The cultures represented a mixture of morphologically heterogeneous cells, with a portion (up to 10%/mm^2) of cells resembling fibroblasts. Higher cell yields were obtained when CECs derived from younger donors were propagated. None of the used culture media could revert severe EnMT or senescence, if they occurred. This is consistent with results published in other studies (Peh et al., 2015; Parekh et al., 2019b). Probable causes of a large cell loss during the culture were initial tissue quality, CECs isolation method, as well as activation of EnMT and senescence signaling pathways during prolonged culture in PM (Frausto et al., 2020). Therefore, this culture protocol will need to be significantly modified in the future, for example by adding inhibitors of senescence and EnMT. Due to lack of sufficient numbers of CECs we did not validate our results by PCR or western blot methods. Besides that, there is no consensus about a correct genotypic expression profile for healthy CECs (Van den Bogerd et al., 2019).

<u>Corneal endothelium (H1):</u> Biomarkers in corneal endothelium

In response to the need to identify the CE phenotype *in vivo* and *in vitro*, we examined healthy corneal tissue for the presence of a number of selected molecules that have been reported by recent studies as markers expressed only by CECs and no other corneal cells. The CEC-specific markers are necessary for characterization of native/intact CECs as well as for in vitro prepared cultures.

Our relatively extensive immunocytochemical study, showed that none of the selected markers is specific for native CE and its close vicinity, where stem cell for CE can be located (TZ, TM) but can be found in other corneal regions.

The stem cell markers, Sox2 and Lgr5, were expressed especially in the peripheral CE, TZ and TM of control corneas. The expression of these markers was found by previous studies that have reported the presence of stem-like cells in peripheral parts of the CE (McGowan et al., 2007; Yam et al., 2019). As the Sox2, which regulates other TFs (Oct4 and Nanog), was shown to promote proliferation and maintain the stemness (Basu-Roy et al., 2015), as well as it is involved in differentiation and wound healing (McGowan et al., 2007; Han et al., 2014), question remains, if we detected the stem CECs or only the CECs responding to wound healing processes occurring as a response to cell loss during hypothermic storage after their transfer to organ culture (performed with some corneas). The Lgr5 positive staining of CECs in our samples is in accordance with other observations (Hirata-Tominaga et al., 2013; Yam et al., 2019). The Lgr5 is a well-established epithelial intestinal stem cell marker (Espana et al., 2015). It may suggest presence of Wnt-driven adult stem cell- populations within peripheral, but also central, CE and TZ. As the selected antibody produced a weak signal, it is necessary to confirm our results using other antibody.

The Ki-67 positive CECs were found predominantly in the periphery of CE but also in the central CE, especially near the wounded CE or denuded DM. More Ki-67 positive CECs were detected in OC corneas than in HT stored corneas. In this case it should be considered that although widely used as a proliferation marker, the Ki-67 protein levels in a cell depends on the current cell-cycle phase and Ki-67 is undetectable at the G1/S transition in slowly

cycling cells that spent a long time in quiescence before re-entering the cell cycle (Miller et al., 2018**)**, the description that applies for CECs **(**Joyce, 2012; Espana et al., 2015).

The CD166 was reported to be expressed in CECs and not keratocytes or epithelial cells, by three independent groups, using three different methods (Ding et al., 2014; Okumura et al., 2014; Dorfmueller et al. 2016). However, the CD166 was detected in other cell types, such as lung fibroblasts or embryonic stem cells (Ding et al., 2014; Dorfmueller et al., 2016), epithelial limbal stem cells (Albert et al., 2012), as well as in corneal stromal stem cells (Funderburgh et al., 2005; He et al., 2016) and corneal epithelium (He et al., 2016). The last finding is in accordance with our observation. The anti-Prdx6 monoclonal antibody TAG-2A12 developed by Ding et al. (2014) was reported to bind CECs only, but the recent transcriptomic study found Prdx6 also in *ex vivo* epithelial cells and keratocytes (Frausto et al., 2020), which is consistent with our observation. The GPC4 was originally reported as a surface-specific marker of CECs, discriminating them from stromal fibroblasts (Cheong et al., 2013). Our data show that GPC4 is not specific to CECs. Expression of GPC4 in *ex vivo* epithelial cells and keratocytes was reported (Frausto et al., 2020).

In our study none of the three CEC-specific markers, 5HT1D/HTR1D, ZP4 and MRGX3/MRGPRX3, originally reported by Yoshihara and colleagues (2015), was found exclusively in CECs. The positivity was found throughout the all corneal layers. Weak expression of these three markers in ex vivo keratocytes and epithelium was reported (Frausto et al., 2020). The CD44 and α-SMA are members of a panel of negative markers, i.e. molecules not expressed in CECs (Van den Bogerd et al., 2020) which, together with positive markers, serve for characterization of CEC phenotype and their discrimination from other cell types. The CD44 was found to be expressed by corneal epithelium and keratocytes (Zhu et al. 1997; Okumura et a. 2014), which is like our observation. We detected weak expression of the α-SMA, highly conserved microfilament, in limbus, stroma and in some EP cells. As the α-SMA is usually found in stroma and epithelium of wounded corneas (Esquenazi et al., 2009), more experiments are necessary to confirm or refute our results.

We also examined two cytoskeletal filaments, typical for epithelial cells, keratin 7 (K7) and keratin 19 (K19) and we detected their expression in native CE as well in *in vitro* cultured CECs. The expression of K19 was more prominent than K7, especially on endothelial imprints. The staining signal for K7 was very weak, showing positivity in minority of CECs. The expression of smaller keratins (K7, K8, K18, K19) was previously described in human CECs (Foets et al., 1990; Merjava et al., 2009; Nagymihály et al., 2017). The K7, is specifically expressed in the simple epithelia lining the cavities of the internal organs, in the gland ducts and blood vessels. The K19 is type I cytokeratin, specifically expressed in the developing epidermis, conjunctiva and limbus (Jain et al., 2010). We also detected expression of K7 and K19 in our *in vitro* cultured CECs, during proliferative phase and after stabilization phase. The expression of K7, K8, K18 and K19 in normal CE shows that the CE has characteristics of a simple epithelia, which usually express these keratins during cell differentiation following cell division (Bragulla and Homberger, 2009). Recently, the K19 expression was associated with increased proliferation of breast cancer cells, where K19 regulates expression of transcription factor E2F1, which plays a key role in transition of cell into S phase of cell cycle (Sharma et al., 2019). Further examination of K7, K19, particularly those in *in vitro* expanded CE, is necessary.

Although several markers have been proposed as endothelium-specific in the last twenty years or so (Engelman et al., 2001), none have been confirmed as such. When characterizing the endothelium in culture, it is best to use a non-specific Na+/K+-ATPase, which, however, corresponds to the functionality of the endothelium, and at the same time well displays its characteristic hexagonal shape. The N-cadherin (He et al., 2016), which was

not investigated in this study, also appears to be a promising marker, as a growing number of studies demonstrate its specific expression in CECs and no other eye cells (Frausto et al., 2020).

Corneal endothelium (H2): Endothelial wound repair of pig endothelium (Paper 1)

Due to lack of donor corneas for research, we introduced porcine CE as a substitute for scarce human tissue to study remodeling of CE layer after induced mechanical injury, as it has similar features as human CE, especially the non-proliferative nature (**Fujita et al., 2013**). The results of the presented study indicate that the porcine CE of all large discs had during repair numerically higher LECD, compared to small discs, but the differences were not statistically significant, suggesting that large discs did not repair/regenerate significantly better compared to the smaller discs. This could be explained that more cells surrounding the lesion (including those in the periphery) could actively participate in wound healing in large discs, compared to small discs. As we did not assess CE parameters in far-periphery of CE, we could not confirm/exclude the participation of stem cells in the periphery to increased LECD in large discs. Additionally, we presume, that endothelial parameters were negatively influenced by the softness of porcine cornea (Elsheikh et al., 2008), as it tends to flatten after trephination from eye, and also by manipulation.

The higher LECD values were obtained in corneas cultured in 10% (v/v) FBS and after prolonged culture (9 days). The injured discs cultured in medium with 10% FBS had mostly higher values of LECD than those cultured in 2% FBS but statistically significant difference was found only in two cases. The positive effect of serum and its components on CE repair/regeneration, particularly on migration and proliferation of CECs, has been reported previously (Ayoubi et al., 1996; Nejepinska et al. 2010). The final LECD values in the central CE of injured corneas after 9-day incubation did not reach those assessed in central part of native corneas, indicating that more than 9-day incubation period is needed for the full re-establishment of CE hexagonal mosaic, or that the CE proliferation/migration was slowed down by intercellular contact inhibition. Storage induced DM folds and natural CE loss during organ culture could counteract the positive effects of storage on regeneration of CE. It is possible that other not yet identified mechanisms might also prevent the full restoration of the ECD.

The wound repair rate could be influenced by the qualioty of tissue and size of the wound (Bhogal et al., 2016), i.e. 6.8 mm^2, representing 5% of the CE surface, which could be too small for a successful activation of putative porcine CE stem cells. It was reported that in humans, the CE with lesion covering 4.4–11% surface of CE was completely closed after 5–7 days (Doughman et al., 1976; Hoppenreijs et al., 1992). In our porcine model, the wound was closed after 9 days of OC in most of the corneas. Thus, we can conclude that for endothelial repair the time is more important compared to serum concentration. As in humans, porcine corneal CECs spread out, proliferate and migrate quite slowly and thus need prolonged time for re-establishment of the CE monolayer.

The proliferation capacity of porcine CEs has been confirmed by the presence of Ki-67-positive CECs in the C and PC endothelium of all injured discs particularly after 9-day OC. No expression was detected in control discs. This is in accordance with previous studies where no cells expressing Ki-67 were present in healthy human CE, but Ki-67 positive cells were detected at the border of healing lesions (Mimura and Joyce, 2006). This suggested that not only migration and cell surface enlargement but also proliferation of CECs contributes to the wound closure under OC. Our findings support the recent trend in tissue banks to store the large discs for ocular transplantations by OC, particularly for a lamellae preparation.

Part II: Amniotic membrane

Amniotic membrane (H3): Decontamination solutions (Paper 2 and 3)

In fresh decontaminated AM, the percentage of dead amniotic epithelial cells (AECs), varied between 4.8–28.1%, which was similar to other studies (Hennerbichler et al., 2006; Laurent et al., 2014). We suppose that this could be the result of the inherent tissue variability, the manipulation with placenta and environmental stress applied on AECs. We showed that there are only small differences in cell survival between the application of BASE128 and LDS. The higher viability of AECs was observed using BASE128 after the treatment of AM for 6 h at 37 °C. Storage for 24 h was less beneficial to the quality of the tissue than storage for 6 h, independent of the type of decontamination solution. The worst survival was observed after decontamination of control AM for 24 h at 4°C. This observation is in accordance with some other studies **(**Jackson et al. 2016**)**, where the best preservation of the cell/tissue morphology was observed at temperatures between 12 and 24 °C.

In fresh and decontaminated tissue, only a small portion of apoptotic AECs was observed (<2% apoptotic cells), while up to 88% of AMCs were stained positively by TUNEL. This suggests that stromal cells are more susceptible to external stress stimuli than AECs. Partially, this result could be explained by the fact, that AECs are continuously released from the basement membrane (Kumagai et al., 2001), whilst dead stromal cells remain in the stroma. The other plausible explanation is that the cells die by other, rather fast, mechanism than apoptosis (necrosis) and therefore cannot be identified using TUNEL assay. The potential effect of AA on induction of apoptosis was thus not confirmed.

The LDS showed higher toxicity on cells, despite having the composition and concentration of antibiotics/antimycotics (AA) like BASE128, the latter being enriched with glucose and vitamins (Gatto et al., 2013). Lack of nutrients in LDS partially explains the worse survival of AECs, compared to BASE128. It was shown that cells exposed to the stress, such as nutrient deprivation, accumulate reactive oxygen species and eventually die after a relatively short time (Altman and Rathmell, 2012; Cabodevilla et al., 2013).

Focused mainly on AA, we used LDS of simplified composition of the decontamination solution in the study to test impact of antibiotics on AM, prior testing another additive. As we demonstrated, such solution has no dramatic impact on %DEC and is simple to prepare at any time, when commercial solution is not available. This solution has been approved by the national authority, the State Institute for Drug Control and is used in a case of supply outage of commercial solution.

The differences in antimicrobial efficiency against five human pathogens and stability (pH and osmolarity) of both decontamination solutions after 1-, 3- and 6-months storage were mostly not statistically significant, with few exceptions. Both solutions were the most effective at elimination of *Proteus mirabilis*. The decontamination efficacy of the two solutions against the remaining three common bacterial species was as follows: *Pseudomonas aeruginosa* > *Escherichia coli* > *Enterococcus faecalis.* Despite small numerical differences, the efficacy of both solutions against *P. aeruginosa* was similar. Both tested solutions were efficient against the *E. coli* and *E. faecalis*. However, the LDS was more efficient against the *E. coli* and *E. faecalis* than BASE128, and this difference was statistically significant. Both tested solutions showed the lowest efficacy against S. aureus. BASE128 was slightly more efficient in elimination of *S. aureus* than LDS, and the efficacy of BASE128 decreased after cold storage. We observed the changes in pH and osmolarity of the two decontamination solutions, which are standardly cold stored as batches before use. The reason of such storage is primarily the preservation of the substances, such as antibiotics or vitamins, in their active state. The osmolarity value of LDS decreased with cold storage,

the osmolarity values of the BASE128 could not be measured as they were below the range of the osmometer. Slight changes were found in pH of the less stable LDS solution, whose pH increased from initial value 7.36 ± 0.07 to 7.72 ± 0.19 after 6 months-storage. We verified that BASE128 expresses similar efficiency in elimination of possible placental bacterial contaminants as LDS and both may be successfully used for decontamination of various tissues.

Amniotic membrane (H 4): De-epithelialization of amnion (Paper 4).

As the results of this work were discussed in a Ph.D. thesis of the first author of this study, Peter Trosan, here we discuss the results very briefly.

We examined three enzymatic method for AM de-epithelialization and chose protocol, including Trypsin/EDTA treatment in combination with mechanical scraping as the most successful method for this purpose. Using this method, we obtained denuded AM with well-preserved BM, as well as about 60% viable multipotent AECs, which can be used for research purposes or tissue engineering (Miki et al., 2005). The worst results, with respect to BM integrity and cell viability were observed with thermolysin use, which is in accordance to previous reports (Siu et al., 2008; Saghizadeh et al., 2013). Further propagation of isolated AECs led up to five successful passages of viable cells, with positive expression of stem cell markers (SOX2, NANOG), which decreased with passaging. This suggests that stem cell characteristics of AECs decrease with each passage. The cell viability correlated well with level of BM damage. Therefore, we suggest that the trypsin/EDTA method is the method of choice when both intact AM and viable AECs are needed for subsequent use.

Amniotic membrane (H5): Amnion in healing of chronic skin wounds

We have been observing the effect of prepared AM grafts on healing of chronic (non-healing) wounds of patients included in an ongoing multicenter study (Project No. NV18-08-00106: Amniotic membrane in the treatment of non-healing wounds) to assess the benefits of AM in diverse groups of non-healing wounds. Preliminary data indicate that AM in most cases initiates the treatment of wounds in which standard treatment procedures (SOCs) have failed. From preliminary results we can clearly see that the application of cryopreserved AM was successful in the treatment of non-healing wounds. The application of AM to non-healing wounds led to an acceleration of healing, which on average started to slow down after 70% wound area reduction. Further healing then proceeded much more slowly. The rate of the treatment of a patient with air-dried AM was slow and after several weeks the treatment was continued using cryopreserved AM instead on the air-dried AM. However, it is too early to make the conclusion, that cryopreserved AM is better for this purpose than dried AM. As the clinical study is ongoing, the complete results will be published elsewhere and thus will not be discussed in this study. Finally, we are currently working on the biochemical analysis of AM, focusing on active substances that contribute to the therapeutic effects of AM, and we are also optimizing preparation of air-dried and lyophilized AM allografts. The proper lyophilization method can lead to AM graft with features and healing efficiency comparable to cryopreserved AM (Dhall et al., 2018). Moreover, dried grafts can be stored without special deep-freeze facility.

Conclusions

Hypothesis 1:

- Although we detected some markers of stem cells and proliferation in the periphery of the cornea, we could not definitively confirm the presence of stem/progenitor cells in the area. We found that corneoscleral rims with preserved peripheral endothelium are a sufficient source of cells to establish endothelial cell culture. The resulting cultures are equivalent to cultures derived from corneas with a complete (or intact) endothelium.
- We have established a protocol for *in vitro* propagation of corneal endothelial cells from discarded corneas or rims with preserved peripheral endothelium. Corneal tissue that would otherwise be discarded can thus be further used to increase pool of endothelial cell for research purposes or for grafting, as it still contains viable cells capable of proliferation. This protocol will be further optimized to maximize cell yields and to maintain their canonical phenotype during *in vitro* culture (hexagonal shape, endothelial marker expression).
- We have clarified the localization of some endothelial markers in the cornea. None of these markers were found to be specific for endothelial cells. The expression of the most promising markers, such as CD166, in *in vivo* and *in vitro* corneal endothelium will be verified in future research.

Hypothesis 2:

- No significant difference in endothelial parameters was found between small (corneal) and large (corneoscleral) porcine cultured discs, although the mean LECD values of large damaged and cultured discs were numerically higher than for small discs. Albeit the biochemical properties of the human and pig corneas are similar, the structure and strength of their cornea are different. The lower stiffness of the porcine cornea could adversely affect the results. It would be appropriate to repeat the presented type of experiments on a model of the human cornea, if a sufficient amount of tissue could be obtained in the future.
- We found that not only migration and flattening of endothelial cells but also their proliferation, which are supported by prolonged organ culture with higher serum levels, contribute to wound healing.
- We introduced the pig model as an *ex vivo* model suitable for research on endothelial repair/regeneration capacity.

Hypothesis 3:

- We developed our own solution for decontamination of amnion, which was introduced into the preclinical practice of a tissue bank. This solution has toxicity and antimicrobial efficacy similar to commercially available BASE128.

Hypothesis 4:

- We have developed and implemented a protocol in which an intact de-epithelialized amnion can be obtained in one step with viable amnion epithelial cell, and they can be further used in basic and preclinical research.

Hypothesis 5:

- We developed a protocol for the preparation of cryopreserved and air-dried amniotic grafts, which have been very successfully used to treat chronic long-term non-healing wounds of patients included in a multicenter preclinical study.

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A list of publications related to the Thesis

1. **Smeringaiova I**, Reinstein Merjava S, Stranak, Z, Studeny P, Bednar J, Jirsova K (2018). Endothelial wound repair of the organ-cultured porcine corneas. Curr Eye Research, 43(7), 856-865.
2. **Smeringaiova I**, Trosan P, Mrstinova MB, Matecha J, Burkert J, Bednar J, Jirsova, K (2017). Comparison of impact of two decontamination solutions on the viability of the cells in human amnion. Cell Tissue Bank, 18(3), 413-423.
3. **Smeringaiova I**, Nyc O, Trosan P, Spatenka J, Burkert J, Bednar J, Jirsova K (2018). Antimicrobial efficiency and stability of two decontamination solutions. Cell Tissue Bank, 19(4), 581-589.
4. Trosan P, **Smeringaiova I**, Brejchova K, Bednar J, Benada O, Kofronova O, Jirsova K (2018). The enzymatic de-epithelialization technique determines denuded amniotic membrane integrity and viability of harvested epithelial cells. PloS one, 13(3), e0194820.

Publication not directly related to this Thesis

Azqueta A, Rundén-Pran E, Elje E, Nicolaissen B, Berg KH, **Smeringaiova I**, Jirsova K, Collins AR (2018). The comet assay applied to cells of the eye. Mutagenesis, 33(1), 21-24.