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# Comparative analysis of the skin decellularization methods

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## Abstract

**Background:** The extracellular matrix plays an important role in the promoting the tissue regeneration and repair. Decellularization or removal of the cells from the complex mixture of the structural and functional proteins that constitute the extracellular matrix (ECM) can be done by the physical (agitation, sonication, freeze and thaw), chemical (alkaline orchids, ionic detergents, nonionic, tri-n-butyl phosphate (TBP), hypotonic or hypertonic treatments, chelating agents), and enzymatic methods (trypsin or protease inhibitors). However, complications associated with the use of the decellularized skin have been reported, which are widespread and poorly understood. In this synthesis have been included publications, identified by the Google Search engine, National Bibliometric Tool (NBT), Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry. The results of the decellularization were reported in terms of the number of cells remaining in the collagen fibers depending on the duration of exposure to chemical agents.

**Conclusions:** The natural matrix is more widely used than synthetic material, because it has the natural structure and composition of the ECM, it naturally stimulates cell development and allows the incorporation of the growth factors and other proteins increasing cell proliferation.The assessment of the quality of decellularization techniques is done by evaluating the necrosis of the extracellulare matrix, the depletion of the collagen fibers and the remaining amount of genetic material.

**Key words:** decellularization, extracellular matrix, cell proliferation.

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## Introduction

The extracellular matrix plays an important role in the promoting of the tissue regeneration and repair [1]. With the development of the decellularization technology, the extracellular matrix in the form of a new biomaterial has attracted the attention of many researchers. The extracellular scaffold is the ideal substrate for the tissue engineering, presenting a carcass on which the body's cells survive [2, 3]. The functions of the extracellular matrix as a support material and biological substrate are the regulation of the cellular metabolism, such as the cell proliferation, morphogenesis and differentiation [4, 5]. Norton L. et al. described the decellularization technology of the use of the non-denaturing anionic detergent [2, 6]. The objectives of the decellularization process are to remove the potential immunogenic material and to obtain a biocompatible carcass for the growing cell host. Decellularization or removal of the cells from the complex mixture of the structural and functional proteins that make up the extracellular matrix can be done by the physical methods (agitation, sonication, freezing and thawing) [7, 8], chemical methods (alkaline oracids, ionic detergents, non-ionic, tri-n-butyl phosphate, treatments with hypotonic, hypertonic substances [8], (EDTA) tetrasodium salt dihydrate chelating agents), enzymatic methods (trypsin) and protease inhibitors [9].

Following the decellularization of the skin, the remaining extracellular matrix is used in the tissue engineering [10], creating a skin graft used for the wound healing, the soft tissue reconstruction in the sports medicine [11–14]. Decellularized tissue samples are frequently used with a variety of the clinical applications. In the plastic surgery, the human acellular skin matrix was used for the tear duct repair [15–18], breast reconstruction [19–21], hernia repair [22–25], in the treatment of the chronic wounds, such as trophic ulcer of the diabetic foot [26–28]. With the development of the tissue engineering, the use of decellularized products is gradually expanding, as they can function as substitutes for the traditional biomaterials (e.g., polyurethanes, PLGA (poly (lactic-co-glycolic acid)), etc.) [29, 30]. Thus, they can serve as the inductive materials for the cell invasion. Therefore, the decellularization methods fall into three broad categories: physical, chemical and enzymatic [31, 32]. Most samples are prepared using a combination of the reagents, the most popular being chemical and enzymatic techniques [33, 34]. The effectiveness of the decellularization procedure

is characterized by the following parameters: the complete character of the removal of the cells and nuclear debris, the preservation of the matrix integrity, the tissue density and the ability of the cell repopulation. The decellularized matrix must be compatible for the cells and have a repairing phenotypic building material [35]. Natural scaffolding allows the invasion, proliferation and proper secretion of the cells, which is important for their survival and regeneration of the affected tissue [36–41]. The shortcomings of some decellularization methods are: the persistence of the residual deoxyribonucleic acid (DNA), which has a significant proinflammatory effect [37], inhibitory response on the cell proliferation and the cytotoxic effect [38, 40]. Researchers have described the factors that can lead to these negative effects on the matrix, being the residual detergents, sterilizing chemicals that change the structure of the scaffold [42]. The material was synthesized based on the randomized studies, clinical and preclinical experimental cases, published between 2003 and 2020, which aimed to elucidate the results of the skin decellularization. In this synthesis, the publications accessible in English, identified by the Google Search engine, National Bibliometric Tool, Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry, from the databases of the life science journals and online books by the keywords are exposed in the table 1.

The literature synthesis was performed using 61 sources from PubMed, 11 – through the National Biometric Tool, 5 – Springer, 2 from ScienceDirect, 2 – Web of Science, 1 from Biosience, Biotechnology and Biochemistry, 1 – Wiley Online Library and 1 from Elsevier.

For the advanced selection of the bibliographic sources, the following filters were applied: papers published until September 2020, articles in Romanian and English. Original research journals were selected informing about studies, conducted in the clinical, preclinical and experimental conditions. After examining the titles of the articles obtained, only works containing relevant information on the skin decellularization methods were selected. The bibliography of the selected articles was also studied, in order to find all potentially significant sources of the intended purpose. The information was systematized, highlighting the main aspects of the contemporary vision on the obtaining of the extracellular dermal matrix. If necessary, the additional sources of the information were consulted to clarify some notions. Duplicate publications, articles that did not correspond to the purpose of the paper and were not accessible for viewing, were excluded from the list of the publications generated by the Search engine.

The evaluation of the decellularization of the biological scaffold for the tissue engineering was based on the cellularity of the matrix [43]. The studies were performed on the fragments of the rat, pig and human skin, taken from the back and abdomen in the first 24 hours after the euthanasia of the animals or the death of the donor. Prior to processing, in order to wash the tissue of impurities, the skin was stored in cold phosphate buffer (PBS) with antibiotic (0.1% Amikacin). Thereafter, the skin was rinsed with PBS to remove the blood residue. The maximum time between the skin sampling and tissue decellularization initiation was up to 4 hours. Deepithelated skin can be obtained by different methods: the mechanical removal of the epidermis [44], osmotic method, enzymatic method with trypsin (0.5%) and 2M sodium chloride [45, 46]. All decellularization reagents were evaluated by point of view of the mechanism of the action and the effect on the extracellular matrix [2, 44, 47]. The isolated material was characterized by the histological examination by eosin and hematoxylin staining, Massom trichrome and spectrophotometric quantification of the nucleic residue.

There are the different reagents and techniques (chemical, physical and enzymatic) of the skin decellularization and usually these methods are used in the combination to increase the effectiveness of the decellularization process. A dermal matrix is made using a three-step method. First, the epidermis is removed using a chemical process. The next decellularization process consisting of the breaking of the lipid-lipid, lipid-protein bonds, solubilization of the cell membranes, osmotic lysis of cells, dehydrating and rupture of the cell membrane finally to varying degrees, will dissociate DNA. The last one, a subsequent washing will remove any residual cellular elements or chemicals [48].

Kumar N. et al. [44] developed the successful techniques for the deepithelialization of the skin by the hypertonic solution. The composition of the substances dissolved in 100 ml of the phosphate buffer solution was as follows: 605 mg of tris base, 4 grams of sodium chloride, 202.5 mg of

**Table 1. Search engines and the keywords used in the synthesis**

| Key words | NBT | PubMed | Web of science | Elsevier | Springer | Wiley Online Library | BBB | Science Direct |
|---|---|---|---|---|---|---|---|---|
| skin biomaterials | 23 | 7468 | 84 | 2482 | 3518 | 4 | 20 | 28766 |
| skin tissue engineering | 48 | 479 | 2752 | 17308 | 40967 | 465 | 49 | 77409 |
| decellularization | 8 | 1873 | 1697 | 1900 | 20 | 2624 | 4 | 5894 |
| skin DNA | 2 | 57 | 569 | 1826 | 9 | 611 | 193 | 250463 |
| ECM | 32 | 27909 | 1256 | 107 | 130 | 51386 | 495 | 652 |
| skin | 634 | 791091 | 2200 | 1826 | 2562 | 1706 | 938 | 100000+ |
| skin cellularity | 2 | 864 | 77 | 5118 | 2717 | 6 | 214 | 332168 |

EDTA. The skin was washed on an orbital shaker at 37° C for 8 hours at 150 rpm. The solution was changed every 4 hours. Macroscopic and microscopic examination was performed at intervals of 4 and 8 hours [44].

Obtaining of the qualitative matrix required the combination of the reagents or the independent action of ones: 1) 0.5% sodium dodecyl sulfate (SDS) with 0.1% EDTA [3]; 2) trypsin combined with EDTA assisted of 1% triton X-100 and 0.26% tris (2-Amino-2-(hydroxymethyl)-1.3-propanediol) [46]; 3) hypertonic solution with 2-Amino-2-(hydroxymethyl)-1.3-propanediol (tris), sodium chloride and EDTA; 4) 1% triton X-100 combined with 0.25% TBP; 5) 0.5% sodium dodecyl sulfate (SDS) with 0.25% tri-n-butyl phosphate; 6) 1% or 2% sodium deoxycholate (SD) folloved by the action of the deoxiribonuclease [44]; 7) hypertonic solution of 1M sodium hydroxide (NaOH) perfects the decellularization action of the 0.25% trypsin – EDTA solution [47]; 8) freeze-thaw cycling (-80°C, six times) with ammonia water (25 mM); 9) 0.1% triton X-100 with 1.5M K Cl aqueous solution; 10) freeze-thaw cycling alone; 11) ammonia water alone; 12) triton X-100 extraction; 13) osmotic shock with 1.5M K Cl; and 14) and freeze-thaw cycling with 3M NaCl [49].

All reagents were helped by the continuous agitation at the room temperature or 37° C in a thermostat for 24 and 48 hours on an orbital shaker at 150 rpm. The solutions were changed at different intervals of 6, 12, 24 and 48 hours. Finally, the tissues were rinsed thoroughly several times with sterile buffer or the distilled water on the orbital shaker. Macroscopic and microscopic examinations were performed at 12 and 48 hours.

## Results and discussion

As a result of the processing of the information identified by Google Search engine, National Bibliometric Tool, Pub Med databases, Web of Science, Springer, Elsevier, Wiley Online Library, Science Direct and Biosience, Biotechnology and Biochemistry, from the databases of the life science journals and online books according to the search criteria 879664 articles were found that address the issue of the skin decellularization. After the primary analysis of the titles, 201 articles were qualified as possibly relevant for the given synthesis. After their repeated review, 84 publications relevant to the stated purpose were finally selected.

As a result of the systematization of the literature data, it was highlighted that the normal structure of the skin served as a template after which the decellularized sample was evaluated according to twenty-one decellularization methods compared for their decellularization effects during skin scaffold preparation (table 2). The authors described the thick epidermis followed by the cellular dermal matrix. Masson trichrome staining showed a preserved cell epidermis, the dermis – with an abundance of the collagen fibers [44].

The final bibliography of the paper included 84 publications. As a result these methods were combined in fourteen separate decellularization protocols [2, 44–47, 49].

The macroscopic estimation of the deepithelialized rat skin with the hypertonic solution for an interval of 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 spontaneously separated with minimal mechanical effort and resulted in an incompletely epithelialized dermal matrix [47].

In this study, twenty-one decellularization methods were evaluated by which the incomplete acellular dermis was obtained (table 2). The treatment of the skin with the hypertonic saline after 24 hours resulted the cell-less matrix with collagen fibers with insignificant thickness. The deepithelialized skin treated with triton X-100 was characterized by more significant cell-less and with the thick collagen fibers. There were cell debris between the interstitial spaces of the collagen fibers [47]. At 48 hours, the complete acellular dermis with increased porosity was described. The treatment of the skin by SDS over 24 hours showed the cell-less membrane with the collagen fibers with the significantly preserved thickness [2]. At 48 hours, the collagen fibers were more fragile with large spaces between them. Treatment with 1% SD effectively removed the cell debris at 48 hours. Increasing of the concentration from 1% to 2% of SD, led to the expansion of the spaces between the collagen fibers. No cell nuclei were observed, and the tissue was composed of the extracellular matrix. At 48 hours,

**Table 2. Overview of the techniques used in the skin decellularization**

| Methods | Mechanism | The effect on ECM | References |
|---|---|---|---|
| **Chemical** | | | |
| Alkaline and acids: ammonium hydroxide, hydrochloric acid, sulfuric acid | Solubilizes the cytoplasmic components of cells; disrupts nucleic acids | Eliminate glycosaminoglycans (GAGs), Dissociates GAGs from collagenous tissues | [50–52] |
| Peracetic acid | More effectively disrupts cell membranes | Preserves many of the native GAGs, preserves the structure and function of many growth factors that are resident in the ecm, including transforming growth factor-β, essential fibroblast growth factor and vascular endothelial growth factor, highly efficient in removing cellular material | [53, 54] |

| | | | |
|---|---|---|---|
| **Non-ionic detergents** | | | |
| Triton-X-100 | Breaks the lipid-lipid and lipid-protein bonds, leaving the protein-protein bonds intact | Almost completely eliminates GAGs, reduced the laminin and fibronectin content | [50–52] |
| **Ionic detergents** | | | |
| Sodium duodecyl sulfate (SDS) | Solubilizes cytoplasmic and nuclear cell membranes | Removes nuclear debris and cytoplasmic proteins (vimentin); tends to disrupt the structure of the native tissue, reduced concentrations of GAG and loss of the collagen integrity | [50–52] |
| TBP | Tend to distort proteins | Keep resistance of of the collagen fibers, but reduce in the collagen content More effective than detergents such as Triton X-100 and sodium dodecyl sulfate (SDS), with varying effects on the preservation of ECM constituents and its native mechanical properties | [55] |
| Sodium deoxycholate | Solubilizes the cell and cytoplasmic membranes | Disrupts ECM components more strongly than SDS | [56] |
| Hypotonic and hypertonic solutions | Cell lysis by osmotic shock | Effective for cell lysis, but do not effectively remove cell debris | [50–52] |
| EDTA | Chelating agents that bind divalent metal ions, thereby disrupting cell adhesion to ECM | Relatively reduce the cellularity of the ECM | [57] |
| **Solvents** | | | |
| Alcohol | Dehydrating and lysing of the cells during tissue decellularization | Decreases levels of structural proteins involved in the interstitial matrix and basement membrane, with a concomitant increase in proteolytic enzymes that degrade these components | [58–60] |
| Glycerol | Dehydrating and lysing of the cells during tissue decellularization | Collagen fiber reassembly, increases the tissue transparency | [61] |
| Acetone | Removes lipids during decellularization | Damages the ECM ultrastructure | [62, 63] |
| **Enzymatic** | | | |
| Trypsin | Removes the peptide bonds of arginine and lysine | Disruption of the ECM, prolonged exposure eliminates laminin, fibronectin, elastin and GAGs, does not influence the amount of collagen in the tissue, decreases tensile strengthn of the collagen fibres | [50–52] |
| Endonuclease | Cleaves phosphodiester bonds within a ribonucleotide and deoxyribonucleotide chains | Difficult to remove from tissue and can invoke an immune response | [50–52] |
| Exonuclease | Cleaves nucleotides from the end of a nucleic acid chain | Slightly reduces ECM cellularity | [52] |
| Dispase | Cleaves specific peptides, especially collagen IV and fibronectin | Slightly reduces ECM cellularity | [64] |
| Phospholipase A2 | Hydrolyzes the phospholipid component | Catalyzes the release of arachidonic acid in the cells, phospholipase A2 -arachidonic acid system is involved in the matrix-initiated signal transduction pathway in ECM, stimulates the ECM cell proliferation by homologous lectin | [58] |
| **Physical** | | | |
| Freezing | Intracellular ice crystals disrupt the cell membrane | Relative ECM disturbance | [59, 60, 51] |
| Freeze–thaw cycling | Cell lysis | Freeze–thaw cycling alone could not remove all the cell nuclei | [49] |
| Pressure | Rupture of the cell membrane | Relative disturbance of ECM | [65] |
| Agitation | It is used to facilitate chemical exposure and removal of cellular material | Aggressive agitation or sonication may disrupt ECM | [66] |
| Electroporation | The oscillation of the electric field disturbs the cell membrane | Partial cell membrane lysis | [49] |

all samples treated according to the protocols showed the complete cell-less matrix with the removal of the cellular debris from the tissue [64].

The hypotonic and hypertonic solutions have been reported as the ineffective decellularizing agents [64–66]. They caused the cell lysis but did not remove the cell debris from tissues [64]. TBP treatment has led to a complete removal of the nuclear waste. TBP did not affect the resistance of the collagen fibers but led to decrease the GAGs content [67].

The cellular content of ECM has the potential to cause rejection when is grafted, therefore it must be removed before the transplantation. DNA and Gal-epitope are two main reasons why the host can respond. The epitope Gal, the α-Gal oligosaccharide (Galα1.3-Galβ1–4GlcNAc-R), is a membrane antigen present in all species, except for Old World monkeys and humans. The absence of α-gal expression in the humans and non-human primates is related to the defects in the α1.3-galactosyl-transferase gene, which catalyzes the assembly of the α-gal molecule in other animals [68]. Because humanity does not have this antigen, transplanting a xenograft leads to a host reaction, causing the graft to be rejected. Therefore, the Gal epitope must be removed from the xenografts before being transplanted [31]. Because the DNA left in the graft can cause inflammatory reactions in the host it must be removed during decellularization. Another reason why DNA needs to be removed is that it causes calcification after implantation. However, because most tissues are very dense, DNA is almost impossible to be 100% removed. Therefore, DNA remaining after the decellularization should be examined quantitatively or qualitatively, that is no image should be obtained after the staining with 4',6-diamidino-2-phenylindole (DAPI) or hematoxylin and eosin (H&E) [42, 69].

Criteria for the assessing the effectiveness of the removal of these components are suggested as follows: Decellularized ECM must have (1) less than 50 ng double-stranded DNA per mg dry weight, (2) less than 200 bp DNA length fragment and (3) no nuclear material visible by 4', 6-diamidino-2-phenylindole or hematoxylin and eosin staining [70]. In addition, the protein content remaining in the ECM must be assessed, focusing on structural proteins, such as collagen, fibronectin, laminin, GAGs and growth factors. Furthermore, the mechanical properties, including elastic recoil and tensile strength, depending on the application, must match the original tissue. In this review, decellularization techniques are evaluated for their effectiveness in these four areas, to rule out failure of complete tissue decellularization, leading to negative results after *in vivo* implantation, including a pro-inflammatory response with M1 macrophage recruitment and subsequent fibrosis [71].

The most decellularization efficacy test reports revealed positive the data on X-100 triton treatment compared to 0.1% SDS and 0.1% trypsin solutions [72–76]. Woods T. and co-authors demonstrated that the X-100 triton decellularization method in the combination with SDS or tri-n-butyl phosphate solution was the most effective, but also the most destructive in terms of the depletion of glycosaminoglycan and collagen [66]. This phenomenon was also shown in the study of Purohit S. [45] who decellularized the skin with trypsin, triton and sodium hydroxide and observed the fibrinoid necrosis, fragmentation and undulation of the fibrillar structures in the dermis which states the depletion of the dermal matrix. Although, from the protocols tested in the study [77], triton X-100 had the least harmful effect on the content of glycosaminoglycans. Crapo P. et al. [69, 78] have suggested that the denser tissues, such as the dermis, tendon and trachea require the decellularization protocols by the continuous agitation, which last from days to months. However, in the present study, the desired results were obtained after 48 hours of the treatment with the biological detergents.

Badylak S. and Gilbert T. [31] have shown that cells and cellular products cannot be completely removed from dense tissues, such as the dermis, even with the most rigorous processing methods. However, in the present study, the complete cell-less membrane was observed after 48 hours of the treatment, although SDS solubilized the cell membranes and dissociated DNA. Therefore, it is effective in the removing of the cellular material from the tissues. Sodium dodecyl sulfate has been more effective in the removing of the cell residues and cytoplasmic proteins such as vimentin from the tissue compared to other detergents, but is more aggressive for ECM [79, 80]. Dodecyl sulfate was more effective than Triton-X 100 in the removing of the nuclei from the dense tissues. SDS disrupted the native tissues and caused a decrease of GAG concentration and depleted collagen [9]. SD is very effective for the removing cellular debris. SD has been shown not to alter the structural properties of the ECM structure, as observed in Kasimir M's study [70] but tends to disrupt the structure of the tissue itself, so it should be used in a lower concentration.

Among the methods described by Hongxu Lu et al., the methods of freeze-thaw cycling with NH(4) OH and triton X-100 with 1.5M K Cl showed the best effect on the removal of cellular components from the complexes, while the other five methods could only partially remove cellular components. The ECM scaffolds prepared by these two methods had similar gross appearances and microstructures [49].

Qi Xing et al. investigated three decellularization methods: high concentration (0.5wt.%) of sodium dodecyl sulfate (SDS), low concentration (0.05wt.%) of SDS, and freeze-thaw cycling method. They found that the high SDS treatment could efficiently remove around 90% of DNA from the cell sheet, but significantly compromised their ECM content and mechanical strength. The elastic and viscous modulus of the ECM decreased around 80% and 62%, respectively, after the high SDS treatment. The freeze-thaw cycling method maintained the ECM structure as well as the mechanical strength, but also preserved a large amount of the cellular components in the ECM scaffold. Around 88% of DNA was left in the ECM after the freeze-thaw treatment. *In vitro* inflammatory tests suggested that the amount of DNA fragments in ECM scaffolds does not cause a significantly different immune response. All three

ECM scaffolds showed comparable ability to support *in vitro* cell repopulation [63, 81].

Haozhen R. et al. described the successful results if the decellularization after the treatment with SDS and Triton X-100. The total absence of the nuclear structures and removal of viable cells were confirmed by hematoxylin-eosin staining and scanning electron microscopy. Collagen was preserved after both treatments. However, the elastin content decreased to about 20% and 60%, the GAGs content decreased to about 10% and 50% and the HGF content decreased to about 20% and 60% of the native liver level after SDS and Triton X-100 treatment respectively. The Triton X-100-treated scaffolds were much superior to SDS-treated scaffolds in the supporting liver-specific function, including albumin secretion (P=0.001), urea synthesis (P= 0.002), ammonia elimination (P=0.007) and mRNA expression levels of the drug metabolism enzymes [82–84].

However, there are studies [55-61], that the enzymatic removal of epitopes from the cell surface reduces the immunogenicity of the xenograft. Thus, in the research [47–55], it has been shown that the presence of the cells in the interstitium and their necrosis after the transplantation can delay the infiltration of the host cells that affect the regeneration. It is obvious, that the specifics of the interaction between the matrix and the cells of the recipient remain to be addressed in the following researches.

## Conclusions

1. The removal of the cells, proteins, DNA from the skin and the porosity of the samples, directly correlate with the exposure time in the decellularization solution.

2. The highest quality extracellular matrices were prepared using a combination of chemical and enzymatic methods.

3. It is clear that skin decellularization with sodium dodecyl sulfate was the most successful and safe method in terms of minimizing the amount of DNA and the risks of the graft rejection after the transplantation.

4. Probably the pig skin could be considered as a study substrat, being available and easily processable, taking into consideration ethical aspects and grafts safety.

5. It has been found that the tissue decellularization probably inhibits the subsequent proliferation of the fibroblasts in the matrix, this fact requires further research.

6. The natural matrix is more widely used than synthetic material, because it has the natural structure and composition of the ECM, it naturally stimulates cell development and allows the incorporation of the growth factors and other proteins increasing cell proliferation.The assessment of the quality of decellularization techniques is done by evaluating the necrosis of the extracellular matrix, the depletion of the collagen fibers and the remaining amount of genetic material.

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MO and DR proposed the concept and design of the study, selected the literature and contributed to the elaboration and writing of the manuscript text. CA and NV performed a critical analysis and helped draft the manuscript. The approval of the final version of the manuscript was read and confirmed by all the authors.

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**Conflict of interests**
The authors declare the absence of a conflict of financial or non-financial interests.