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Morphological evaluation of the amniotic membrane decellularization

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Abstract

Background: Biological materials derived from decellularized tissues could be a good basis for progress in regenerative medicine while maintaining the main components of the extracellular matrix. A promising scaffold for tissue-engineered is the human amniotic membrane. It is one of the oldest biomaterials used for scaffolds.
Material and methods: 3 placentas were obtained through Human Tissue Bank. Under sterile condition human amniotic membrane was collected. The human amniotic membrane was treated with 0.5% of sodium dodecyl sulphate (SDS), 1% Triton for 24 and 5 hours. Amniotic membrane decellularization was also carried out in combination with ultrasound bath for 20 minutes 3 times. For morphological and structure evaluation of human amniotic membrane the scanning electron microscopy of native amniotic membrane and histology of decellularized and native amniotic membrane were performed.
Results: The human amniotic membrane decellularization process with 0.5% SDS solution and 1% Triton solution showed that decellularization for 24 hours is too aggressive for human amniotic membrane structure. The decellularization for 5h with 1% Triton solution was incomplete.
Conclusions: The method of decellularization with 0.5% SDS solution is more suitable for amniotic membrane decellularization and can be performed in only 5 hours. The use of ultrasound bath did not have a significant effect on the obtained results.
Key words: amniotic membrane, decellularization, morphology.

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Introduction

Tissue engineering aims at replacing or regenerating human tissues or organs in order to restore or establish normal function [1]. The tissue engineering triad consists of three main factors: the cells, signaling molecules, and scaffold, which support and rely upon one another. The scaffold, together with integrated signaling molecules, provides structural, biochemical, and biomechanical cues to guide and regulate cell behavior and tissue development [2].

Scaffolds can be prepared through synthetic or natural materials. Synthetic scaffolds are beneficial in that their structure and mechanical properties can be manipulated and controlled with the goal of producing an optimal environment for a particular cell type or cell set. Among these properties, matrix stiffness and topography show profound influences on cell growth and differentiation [2].

Biomaterials have important roles as mimicking the natural environment and providing the physical and biological helpers to the attached cells during the *in vivo* and *in vitro* cultivation [3]. Cellular adhesion is one of the most undesirable properties for biomaterials. There are many studies in progress about surface modification of biomaterials [4]. Furthermore, optimal biomaterials should degrade without toxicity and must control degradation rate [5] and have excellent swelling and biodegradation behaviors [6]. Ideal scaffolds for tissue engineering should provide such properties as:

- Biocompatibility
- Biodegradability (tissue and damaged based)
- Support cell adhesion
- Non-immunogenicity
- Non-toxicity
- Easy obtainable
- Controllable porosity
- Provide vasculature for oxygen and nutrients delivery
- Provide microenvironment and promote cells growth
- Possess proper biomechanical strength [7].

Because many challenges are associated with preparing synthetic scaffolds that recapitulate the complexity of the cell microenvironment, there has been increasing interest in utilizing naturally derived extracellular matrix (ECM)

itself. This biologic scaffold is obtained through the process of decellularization. The ultimate goal of decellularization is to rid the ECM of native cells and genetic materials such as DNA while maintaining its structural, biochemical, and biomechanical cues [2]. The decellularized ECM can then be repopulated with a patient's own cells to produce a personalized tissue and be used to improve, maintain or restore damaged tissues or whole organs. The rationale for decellularization is related to the adverse response that cell waste may induce when tissue-derived material is used for implantation procedures, including immune reaction and inflammation, leading to implant rejection. Therefore, decellularized ECM is usually obtained by different decellularization methods, developed to eliminate the cells and their waste, mainly DNA [8]. As for all biological scaffolds, gentle but complete decellularization is a critical step in removing allogenous cells, and various methods have been described [9].

Various natural structures have the required therapeutic potential to be used as a tissue-engineered structure. Among them are the inner body membranes. Membranes actually consist of thin layers of cells or tissues that envelope the body, its internal organs, and cavities. Amniotic membrane, mesentery, omentum, pericardium, peritoneum, and pleura are all examples of these membranes with therapeutic applications [10].

A promising scaffold for tissue-engineered is the human amniotic membrane. It is one of the oldest biomaterials used for scaffolds and it has many characteristics that make it attractive as a biomaterial [11]. Human amniotic membrane is a thin semitransparent membrane normally 20 μm to 500 μm in thickness. It is tough and is devoid of blood vessels, lymphatics, and nerves [9, 12]. Amniotic membrane lines the amniotic cavity; its apical surface is bathed in amniotic fluid, whereas the basal surface lies on top of the chorion [13]. The amnion basement membrane is largely composed of collagen I, collagen III, collagen IV, laminin, and fibronectin. It is inexpensive and easily takes, and its availability is virtually unlimited, negating the need for mass tissue banking [11].

The human amniotic membrane has been widely used in tissue engineering and regenerative medicine not only due to its favorable biological and mechanical properties but also as its usage has low ethical problems. In general, for amniotic membrane clinical applications or its preservation in tissue banks, it is crucial to perform donor screening and selection, procure the membrane, wash it, and perform additional processing steps. It is common to treat the amniotic membrane chemically or with antibiotic substrates, preserve, sterilize, package, and store it [14]. The reasons for epithelial layer removal, amniotic membrane sterilization, and its preservation are, respectively, to reduce graft rejection, minimize the risk of disease transmission, and store it more quickly for a more extended period [15]. Elasticity, stiffness and other biomechanical properties also make it possible to use the amniotic membrane for various medical purposes. Amniotic membrane is almost always considered as discarded substance; it satisfies most of the criteria of an ideal biological tissue and shows almost zero rejection phenomenon [16].

This paper aim is to evaluate the morphology and structure of decellularized human amniotic membrane by different methods.

Material and methods

Human amniotic membrane collection

Human placentas were obtained through Human Tissue Bank, the Republic of Moldova. After written informed consent was obtained, 3 placentas were obtained after caesarean section. The research was approved 15.03.2019 No 14, by Ethical Committee of Research at *Nicolae Testemitanu* State Medical and Pharmaceutical University of the Republic of Moldova, Chisinau. According to Standard Operating Procedure (SOP) the screening to exclude any risk of transmissible infections, such as human immunodeficiency virus, hepatitis virus types B and C, and syphilis was done. Under sterile conditions the placentas were decontaminated to remove pathogens by rinsing several times with sterile saline solution, and finally, the amnion and chorion were separated manually and rinsed with the saline solution containing antibiotics and antimycotics. A part of human amniotic membrane was decellularized.

Decellularization of human amniotic membrane

Sodium dodecyl sulphate (SDS) decellularization. Human amniotic membrane was treated with 0.5% SDS solution for 24h and 5h at room temperature. To help remove cells from the substrate additional mechanical scraping was applied. After the decellularization process human amniotic membrane was washed three times with phosphate buffered saline (PBS) solution (pH 7.4) for 15 min each with gentle agitation.

Triton X-100 decellularization. The tissue was placed in a solution of 1% Triton X-100 for 24h and 5h at room temperature. The scraping for cell removal was performed, followed by a final wash using sterile PBS (pH 7.4) for 15 min each with gentle agitation.

Ultrasound bath decellularization. The amniotic membrane placed in tubes with 1% Triton and 0.5% SDS solutions was additionally placed in ultrasound bath for 20 minutes 3 times. After that, the solutions with human amniotic membrane were left for 24 and 5 hours at room temperature. After the decellularization process the mechanical scraping was applied and amniotic membrane was washed three times with PBS solution (pH 7.4) with gentle agitation for 15 min each.

Morphological characterization of human amniotic membrane

Scanning electron microscopy analysis

The samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 24 hours and post-fixed in 1% osmium tetroxide for 1 h, dehydrated with a series

of ethanol solutions of increasing concentrations (30%, 50%, 70%, 90%, and 100%) for 10 min each and critical point dried. For SEM, the amniotic membrane (AM) was further dried with carbon dioxide in a critical point dryer and coated with gold in a sputter coater. Then, the samples were subjected to scanning electron microscopy Zeiss EM 900 (MHH, Hannover).

Histological analysis

Native and decellularized human amniotic membranes were fixed in 10% formalin, dehydrated with an increasing series of ethanol solutions and embedded in paraffin wax. The samples were sectioned with each section having a thickness of 4 µm. The native amniotic membrane was stained with hematoxylin and eosin (H&E) and Van Gieson staining. Decellularized amniotic membrane was stained with H&E staining. Samples (n=3) were viewed by microscope (Leica S 80/0.30).

Results and discussion

Human amniotic membrane is one of the thickest membranes in the human body. It consists of a thin epithelial layer, a thick basement membrane and avascular stroma consisting mainly of collagen [17].

SEM was performed to study the surface morphology of the outer and inner layers of native amniotic membrane (fig.1). The cuboidal epithelial cells with irregular shape were observed on the outer layer of the amniotic membrane (fig. 1(a)). It was possible to see the direction of collagen fibers, which are an indicator of mechanical properties of the human amniotic membrane. Collagen fibers directed unregularly were clearly visible on the inner layer of human amniotic membrane (fig. 1(b)).

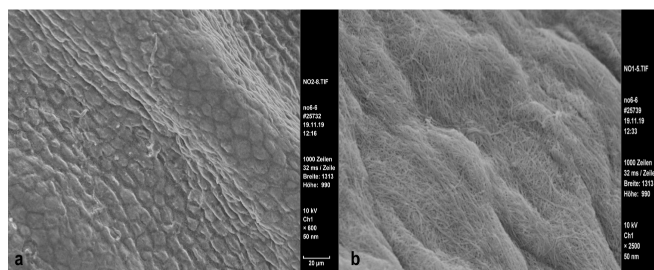

Fig. 1. SEM of native amniotic membrane. (a) the outer layer of amniotic membrane consists of epithelial cells, (b) collagen fibers on the inner layer of the amniotic membrane

Different direction of collagen fibers suggests that human amniotic membrane has good mechanical properties that allow using it in various fields of medicine and especially in tissue engineering.

Epithelium is a monolayer of metabolically active cuboidal cells with microvilli present on its apical surface [17] and uniformly arranged on the basement membrane. *Basement membrane* is made up of type IV, V and VI collagen in addition to fibronectin and laminin [18]. *Stroma* is further divided into three contiguous but distinct layers: the inner compact layer which is in contact with the basement membrane and contributes to the tensile strength of the membrane, middle fibroblast layer which is thick and made up of a loose fibroblast network and the outermost spongy layer [17].

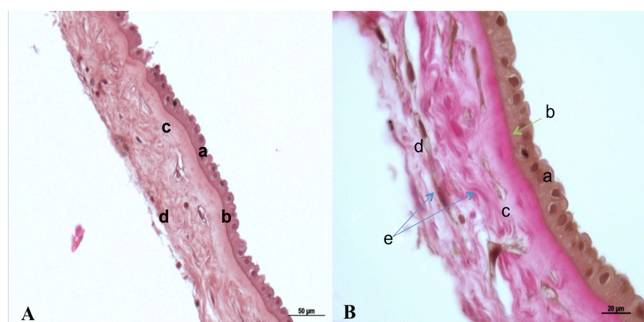

Fig. 2. (A) H&E stained histological image of native amniotic membrane: uniform epithelial layer (a), intact basement membrane (b), unmodified compact layer (c), preserved fibroblastic layer (d). H-E, × 100.

(B) Van Gieson stained histological image of native hAM: uniform epithelial layer (a), intact basement membrane (b), unmodified compact layer (c), preserved fibroblastic layer (d), collagen fibers (e). Picrofuxin, × 400

On histological examination of native human amniotic membrane (fig. 2) all the layers were found: uniform epithelial layer, basement membrane, compact layer and fibroblastic layer. In Van Gieson-stained slides the collagen fibers can be clearly seen (fig. 2A).

Figure 3 shows the histology of decellularized human amniotic membrane. The human amniotic membrane decellularization process with 0.5% SDS solution and 1% Triton solution showed that decellularization for 24 hours is too aggressive. The amniotic membrane structure was damaged with many gaps. In samples were used 1% Triton solution fig. 3 (A) could be seen disappearance of the epithelium (a); continuous basement membrane (b); disorganization of the fiber architecture of the compact layer (c); cellular debris (d). The decellularization in 0.5% SDS solution for 24 hours shows the disappearance of the epithelium (a); continuous basement membrane (b); disorganization of the architecture of the compact layer (c); cellular debris (d). The decellularization in the same solutions in complex with the ultrasound bath showed the same results (fig. 3 (C), (D)), which means that ultrasound had no significant effect on the amniotic membrane decellularization procedure. Decellularization with 1% Triton solution with ultrasound (fig. 3 (C)) shows some fragments of epithelial cells (a); continuous basement membrane (b); disorganized compact layer (c); cellularity (d). 0.5% SDS solution with ultrasound (fig. 3 (D)) shows the disappearance of the epithelium (a); basement membrane discontinuity (b); disorganized compact layer with fragmentation (c); and acellularity (d).

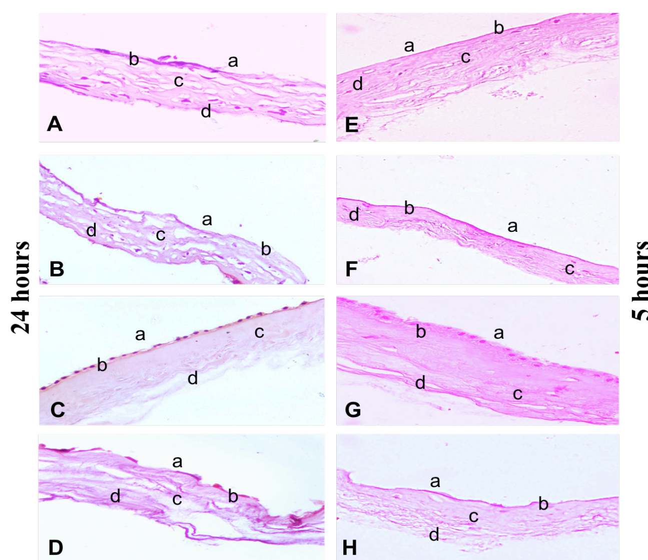

Fig. 3. Histological staining of decellularized amniotic membrane in:

(A) 1% Triton solution for 24h. H-E, × 100,
(B) 0.5% SDS solution for 24h. H-E, × 100,
(C) 1% Triton solution with ultrasound for 24h. H-E, × 100,
(D) 0.5% SDS solution with ultrasound for 24h. H-E, × 100,
(E) 1% Triton solution for 5h. H-E, × 100,
(F) 0.5% SDS solution for 5h. H-E, × 100,
(G) 1% Triton solution with ultrasound for 5h. H-E, × 100.
(H) 0.5% SDS solution with ultrasound for 5h. H-E, × 100.

The treatment of human amniotic membrane with 1% Triton solution for 5 hours (fig. 3 (E)) was incomplete with some visible cells. The histology shows the disappearance of epithelium (a); contoured basement membrane (b); compact layer preserved (c) and solitary cellular debris (d). Decellularization with 0.5% SDS solution of human amniotic membrane for 5 hours showed better results (fig. 3 (F)). The tissue remained undamaged with no visible cells. The histology shows the disappearance of the epithelium (a); continuous basement membrane (b); disorganized compact layer (c) and acellularity (d).

1% Triton solution ultrasound decellularization (fig. 3 (G)) shows the atrophy with disappearance of the epithelium (a); disappearance of the basement membrane (b); disorganized compact layer (c) and cellular debris (d). The same procedure of decellularization with 0.5% SDS solution and ultrasound bath (fig. 3 (H)) shows the disappearance of the epithelium (a); continuous basement membrane (b); disorganized compact layer (c) and acellularity (d). Based on the decellularization results, it can be assumed that the best decellularization method is with 0.5% SDS solution for 5 hours.

Tissue decellularization is a promising method for the preparation of bio-scaffolds for regenerative medicine. Removing cellular components from tissue or organs produces an ECM consisting of active structural proteins that can be used in tissue engineering. The most effective method of tissue and organ decellularization depends on many factors, such as cell (tissue) type, cell density, tissue's thickness, and lipid content [19]. Different decellularization protocols describe combinatorial and sequential use of different physical, chemical, and enzymatic techniques (tab. 1).

Table 1. Known used decellularization methods [7]

Chemical	Biological	Physical
*Alkaline-acid treatment *Non-ionic detergents *Ionic detergents *Zwitterionic detergents *Tri(n-buyl) phosphate *Hypotonic and hyper-tonic treatments *Chelating agents	*Protease inhibitors *Calcium chelating agents *Nucleases *Antibiotics	*Freezing & Thawing *Mechanical force *Sonication *Mechanical Agita-tion *Hydrostatic pres-sure

In general, chemical and enzymatic techniques are mainly responsible for successful decellularization in commonly used protocols. Physical techniques are generally used to complement chemical and biological techniques and therefore increase the decellularization effects. Physical techniques can produce damage in the matrix, while chemical techniques can produce reactions that change the chemical composition of the ECM [8, 20, 21].

Detergents are chemical agents used to solubilize cell membranes and to dissociate their inner structure. Among them, Triton X-100 is the most commonly used detergent in decellularization processes. It targets the lipid–lipid and lipid–protein interactions, but it leaves the protein–protein interaction intact [22, 23]. It is a very useful agent in those tissues where the key matrix components are primarily proteins. It is an effective detergent to eliminate cells from many tissues, but it is generally avoided in tissues with glycosaminoglycans as a key component in their matrix. Side by side with the Triton X-100, SDS is the other most commonly used detergent in decellularization procedures. SDS solubilizes both the external and nuclear membranes, but also tends to denature proteins and may alter the native structure of the matrix [24, 25]. For these reasons, short time SDS treatment is the most common, aiming to minimize the possible damage to proteins and the overall matrix structure. Nevertheless, it is very efficient in removing nuclear and cytoplasmic waste [26].

As previously mentioned, physical techniques are not enough to decellularize the tissue, but they can help in combination with chemical and enzymatic processes. For example, when big tissue pieces or whole organs are the target of decellularization, perfusion is recommended in order to better reach all tissue areas [27].

Superficial cells of a tissue can be effectively eliminated by physical scraping with a sharp tool or abrasive accompanied by enzymes or salt solution. Physical removal of the extra layers initially helps to make the decellularization regimen more efficient. However, the amount of force required must

be precise because the underlying structure and membrane attachment are vulnerable to any kind of direct mechanical stress [9, 21].

Mechanical agitation and sonication are useful in combination with a chemical treatment to assist in cell lysis and the removal of cellular debris [28, 29]. Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low-profile roller. There are no studies to determine the optimal magnitude or frequency of sonication for the disruption of cells. However, the standard ultrasonic cleaner appears to be as effective at removing cellular material as the movement of an orbital shaker. In all of these procedures, the optimal speed, the volume of reagent, and the length of mechanical agitation are dependent on the composition, volume, and density of the tissue [30]. In Forouzesh F. et al. study [31], direct and indirect ultrasonic waves were accompanied by SDS with 0.1% and 1% concentrations as chemical agents to decellularize cartilage tissue. The decellularization process was investigated by nucleus staining with H&E, and by glycosaminoglycans and collagen staining. Results of this study showed that H&E staining indicated that 1% SDS, in addition to ultrasonic bath for 5 h, significantly decreased the cell nucleus remnant to the lacuna ratio by 66%. It is declared that ultrasonic bath helps to a better infiltration of decellularization agents, moreover, it is mentioned that the process time has decreased due to this method and no significant defect has been seen on the structure of the tissue.

In order to select the appropriate decellularization method, the structure of the tissue must be taken into account. These decellularization methods must be carefully selected and tested. If it is necessary to keep the tissue structure intact, the chemical methods must be carefully selected in order not to damage the ECM.

Conclusions

Based on the SEM of the human amniotic membrane, it is possible to assume that the collagen fibers arranged in different directions are an indication that the human amniotic membrane has good mechanical and tensile properties.

The histological examination showed that the membrane consists of three main layers, such as epithelium, basement membrane and stroma, which were previously described in the literature.

The human amniotic membrane decellularization process with 0.5% SDS solution and 1% Triton solution showed that decellularization for 24 hours is too aggressive for the amniotic membrane structure. It shows the disappearance of the epithelium, obliterated basement membrane and disorganization of the architecture of the compact layer. The decellularization methods with 1% Triton solution for 5 hours show incomplete decellularization with some visible cells.

The method of decellularization with 0.5% SDS solution is more suitable for amniotic membrane decellularization and can be performed in only 5 hours.

The use of ultrasound during one hour did not have a significant effect on the decellularization procedure. Based on these results and literature data, it is possible that ultrasound should be used more time in combination with chemical decellularization.

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Authors' contributions

OI conducted literature review, obtained the necessary data and wrote the manuscript; AC monitored the experiment and critically revised the manuscript; OP interpreted the data and drafted the manuscript; VN revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate

The project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No14, 15.03.2019).

Conflict of interests

No competing interests were disclosed.