MEDICAL UNIVERSITY
„PROF. DR. PARASKEV STOYANOV" – VARNA

FAKULTY OF „MEDICINE"
DEPARTMENT OF OPHTHALMOLOGY AND VISUAL SCIENCES

Catherina Meglena Bommert, M.D.

Therapeutic approach of the anterior ocular surface – Evaluation with in vivo confocal microscopy

Supervisor:

Prof. Christina Nikolova Grupcheva, MD, PhD, DSc, FEBO, FICO(Hon), FBCLA, FIACLE

AUTOREFERAT
of the dissertation for obtaining an educational and scientific degree „PhD",
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МЕДИЦИНСКИ УНИВЕРСИТЕТ
„ПРОФ. Д-Р ПАРАСКЕВ СТОЯНОВ” – ВАРНА

ФАКУЛТЕТ „МЕДИЦИНА”
КАТЕДРА „ОЧНИ БОЛЕСТИ И ЗРИТЕЛНИ НАУКИ”

Д-р Катерина Меглена Боммерт

Терапевтичен подход към предна очна повърхност-проследяване с ин виво конфокална микроскопия

АФТОРЕФЕРАТ

на дисертационен труд за придобиване на образователна и научна степен „Доктор”,
Научна специалност „Офталмология”,

Научен ръководител:

Проф. д-р Христина Групчева, д.м.н., FEBO, FICO(Hon), FBCLA, FIACLE

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Доц. д-р Весела Иванчева Любенова, д.м. FEBO

Варна 2021

The dissertation contains 159 pages and is illustrated with 15 tables and 24 figures. The bibliography includes 310 literature sources. The study was conducted at the Specialized hospital for eye diseases with active treatment - Varna.

The dissertation was discussed and proposed for defense by the Department Council of the Department of Ophthalmology and Visual Sciences at the Medical University „Prof. Dr. Paraskev Stoyanov" - Varna.

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The defense of the dissertation will take place on 05.03.2021. The materials on the defense are available in the library of the Medical University „Prof. Dr. Paraskev Stoyanov" - Varna.

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LIST OF ABBREVATIONS

AAS	Antibiotic Antimycotic Solution
AM	Amniotic Membrane
ATP	Adenosine Triphosphate
CNS	Central Nervous System
CO2	Carbon Dioxide
Cx43	Connexin 43 Gap Junction Protein
DAM	Denuded Amniotic Membrane
DMEM	Dulbecco's Modified Eagle's Medium
HAM	Human Amniotic Membrane
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HE	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HTLV	Human T-cell Leukemia-lymphoma Virus
IL	Interleukin
IVCM	In Vivo Confocal Microscopy
LSC	Limbal Stem Cell
NaCl	Sodium Chloride
OCT	Optical Coherence Tomograph
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
SD	Standard Deviation
SEM	Standard Error of the Mean
SOP	Standard Operating Procedure
TAC	Transient Amplifying Cell
TGF	Transforming Growth Factor
THBS1	Thrombospondin 1
TIMP	Tissue Inhibitor of Metalloproteinase
TPHA	Treponema Pallidum Hemagglutination

1 INTRODUCTION

The human amnion or human amniotic membrane is the innermost portion of the human fetal membranes of the placenta (Bourne, 1962), a multilayered membrane, partitioned into the main histological layers that include the inner epithelial layer, the intermediate basement membrane and the outer avascular mesenchyme (Toda, Okabe, Yoshida, & Nikaido, 2007) (George, Dalvi, Balram, KJ, & Anil, 2018). The healthy human amnion is a thin translucent tissue of fetal origin with a varying thickness of 0.02 mm to 0.5 mm (Bourne, 1962) (Malhotra & Jain, 2014) (M. Kesting, Wolff, Nobis, & Rohleder, 2014) (Gupta, Kedige, & Jain, 2015) which on reflection has a bluish sheen (Ana Catarina Mamede & Botelhoeditors, 2015) and envelops and secures the embryo during embryonic development in the uterus. The amniotic membrane and its structure with its outstanding beneficial properties (Zeng, Li, Zeng, Yang, & Zhu, 2014) and therapeutic potential have a long tradition in medicine, especially in the field of ophthalmology. The initial inducement of fresh human fetal membranes in literature was over 110 years ago, when Dr. JW Davis reported in the beginning of 1900s the therapeutic application of fetal membranes for human skin transplantation (Davis, 1910). In the field of ophthalmology the first scientific mentioned amniotic membrane application was in 1940 by De Rotth (de ROTTH, 1940) for the course of ocular burn treatment in form of a bandage. Ever since its first application in ocular treatment by de Rotth (de ROTTH, 1940), Sorsby (Sorsby & Symons, 1946) (Sorsby et al., 1947) and over the course of time as a graft by Kim and Tseng (J. C. Kim & Tseng, 1995b), with the implementation of the cryopreservation the human amniotic membrane has attracted the focus of attention for its beneficial properties (Zeng et al., 2014) and therapeutic potential and has been implemented in various medical disciplines and broadly in the treatment management of ocular surface disorders (Malhotra & Jain, 2014). With the introduction of the preparation method by Tseng and coworkers (J. C. Kim & Tseng, 1995b) (Lee & Tseng, 1997) the preserved amniotic membrane contributed to the advancement of reconstructive medicine. Transplantation of the human amniotic membrane, whether used as a patch or graft, has been demonstrated in numerous studies to be effective in ocular surface reconstruction of corneal ulceration (Jinghua Liu, Li, & Li, 2019) (Hanada, Shimazaki, Shimmura, & Tsubota, 2001), persistent epithelial defects and perforations (Azuara-Blanco et al., 1999) (C. Y. Su & Lin, 2000) (S. H. Lee & Tseng, 1997) (Letko et al., 2001) , chemical and thermal burns (J. S. Kim, Kim, Na, Jeong, & Song, 2000) (M. S. Sridhar, Bansal, Sangwan, & Rao, 2000), bullous keratopathy (Espana, Grueterich, et al., 2003) (David F Anderson, Prabhasawat, Alfonso, & Tseng, 2001) (Pires et al., 1999), pterygium (Prabhasawat et al., 1997)

(Jain, Bansal, & Sukhija, 2008) and partial limbal stem cell deficiency (Sangwan et al., 2004) (D. F Anderson et al., 2001) (Scheffer C.G. Tseng, Prabhasawat, Barton, Gray, & Meiler, 1998). The amniotic membrane with its two kinds of cells, the human amniotic epithelial cells and human amniotic mesenchymal cells (Koike et al., 2014) (Wassmer & Berishvili, 2020) and specific structure, offers beneficial properties that make the membrane particularly suitable for applications in ocular surface reconstruction. Its success with favorable clinical outcomes in corneal surface management are attributable to the membrane's biomaterial properties of anti-inflammatory effect (Shimmura, Shimazaki, Ohashi, & Tsubota, 2001) (Harminder S Dua, Gomes, King, & Maharajan, 2004) (Solomon et al., 2001) (Hao, Ma, Hwang, Kim, & Zhang, 2000), low immunogenicity without signs of immune rejection when transplanted (Akle, Welsh, Adinolfi, Leibowitz, & Mccoll, 1981) (Malhotra & Jain, 2014) (Bailo et al., 2004), anti-microbial properties (Talmi, Sigler, Inge, Finkelstein, & Zohar, 1991), anticarcinogenic properties (Seo, Kim, & Kim, 2008) (J. C. Kim & Tseng, 1995a) (Hao et al., 2000) , anti-scaring effect (S. C. G. Tseng, Li, & Ma, 1999), enhanced epithelization (M. R. Kesting, Wolff, Hohlweg-Majert, & Steinstraesser, 2008) and also its ethically unobjectionability, as it is usually discarded after delivery (Koike et al., 2014), that contribute to its widespread use. On the basis of these attributes, indications for the amniotic membrane use in ophthalmology is expanding and the membrane has emerged as an ideal source for ocular surface reconstruction of epithelial defects unresponsive to conventional management strategies including corneal degenerative disorders with associated limbal stem cell deficiency (Malhotra & Jain, 2014). Though reconstructive surgery, especially in the field of ophthalmology, has experienced enormous progress in the past decades by new innovative treatment perspectives, the ocular surface reconstruction and integrity restoration of severe ocular surface disorders, is nevertheless a considerable challenge (Kruse, Rohrschneider, & Völcker, 1998) (Haagdorens et al., 2016).With the introduction of the cryopreserved amniotic membrane in ophthalmic reconstructive surgery (J. C. Kim & Tseng, 1995b) and quite recently in tissue engineering, the human amniotic membrane has acquired an essential component in the treatment of various diseases of the ocular surface (Daniel Meller, Pauklin, Thomasen, Westekemper, & Steuhl, 2011). Approaches to treat severe limbal stem cell deficiency have been gaining awareness since the pivotal role of limbal stem cells in maintaining ocular surface integrity and transparency (Vemuganti, Kashyap, Sangwan, & Singh, 2004) was discovered. The human amniotic membrane as the culture substrate has shown in experimental and clinical trials its abundant characteristics for the ex-vivo expansion of limbal epithelial cells in limbal stem cell deficiency treatment (Noriko Koizumi, Inatomi, Quantock, et al., 2000) (R. J.-F. Tsai, Li, &

Chen, 2000). Thoroughly processed by cryopreservation, the biological properties are retained and making the human amniotic membrane the optimal candidate for application in ocular surface reconstruction by promoting epithelialization (M. R. Kesting et al., 2008), reducing the risks of postoperative infection by its antimicrobial properties (Talmi et al., 1991), its low immunogenicity without expressing cell surface proteins HLA-A, B, C, DR antigens or β2-microglobulin, responsible for the regulation of the immune reaction (Akle et al., 1981) , and suppressing stromal inflammation, angiogenesis and scarring (Jingbo Liu, Sheha, Fu, Liang, & Tseng, 2010). This opened the possibility to safely utilize the pretreated amniotic membrane in ophthalmic transplantations and made it to date the leading biological scaffold for ex-vivo cultivation of limbal epithelial stem cells (Shortt, Secker, Notara, et al., 2007) (I R Schwab, 1999) (Noriko Koizumi, Inatomi, Suzuki, Sotozono, & Kinoshita, 2001) (Grueterich, Espana, Touhami, Ti, & Tseng, 2002) (Nakamura, Inatomi, Sotozono, Koizumi, & Kinoshita, 2004) (Shortt et al., 2008) (Zakaria et al., 2014). Since the emergence of such new promising techniques in means of tissue engineering, the amniotic membrane has proven in several studies to be the most promising carrier for human corneal endothelial cells so far (Ishino et al., 2004) (Hassan Niknejad et al., 2008) (Liying Zhang et al., 2016). However, with the progression of the amniotic membrane serving as a carrier for cell cultivation, its handling as a biomaterial in everyday clinical practice gives rise to new requirements and fundamental challenges commencing from basic management with the demand for an appropriate mounting device, suitable for simplified and safe handling in cell seeding and operative procedure. Choosing an appropriate mounting device to ensure quality for optimal cell growth and further use is a significant and still pending problem. To date, there are still no available standardized protocols to ensure optimal graft preparation, handling and mounting for transfer, what makes comparison of current studies more intricate (Daniel Meller et al., 2011). We have set ourselves the task to integrate the readily available, inexpensive, and naturally biocompatible amniotic membrane for ex vivo cultivation of limbal stem cells in ophthalmic tissue engineering and regenerative medicine in Bulgaria. We successfully prepared the human amniotic membrane for the use as a scaffold for ex vivo limbal stem cell cultivation by cryopreservation and de-epithelization by a safe and efficient method to contribute to research in ophthalmology. Alloheal® is the first bioproduct in Bulgaria that is manufactured by cultivated limbal epithelial stem cells derived from allogenic, corneoscleral donor tissue on cryopreserved thermolysin treated human amniotic membranes for the management of anterior ocular surface disorders with a self-customized flexible carrier device. The wrinkle-free bioproduct prototype carrier shaped out of a flexible ring composed of convenient everyday clinical equipment, irrespective

of commercially available products was designed to be compatible for our demands for optimal viable cultivation, transportation, and surgical application with achieved decreased surgery time by reducing trauma to the seeded cells and the ocular surface. Further utilization was evaluated before and after biological therapy with regard to the microstructural level by utilizing in vivo confocal microscopy.

We are convinced that the amniotic membrane serves as a perfect scaffold material for ex vivo expansion of limbal epithelial cells with a promising future in tissue engineering and that we use the amniotic membrane far from its available potential. In the near future, we desire to recast and further develop our knowledge with advanced preparation techniques and scaffold strategies to optimize the utilization of human amniotic membrane and its beneficial properties in the field of ophthalmology.

2 AIM

The aim of the current studies is to develop and utilize the contemporary biological therapies and apply them to the anterior ocular surface in order to achieve microstructural integration proven by in vivo confocal microscopy

1. To study the literature and summarize the current achievements in new biological therapies applied on the ocular surface for structural restauration.

2. To master the in vivo confocal microscopy technique and apply it in difficult patients with ocular surface disorders.

3. To develop a prototype of a medical device/biological therapy, easy for application in standard surgical procedure

4. To follow up patients with significant ocular surface disintegration before and after application of biological therapy at microstructural level, utilizing in vivo confocal microscopy.

5. To perform a comparative analysis between classic amniotic membrane transplantation and biological therapy based on amniotic tissue.

3 MATERIAL AND METHADOLOGY

3.1 TISSUE AQUISITATION AND ETHICS DECLARATION

The present study was conducted in the Department of Ophthalmology and Visual Sciences of the Medical University „Prof. Dr. Paraskev Stoyanov", Varna and the Specialized University Hospital for Ophthalmology Diseases for Active Treatment, Varna („Специализираната болница за очни болести с активно лечение – Варна" ЕООД).
The study was approved by the local Ethics Committee. The usage of the amniotic membrane for transplantation in ophthalmology was according to the procedures of the University Hospital for Active Treatment „St. Marina", Varna.

The human term placentas were provided by the Specialized Hospital of Obstetrics and Gynecology for Active Treatment „Prof. Dr. D. Stamatov", Varna (СБАГАЛ „Проф. д-р Д. Стаматов" ЕООД – Варна), from consenting donors undergoing elective caesarean sections. The tissues of the amniotic membrane were subsequently processed and stored at the Center for Translational Medicine and Cell Therapy, University Hospital for Active Treatment „St. Marina", Varna.
Laboratory screening was provided by the Laboratory of Clinical Immunology at the University Hospital for Active Treatment „St. Marina", Varna. Patients not complying with the inclusion criteria for tissue donation were excluded.

3.2 MATERIAL

3.2.1 AMNIOTIC MEMBRANES

The human placentas / amniotic membranes were retrieved from the Specialized Hospital of Obstetrics and Gynecology for Active Treatment „Prof. Dr. D. Stamatov", Varna (СБАГАЛ „Проф. д-р Д. Стаматов" ЕООД – Варна). The donor mothers were informed and asked for signed consent for amniotic membranes donation. Criteria for obtaining the amniotic membrane was performed in accordance with EU standards and guidelines following the SOP and referred to serology screening for hepatitis B and hepatitis C, syphilis, HIV, and additional serology subsequent to selection from healthy maternal donors with exclusively elective caesarean section with appropriate social and medical history.

3.2.2 CORNEOSCLERAL RINGS

Human corneoscleral rings were sourced from remnants after corneal transplantation from donated corneas. The stem cells were obtained with the consent of the donors or their authorized representatives. The corneas / corneoscleral rings were mainly obtained from the International Corneal Bank.

Preparation was performed in the Laboratory for Translational Medicine at the University Hospital for Active Treatment St. Marina Hospital, Varna.

3.2.3 PREPARATION EQUIPMENT AND INSTRUMENTS

Description	Manufacturer
Anatomical forceps	Geuder
Cell Culture Plates, 6-well plates	Biologix
CellCrown ™ inserts for 6-well plates	Sigma-Aldrich
Centrifuge	Eppendorf
Centrifuge tubes 50 ml	Deltalab
CIRRUS 5000 HD-OCT	ZEISS
Disposable gloves	Hartmann
Electric pipettor	Dans Pharma
Gauze Swabs, sterile	Lohmann & Rauscher
Heidelberg Retina Tomograph - HRT3 RCM	Heidelberg Engineering GmbH
Incubator for cell cultures	Panasonic
Laminar Flow Cabinet	Biobase
Light microscopy	Zeiss
Manipler® AZ skin stapler	B. Braun Melsungen
Micro needle holder	Geuder
Microscope Slit Lamp HS-7000	Huvitz
Petri dishes, glass	Deltalab; Falcon; Dans Pharma
Pipettor, 1ml	Eppendorf
Scalp vein set, Butterfly with needle	B. Braun Melsungen
Serological pipette, sterile 10 ml	Deltalab
Serological pipette, sterile 25 ml	Deltalab

Serological pipette, sterile 5 ml	Deltalab
Statistical program SPSS	IBM
Sterile latex surgical gloves	Top Glove Europe
Sterile nitrocellulose paper	Merck Millipore; Sartorius
Surgical forceps	Geuder
Surgical scissors, Peha®	Hartmann
Surgical sutures, 8/0 Polyglycolic Acid	FSSB Chirurgische Nadeln
Surgical tweezers	Geuder
Tenotomy Scissors	Westcott
TomoCap	Heidelberg Engineering
Tube from central venous catheter	B. Braun Melsungen
3D OCT 2000 Topcon	Topcon

3.2.4 SOLUTIONS AND CHEMICALS

Description	**Manufacturer**
6% hydrogen peroxide solution	Pharmacy
70% alcohol solution	Pharmacy
Antibiotic / antifungal solution 100X	Gibco, life technologies; Sigma-Aldrich; Capricorn
DMEM / HAM F12 medium with stable glutamine	Merck
Dulbecco's Modified Eagle's medium (DMEM)	Invitrogen / Life Technologies; Sigma-Aldrich
Glycerol	Sigma-Aldrich
Hanks 'balanced salt solution (HBSS)	Life Technologies
Human serum (HS)	Sigma-Aldrich
Isotonic Saline Solution 0.9% (NaCl)	B. Braun Melsungen; Baxter
Phosphate-buffered saline (PBS)	Sigma-Aldrich; Life Technologies
Thermolysin (Thermolysin from Geobacillus stearothermophilus # T7902)	Sigma-Aldrich

3.2.5 SURGICAL EQUIPMENT AND MEDICATIONS

Description	Manufacturer
ALCAINE® Solution (proparacaine hydrochloride 0.5%)	Alcon
Braunol®	B. Braun Melsungen
Corneal Scarifier/ Corneal Dissector	Geuder
Corneregel®	Bausch & Lomb
Curved Micro Scissors	Geuder
Eyelid Retractor	Geuder
Isotonic Saline Solution 0.9% (NaCl)	B. Braun Melsungen
Lidocaine (Lidocaine Hydrochloride)	Sopharma
PUREVISION®2, (Bandage Contact Lens)	Bausch & Lomb
Sutures 8/0 Polyglycolic Acid	FSSB
Tobrex® Eye Drops (Tobramycin 0.3%)	Alcon
Weck-Cel® Cellulose Eye Spears	BVI

3.3 COLLECTION AND PROCESSING OF THE HUMAN AMNIOTIC MEMBRANE

3.3.1 SELECTION OF THE DONOR

The donation of the placenta / amniotic membrane was based on a voluntary basis for all donor mothers. Women who decided to donate their placenta postpartum, have been fully informed beforehand of the intended purpose and application of the therapeutic amniotic membrane transplantation in ophthalmology. The evaluation and selection of a potential amniotic membrane donor must be comprehensive and thorough to exclude and minimize the risk of disease transmission in human tissue transplants of recipient patients. With signed consent of the donor, donor mothers were listed in the Executive Agency for Transplantation (EAT).
Besides the medical history and the data of the general examination, the blood of the suitable donor mothers was serologically and / or molecularly tested for the absence of infectious diseases, including HIV, Hepatitis B and C as well as Syphilis (Treponema pallidum). Exclusively amniotic membranes of the mother donors, which have negative results for all tested diseases are further used for allograft preparation. The placentas were obtained sterile after scheduled successful cesarean section. Donor screening was provided in accredited laboratories. The inclusion criteria were based on an elective caesarean section by a healthy donor mother with confirmed negative test results for all required diseases.

3.3.2 DONOR EXCLUSION CRITERIA

Donors with the following findings in medical history or laboratory results were excluded as potential placenta donors.
Prospective donors with preexisting systemic bacterial, viral, fungal, parasitic, or idiopathic infections or history of past diseases with unclear etiology were considered unacceptable and are routinely excluded at this point. Donors with recent history of vaccination for yellow fever, poliomyelitis, measles, mumps, rubella in the last 4 weeks and rabies vaccine in the last 12 months were excluded. Women with local diseases of the female genital organs, premature rupture of the membrane, amniotic infection syndrome, fetal malformation, or visible pathological alterations, as seen in Figure 2, have not been allowed to donate. With diagnosed

diseases of the CNS such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and inflammatory diseases of the central nervous system or risk of Creutzfeldt - Jakob disease the women were excluded. Prospective donors with consumption of hazardous substances, for instance, mercury, cyanide, or lead were declined. Women with positive serologies of the infectious markers of human immunodeficiency viruses (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell leukemia-lymphoma virus (HTLV), Treponema pallidum hemagglutination assay (TPHA) are contraindicated as potential donors.

To safely expand the pool of women donors, women with any disease with harmful potential for the recipient, were withdrawn before completion of the evaluation process.

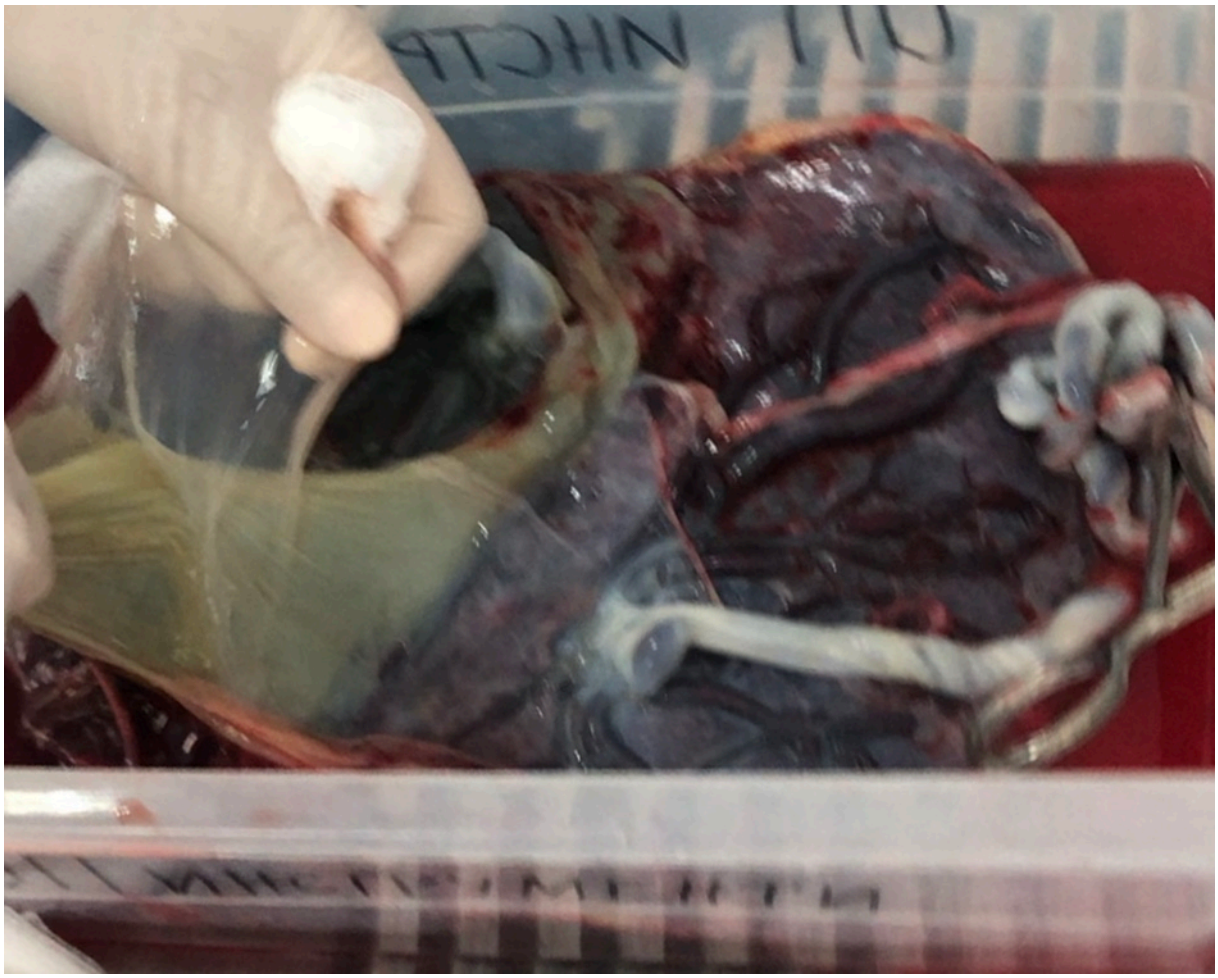


Figure 1: Discarded donor material

Placenta with pathological changes of greenish discoloration and multiple tears of the amniotic membrane from delivery that was discarded

3.3.3 CONTROL AND MONITORING OF THE INFECTIOUS STATUS

For utilization as a surgical graft for ophthalmic indications and ocular reconstruction, including persistent epithelial defects and non-healing corneal ulcers as well as corneal disorders according to limbal stem cell deficiency, conjunctival reconstruction, corneoscleral perforations and more (Y. Shao et al., 2020), the minimization of the risk of infection by monitoring the infectious status with PCR and serological tests has the highest priority.
To ensure minimal potential risk for transplantation recipients, the donor microbiological status and virologic status of the donor was tested for:

- HIV I and II antibodies
- Hepatitis B surface antigen (HBsAg)
- Antibodies to hepatitis B core antigen (anti-HBc)
- Anti - HCV antibodies
- Rapid plasma test for syphilis (Treponema pallidum)

According to the norm serological tests were repeated after 3-6 months in seronegative individuals in order not to miss possible infectious diseases during the time interval of the “window period”. The medical test results of the donor women are kept for 10 years after transplantation. The amniotic membrane is stored and not authorized for use until seronegative status is recognized according to the SOP.

3.3.4 HARVESTING THE HUMAN AMNIOTIC MEMBRANE

The placentas for donation were exclusively harvested from healthy women with signed consent, negative serological screening for hepatitis B, hepatitis C, HIV, and syphilis. Positive test results were a criterion for exclusion. The placentas were harvested by a specialist in the Specialized Hospital of Obstetrics and Gynecology for Active Treatment „Prof. Dr. D. Stamatov”, Varna (СБАГАЛ „Проф. д-р Д. Стаматов” ЕООД – Варна) under strict aseptic conditions with a planned caesarean section of a term pregnancy. After the umbilical stump was clamped the placenta was placed in a sterile organ box and washed under sterile conditions with sterile 0.9% sodium chloride solution until blood clots were removed. The harvested fetal membranes were macroscopically evaluated to exclude possible visible pathologic alterations.

On site, the amnion was separated from the chorion by finger blunt dissection and surgical tweezers when needed and placed in a 125 ml sterile transport container with 50 ml transport medium with Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture *(Merck)* with 1% broad-spectrum Antibiotic / antifungal solution *(Capricorn)*.

All procedures took place under strict sterile conditions and the amniotic membrane was ensured to remain moist without risk of desiccation. The membrane was transported directly to the laboratory for further preparation. At the laboratory the amniotic membrane was placed for 12-24 hours at + 4 / + 8 ° C and a transport medium sample for microbiological examination extracted.

3.4 CRYOPRESERVATION OF THE HUMAN AMNIOTIC MEMBRANE

3.4.1 PREPARATION OF THE CRYOPRESERVATION MEDIUM

The cryopreservation medium was prepared from 250 ml Dulbecco's Modified Eagle's medium *(Sigma-Aldrich)* mixed with 1 % Antibiotic-Antimycotic solution containing penicillin, streptomycin, and Amphotericin B *(Gibco or Sigma-Aldrich)*. Disposable 50 ml centrifuge tubes were pipetted with 20 ml of the prepared Dulbecco's Modified Eagle's medium with 1 % Antibiotic-Antimycotic solution and 20 ml of sterile Glycerol *(Sigma-Aldrich)* in a ratio of 1:1 to a total of 40 ml.

3.4.2 CROYPRESERVATION

The cryopreservation of the amniotic membranes was performed in a laminar flow hood in order to maintain the hygienic and aseptic conditions to prevent contamination with preliminary sterilization of the laboratory room with UV. The amniotic membrane was washed with sterile saline solution *(B. Braun Melsungen)* for three times to achieve the greatest possible germ-free condition.

The prepared amniotic membrane was carefully placed in the prepared centrifuge tubes with cryopreservation medium with 1:1 ratio of sterile Glycerol *(Sigma-Aldrich)* (sterilized by autoclave) and DMEM as mentioned in the preparation of the cryopreservation medium and stored at -78 / -85 ° C in a freezer. The cryopreserved amniotic membranes were stored in the

freezer until microbiological and virologic results were released. For the cryopreserved amniotic membrane, the storage period is from twelve months to two years. For further processing the membranes were defrosted at room temperature or in a water bath at 28°C.

3.5 DECELLULARIZATION OF THE HUMAN AMNIOTIC MEMBRANE

The cryopreserved human amniotic membrane is decellularized to remove amniotic epithelial cells without performing mechanical scraping procedure, to use the denuded amniotic membrane as a carrier for the ex-vivo expansion of limbal epithelial stem cells. All procedures were performed in an aseptic environment under sterile conditions, following the guidelines of the SOP. The sterile laminar flow box was surplus cleaned with solutions of 6 % hydrogen peroxide and 70 % ethanol to ensure sterility.

The cryopreserved human amniotic membrane was defrosted at room temperature. Aliquots of 25 ml sterile saline with 0.9% NaCl, *(B. Braun Melsungen)* and sterile phosphate buffer *(PBS, Sigma-Aldrich)* in 50 ml sterile centrifuge tubes *(Deltalab)* were prepared. When defrosted, the cryopreserved amniotic membrane was washed repeatedly 3 times for 10 minutes with sterile saline, 0.9% NaCl *(B. Braun Melsungen)*, at room temperature with even circular shaking motion by the rotary shaker to rinse from cryopreservation medium, followed by incubation of the amniotic membrane with a 1% (1 ml) solution of the proteolytic enzyme thermolysin *(Sigma-Aldrich)* with (9 ml) Sterile phosphate buffer *(PBS, Sigma-Aldrich)* for eight minutes at room temperature with uniform shaking on the rotary shaker. Subsequently the membrane was washed repeatedly 3 times for 10 minutes with sterile phosphate buffer (*(PBS, Sigma-Aldrich)*.

The decellularized amniotic membrane was stretched out on a 10 cm sterile petri dish with the help of surgical forceps. The amniotic membrane was evenly spread to determine the direction of the amniotic membrane. It was important to be aware of the correct orientation of the membrane, which was the epithelial or upper side and which was the bottom side facing the chorion. An easy swab test was performed for confirmation. If the swab stuck to the membrane, it was known that it was the bottom sticky fibroblastic side of the amniotic membrane. With the epithelial / basement layer surface up, and the stroma / sticky fibroblastic side facing down, the membrane was placed on the nitrocellulose paper. The membrane was left to adhere to the

nitrocellulose paper, as shown in Figure 3, and then carefully cut in 45 mm x 45 mm square pieces (20, 25 cm²). The prepared amniotic membrane patches were placed using sterile forceps in the sterile tubes ensuring that the amniotic membrane was oriented towards the cryopreservation medium and stored in a freezer at -78 / -85 ° C. To exclude the risk of media contamination with bacteria or fungi, a media sample from a random tube was collected for a microbiological examination to consider the batch as free of cultivable microbial agents.

Figure 2: Amniotic membrane on the nitrocellulose paper

The human amniotic membrane is oriented with the epithelial / basement layer surfacing up and stromal side down in direct contact with the nitrocellulose paper.

3.6 HISTOLOGICAL ANALYSIS OF THE HUMAN AMNIOTIC MEMBRANE

The samples were prepared in the Laboratory for Translational Medicine at the University Hospital for Active Treatment St. Marina Hospital, Varna and analyzed under the light microscope.

The cryopreserved intact human amniotic membrane and the cryopreserved decellularized human amniotic membrane were stained by Diff-Quik and Hematoxylin and Eosin staining to compare and confirm decellularization of the denuded human amniotic membrane by histological observation.

For the histological analysis preparation, the samples of the intact human amniotic membrane and the decellularized human amniotic membrane have been defrosted at room temperature. The membranes were cut in sample sizes of 1x1 cm and placed with the fibroblast, sticky layer on the microscope slide for light microscopy. With the epithelial layer facing upwards the samples were left to dry shortly on the microscope slide and exposed to Hematoxylin and Eosin staining and Diff-Quik staining according to standard methods and analyzed under a light microscope.

3.7 CORNEOSCLERAL RINGS AND LIMBAL STEM CELL PREPARATION FOR EX-VIVO CULTIVATION

Corneoscleral rings were sourced from remnants after corneal transplantation at the University Hospital for Ophthalmology for Active Treatment, Varna. The cadaveric buttons were obtained from the operating room after keratoplasty for cultivating allogenic limbal epithelial stem cells obtained from residual corneoscleral donor tissue on cryopreserved denuded amniotic membrane patches serving as a biological scaffold for the manufacture of the bioproduct Alloheal®.

The cadaveric corneoscleral rings are a benefit in terms of resources and ethics for obtaining the important limbal tissue, which is preserved after corneal transplantation, where after trepanation only the central corneal part is transplanted and the ring with the limbal region generally discarded.

The residual corneoscleral rings used as a donor for limbal epithelial progenitor cells were processed in the Laboratory for Translational Medicine at the University Hospital for Active Treatment St. Marina Hospital, Varna.

For transportation of the residual corneal button the required transport medium was prepared with Dulbecco's Modified Eagle's medium *(Sigma-Aldrich)* mixed with 1 % Antibiotic-Antimycotic solution containing penicillin, streptomycin, and Amphotericin B *(Gibco)*. The transport medium was produced in sterile conditions not more than one day before scheduled operation and transfer to the laboratory and stored at a temperature of + 4 to + 8 ° C. The corneal residual button was placed in the transport medium centrally with distance to the container wall, tightly closed, labeled with the identifiable data in the manner indicated in the SOP and transported refrigerated between 4-8 °C to the laboratory within 1 hour. Under sterile conditions with previous sterilization of the laboratory room and laminar flow box with UV for professional disinfection and surplus preparation of the laminar flow box with 6% hydrogen peroxide and 70% ethyl alcohol the cadaveric buttons was placed in in a 50 ml vial *(Deltalab)* with 10 ml Dulbecco's Phosphate Buffered Saline *(Sigma Aldrich)* with 1 % Antibiotic-Antimycotic solution *(Capricorn)* and then prepared by removing redundant parts of the central cornea, conjunctiva and iris, until only parts of the limbal region were left. The rim was cut with a sterile scalpel in sizes of 1 mm to 2 mm. Up to 20 pieces are possible to generate depending on the donor characteristics. For isolation and growth in a culture dish, the corneal limbal tissue pieces were placed in 6-well plates with 2 ml culture media to generate and seed a cell suspension from the explant that would grow out and that than can be explanted on the prepared amniotic membrane. For this purpose, the culture media to cultivate limbal epithelial cells was prepared with the use of an automatic pipettor with a combination of 500 ml Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture with stable glutamine in a ratio of 1:1 *(Merck)* with 2% (10 ml) human serum *(Sigma-Aldrich)* and 1 % (5 ml) Antibiotic-Antimycotic solution *(Capricorn)*. In each well up to 5 pieces of the limbal tissue containing the limbal epithelial progenitor cells were placed in 2 ml of culture media. The 6-well plates were incubated in the thermostat with 37 ° C, 5% CO2 and 95% air.

The culture medium was renewed every second day by supplying an additional 1 ml culture medium with additionally daily microscopic observation. In the presence of cell migrating, inoculation of amniotic membrane scaffold was suitable.

3.8 MANUFACTORING THE BIOLOGICAL PRODUCT ALLOHEAL®

3.8.1 MOUNTING THE AMNIOTIC MEMBRANE FOR EX VIVO EXPANSION

The Alloheal® bioproduct was established by cultivating limbal epithelial stem cells derived from cadaveric, allogenic corneoscleral donor tissue, on a cryopreserved denuded amniotic membrane mounted on a suitable carrier device. The principal challenge was to develop a suitable uniform carrier for anchoring the bioproduct Alloheal® for tissue engineering and then to develop a surgically acceptable modality for transfer. Different methods have been investigated to secure the amniotic membrane as a carrier for the ex-vivo expansion of limbal epithelial stem cells with the focus on improving the amniotic membrane fixation method in regard to cell growth and surgical handling of the transplantation process.
For achieving the most suitable mounting method for our purposes, we compared the by anchoring the amniotic membrane with the commercially available mounting device CellCrown™ and a self-customized ring device. The mounting of the amniotic membrane on the ring or insert was prepared 24 hours before inoculation of the membrane with the limbal tissue pieces.

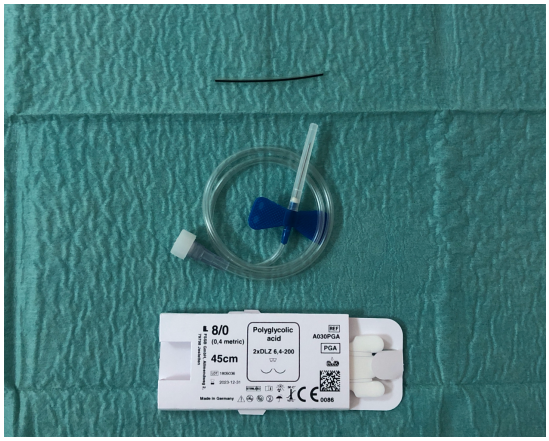
3.8.2 MOUNTING THE AMNIOTIC MEMBRANE WITH A COMMERCIALLY AVAILABLE MOUNTING DEVICE

After thawing, the amniotic membrane was washed from the cryoprotectant with sterile saline of 0.9% NaCl (*Baxter)* at room temperature. On a sterile petri dish, the amniotic membrane was placed for determining the orientation using surgical forceps and sterile gauze. With surgical forceps the denuded amniotic membrane was carefully removed from the nitrocellulose paper and fixed in a firm position between the components of the CellCrown™ inserts *(Sigma-Aldrich)* in a way that the migrating cells can later be placed on the decellularized epithelial surface. The carrier membrane is sealed by placing the ring component and the excess tissue is removed. The anchored amniotic membranes were placed in 6-well cell culture plates medium *(Biologix)* with 3 ml transport media and incubated 24 hours before ex-vivo cultivation.

3.8.3 PREPARATION OF THE SELF CUSTOMICED RING FOR MOUNTING THE AMNIOTIC MEMBRANE

The self-customized ring, for mounting the amniotic membrane for ex-vivo cultivation, was formed from products that are available in the clinical field in daily use, easily accessible and inexpensive. For the production of the ring, as demonstrated, only a butterfly needle infusion set, a tube piece, suture, micro needle holder and a simple sterile pair of scissors were needed. All components and devices used during manufacturing were sterile. Under the laminar flow hood the self-customized carrier was prepared, as visualized in the Figure 4 below.

The butterfly needle catheter was opened and the hypodermic needle was removed by cutting the tube at the neck of the flexible wings. Also, the connector was truncated, so only the transparent flexible tube was left. The tube with a lumen of 2 mm was cut to a length of 9 cm. The ends of the flexible tube were merged and stabilized by a small plastic tube of 4 cm length in the inner part of the butterfly tube. With a smaller diameter of the small plastic tube, which was taken from a central venous catheter, it was placed in the inner part the butterfly tube. The end parts of the tube were connected by needle stitching forming an applicable, flexible ring-shaped device with a surface area of 6, 44 cm² and a diameter of 2,86 cm.



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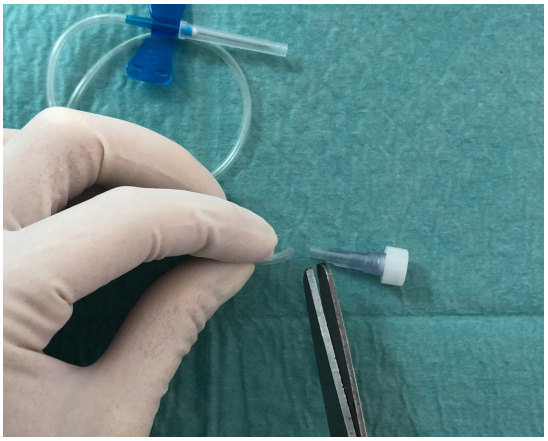

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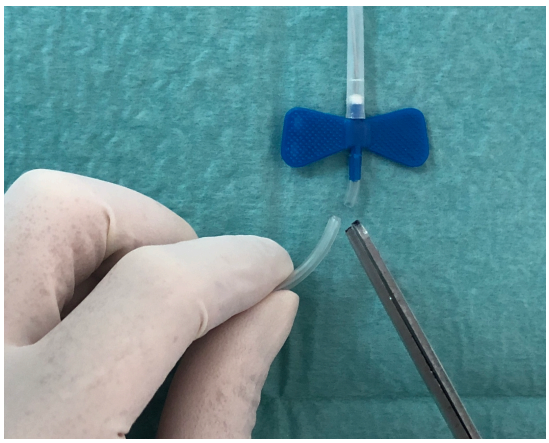

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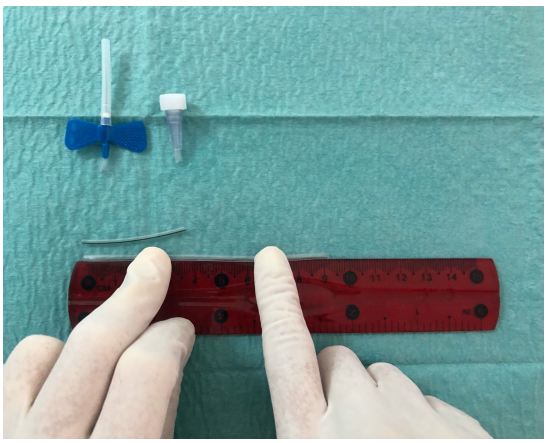

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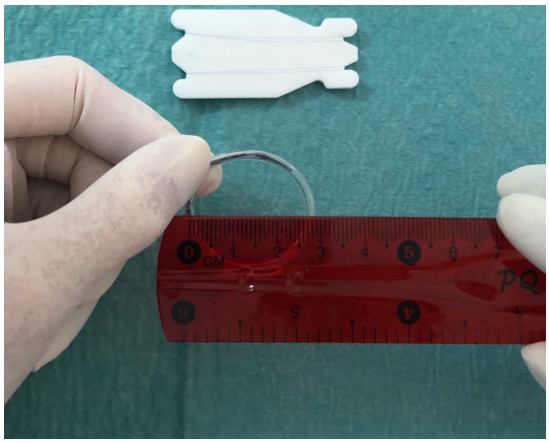

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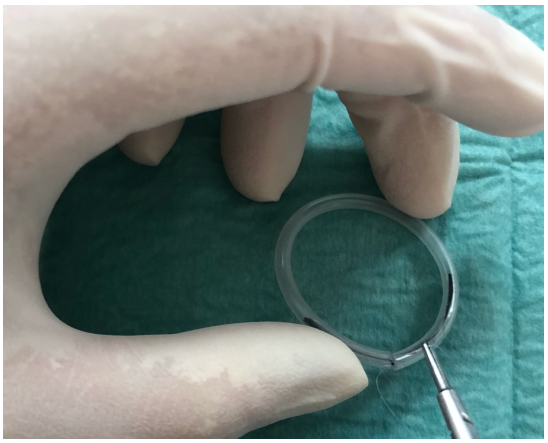

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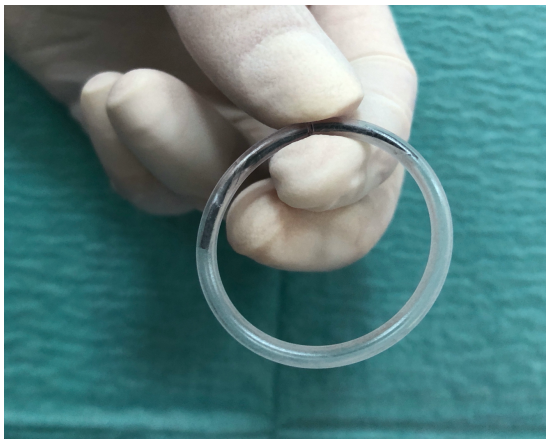

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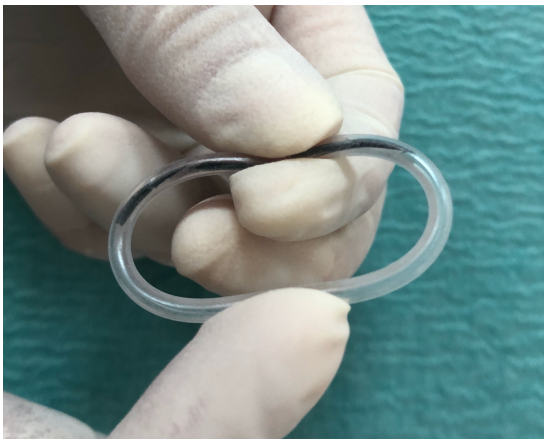

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Figure 3: Preparation of the self-customized ring device for mounting the amniotic membrane for ex-vivo cultivation

a) The components of the ring-device: butterfly needle infusion set, a tube piece, suture

b) The butterfly needle set was opened and the connector removed by cutting

c) Hypodermic needle and flexible wings were cut, so only the transparent flexible tube was left

d) The flexible butterfly tube was cut to a length of 9 cm

e) The small tinier plastic tube was cut to 4 cm length and placed in the inner part of the flexible tube for stabilization forming a carrier ring device with a surface area of 6, 44 cm²

f) Terminal ends of the butterfly tube were sutured to give the ring more resistance and to prevent to disintegrate

g) Customized ring device

h) Flexibility of the ring device

3.8.4 MOUNTING THE AMNIOTIC MEMBRANE WITH A SELF CUSTOMICED RING

In the laminar flow cabinet, the cryopreserved denuded amniotic membrane was defrosted at room temperature and washed from the cryopreservation media with sterile saline of 0.9% NaCl *(Baxter)*. After thawing the membrane was ready to be merged together with the flexible self-customized ring device, as shown in Figure 5.

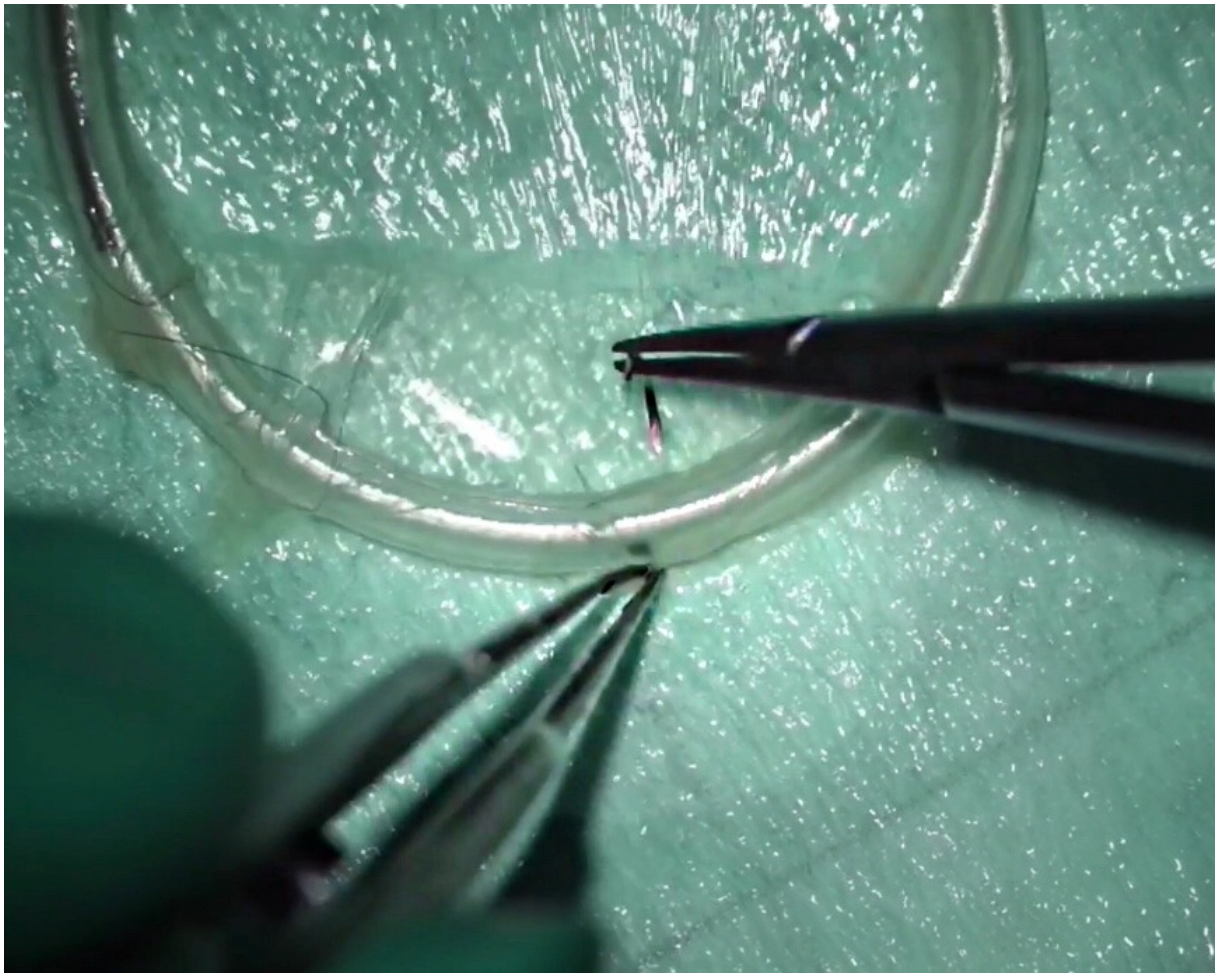


Figure 4: Stitching the amniotic membrane to the self-customized ring

The amniotic membrane was expanded over the carrier device and sutured with 12 uninterrupted stiches to the flexible ring device

The placement of the membrane for anchoring was performed in two different variations to determine the more advantageous method.

On the first experiment, the membrane was carefully removed from the nitrocellulose paper and carefully stretched out on a petri dish. With attention to the orientation of the membrane, it was merged with the flexible self-customized ring by lifting it on top of the device with the help of surgical forceps. With the epithelial side up the membrane was sutured continuous with 8/0 vicryl sutures on the ring device.

On the second experiment, the membrane was not fully removed from the nitrocellulose paper. Here, the membrane was slightly lifted in one corner from the nitrocellulose paper. The ring was carefully pushed underneath, in which the membrane detaches from the paper and wraps itself around the ring with the relevant, epithelial side up. The membrane was secured with sutures to the ring by continuous stitches.

For the fixation of the membrane to the holder two variants were experimented. The amniotic membrane was sutured to the ring device in 5 trials with 6 uninterrupted sutures and in 5 trials with 12 uninterrupted sutures.
In contrast to the CellCrown ™ mounting, it was possible to suture the amniotic membrane in a kind of way, that the membrane is not tightly expand over the carrier device, but with a slight downward curved arrangement.

A further attempt was made to secure the membrane with sterile single-use skin stapler *(Braun)* to the ring device. Due to lack of success, this approach was not pursued further.

The manufactured rings with the amniotic membrane were placed in 6-well cell culture plates *(Biologix)* filled with 3 ml transport medium per well (Figure 6) and incubated 24 hours before ex-vivo cultivation.

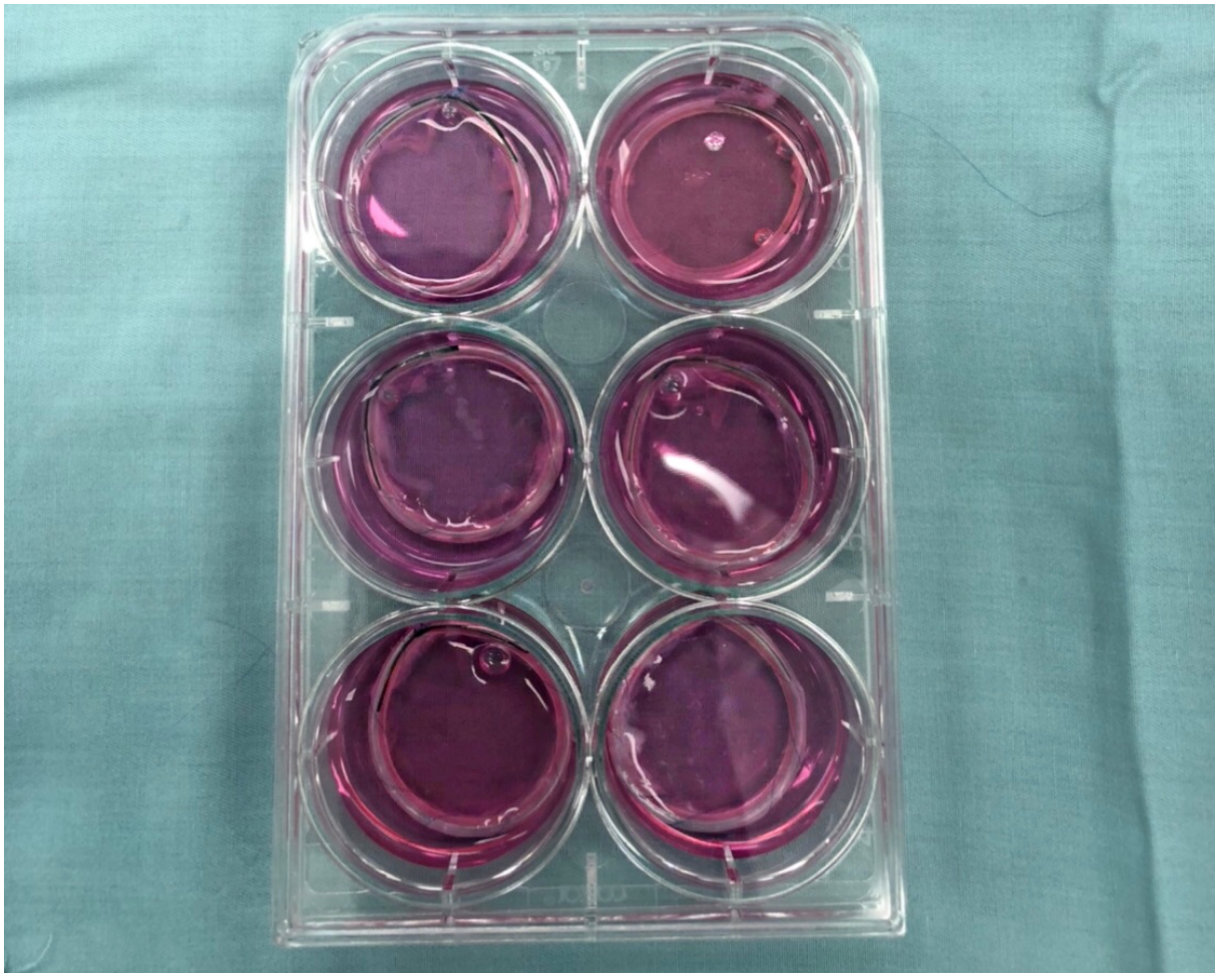


Figure 5: Amniotic membrane mounted on the flexible self-customized ring device

Rings placed in the cell culture plates in transport medium with the basement membrane facing up

3.8.5 EX-VIVO CULTIVATION OF LIMBAL EPITHELIAL CELLS ON THE AMNIOTIC MEMBRANE

For the ex-vivo cultivation the previously cryopreserved denuded amniotic membrane anchored with the CellCrown ™ *(Sigma-Aldrich) and* with the self-customized ring devices were utilized. The incubated corneal limbal tissue pieces that were placed in a culture medium-filled plate were approved for ex-vivo cultivation when cell migration was observed. The transport medium of the 6-well plates with the anchored amniotic membranes was completely discharged.

Analogous to the mounting of the amniotic membrane on different carrier devices, the ex-vivo cultivation behavior on two differently anchored amniotic membranes was visualized. The explant pieces were placed with the help of surgical tweezers on the decellularized epithelial surface of the denuded amniotic membranes. Culture media containing a combination of Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture with stable glutamine 1:1

(Merck) with 2% human serum *(Sigma-Aldrich)* and 1 % Antibiotic-Antimycotic solution *(Capricorn)* was added. In order to prevent the corneal limbal pieces from floating, the limbal tissue pieces were left for 10 minutes to adhere on the surface of de-epithelized human amniotic membrane and then only the amount of medium was used, that the corneal pieces were just completely covered. After incubation at 37 °C with 5% CO2 for 12-24 hours the establishment of cell explant adhesion to the epithelial side on the scaffold was observed on an inverted light microscope. The culture media was increased after attachment of the explant pieces. The bioproducts were re-incubated in cell culture incubator for 21 days until 80 % (¾) of the surface was observed with a monolayer of cells using an inverted microscope. The explants were carefully removed with surgical tweezers from the surface with preserving the integrity of membrane. The culture media was changed every 3 days. The bioproducts were approved when at least ¾ of the surface was covered with cells, no evidence for microbiological contamination existed and the integrity of the surface was intact.

3.9 OPERATIVE PROCEDURE

The bioengineered grafts of allogenic ex-vivo cultivated corneal limbal stem cells explanted on cryopreserved deepithelialized amniotic membrane and mounted on the self-customized ring forming the bioproduct Alloheal® or the CellCrown ™ *(Sigma-Aldrich)* were transported from the laboratory to the Specialized University Hospital for Ophthalmology for Active Treatment, Varna with the required sterility conditions and a stable temperature of 37 ° C. All surgeries were performed by Prof. Christina Nikolova Grupcheva, MD, PhD, DSc, FEBO, FICO(Hon), FBCLA, FIACLE. All grafts were transplanted to the patients under local anesthesia with proxymetacaine hydrochloride *(Alcaine 0.5%, Alcon)* and/or peribulbar anesthesia with lidocaine hydrochloride *(Lidocaine 0,2%, Sopharma)*.

The grafts were sutured with six to eight continuous 8-0 Vicryl sutures *(FSSB)*. The amniotic membranes functioning as a biomatrix were placed on the surface of the eye, together with the cultivated cells, with epithelial/basement membrane side facing up and the mesenchymal surface coating the ocular surface to promote membrane adhesion.

Preliminary steps included washing the membrane to remove glycerin, cleansing the operation site with Braunol *(B. Braun Melsungen)* as an antiseptic, removal of excessive tissue by superficial keratectomy removed and Bauman's membrane polished based on the individual medical condition of the patient, along with the preparation for transplantation were performed. In the presence of corneal ulcerations and stromal thinning, necrotic debris was cleared from the ulcer walls.

3.9.1 TRANSPLANTATION OF THE BIOPRODUCT MOUNTED ON A COMMERCIALLY AVAILABLE MOUNTING DEVICE

Preoperative procedures were performed according to the same principles. Superficial keratectomy was performed according to the extent of the preoperative status. The amniotic membrane mounted on the CellCrown cell culture inserts was carefully rinsed with sterile saline from the media and unlocked from the counting device. The membrane was placed with the help of forceps on the eye of the patient, controlled for correct side alignment, and trimmed to the appropriate size.

The membrane was sutured to the conjunctiva, overlapping the entire corneal surface. A therapeutic bandage contact lens *(PUREVISION2, Bausch & Lomb)* was placed for comfort. Topical antibiotic drops Tobramycin *(Tobrex 0.3%, Alcon),* was administered to the patient eye and covered with sterile dressings.

Postoperatively, the patients were treated with topical antibiotic drops Tobramycin in combination with Dexamethasone *(Tobradex, Alcon)*, Vigamox *(Moxifloxacin 0.5%, Alcon)* and Dexpanthenol gel *(Corneregel, Bausch & Lomb)*. After the amniotic membrane removal, the therapy was adapted in accordance with the current ophthalmic status of the patient. Preservative-free lubricants were kept as part of the regimen of the patient.

3.9.2 TRANSPLANTATION OF THE TISSUE ENGINEERED BIOPRODUCT ALLOHEAL®

Preoperatively, the patients were given a local anesthetic with proxymetacaine hydrochloride *(Alcaine 0.5%, Alcon)* drops into the eye for corneal anesthesia. Peribulbar anesthesia was performed with lidocaine hydrochloride *(Lidocaine 0,2%, Sopharma*). The eyelids were fixed with an eyelid retractor.

The Surgical steps utilizing the bioproduct for ocular surface reconstruction are show in the Figures 7 to Figure 11.

The first surgical step involved the superficial keratectomy in patients aligned to level and extent of the preoperative status as shown in Figure 7.

In the second surgical step the bioproduct Alloheal® was gently rinsed in sterile saline to remove residues of the medium and placed on the ocular surface of the eye with the epithelial/ basement membrane side facing up (Figure 8).

In the third surgical step the amniotic membrane mounted on the flexible ring device was atraumatically excised with the scalpel at the rim of the ring edge (Figure 9).

In the fourth surgical step the bioproduct is cut and released from the mounting device, adjusted to the required size and width (Figure 10). Without the need of renewed orientation or renewed cutting the required graft size is determined on site, matching correctly aligned the eye width of the receiver.

In the fifth surgical step the bioproduct Alloheal® is sutured to the perilimbal episclera and to the edge of the conjunctiva, overlapping the entire corneal surface as demonstrated in Figure 11.

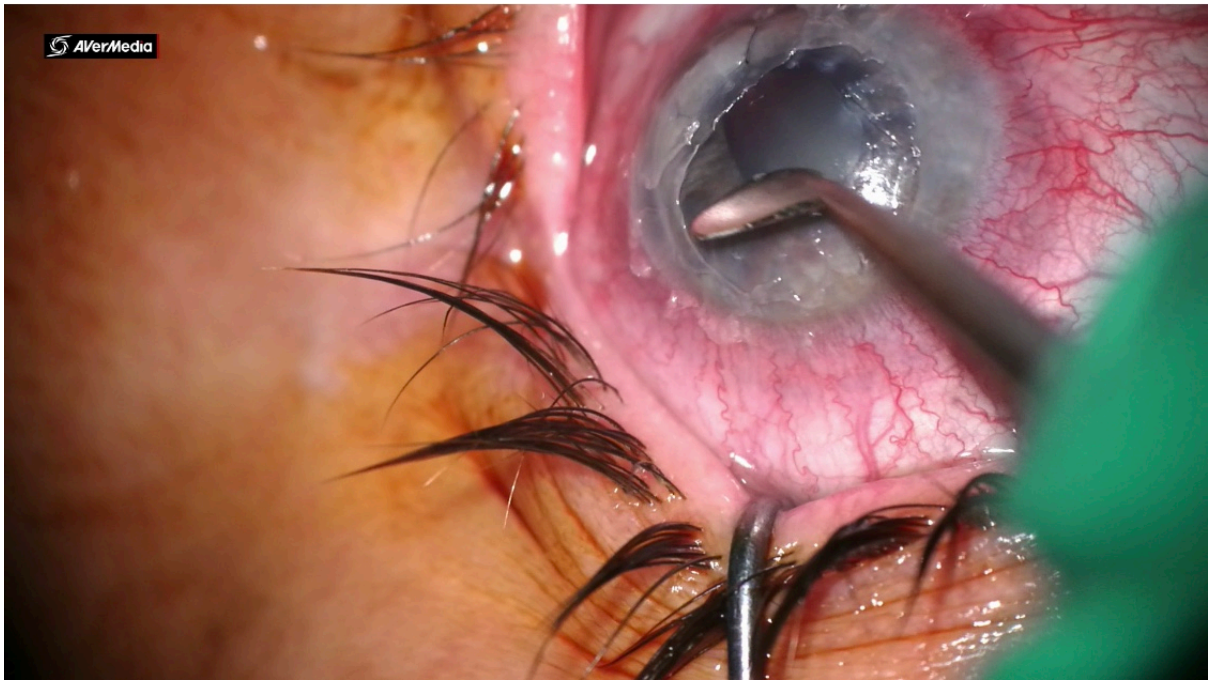


Figure 6:Fist Surgical Step in Transplantation of the bioproduct Alloheal®: Superficial keratectomy

Superficial keratectomy with removal of the corneal epithelium down to the level of Bowman membrane

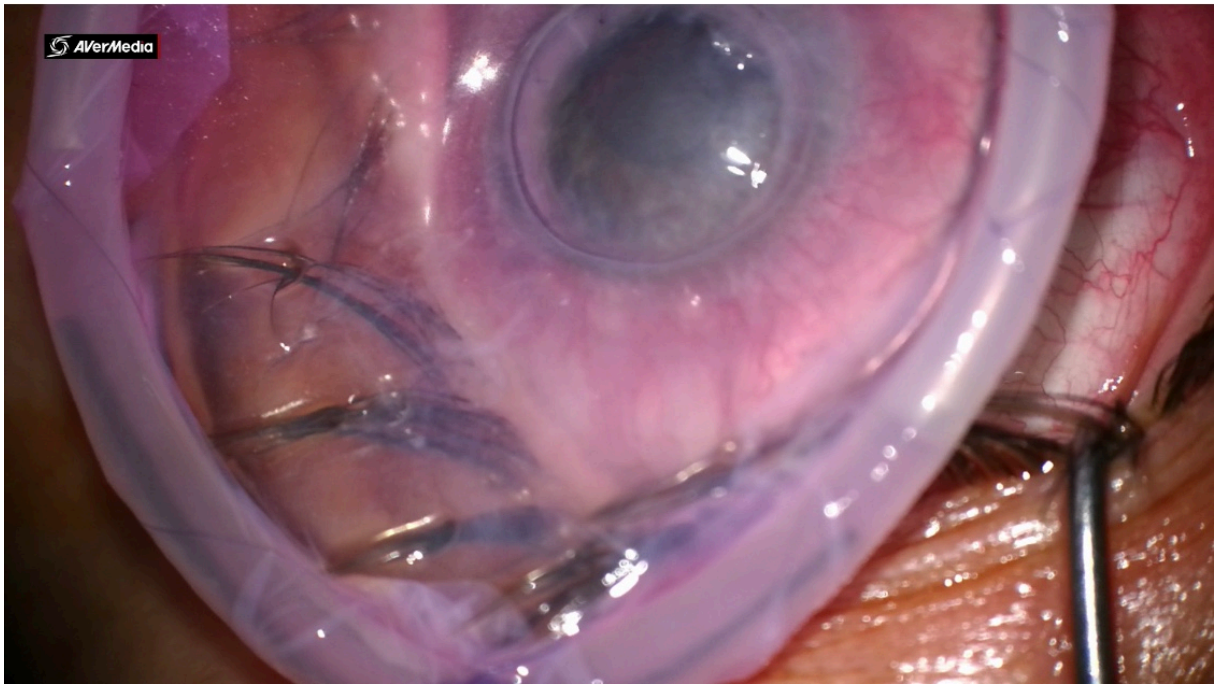


Figure 7: Second Surgical Step in Transplantation of the bioproduct Alloheal®: Positioning of the bioproduct Alloheal® on the ocular surface

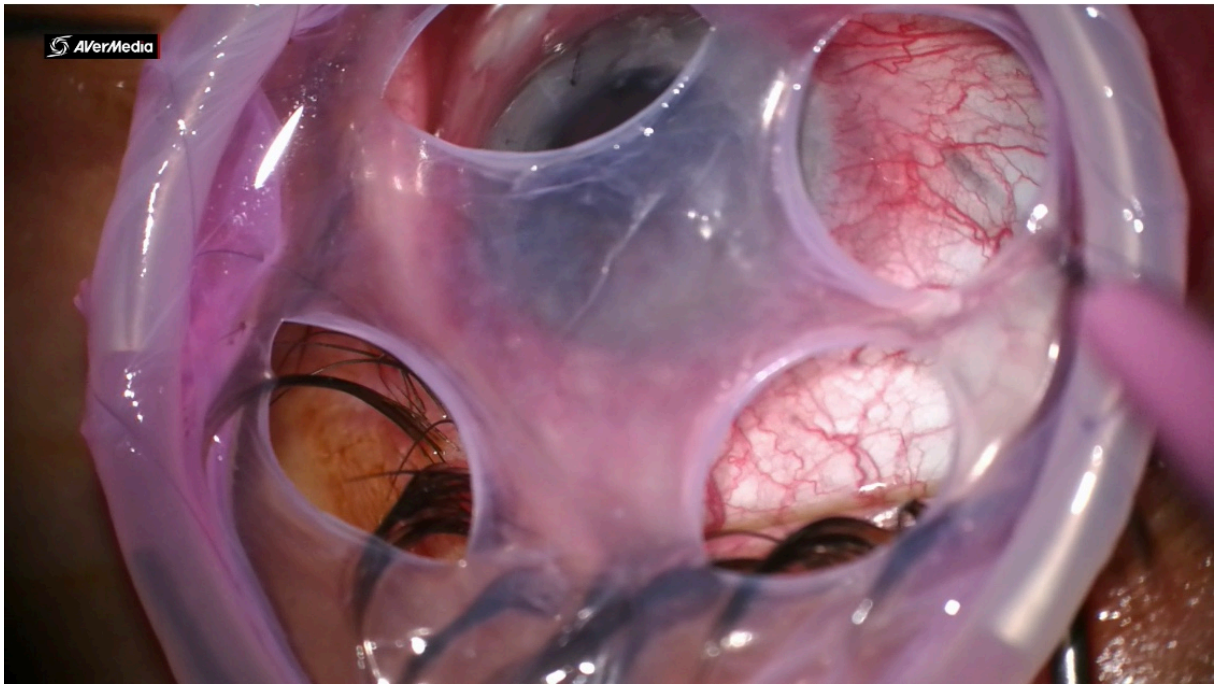


Figure 8: Third Surgical Step in Transplantation of the bioproduct Alloheal®: Atraumatic excision of the product from the mounting device

Amniotic membrane is atraumatically incised at the rim with the preferred size, for releasing the bioproduct from the mounting device

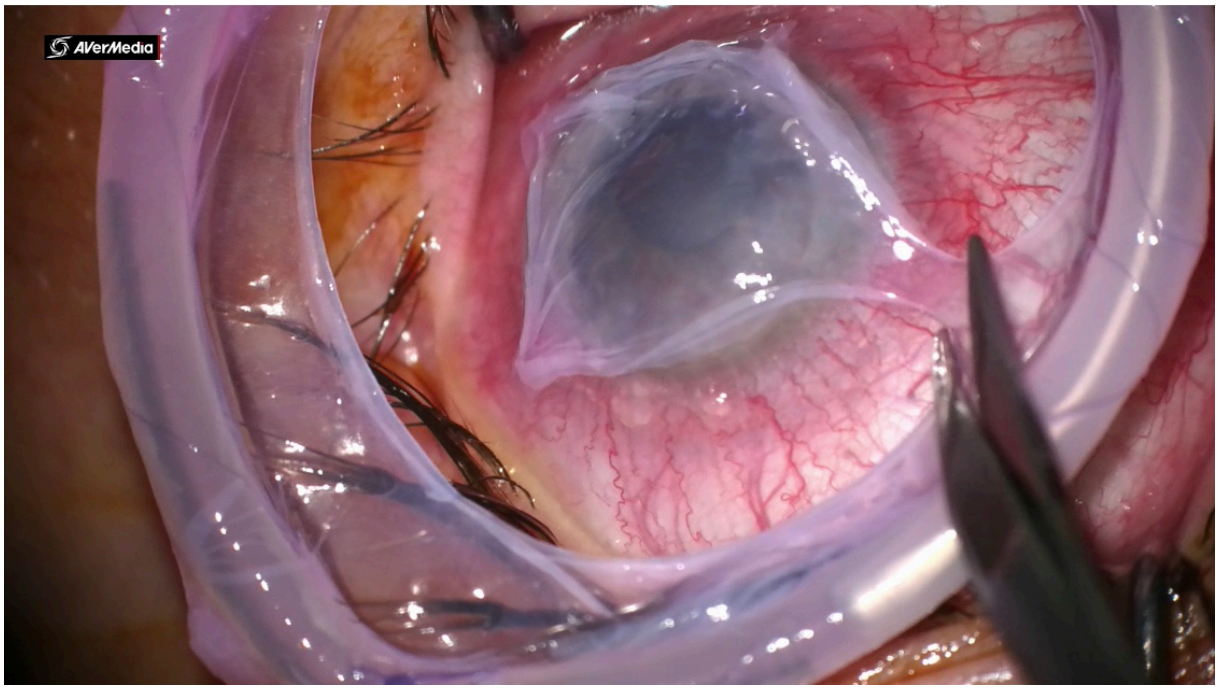


Figure 9: Fourth Surgical Step in Transplantation of the bioproduct Alloheal®: Full release of the bioproduct from the mounting device

The bioproduct is cut and released from the mounting device, adjusted to the required size and width

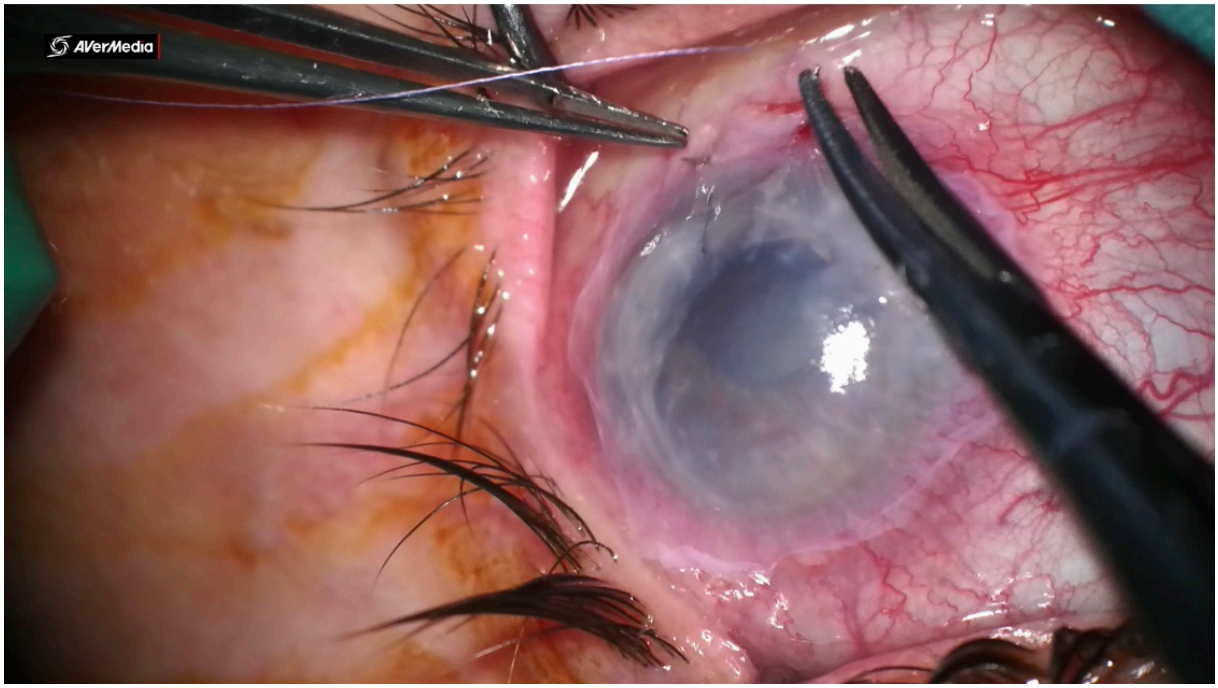


Figure 10: Fifth Surgical Step in Transplantation of the bioproduct Alloheal®: Suturing the bioproduct Alloheal® with 6 continuous sutures

The bioproduct Alloheal® is sutured in continuous sutures, overlapping the entire corneal surface

A therapeutic bandage contact *lens (PUREVISION2, Bausch & Lomb)* was placed over the membrane for comfort of the patient. Topical antibiotic drops Tobramycin *(Tobrex 0.3%, Alcon)* were administered to the patient before covering the eye with sterile dressings.

Postoperative treatment included nonsteroidal drops and antibiotic drops, topical therapy with steroid and preservative–free lubricants.

Postoperatively medication included same therapy as patients transplanted with classic amniotic membrane transplantation with topical antibiotic drops Tobramycin in combination with Dexamethasone *(Tobradex, Alcon)*, Vigamox *(Moxifloxacin 0.5%, Alcon)* and Dexpanthenol gel *(Corneregel, Bausch & Lomb)*. After the amniotic membrane removal, the therapy was adapted in accordance with the current ophthalmic status of the patient. Preservative-free lubricants were kept as part of the regimen of the patient.

3.9.3 STATISTICAL ANALYSIS OF OPERATIVE HANDLING

To what extent the different bioproduct mounting devices have an influence on the successful operation handling of the cryopreserved deepithelialized amniotic membrane mounted on the self-customized ring forming the bioproduct Alloheal® and the amniotic membrane mounted on the commercially available mounting device *(CellCrown, Sigma-Aldrich)* was the focus of the first statistical study.

These two approaches for membrane fixation were evaluated in their operative handling according to five key steps in membrane transplantation steps: (1) handling from the container, (2) washing before transfer to the ocular surface, (3) transfer to the ocular surface on operation site, (4) adjustment for cutting, (5) cutting and adaptation. The surgical application of the cryopreserved amniotic membrane anchored was assessed using the 2-6-point grading scale. The evaluation scale included the assessment as follows: 2 points = poor, 3 points = sufficient, 4 points = good, 5 points = very good, 6 points = excellent. A distinction regarding the number of sutures used to attach the amniotic membrane to the self-customized was not made because only amniotic membranes with 12-sutures attached to the ring were used for further utilization.

The data acquisition and statistical analysis was conducted with the statistical program SPSS vol.19.0 *(SPSS, IBM, USA)*. The measured data and results were transferred to the program Excel *(Microsoft Excel, USA)* and displayed as histograms. For the statistical analysis we used the Kolmogorov-Smirnov Test (KS-Test) to evaluate the normality of distribution. The Descriptive statistics was used to summarize the collected variables and to determine a presence of a normal distribution of the data. In case of normal distribution, the results for quantitative variables are presented by arithmetic mean and standard deviation. Variables with different from the normal distribution are presented with Median. Qualitative variables are represented by relative shares (%). For the comparisons of mean values of variables, the Independent Samples (Student) t-test and for comparison of the average values between several groups, the ANOVA-parametric method could be used. For non-parametric method for comparison of a given indicator between two groups the Mann-Whitney U test and Two-Sample Kolmogorov – Smirnov Test could be the test of choice. Values with a $p < 0.05$ were determined as statistically significant. A p-value higher than > 0.05 was determined as not statistically significant. For a null hypothesis significance level, $p < 0.05$ was assumed at a confidence interval of 95%.

3.10 CLINICAL MICROSTRUCTURAL EVALUATION OF THE CORNEA AFTER TRANSPLANTATION

Patients with significant ocular surface disintegration were examined before and after application of biological therapy with the bioproduct Alloheal® at microstructural level. Comparative analysis between patients transplanted with classic amniotic membrane transplantation and biological therapy based on amniotic tissue was performed.

Since the examination of the ocular surface through the coating layer of the transplanted amniotic membrane by slit-lamp biomicroscopy *(Microscope Slit Lamp HS-7000, Huvitz)* is difficult to accomplish, we have applied the use of in vivo confocal microscopy *(Heidelberg Retina Tomograph - HRT3 RCM, Heidelberg Engineering GmbH, Dossenheim, Germany)* and of optical coherence tomography *(CIRRUS 5000, ZEISS)* for the observation of the corneal structures covered by classic human amniotic membrane transplantation and biological therapy.

Amniotic membranes and the biological products were provided and prepared by the Center for Translational Medicine and Cellular Therapy at the St. Marina University Hospital (Varna, Bulgaria) according to all safety requirements of SOP.

3.10.1 EXAMINATION PROCEDURE

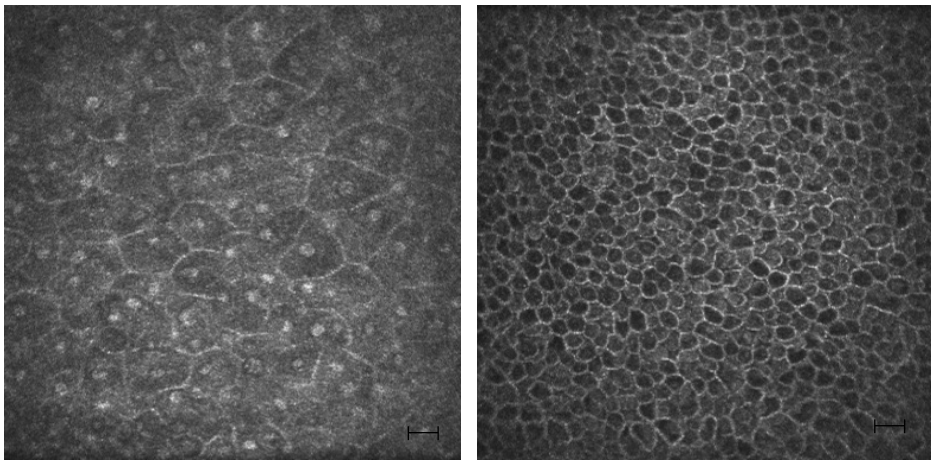
To perform comparative analysis at microstructural level between classic human amniotic membrane transplantation and biological therapy a total of 22 eyes of 19 patients were monitored. The examination procedure included slit-lamp biomicroscopy, AS-OCT and IVCM. The preoperative examination of patients included the collection and evaluation of clinical history of the patient, as well as pre-existing and chronic diseases.

For confocal microscopy of the cornea the Heidelberg Retina Tomograph (HRT3) *(Heidelberg Engineering GmbH, Dossenheim, Germany)* confocal scanning laser ophthalmoscope in combination with the Rostock Cornea Module (RCM) attached to the HRT had been used. Cellular structures were imaged through the layers of the entire cornea, from the epithelium to the endothelium with an image size of 400 × 400 μm. Through the CCD camera a seamless

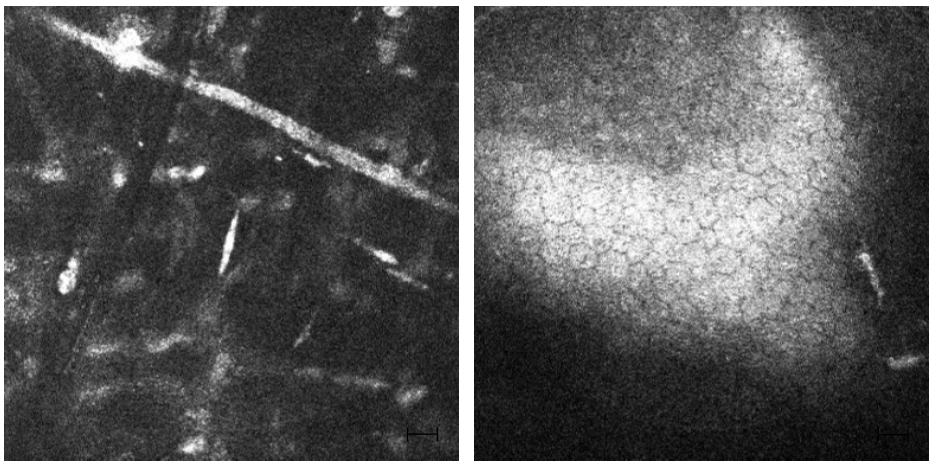
corneal contact was monitored, which allows control of the position without direct access to the examination site.

Patients were informed in advance in detail about the examination procedure. The patients were examined by IVCM the day pre-surgery and 7th day post-surgery. The examination was performed identically for patients with classic human amniotic membrane transplantation and patients with transplantation of the biological product.

The ocular surface of the patient was anaesthetized with one drop of local anesthetic with proxymetacaine hydrochloride *(Alcaine 0.5%, Alcon)* and the examination performed through a therapeutic contact lens on the cornea. A small amount of highly viscous gel *(Corneregel, Bausch & Lom)* and the sterile TomoCap *(Heidelberg Engineering)* form the contact with the locally anesthetized cornea. A small amount of highly viscous gel was placed in in the TomoCap to form a bridge between the cap and the lens. Special attention was paid to the avoidance of air bubbles in the gel. The additional fixation lamp on the confocal device was positioned in front of the patient to define viewing angle for maintaining eye position and to avoid eye movement. The imaging was performed through the layers of the cornea with special attention to the surface epithelium cells and the basal cells as demonstrated in representative in vivo confocal microscopic images of the cornea in Figure 12.



a) Superficial epithelial cells *b) Basal epithelial cells*



c) Mid stroma with nerve trunk *d) Corneal endothelial cell layer*

Figure 11: Representative in vivo confocal microscopy of the cellular corneal layers

Representative IVCM images of cellular corneal layers using Heidelberg Retina Tomography 3 Rostock Corneal Module (Heidelberg Engineering, Heidelberg, Germany), a) superficial epithelial cells b) basal epithelial cells c) mid stroma with nerve trunk d) corneal endothelial cell layer. All images are 400 × 400µm. Bar represents 50 µm.

The in vivo confocal microscopy was used to classify the corneal epithelium cells. The cellular density was evaluated at the level of the superficial epithelium and the basal epithelial layer by counting the cells present in one image, counted using point-and-click software with the built-in cell counting software. The results are expressed in cells per square millimeter (Figure 13). The data regarding the corneal cellular density at the level of the superficial epithelium and the basal epithelial layer were calculated as means ± standard deviations.

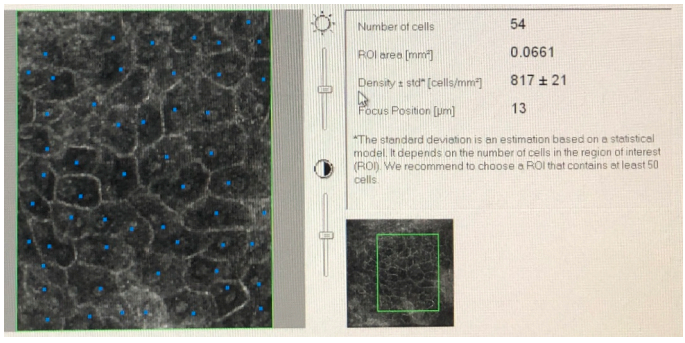


Figure 12: Program for evaluation of the corneal epithelial cell density

The corneal thickness and the amniotic membrane thickness were exanimated with the help of the optical coherence tomography *(CIRRUS 5000, ZEISS)* the day pre-surgery, 1st and 7th day post-surgery.

3.10.2 STATISTICAL ANALYSIS OF MICROSTRUCTUAL CHANGES

The obtained data and statistical analysis were carried out using the SPSS statistics program vol. 19.0 (SPSS, IBM, USA). All clinical obtained data and results were transferred to the program Excel (Microsoft Excel, USA) and displayed as circle graph chart or Bar charts. The results underwent a descriptive statistical analysis based on the software SPSS vol. 19.0 (SPSS, IBM, USA) to summarize the collected variables, from which Means and standard deviations were calculated.

Due to the importance of the presence of SD in this group, although of absence of normal distribution, the data was presented with their means and SD.
Without the presence of normal distribution of the indicators in the study group, a nonparametric statistical analysis was conducted. The Wilcoxon rank sum test was used to compare the density of corneal superficial epithelial cells and corneal basal epithelial cells between the patients transplanted with classic AMT and the bioproduct. It is also used to compare the corneal thickness and amniotic membrane thickness, pre- and postoperatively in the groups. Values with a $p < 0,05$ was considered to have statistical significance.

4 RESULTS

4.1 CLINICAL EVALUATION OF MOUNTING THE HUMAN AMNIOTIC MEMBRANE WITH DIFFERENT MOUNTING DEVICES

The unmounted human amniotic membranes were difficult to process. The amniotic membranes immediately curled up and stuck together, unsuitable for further processing as a scaffold for ex vivo cultivation. Unfixed the amniotic membrane had always the tendency to contract and adhere to itself, which complicated the correct orientation of the membrane. With mounting the human amniotic membrane on the CellCrown and the self-manufactured ring, the immobilization ensured required stability of the membrane. We compared the bioproduct Alloheal® mounted on the self-customized ring and the membrane anchored by a universally available mounting device *(CellCrown, Sigma-Aldrich)*.

In the course of the manufacturing it became apparent that the positioning of the membrane on the self-customized device was easier archived, with faster orientation of the desired membrane alignment. This was accomplished by detaching a small corner of the cryopreserved denuded amniotic membrane from the nitrocellulose paper and pushing the ring between the nitrocellulose paper and the amniotic membrane, wherewith in this movement, the membrane simultaneously detaches and clings to the ring in the correct surface showing upward. The membrane was placed on the ring with the desired decellularized epithelial surface side up, without fully releasing the membrane from the nitrocellulose paper and without the need to reposition it anew due to its stickiness.

In the case of the envisaged CellCrown *(Sigma-Aldrich)*, the need of the complete disconnection of the cryopreserved denuded amniotic membrane from the nitrocellulose paper, to place the membrane on the CellCrown *(Sigma-Aldrich)* led to the difficulty described above. Removed from the nitrocellulose paper, with its elastic nature, the membrane constantly wrinkled when placed on the CellCrown *(Sigma-Aldrich)*, complicating the procedure of correct positioning without tearing, and making differentiation of the sides with repeated testing for correct alignment difficult.

In turn, suturing the amniotic membrane to the self-customized ring was more time-consuming than clamping the membrane with the CellCrown. However, when suturing the amniotic membrane to the self-customized flexible ring, we had the opportunity to determine the

tightness with which the membrane was secured to the carrier device, which showed to have a profound effect on cultivation behavior and adhesion of the explant corneal limbal pieces, as well as other advantages in handling in operation site, detailed in the results of the operative handling.

When attaching the amniotic membrane to the flexible ring, different approaches were applied to ideally mount the amniotic membrane to the ring device. The attachments were compared with a 6-suture attachment and a 12-suture attachment, as well as a further attachment of the amniotic membrane by clasping the amniotic membrane by a single-use skin stapler to the ring as listed in Table 1.

Table 1: Overview of the attachment trials of the amniotic membrane to the mounting device

	Amniotic membrane sutured with 6 stiches to the ring device					**Amniotic membrane sutured with 12 stiches to the ring device**					**Sterile single-use skin stapler**
Trial No.	1	2	3	4	5	1	2	3	4	5	1
Time for suturing	4 min 15 sec	3 min 45 sec	4 min 20 sec	3 min 10 sec	3 min 20 sec	6 min 00 sec	5 min 30 sec	4 min 45 sec	5 min 10 sec	4 min 40 sec	No fixation possible
Durability on the ring	no	yes	no	no	no	yes	yes	yes	yes	yes	no

In the present study, three different approaches were used to attach the amniotic membrane to the mounting device. In 10 approaches were 5 cryopreserved amniotic membranes were fixed with a 6-suture attachment and 5 cryopreserved amniotic membranes were fixed with a 12-suture fixation to determine which attachment is durable and so suitable for the later use in ex vivo cultivation of limbal epithelial stem cells, it was clearly demonstrated that the fixation with 12 stiches firmly mount the membrane to the device. The approach with 6 sutures was insufficient. Only one of the five membranes (20%) could be attached to the ring with 6 sutures. Here, the membrane contracted due to its flexibility and could not be used for optimal cell seeding. With the 12-stitch fixation, all amniotic membranes (100%) were fixed well to the flexible ring.

Amniotic membrane fixation to the ring device with sterile single-use skin stapler was not possible. It became apparent that tacking through the ring was not possible and the approach was not pursued further.

Though the fixation of the amniotic membrane with an average time of 3 min 46 sec. with a 6-suture fixation is faster than the average time of 5 min 13 sec. with a 12 -suture fixation, on account of inadequate fixation only amniotic membranes with the 12-suture fixation were used for further processing.

4.2 CLINICAL EVALUATION OF EX-VIVO EXPANSION OF CORNEAL LIMBAL STEM CELLS ON THE AMNIOTIC MEMBRANE WITH DIFFERENT MOUNTING DEVICES

Allogenic limbal epithelial stem cells were cultivated in vitro on the human amniotic membrane fixed by a suitable carrier device according to all safety requirements of SOP. The human amniotic membranes were mounted on the carrier prototype a flexible ring and on a universally available mounting device *(CellCrown, Sigma-Aldrich)*. The two different methods for mounting the amniotic membrane for ex-vivo cultivation was performed. The first difference became evident in placing the corneal limbal tissue pieces on the denuded human amniotic membrane. Spanned within the prototype of a flexible ring device the human amniotic membrane was anchored with a slight convex down position not tightly fixed, whereby the limbal pieces adhere better without floating. The membrane which was firmly embedded within the commercially available mounting device *(CellCrown, Sigma-Aldrich)* proved to be disadvantaged with the limbal pieces floating by the addition of the growth medium.

The progression in the explant culture was impressively observed in both methods. Control was exercised in two ways, by phase contrast microscopy and by H&E staining. After a few days, the growth of cells from the explant could be observed. Corneal limbal epithelial cells cultured on the denuded human amniotic membrane forming the bioproduct Alloheal® were shown in Figure 14. Also, macroscopically the spread across the amniotic membrane was visible forming spindle-like long-stretched cells. H&E staining of the bioproduct Alloheal® showed vital, well differentiated cells (Figure 15).

Particularly noteworthy was the recognition that the growing corneal limbal epithelial cells pulled on the membrane during the cultivation forming holes in the human amniotic membranes when to tightly spanned, especially within the commercially available mounting device *(CellCrown, Sigma-Aldrich)*. Here it became apparent, that the denuded membranes anchored with the self-customized flexible ring device were superior due to the not too tight mounting, giving the membrane the required tension. In contrast, the membrane anchored in the CellCrown had significantly more frequent holes due to lack of flexibility. The amniotic membranes with multiple holes were not suitable for further use in ocular surface reconstruction and were discarded. The cultivation behavior on the two differently anchored amniotic membranes visualized that the self-manufactured flexible ring device was for our purposes superior to the commercially available mounting device.

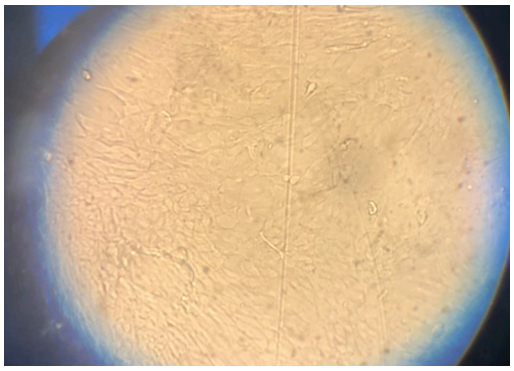


Figure 13: Corneal limbal epithelial cell culture on the denuded human amniotic membrane of the bioproduct Alloheal®

Corneal limbal cell population on the bioproduct Alloheal® after day 20 of cultivation

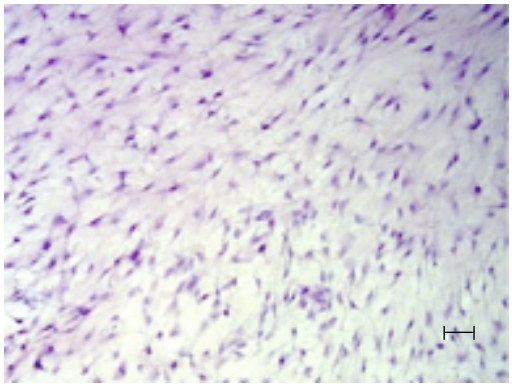


Figure 14: H&E staining of the bioproduct Alloheal®

H&E staining of the bioproduct Alloheal®, showing vital, well differentiated cells (Magnification x10; *Bar represents 100 µm)*

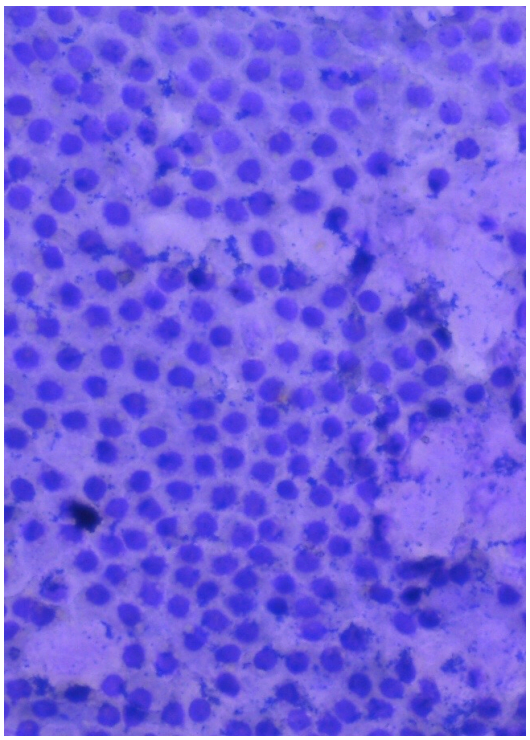
4.1 HISTOLOGICAL ANALYSIS

The histological analysis was performed for the comparison of a cryopreserved intact human amniotic membrane and a cryopreserved denuded, decellularized human amniotic membrane, to evaluate the feasibility of the method of decellularization of the human amniotic membrane utilized as a matrix for the ex-vivo cultivation of allogenic limbal stem cells.

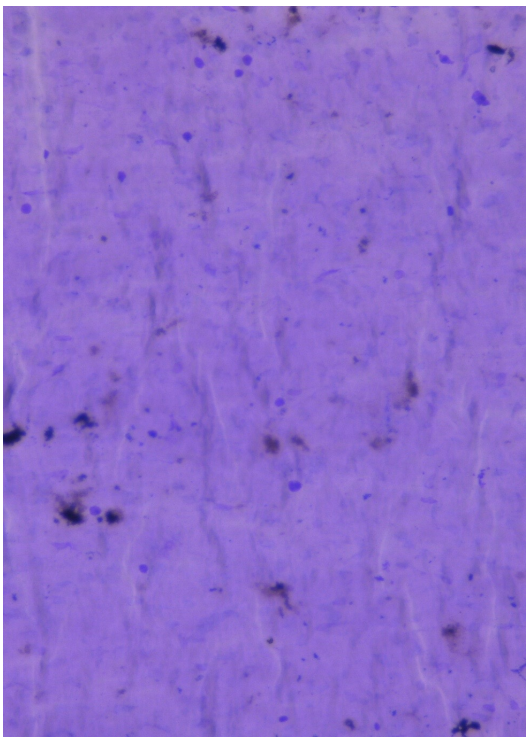
The process of decellularization was examined microscopically with light microscopic studies of the human amniotic membrane stained with Hematoxylin and Eosin staining and Diff-Quik staining. The intact human amniotic membrane showed characteristic organized epithelial layer of the epithelial surface with cells of all about the same size containing a roundish to oval, centrally located nucleus (Figure 16 a on the left and Figure 17 a on the left), where the denuded human amniotic membrane showed absence of nuclear structures (Figure 16 a on the right and Figure 17 b on the right) with otherwise intact membrane structure.

Hematoxylin and Eosin staining and Diff-Quik staining were prepared to visualize cellular components of the amniotic membrane. The hematoxylin and the eosin used for counterstaining stained the cell nuclei blue, the cytoplasm pinkish. The Diff-Quik staining expressed the cell nuclei dark blue and the cytoplasm light blue/violet. Unfortunately, after one day the mounting glue has formed many dark blue bubbles as seen on Figure 16 b left and Figure 17 b left.

Based on these observations we conclude that the process of decellularization of the amniotic membranes we utilized in tissue engineering is effective and that the amniotic membranes used for the bioproduct Alloheal® are decellularized without visual structural disruption.



a) Diff-Quik staining of intact AM

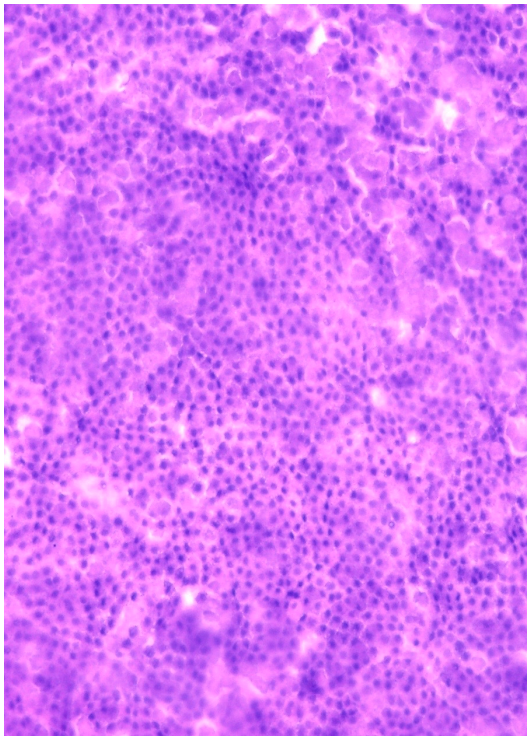


b) Diff-Quik staining of denuded AM

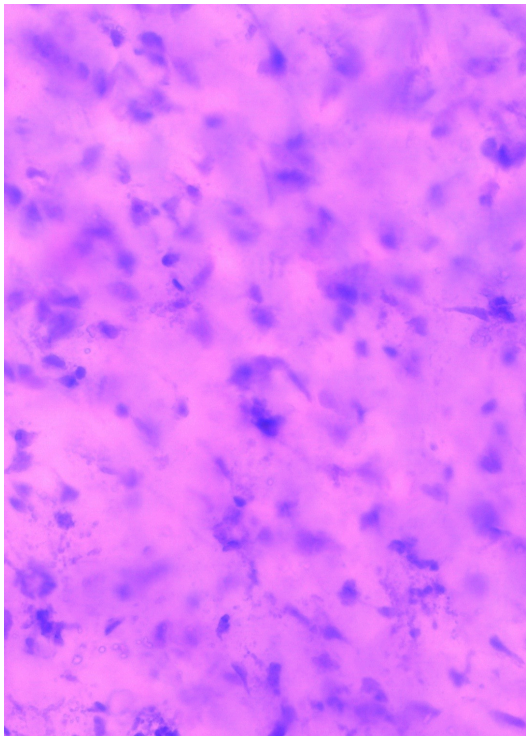
***Figure 15:*Diff-Quik staining of human amniotic membrane**

a) Diff-Quik staining shows an intact human amniotic membrane covered with a layer of epithelial cells

b) Diff-Quik staining shows a denuded human amniotic membrane with decellularization



a) H&E staining of the intact AM



b) H&E staining of the denuded AM

Figure 16: Hematoxylin and eosin staining of human amniotic membrane

a) H&E staining shows the intact amniotic membrane covered with a layer of epithelial cells. (Magnification ×10)

b) H&E staining shows the denuded human amniotic membrane with confirmed decellularization by the absence of cellular components. (Magnification ×20)

4.2 STATISTICAL ANALYSIS OF OPERATION HANDLING WITH DIFFERENT MOUNTING DEVICES

The main focus of the statistical evaluation was to determine if there is a statistical significance difference between the two types of mounting devices and whether the self-customized ring forming the bioproduct Alloheal® is superior to the other commercially available mounting method in terms of operational handling.

The statistical evaluation of the 40 operations underlined our clinical experience. The statistical analysis showed great differences in three aspects with regard to transfer to the ocular surface, adjustment for cutting, cutting and adaptation, whereas two aspects the handling from the container and the washing before transfer to the ocular surface did not show significant variations for operative handling.
However, based on the Kolmogorov-Smirnov Test (KS-Test) there was no normal distribution in the variables, therefore all variables were presented with their medians in Table 2 and Figure 18.

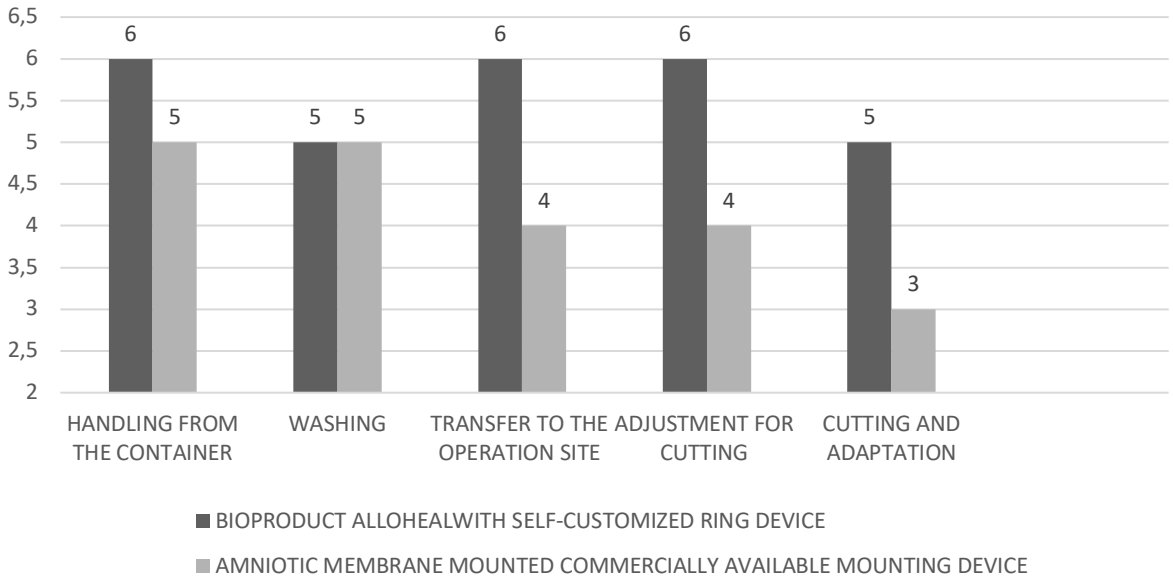


Figure 17: Graphical evaluation of the median of the grading in terms of operational handling of the two mounting, the self-customized ring device, and the commercially available mounting device

Table 2: Grading of the two mounting devices with the regard to the operative handling steps and the resulting median

Operation handling			Handling from the Container	Washing	Transfer to the Operation Site	Adjustment for Cutting	Cutting and Adaptation
Self-customized mounting device - Bioproduct Alloheal®	N	Valid	20	20	20	20	20
		Missing	0	0	0	0	0
	Median		6.00	5.00	6.00	6.00	5.00
Commercially available mounting device- CellCrown	N	Valid	20	20	20	20	20
		Missing	0	0	0	0	0
	Median		5.00	5.00	4.00	4.00	3.00

Furthermore, the statistical analysis of the frequency of evaluations for both methods in comparison clearly highlighted that the self-customized ring forming the bioproduct Alloheal® was superior to the commercially available devise in the operations handling steps transfer to the ocular surface, adjustment for cutting and cutting and adaptation. The frequencies and

percentages are presented in detail in the Table 3, Table 4, and Table 5 for this surgical handling steps.

Table 3: Results of the grading frequency and percentage for transfer to the ocular surface

TRANSFER TO THE OCULAR SURFACE			
Mounting device	**Grading**	**Frequency**	**Percent**
Self-customized mounting device -Bioproduct Alloheal®	poor	0	0 %
	sufficient	0	0 %
	good	0	0 %
	very good	8	40 %
	excellent	12	60 %
	total	20	100 %
Commercially available mounting device - CellCrown	poor	0	0 %
	sufficient	2	10 %
	good	10	50 %
	very good	8	40 %
	excellent	0	0 %
	total	20	100%

Table 4: Results of the grading frequency and percentage for adjustment and cutting

ADJUSTMENT FOR CUTTING			
Mounting device	**Grading**	**Frequency**	**Percent**
Self-customized mounting device - Bioproduct Alloheal®	poor	0	0 %
	sufficient	0	0 %
	good	0	0 %
	very good	8	40 %
	excellent	12	60 %
	total	20	100 %
Commercially available mounting device - CellCrown	poor	0	0 %
	sufficient	9	45 %
	good	10	50 %
	very good	1	5 %
	excellent	0	0 %
	total	20	100%

Table 5: Results of the grading frequency and percentage for cutting and adaption

CUTTING AND ADAPTATION			
Mounting device	**Grading**	**Frequency**	**Percent**
Self-customized mounting device - Bioproduct Alloheal®	poor	0	0 %
	sufficient	0	0 %
	good	5	25 %
	very good	12	60 %
	excellent	3	15 %
	total	20	100 %
Commercially available mounting device - CellCrown	poor	3	15 %
	sufficient	14	70 %
	good	3	15 %
	very good	0	0 %
	excellent	0	0 %
	total	20	100%

To compare the different surgical steps of the two mounting methods, and to verify the statistical significance of the observations, the Mann–Whitney U test and the Two-Sample Kolmogorov– Smirnov Test showed to be the most appropriate statistical methods. These nonparametric methods were used to compare the two mounting devices with regard to the operative handling steps. For a null hypothesis significance level, $p < 0.05$ was assumed at a confidence interval of 95%. This resulted in 2-tailed significance with a value $p < 0.05$ for the transfer to the ocular surface, the adjustment for cutting and the cutting and adaptation steps (Table 6 and Table 7). The Z-value with the direction of the difference that exists between the two groups studied was also displayed in the Table 6 and Table 7.

This also statistically confirms, in accordance to our clinical experience, that the self-customized ring device was in three steps of the operation handling statistically significant superior to the commercially available mounting device *(CellCrown, Sigma-Aldrich)*.

Table 6: Statistical analysis of the operational handling of the two mounting devices (self-customized ring device and the commercially available mounting device) by Mann-Whitney U Test

	Handling from the Container	**Washing**	**Transfer to the Operation Site**	**Adjustment for Cutting**	**Cutting and Adaptation**
Z	-1.574	-0.576	-4.807	-5.482	-5.423
p-value	0,165	0,659	0,000	0,000	0,000

Table 7: Statistical analysis of the operational handling of the two mounting devices (self-customized ring device and the commercially available mounting device) by Two-Sample Kolmogorov– Smirnov Test

	Handling from the Container	Washing	Transfer to the Operation Site	Adjustment for Cutting	Cutting and Adaptation
Kolmogorov-Smirnov Z	0,632	0,158	1,897	3,004	2,688
p-value	0,819	1,000	0,001	0,000	0,000

Though the preparation of the self-customized flexible ring with stitching the membrane to the ring was more time consuming than using a preexisting commercially available anchoring device, the flexible ring provided better surgical handling, resulting in shorter operation time, in favor of the patient and the surgeon. In addition, the self-customized ring device provided easier handling with safer transfer of the membrane to the operation site (Table 8), without the need to unlock the membrane from the anchoring device before aligning it to the ocular surface. With the need to unlock the anchored membrane from the CellCrown before transferring and adjusting it to the ocular surface, the membranes repeatedly contracted and stuck together. This further contributed to the fact that the required membrane side was obscure and required repeatedly realignment and testing for the correct positioning what required considerable time. The flexibility and adjustability of the biological product Alloheal® and its easy use were significant advantages, these combined with the precondition for no-touch technique with reduced risk of membrane contraction during adjustment for cutting and cutting and adaptation for suturing, provided easy handling and ocular surface adaptation with less trauma to the membrane, to the cultivated stem cells as well as to the ocular surface of the patient.

Table 8: Significance of the individual operations steps of the operation handling based on p-values

	p-value	significant (p<0,05)
Handling from the Container	0,165	no
Washing	0,659	no
Transfer to the Operation Site	0,000	yes
Adjustment for Cutting	0,000	yes
Cutting and Adaptation	0,000	yes

4.3 MICROSTRUCTURAL EVALUATION

4.3.1 ANALYSIS OF CLINICAL DATA

Examination of the ocular surface through the coating layer of the transplanted amniotic membrane by slit-lamp biomicroscopy is difficult to accomplish, consequently in vivo confocal microscopy *(Heidelberg Retina Tomograph - HRT3 RCM, Heidelberg Engineering GmbH, Dossenheim, Germany)* and OCT was used for the observation of the corneas covered with amniotic membrane.

A total of twenty-two patients (25 eyes), including 11 males (50 %) and 11 females (50%), were enrolled in this study. Fifteen eyes of 13 patients were transplanted with classic human amniotic membrane transplantation. Of these 7 (46,7%) were the right and 8 (53,3%) the left eyes (Table 9). The average age of the patients was 52.53 ± 22.09 years, with the youngest patient of the age of 8 and the oldest patient with the age of 86 years.
Ten eyes of 9 patients were treated with bioproduct transplantation. In 60% (6 eyes) of the cases the right eye was operated and in 40% (4 eyes) of the cases was the left eye operated (Table 9). The average age of the patients was 60.10 ± 22.95 years, with the youngest patient of 21 years and the oldest with 82 years of age.

Table 9: Frequency and percentage of the distribution of the operated eye

Treatment Type	Eye	Frequency	Percentage
Classic AMT	right	7	46,7%
	left	8	53,3%
	Total	15	100%
Bioproduct Alloheal®	right	6	60%
	left	4	40%
	Total	10	100%

The most common surgical indications for classic amniotic membrane transplantation were corneal ulcerations (20%) and Keratoconus (20%), while the most common surgical indications for bioproduct treatment (Table 10) were corneal burns (30%), corneal ulceration (20%) and trauma with persistent corneal epithelial defects (20%).

Table 10: Patients receiving bioproduct transplantation - distribution by disorders

Disorder	Frequency	Percentage
Corneal burns	3 Eyes	30 %
Corneal Ulceration	2 Eyes	20 %
Trauma	2 Eyes	20 %
Lattice corneal dystrophy	1 Eye	10 %
Salzmann's corneal dystrophy	1 Eye	10 %
Persistent corneal epithelial defects due to Neurotrophic keratitis	1 Eye	10 %

Secondary findings showed that 16 of all transplanted patients suffer from concomitant systemic diseases, as graphically demonstrated in Figure 19. In total, 9 patients did not have concomitant diseases. The two most common concomitant diseases found in the order of their occurrence were hypertension in 9 patients (36%), Diabetes mellitus in 5 patients (20%). Dysplastic coxarthrosis (4%) and prostate cancer (4%) were the least frequent concomitant diseases.

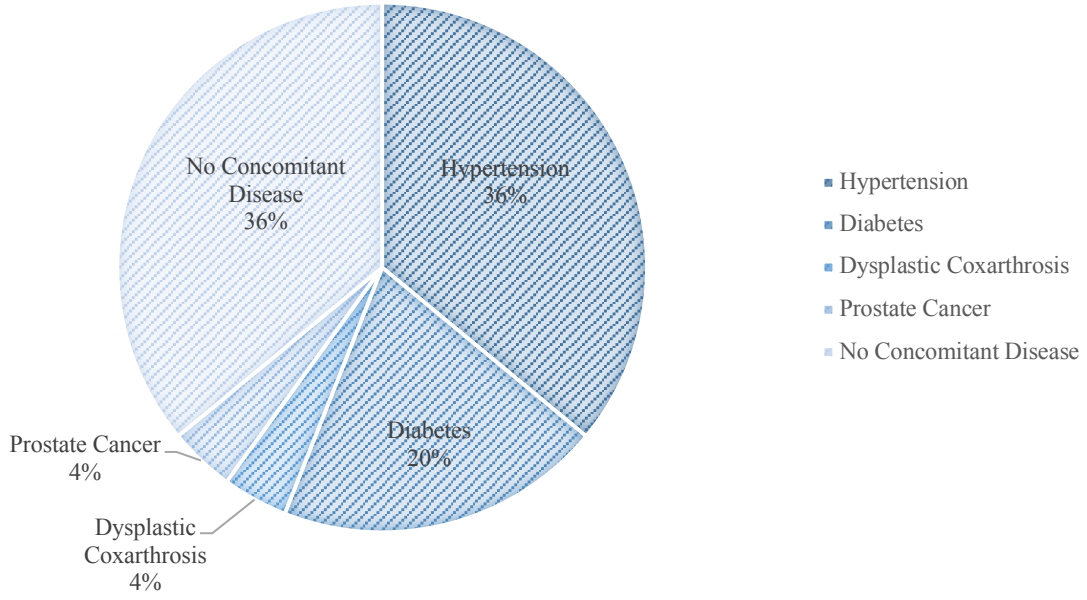


Figure 18: Concomitant diseases with their frequencies in the patient population

4.3.2 MICROSTRUCTURAL EVALUATION

To evaluate early corneal epithelial healing in human eyes after amniotic membrane transplantation and after bioproduct therapy with amniotic membrane cultivated limbal stem cells, in vivo confocal microscopy of the human cornea was performed in total of 25 eyes of 22 patients.

In all patients of both groups, patients treated with classic amniotic membrane transplantation and patients treated with the bioproduct, it was possible to visualize the corneal layers with the in vivo confocal microscope *(Heidelberg Retina Tomograph - HRT3 RCM, Heidelberg Engineering GmbH, Dossenheim, Germany)* and were not limited severely by the transplanted semi-transparent amniotic membrane.

By in vivo confocal microscopy of patients treated with classis amniotic membrane transplantation and treated with the bioproduct, corneal structures before and after transplantation could be analyzed at microstructural level. Morphological and microstructural changes of the epithelium of the cornea and changes in the morphology of the amniotic membrane transplanted were assessed in all patients. Under the transplanted amniotic membrane, the regeneration of the corneal epithelial cells was verifiable through IVCM. The

average density of corneal epithelial superficial cells pre-operative was measured in patients treated with classic amniotic membrane transplantation 852,47 ± 314,342 cells/mm² and 638.50 ± 162.275 cells/mm² in patients treated with the bioproduct Alloheal® (Table 11).

Post-operative an average density of 905.93 ± 314.197 cells/mm² was observed in classic amniotic membrane transplantation. Patients treated with the bioproduct, an increase to 718.10 ± 142.004 cells/mm² of the corneal superficial epithelial cell density could be verified. The cell density of corneal superficial epithelial cells was compared preoperative to postoperative on the 7th day in patients treated with classic amniotic membrane transplantation (p=0,001) and in patients treated with the bioproduct Alloheal® (p=0,025) were considered to have statistically significant difference (Table 15). When IVCM performed after transplantation new immature superficial corneal epithelial cells were large, flat, and polygonal in shape (Figure 20). Bright cell borders and identifiable bright nuclei have been determined.

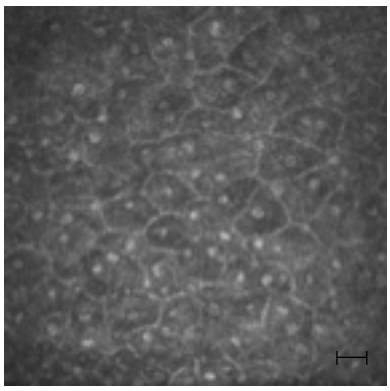


Figure 19: In vivo confocal microscopy of superficial corneal epithelium cells on the 7th. day post Alloheal® transplantation

The average density of the superficial corneal epithelium cells on the 7th postoperative day was 718.10 ± 142.004 cells/mm². Bar represents 50 µm.

Also, the corneal basal epithelial cells showed signs of immaturity in a compact regular pattern after amniotic membrane transplantation. From 4832.33 ± 1104.259 cells/mm² cell density of the basal epithelial cells in patients treated with classic AMT and 4647.40 ± 652.398 cells/mm² in patients treated with the bioproduct Alloheal® the average density increased in both groups with statistical significance difference ($p < 0,05$) to 5235.80 ±1200.790 cells/mm² and 5272.30 ± 642.356 cells/mm².

Especially corneal superficial epithelial cell density and basal epithelial cell density was lower in the patients with severe corneal disintegrations, in which classic amniotic membrane

transplantation would not be sufficient, and required transplantation of the bioproduct Alloheal® with cultivated limbal stem cells, compared with the patients treated with classic amniotic membrane transplantation.

Table 11: Density of superficial epithelial cells of the treated corneas

Treatment		Superficial epithelial cell density (cells/mm2)	
		Pre-operative	7th day postoperative
Classic AMT	Mean	852.467	905.933
	SD	314.342	314.197
Bioproduct Alloheal®	Mean	638.500	718.100
	SD	162.274	142.004

Table 12: Density of basal epithelial cells of the treated corneas

Treatment		Basal epithelial cell density (cells/mm2)	
		Pre-operative	7th day postoperative
Classic AMT	Mean	4832.333	5235.800
	SD	1104.259	1200.790
Bioproduct Alloheal®	Mean	4647.400	5272.300
	SD	652.398	642.356

AS-OCT showed that the preoperative corneal thickness at the corneal defect measured point was 455.93 ± 115.54 μm in patients treated with the classic AMT and 355.70 ± 97.75 μm in patients requiring Alloheal® treatment. The amniotic membrane thickness on the first day postoperatively was measured with 161.40 ± 143.87μm in patients treated with the classic AMT and 252.90 ± 116.90 μm in patients with bioproduct treatment.

The first day after transplantation the amniotic membrane is distinguishable in IVCM and AS-OCT. Patients with deep defects larger than 20% - 25% from the overall corneal thickness were treated with combined procedure which adds to the variations in membrane thickness. The thickness of the thinnest amniotic membrane layer of the transplanted population was measured to be 58 μm and the thickest 487 μm. The average preoperative thickness was 161.40 ± 143.87 μm in patients treated with the classic AMT and 252.90 ± 116.90 μm in patients treated with the bioproduct (Table 13). On the 1st day post-operation the mean corneal thickness amounted in patients treated with the bioproduct increased to 357.00 ± 96.77 μm, however, no significant

difference was found in the corneal preoperative thickness the thickness 1st day post operation in these patient population (p=0,119).

Table 13: Corneal Thickness AS-OCT measurements before surgery, on the 1st and 7th day post-surgery

	Corneal Thickness (µm)		
Treatment	**Preoperative**	**1st day post-operative**	**7th day post-operative**
Classic AMT	455.93 ± 115.54	459.67 ± 115.81	479.27 ± 121.72
Bioproduct Alloheal®	355.70 ± 97.75	357.00 ± 96.77	385.40 ± 101.40

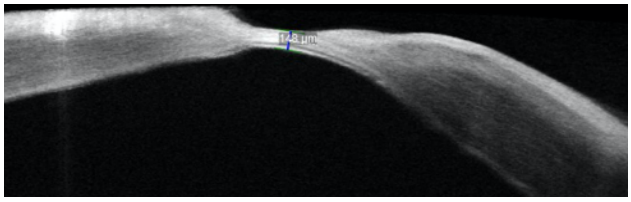


Figure 20: Representative OCT image of persistent epithelial defect preoperative Alloheal transplantation?

Representative case of a patient with persistent epithelial defect. The thinnest point preoperatively was measured 148 µm

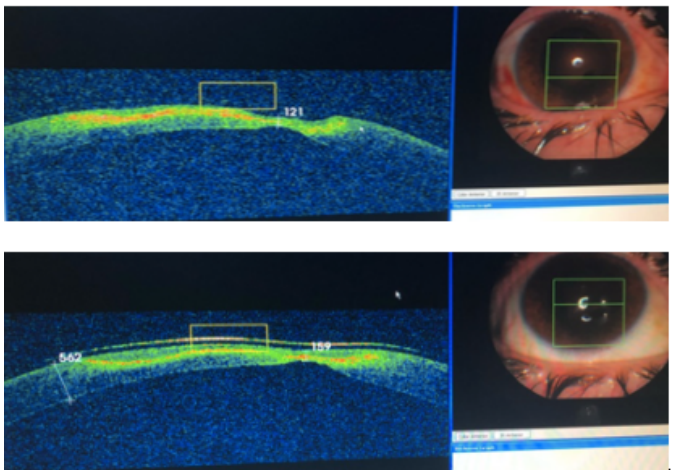


Figure 21: Structured reinforcement of cornea with impeding perforation, AS-OCT images 20 days after bioproduct transplantation

Dynamics after 20 days, representative case of a patient with persistent epithelial defect by AS-OCT (Topcon, Hasunumacho, Itabashiku, Tokyo, Japan). The thinnest point of the ulcer bed was measured 121 µm. On the 20th day postoperative the bandage contact lens and the amniotic membrane filling the ulcer were clearly identifiable and the corneal thickness at the ulcer measured 159 µm.

On the 7th day postoperative, the corneal thickness of patients treated with classic amniotic membrane transplantation and the bioproduct the corneal thickness was significantly higher compared to the corneal thickness preoperative, and the difference was statistically significant with (p=0,001) and (p=0,037) (Table 15). Consequently, post-surgery, the amniotic membrane was visualized with confocal microscopy. A progressive degradation of the membrane with patchy remnants was detected in the examined population, as seen in Figure 22. Also, the corneal stroma of the patient population showed morphological changes by in vivo confocal microscopy with activated stromal cells. The average thickness of the amniotic membrane, measured on the 7th day after classic amniotic membrane transplantation, was reduced to 92.93 ± 46.88 μm. In patients treated with the bioproduct the thickness was reduced to 166.00 ± 72.74μm (Table 14).

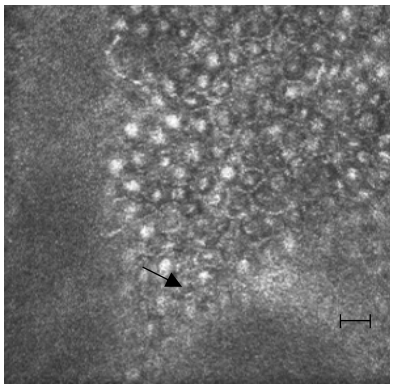


Figure 22: The in vivo confocal microscopic image with progressive degradation of the amniotic membrane with patchy remnants 7 days after operation.

Progressive degradation of the amniotic membrane with patchy remnants (marked with black arrow) 7 days after operation. Bar represents 50 μm.

Table 14: Amniotic Membrane Thickness AS-OCT measurements on the 1st and 7th day post-surgery

	Amniotic Membrane Thickness (μm)	
Treatment	**1st day post-operative**	**7th day post-operative**
Classic AMT	161.40 ± 143.87	92.93 ± 46.88
Bioproduct Alloheal®	252.90 ± 116.90	166.00 ± 72.74

Table 15: Summary of the statistical analysis demonstrating the comparison of the of the Z and p-values of classic AMT and the bioproduct

Treatment		Superficial epithelial cell density Preoperative - Postoperative 7th day	Basal epithelial cell density Preoperative - Postoperative 7th day	Corneal Thickness Preoperative - Postoperative 1st day	Corneal Thickness Preoperative - Postoperative 7th day	AM Thickness Postoperative 1st day- Postoperative 7th day
Classic AMT	Z	-3,408	-3,408	-3,305	-2,443	-3,408
	p	0,001	0,001	0,001	0,015	0,001
Bioproduct Alloheal®	Z	-2,244	-2,803	-1,559	-2,805	-2,090
	p	0,025	0,005	0,119	0,005	0,037

In patients with significant ocular surface disintegration after ocular burn the normally avascular cornea tissue showed the presence of blood vessels, a result of corneal neovascularization. The neovascularization was clearly visible in these group of patients with in vivo confocal microscopy (Figure 23). Newly formed corneal neovascularization could not be detected in any patient postoperatively during the follow-up interval.

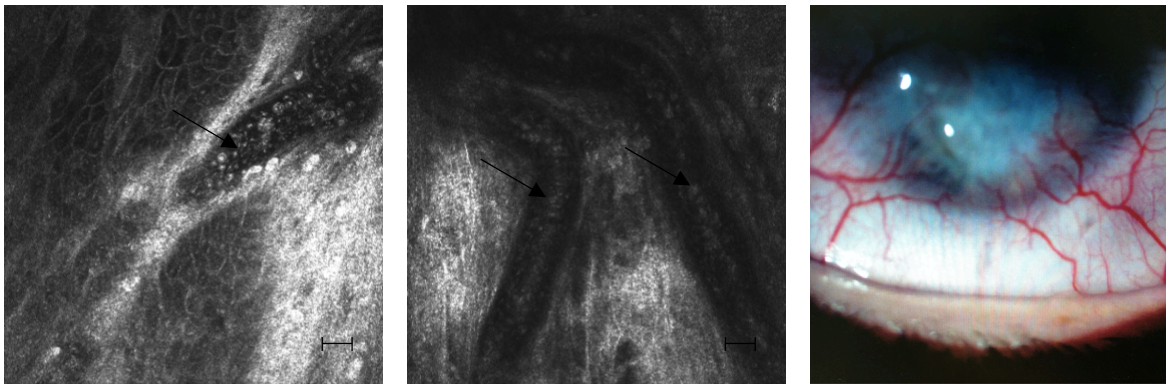


Figure 23: Corneal neovascularization

Corneal neovascularization (marked by black arrow) preoperative with significant ocular surface disintegration. Bar represents 50 µm.

Our findings indicate that all layers of the cornea could be visualized at any time of examination and were not drastically restricted by the amniotic membrane, which positions confocal

microscopy as a useful tool for future examinations, the diagnosis and follow up after amniotic membrane transplantation.

No complications were encountered for the experimental period. Interestingly, further improvement was detected with some degree of stromal regeneration also between the 7th and the 20th day post operation with significant increase of the corneal thickness (Figure 24).

The results of slit-lamp biomicroscopy and according to the data obtained from the IVCM and AS-OCT demonstrate that that patients with serious significant ocular surface disintegration, where classic amniotic membrane transplantation would not be sufficient, can profit of corneal stabilization and successful epithelization when transplanted with the bioproduct Alloheal®.

5 DISSCUSSION

5.1 AMNIOTIC MEMBRANE

The human amniotic membrane possesses properties that has made it sought-after. The amnion and chorion are part of the extraembryonic membranes that protect the fetus inside the womb. The amnion is the most inner semitransparent layer that covers the placenta on the side of the umbilical cord and serves as a nonimmunogenic barrier between the mother and fetus during pregnancy, which includes the absence of inducing an immune reaction and having an anti-inflammatory effect which lately makes transplantations without host rejection possible (Lei, Priddy, Lim, & Koob, 2017).

Due to its multiple unique medicinal properties, the human amniotic membrane was applied in variety of medical fields, but with increasing emerging knowledge with regard to the risks associated with the potential for infectious disease transmission and storage difficulties, its use declined (Zelen, Serena, Denoziere, & Fetterolf, 2013). Also in ophthalmology the amniotic membrane transplantation for ocular surface reconstruction, that was first described by DeRötth in 1940 (de ROTTH, 1940), was most likely abandoned due to difficulties in safely harvesting the membrane and due to the only possibility to use the membrane as fresh untreated material. However, on the credit of Tseng and his research group in 1995 (J. C. Kim & Tseng, 1995b), this method was again perceived and optimized. In that respect with current understanding, the newly preservation methods and knowledge based on extensive studies, the use and preparation of the human amniotic membrane become safer with guaranteed quality (H S Dua, Saini, Azuara-Blanco, & Gupta, 2000) (S. C. Tseng, Prabhasawat, Barton, Gray, & Meller, 1998). Although regrettably there is still no gold standard or defined standardized protocol for the collection, preparation and preservation of human amniotic membranes for further use in regenerative medicine, additional studies with more precise experimental designs and larger case numbers are necessary to determine the most accurate and best technique for preparing and preserving human amniotic membrane while maintaining its profound properties (Ramuta & Kreft, 2018).

With the increase in knowledge concerning the amniotic membrane and success in repairing corneal surface defects the spread of amniotic membrane transplantation in ophthalmic practice has been ascending over the past two decades. The membrane has been applied in the treatment

of various diagnosed corneal diseases as persistent epithelial defects, band keratopathy, bullous keratopathy, chemical or thermal ophthalmic burns, pterygium, Stevens-Johnson syndrome, corneal ulcers in which amniotic membrane transplantation displayed a positive effect (Sippel, Ma, & Foster, 2001) (Gabrić, Mravičić, Dekaris, Karaman, & Mitrović, 1999) (Harminder S Dua & Azuara-Blanco, 1999) (Azuara-Blanco, Pillai, & Dua, 1999) (Kruse & Meller, 2001) (Honavar, Bansal, Sangwan, & Rao, 2000). In partial limbal stem cell-deficient corneas human amniotic membrane has been able to replace a limbus transplantation for reconstructing the corneal epithelial surface (Sangwan, Matalia, Vemuganti, & Rao, 2004).

Particularly suitable for the use as patch or graft material is the amniotic membrane due to its low immunogenicity (Kubo, Sonoda, Muramatsu, & Usui, 2001) with facilitation of epithelial healing (Insausti et al., 2010) by the secretion of epithelialization growth factors like epidermal growth factor (EGF), TGF-α, KGF, HGF, bFGF and other factors (Noriko Koizumi, Inatomi, Quantock, et al., 2000) (Jin, Kim, Han, & Kim, 2016). Recently the human amniotic membrane has been further utilized in regenerative medicine as natural cell seeded scaffold material for the ex vivo cultivation and transplantation of corneal limbal epithelial cells (Sippel et al., 2001). The fundamental concept behind this emerging technology is to transplant laboratory cultured human amniotic membranes which carry as many limbal stem cells as possible to compensate for the stem cell deficiency of the patient and to prepare a basis for ocular surface reconstruction, to replenish the limbal epithelial cell population and to achieve subsequent permanent epithelialization, where conventional amniotic membrane transplantation is unable to provide adequate therapy.

The application technology of transplantation of ex vivo expanded human limbal epithelial cells on human amniotic membrane has shown success in restoring the structural and functional integrity of the corneal surface in patients with unilateral total limbal stem cell deficiency (Shortt, Tuft, & Daniels, 2010). Further studies of this new technique of transplantation of limbal epithelial cells cultured on amniotic membrane have shown similar promising success, but at this juncture it must be considered that the number of study cases is small and further larger studies are needed (R. J.-F. Tsai et al., 2000) (Nakamura, Inatomi, et al., 2004) (Ivan R Schwab, Reyes, & Isseroff, 2000) (Shimazaki et al., 2002) (Grueterich, Espana, & Tseng, 2003). An essential foundation for this dynamic development has been provided by the cryopreservation preparation method within the past 25 years, that enabled to preserve the biological properties of the human amniotic membrane, to maintain the native tissue integrity decisive for clinical success (E. K. Tan et al., 2014) and to decreased the risk of disease transmission and to form an optimal biological graft material (Maral et al., 1999). Next to

cryopreservation, which has become widely accepted as it meets the preparation requirements (Le & Deng, 2019), also other preparation methods as sterilization, freeze-dried (lyophilized) (Nakamura, Yoshitani, et al., 2004) air-drying (Singh, Gupta, Kumar, Kumar, & Chacharkar, 2003), or use of unpreserved fresh amniotic membrane (Chugh, Jain, & Sen, 2015) are mentioned. However, fresh untreated amniotic membrane cannot be applied in clinical practice due to the risk of infection with blood transmitted infections.

On the account that the amniotic epithelial cells are avital after cryopreservation and the cryopreservation method preserves the biochemistry and structural integrity of amnion (Huang, Tsai, Wang, & Chen, 2020) with continuity of the basement membrane that provides support of epithelial differentiation and influences the adhesion of the epithelial cells (von Versen-Höynck, Syring, Bachmann, & Möller, 2004) the cryopreserved amniotic membrane has shown to be clinically effective and safe to suit our and also from other researchers the requirements as an excellent substrate for the expansion of epithelial cell (Thomasen, Pauklin, Steuhl, & Meller, 2009).

Unfortunately, the methods for human amniotic membrane preparation for the cultivation and transplantation of human limbal epithelial stem cells differ and no standardized protocol is defined as gold standard, which is objected by several research groups (Shortt et al., 2009) (Shahdadfar et al., 2012) (Dhamodaran et al., 2016) (von Ferenczy & de Souza, 2020).

Differences, which also complicate the further development of the bioproduct, include variations in cryopreservation techniques, mounting the amniotic membrane, uncertainty about ideal technique for harvesting limbal epithelial cells and cultivating limbal epithelial cells on the amniotic membrane. Furthermore, there is the fundamental consideration if decellularisation, removal of the amniotic epithelial cells of the human amniotic membrane for ex- vivo limbal epithelial stem cell expansion, influences the growth conditions of the limbal cells. Over the past decades numerous variations have been tested by removing the amniotic epithelium mechanically or enzymatically and preparing procedures are highly variable (Hopkinson et al., 2008) (Noriko Koizumi et al., 2007) (Li et al., 2006) (N. Koizumi et al., 2000) (Fatima et al., 2006) (Shimazaki et al., 2002), whereby some studies have reported benefits of using intact human amniotic membrane for ex vivo cell expansion for ocular surface reconstruction processes (Grueterich & Tseng, 2002) (Touhami, Grueterich, & Tseng, 2002) (C. Shao et al., 2004) with the intact membrane epithelium containing higher levels of epidermal , keratinocyte , hepatocyte and fibroblast growth factors than the amniotic membrane that has been decellularized (Burman & Sangwan, 2008). To date, the information available in the literature is not fully conclusive in this respect and both decellularisation and intact human

amniotic membrane seem to be utilizable for this process (von Ferenczy & de Souza, 2020) (Ana C. Mamede et al., 2012). According to Koizumi and colleagues (N. Koizumi et al., 2000), naked human amniotic membrane provides superior corneal epithelial cell colonization, with faster cell migration and more regular growth than on intact amniotic membrane.

Since the decellularized human amniotic membrane appears to be with the present state of knowledge the superior method of choice for the generation of tissue-engineered amniotic membrane substrate for ex-vivo cultivation (von Ferenczy & de Souza, 2020) with the capability to maintain the structural and molecular integrity of the basement membrane in contrast to EDTA- and dispase-based decellularization techniques (Hopkinson et al., 2008), we have applied thermolysin denuded cryopreserved human amniotic membrane in this study. To retrain the structural and functional component of the extracellular matrix for ideal promotion of cell migration, cell differentiation and cell adhesion with uniform pattern of growth, also other studies demonstrated superiority with the use of decellularized human amniotic membrane (N. Koizumi et al., 2000) (Noriko Koizumi et al., 2007) (Shortt et al., 2009). Although there are various methods for de-epithelialization with agents such as ethylenediaminetetraacetic acid (EDTA) and Trypsin-EDTA, dispase, urea (Bandeira et al., 2019) or ethanol, the method of de-epithelialization with thermolysin has gained widespread acceptance (Hopkinson et al., 2008) (von Ferenczy & de Souza, 2020). Microscopically, we can confirm the observation of numerous authors, that the thermolysin-based technique effectively denudes the human amniotic membrane. The H&E and Diff-Quik staining's performed in this study showed that a monolayer of amniotic epithelial cells was well kept on the untreated intact human amniotic membrane, whereas these cells were completely removed on the thermolysin-treated amniotic membrane. In view of all of the remarkable properties the human amnion entails, it may provide the ideal support scaffold for further development of new treatment options in tissue engineering. With its mentioned properties of antibacterial, anti-inflammatory, antiviral, anti-angiogenic, antifibrotic, anticancer and immunological characteristics, the membrane can be used in a wide range of clinical applications, and there is little doubt that with continued studies we will learn more and see that the full potential of the amniotic membrane has not yet been completely realized. What is especially important and should not be underestimated is that the amniotic membrane, along with its human amniotic mesenchymal stromal cells (hAMSCs) and human amniotic epithelial cells (hAECs) with pluripotent properties (Pozzobon, Piccoli, & De Coppi, 2014), does not arouse ethical conflicts when deployed as a biological transplant product (Ghamari, Abbasi-Kangevari, Tayebi, Bahrami, & Niknejad, 2020) (Ana C. Mamede et al., 2012).

The fact that this unlimited source of biological tissue and cells are harvested from usually discarded material causing no ethical considerations and are inexpensive without causing harm to a single living organism, makes the human amniotic membrane unique to contribute new approaches in regenerative medicine for the development of novel therapeutic approaches.

Over the last decades the application range of amniotic membrane transplantations within ophthalmic treatments has expanded, with increasing understanding and improvement in ocular surface management. The human amniotic membrane and the human amniotic membrane derived cells have contributed to this, with its characteristic and properties that make them qualified for application in regenerative medicine in ocular surface reconstruction surgery. However, understanding of human amniotic membrane is not yet complete but shows beneficial usage in challenging ophthalmic situations. Unfortunately, treating severe anterior ocular surface disorders especially in combination with limbal stem cell deficiency is still a struggling challenge, which makes the quest for new approaches in regenerative and reconstructive surgery essential and indispensable.

We have set ourselves the task to integrate the readily available, inexpensive, and naturally biocompatible amniotic membrane for ex vivo limbal stem cell cultivation in ophthalmic tissue engineering and regenerative medicine in Bulgaria. We have successfully prepared the human amniotic membrane for the use as a scaffold for ex vivo limbal stem cell cultivation by cryopreservation and de-epithelization by a safe and efficient method to contribute to research in ophthalmology. This is the first study in Bulgaria to present the human amniotic membrane as a scaffold for ex vivo limbal stem cell cultivation on cryopreserved denuded human amniotic membrane and forming the bioproduct Alloheal® for the treatment in anterior ocular surface disorders. We are convinced that the amniotic membrane serves as a perfect scaffold material for ex vivo expansion of limbal epithelial cells with the ability to be transplanted collaborative with cultivated limbal stem cells with a promising future in tissue engineering and that we use the amniotic membrane far from its available potential. We desire, that in the near future we are able to recast and further develop our knowledge with further elaborated preparation techniques and scaffold strategies to optimize the use of the human amniotic membrane and its beneficial properties in ophthalmology and other medical fields.

5.2 CLINICAL CONCLUSION FOR MOUNTING THE AMNIOTIC MEMBRANE FOR TISSUE ENGENEERING

Alloheal® is the first bioproduct in Bulgaria manufactured by cultivating limbal epithelial stem cells derived from allogenic, corneoscleral donor tissue on cryopreserved denuded human amniotic membranes for treating anterior ocular surface disorders with a self-customized developed uniform carrier. Since the amniotic membrane has been utilized as a carrier in tissue engineering, its handling as a biomaterial in everyday clinical practice presents with fundamental challenges from basic management and the demand for an appropriate mounting device, suitable for simplified and safe handling in cell seeding and operative procedure. Choosing an appropriate mounting device to ensure quality for optimal cell growth and further use is a significant and still pending problem. We provided a collection of comparative approaches and an alternative for amniotic membrane fixation by mounting the amniotic in a self-customized carrier device. In summary, the results presented here, show that the use of a relatively expensive, commercially available mounting device is not entirely necessary. An equal comparison of a uniform carrier and commercially available mounting device regarding safety, less traumatic mounting procedure, stability for viable cultivation, transportation, and surgical application has not been performed to date in Bulgaria. Though, the demand and interest for biomaterials and tissue engineering is increasing, promoted by promising results, the mounting devices are given little attention or are not discussed in detail in multitude of publications. Only a few have addressed this issue and developed new alternatives (Harkin et al., 2017). This makes comparisons and further development for optimal handling of the amniotic membrane difficult. Therefrom we conclude that the most research groups use the conventional devices available on the market. The few commercially available products for biomaterial fixation that are readily available are the CellCrown cell culture inserts from Scaffdex and Snapwell cell culture inserts from Corning/Costar (New York, NY, USA) that are provided by Sigma-Aldrich (St Louis, MO, USA) (Harkin et al., 2017). However, the two widely available products (e.g. Snapwell and CellCrown) have restrictions regarding our purpose by application of the selected tissue, sealing properties, cell seeding. The restrictions noticed according to our required purposes with the universally available mounting device *(CellCrown, Sigma-Aldrich)* encouraged us to design our own prototype model for the attachment of the amniotic membrane for properly mounting and cell seeding and to form a bioproduct composed of a cryopreserved denuded human amniotic membrane with seeded

allogenic limbal epithelial stem cells supported by a flexible ring device, not only for safe and easy mounting and cell cultivation but also suitable for safe and handy operative handling for ocular alignment.

Our aim was to develop a mounting device for the human amniotic membrane carrier for the bioproduct Alloheal® with regard to our demands, that is suitable for every day clinical practice for easy and safe handling, hold the membrane and keep the limbal epithelial stem cell during culture, transportation, and surgical transplantation. The commercially available cell culture inserts *(CellCrown, Sigma-Aldrich)* was selected by us because it came closest to our requirements as a mounting device available in advance with its removable collar for securing the membrane and access to the culture surface. It was not only important to us to develop a compatible fixation device for human amniotic membrane in tissue engineering, but also to keep the expenses low and to make ourselves independent by using basic sterile materials which are available in every day clinical practice.

Although this was far from our imagination when we started this development a few years ago, we were faced with challenges in year 2020/2021 with the existing pandemic and suddenly the global world's ability to order materials quickly was no longer accessible as it used to be. Our flexible mounting ring device consists of a simple tube of a standard butterfly needle set, cut in the appropriate length, and connected by surgical sutures. The size of the ring mounting device was therefore easier to personalize compared to prefabricated devices, adaptable to the eye disease being treated and in this regard the size required.

The diameter of the ring we selected with 2,86 cm and a surface area of 6,44 cm² was able to be fitted in appropriately sized 6-well cell culture plates and subsequently seed with limbal epithelial stem cells. With the pull over technique of the pre-aligned membrane on the nitrocellulose paper twisting and realignment as well as searching the correct membrane side is prevented. This reduces the risk of tearing of the membrane and the membrane can be aligned with the appropriate stress across the membrane surface for cell cultivation. The membrane is sutured to the flexible ring device without strong tension allowing the membrane to form a slight downward bent position with the limbal tissue piece placed at the liquid-air interface, prevented from floating away and giving the membrane a degree of flexibility to the cells proliferating along the membrane surface to avoid the formation of holes. This mounting ensures a stable anchored membrane during culture, inspection, and transportation to the operating site with sufficient yielding to prevent the membrane from tearing through the pulling force of the proliferating cells. We provided a collection of comparative approaches and an alternative for amniotic membrane fixation within a self-customized, flexible carrier device.

The ability to directly fit the designed mounting device with the membrane inside the cell culture plate and also to have the possibility to adjust the size of the ring is a very desirable option. A significant benefit is the direct access to examine the cell proliferation within these culture plates macroscopically and microscopically, thus contributing to reduce the risk of contamination associated with repeated contact for inspection or transfer to the operation site. The round, flexible shape of the amniotic membrane carrier device also showed to be superior to the other commercially available mounting method in terms of operational handling with regard to transfer to the ocular surface, adjustment for cutting and cutting and adaptation. Even forming the self-customized flexible device was more time-consuming, it provided better surgical handling. Considering the importance of reduction of the surgical time, this method was seen as beneficial investment. With the flexibility and adjustability, it improved surgical handling and the rapid transplantation of a wrinkle-free bioproduct carries a decreased risk of trauma to the cultivated limbal stem cells and the ocular surface.

We see the research and further development on the fixation device as the essential basic and fundamental framework for the success of the novel technology in tissue engineering. In our hands, with achieving the requirements for good handling, we see this as a prototype of a clinically applicable device that supports the ex vivo cultivation of limbal stem cells on the amniotic membrane and accelerates further development.

5.3 ALLOHEAL®

Ever since the role of the amniotic membrane in management of ocular surface reconstruction surgery was established, a variety of concepts have been introduced to treat ocular surface disorders and limbal stem cell deficiencies. Since the first amniotic membrane application in 1910 (Davis, 1910) and 1940 (de ROTTH, 1940) in the field of ophthalmology, consistently improving results were achieved by various investigators in the demanding treatment of ocular surface disorders. With the establishment of tissue cryopreservation in the mid-1900s (J. C. Kim & Tseng, 1995b), the new possibility to preserve the amniotic membrane for long-term material storage opened a new opportunity for not only application on request, but also the possibility to transform the amniotic membrane into a variety of bioproducts and medical devices. Application of fresh tissue has been limited to short usage time, allograft unsuitable, and in respect of diseases transmission not applicable. Different approaches were investigated to preserve amniotic membrane properties and to ensure longer storage, such as dehydration, freeze-drying, fresh storage, and cryopreservation to create reliable and consistent quality and preservation of the tissue. Consequently, cryopreservation enabled the amniotic membrane to be kept after preservation, with the possibility to preserve histological, biochemical and functional properties of the membrane (Kannaiyan et al., 2016). This led to the rising interest and progression in transplantation medicine, providing a basic foundation for ophthalmic indication (Y. Shao et al., 2020). Beneficial properties of the amniotic membrane make the membrane particularly suitable for applications in ocular surface reconstruction. On the basis of these properties, the amniotic membrane has emerged as an ideal source for ocular surface reconstruction of epithelial defects including corneal degenerative disorders with associated limbal stem cell deficiency. Approaches to treat severe limbal stem cell deficiency have been gaining increasing attention since the pivotal role of limbal stem cells in maintaining ocular surface integrity and transparency (Vemuganti et al., 2004) was discovered. With the newly advancements in understanding corneal epithelial stem cell growth and ocular surface restoration, new therapeutic approaches came into view with the aim to treat limbal stem cell deficiency by restoring the stem cell function, ocular surface integrity and vision with performing reconstruction surgeries like cadaveric allogenic limbal transplantation (D. T. H. Tan, Ficker, & Buckley, 1996), living related or unrelated allogenic limbal transplantation(R. J. F. Tsai & Tseng, 1994), autologous limbal transplantation (Keivyon & Tseng, 1989), amniotic membrane transplantation (Kheirkhah, Casas, Raju, & Tseng, 2008) (Honavar et al.,

2000) and lately transplantation of ex-vivo cultured corneal epithelial stem cells (Ivan R Schwab et al., 2000) (Sangwan et al., 2011). Transplantation of autologous limbal tissue in patients with unilateral limbal stem cell deficiency has been proven to be a useful option in ocular surface reconstruction with reported successful results (Kenyon, 1989) (Copeland & Char, 1990) (Morgan, 1996). However, required large limbal-cell grafts collection carries the potential risk of limbal cell deficiency in the unaffected eye when the central corneal epithelium is removed (R. J.-F. Tsai et al., 2000), demonstrated by a study of Chen and Tseng (Chen & Tseng, 1990) in a rabbit model. To circumvent complications of contralateral eye and to treat severe stem cell deficiencies where limbal stem cell biopsy is not possible in bilateral ophthalmic involvement, allogenic cultivated corneal epithelial transplantation is the considered option (Noriko Koizumi et al., 2001). Since the emergence of such new promising techniques in means of tissue engineering, the amniotic membrane has proven in several studies to be the most promising carrier device (Ishino et al., 2004) (Niknejad et al., 2008) (Zhang et al., 2016). Unfortunately, treatment of severe anterior ocular surface disorders especially in patients with limbal stem cell deficiency is nowadays still a challenging topic for the physician and the patient. Previous studies showed that ex vivo expanded limbal epithelial cells on human amniotic membrane have the ability to recover the corneal surface in these patients (Ivan R Schwab et al., 2000) (R. J.-F. Tsai et al., 2000) (Noriko Koizumi, Inatomi, Quantock, et al., 2000). Therapeutic approaches for treating patients with complex corneal surface disorders have been revolutionized by new opportunities with the introduction of tissue engineered bioproducts. Confronting the emerging challenges in structural ocular surface restoration of significant ocular surface disintegrations the current study tried to utilize the current biological therapies and to apply them to the anterior ocular surface and compare them with conventional amniotic membrane transplantation by in vivo confocal microscopy. The bio-engineered bioproduct manufactured by cryopreserved denuded amniotic membrane with cultured cadaveric allogenic limbal stem cells fixed on a self-customized prototype mounting device was transplanted to the anterior ocular surface for corneal surface reconstructing. Due to difficulties to observe the cornea through the amniotic membrane by slit-lamp biomicroscopy in vivo confocal microscopy for observation of the corneal structures covered by the amniotic membrane has been performed. In vivo confocal microscopy has demonstrated to have advantages for clinical examination and compare the corneal healing response, without being limited by the opaque human amniotic membrane. The layers could be clearly observed through the amniotic membrane by confocal microscopy. At microstructural level, therapy with the bioproduct showed an overall increase in corneal superficial epithelial cell density and in

corneal basal epithelial cell density with an increase in corneal thickness with some degree of stromal regeneration. In this study we have provided prove, also on microstructural level, that the cryopreserved denuded human amniotic membrane is an optimal innovative matrix for ex vivo cultivation of limbal epithelial stem cells. When transplanted jointly to the anterior ocular surface Alloheal® shows to be a promising method getting towards the approach of the tissue engineering application for reconstruction of complex anterior surface disorders complicated by limbal stem cell deficiency.

6 CONCLUSION

Persistent anterior ocular surface disintegrations still remain a therapeutic challenge in the field of ophthalmology where surgical intervention is frequently required (Kruse et al., 1998). With the introduction of human amniotic membrane transplantation, a valuable tool for new approaches for enhanced therapy have been disclosed. Studies proved that epithelial wound healing is facilitated and stromal inflammation and scarring as well as new blood vessel formation is reduced by amnion graft transplantation (Kruse et al., 1998) (Harminder S Dua et al., 2004) (Harminder S Dua & Azuara-Blanco, 1999) (Sippel et al., 2001). However, several studies were conducted (D. F Anderson, Ellies, Pires, & Tseng, 2001) (Daniel Meller et al., 2000) describing the necessity of combined limbal epithelial stem cell transplantation with classic human amniotic membrane transplantation in case of a present total limbal stem cell deficiency, were classic amniotic membrane transplantation may not achieve satisfactory success. These have given rise to a demand for novel approaches of treatment concepts on the basis of cell biology with focused glance at tissue engineering and its potentials. In this respect, development of tissue engineering products showed a great upswing in recent years (Karamichos, 2015) (Akter, 2016).

In this framework, the aim was to further refine the new cell- and scaffold-based therapies with the development of a prototype of a medical device/biological therapy and to apply the bioproduct to the ocular surface for structural restauration.

Most reported studies in literature indicate promising results of amniotic membrane use as a substrate for ex-vivo expansion of corneal and conjunctival epithelium (Shortt et al., 2009) (Noriko Koizumi, Inatomi, Quantock, et al., 2000) and also of limbal stem cells transplantation (Ivan R Schwab et al., 2000) (Noriko Koizumi et al., 2001) (Pellegrini et al., 1997) (Nakamura et al., 2003) (Shortt et al., 2008) according to the cultivation of corneal epithelium as an alternative in patients with difficult ocular surface disease, which corresponded to our result.

To our knowledge, this study is the first one in Bulgaria forming a tissue engineered bioproduct composed of ex-vivo expanded cadaveric corneal limbal stem cells on cryopreserved thermolysin treated human amniotic membrane mounted on a feasible carrier device for ocular surface reconstruction.

We see the research and further development on the fixation device as the essential basic and fundamental framework for the success of the novel technology in tissue engineering. In our

hands, with achieving the requirements for good handling, we see the customized ring as a prototype of a clinically applicable device that supports ex vivo cultivation of limbal stem cells on the amniotic membrane and accelerates further development.

Further we demonstrated, that application of the tissue engineered bioproduct Alloheal® has a positive effect on the healing process of severe damaged ocular surface and its application is indispensable and promising for corneal reepithelization and stabilization with increase in superficial and basal epithelial cell density. After comparing the bioproduct and classic amniotic membrane transplantation on microstructural level, the bioproduct showed that in patients were amniotic membrane transplantation would not be sufficient, an increase in corneal thickness and stabilization of the epithelium could be achieved. With the present number of cases and constellation, the Wilcoxon rank sum test was the test of choice to asses these differences for significance. This could be shown with a $p < 0,05$. Patients transplanted with Alloheal® showed in the preoperative with the 7^{th} day postoperative comparison an increase in corneal thickness with a $p = 0,005$, increase in basal epithelial cell density ($p = 0,005$) and an increase in superficial epithelial cell density ($p = 0,025$) concluding a successful treatment outcome with corneal reepithelization and thus stabilization of the epithelium.

Further the results of present studies highlight the important role of the amniotic membrane for the treatment of anterior ocular surface degenerations and dystrophies, and like further studies (Jalbert, Stapleton, Papas, Sweeney, & Coroneo, 2003) (Shortt, Secker, Munro, et al., 2007), this study results concurs that the diagnostic modality utilizing in vivo laser-scanning confocal microscopy (IVCM) and anterior segment optical coherence tomography (AS-OCT) are prospective techniques that support the guidance and the therapeutic management of severe anterior ocular surface disorders in particular in correlation with limbal stem cell deficiency. The IVCM demonstrated to be a valuable method for corneal cell density evaluation preoperative but also postoperatively for treatment response evaluation with human amniotic membrane transplanted to the ocular surface.

Altogether, others and our work demonstrated that the application of tissue engineered bioproducts has brought about essential advances in the reconstructive surgery of the ocular surface. The impact of amniotic membrane transplantation with ex vivo cultivated limbal epithelial stem cells can be effectively assessed by in vivo confocal microscopes.

This study demonstrated, in vivo confocal microscopic parameters, including the corneal superficial epithelial cell density and basal epithelial cell density were improved after treatment with the biological therapy.

The Alloheal® graft shows to be effective in promoting re-epithelization, which results in stabilization of the epithelium and increase in corneal thickness, proving that Alloheal® is an indispensable, efficient, and effective promising therapeutic approach for the management of nowadays still challenging anterior ocular surface disorders.

In the future, in vivo confocal microscopic parameters, in combination with optical coherence tomography, may assist in assessing the effectiveness of the treatment for severe anterior ocular surface disintegration. Furthermore, in vivo confocal microscopy can be used to evaluate the efficacy of other new treatment options of tissue engineered bioproducts.

We are convinced that the amniotic membrane has a promising future in tissue engineering especially in regard of cell- and scaffold-based therapies and that we use the amniotic membrane far from its available potential. With a glimpse to the near future, we see an emergence of tissue engineered bioproducts not only in ophthalmology, but also in a wide range of medical specialties and a further development in our knowledge with advanced elaborated preparation techniques and scaffold strategies with optimized utilization of the human amniotic membrane.

7 SUMMARY

In recent decades, a high level of interest has been obtained in the development of tissue-engineered products in various fields of medicine (Akter, 2016). Interest in the development of tissue-engineered products has been particularly high in the field of ophthalmology, with considerable advancement (Sun, O'Connor, Wood, Casson, & Selva, 2017). In the field of corneal reconstructive surgery, novel tissue engineering (TE) approaches with innovative cell-based therapies have recently been emerging to compensate or replace specific cells within the environment. Impaired vision and blindness are a worldwide public health issue that impacts quality of life and increases the need for progressed treatment therapies (Adelson et al., 2021).

Ever since the role of the amniotic membrane in management of ocular surface reconstruction surgery was established, a variety of concepts have been introduced to treat ocular surface disorders and limbal stem cell deficiencies.

The amnion, or amniotic membrane, is the innermost layer of the placenta, is derived exclusively from fetal origin (Harminder S Dua et al., 2004) and consists of three main histological layers partitioned into an inner simple epithelium, an intermediate thick basement membrane, and an outer avascular stroma (S. C. G. Tseng et al., 2004) (Toda et al., 2007) (George et al., 2018). Since its first application as a dressing material in ocular treatment by de Rotth (de ROTTH, 1940) and Sorsby (Sorsby & Symons, 1946) (Sorsby, Haythorne, & Reed, 1947) and over the course of time as a graft by Kim and Tseng (J. C. Kim & Tseng, 1995b) the human amniotic membrane has been broadly implemented for a wide variety of ocular surface disorders (Malhotra & Jain, 2014). Transplantation of the human amniotic membrane, whether used as a patch or graft, has been demonstrated in numerous studies to be effective in ocular surface reconstruction of corneal ulceration (Jinghua Liu, Li, & Li, 2019) (Hanada, Shimazaki, Shimmura, & Tsubota, 2001), persistent epithelial defects and perforations (Azuara-Blanco et al., 1999) (Su & Lin, 2000) (Lee & Tseng, 1997) (Letko et al., 2001) , chemical and thermal burns (J. S. Kim, Kim, Na, Jeong, & Song, 2000) (Sridhar, Bansal, Sangwan, & Rao, 2000), bullous keratopathy (Espana et al., 2003) (David F Anderson, Prabhasawat, Alfonso, & Tseng, 2001) (Pires et al., 1999), pterygium (Prabhasawat, Barton, Burkett, & Tseng, 1997) (Jain, Bansal, & Sukhija, 2008) and partial limbal stem cell deficiency (Sangwan et al., 2004) (D. F Anderson et al., 2001) (S. C. G. Tseng, Prabhasawat, Barton, Gray, & Meiler, 1998). Its success

with favorable clinical outcomes in corneal surface management are attributable to the amniotic membrane's biomaterial properties of anti-inflammatory (Shimmura et al., 2001) (Harminder S Dua et al., 2004) (Solomon et al., 2001) (Hao et al., 2000), anti-angiogenic (J. C. Kim & Tseng, 1995a) (Hao et al., 2000) , anti-scaring effect (S. C. G. Tseng et al., 1999) and enhanced epithelization (M. R. Kesting et al., 2008) that contribute to its widespread use. Due to its unique biochemical and mechanical properties the cryopreserved human amniotic membrane is gaining increasing significance in the field of tissue engineering. There is a rising demand in novel materials for biological scaffolds in regenerative medicine and tissue engineering. With its unique characteristics, the human amniotic membrane moreover was identified to a conducive biological scaffold for the ex vivo expansion of limbal epithelial cells (Pellegrini et al., 1997) (Grueterich et al., 2002) (Shortt et al., 2008) (Nakamura et al., 2003) (I R Schwab, 1999) . The possibility of applying the human amniotic membrane as a scaffold for ex vivo cultivation of corneal epithelial stem cells (Sippel et al., 2001), which through progressive emerging knowledge is determined as a promising source for corneal regeneration, has transformed the human amniotic membrane at this point in time to the most widely used substrate (Zhang et al., 2016) in the field of ophthalmology. The transplantation of limbal stem cells cultivated on a substrate, first mentioned by Pellegrini et al. in 1997 (Pellegrini et al., 1997), is the novel promising treatment of total limbal stem cell deficiency.

Though reconstructive surgery, especially in the field of ophthalmology, has experienced enormous progress in the past decades by new innovative treatment perspectives, the ocular surface reconstruction and integrity restoration of severe ocular surface disorders, is nevertheless a considerable challenge (Kruse et al., 1998) (Haagdorens et al., 2016).With the introduction of the cryopreserved amniotic membrane in ophthalmic reconstructive surgery (J. C. Kim & Tseng, 1995b) and more recently in tissue engineering, the human amniotic membrane has acquired an essential component in the treatment of various diseases of the ocular surface (Daniel Meller et al., 2011). This surge is attributed to the human amniotic membrane's unique and exclusive properties, which cannot be attributed to another membrane. Its inherent characteristics of stem cells with multipotent differentiation ability(Koike et al., 2014) (Wassmer & Berishvili, 2020), which contributes to its superior physical and biological properties, provide a basic foundation for ophthalmic utilization (Y. Shao et al., 2020). The amniotic membranes with its two kinds of cells, the human amniotic epithelial cells and human amniotic mesenchymal cells (Koike et al., 2014) (Wassmer & Berishvili, 2020) and specific structure, offers beneficial properties that make the membrane particularly suitable for applications in ocular surface reconstruction. However, not to be underestimated is the fact that

amnion-derived cells are considered to be a highly beneficial cell source for cell therapy in future clinical application in regenerative medicine (Parolini, Soncini, Evangelista, & Schmidt, 2009). The human amniotic membrane has shown to be a desirable source for stem cells with multi-differential potential (Koike et al., 2014) (Wassmer & Berishvili, 2020), immunomodulatory characteristics (Parolini et al., 2009), with low immunogenicity without signs of immune rejection when transplanted (Akle et al., 1981) (Malhotra & Jain, 2014) (Bailo et al., 2004), with anti-inflammatory (Shimmura et al., 2001), anti-microbial (Talmi et al., 1991), anticarcinogenic properties (Seo et al., 2008) and ethically unobjectionability, as it is usually discarded after delivery (Koike et al., 2014). On the basis of these properties, the amniotic membrane has emerged as an ideal source for ocular surface reconstruction of epithelial defects, including corneal degenerative disorders with associated limbal stem cell deficiency. Approaches to treat severe limbal stem cell deficiency have been gaining increasing attention since the pivotal role of limbal stem cells in maintaining ocular surface integrity and transparency (Vemuganti et al., 2004) was discovered. Thoroughly processed by cryopreservation, the biological properties are retained and making the human amniotic membrane the optimal candidate for application in ocular surface reconstruction by promoting epithelialization (M. R. Kesting et al., 2008), reducing the risks of postoperative infection by its antimicrobial properties (Talmi et al., 1991), its low immunogenicity without expressing cell surface proteins HLA-A, B, C, DR antigens or β2-microglobulin, responsible for the regulation of the immune reaction (Akle et al., 1981), and suppressing stromal inflammation, angiogenesis and scarring (Jingbo Liu et al., 2010). This opened the possibility to safely utilize the pretreated amniotic membrane for the successful use in variety of medical fields. To date, the human amniotic membrane is most widely used in ophthalmic transplantations and the leading biological matrix in ex-vivo cultivation of limbal epithelial stem cells (Shortt, Secker, Notara, et al., 2007) (I R Schwab, 1999) (Noriko Koizumi et al., 2001) (Grueterich et al., 2002) (Nakamura, Inatomi, et al., 2004) (Shortt et al., 2008) (Zakaria et al., 2014). The human amniotic membrane transplantation had proved to be successful for corneal surface restoration in partial limbal stem cell deficiency (D. F Anderson et al., 2001) (Nakamura, Inatomi, et al., 2004), maintaining the surface integrity. Though, once these stem cells, described by Tseng (S. C. G. Tseng, 1996) as the ultimate source of corneal regeneration, are depleted, the state of total limbal stem cell deficiency with pathological signs of conjunctival epithelial cell ingrowth with goblet cells and vascularized corneal stroma with chronic inflammation occurs and amniotic membrane transplantation alone is not sufficient to restore the corneal surface (D. F Anderson et al., 2001) (Daniel Meller et al., 2000). Since the results of conventional limbal transplants

are not entirely satisfactory (Cauchi, Ang, Azuara-Blanco, & Burr, 2008), there was a request for enhanced approaches of amniotic membrane transplantation for ocular surface reconstruction. With the ability of the amniotic membrane to promote epithelization (Noriko Koizumi, Inatomi, Sotozono, et al., 2000) (Lo & Pope, 2009) and the ability of the stem cells cultured on the membrane to retain their in vivo properties of slow cycling, label retaining, and undifferentiation with prolonged epithelial stem cell survival, the amniotic membrane became not only indispensable in a wide-range of ophthalmic applications, but also became the leading substrate for ex-vivo cultivation of the epithelial stem cells of the corneal epithelium (D Meller, Pires, & Tseng, 2002). Since the emergence of such new promising techniques in means of tissue engineering, the amniotic membrane has proven in several studies to be the most promising carrier for human corneal endothelial cells so far (Ishino et al., 2004) (Hassan Niknejad et al., 2008) (Liying Zhang et al., 2016). However, with the progression of the amniotic membrane serving as a carrier for cell cultivation, its handling as a biomaterial in everyday clinical practice gives rise to new requirements and fundamental challenges commencing from basic management with the demand for an appropriate mounting device, suitable for simplified and safe handling in cell seeding and operative procedure. Choosing an appropriate mounting device to ensure quality for optimal cell growth and further use is a significant and still pending problem. To date, there are still no available standardized protocols to ensure optimal graft preparation, handling and mounting for transfer, what makes comparison of current studies more intricate (Daniel Meller et al., 2011).

Facing the arising challenges in structural ocular surface restauration of significant ocular surface disintegrations the aim of the current studies is to implement the novel contemporary biological therapies and selectively apply them to the anterior ocular surface in form of a bio-engineered bioproduct applicable in everyday clinical practice to achieve microstructural integration, proven by in vivo confocal microscopy. One of the important aspects was to develop a suitable uniform carrier and to develop a surgically acceptable modality for transfer and form a bioproduct easy to handle for every day clinic use that is safe, effective, and simple to adjust on the ocular surface and that is an advancement for management complex anterior ocular surface disorders.The bioproduct was manufactured by cultivating limbal epithelial stem cells derived from allogenic, corneoscleral donor tissue on cryopreserved denuded amniotic membranes acting as a matrix, forming Alloheal®, the first bioproduct in Bulgaria for managing complex anterior ocular surface disorders. To develop a suitable mounting device for the human amniotic membrane acting as a scaffold for ex vivo expansion for limbal stem cell

cultivation and further to transplant the membrane with the seeded cells to the ocular surface basic knowledge of the properties of amniotic membrane and of current achievements in novel biological therapies applied on the ocular surface for structural restauration is essential for successful tissue engineering. According to our perspective, in order to provide consistent quality and preservation of the human amniotic membrane for TE, not only is the uniform processing of the amniotic membrane important, but also providing an optimal framework, which contributes to the overall success of ex vivo expansion of limbal epithelial stem cells and surgical handling. A functional framework in form of a mounting carrier device for the scaffold is needed as a support for a suitable transplant membrane providing adequate cell-scaffold interactions to facilitate the processes of the cultivation of limbal epithelial stem cells. With recent advances and expanding presence in the field of corneal epithelial tissue engineering, new approaches have been developed, but also new requirements and questions have emerged alongside seeking responses.

Our requirements were not addressed by conventional mounting devices; moreover, there are a few prototypes but little mature mounting solutions. Success requires that mounting devices for biomaterials for the use in ophthalmic tissue engineering are not only convenient to use, but also cost-effective in manufacture and preferably not dependent in production. With the ambition to develop for novel ophthalmic tissue engineering an innovative mounting device for cell proliferation, we provided an alternative prototype for mounting the human amniotic membrane in a flexible self-customized cost-efficient carrier for every day clinical practice. Consideration was given to the fact that the components of the mounting device were manufactured from easily accessible and cost-effective materials, what we succeeded. At present, in times of a pandemic, this issue of the independence of the required components is an even more prevailing aspect with which we have not recently addressed to this extent. From two sterile everyday medical instruments an amniotic membrane mounting device was created that in our eyes is superior in amniotic membrane positioning and handling with a lower risk of damaging the amniotic membrane and injuring the ex vivo cultured limbal epithelial stem cells. According to the way the biomaterials are mounted, it may be possible to optimize handling and preserve the human amniotic membrane from trauma. The flexible mounting device for ex vivo cultivation of limbal epithelial cells was made from readily obtainable and inexpensive items used in everyday clinical practice. Items needed were the tube from a sterile butterfly needle infusion set, suture material, and optionally, but not necessary, a small piece of wire or a plastic tube, e.g., from a venous catheter set, with a smaller diameter of the tube from a sterile butterfly needle infusion set. Under the laminar flow hood, the homemade carrier was prepared

in 3 steps and formed a ring device with a surface area of 6.44 cm² and a diameter of 2.86 cm. The fixation of the amniotic membrane on the mounting device was prepared 24 hours before inoculation of the membrane with the limbal tissue pieces. Different methods have been investigated to position the amniotic membrane on the device and then to secure the amniotic membrane for the ex-vivo expansion of limbal epithelial stem cells to the customized anchoring device with the focus on improving the amniotic membrane fixation method in regard to further handling in tissue engineering. It was clearly observed that the unmounted human amniotic membrane was difficult to be handled due to its flexibility, therefore, ex vivo cultivation is preferentially performed in a culture mounting system that secures the amniotic membrane in a desired wrinkle-free, stable, horizontal position. Positioning the membrane on the flexible mounting device showed that the approach of not detaching the membrane from the nitrocellulose paper and sliding the flexible ring fixture between the membrane and the paper resulted in the best positioning solution. It eliminated the need for repositioning for the correct membrane side and thus eliminated the risk of trauma and perforation of the membrane, and also saved time lost for repositioning. The outstanding feature of our developed prototype is that it is shaped out of a flexible ring which provides enough stability, but offers adequate flexibility and is made out of convenient everyday clinical equipment, irrespective of commercially available products and designed to be compatible for our requirements for best viable cultivation, transportation, and surgical application. In order to define the carrier devices and their handling in operational situations, we compared a commercially existing insert crown with the customized flexible ring device. To determine the operation handling of the devices, we compared them in the essential steps of transplantation. Even though the self-customized flexible ring device was more time-consuming in anchoring the membrane than using an existing insert crown, the flexible ring saved time by providing more precise surgical application, transfer, and better adjustment at cutting. Decreased surgery time was achieved by overall easier handling and a better surgical outcome was accomplished by reducing complications and trauma to the seeded cells and the ocular surface. Considering the importance of reduction of the surgical time, this method was seen as beneficial investment. Its flexibility and adjustability improved surgical handling and the rapid transplantation of a wrinkle-free bioproduct carries a decreased risk of trauma to the cells and the ocular surface.

In a second part we evaluated utilization before and after biological therapy with regard to the microstructural level by utilizing in vivo confocal microscopy and performed a comparative analysis between classic amniotic membrane transplantation and biological therapy based on amniotic tissue. Patients were examined pre-operative, 1st day post-operative and 7th day post-

operative. Special attention was paid to the corneal superficial epithelial cell density and the corneal basal epithelial cell density, as well to the corneal thickness and amniotic membrane thickness. By in vivo confocal microscopy all layers of the cornea could be visualized at any time of examination and were not drastically restricted by the amniotic membrane. Over the course of the study, all patients showed an increase in corneal superficial epithelial cell density and the corneal basal epithelial cell density and increase in corneal thickness. Patients treated with the bioproduct Alloheal® showed an average increase of corneal thickness of 29 µm with some degree of stromal regeneration. With follow-up examination performed by slit-lamp biomicroscopy, AS-OCT and IVCM we explore the changes of the corneal surface under amniotic membrane transplant and determine a promising therapeutic outcome through corneal epithelialization and stabilization of the corneal thickness.

The promising results demonstrate that patients with serious significant ocular surface disintegration, where classic amniotic membrane transplantation would not be sufficient, can profit of corneal stabilization and successful epithelization after bioproduct transplantation.

With a glance to the future with the new possibilities available through tissue engineering, bioproducts like Alloheal® are a novel promising therapeutic approach for the management of nowadays still difficult treatable ocular surface disorders

8 ABSTRACT

OBJECTIVE/PURPOSE:

The transplantation of the human amniotic membrane has a long tradition in the field of ophthalmic surgery and has become a favorable treatment option in ocular surface reconstruction based on newly evolved knowledge of tissue preservation.
As a biological therapy the human amniotic membrane has recently been introduced in tissue engineering, as a biomatrix for expansion and culturing limbal epithelial stem cells to treat severe ocular surface disintegration for structural restauration.
Our aim was to develop and utilize the contemporary biological therapies forming a prototype of a medical device for easy and safe application in standard surgical procedure and apply them to the anterior ocular surface in order to achieve microstructural integration and describe corneal changes by in vivo confocal microscopy.

METHODS:

The literature was studied on latest human amniotic membrane transplantation evolvements and novelties in tissue engineering for amniotic membrane developments.
The bioproduct Alloheal® was manufactured by cultivated limbal epithelial stem cells derived from allogenic corneo-scleral donor tissue on cryopreserved and thermolysin treated human amniotic membranes. These were mounted within a self-customized flexible ring device and a commercially available insert ring construct to compare surgical handling and to assess a surgically acceptable modality for transfer.
Patients with significant ocular surface disintegration before and after application of the biological therapy Alloheal® and transplantation with classic human amniotic membrane transplantation were accessed and compared at microstructural level, with the utilization of in vivo confocal microscopy *(Heidelberg Retina Tomograph - HRT3 RCM, Heidelberg Engineering GmbH, Dossenheim, Germany)*.

RESULTS:

The customized flexible prototype device for mounting the human amniotic membrane, provided despite the more time-consuming preparation than using an existing insert crown, better requirements for cultivation, transportation, and in essential steps of surgical handling. The flexibility and adjustability improved surgical handling, decreased surgery time resulting in rapid transplantation of a wrinkle-free bioproduct with decreased risk of trauma to the cells and/or to the ocular surface and with considering the importance of reduction of the surgical time the longer preparation time was encountered as beneficial investment.
The transplantation of the bioproduct Alloheal® resulted in a regain in corneal thickness and thus promoting reepithelization and stabilization of the epithelium. Preoperative IVCM displayed an average superficial corneal epithelium density of 638,500 ± 162,274 cells/mm² and an average basal epithelial cell density of 4647,400 ± 652,398 cells/mm². The cells showed immaturity and on the 7th day post-operative the average superficial corneal epithelium density increased to 718,100 ± 142,004 cells/mm². The basal epithelial cell density also increased to 5272,300 ± 642,356 cells/mm². The preoperative corneal thickness was displayed by AS-OCT and was 355,70 ± 97,75 μm in average in patients treated with Alloheal®. The average corneal thickness increased to 385,40 ± 101,40 μm on the 7th day postoperative. The amniotic membrane (AM) thickness was measured to be 252,90 ± 116,90 μm the first day postoperative and decreased to 166,00 ± 72,74 μm on the 7th day. Compared to classic human amniotic membrane transplantation, a greater increase in average superficial cell density and basal epithelial cell density was observed.

CONCLUSION:

Though reconstructive surgery in the field of ophthalmology, has experienced enormous progress in the past decades through novel innovative therapeutic perspectives and tissue engineering, ocular surface reconstruction and integrity restoration of severe ocular surface disintegration, is nevertheless a demanding challenge. In our hands Alloheal® is a safe, effective, and efficient method, clinically meaningful for future advancement for managing complex anterior surface disorders. So far, our proprietary Alloheal® is the first biological product on Bulgarian market and is not perceived as a medical device, because of its specific characteristics. The promising results demonstrate that patients with serious significant ocular

surface disintegration, where classic amniotic membrane transplantation would not be sufficient, can profit of corneal stabilization and successful epithelization after bioproduct transplantation. With a glance to the future with the new possibilities available through tissue engineering, bioproducts like Alloheal® are a novel promising therapeutic approach for the management of nowadays still difficult treatable ocular surface disorders even though further research and development will be needed for better and even more universal products for corneal regeneration.

9 РЕЗЮМЕ

ЦЕЛ:

Трансплантацията на амниотична мембрана е с дълга традиция в областта на очната хирургия и се е превърнала в предпочитан метод за лечение при реконструкция на предната очната повърхност, базиран на новостите в съхранение на тъкани.
Като биологична терапия в лечението и възстановяването на тежки нарушения на предната очната повърхност, амниотичната мембрана наскоро бе въведена в тъканното инженерство, като биоматрица за разтеж и култивиране на лимбални епителни стволови клетки.
Основавайки се на съвременните методи на лечение, нашата цел бе разработването на прототип на медицинско изделие за лесно и безопасно приложение на стволови клетки в стандартна хирургия на предната очна повърхност, за да постигнем микроструктурна интеграция и да опишем промените в роговицата чрез in vivo конфокална микроскопия.

МЕТОДИ:

Проучиха се новостите в тъканното инженертсво при изготвянето и трансплантацията на амниотична мембрана.
Разработи се биопродуктът Alloheal®, произведен от култивирани лимбални епителни стволови клетки, получени от алогенна донорска корнео-склерална тъкан върху криоконсервирана и термолизирана амниотична мембрана.
За оценка на хирургически приемлив начин за трансфер и удобството при манипулиране се сравни специално конструирано, гъвкаво устройство за фиксиране на Alloheal® с предлагани в търговската мрежа пръстеновидни устройства.

Чрез in vivo конфокална микроскопия (Heidelberg Retina Tomograph - HRT3 RCM, Heidelberg Engineering GmbH, Dossenheim, Германия) се извърши сравнение на микроструктурно ниво на пациенти с тежки нарушения на предната очната повърхност преди и след прилагането на биологичната терапия с Alloheal® спрямо прилагането на амниотична мембрана.

РЕЗУЛТАТИ:

Въпреки по-трудоемката подготовка, необходима за адаптиране на амниотичната мембрана към конструираното гъвкаво устройство в сравнение с използването на твърд пръстен,се установиха по-добри условия за култивиране, транспортиране и най-вече за хирургично приложение. Гъвкавостта и възможността за лесна манипулация намаляват оперативното време, спомагат за добра адаптация на биопродукта без гънки и с намален риск от травмиране на клетки и / или на очната повърхност. Вземайки под внимание важността на намаленото хирургично време, по-дългата подготовка на пробукта бе оправдана.

Трансплантацията на биопродукт Alloheal® доведе до възстановяване на дебелината на роговицата и по този начин стимулира реепителизацията и стабилизирането на епитела. Предоперативната IVCM показва средна плътност на повърхностния епител на роговицата от 638 500 ± 162 274 клетки / mm² и средна плътност на базалния епител от 4647 400 ± 652 398 клетки / mm². Клетките бяха незрели и на 7-ия ден след операцията, средната плътност на повърхностния епител на роговицата се увеличи до 718 100 ± 142 004 клетки / mm². Клетъчната плътност на базалните клеки също се е увеличи до 5272 300 ± 642 356 клетки / mm². Предоперативната дебелина на роговицата се изобрази чрез AS-OCT и беше средно 355,70 ± 97,75 μm при пациенти, лекувани с Alloheal®. Средната дебелина на роговицата се е увеличи до 385,40 ± 101,40 μm на 7-ия ден след операцията. Дебелината на амниотичната мембрана (AM) бе измерена на 252,90 ± 116,90 μm през първия ден след операцията и намаля до 166,00 ± 72,74 μm на 7-ия ден. В сравнение с класическата трансплантация на амниотична мембрана се установи по-голямо увеличение на средната плътност на повърхностните клетки и на базалните епителни клетки.

ЗАКЛЮЧЕНИЕ:

Днес реконструкцията на предната очна повърхност и възстановяването на целостта й след тежка дезинтеграция остава предизвикателство, въпреки напредъка в тъканното инженерство и иновативните терапевтични постижения в реконструктивната хирургия.

В нашите ръце Alloheal® е безопасен, ефективен и ефикасен метод, клинично значим в лечението на тежките нарушения на предната очна повърхност. Поради специфичните

си характеристики Alloheal® е първият патентован биологичен продукт на българския пазар, които не е медицинско изделие. Обещаващите резултати показват, че при пациенти с тежко нарушение на предната очната повърхност, при които класическата трансплантация на амниотична мембрана не би била достатъчна, може да бъде постигната стабилизацията на роговицата и успешна епителизация след трансплантация на биопродукта.

С поглед към бъдещето и новите възможности за по-добри и универсални продукти за регенерация на роговицата, достъпни чрез тъканното инженерство, биопродукти като Alloheal® са нов обещаващ терапевтичен подход за лечение на тежките нарушения на предната очната повърхност, въпреки, че ще са необходими допълнителни изследвания.

10 ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Prof. Christina Nikolova Grupcheva, MD, PhD, DSc, FEBO, FICO(Hon), FBCLA, FIACLE for giving me the opportunity and support during these past years.
I will forever be thankful for her guidance and personal encouragement but also for her generosity with which she guided me the entire time. I am grateful for the knowledge she taught me and above all she taught me that only with the heart one can see rightly, and that what is essential is invisible to the eye.

Especially, I would like to thank my colleagues Dr. Mladena Radeva, Dr. Mariya Boyadzhieva, Dr. Dimitar Grupchev and all my colleagues from the Specialized Hospital for Active Treatment in Ophthalmology, Varna who contributed to the project from both the professional and the personal side during the time of my doctoral education and residency with their continuous support. Many special thanks go to Dr. Elitsa Hristova for her professional and competent assistance regarding the statistical part of this work.
I would like to extend my sincere thanks to The Department of Science and Research of Medical University "Prof. Dr. Paraskev Stoyanov" – Varna for their continuous support. I would like to thank all current and former lab members of the Laboratory for Translational Medicine at the University Hospital for Active Treatment St. Marina Hospital, Varna.
Many thanks go also to my friends who were always there in good and in bad times.
I would like to thank my boyfriend for his love and constant support.
And at least I would like to thank my parents, Meglena and Peter Bommert, who have made my studies and thus this dissertation possible and have encouraged and supported me with all their love and strength for which I am forever grateful.

11 RESEARCH AWARD IN RELATION WITH THE DISSERTATION

Laureate of the „Professor S. Dubov" Award of a YOUNG OPHTHALMOLOGIST FOR SCIENTIFIC CONTRIBUTION at the XXXIII National Conference „Innovations in Ophthalmology 2018", 23-25.11.2018, Pravets, Sofia

Topic: Experimental Considerations ad Clinical Applications of the Innovative Bioproduct „ALLOHEAL"

Лауреат за награда „Професор Стоимен Дъбов" на МЛАД ОФТАЛМОЛОГ ЗА НАУЧЕН ПРИНОС – XXXIII Национална Конференцията „Новости в офталмологията 2018", 23-25.11.2018г. РИУ Правец гр. Правец, София

Тема: Експериментални съображения и клинични приложения на иновативен биопродукт „АЛОХИЛ"

12 PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS

PUBLICATIONS

October 2020

ИЗКУСТВЕНИ МИГЛИ – КРИЯТ ЛИ РИСК ЗА НАШИТЕ ОЧИ?

Ас. д-р М. Радева, Д-р М. Стоева, Ас. д-р Д. Групчев, Д-р К. Бомерт, Проф. д-р Хр. Групчева, д.м.н. FEBO, FICO (hon), FBCLA, FIACLE,
Катедра „Очни болести и зрителни науки", Медицински Университет – Варна
Изкуствени мигли – кият ли риск за нашите очи? https://gpnews.bg/изкуствени-мигли (P News – Новини за общопрактикуващия лекар, Date: 27.01.2021)

June 2020

SLEEP APNEA AND THE DRY EYE – HOW DOES SLEEP APNEA AFFECT THE EYE SURFACE

Bommert, C.M., Grupcheva, C., Radeva, M.N., Grupchev, D.I., & Boyadzieva, M.R. (2020). Sleep apnea and the dry eye – how does sleep apnea affect the eye surface:.DOI:10.24292/01.ot.300620.3

October 2019

CATARACT SURGERY - BEHIND THE NUMBERS

Radeva, M., Boyadzhieva, M., Bommert, C., Hristova, E., Boyadzhiev, D., Grupchev, D., Neshkinski, E., & Grupcheva, C. (2019). Cataract surgery - behind the numbers. Bulgarian Review Of Ophthalmology, 63(2), 34-41. doi:http://dx.doi.org/10.14748/bro.v63i2.6097

November 2018

EXPERIMENTAL CONSIDERATIONS AND CLINICAL APPLICATIONS OF THE INNOVATIVE BIOPRODUCT ALLOHEAL

Bommert, C. (2018). Experimental considerations and clinical applications of the innovative bioproduct Alloheal. Bulgarian Review Of Ophthalmology, 62(3), 9-15. doi:http://dx.doi.org/10.14748/bro.v0i3.5547

ORAL PRESENTATIONS

March 2020	Sleep apnea and the dry eye, how does sleep apnea affects the eye *Fourth National Conference on Sleep Medicine, with international participation, Aqua Hotel, Varna*
August 2019	Biomaterials – and bioproducts where is the place of the amniotic membrane? *Fifth Audio-Vestibology and Otology days with international participation Comorbidity in audio vestibular Medicine Second symposium on biomaterials and implants in head and neck region - St. St. Constantine and Elena Resort, Varna*
November 2018	Experimental Considerations and Clinical Application of Alloheal *Конференциа Новости в Офталмологията 2018, Pravez (Sofia)*
October 2018	Management of Floppy Eyelid Syndrome as a Result of Sleep Apnea *Third National Conference on Obstructive Sleep Apnea and Snoring with International Participation - St. St. Constantine and Elena Resort, Varna*

13 CURRICULUM VITAE

PERSONAL INFORMATION

Catherina Meglena Bommert, M.D.

Sachsenring 38, 65843 Eppstein, Germany

00491623300391

catherina@bommert.de

cathi@bommert.de

Sex female | Date of birth 27 February1989| Place of birth Frankfurt am Main

WORK EXPERIENCE

November 2017- Present

PhD Student

Medical University "Prof. Dr. Paraskev Stoyanov" – Varna

Faculty of Medicine, Department of Ophthalmology and Visual Sciences

- Therapeutic approach of the anterior ocular surface – Evaluation with in vivo confocal microscopy

July 2020- Present

Resident Doctor in Ophthalmology

Specialized eye hospital for active treatment – Varna

Специализираната болница за очни болести с активно лечение (СБОБАЛ)

- Supervisor: Prof. Christina Nikolova Grupcheva, MD, PhD, DSc, FEBO, FICO(Hon), FBCLA, FIACLE

March 2018 – July 2020

Resident Doctor in Ophthalmology

Specialized eye hospital – Burgas

Очна Болница "Бургас" - Д-р Иванови, Медицински "Младост"

April 2015- September 2017

Resident Doctor in Internal Medicine

Hochtaunuskliniken – Bad Homburg vor der Höhe

Hochtaunuskliniken – Usingen

- 01.04.2015 – 31.03.2016 Department of Gastroenterology & Hepatology and Infectious Diseases
- 01.04.2016 – 30.09.2016 Emergency Unit Department
- 01.10.2016 – 31.03.2017 General Internal Medicine (Usingen)
- 01.04.2017 – 30.09.2017 Department of Geriatrics

EDUCATION AND TRAINING

21 December 2017

Specialization as Emergency Specialist

Acquisition of the additional title of emergency medicine

15 September 2008 – 29 November 2014

Master Degree in Medicine

Physician in Medicine, M.D.

Medical University "Prof. Dr. Paraskev Stoyanov" – Varna

- MAGNA CUM LAUDE
  Grade point average degree program: very good 4,82
  Grade point average state exams: excellent: 5,8

- Approbation, State Medical Association Hessen Germany
  (Landesärztekammer Hessen, December 2014)
  Approbation *Admission to the National Medical Association Varna, Bulgaria (Български Лекарски Съюз Рк На Блс Варна, November 2017)*

June 2008

Gymnasium Diploma

Friedrich-Dessauer-Gymnasium, Oberstufengymnasium, Frankfurt am Main

- Major subjects with a focus on English and Biology

June 2005 – June 2006

High School Year

Calhoun High-School, Port Lavaca, Texas

PERSONAL SKILLS

Mother tongue(s): German

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
	-				
Bulgarian	95%	100%	92%	92%	48%
	European Consortium for the certificate of Attainment in modern languages – ECL Level B1				

Driving license

- Class B driving license
- American driver's license

AWARD

November 2018

Award in Young Research Competition

Competition for a young researcher "Prof. Dubov "at the conference "Innovations in Ophthalmology 2018"

- Награда в Конкурс за Млад Изследовател, на конференцията „Новости в офталмологията 2018" American driver's license
- На Софийското Офталмологично Дружество Бе Проведен Конкурса за Млад Изследовател „Проф. Дъбов".
- Topic: Experimental Considerations ad Clinical Applications of the Innovative Bioproduct ALLOHEAL
- *https://www.mu-varna.bg/BG/Pages/Bomert-mlad-izsledovatel.aspx* (Medical University, Varna, Date: 27.01.2021)

PUBLICATIONS

October 2020

ИЗКУСТВЕНИ МИГЛИ – КРИЯТ ЛИ РИСК ЗА НАШИТЕ ОЧИ?

*Ас. д-р М. Радева, Д-р М. Стоева, Ас. д-р Д. Групчев, **Д-р К. Бомерт**, Проф. д-р Хр. Групчева, д.м.н. FEBO, FICO (hon), FBCLA, FIACLE,*
Катедра „Очни болести и зрителни науки", Медицински Университет – Варна
Изкуствени мигли – крият ли риск за нашите очи?
https://gpnews.bg/изкуствени-мигли (P News – Новини за общопрактикуващия лекар, Date: 27.01.2021)

June 2020

SLEEP APNEA AND THE DRY EYE – HOW DOES SLEEP APNEA AFFECT THE EYE SURFACE

***Bommert, C.M.**, Grupcheva, C., Radeva, M.N., Grupchev, D.I., & Boyadzieva, M.R. (2020). Sleep apnea and the dry eye – how does sleep apnea affect the eye surface:.DOI:10.24292/01.ot.300620.3*

October 2019

CATARACT SURGERY - BEHIND THE NUMBERS

*Radeva, M., Boyadzhieva, **M., Bommert**, **C.**, Hristova, E., Boyadzhiev, D., Grupchev, D., Neshkinski, E., & Grupcheva, C. (2019). Cataract surgery - behind the numbers. Bulgarian Review Of Ophthalmology, 63(2), 34-41. doi:http://dx.doi.org/10.14748/bro.v63i2.6097*

November 2018

EXPERIMENTAL CONSIDERATIONS AND CLINICAL APPLICATIONS OF THE INNOVATIVE BIOPRODUCT ALLOHEAL

***Bommert, C.** (2018). Experimental considerations and clinical applications of the innovative bioproduct Alloheal. Bulgarian Review Of Ophthalmology, 62(3), 9-15. doi:http://dx.doi.org/10.14748/bro.v0i3.5547*

CONFERENCE PRESENTATIONS

March 2020 — Sleep apnea and the dry eye, how does sleep apnea affects the eye

Fourth National Conference on Sleep Medicine, with international participation, Aqua Hotel, Varna

August 2019 — Biomaterials – and bioproducts where is the place of the amniotic membrane?

Fifth Audio-Vestibology and Otology days with international participation Comorbidity in audio vestibular Medicine Second symposium on biomaterials and implants in head and neck region - St. St. Constantine and Elena Resort, Varna

November 2018 — Experimental Considerations and Clinical Application of Alloheal

Конференциа Новости в Офталмологията 2018, Pravez(Sofia)

October 2018 — Management of Floppy Eyelid Syndrome as a Result of Sleep Apnea

Third National Conference on Obstructive Sleep Apnea and Snoring with International Participation - St. St. Constantine and Elena Resort, Varna

CONTRIBUTED PRESENTATIONS

October 2018 — MANAGEMENT OF FLOPPY EYELID SYNDROME AS A RESULT OF SLEEP APNEA

Third National Conference on Sleep Apnea and Snoring, St. Constantine and Elena Resort, Varna

October 2018 — Alloheal – The First Bulgarian Bioproduct for Treatment of Anterior Ocular Surface Disorder

Third National Conference on Sleep Apnea and Snoring, St. Constantine and Elena Resort, Varna

ADDITIONAL INFORMATION

Courses

February 2018 — ICH-E6 (R2) GCP INVESTIGATOR SITE PERSONAL TRAINING, *Bulgarian Association of Clinical Research*

December 2017 — CRITICAL APPRAISEL COURSE, *Berlin*

March 2017 — ADVANCED CARDIOVASCULAR LIFE SUPPORT PROVIDER Course, *Berlin*

March 2017 — EMERGENCY MEDICINE COURSE, *Berlin*

November 2016 — SPECIALIZED COURSE FOR X-RAY

DIAGNOSTICS, *Bad Homburg*

June 2016 BASIC COURSE IN RADIATION SAFETY EXPERTISE, *Bad Homburg*

April 2016 REANIMATION TRAINING, *Bad Homburg*

Participated Competitions

2019-2020 Finalist in the Thea Trophy Contest

- Finalist in the international contest of clinical cases in pathologies of the eye, Thea Trophy Contest 2019-2020
- Topic: Severe dry eye after molten metal ocular burn. Treatment with cryopreserved amniotic membrane transplantation and limbal stem cell transplantation

November 2018 Winner of the Competition for a young researcher "Prof. Dubov "at the conference "Innovations in Ophthalmology 2018"

- Topic: Experimental Considerations ad Clinical Applications of the Innovative Bioproduct ALLOHEAL