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The effectiveness of the tissue engineering in the obtaining of the biological materials from the extracellular matrix

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Abstract

Background: The present work describes the possibility of manufacturing biomaterials from the extracellular matrix for the treatment of the skin wounds. Biomedical collagen-based materials are clinically effective. Collagen is the most abundant and major component of the skin. Porcine collagen is almost similar to the human collagen, it is not immunogenic when used for the therapeutic purposes. Biomaterials can be obtained from the decellularized dermis, being a matrix rich in the collagen and glycoproteins.
Material and methods: 3 parallel groups of biomaterials were established and the average value was calculated. To ensure the effectiveness of the decellularization process, the decellularized porcine dermis was compared with the intact sample using qualitative and quantitative criteria.
Results: Histologically, the decellularized tissues revealed the presence of fewer cells. As a result, were removed approximately 80.5% of the genetic material from porcine dermal structures, demonstrated by the spectrophotometric quantification of deoxyribonucleic acid. *In vitro* graft degradation study in 0.01 M phosphate buffer pH 7.4 combined with collagenase, demonstrated a significant ($p < 0.05$) loss of collagen sponge mass by 100% over one hour in the group II compared to the decellularized dermis in group I which decreased in the weight by 91.3% during 35 hours.
Conclusions: Acellular biomaterials are immunologically inert, have hydrophilic and biodegradable properties, thus they can play a key role in the wound care, exerting the transfer of the bioactive molecules and drugs directly into the wound.
Key words: porcine acellular dermis, collagen sponge, biomaterials, tissue engineering.

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Introduction

The extracellular matrix (ECM) is a structural support network made up of various glycoproteins. It influences a large number of cellular processes, including migration, wound healing, and differentiation, all of which are of particular interest to tissue engineering researchers [1]. Collagen-based biomedical materials are clinically important and effective [2]. Collagen is the most abundant structural protein in animals. It is the major component of the skin [3, 4]. Porcine collagen is almost similar to human collagen, it is not immunogenic when used for therapeutic purposes. The dermis has been used for tendon reconstruction, hernia repair, skin and wound healing in plastic and reconstructive surgery (Belviso I., et al. 2020) [5]. Decellularization or removal of cells from the complex mixture of structural and functional proteins that make up the extracellular matrix can be done by physical (shaking, sonication, freezing and thawing), chemical (alkaline oracids, ionic, nonionic detergents, tri-n-butyl phosphate (TBP), hypotonic or hypertonic treatments, chelating agents) and enzymatic methods (trypsin or protease inhibitors) [3]. The effectiveness of the decellularization procedure is characterized by the following parameters: the absence of cells and nuclear debris, the preservation of matrix integrity, tissue density and the ability of cellular repopulation. The acellular matrix must be compatible with cells and possess phenotypic building material [7]. Scientific articles describe different techniques for extracting collagen from the skin of animals eaten for meat. The journals address the pretreatment and extraction methods that have been investigated for the production of collagen from animal skin. Enzymatic, acid or alkaline processing was used. Chemical hydrolysis extraction, salt solubilization, enzymatic hydrolysis, ultrasound-assisted extraction, and other methods are described. Post-extraction purification methods are also explained. Natural scaffolds allow proper cell population, proliferation and secretion, which is important for their survival and regeneration in the affected tissue [8-10]. The shortcomings of some decellularization methods are: persistence of residual deoxyribonucleic

acid, which has a significant proinflammatory effect [11], inhibitory response on cell proliferation and cytotoxic effect. Researchers have described the factors that can lead to these negative effects on the matrix, being residual detergents, sterilizing chemicals that modify the structure of the scaffold. The behavior of the acellular scaffold applied to the wound will be different depending on the hydrogen indicator in the lesion [12]. Cellular content in the ECM has the potential to cause graft rejection when grafted, so it should be removed prior to transplantation. With the development of decellularization technology, extracellular matrix as a new biomaterial has attracted the attention of many researchers. In the present work will be examined the effects of tissue engineering methods in obtaining biomaterials and the factors that influence the preservation of bioactive properties for the development of biological dressings for skin wounds: (1) Decellularized ECM must have less than 50 ng of double-stranded DNA per mg weight dry, (2) lack of visible nuclear staining when treated with DAPI or H&E. Furthermore, the mechanical properties, including (3) biodegradable and (4) hydrophilic properties, will be considered as well.

Material and methods

Skin preparation. To achieve the aim of the study were examined 30 decellularized porcine dermal grafts and 30 non-cross-linked porcine dermal collagen scaffold fragments (fig. 1, 2). The samples were obtained from piglets weighing up to 10 kg euthanized by blunt trauma, following the recommendations of the university ethics committee (decision No 41 of 03.02.2020). The dimensions of the tissue fragments being 5×5×2 mm and the weight of 87.9 ± 3 mg for the acellulardermis and 15.26 ± 5.0 mg for the collagen sponge.

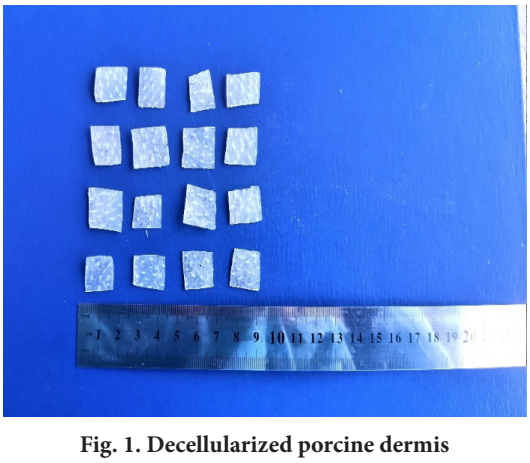

Fig. 1. Decellularized porcine dermis

Separation method. By treating the tissues with 0.3% trypsin solution at 37°C for 30 minutes with minimal mechanical effort, the dermo-epidermal separation of the grafts was obtained, according to the protocol (Wilkinson D., et al. 1974) [13].

Decellularization method. Tissue decellularization was performed by treating porcine dermis with 1% Triton

Fig. 2. Porcine dermal collagen sponge

X-100, 4% sodium deoxycholate and washing thoroughly in sodium phosphate buffer [14].

Staining method with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI). DAPI staining was performed to visualize the presence of residual nuclei in normal and decellularized porcine dermis and to qualitatively assess the effects of decellularization, using confocal fluorescence microscopy (Optica, Italy) [15]. Samples were fixed in 4% paraformaldehyde, permeabilized, sectioned with a microtome (7 μm) and stained with DAPI (fig. 3).

Fig. 3. Staining of the dermis with 4′,6-Diamidino-2-phenylindole dihydrochloride

Morphological assessment. Examination of the decellularized samples was performed by the histological examination with hematoxylin-eosin (H&E). Samples were fixed in 4% buffered formaldehyde [15].

Spectrophotometric method. The spectrophotometric method was applied for DNA quantification. Decellularized and native tissues were quantified using a kit (DNA Extraction Kit, Cygnus Technologies, USA). The extracted DNA was quantified spectrophotometrically in a microplate reader at a wavelength of 260/280 nm (NANODROP 2000C). The value obtained was represented as a function of the weight of dry samples ng/mg [16, 17].

Obtaining collagen scaffolds. Collagen was most commonly extracted from the skin through a hydrolysis treatment involving the use of acidic or alkaline solutions. Under acidic conditions, collagen molecules have a net positive shift, and the resulting electrostatic repulsive

force between them facilitates molecular separation [3]. Commonly used organic acids are acetic, chloroacetic, citric and lactic acids. Acetic acid has been widely reported for collagen extraction [2]. Proteolytic enzymes are used in collagen extraction. These enzymes can be of animal origin (trypsin, pepsin), plant origin (e. g. bromelain, papain, ficin) or single or mixed enzymes microbial products (e. g. collagenase, proteinase K, Alcalase® (Novozymes, Bagsværd, Denmark), Nutrase® (Nutrex, Hoogbuul, Belgium), Flavourzyme® (Novozymes, Bagsværd, Denmark) and Protamex® (Novozymes, Bagsværd, Denmark)). Pepsin from animal sources is most commonly used in collagen extraction [3]. Thus, 30 fragments of further decellularized porcine dermis were morcellated and subjected to successive treatment with 0.5 M acetic acid solution and 5% w/w pepsin based on dry tissue weight. Neutral salt solutions are effective in solubilizing collagen and are commonly used in extraction. Examples of salts used are citrates, phosphates, sodium chloride and Tris-HCl. Collagen suspension was filtered and repeatedly solubilized with 2.5 M sodium chloride in 0.5 M acetic acid solution. Afterwards, the obtained collagen was lyophilized for 72 hours until the porous collagen scaffold was obtained (Hakim T., et al. 2021) [18].

***In vitro* degradation of dermal grafts.** Each phase of skin wound epithelialization is influenced by exogenous and endogenous factors. One of the significant endogenous factors that determine the rate and outcome of biochemical reactions during wound recovery is wound pH [12]. A number of the authors claim that the pH changes during the epithelialization process. During the inflammatory phase, the hydrogen value moves to the acidic side and varies from 5.4 to 6.9. During the proliferative phase the pH becomes neutral or alkaline and ranges from 6.9 to 9.0. And in the final phase of healing, the pH takes on the value of healthy skin, which normally ranges from 4 to 6. Thus, the *in vitro* degradation behavior was analyzed by following the weight loss of the biomaterials in 0.01 M phosphate buffer solution (PBS) in acidic, neutral and basic environments. The freeze-dried scaffold was weighed (m0), immersed in a centrifuge tube containing PBS. The pH of the buffer was 7.4, 4.0 and 10.0, the exposure time being 1, 7, 14, 21 and 28 days in the incubator conditions at t 37°C. Comparatively, graft degradation was monitored in 0.01 M PBS pH 7.4 solution combined with collagenase from Clostridium histolyticum (≥250 CDU/mg solid, Sigma-Aldrich, UK) 10 U/ml, the follow-up period being of 1, 5, 8, 24 and 35 hours at t 37°C. The remaining mass fraction (D, %) was calculated using the following formula: $D = mx/m0 \times 100\%$, where mx is the final tissue mass. Four parallel groups were established and the mean value was calculated.

Water absorption test. In the local treatment of skin wounds, it is important to rely on the use of dressing material impregnated with antiseptic drugs. Therefore, this test allowed studying the hydrophilic properties of the scaffolds. The absorption of water revealed the diffusion of the medium into the tissues being necessary for the resorption of exudate from the wound and the cultivation of cells on the ECM obtaining essential nutrients. 0.01 M PBS pH 7.4 was used in the fluid absorption test. The time required to follow the weight dynamics of the samples being 1, 2, 4, 8, 12 and 24 hours at t 25°C. The immersed samples were then removed from the solution and weighed, and the excess water on the surface of the samples was gently blotted with a filter paper to obtain the W_{WET}. The percentage of water absorption for the samples at different times was calculated as follows:

$$\text{Water absorbtion (\%)} = \frac{100\times W_{WET} - W_{DRY}}{W_{DRY}},$$

where W_{DRY} and W_{WET} are the weights of the dry and wet scaffolds respectively at the required times [19]. Four samples were tested for each scaffold and the average values were recorded.

Scanning electron microscopy. The inside of the graft is crucial for appreciating the "microenvironment". Pore size and connectivity affect cell adhesion, nutrient exchange and metabolic waste removal, and skin regeneration. The morphological characteristics of the acellular dermis scaffolds were observed using scanning electron microscopy (SEM) [20]. After washing with phosphate buffer, the decellularized dermis samples were dehydrated and dried under vacuum. Then the dried sample was cut and the cross section was coated with 10 nm of Au. For scanning electron microscopy was used a representative tissue sample from each study group and a non-degraded control sample to monitor ECM disorganization leading to tissue weight loss.

Results

DAPI and hematoxylin eosin analysis

To create the porcine extracellular matrix biomaterials, decellularization was performed to remove only porcine resident cells from the dermal skin devoid of epidermis and hypodermis. To verify the efficiency of decellularization, DAPI and H&E staining was performed. As shown in figure 4, the nuclei of the cells in the normal dermis are stained compared to the image (figs. 5 and 6) where the cells of the decellularized dermis were not highlighted by staining, while the ECM surrounding the cells was maintained.

DNA quantification by spectrophotometry

To quantitatively characterize the effects of decellularization, DNA was quantified from normal and decellularized dermis. Figure 7 shows the result. Residual DNA in the extracellular matrix was 2.43 ± 0.5 ng/µl ng/mg, which was significantly different from 17.43 ± 3.4 ng/µl determined from normal dermis from which the epidermis and adipose tissue have been removed.

Thus, it was managed to remove about 80.5% of the genetic material from the porcine dermal structures according to spectrophotometric DNA quantification. As a

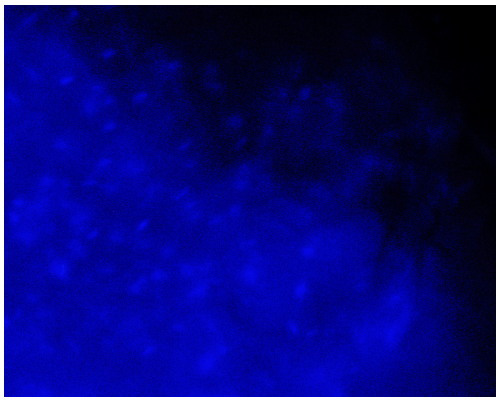

Fig. 4. Normal porcine dermis stained with DAPI

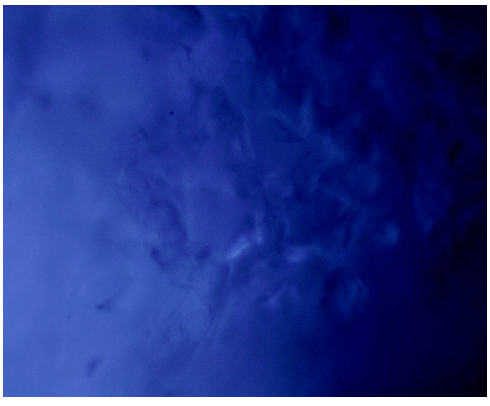

Fig. 5. Decellularized porcine dermis stained with DAPI

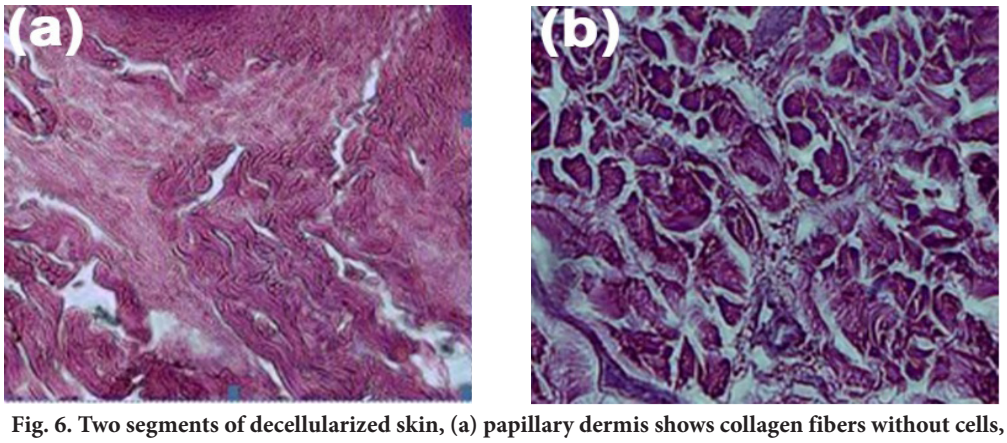

Fig. 6. Two segments of decellularized skin, (a) papillary dermis shows collagen fibers without cells, (b) reticular dermis shows collagen fibers without cells, H-E×140

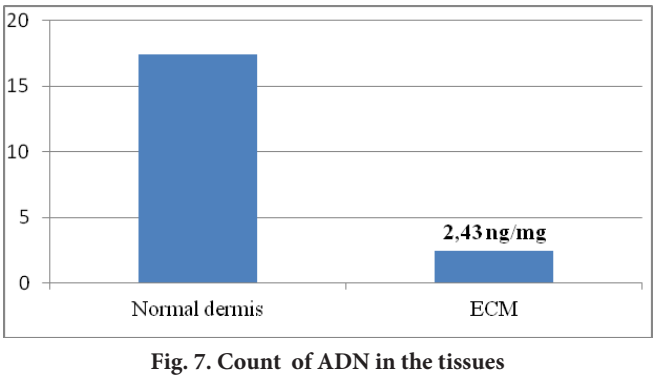

Fig. 7. Count of ADN in the tissues

result, similar to the results of DAPI and H&E staining, the extraction of dermal extracellular matrix was confirmed.

***In vitro* degradation of the biomaterials**

The rate of biomaterial degradation in the skin wound should ideally match the rate of wound regeneration. Thus, if the acellular scaffold degrades rapidly during the early stage of wound regeneration, it will not provide a good barrier for regeneration itself, and this will eventually lead to soft tissue extension into the skin defect, which is not welcome for organized soft tissue regeneration. As shown in Figure 8 and 9, the degradation rate of dermal

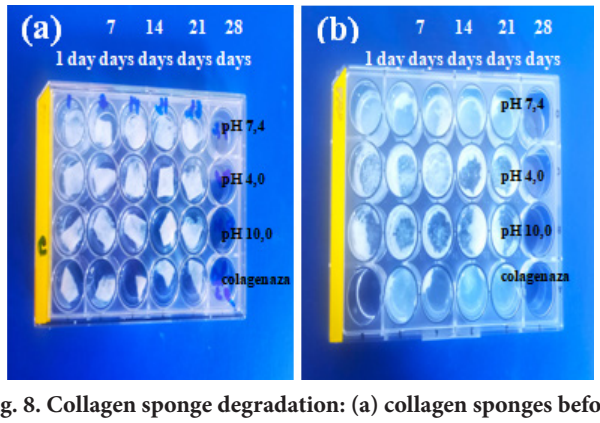

Fig. 8. Collagen sponge degradation: (a) collagen sponges before degradation, (b) collagen sponges after degradation.

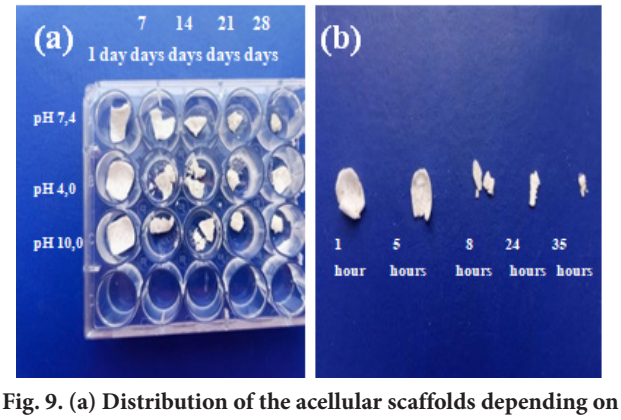

Fig. 9. (a) Distribution of the acellular scaffolds depending on the duration of exposure and the pH of PBS chosen for the study. (b) Degradation behavior of acellular dermis in the presence of collagenase (10 U/ml, PBS pH 7.4, 35 hours).

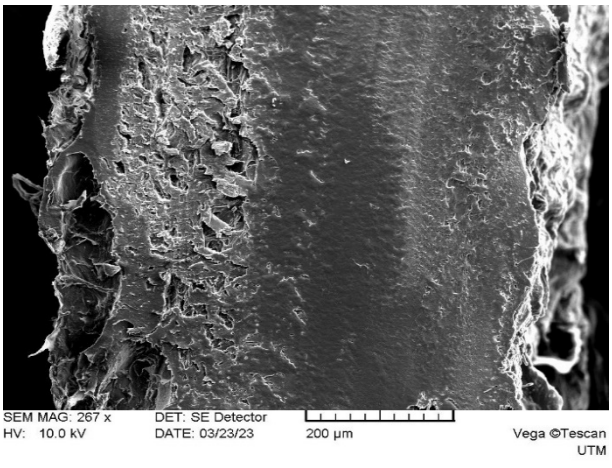

Fig. 10. ECM from the decellularized porcine dermis.

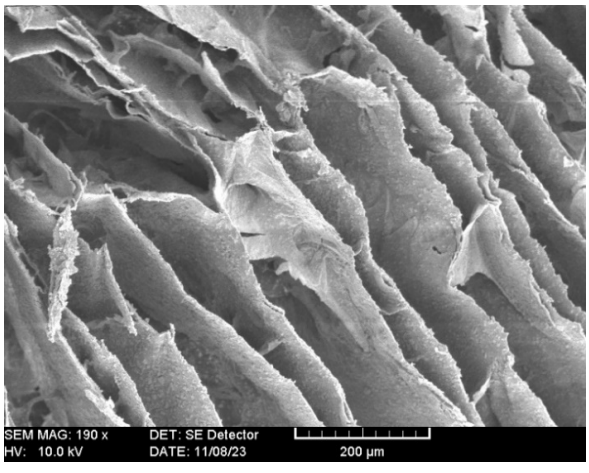

Fig. 11. Collagen sponge from porcine dermis.

collagen sponge in 0.01M PBS pH 7.4 combined with collagenase from Clostridium histolyticum was the fastest, reaching a degradation rate of 100% in the first hour of contact with fluid compared to 91.3% of the extracellular matrix that degraded within 35 hours. At the same time, the degradation volume of collagen sponges in 0.01 M PBS solution with pH 7.4 and 10.0 was 100% and 95% in pH 4.0 at 7 days. Compared to acellular scaffolds that performed differently in 0.01 M PBS solution pH 4.0 and 10 without enzymes, it accounted for 79.8% and 74% of the total sample from the 21st to the 28th day, and then the degradation tended to be slow. In 0.01 M PBS solution with pH 7.4, the degradation of acellular dermis reached 90.3% on the 28th day.

Evaluation of hydrophilic properties

The hydrophilic properties of the biomaterials were evaluated by the absorption test of 0.01 M PBS solution with pH 7.4. A variable depending on the exposure time was obtained, namely the acellular dermis immersed in the solution reached 350 mg at the 4th hour of immersion in the liquid, the initial mass being 87.9±3 mg. The absorption rate of the collagen sponges was nil. The collagen samples hydrolyzed in the PBS solution during the first hour of immersion.

Scanning electron microscopy

Decellularization of the dermis with Triton/SDS removed cells, resulting in a porous appearance of the ECM (fig. 10) and preservation of dermal structures, which is a hallmark of decellularization. The ECM is mostly composed of fibers, has a more dense structure and small pores. Scanning electron microscopy image of the sponges showed collagen-based membrane and fibrous components. It has an irregular and relatively fluffy structure, with large pores (fig.11).

In vivo recruitment of the tissue-forming cells into scaffolds is closely regulated by the physicochemical properties of the scaffolds, e.g., pore size, porosity, bioactivities, stiffness, etc. The dermis, having dense ECM with inherent small pores, would inhibit cellular infiltration and lead to poor tissue regeneration. Therefore, the development of naturally occurring scaffolds with three-dimensional structure of large interconnected pores would be essential for cell infiltration and functional tissue regeneration [21]

Discussion

The skin is the natural barrier between the human interior and the external environment, consisting of the epidermis, dermis and subcutaneous tissue. In daily life, the skin is easily damaged, and the human body has a certain reconstruction function to realize the self-repair of the damaged skin. However, when the affected skin area is large and the degree of damage is severe, such as skin defects caused by severe burns, trauma or some chronic wounds that cannot heal, they may be complicated by infection, and the reconstruction of the structure and skin function can only be ensured by autologous skin transplantation [22-25]. Autograft requires a sufficient supply of skin, however the amount of autologous skin available to the patient is quite limited and the process of removing the skin will cause additional pain and secondary trauma to the patient. Another type of implantable skin is derived from cadaver skin. However, corpse skin is limited and mostly aged and unhealthy. More importantly, the use of cadaveric skin carries the risk of transmitting contagious diseases and is ethically restricted [26]. Therefore, the development of artificial skin has become a hot spot in the field of medical skin tissue engineering. An acellular dermal matrix is used as a skin substitute. For this, it must be de-epithelialized and decellularized removing the cellular components and preserving the three-dimensional collagen and reticular structures in the dermis [27, 28]. Thus, the extracellular matrix reduces the rejection reaction while preserving the native dermal structure, thereby inducing the growth of cellular components, supporting fibroblast infiltration, the formation of new blood vessels, promoting the gradual fusion of fibroblasts and transplanted autologous epidermal components, finally, the complete structure of the skin is formed,

and the original functions of the skin are practically recovered. In the process of preparing the acellular dermal matrix, pig skin is usually selected and treated by a physical, chemical or biological method to obtain the porcine acellular dermal matrix [28]. A key parameter of decellularized ECM materials is balancing strength and biodegradability properties. Decellularized ECM materials have been shown to retain a complex array of proteins present in the original tissue being the cytokines that are preserved at the time of decellularization. Among the several cytokines whose levels are quantified are vascular endothelial growth factor and transforming growth factor beta that are retained in the ECM [29, 30]. After decellularization of the dermis, many ECM proteins remain in the material, including collagen III, collagen IV, collagen VII, laminin, and fibronectin. Glycosaminoglycans are also preserved, including hyaluronic acid. They ensure the bioactive properties of ECM for recellularization, promoting rapid integration and repair in clinical applications [29]. Most decellularization efficacy test reports revealed positive data on X-100 triton treatment compared to 0.1% sodium dodecyl sulfate (SDS) and 0.1% trypsin solutions [32-32]. It was demonstrated that the X-100 triton decellularization method in combination with SDS or tri-n-butyl phosphate solution was the most effective, but also the most destructive in terms of glycosaminoglycan and collagen depletion. This phenomenon was also shown in the study where the skin was decellularized with trypsin, triton, and sodium hydroxide and observed fibrinoid necrosis, fragmentation, and undulation of fibrillar structures in the dermis affirming depletion of the dermal matrix. Although, from the protocols tested in the study, triton X-100 had the least harmful effect on glycosaminoglycan content [35]]. Crapo P., et al. suggested that the densest tissues, such as dermis, tendon, and trachea require decellularization protocols by continuous agitation lasting from days to months [32, 36, 37]. However, in the present study, the desired results were obtained after 48 hours of treatment with biological detergents. Gilbert T. et al. have shown that cells and cell products cannot be completely removed from dense tissues such as dermis, even with the most rigorous processing methods [7]. However, in the present study, complete cell-free membrane was observed after 48 h of treatment, although SDS solubilized cell membranes and dissociated DNA. It is therefore effective in removing cellular material from tissues. Sodium dodecyl sulfate was more effective in removing cell debris and cytoplasmic proteins such as vimentin from the tissue compared to other detergents, but is more aggressive to ECM [35, 38, 39]. Dodecyl sulfate was more effective than Triton-X 100 in removing nuclei from dense tissues. SDS disrupted native tissues and caused a decrease in the concentration of GAGs and depleted collagen. Sodium deoxycholate (SD) is very effective at removing cellular debris. SD has been shown not to alter the structural properties of the ECM but it tends to disrupt the structure of the tissue itself, so it should be used in a lower concentration. Among the freeze-thaw cycle methods with NH (4) OH and triton X-100 with 1.5 M K Cl showed the best effect on the removal of cellular components from the complexes, while the other five methods could only partially remove the component cells. The freeze-thaw method maintained the ECM structure as well as the mechanical strength, but retained a large amount of the cellular components of the ECM scaffold. About 88% of the DNA was left in the ECM after freezing defrosting treatment. *In vitro* inflammatory assays suggested that the amount of DNA fragments in the ECM scaffolds did not elicit a significantly different immune response. All three ECM scaffolds showed a comparable ability to support cell repopulation. There were described the successful results of de-cellularization with SDS and Triton X-100. Total absence of nuclear structures and removal of viable cells was confirmed by hematoxylin-eosin staining and scanning electron microscopy [39]. Macroscopic evaluation of de-epithelialized rat skin with the hypertonic solution for 4 hours found that the epidermis was not separated from the dermis. Thus, after 6 hours, the multilayered epithelium was removed more easily. However, after another 8 hours the epidermis was separated spontaneously with minimal mechanical effort and a completely de-epithelialized dermis was obtained. Treatment of the skin with hypertonic saline for 24 hours resulted in an acellular matrix with collagen fibers of insignificant thickness. In de-epithelialized skin treated with triton X-100, cell debris was detected between the interstitial spaces of the thicker collagen fibers [11]. At 48 hours after treatment with triton X-100, the acellular dermis became more porous. Treatment of skin with sodium dodecyl sulfate (SDS) for 24 hours resulted in a membrane with fewer cells and collagen fibers with significantly preserved thickness. At 48 hours after immersing the dermis in SDS, the collagen fibers became more fragile with large spaces between them. Treatment with 1% sodium deoxycholate (SD) effectively removed cellular debris at 48 hours. Increasing the concentration from 1% to 2% of SD, led to the expansion of the spaces between collagen fibers. No cell nuclei were observed and the tissue was composed of the more porous extracellular matrix. Hypotonic and hypertonic solutions have been reported as ineffective decellularizing agents [39]. 48 hours after immersing the dermis in SDS, the collagen fibers became more fragile with large spaces between them. Treatment with 1% sodium deoxycholate (SD) effectively removed cellular debris at 48 hours. Increasing the concentration from 1% to 2% of SD, led to the expansion of the spaces between collagen fibers. No cell nuclei were observed and the tissue was composed of the more porous extracellular matrix. Hypotonic and hypertonic solutions have been reported as ineffective decellularizing agents. TBP treatment resulted in a displacement of nuclear waste. This led to a decrease in the content of glycosaminoglycans. However, because most tissues are very dense, deoxyribonucleic acid (DNA) is almost impossible to remove 100%. Therefore, DNA remaining after de-

cellularization should be examined quantitatively or qualitatively, that should not yield any staining after treatment with DAPI or H&E [15].

Conclusions

1. The variability of the hydrogen indicator and the involvement of the proteolytic enzyme that is secreted in significant quantities in all epithelial lesions and causes the degradation of collagen polypeptide fibers is important in the resorption of acellular dermal grafts.

2. The samples degraded in contact with the fluids, result in a significant reduction in weight. Non-cross-linked sponges hydrolyze immediately on contact with fluids.

3. Evaluation with DAPI, H&E and SEM assesses the effectiveness of tissue engineering in obtaining biocompatible materials.

4. Obtaining smart biological grafts for skin wound regeneration is one of the research directions of modern tissue engineering and represents an attractive segment of regenerative medicine.

5. Xenograft is easier to obtain in commercial quantities that would cover clinical needs. Scaffolds obtained from decellularized porcine dermis have great potential as a source of bioactive molecules with biocompatible, hydrophilic and biodegradable properties.

6. To show the quality of the scaffolds, it is necessary to perform immunohistomic studies to determine the growth factors on the acellular membrane, cross-linking, recellularization and cytocompatibility of the grafts.

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OM proposed the concept and design of the research, selected the literature and contributed to the elaboration and writing of the manuscript. AC, VC and TB performed microscopic images and helped draft the manuscript. VN conceptualized the idea, designed the research and monitored the experiment. All the authors 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 No 41 of 03.02.2020).

Conflict of interests
No competing interests were disclosed.