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An overview of animal tissue decellularization techniques and clinical applications

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ABSTRACT

There is a persistent an urgent need to solve tissue and organ donor shortage issues. Decellularized tissues represent a promising alternative to other biologic and synthetic treatments that have been developed, since they aim to retain native tissue characteristics that would help in the regenerative processes such as proliferation, cellular adhesion, and the presence of growth factors, while minimizing the chances of an unwanted host immune response. In the present review, we describe the most common methodologies for decellularization processes, as well as the clinical applications of these biomaterials.

Keywords: Decellularization process, extracellular matrix, tissue, detergent, clinical applications.

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UNA VISIÓN GENERAL DE LAS TÉCNICAS DE DESCELULARIZACIÓN DE TEJIDOS ANIMALES Y APLICACIONES CLÍNICAS

RESUMEN

Existe una necesidad urgente y persistente de resolver los problemas de escasez de donantes de tejidos y órganos. Los tejidos descelularizados representan una alternativa prometedora a otros tratamientos biológicos y sintéticos que se han desarrollado, ya que pretenden retener las características del tejido nativo que ayudarían en los procesos regenerativos como la proliferación, la adhesión celular y la presencia de factores de crecimiento, al tiempo que minimizan las posibilidades de una respuesta inmunitaria no deseada del huésped. En la presente revisión, describimos las metodologías actuales más comunes para los procesos de descelularización, así como las aplicaciones clínicas de estos biomateriales.

Palabras claves: *Proceso de descelularización, matriz extracelular, tejido, detergente, aplicaciones clínicas.*

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1. INTRODUCTION

Organ failure, tissue damage, degenerative and autoimmune diseases, and the decreasing supply of tissue and organ donors, alongside the risks associated with these procedures, have challenged the human life for decades, affecting hundreds of thousands of people every year [1]. The interest and need to find alternative strategies to treat these issues has been a priority for researchers, health physicians and engineers, with the mindset of possibly achieving complete organ and tissue regeneration [2].

Mitigating the donor shortage and the prominence of degenerative diseases has been a goal of tissue engineering [3]. Tissue engineering is one of the most interdisciplinary fields in health research, focused on combining cell biology, materials science, and engineering concepts to develop biologic substitutes that mimic the native tissue's anatomy, physiology, mechanical, and biochemical properties for restoring or maintaining its normal function [4, 5]. In particular, the development of highly complex products has proven to be effective in many areas of biomaterial-based therapies, increasing the effectiveness of wound and tissue healing thanks to their interaction with cells in vivo [6]. Biomaterials used for tissue engineering application can be divided in two categories depending on the material's source: synthetic (e.g., metallic materials, ceramics and synthetic polymers) and naturally-derived biomaterials (e.g., plant-, animal-, and microorganisms-derived polymers) [7]. The former presents advantages in their elasticity and mechanical properties, the latter has shown advantages in terms of its interactions with the cellular components [7]. Some combinations of synthetic and natural materials have been made aiming to improve the effectiveness and reliability of matrix - cell interactions, the biocompatibility, and mechanical plasticity of biomaterials [8, 9].

A cutting-edge approach to crafting naturally-derived biomaterials sourced from animal or plant extracellular matrices involves the technique of decellularization. Decellularization is the meticulous process of eliminating cellular and genetic elements from tissues or organs, yielding a matrix enriched with proteins, microfibrils, growth factors, and glycosaminoglycans (GAGs). The primary objective of this method is to maintain the original 3D structure and mechanical properties, achieved through the use of diverse agents [10, 11]. These biomaterials are essential in tissue engineering due to their biocompatibility and preservation of vascular structures, providing vital cues for cell recruitment and tissue repair [12, 13].

Given the importance of utilizing appropriate and effective protocols while researching and manufacturing products for clinical applications in tissue repair, a knowledge of the different methods and agents commonly used and their effects in the overall structure and complexity of the tissues is crucial. The present review presents some of the components and techniques used for developing extracellular matrix-derived biomaterials as an alternative in regenerative medicine applications.

2. COMPOSITION AND FUNCTIONS OF THE EXTRACELLULAR MATRIX

The extracellular matrix is an active interconnected multimolecular network forming all tissues in the organism, where neuro-immune-endocrine communication takes place between the cells and the extracellular environment [14, 15]. The ECM is produced by cells and secreted to the extracellular space, giving elasticity and strength to the biologic structure [16]. There are four types of tissues: muscular, nervous, epithelial, and connective [17, 18]. To differentiate the components of each tissue, the ECM is fine-tuned and remodeled by mechanical and biochemical signals [19, 20]. Topographically, there are two main types of ECM: the interstitial one, surrounding the cells for tissue integrity, and the pericellular, which is in close contact with the cells for better attachment (e.g., basement membrane that anchors the parenchyma with the connective tissue) [15, 19].

In general, the composition of the matrix is based on water, proteins, and polysaccharides [18]. Specifically, the macromolecular core components are: collagen, glycosaminoglycans, proteoglycans, elastin, laminin, fibronectin and growth factors. The variability in concentration of each component and their molecular organization, determine the meshwork type with its mechanical, functional, and host cell properties [18, 20, 21]. Because of the continuous remodeling requirements, either in normal or pathological processes (e.g., wound healing), the ECM-secreting cells coexist with proteolytic enzymes, some of them called metalloproteinases [20].

The functions of the ECM can be elucidated through two fundamental concepts: biotensegrity and mechanotransduction. Biotensegrity emphasizes the dynamic nature of functional tension, highlighting its active role rather than a passive one [14, 22]. Mechanotransduction, on the other hand, involves the conversion of mechanical energy into chemoelectrical signals, creating a continuous regulatory feedback state known as dynamic reciprocity [23-26].

In this intricate interplay, ECM mechanical forces play a pivotal role. They can modify gene expression, facilitate proper interstitial fluid flow, and release bioactive molecules such as growth factors, hormones, cytokines, proteins, and immune cells like mastocytes, macrophages, granulocytes, and lymphocytes [25, 27]. To summarize, the ECM functions encompass the modulation of signaling pathways, release of functional fragments (such as cryptic molecules), and adjustment of biomechanical properties. These functions collectively regulate crucial cellular processes including growth, homeostasis, migration, differentiation, and adhesion [14, 22].

3. EXTRACELLULAR MATRIX-DERIVED BIOMATERIALS

Removal of cellular components through decellularization techniques is a method for producing ECM-derived biomaterials. The goal of the process is to minimize the host innate immune response to implanted medical devices and biomaterials [28]. In the context of tissue engineering, ECM-derived scaffolds promote angiogenesis, cell proliferation and differentiation, due to the growth factors and other bioactive components remaining in the scaffolds [29], becoming a major player in tissue remodeling, repair, and regeneration [30, 31]. These scaffolds can be obtained from different tissues, such as small intestine, bladder, placenta, among others [21].

It has been reported that decellularized ECM-derived scaffolds can be used for the development of hydrogels and scaffolds, helping with regulation and treatment of different pathologies like cancer and chronic inflammatory disorders, as well as functioning as a cell and drug delivery vehicle, producing bioinks and controlling stem cell behavior in cellular therapies [32-34].

Multiple studies have shown the effectiveness of these novel materials to improve the regeneration rate of cardiac tissue when compared with traditional outcomes [35, 36]. Likewise, ECM-derived scaffolds have been used for cartilage and bone repair applications, showing improvement of strength, physical resistance, and overall movement in comparison with other conventional therapies [37, 38]. Other studies are focusing on making new ways of modeling diseases and tumors for future analysis, preparations for surgery and other kinds of treatments [39]. It is important that these applications, as well as other ECM-based therapies in development, are furtherly studied to determine their effectiveness in humans or other species.

4. DECELLULARIZATION AGENTS AND METHODS

Decellularization protocols involve a strategic combination of physical, chemical, and biological agents applied to tissues. The goal is to eliminate cellular and genetic elements that might trigger immune rejection upon implantation [40]. The selection of agents is contingent upon the tissue's origin, density, vascular composition, biochemical contents, and the intended purpose of the final product.

The efficacy of these protocols hinges on the careful choice of agents and their impact on the resulting product. This determination is made through the quantification of cellular and extracellular matrix components, DNA fragments, growth factors, and other biomolecules. Additionally, rigorous physical and mechanical testing is conducted to ensure the product possesses appropriate mechanical integrity, density, porosity, elasticity, tension, and compression resistance tailored to the specific application. In the following sections, widely utilized methods for tissue decellularization will be explored.

4.1. Chemical agents

A variety of chemical agents are used to facilitate decellularization given their capabilities to disrupt phospholipidic membranes, dehydrate, remove lipids and nucleic acids, and induce lysis of cells. Among these agents the most commonly used are detergents, acids and bases, and alcohols, which are all effective for cell removal [2, 41]. All protocols using chemical agents must include extensive rinsing and washing procedures to remove remnant compounds, thus avoiding degradation of essential ECM components and toxicity effects in vivo.

4.1.1. Detergents

Detergents are soluble amphipathic molecules usually with a phospholipidic head with hydrophilic properties and a non-polar hydrophobic chain capable of interacting with the lipid bilayer membrane [42]. Detergents induce cell membrane solubilization, contributing to the process of cell removal from the ECM while maintaining structural, biochemical, and biomechanical properties [2, 11, 41, 43]. Moreover, the use of detergents has shown to contribute to DNA and protein dissociation, improving their effectiveness as decellularization agents [43]. Three types of detergents can be identified based on their head's polarity: ionic, non-ionic and bipolar or zwitterion [41].

4.1.1.1. Ionic Detergents

Ionic detergents are the most commonly used detergents in tissue decellularization given their hydrophilic polarized head group [44]. Studies have also shown effectiveness when used in dense and thick tissues, removing most cellular debris such as nuclear and cytoplasmic compounds [41]. Sodium dodecyl sulphate (SDS), Triton X-200 and sodium deoxycholate (SDC) are among the ionic detergents used for tissue decellularization [45].

Ionic detergents have proven to be stronger than other detergents, however, many issues associated to the loss of mechanical properties and removal of ECM components can also be attributed to them. Studies have shown a decrease in GAG and growth factor count in ECM-derived scaffolds, decreasing mechanical stability and healing potency [40, 41, 46]. Furthermore, an extreme disruption of protein-protein interaction can occur when exposed to ionic detergents, which can lead to a loss of collagen integrity [40, 41].

4.1.1.2. Non-Ionic Detergents

Non-ionic detergents have an uncharged hydrophilic head group and have a lower destruction rate of the ECM, producing mild effects to its structure and composition [40, 44]. By disrupting lipid-lipid and lipid-protein interactions while maintaining protein-protein interactions, cell removal can be achieved preserving tissue architecture and protein functionality. Triton X-100 is the most commonly used non-ionic detergent for decellularization processes [41].

Due to its mild nature and activity, non-ionic detergents are mostly used in low-density tissues. Given Triton X-100's ability to remove GAGs from the ECM's surface, it is worth noting that exposure times should be controlled depending on the application [40].

4.1.1.3. Zwitterion Detergents

Zwitterion detergents exhibit properties of both ionic and non-ionic detergents, carrying a positively and negatively charged head group, producing mild effects in ECM architecture and composition while still being effective for thin tissue decellularization [45]. They protect the native state of proteins while effectively removing cellular content [44]. CHAPS ((3-((3-cholamidopropyl) dimethylammonium)-1-propanesulfonate) is the most commonly used bipolar detergent in decellularization protocols and has proven to be ideal for collagen and elastin preservation. However, it can affect mechanical properties needed for structural support [41].

4.1.2. Acids and Bases

Acidic and alkaline solutions play a crucial role in tissue decellularization due to the pH sensitivity of intracellular biochemical processes. These solutions facilitate decellularization by inducing the hydrolysis of cytoplasmic components and degrading or denaturing nucleic acids [11, 47]. It is vital to carefully control the concentration and exposure time of these solutions to prevent toxic effects on the tissue and preserve essential elements like GAGs and mechanical cues [40].

Acids catalyze the hydrolytic degradation of biomolecules, disrupting nucleotide sequences to remove genetic contents effectively [45]. However, the use of acidic solutions must be regulated to prevent dissociation of DNA from the ECM, avoiding denaturation of growth factors, GAGs, and collagen [47]. While acetic acid is commonly employed, it can negatively impact the ECM's structure by denaturing or removing collagen [45]. Peracetic acid is a frequently used agent in whole-organ perfusion protocols, efficiently removing residual nucleic acids while preserving growth factors and the mechanical properties of the ECM. Additionally, it contributes to tissue disinfection [40, 41, 43]. Deoxycholate acid is proven effective without affecting the ECM and is commonly used in tissues like the urinary bladder and small intestine submucosa [48].

Bases exert a strong effect on tissues, denaturing nucleic acids and disrupting the double helix structure of DNA. Due to their potency, bases are often applied to dense tissues like dermis to remove hair follicles before the application of other decellularizing agents [40, 47]. However, the use of bases can significantly impact the ECM's me-

chanical properties by affecting collagen fibers and removing growth factors [41]. Commonly used bases in research and biologic scaffold production include ammonium hydroxide, calcium hydroxide, and sodium hydroxide [45]. Careful consideration of these agents and their effects is vital in optimizing tissue decellularization processes for various applications.

4.1.3. Alcohols

Alcohols effectively remove lipid contents by diffusing into cells and replacing the intracellular water of GAGs and proteoglycans, causing cell lysis and dehydration in treated samples [40, 47]. The solubilization and extraction of lipids allows for the removal of cells from dense tissues, producing stronger effects when compared with enzymatic decellularization approaches, while also preventing calcification. Using alcohols such as ethanol and methanol may result in protein precipitation, which affects the structural and mechanical properties of the extracellular matrix [41]. Furthermore, it can induce collagen fiber crosslinking that results in higher mechanical stability and stiffness while also affecting the scaffold's degradation rate and renovation capacity when implanted [49].

Glycerol is commonly used given its capabilities of removing lipids and lysing cells, and it also serves as a cryoprotectant for long term tissue storage. Ethanol also can be useful for tissue preservation in low temperatures and act as a disinfectant thanks to its antimicrobial, antifungal, and antiviral properties, but may damage the ECM's ultrastructure [50]. This last one and isopropanol are commonly used in adipose tissue, liver and corneas [47].

4.1.4. Hypotonic and Hypertonic Solutions

Hypotonic and hypertonic solutions disrupt DNA-protein interactions by separating genetic contents from the main structure and create an osmotic shock reaction that removes intracellular content with a significant low time consumption [32, 51]. These agents do not affect the ECM's microstructure and preserve most mechanical and physical cues [41]. Washing and perfusion protocols using hypotonic/hypertonic treatments promotes cell lysis and effectively removes cells [52]. It is common practice to alternate between hypotonic and hypertonic exposure through several cycles to achieve the best results [49]. Hypotonic solutions such as Tris-HCl and isotonic PBS produce cell lysis thanks to a large influx of water and are used on dense articular cartilage and skeletal muscle. Sucrose, saline, potassium chlorine and potassium iodine are some of the most commonly used hypertonic solutions to shrink epithelial cells and detach them from the lamina propria [47].

4.1.5. Other Solvents

Acetones serve as effective decellularization agents due to their dehydration mechanism and their ability to induce osmotic shock, leading to cell lysis [32]. However, they have the drawback of destroying the ECM microstructure by fixing the tissue and increasing its stiffness, deviating from the natural mechanical properties of native tissue [43, 45].

Tributyl phosphate (TBP) is an organic solvent with antiviral properties commonly utilized in tissue decellularization. It forms stable complexes and disrupts protein-protein interactions, leading to the destruction of the ECM protein structure. While TBP is predominantly employed in dense tissues, it has minimal impact on the ECM's mechanical properties. Nonetheless, some studies have indicated potential collagen loss in tendons [43, 51]. Although TBP has been utilized in protocols for ligaments and tendons, further research is essential to assess its effects on various tissue types, ensuring comprehensive evaluation of its efficacy and reliability [41].

4.2. Physical and Mechanical Agents

The use of physical and mechanical agents is known to be an alternative for decellularization protocols to reduce the residual content of chemical and enzymatic agents. These methods increase inner cell pressure to promote cell lysis, slicing or mincing to increase the surface area and promoting the removal of cellular components in wash cycles, using supercritical fluids to remove residues, creating ice crystals to destroy the phospholipidic membrane, among others [10, 53]. In this section, different physical decellularization agents will be discussed and explained as well as some of the different methods used in laboratory studies.

4.2.1. Immersion and Agitation

Agitation is the most commonly used method for decellularization protocols, specifically for thin tissues without a complex vascular system [45]. Samples are immersed in a decellularization solution to allow free roaming of the tissue while maintaining full contact with the agent, allowing all sections to be equally exposed to its effects. Utilizing a centrifugation device, a vortex shaper or a magnetic stirrer, the samples are then subjected to a controlled mechanical agitation that can be carried out through a single cycle or multiple cycles [54]. The length of time for each

cycle depends on tissue properties such as density, thickness and mechanical stability, the decellularization agent utilized and the agitation rate and speed [47].

Immersion and agitation protocols have shown positive results for numerous types of tissues, such as small intestine, urinary bladder, esophagus, dermis, cartilage, skeletal muscle, peripheral nerves and spinal cord, but it is mostly recommended for thinner specimens to avoid agent overexposure [47, 55].

4.2.2. Freeze-Thaw cycles

Freeze-thawing refers to the process of freezing and defrosting tissues by cycling samples between very low and regular preservation temperatures, usually alternating between −80°C and 37°C, but lower temperatures are also used in thawing when additional tissue preservation is needed [2]. Temperature cycling disrupts the integrity of the cell membrane by creating intracellular ice crystals, which tear up the membrane and create small holes that allow the removal of cellular components with further processing [45]. The application of other decellularization agents is needed to fully remove all intracellular contents since freezing and thawing only fractures the membrane.

This process has proven to be effective for tissue decellularization due to its capabilities of inducing and improving cell lysis. It has been shown that this method reduces adverse immune responses and makes other decellularization protocols more effective, while still preserving most of the mechanical and physical properties associated with the tissue [43, 56].

4.2.3. High Hydrostatic Pressure

Pressure gradients are used for developing decellularization protocols to improve the efficiency of other methods. The application of high hydrostatic pressure (HPP) affects the integrity of the membrane and ultrastructure, allowing for an improved effect of cell lysis and chemical/enzymatic exposure by forcing them into the tissue and elevating intercellular pressure that results in cellular residues removal [43, 45]. Studies have shown positive results regarding the preservation of molecular components and most mechanical properties, as well as sterilization benefits thanks to the destruction of viral and bacterial membranes [2, 47]. HPP also helps to remove immunogenic agents and inflammation substances, which can be helpful for further material purification [57]. However, it is worth noting that this method affects the integrity of the membrane and limits exposure times to other chemical agents, which can affect the final product and the efficiency of each application [2].

4.2.4. Perfusion

Perfusion protocols take advantage of vascular structures or a tissue's lumen to expose cells to different decellularization agents through a constant flow, while also allowing for the transport of cellular residues for their disposal [43]. The technique largely preserves the three-dimensional architecture of the native organ from which the extracellular matrix was extracted from, making the successful decellularization of complex organs such as the heart, lungs, kidneys and liver very feasible [47, 58]. Multiple variables must be considered when developing perfusion protocols to optimize the decellularization process, including temperature, pressure gradients and flow rate [45].

Whole-organ scaffolds are one of the most promising clinical applications for regenerative medicine. These are ideal since all micro and macrostructures of the organ are preserved, including complex vascular networks and other essential structures that allow for their correct functionality, while also allowing for future cellular repopulation to develop fully functioning organs for organ transplantation, drug developing, disease modeling and many others [45, 47].

Antegrade and retrograde perfusion are used for decellularization. These techniques are usually accompanied by the cannulation of an opening of the vascular network, such as the aorta for the heart and the coronary artery for the lungs, to allow the agent to flow through the entirety of the organ [47]. Washing protocols must be included when developing these techniques, as the complete removal of chemical agents and debris is critical.

4.2.5. Supercritical Fluid

Supercritical carbon dioxide (CO_2) induces a low viscosity, fluid-like density, and high diffusivity rate producing quick and effective results when used in decellularization protocols [59]. CO_2 can be quickly released and does not require washing procedures to remove the molecule out of the processed tissue [60]. The use of an inert substance also preserves the mechanical and biochemical properties of the ECM by not reducing collagen and elastin content, while also achieving effective cell removal [2, 47]. The decellularization occurs when the fluid is passed through tissues at a controlled rate similar to critical point drying, removing all cell residues found within the structure [43].

4.2.6. Electroporation

Non-thermal irreversible electroporation consists in the application of microsecond electrical pulses across a tissue and its resident cells to cause the destabilization of the cell membrane's electrical potential. This method uses pulsed electrical fields capable of membrane disruption, which results in the formation of irreversible nanoscale pores in the lipid bilayer that promote cell lysis [45, 61]. These micropores generate the loss of cell homeostasis, leading into cell death that can be effectively applied in different decellularization protocols. However, this technique is limited by the size of electric probes and can only be used in smaller tissues, while also the exposure to these effects must be limited to fully preserve ECM's integrity [43].

4.3. Biologic agents

These agents can be mainly divided in proteins with enzymatic and non-enzymatic activity, with functions of degrading macromolecules [43, 53, 62].

4.3.3. Enzymes

Enzymatic techniques include the use of proteases (e.g., trypsin, dispase, thermolysin, collagenase), lipases, esterases and nucleases, which have great affinity to the biologic substrate allowing them to eliminate undesirable cellular debris or ECM fragments [43]. Enzymatic remnants could generate adverse effects in applications of the scaffold [43, 63].

Proteases recognize specific sequences inside (endoproteases) or outside (exproteases) proteins and cut those sections [45]. Some enzymes that belong to this group of agents are trypsin, collagenase, dispase and phospholipase A2. Trypsin, for example, is a highly used proteolytic enzyme for cleaving peptide bond or cell-matrix interactions, but it can also be harmful to elastin and collagen [13]. On the other hand, collagenase is used during ECM decellularization protocols, but it does not allow to preserve the tissue ultrastructure or high collagen levels [13]. Lipases, which work by hydrolysis of triglycerides [64], do not work well alone [13]. Phospholipase A2 (an esterase), which hydrolyzes phospholipids present in the tissue, preserving the structure of collagen and proteoglycans, although it can also reduce glycosaminoglycans [43].

Nucleases divide and degrade genetic material by recognizing nucleotide fragments. Endonucleases cut into the chain; therefore, they are more efficient than exonucleases that catalyze the hydrolysis of the terminal bonds [63]. Removing their remnants can be a challenge, causing undesirable immune reactions [13].

4.3.1. Non enzymatic agents

Chelating agents possess a high affinity for metal ions, and their application disrupts protein activity significantly. Proteins, for their stability, rely on cations like calcium and magnesium to maintain their bonds with the ECM. Chelating agents sequester these cations, leading to the destabilization of bonds and the subsequent removal of cell/matrix adhesions. However, to ensure complete cell elimination, these agents must be combined with enzymes or detergents. Among the commonly used chelating agents are EDTA and EGTA [43, 53].

In dense tissue decellularizations, specific toxins such as Latrunculin B have found utility when used in conjunction with DNases. These compounds work by degrading actin, a crucial component present in cells. While effective, this method may result in a reduction of certain GAGs [63]. Careful consideration of these techniques is essential in optimizing the decellularization process for various tissue types and applications.

4.4. Further processing of ECM-derived scaffolds

Following cell lysis processes, inhibition of resealed proteases should be completed to reduce damage of the ECM [65]. Retention and functional activity ECM components (e.g., collagen, fibronectin, growth factors, elastic fibers, glycosaminoglycans, etc.) should be evaluated after a decellularization process [66]. Finally, disinfection and sterilization processes should be included in any protocol to reduce contaminants from the scaffold [67].

5. ADVANCES IN DECELLULARIZATION STRATEGIES FOR DIFFERENT TISSUES

5.1. Bone tissue

Bone, a fundamental component of the skeletal system, offers vital support and facilitates movement. Within its marrow, both red and white blood cells are produced [53]. Structurally, bone comprises an organic phase, primarily consisting of collagenous proteins such as collagen types I, III, and V. These proteins provide essential mechanical

support to the matrix. Additionally, non-collagenous proteins like proteoglycans contribute to fiber assembly [68]. The inorganic phase contains hydroxyapatite, serving as a storage site for minerals and metallic ions [68, 69].

Defects in bone structure and function can arise from tumors, trauma, congenital disorders, or infections. Consequently, bone transplantation stands as the second most common procedure after blood transfusion [68]. The age of the donor plays a crucial role in the efficiency of decellularization protocols and subsequent osteogenic differentiation within the scaffold. Older donor scaffolds exhibit increased porosity and enhanced osteogenic differentiation. However, bone mineral density, calcium, and phosphate levels remain similar to those from younger donors [68]. Coatings incorporating substances such as hyaluronic acid, platelet-rich plasma, or collagen can augment bone formation, stiffness, and osteogenic differentiation [68]. Careful consideration of these factors is essential in optimizing bone tissue engineering strategies for effective clinical applications.

Various methods exist for bone decellularization, including the use of chemical agents like EDTA or SDS, in combination with supercritical carbon dioxide or ammonium hydroxide. Alternatively, thermal shock combined with Triton X-100 has proven effective [53].

5.2. Cartilaginous tissue

Cartilage, a resilient connective tissue primarily found in bone junctions, serves as a crucial element in providing flexibility and preventing friction [53]. There are three types of cartilage—hyaline, fibrous, and elastic—yet hyaline cartilage is the most frequently employed in regenerative therapies, especially considering the prevalent joint injuries resulting from trauma or pathology [71]. Avascular in nature, cartilage comprises essential components such as collagen, laminin, and GAGs, which contribute significantly to its mechanical properties [53, 72].

Joint damage, often stemming from trauma or underlying pathologies, can be temporarily resolved through surgical interventions. However, regenerative medicine offers a long-term solution, despite the challenge posed by cartilage's limited vascularization, which hinders its natural regeneration [53].

In the process of decellularizing cartilage tissue, a key objective is to preserve as many GAGs as possible [53, 72]. Before decellularization, the tissue undergoes specific treatments like freezing and thawing, along with exposure to hypo- and hypertonic solutions. These steps induce lysis and facilitate effective agent penetration [53]. The actual decellularization procedure involves chemical or enzymatic methods, such as the trypsin-EDTA protocol with controlled exposure to prevent degradation of proteins of interest. While Triton X-100 and SDS have been utilized, they tend to deteriorate GAGs [53, 72].

5.3. Adipose tissue

White adipose tissue stores energy through lipids, providing insulation, cushioning and supporting both the subcutaneous tissue and internal organs [53]. Adipose tissue also participates in metabolic mechanisms of lipid and glucose homeostasis [73]. The ECM composing the adipose tissue is formed by collagen and laminin, which are key for anchorage sites and adipocyte barriers [53, 70].

For an optimal decellularization process of adipose tissue, a de-lipidation (lipid removal) using organic solvents, such as alcohols and acetone, is first required [74]. Removal of cell components is completed by freezing and thawing cycles using hypotonic buffer, isopropanol, an enzymatic digestion using trypsin-EDTA, DNAase, RNAse and lipase [53, 70, 75].

Adipose ECM-derived scaffolds have proven to be effective tools to promote repair and tissue regeneration in breast cancer treatments, providing appropriate microenvironments in vivo [2, 53]. In addition, as adipose tissue is a good reservoir of stem cells, using ECM-derived scaffolds to seed the tissue's own stem cells has worked for reconstructive surgeries for structural or aesthetic purposes [2].

5.4. Skeletal muscle and tendons

Skeletal muscles transform neural signals into forces to perform a coordinated motor task [76]. Skeletal muscles are attached to bones through tendons which have 5% of their volume as cellular component and 95% as ECM [53, 77].

For muscle decellularization, physical methods (e.g., freezing and thawing) or proteases have been used but they can be very aggressive, therefore using detergents (e.g., sodium deoxycholate, Triton X-100, and SDS), saline solutions, or specific enzymes (e.g., DNAse) have been more efficient [77]. Some studies have shown that trypsin at low concentrations and for short periods of time could be useful [78].

Tendon ECM is composed of collagen type I, elastin and proteoglycans that provide mechanical and elastic capabilities [53, 77, 78]. Although tendons have a self-regenerative ability, when severe trauma or repetitive injuries happen, they can lose this function [77, 78]. Tendon decellularization includes a cycle of freezing and thawing prior to the use of detergents (e.g., Triton X-100 and SDS) [79]. Tri-n-butyl phosphate (TnBP) has also been used and has shown better results than other agents [53, 77, 78].

5.5. Cardiovascular tissue

The heart, a muscular organ responsible for pumping blood throughout the circulatory system, relies on a complex ECM predominantly composed of collagen, fibrillin, laminin, fibronectin, and proteoglycans. These elements provide the heart with the necessary strength, flexibility, and durability [11, 53, 80].

In the pursuit of alternatives to transplantation, regeneration and repair have emerged as promising approaches, with decellularization serving as a fundamental technique to preserve native properties, including elasticity [11, 53]. During the decellularization process, the initial step involves inducing the loosening of the cardiac ECM through osmotic shock or the use of a trypsin-EDTA solution. Subsequently, cardiac ECM decellularization is typically achieved through perfusion of detergents such as SDS, sodium deoxycholate, polyethylene glycol (PEG), or Triton X-100, connecting various cardiac vessels like the superior vena cava, the ascending aorta, the pulmonary artery, or the pulmonary vein [70]. To enhance porosity, allowing increased cell migration and nutrient transport, the decellularized tissue is lyophilized [11, 53, 80].

Initially limited to heart valves, decellularization techniques have evolved to encompass whole hearts. In the case of heart valves, enzymes are employed to eliminate residual nucleic acid remnants after detergent treatment, ensuring a thorough decellularization process [53, 70, 80]. These advancements hold immense potential in reshaping cardiac tissue engineering and regenerative medicine.

5.6. Vascular system

Vascular tissues are responsible for transporting nutrients, hormones, blood cells, oxygen and CO_2 throughout the body. The vascular system is composed of arteries, arterioles and veins, with arteries as the main focus in decellularization protocols [81]. Arteries are composed of three tissue-layers: intimate, media and adventitia. The intimate layer of arteries contains laminin and collagen, the media is composed of type III collagen, elastin, glycoproteins and GAGs, while adventitia has type I collagen, elastin and proteoglycans [81]. It is important to preserve the elasticity and strength properties of these layers during the decellularization protocol since they play a key role in the normal vascular function [82].

Decellularized artery grafts are gaining more research attention [53, 81]. The process starts with lysis of blood cells using washes of distilled water or EDTA freeze-thaw cycles. The decellularization step can be completed by using trypsin and hypo- and hypertonic solutions combined with detergents, although enzymatic removal of genetic material is also suggested to finalize the protocol [53]. Using supercritical and pressurized CO_2 and endonucleases to eliminate residual genetic material has also been used [53, 81].

Preservation of the mechanical properties is the main challenge for using vascular ECM-derived scaffolds [83]. Therefore, bioreactors are being considered as an option to let decellularized vascular tissues mature and gain these mechanical properties before implanting [84].

5.7. Dermal tissue

Protection, thermoregulation and perception are among the functions of the skin [53]. The dermis is the middle tissue of the skin, that minimizes stress and tension by providing perception, elasticity and warmth [78]. Several proteins are part of the dermal tissue, like elastin and fibrin, vitronectin, and fibronectin. The dermal ECM also contains proteoglycans and GAGs responsible for hydration and osmotic balance [53, 78].

Dermal ECM-derived scaffolds, from human or animal origin, are used for non-self-healing skin injuries [53, 78, 85]. For decellularization purposes, a mechanical isolation of the dermis, from the epidermis and hypodermis, should be completed. The dermal tissue is then incubated in hypotonic buffer to lyse the cells. The decellularization process can be carried out with different detergents, such as Triton X-100, sodium deoxycholate, N-lauroylsarcosinate (NLS) or SDS, in combination with trypsin, bovine serum albumin (BSA), EDTA and/or dispase. Some acids and bases can be used to degrade nucleic acids and hair [53, 78, 85].

The use of human dermis confers positive results in the healing of skin ulcers, wounds and other traumatic skin injuries, acting as a substitute and promoting vascular and cellular growth. It has also been used on breast reconstructive surgery [53, 78, 85]. However, some challenges remain like preserving or reconstructing important components of the skin, such as sweat glands and hair follicles [34].

5.8. Respiratory system

The respiratory system is composed of various organs, from which trachea, lungs and diaphragm have been therapeutic targets in regenerative medicine [86, 87]. The trachea is formed by alternating rings of cartilage supported by tendons, and covered by a mucosal membrane at the lumen, to support the air exchange between nose and mouth and the lungs [88]. If damaged, a tracheal replacement surgery could be needed [89]. The decellularization process of the trachea is carried out by lyophilization, with subsequent detergent and DNAse treatments [11, 53, 86, 87].

Lungs, on the other hand, have a low regenerative capacity [86]. Decellularized tissue is a novel alternative to transplantation and can be performed by perfusion with detergents such as SDS and Triton X-100, while using enzymes for vascular structures and the respiratory tract. This alternative intends to fill the gap in the high demand for lung donors [11, 53, 86, 87].

5.9. Gastrointestinal tract

The anatomy of the gastrointestinal tract is complex; thus, the use of bio-scaffolds is difficult because it implies the process for several tissue layers and recellularization with different cell types [11]. Liver and intestine have been the main focus in decellularization research with regenerative applications, while the esophagus and pancreas have been getting more attention recently [11, 53, 80].

Given the complex cellular and matrix structure of the intestine, a combination of chemical and enzymatic solutions is used to perform a decellularization process, which include perfusion with sodium deoxycholate, DNAse, immersion in hypotonic solutions, among others [56, 62]. Intestinal ECM-derived scaffolds have been used in regenerative medicine to reconstruct urethra and esophagus, as well as a vascular and intestinal graft, dermal substitute, among others [30, 62].

Liver decellularization methods involve intravascular perfusion of detergents such as dodecyl sulfate (SDS) and/or Triton X-100, hyperosmotic solutions such as NaCl and enzymatic solutions such as DNAse. The resulting scaffolds are used as a replacement for the dysfunctional organ [11, 53, 80].

5.10. Nervous system

The nervous system is responsible for receiving and responding to different stimuli [90, 91]. Nervous ECM is composed of collagen, laminin, fibronectin that guides the growth of axons, and acetylcholinesterase that regulates cell/cell and cell/ECM signals [16]. Nervous tissue damage can occur from diseases, conditions and injuries that affect motor functions; therefore, damaged nerve connections should be reestablished [92]. Neural ECM-derived scaffolds and Schwann cells could be an option for neuronal functional repair [53]. For nervous tissue decellularization process, washes with detergents are commonly used, such as Triton X-200, SDS or Triton X-100 combined with osmotic shock [53, 93].

Notably, several scaffolds are derived from central nervous system tissues such as the optic nerve, spinal cord, and brain, which retain supporting proteins and growth factors that modulate cell behavior [53, 94]. Nevertheless, decellularized nerves present different limitations, which recent studies in the field have tried to overcome. Grafts using these decellularized tissues sometimes have low regeneration in situ or do not regenerate long enough for them to be viable in long-gap injuries [94, 95].

5.11. Cornea

The cornea protects, refracts and focuses light in the eye [96]. Corneal tissue is avascular and highly innervated, acting as a barrier against infection. The human cornea is organized into five layers, three of which are cellular (epithelium, stroma and endothelium) and the other two layers serve as interfaces (Bowman and decemet membranes) [11, 53]. Corneal ECM contains water, inorganic salts, proteoglycans, glycoproteins and collagen [53, 97]. Some eye disorders require cornea transplantation to improve or restore visual acuity, but its clinical utility is limited which is why cadaveric, porcine and bovine cornea have been used, as well as the use of replacement treatment with decellularized intestinal submucosa and amniotic membrane [98].

Corneal decellularization includes the use of detergents, such as SDS and Triton X-100. When the resulting scaffolds remains opaque, transparency is restored by immersing the matrix in glycerol. Another alternative could be replacing detergents with NaCl or with a homologous nuclease to benzonase so the transparency is not affected [11, 53, 98].

Some challenges in the clinical application of corneal ECM-derived scaffolds include the recellularization of the stroma, since the cells should be evenly dispersed and the methods used do not guarantee proper cell filtration [50, 97, 99].

5.12. Whole-organ decellularization

Currently, transplant is the most used treatment option for patients with organ failure [100]. Decellularized organs and their subsequent recellularization have been at the forefront in recent decades as possible treatments [100, 101]. Specific cell types or stem cells are used in order to reconstruct the microstructure and recreate the specific function of the organ [102]. After recellularization, maturation of the neo-organ is carried out so the organ can be implanted without the need of immunosuppression [62].

6. CONCLUSIONS

The efficiency of a decellularization process crucially hinges on the thorough removal of cellular components capable of eliciting adverse host responses [66]. Despite the absence of a standardized protocol, the careful consideration of tissue/organ type is paramount in selecting the appropriate method and agent to ensure successful decellularization [102]. Equally essential is the preservation of the ECM structure. Mishandling of the tissue or excessive use of chemical agents can result in the loss of vital protein components necessary for scaffold anchoring, cell seeding, and proliferation [66].

The advent of ECM-derived scaffolds, generated through the decellularization of tissues, presents a groundbreaking opportunity for diverse clinical applications that have long remained stagnant in the field of tissue engineering. These scaffolds, designed to retain natural tissue components as much as possible, offer the fundamental characteristics essential for regeneration. Simultaneously, they eliminate elements that might provoke an undesirable immune response in the host [66].

Despite the need for further refinement, these matrices signify a significant advancement in addressing the ongoing crisis of organ and tissue donation, marking a substantial step forward in medical science. However, it is imperative to acknowledge the challenges inherent in the decellularization process, such as the lack of standardization, the need for careful tissue-specific considerations, and the preservation of ECM integrity. Researchers and clinicians must navigate these challenges to unlock the full potential of decellularized ECM in regenerative medicine, ultimately revolutionizing the landscape of organ transplantation and tissue engineering.

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