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Research Article

Modification of a Decellularized Lyophilized Amniotic Membrane for Ophthalmology

Milyudin Evgeny[1]*, Volova LT[1], Kuchuk KE[2] and Pavlova OV[2]

[1]*Research Institute of BioTech, Samara State Medical University, Russian Federation*

[2]*Samara Regional Clinical Ophthalmological Hospital named after T.I. Eroshevsky, Russian Federation*

***Corresponding Author:** Milyudin Evgeny, Research Institute of BioTech, 1Samara State Medical University, Russian Federation.

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Abstract

Introduction: Corneal epithelial regeneration, especially when the basement membrane is damaged, is accompanied by the initiation of angiogenesis and the formation of corneal opacity. To reduce the likelihood of developing pathological changes in the cornea, many authors use amniotic membrane coating. Consequently, the therapeutic effect of amniotic membrane application should result in the absence of newly formed vessels either on the surface or within the cornea, while simultaneously activating proliferation and forming a specific epithelial coating. However, the use of various amniotic membrane preservation methods does not always guarantee the preservation of biologically active substances and the absence of newly formed vessels.

Purpose of the Study: The purpose of this study was to evaluate the feasibility of using decellularized lyophilized amniotic membranes pretreated with glycerol in ophthalmological practice.

Materials and Methods: An experimental study was conducted on two groups of experimental animals. A fragment of decellularized, lyophilized human amniotic membrane was implanted into a subcutaneous pocket in the withers. The first group received a fragment additionally treated with glycerol, while the second group did not. The fragments of decellularized, lyophilized amniotic membrane were subjected to proteome analysis.

Results: A significant difference in the proteomic composition of amniotic membrane treated with glycerol before decellularization and lyophilization was revealed, compared to that not treated with glycerol. Histological examination of the preparations revealed a significant increase in newly formed vessels in animals implanted with a fragment of decellularized, lyophilized amniotic membrane not treated with glycerol.

Conclusion: Therefore, pre-treatment of biomaterial with glycerin and subsequent decellularization using physical methods promotes the preservation of various protein compounds within the tissue structure, which determine biological activity. Specifically, biomaterial not impregnated with glycerin contains a higher protein content, promotes greater cellular infiltration, and activates neoangiogenesis. In contrast, amniotic membrane pre-treated with glycerin does not cause an inflammatory reaction and does not lead to the development of new vessels.

Consequently, in corneal surgery, it is necessary to use lyophilized amniotic membrane pre-treated with glycerin, which has a greater ability to prevent neoangiogenesis.

Keywords: *Decellularized Lyophilized Amniotic Membranes; Proteomic Analysis; Morphological Study; Neoangiogenesis*

Introduction

Corneal epithelial defects heal fairly quickly; however, pathological changes in the basement membrane or limbal stem cell deficiency lead to recurrent erosion [1]. One of the main causes of recurrent disease is impaired adhesion of epithelial cells to the basement membrane [2]. Proteoglycans contained in the basement membrane play an important role in the anchoring of regenerating epithelium, facilitating the formation of anchoring fibrils in newly formed epithelial cells [3,4].

The basement membrane of the corneal epithelium is an extracellular matrix, a transparent, acellular structure whose main components are type I collagen, collagen IV, laminin, heparan sulfate proteoglycan, and perlecan [5-8]. Desmosomes, which play an important role in the adhesion of epithelial cells to the basement membrane, are present in significant quantities in the alar cell layer [9]. Epithelial cells of the basal layer firmly attach to the underlying basement membrane via hemidesmosomes and anchoring fibrils, forming an anchorage complex between the basal epithelial cells and the basement membrane [10,11].

With an intact basement membrane, complete restoration of the corneal epithelium occurs within 5 - 7 days; with damage, it takes at least 6 weeks [12,13]. In the absence of a basement membrane, epithelialization of the defect proceeds from the edge to the center of the defect. As the basement membrane recovers, epithelial cells attach to it, initially forming temporary connections and subsequently permanent anchorage complexes. However, insufficiently strong temporary connections between epithelial cells and the underlying layer lead to the development of recurrent erosion [14,15]. Therefore, it is necessary to use a biological substrate that acts as a basement membrane. Noriko Koizumi., *et al*. believe decellularized amniotic membrane is the most suitable material for these purposes [16].

Amniotic membrane, when used as a therapeutic covering, is extremely attractive because the biological material made from it is transparent and bioresorbable, allowing for monitoring of the pathological lesion. At the same time, the biomaterial used for corneal pathology must specifically react with the tissue, as the cornea is avascular. Consequently, the therapeutic effect of the biological tissue should not result in the formation of new vessels either on the surface or within the cornea, but should instead activate proliferation and form a specific epithelial covering [17-19].

Native amniotic membrane contains a range of biologically active substances, which are preserved to some extent during preservation [20-22]. Biomaterial is used, both preserved with viable cellular structures and preserved with impaired vitality.

The most common cryopreservation technique preserves both the cellular structures and the anatomical integrity of the amniotic membrane, thereby preserving biologically active substances. Researchers believe that cryopreserved amniotic membrane prevents the formation of new blood vessels in the implantation zone [23,24].

However, some researchers who prefer to use lyophilized biomaterial have expressed doubts about the preservation of biologically active properties in amniotic membranes without viable cellular structures and believe additional research is necessary [25-28]. In particular, cryopreservation involves the use of glycerol as part of the preservative medium, whereas lyophilization does not require the use of glycerol [29,30].

Based on the Samara Tissue Bank's experience in developing lyophilization methods for biological tissues, we have developed a method for processing and lyophilizing amniotic membranes for ophthalmology. This method involves the use of physical decellularization methods and a special freeze-drying regimen [30].

Purpose of the Study

The purpose of this study was to evaluate the feasibility of using decellularized lyophilized amniotic membranes pretreated with glycerol in ophthalmological practice.

Materials and Methods

The study involved human amniotic membranes received by the tissue bank from a donor. The amniotic membrane was mechanically separated from the chorion and washed to remove blood clots in saline (0.9% NaCl, pH 5-7.5) for at least 30 minutes. Then the donor biomaterial fragments were divided into two groups. During pre-treatment, the first group of biomaterial fragments is placed in a 10% glycerol solution for 20 minutes, followed by 15 minutes of ultrasound at a frequency of 35 ± 10% kHz. After that the fragments are placed in a chamber for 15 minutes under vacuum with a residual pressure of 1-2 Pa. Being removed from the chamber, the biomaterial is washed again in a 0.9% NaCl solution, frozen at -20°C to -60°C, lyophilized, hermetically sealed, and sterilized using radiation method (gamma rays or fast electrons).

The second group of biomaterial fragments, without prior glycerol impregnation, was also exposed to low-frequency ultrasound, placed in a vacuum chamber, and, after repeated washing and freezing, lyophilized. The lyophilized biomaterial was packaged in sealed bags and sterilized using radiation.

Proteomic analysis of three fragments from each group of lyophilized amniotic membrane was performed at the Federal Research Center "Fundamentals of Biotechnology" of the Russian Academy of Sciences. High-performance liquid chromatography with mass spectrometry was performed on an Impact II high-resolution quadrupole-TOF mass spectrometer (Bruker Daltonik, Germany) equipped with an Apollo II electrospray ionization source (Bruker Daltonik, Germany). Elute ultra-high-performance liquid chromatography (Bruker Daltonik, Germany) was used on a Waters Acquity HSS reversed-phase column (Waters, Ireland). Spectral processing and protein identification were performed using BioPharma Compass 3.1.1 (Bruker Daltonik, Germany) and Mascot 2.8.1 (Matrix Science, UK) software packages.

The biologically active properties of the polymeric material were studied in an animal model. The research was carried out on 28 Wistar rats of both sexes, divided into two groups. When performing surgical interventions on animals, as well as their maintenance in the vivarium of the Research Institute of BioTech, Samara State Medical University, Ministry of Health of the Russian Federation, we were guided by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, March 18, 1986); the "Principles of Good Laboratory Practice" of the Russian Federation National Standard GOST No. 33044-2014, introduced on August 1, 2015; and the "Sanitary and Epidemiological Requirements for the Design, Equipment, and Maintenance of Experimental Biological Clinics (Vivariums)" (SP 2.2.1.3218-14).

The following animal selection criteria were used: age 5-6 months, absence of disease. Before the experiment, the animals were kept in an isolation ward for 14 days and treated for ecto- and endoparasites. The rats were maintained under a balanced lighting regimen, with free access to water and standard laboratory chow. All animals underwent surgery between September and December 2023. All surgical interventions were performed under intramuscular anesthesia with a mixture of the anesthetics "Zoletil 100" (Virbac C.A., France) at a dosage of 0.5 mg/100 g of body weight and "Solutionis rometarum 20 mg/ml" (Bioveta, Czech Republic) at a dosage of 0.6 mg/100 g of body weight. The onset of surgical anesthesia was assessed by the presence of ciliary, corneal, and pedal reflexes. The experiment was performed in compliance with aseptic and antiseptic rules. After hair removal, the surgical field was treated with an antiseptic and isolated with sterile napkins. Scalpel was used to make a 1-1.5 cm long skin incision in the withers area. Blunt dissection was used to create a pocket into which a 2 x 2 cm fragment of lyophilized amniotic membrane was placed. The wound was tightly sutured. The animal was placed in a carrier with heating pads. After the surgery the rat's condition was checked every 10 minutes. After the animal assumed a natural body and head position, it was transferred from the surgical unit to the permanent holding room. Physical examination in the cage was performed daily, and all abnormalities were recorded in the examination log. At the end of the experiment, the animal was sacrificed by intracardiac injection of an overdose of anesthetic. The excised tissue fragment from the implantation area was placed in fixative fluid (10% buffered formalin) at room temperature for 24-48 hours. After that histological preparations were made using standard techniques and stained with hematoxylin and eosin; hematoxylin and picrofuchsin.

The analysis of histological preparations was carried out using a visualization system based on an Olympus BX41 research microscope, a ProgRes CF color digital camera and a stationary computer, with the Morphology 5.2 software.

This experiment was performed with the permission of the Bioethics Committee of Samara State Medical University (Extract from Protocol No. 206 dated March 18, 2020).

Results and Discussion

The method of preserving amniotic membranes using freeze-drying with preliminary decellularization using physical stimulation, used in our tissue bank, allows for the storage of preserved biomaterial for 3 years under room conditions. Decellularization and freeze-drying, in turn, lead to structural changes and even possible denaturation of the proteins that provide biological activity to the native amniotic membrane [31-33]. In order to stabilize proteins and preserve the biological activity of the lyophilized amniotic membrane, the biomaterial was pretreated with glycerol. However, previously performed Raman spectroscopy showed the preservation of amides, polysaccharides, and other protein compounds in both glycerol-treated and untreated amniotic membrane fragments prior to lyophilization. A comparative analysis of the Raman spectra revealed minimal differences between the spectra of native amniotic membrane and amniotic membrane lyophilized without glycerol treatment, and significant differences with the spectra of the amniotic membrane treated with glycerol prior to lyophilization. The identified changes in Raman spectroscopy concerned the degree of spectral intensity [31] and did not allow a specific determination of the effectiveness of glycerol treatment of the biomaterial. In this regard, we conducted a comparative study of the proteomic composition of lyophilized amniotic membranes treated with glycerol (group I biomaterial fragments) and without treatment (group II biomaterial fragments). This study identified different numbers of proteins. A total of 153 proteins with molecular weights ranging from 8.4 kDa to 628.7 kDa were identified with high reliability in the human amniotic membranes not treated with glycerol prior to lyophilization. In contrast, 121 proteins with molecular weights ranging from 11.4 kDa to 628.7 kDa were identified in the amniotic membranes treated with glycerol prior to lyophilization. It should be noted that the protein composition in the samples treated and not treated with glycerin differs significantly. In group I of biomaterial fragments, 88 protein structures were not identified from those present in group II of biomaterial fragments. Accordingly, in the samples of lyophilized amniotic membrane not treated with glycerin, 28 proteins were missing that were present in the fragments impregnated with glycerin before lyophilization. Considering that both groups of biomaterial were subjected to physical impact, which resulted in the destruction of cellular structures, it is likely that proteins present in the nuclei, cytoplasm, or were integrated into the cell membrane partially ended up in the extracellular matrix [31,34]. The smaller number of identified proteins in the structure of lyophilized amniotic membrane samples treated with glycerin is explained by the stabilizing function of glycerin, which, by interacting with proteins, forms three-dimensional structures and changes the characteristics of protein compounds [34].

Given the observed differences in proteomic composition, it's likely that the biomaterial processed using different methods has different properties. To clarify this hypothesis, we conducted an experimental study on two groups of animals. Animals were withdrawn from the experiment on the 12th day of the postoperative period and the excised tissue fragments with the implanted biopolymer were subjected to morphological examination. As a result of the study of histological preparations, we obtained convincing data on the different effects on tissues around implanted fragments of lyophilized amniotic membrane treated with glycerin (Group I) (Figure 1) and fragments of amniotic membrane without treatment with glycerin before lyophilization (Group II).

Lyophilized amniotic membrane in tissue slides from animals in Group I (the biomaterial was treated with glycerin before lyophilization) is clearly visible, its fibrous structure is preserved, and slight edema is noted. The surrounding tissue is slightly edematous, infiltrated by isolated mast cells, macrophages, and fibroblasts. Isolated blood-filled vessels are present (Figure 2).

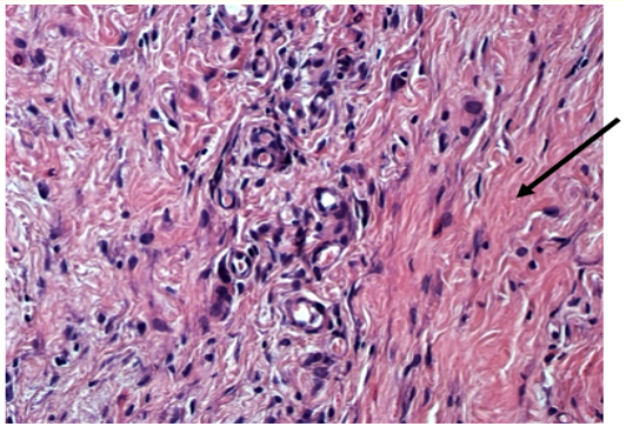

Figure 1: *Histological preparation of the implantation zone of a lyophilized amniotic membrane fragment treated with glycerin. Stained with hematoxylin and eosin. Magnification - 400x. The arrow indicates the preserved amniotic membrane.*

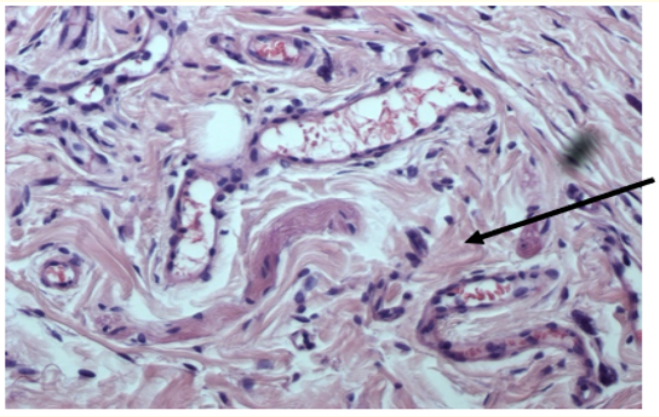

Figure 2: *Histological preparation of the implantation zone of a lyophilized amniotic membrane fragment without glycerin treatment. Hematoxylin and eosin staining. Magnification: - 400x. The arrow indicates the amniotic membrane being replaced.*

Group II preparations (biomaterial without glycerin treatment before lyophilization) show more pronounced lysis and replacement of the lyophilized amniotic membrane, as well as macrophage infiltration. The surrounding tissue is infiltrated with a large number of mast cells. Numerous newly formed vessels are also present.

A distinctive feature of the pre-processing technology for biomaterial subjected to lyophilization in the BioTech tissue bank of Samara State Medical University is the use of physical methods. The main difference between the processing of the two groups of amniotic membrane fragments obtained from the same donor was the treatment of Group I fragments with glycerin. Glycerol is often used in the preservation of amniotic membrane [28,34,36]. Therefore, the differences we identified in the proteome composition of the studied fragments are determined by the use of glycerol during the pre-treatment of the amniotic membrane. In Group I biomaterial fragments, 88 protein structures were not identified among those present in Group II fragments. Accordingly, lyophilized amniotic membrane samples not treated with glycerol lacked 28 proteins present in fragments soaked in glycerol before lyophilization.

The majority of identified proteins in Group I biomaterial fragments were classified as intracellular—70 proteins (57.8%), while 51 proteins (42.2%) were classified as extracellular. The majority of identified proteins in Group II biomaterial fragments (not soaked in glycerol during pre-lyophilization processing) were intracellular—97 proteins (63.4%), while the proportion of extracellular proteins was 56 proteins (36.6%). Considering that both groups of biomaterial were subjected to physical impact resulting in the destruction of cellular structures, it is likely that proteins present in the nuclei, cytoplasm or were integrated into the cell membrane ended up in the extracellular matrix [36,37]. The smaller number of identified proteins in the structure of lyophilized amniotic membrane samples treated with glycerol is explained by the stabilizing function of glycerol, which, interacting with proteins, forms three-dimensional structures, changes the characteristics of protein compounds and prevents them from interacting with other substances [36-38]. The results of the morphological study showed a more pronounced reaction to the biopolymer without the use of glycerol in its preparation. In particular, in the micropreparations of the 1st study group, there is significantly less infiltration by mast cells and no newly formed vessels are observed. Whereas on the micropreparations where the biopolymer not impregnated with glycerin was implanted, there was significant infiltration of mast cells and numerous newly formed vessels.

Protein analysis revealed that glycerol-impregnated biopolymer fragments contain 13 proteins that directly regulate angiogenesis. According to data from the open-source PANTHER and neXtProt resources, six proteins are inducers, and seven proteins inhibit angiogenesis.

Fragments of lyophilized amniotic membrane not treated with glycerol also revealed 13 proteins involved in the regulation of angiogenesis, eight of which are inducers, and five are inhibitors.

Both groups I and II samples contained similar proteins, including angiogenesis mediators—Annexin A1, 14-3-3 protein zeta/delta, Annexin A2 (ANXA2), Tenascin-X, and Transforming growth factor-beta-induced protein βig-h3 (Beta βig-h3)—and angiogenesis inhibitors—Thrombospondin-1; Collagen alpha-2(IV) chain; Fibronectin; and Decorin.

At the same time, only lyophilized amniotic membrane pre-treated with glycerol contained Collagen alpha-1(IV) chain (COL4A1), also an angiogenesis inhibitor. Collagen chains α1(IV) and α2(IV) are present in the basement membranes of all tissues and are an important component in cell adhesion and differentiation. The interaction of these proteins is crucial for many biological processes. Including migration, survival, and angiogenesis [33,34,39-41].

Conclusion

Our study demonstrated more pronounced biological properties of the biomaterial not treated with glycerol prior to preservation, manifested by significant mast cell infiltration and neoangiogenesis. In contrast, implantation of amniotic membrane pretreated with glycerol did not cause an inflammatory response or activate neoangiogenesis.

Therefore, for use in corneal surgery, it is necessary to use lyophilized amniotic membrane pretreated with glycerol, which better ensures adhesion of epithelial cells to the corneal stromal surface and prevents neoangiogenesis.

Financial Interest and Conflict of Interest

None declared.

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