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Morphological evaluation of decellularized lyophilized amniotic membrane

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Abstract

Aim – to study the morphological structure of lyophilized amniotic membrane preliminarily subjected to physical decellularization.

Material and methods. An experimental study of the preservation of the anatomical structure of lyophilized amniotic membrane was performed on four groups of amniotic membrane fragments. Group 1: AM impregnated with glycerin and dried over silica gel; Group 2: AM impregnated with glycerin, treated ultrasonically and lyophilized; Group 3: AM treated ultrasonically and lyophilized; Group 4: native AM without preservation. The biomaterial was studied using light microscopy and scanning electron microscopy.

Results. Physical methods of influencing biological tissue have an expected effect on cell viability and allow obtaining a completely decellularized amniotic membrane. Additional treatment with glycerol before physical action on biological tissue for the purpose of decellularization does not have a significant effect on the preservation of cellular structures. It should only be noted that in the amniotic membrane impregnated with glycerol, more fragments of epithelial cell membranes are preserved and the basement membrane is more preserved.

Conclusion. The decellularization method developed by us using physical methods does not introduce any chemicals into the processed biomaterial that can have an unpredictable effect on regenerating tissues. Preservation of the amniotic membrane by lyophilization allows obtaining a morphologically integral, elastic and durable biomaterial.

Keywords: amniotic membrane, decellularization, lyophilization, morphology of lyophilized amniotic membrane.

Conflict of interest: nothing to disclose.

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Оценка морфологической структуры децеллюляризованной человеческой амниотической мембраны

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Аннотация

Цель – изучить морфологическую структуру лиофилизированной амниотической мембраны, предварительно подвергнутой децеллюляризации физическим методом.

Материал и методы. Экспериментальное исследование сохранности анатомической структуры лиофилизированной амниотической мембраны (АМ) было выполнено на четырех группах фрагментов амниотической мембраны. Первая группа – АМ, пропитанная глицерином и высушенная над силикагелем; вторая группа – АМ, пропитанная глицерином и обработанная ультразвуком, лиофилизированная; третья группа – АМ, обработанная ультразвуком и лиофилизированная; четвертая группа – нативная АМ, не консервированная.

Выполнено изучение биоматериала с помощью световой микроскопии и сканирующей электронной микроскопии.

Результаты. Физические методы воздействия на биологическую ткань ожидаемо оказывают влияние на жизнеспособность клеток и позволяют получить полностью децеллюляризованную амниотическую мембрану. Дополнительная обработка АМ глицерином перед физическим воздействием с целью децеллюляризации достоверно не способствует

сохранению клеточных структур. При этом необходимо отметить, что в амниотической мембране, пропитанной глицерином, после физического воздействия сохраняется значимо больше фрагментов мембран эпителиальных клеток и более сохранна базальная мембрана.
Выводы. Разработанный нами метод децеллюляризации с использованием физических методов не подразумевает внесения в обрабатываемый биоматериал каких-либо химических веществ, которые впоследствии могут оказать непредсказуемое воздействие на окружающие имплантированную АМ регенерирующие ткани. Также консервация АМ предложенным способом лиофилизации позволяет получить морфологически целостный, эластичный и прочный биоматериал для регенеративной медицины.
Ключевые слова: амниотическая мембрана, децеллюляризация, лиофилизация, морфология лиофилизированной амниотической мембраны.
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АМ – амниотическая мембрана.

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INTRODUCTION

In regenerative medicine, one of unique biomaterials is used widely: the amniotic membrane (AM). It is a conglomeration of a monolayer of epithelial cells on the basal membrane and the stroma consisting of three layers. The biologically active substances present in all the layers of the AM ensure activation of regenerative processes and cell proliferation, and accelerate their migration [1–4]. Before being used, the donor biomaterial is subjected to compulsory pre-treatment. First, the AM surface is washed from blood and mucus clots. This is generally accepted practice. The subsequent processing of the amniotic membrane with various chemical agents for disinfection (NB: disinfection in this case!), protection of the biomaterial from excessive damage during preservation, and subsequent preservation is differently assessed by researchers in terms of necessity, effectiveness, and impact on the preservation of biologically active substances in the biomaterial [5]. Many specialists prefer using native or cryopreserved AM, as in these cases, the cells remain viable and the anatomical structure of the biomaterial is practically undamaged [6–9]. However, since native AM cannot be stored and there is a risk of using infected material, the method of choice for utilizing AM in regenerative medicine is cryopreservation.

It should be noted that the use of chemical substances during cryopreservation in glycerol-containing media, the application of antibacterial agents for disinfection, as well as freezing and thawing processes may significantly alter both the structure and viability of cryopreserved AM [9]. Other common AM preservation methods (specifically drying over silica gel or lyophilization) entail complete loss of cellular viability, decellularization, and potential disruption of morphological structure while maintaining the biomaterial's anatomical integrity [10, 11], which many authors consider a critical factor for successful application in reconstructive surgeries [11–13].

When creating tissue-engineered constructs, researchers prefer using decellularized AM as a biological scaffold for cultured cells. Decellularization is a process aimed at removing cells from tissue while preserving the extracellular matrix and its three-dimensional structure [7, 14, 15], utilizing various cell-disruption methods. All decellularization methods can be classified into three types based on the primary disruptive factor: physical, chemical and biological. Physical methods are found in the majority of protocols of primary tissue treatment. They entail the use of rotators, shakers or direct perfusion chambers that provide accelerated fluid exchange with an effect on cell membranes to break the cells and their nuclei. More often, researchers use chemical agents: sodium dodecyl sulfate, an anionic surfactant capable of protein denaturation and dissolution of cell membranes. Organic acids are also used, specifically, peracetic acid that decomposes and removes nucleic acids. Decellularization of biomaterial is also possible with the use of spirits and chelating agents [16, 17].

The simplest physical method of decellularization is the process of multiple alternating freezing and thawing, under which cell membranes rupture by crystals of ice, and the cells lose their viability. Immersion in hypertonic solution resulting in osmotic stress and damage to cell membranes is also regarded as a physical method [17, 18]. However, the most efficient physical method of decellularization is ultrasonic impact, since the high efficiency of cell structure destruction by sonic energy is augmented by the mechanical purification of biomaterial from cellular debris [14].

AIM

Study the morphological structure of lyophilized amniotic membrane preliminarily subjected to physical decellularization.

MATERIAL AND METHODS

An experimental study of the anatomical structure preservation of lyophilized amniotic membrane was conducted on four groups of amniotic membrane fragments. The biomaterial, after washing in running water to remove blood clots, was cut into 1×1 cm fragments and divided into four groups. Group 1 (10 fragments): the amniotic membrane was impregnated with glycerol, placed in frames over silica gel and dried. Group 2 (12 fragments): the amniotic membrane was impregnated

with glycerol and treated with low-frequency ultrasound at 24–40 kHz in an ultrasonic bath “Sapfir” TTK (“Sapfir” LLC, Moscow, Russia) with subsequent lyophilization. Group 3(10 fragments): the amniotic membrane was treated with low-frequency ultrasound at 24–40 kHz in an ultrasonic bath “Sapfir” TTK (“Sapfir” LLC, Moscow, Russia) with subsequent lyophilization, without glycerol impregnation. Group 4 (control group) consisted of 10 fragments of native amniotic membrane studied without additional treatment and conservation.

The material was lyophilized (vacuum-dried by sublimation) on the ALPHA2-4LSC sublimation machine (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

Morphological studies were conducted after fixing the biomaterial in 12% neutral buffered formalin, processing through an alcohol series, and embedding in celloidin. No fewer than 500 sections were made from different biomaterial samples. The sections were stained with hematoxylin-eosin or picrofuchsin using Van Gieson’s method. The images of stained preparations were analyzed with the visualization system comprising an Olympus BX41 research microscope (“Olympus”, Japan), digital color camera “ProgRes CF” and a personal computer with Morphology 5.2 software suite (“VideoTesT”, Russia).

Scanning electronic microscopy (SEM) of the amniotic membrane after conservation were performed with the raster electronic microscope JEOLJSM-6390 A Analysis Station (Japan). For the purposes of this study, the fragments of biomaterial were fixed with 2.5% water solution of glutaric aldehyde and processed through an alcohol series. After processing through an ethanol solution of increasing concentration and drying at room temperature for 24 hours, gold or carbon was sputter-coated onto the biomaterial to enhance the required surface conductivity for scanning electron microscopy.

The obtained results were processed with statistical methods in the SPSS_Statistics software suite.

The work was performed with the approval from the Committee for Bioethics of the Samara State Medical University (Excerpt from the Protocol No. 206 dated 18 March 2020).

RESULTS

The active agent for decellularization was selected based on the anatomic and histological structure and dimensions of the biomaterial. The low-frequency ultrasonic impact may remove particles of the blood, and the wave effect and cavitation may likely damage all cellular structures of the amniotic membrane since the thickness of the native biomaterial is not more than 0.5 mm. Considering that the criteria of efficiency of decellularization are not determined at present, we prepared morphological preparations. We believe that decellularized donor organs should not contain unaffected cells and cellular components.

From the macroscopic perspective, the biomaterial of all three experimental groups had the appearance of tissue paper, elastic and velvety to the touch. The samples from Group 3 of lyophilized amniotic membrane without glycerol impregnation turned out to have a more matte and non-uniform surface than the samples from Groups 1 and 2 that had been impregnated with glycerol.

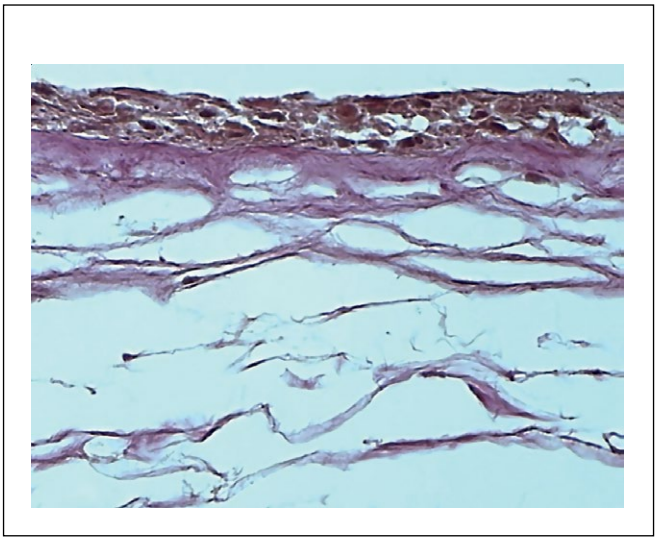

Figure 1. *Native amniotic membrane preparation. Stained with picrofuchsin. Magnification x400.*

Рисунок 1. *Препарат нативной амниотической мембраны. Окраска пикрофуксином. Ув. х400.*

The morphological preparation of the native amniotic membrane shows a completely preserved epithelial layer with viable cells and a multitude of pinosomes (**Fig. 1**). Adjacent to the basement membrane lies the compact layer, composed of tightly interwoven collagen fibers, followed by the fibroblast layer: a loose arrangement with fibroblasts interspersed among reticular fiber networks. The spongy layer consists of delicate, randomly oriented reticular fibers.

In histological preparations of the first group (silica-dried amniotic membrane after preliminary glycerol impregnation), an almost homogeneous band is observed, where distinguishing the epithelial and compact layers becomes difficult (**Fig. 2**). Focal areas show severely flattened cell nuclei. The stromal compact layer appears as a homogeneous acellular oxyphilic band. Nuclear shadows are frequently visible in the fibroblast layer. Persisting fibroblast nuclei exhibit rod-shaped morphology. The spongy layer is markedly flattened, identifiable by multidirectional oxyphilic-stained fibers. Morphometric

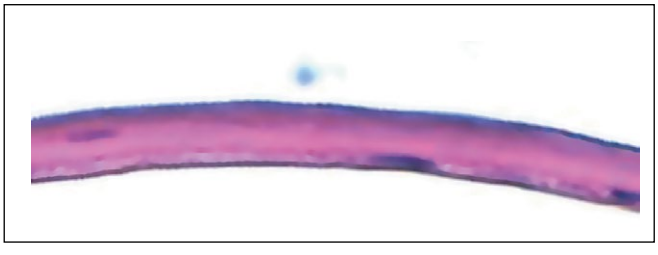

Figure 2. *Amniotic membrane preparation preserved by drying over silica gel after preliminary treatment with glycerol. Stained with hematoxylin and eosin. Magnification x400.*

Рисунок 2. *Препарат амниотической мембраны, консервированной путем высушивания над силикагелем после предварительной обработки глицерином. Окраска гематоксилин-эозином. Ув. х400.*

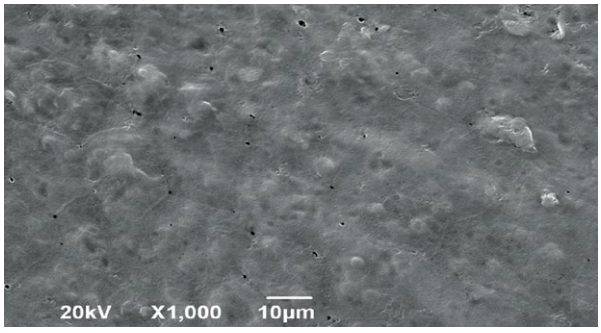

Figure 3. *Electron microscopic image of the amniotic membrane in a scanning electron microscope. Epithelial surface of the amniotic membrane preparation dried with silica and pre-impregnated with glycerol. Magnification x1000.*

Рисунок 3. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Эпителиальная поверхность препарата амниотической мембраны силиковысушенной с предварительным пропитыванием глицерином. Ув. х1000.*

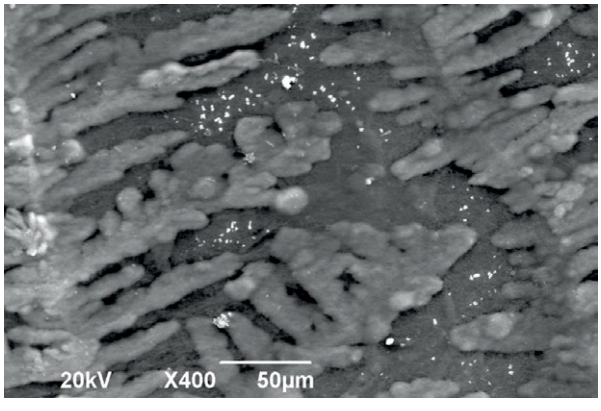

Figure 4. *Electron microscopic image of the amniotic membrane in a scanning electron microscope. Spongy layer of the amniotic membrane preparation dried with silica and preliminary impregnation with glycerin. Magnification x400.*

Рисунок 4. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Спонгиозный слой препарата амниотической мембраны силиковысушенной с предварительным пропитыванием глицерином. Ув. х400.*

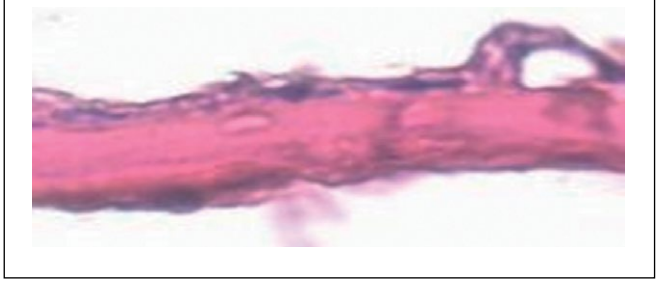

Figure 5. *Amniotic membrane preparation preserved by lyophilization after preliminary treatment with glycerol. Stained with hematoxylin and eosin. Magnification x400.*

Рисунок 5. *Препарат амниотической мембраны, консервированной путем лиофилизации после предварительной обработки глицерином. Окраска гематоксилин-эозином. Ув. х400.*

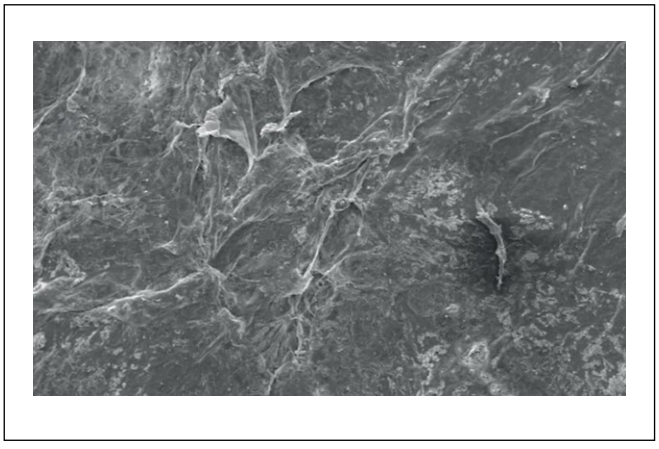

Figure 6. *Electron microscopic image of amniotic membrane in a scanning electron microscope. Epithelial surface of a lyophilized amniotic membrane preparation with preliminary impregnation with glycerol. Magnification x5*

Рисунок 6. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Эпителиальная поверхность препарата лиофилизированной амниотической мембраны с предварительным пропитыванием глицерином. Ув. х50..*

analysis of total graft thickness (n=55) in lyophilized glycerol-impregnated amniotic membrane yielded a mean measurement of 6.9184 μm.

Raster electronic microscopy confirmed preservation of the epithelial layer in samples from Group 1 (**Fig. 3**). The epithelial layer represented by partially affected cells adjoins the substratum on the entire surface of the biomaterial. Individual defects are seen, restricted by cell contours.

Raster electronic microscopy of the amniotic membrane dried over silica gel from the side of the spongy layer confirms that significant changes occur in the loose connective tissue layer of the stroma, specifically, the spongy layer becomes smoothed. It is important to note the presence of homogeneous amorphous substrates forming dendritic patterns along multidirectional reticular fibers (**Fig. 4**).

The detailed study of the histological preparations of Group 2 offers a clear view of the epithelial layer (**Fig. 5**). In isolated areas, focal destruction of epithelial cells is observed. In preserved cells, nuclear pyknosis and chromatin condensation into conglomerates are evident. The basement membrane is damaged in regions of focal epithelial cell destruction. The compact layer appears in some areas as a homogeneous acellular oxyphilic band, while in others fibers are visible. The spongy layer is also preserved but compacted, with loss of structural organization. The morphometry of the total thickness of samples (n=48) of lyophilized amniotic membrane impregnated with glycerol yielded a mean measurement of 10.236 μm.

Scanning electron microscopy revealed, in a clearer way, the destruction of the epithelial layer, absence of viable cellular structures, and damage with partial desquamation of the basement membrane.

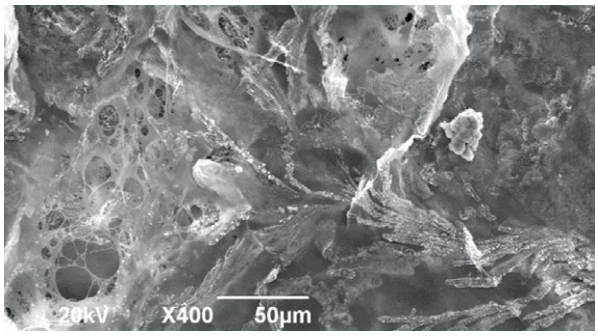

Figure 7. *Electron microscopic image of amniotic membrane in a scanning electron microscope. Spongy layer of a lyophilized amniotic membrane preparation with preliminary impregnation with glycerol. Magnification x400.*

Рисунок 7. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Спонгиозный слой препарата лиофилизированной амниотической мембраны с предварительным пропитыванием глицерином. Ув. х400.*

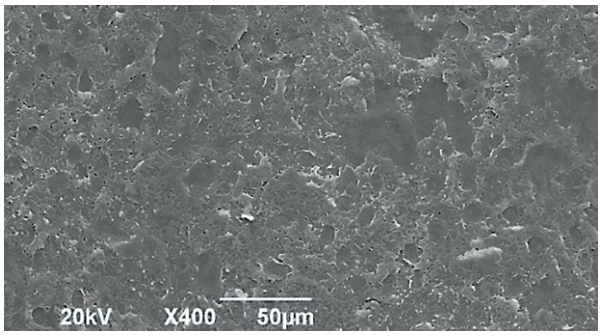

Figure 9. *Electron microscopic image of amniotic membrane in a scanning electron microscope. Epithelial surface of a lyophilized amniotic membrane preparation without preliminary impregnation with glycerol. Magnification x400.*

Рисунок 9. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Эпителиальная поверхность препарата лиофилизированной амниотической мембраны без предварительного пропитывания глицерином. Ув. х400.*

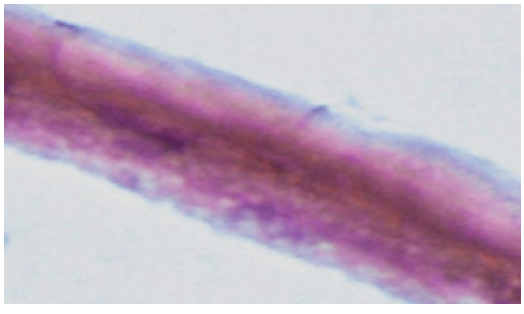

Figure 8. *Amniotic membrane preparation preserved by lyophilization without glycerol treatment. Hematoxylin and eosin staining. Magnification x400.*

Рисунок 8. *Препарат амниотической мембраны, консервированной путем лиофильной сушки без обработки глицерином. Окраска гематоксилин-эозином. Ув. х400.*

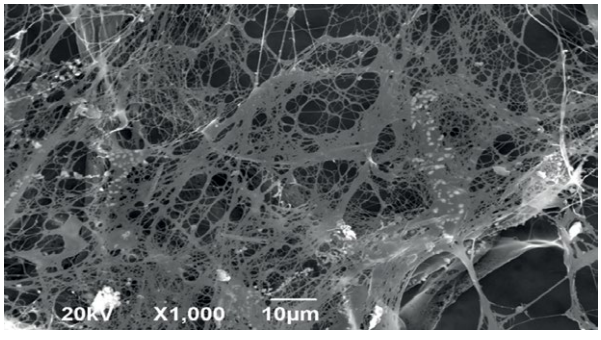

Figure 10. *Electron microscopic image of amniotic membrane in a scanning electron microscope. Spongy layer of the preparation of lyophilized amniotic membrane without preliminary impregnation with glycerol. Magnification x1000.*

Рисунок 10. *Электронно-микроскопическое изображение амниотической мембраны в сканирующем электронном микроскопе. Спонгиозный слой препарата лиофилизированной амниотической мембраны без предварительного пропитывания глицерином. Ув. х1000.*

The spongy layer is represented with denser connective fibers, on which homogenous structure-less substrates are visualized, attached in clusters (**Fig. 7**).

The epithelial layer in histological preparations of Group 3 of samples appears as a flattened homogeneous oxyphilic layer (**Fig. 8**). Isolated chromatin conglomerates from destroyed epithelial cell nuclei are observed.

The basement membrane is uneven in thickness and partially absent. The compact layer exhibits a dense homogeneous structure. The fibroblast layer is nearly devoid of cellular elements. Isolated nuclei of destroyed fibroblasts appear as rod-shaped shadows. No chromatin conglomerates are present. The spongy layer is flattened. Morphometric analysis of total graft thickness (n=44) in lyophilized non-glycerinated amniotic membrane yielded a mean measurement of 10.026 µm.

Scanning electron microscopy of preserved amniotic membrane samples from Group 3 revealed the following: the epithelial layer consists of minor cellular membrane fragments and the basement membrane that is desquamated across most of the examined surface. At higher magnification, the basement membrane appears as sparse fragments with curled edges, exposing the underlying stromal layer (**Fig. 9**).

The spongy layer in this sample group shows the least damage. Collagen fibers are thin, multidirectional, and loosely arranged. This preparation clearly demonstrates the end-to-end porosity of the biomaterial. The isolated homogeneous formations attached to collagen fibers are present (**Fig. 10**).

DISCUSSION

The histological study of the amniotic membrane showed significant differences of the morphological

landscape depending on the preliminary (pre-conservation) treatment of the biomaterial. In those cases where decellularization was not performed and the silica gel drying method was used without biomaterial freezing stages, the epithelial layer with preserved nuclei and cell membranes was visualized.

Studies of the amniotic membrane fragments subjected to decellularization by low-frequency ultrasound in Groups 2 and 3 reveal complete destruction of cellular structures and almost complete removal of cell components. In the histological samples, complete destruction of epithelial layer cells and partial destruction of the basement membrane with exposure of the stroma and formation of trabecular architectonic of the biomaterial are observed. Such changes are more manifested in the preparations not impregnated with glycerol before lyophillization.

In the decellularized tissue, the extracellular matrix remained unchanged. No swelling or other pathological changes in the structure, architectonic, fiber orientation, and tinctorial properties of the connective tissue were observed.

Thus, the methods of physical treatment of biological tissue provide an expected impact on the cell viability and allow production of a completely decellularized amniotic membrane. Impregnation with glycerol before physical treatment of the biological tissue with the aim of decellularization has no significant effect on the preservation of cellular structures. It remains to be noted that the decellularized lyophilized amniotic membrane impregnated with glycerol preserves more fragments of membranes of epithelial cells, and the basement membrane is also preserved to a greater extent.

CONCLUSIONS

The decellularization method we developed using physical approaches introduces no chemical substances into the processed biomaterial that could unpredictably affect regenerating tissues.

AM conservation through lyophilization yields morphologically intact, elastic, and durable biomaterial.

ADDITIONAL INFORMATION	ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ
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Conflict of interest. The authors declare that there are no obvious or potential conflicts of interest associated with the content of this article.	***Конфликт интересов.*** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с содержанием настоящей статьи.
Compliance with Ethical Standards. The study was approved by the Bioethics Committee of Samara State Medical University (extract from protocol No. 206 dated March 18, 2020).	***Соответствие нормам этики.*** Исследование одобрено комитетом по биоэтике Самарского государственного медицинского университета (выписка из протокола №206 от 18 марта 2020 г.).
Contribution of individual authors. K.E. Kuchuk: processing andconservation of biomaterial, text preparation. L.T. Volova: development of the concept and design of the study, analysis of the obtained data. I.V. Novikov: analysis of obtained data, preparation of text. E.S. Milyudin: collection of material, analysis of obtained data, editing of text. The authors gave their final approval of the manuscript for submission, and agreed to be accountable for all aspects of the work, implying	***Участие авторов.*** К.Е. Кучук – обработка и консервация биоматериала, подготовка текста. Л.Т. Волова – разработка концепции и дизайна исследования, анализ полученных данных. И.В. Новиков – анализ полученных данных, подготовка текста. Е.С. Милюдин – сбор материала, анализ полученных данных, редактирование текста. Все авторы одобрили финальную версию статьи перед публикацией, выразили согласие нести ответственность за все аспекты работы, подразумевающую надлежащее изучение и решение вопросов, связанных с точностью или добросовестностью любой части работы.

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