REVIEW ARTICLE

Decellularization of Tissues and Organs: Methods and Assessments

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ABSTRACT

The urgent need for organ and tissue transplants has led to the exploration of alternative sources, such as scaffolds derived from the decellularized extracellular matrix (dECM). These dECMs have their cellular and genetic components removed while retaining their structural integrity. This review discusses and compares various decellularization methods, including physical, chemical, and biological techniques, each employing distinct mechanisms. Additionally, it summarizes the range of tissues and organs that have undergone decellularization. Physical methods, such as the application of high hydrostatic pressure (HHP), are highlighted as a promising approach, with a detailed evaluation of their parameters. Finally, the review assesses dECMs post-decellularization, focusing on risks related to immunogenicity and preservation of mechanical properties.
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INTRODUCTION

Organs and tissue transplantation are crucial for affected patients to replace malfunctions, which is a step toward recovery. These malfunctions can differ based on the organs or tissues, such as bone fracture, skin burns, heart valve leakage, and liver failure. Despite the high demand for organ and tissue donation globally, less than 10% of the annual demand was met [1]. Additionally, there is a significant difference between countries on donation and transplantation activity, equally among nations with comparable socioeconomic policies, especially healthcare systems [2]. Therefore, there is a clinical need for tissue engineering and regenerative medicine in order to fulfil the dire demand for transplantations. It also shows the importance of tissue engineering advancement to increase the rate of patients receiving transplantation with reduced effects from the scarcity of human donors and socioeconomic aspect related to it. Rapid advances in tissue engineering can be one of the solutions for this critical need for organ and tissue transplantations among patients from various backgrounds. The combination of cells, bioactive materials, and scaffolds is the foundation of tissue engineering. Decellularized extracellular matrix (dECM) delivers the criteria of this foundation and is advancing in the field. Extracellular matrices from human or animal donors are decellularized, i.e. native cells removal, and recellularized with the recipient's cells [3]. Based on the methods of decellularization, the two critical criteria of the biological scaffold are achieved using dECM, which involves preserved mechanical properties and biocompatibility through the minimization of immunogenicity and cytotoxicity [4, 5].

ECM of a variety of tissues and organs have been studied, including blood vessels, bone, uterine, and meniscus [6-10]. These studies were mostly conducted in vitro and in vivo, either in animal models or for human allografts [10]. Although the recipient's native cells can be seeded on the dECM, however, it does not guarantee biocompatibility due to ECM native cells residue [11]. Therefore, these studies focused on effective and efficient cell removal to minimize the possibility of rejection and devastating

immune response by the animal recipients [12-14]. Cells removal or decellularization methods generally can be divided into three categories, i.e., physical, chemical, and biological methods, where different principles and mechanisms of decellularization agents are performed [15, 16].

Despite the plethora of available decellularization methods, there is no one method that can fit all types of ECM [17, 18]. Hence, it is important to identify the needs and characteristics of the applied ECM, so that a suitable decellularization method can be employed with good efficiency and the least damage to the ECM's mechanical structure and biochemical composition while minimizing the risk of immunogenicity. In brief, the application of tissue engineering through dECM has the potential to fill the gap for the deficiency of organ and tissue donors through the decellularization of ECM, mainly utilizing xenogeneic sources due to its abundance and inherent structural similarity with human ECM. Therefore, this review has summarized the application of decellularization on a plethora of tissues and organs. Additionally, the primary points of decellularization assessment were also discussed, and the criteria of decellularized dECM were also examined with the focus of comparing chemical and physical methods, highlighting HHP as a promising decellularization approach.

EXAMPLES OF DECELLULARIZED ORGANS AND TISSUES

ECM is composed of a connective network containing collagen, glycoproteins, proteoglycans, and other small molecules [19]. When decellularization methods are imposed on the ECM, cell-free dECM including tissues and organs is the product, where the native cells of the structure are effectively removed through one or a combination of decellularization methods. These dECM can be applied in regenerative medicine and tissue engineering, among other fields. Various types of tissues and organs have been studied in recent years, where decellularization methods were implemented and their effects on the biological scaffold in vitro and ex vivo. Examples of dECM include the meniscus, blood vessels, nerves, uterine, and cancellous bone.

Meniscus

The meniscus is a fibrocartilage of the knee, whose roles include absorbing shock, stabilization, and lubrication. When injured, its poor healing ability is the main challenge in repair efforts [6]. Therefore, the damages or defective menisci can be replaced via the gold standard, meniscus allograft transplantation. In order to procure the menisci grafts, procurement methods include fresh, fresh-frozen, cryopreservation, and lyophilization. Fresh grafts face the challenges of logistics to the recipient, which is associated with a higher possibility of disease transmission. Frozen and cryopreserved grafts might seem to be the more viable options, however lower viability of chondrocytes limits its potential [20]. Therefore, decellularization using physical methods is effective in menisci ECM to create a cell-free environment [6, 12, 21]. Both methods showed good native cell removal while maintaining acceptable biomechanical properties of the menisci ECM. Although high hydrostatic pressure-treated (HHP-treated) menisci were shown to be immunologically superior, despite being biomechanically inferior [6]. Despite this, efforts to optimize the decellularization process should be the main goal to ensure viable and safe recellularization for long-term applications [20].

Blood vessels

Blood vessels, most commonly aortic vessels and arteries were studied for their capabilities to be decellularized and used as biological scaffolds for ex vivo applications [7, 22-26]. Disorders or injuries to the blood vessels have various existing treatments ranging from invasive to non-invasive, such as surgery, medications, and rehabilitation. When faced with severe malfunction of the vessels, surgical interventions are common, where the vessels are harvested from recipients. Unfortunately, the harvesting process could cause more harm than good to the patient, where severe consequences include significant donor site morbidity and other complications [27]. Therefore, decellularization of the blood vessels is a promising approach for vascular replacement. The decellularized blood vessels showed good in vivo performance, given that the studies suggested xenogeneic vascular grafts, due to the limited availability of autologous grafts [7, 23, 24]. Regardless of the good results, there is a limited number of studies elucidating the functionality and ex vivo performances of the xenogeneic vascular grafts [7]. Hence, efforts must be made to ensure the safety and functionality of the decellularized blood vessels, so that its full potential as an alternative to autologous grafts can be achieved.

Nerves

Nerve injuries are devastating due to their severe physiological and psychological effects on the patient. When damage occurs in the nervous system, including the central and peripheral nervous system, repair and recovery are deemed challenging after a reconstructive surgery [28]. Autografts have been the gold standard in tissue reconstructive, including nerve and bone tissue. Nonetheless, limitations including low availability and donor site morbidity are significant, where allografts have been an increasingly promising alternative to the gold standard [29-31]. Application of allografts from the same or different species requires the effective removal of cells from the tissue or organ. Perfusion-mechanical agitation decellularization technique was widely applied in various studies, resulting in good viability of the nerve autografts [8, 32]. Regardless, optimization of the decellularization process was studied to reduce processing time to be cleanroom compliant, as well

as better storage conditions that can preserve the structure of the autografts [8, 33]. Nonetheless, it is important to note that due to the delicate structure of nerves, decellularization methods that include physical methods can significantly damage the ultrastructure [29]. Although this method can provide low immunogenicity, application on nerve grafts may not be the most suitable.

Uterus

The uterus, the female reproductive organ plays a vital role in pregnancy and fetal development. However, significant tissue damage from scarring during uterine resection surgeries or from many cesarean sections lowers fertility and raises the possibility of uterine rupture during a subsequent pregnancy [34]. Therefore, studies have been decellularizing uterine to fabricate a biological scaffold for recellularization, and consequently organ transplantation [9, 14, 35, 36]. In this case, most of the uterines fabricated or reconstructed were from animal models, such as bovine, mice, murine, and rats. Low host immune responses were desirable for safe outcomes of transplantation in humans, thus, a physical method such as high hydrostatic pressure (HHP) was implemented in several studies [14, 35]. Nonetheless, decellularization via a chemical method, especially using Sodium Dodecyl Sulfate (SDS) or Triton-X via perfusion was still applied for uterine tissues. In these cases, pre-conditioning treatment may be carried out before decellularization to minimize the immunogenicity of the dECM in vivo [9, 36].

Bone

Bone is composed of organic and inorganic components. The organic components are primarily collagen, approximately 30% of bone, while the rest is calcium phosphate in the form of hydroxyapatite [3, 37]. Bone can remodel itself when faced with injuries such as fractures, to replace old bone or calcium homeostasis. The bone cells including osteoclasts, osteoblasts, osteocytes, and bone lining cells collaborate to build temporary anatomical structures, which subsequently resorbs to become bone tissue [37]. However, when the fracture is acute and the defect size is critical, bone tissues might face difficulties in regenerating itself. Thus, bone tissue engineering with bone ECM is one of the possible interventions for fracture healing [10]. Nonetheless, the bone ECM must be decellularized before grafted or transplanted into the patient. Hence, the decellularization of bone tissues has been carried out by several studies to fabricate bone scaffolds or grafts [10, 38-42]. In these studies, the type of bones studied involves bone marrow, compact or cortical bone, and cancellous or trabecular bone. The objectives of these studies are similar, which include maintaining the mechanical strength and ultrastructure of the bones while removing the cellular components that might induce an immune reaction to the host. Nonetheless, the search for the method or protocol that can effectively achieve these goals is still ongoing. Most of the methods applied for the decellularization of bones are either chemical methods or compound physicochemical methods [38-40].

CRITERIA OF DECELLULARIZED ECM

There is a plethora of studies on tissue engineering that applied decellularization techniques, however, there has not been a clear and direct criterion for a decellularized ECM. So far, there is only one mention of, to some extent, a criterion, although it mainly focuses on the biochemical aspects of decellularization. It concluded that (1) there should be <50 ng of double-stranded DNA per mg of the ECM's dry weight, (2) DNA fragments should be <200 base pairs, and (3) staining procedure with 4',6-diamidino-2-phenylindole (DAPI) or H&E should reveal the absence of visible nuclear components [1, 43].

As noted, the focus of these criteria is on the nuclear materials of the cell, as the presence of DNA was conjectured to have a direct correlation with adverse host reactions [43, 44]. Nonetheless, histological and mechanical aspects of the ECM should also be taken into consideration when evaluating the decellularized ECM [9, 10, 42]. As for the mechanical properties, it is an indication of the maintenance of the ECM structure, where the compressive strength and collagen content can be evaluated as the hallmarks. However, the tissue of interest differs between studies, which could be why standardization in the focus of decellularization is difficult. Although it was possible, it was deemed challenging due to the variety of tissues decellularized and decellularization techniques available [45]. Therefore, the extent of changes in the benchmarks, i.e. collagen content and compressive strength could be elucidated across various tissues, for comparison.

Furthermore, DNA retention might not be the sole contributor to immunogenicity. Decellularization agents, alteration of the ECM, source of tissue and organs, and implant sites are factors that contribute to immunogenicity, due to the release of specific antigens or ECM components [18]. Additionally, xenoantigens such as α-Gal, which is a unique target of human antibodies in xenotransplantation, where decellularization alone was insufficient to remove it from the dECM. In further studies, the level of xenoantigens in vitro and in vivo could be quantified as an indication of the expected host inflammatory response [46]. Therefore, the focus of decellularization should be equally distributed between removing DNA and cellular components, as well as the changes that may occur within the ECM due to certain steps in the decellularization. These aspects are important in ensuring the application of dECM lacks adverse immune response and rejection, even in pre-clinical application. Therefore, future research should divert attention towards these factors too, rather than focusing on nuclear and cellular material as an

indication of risks.

DECELLULARIZATION METHODS

Decellularization of ECMs from tissues and organs can be achieved with various methods, mainly categorized into chemical, physical, and biological methods, before the ECMs are subjected to assessment post-decellularization (Fig. 1). In the study of creating a cell-free ECM via decellularization, chemical methods using SDS or Triton-X 100 are popular and widely used in the decellularization of a diverse type of tissues and organs [45]. Nonetheless, although the protocols were successful in achieving the intended effect, the set of criteria for dECM is lacking as most studies focused on the tissue-specific criteria of complete decellularization.

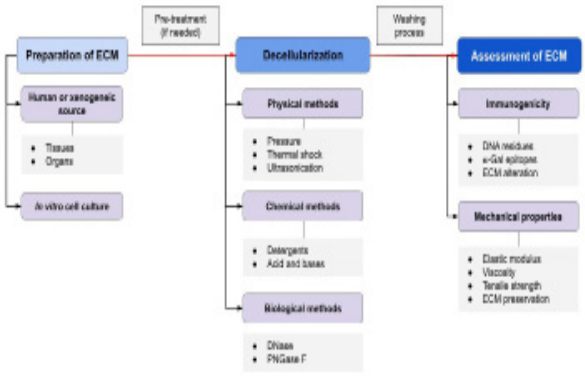

Fig 1. Flowchart of Decellularization and its Assessments.

Chemical approaches to decellularization

Methods of decellularization through the implementation of chemical reagents of various properties are common to achieve cell removal. Chemical properties such as non-ionic, ionic, zwitterionic, acid, and alkaline detergents used are vital for the mechanism of decellularization. Ionic detergent, i.e. SDS, and non-ionic detergent, i.e. Triton X100, are common when decellularizing ECM using a chemical approach. Without altering the ECM protein structures, the ionic detergent decellularizes by disintegrating membrane proteins and lysing cells. Moreover, ionic detergent, particularly SDS, targets protein-protein bonds and efficiently detaches cellular proteins and nuclear materials [4]. Moreover, the efficiency of these detergents for decellularization varies based on the size, density, thickness, number of cells, and lipid content of the ECM [47].

Other than detergents, pH can also contribute to cell removal, where pH can be adjusted via the addition of acid or alkaline. Alkalines such as sodium hydroxide, calcium hydroxide, ammonium sulfate, and ammonium hydroxide have been utilized in the decellularization of animal tissues [48-50]. An increase in pH via the addition of alkaline bases contributes to the denaturation of DNA. Despite this, decellularization using alkaline was paired with other methods such as free-thaw cycles in the decellularization protocol of mesenchymal 3D cell culture. Meanwhile, acids were also previously implemented, which act by solubilizing components in the cytoplasm and disrupting nucleic acids that subsequently led to dissociation of DNA structure. Moreover, acids can denature proteins in the ECM, including collagen and GAGs. This effect of acid emphasizes the importance of optimization in the protocol, such as the dose and exposure time of the ECM to the reagents [51]. Peracetic acid is commonly applied for the decellularization of tissue and organs, in combination with mechanical agitation or subsequent treatment using DNase I, as seen in the decellularization of small intestine submucosa [52, 53].

Biological approaches to decellularization

The specificity of enzymes is advantageous in targeting cellular structures that led to decellularization of the ECM, hence why it can be applied for this purpose in various studies [51]. Enzymes such as deoxyribonuclease I (DNase I) and peptide:N glycosidase F (PNGase F) were previously applied as a component of the decellularization protocol in arterial tissues, pulmonary heart valves, and submillimetre vascular tissues, to be specific [46, 52, 54]. These enzymes were utilized in various methods, including as a step in a decellularization cycle that includes chemical detergents, and singular or dual-enzyme application after decellularization using chemical detergents [46, 52, 54].

Interestingly, in the dual-enzyme application method, inflammatory effects were low, contributing to the removal of xenoantigens through PNGase F treatment, in addition to the breakdown of DNA residues by DNase I. The use of the PNGase F was justified by the previous findings, where almost all known xenoantigens are categorized as carbohydrates. Hence, PNGase F treatment in this study aimed to break the N-glycans and inactivate the xenoantigens for successful xenotransplantation [46]. The use of proteinase such as trypsin has been studied in the decellularization of porcine aortic valves, to optimize the previous protocol of decellularization with enzymes and chemical detergents. The enzymatic treatment was conducted with Tergitol, which functions to maintain the structural integrity and functionality of the valves [55]. Hence, although an enzyme is a great tool to target specific molecules or structures for decellularization and subsequent in vivo applications, it needs to be combined with other approaches, including detergents and freeze-thaw.

Physical approaches to decellularization

Physical methods of decellularization boast the avoidance of chemical detergents, which could lead to immunogenicity of the ECM. Freeze-thaw cycles, pressure, and supercritical fluid are the common physical methods applied to various tissues for cell removal from the native ECM. Mechanical agitation and perfusion of tissues and organs have been employed alongside chemical approaches including detergents,

acids, and bases. Meanwhile, freeze-thaw cycles of very low temperatures at -80°C to -196°C were applied in conjunction with enzymes and detergents, usually for vascular ECM. Implementation of high pressure and supercritical fluid for decellularization are relatively more recent techniques compared to the previous two techniques.

Freeze-thaw method of decellularization involves cell damage during water crystallization during freezing and subsequent osmotic gradients that form due to differences in the rate of ice melts in the thawing process [56, 57]. This method can be independently applied, where the goal is to reduce the adverse effect of chemicals on the structure of ECMs [6, 13, 58]. Nonetheless, there were also studies that utilize freeze-thaw cycles in collaboration with enzymes such as DNase and detergents such as SDS [52, 58].

The decellularization approach using supercritical fluid as a decellularization agent has been recently observed in studies on various tissues. This method utilizes the properties of supercritical fluids, mainly the high transfer rate, permeability, and solvent power to extract the cells from the ECM. The solvent power of the supercritical fluids can be optimized by adjusting the temperature and pressure [59, 60]. Mainly carbon dioxide was utilized as the decellularization agent, where the exposure time could last from hours to days, depending on the target tissue, temperature, and pressure [61]. This method was implemented in the decellularization of porcine sciatic nerve tissues, where supercritical carbon dioxide fluid treatment alone was comparable with using SDS for cell removal [62]. Meanwhile, a hybrid approach was developed by treating the porcine aortic tissue with SDS before it was subjected to decellularization with carbon dioxide fluid and ethanol as co-solvent, and the DNA quantified from the samples was close to none [60]. Nonetheless, supercritical fluid as decellularization shows promising results, although further optimization and standardization of the important parameters are dire for its application.

Finally, ultra-high pressure as a decellularization agent has been employed for the decellularization of tissues from various sources. High hydrostatic pressure was able to shorten exposure time to minutes, while still having similar effects with other decellularization agents [51]. Tissues including vascular tissues, uterine, menisci, and bones were subjected to high hydrostatic pressure for decellularization [6, 14, 22, 26, 42]. The results from these studies were promising, with very low residual DNA when the washing process was emphasized. Recent studies focused on reducing the negative effect of the high pressure on the mechanical properties and the ultrastructure of the ECM [63].

Comparison between biological, chemical, and physical approaches for decellularization of ECM

The biological approach of decellularization refers to the utilization of enzymatic agents to target specific substrates. Enzymes such as deoxyribonuclease I (DNase I) and peptide:N glycosidase F (PNGase F) were previously applied as a component of the decellularization protocol in arterial tissues, pulmonary heart valves, and submillimetre vascular tissues, to be specific [46, 52, 54]. Meanwhile, chemical approaches include the implementation of acid, bases, non-ionic, ionic, and zwitterionic detergents. Ionic detergent, i.e. SDS, and non-ionic detergent, i.e. Triton X100, are the combinations most applied when decellularizing ECM using a chemical approach. Without altering the ECM protein structures, the ionic detergent decellularizes by disintegrating membrane proteins and lysing cells [4]. Moreover, the efficiency of these detergents for decellularization varies based on the size, density, thickness, number of cells, and lipid content of the ECM [47]. On the other hand, physical decellularization methods based on pressure, thermal shock, and ultrasonication are suggested as the more efficient solution to tissue decellularization.

In chemical decellularization, the usage of chemical detergents may result in incomplete removal of cellular debris, such as dsDNA, mitochondrial DNA, mitochondria, and membrane phospholipids. These remnants may compromise the remodeling process due to the concern of their attachment to the ECM [64]. The consequences of these residues are also determined by the type of host tissue, the host's immune function, and the source of the ECM [51]. In a study where both peracetic acid and detergents, i.e. SDS and TX were utilized in the decellularization of small intestine submucosa, peracetic acid-treated specimens were shown to have higher residual DNA at ≤ 60 ng. Meanwhile, the SDS/TX treated tissue showed lower DNA content, i.e. < 30ng/mg. In addition, residues of SDS are also detected in the dECM, which may give rise to an adverse host's immune reaction [43, 44, 65]. For example, the retention of SDS within decellularized tissues increases inflammation and immune response to foreign materials post-implantation [18, 65]. The cytotoxic effect of residual SDS partially dictated this outcome. Hence, an efficient washing process is required to wash out SDS remnants by effectively breaking the bonds between SDS and protein within the dECM [18]. In biological methods, chemical residues and their effect on the ECM may be due to the application of detergents and acid in the decellularization protocol with the addition of enzymes [52, 54]. When compared to a detergents-based method (SDS+TX), the detergent-enzyme (Trypsin+TX) method showed a similar level of residual DNA content after decellularization, i.e. < 5×10^3 ng/mg dry weight of the pulmonary heart valves. Nonetheless, in terms of residual detergents in the same study, the SDS level became the benchmark, as SDS has a cytotoxic effect in the host. The SDS content in the SDS+TX method was higher than that of the SDS-only

method, at < 15 µg/mg and < 12 µg/mg, respectively [46]. Nonetheless, another study that involved aortic blood vessels proved that residual DNA in the physical method, i.e. HHP, was very low, indicating that DNA was removed to the instrument's measurement level. On the other hand, the detergents-based method resulted in higher DNA residual, in the range of 0.3 to 1.3 µg/mg, the and enzymatic method (Trypsin) resulted in 0.8 µg/mg DNA content [22, 26]. Meanwhile, in terms of residual chemicals, i.e. SDS, physical methods such as HHP are advantageous, since minimal to no chemicals are utilized during the decellularization process, except during the washing process [22, 26]. In brief, residual DNA and SDS were detected in chemical and biological methods in varying degrees, though absent in physical methods, such as HHP.

Furthermore, there is an issue with the modulation of detergent concentration to achieve higher efficiency in the decellularization process. The increase in detergent concentration aggravated the issues with poor cell morphology and growth, as shown amidst the in vitro culture of human urothelial cells with SDS implemented as a decellularization agent [65]. Its deleterious effects on the ECM structure such as reduced collagen and elastin content have also become an ongoing concern. Whereas, enzyme applications were optimized to reduce the inflammatory effects, even with the utilization of two chemical detergents such as SDS and Triton X-100 [46]. Meanwhile, physical methods depend on physical parameters such as pressure and temperature, where the modulation of these parameters can contribute to effective decellularization [62].

Another concern involves the depletion of growth factors and elimination of glycosaminoglycans (GAGs) embedded in the matrix of dECM due to the vigorous effect of SDS and Triton-X100 [66, 67]. Therefore, the search for alternatives for these two common detergents has been executed and SLES is one of the promising alternatives. In one study, SLES's performance was compared with SDS's. SLES was able to perform better in preserving the ECM structure of the rat kidney while on par with the decellularization efficiency of SDS. As an anionic detergent, SLES was suggested to be a promising alternative to SDS due to its elevated biodegradability and moderate chemical effect [66]. Despite this, further research to elucidate the effect of SLES on the dECM is needed, where the optimal concentration and the number of residual detergents should be examined.

On the other hand, the diminishing effect of enzymatic methods mainly revolves around the compromised ECM nature, manifested as a ruptured vascular graft, which may be attributed to the long exposure of the tissue to detergent and enzymes through immersion and perfusion [52]. It is also important to note that most applications of enzymes as decellularization agents were mainly for soft vascular tissues, where future studies of its utilization on mineralized and cartilaginous tissues are suggested.

Whereas, employing exclusively physical methods can diminish the risk of chemical residue within the ECM, hence, aiding in the preservation of the structure and lowering the risk of adverse host reactions [11, 18]. The effect of these decellularization methods on the mechanical properties of the tissue can be quantitatively measured via elastic moduli. As an example, the longitudinal elastic modulus of peracetic acid-treated small intestinal submucosa was shown to be significantly higher than that of detergents methods (SDS and TX) and detergent-enzyme method (SDS and DNase I), at 4×10^4 MPa and 0.5×10^4 to 1.5×10^4 MPa respectively [53].

Additionally, physical methods such as HHP are associated with reduced mechanical properties including elasticity, and denaturation of structural proteins due to ultra-high pressure exposure [22]. In terms of elastic modulus, HHP-treated porcine aortic tissue was shown to have a higher longitudinal modulus at 1.9 ± 0.3 MPa compared to SDS-treated aortic tissue, at 0.5 ± 0.2 MPa. Increment in modulus could indicate stiffer tissue, where a high amount of stress can be a result of a small elastic strain [68]. Therefore, treatments in addition to the HHP have been introduced, as shown in the decellularization of human skin fibroblasts, where the cells were subjected to supercooling pretreatment [63]. In brief, chemical and physical methods can be compared based on the decellularization agent, residual DNA and reagent, and immunogenicity.

The effect of these decellularization methods on the mechanical properties of the tissue can be quantitatively measured via elastic moduli. As an example, the longitudinal elastic modulus of peracetic acid was shown to be significantly higher than that of detergents methods (SDS and TX) and detergent-enzyme method (SDS and DNase I), at 4×10^4 MPa and 0.5×10^4 to 1.5×10^4 MPa respectively [53].

The comparison between biological, chemical, and physical approaches in decellularization is summarized in Table I and II.

HIGH HYDROSTATIC PRESSURE: A PROMISING DECELLULARIZATION APPROACH

As its name suggests, high hydrostatic pressure (HHP) subjects the samples to extreme pressure, where the pressure varies from 100 to 800 MPa, depending on the type of ECM. The process may or may not involve heat, measured by the temperature set for each cycle. Compared to other physical methods, HHP stands out as the technique destroys cell membranes uniformly in a short treatment time, commonly 10 minutes per cycle [14, 22, 63]. Subjection of the cells to high pressure will cause apoptosis and necrosis, where lower pressure is shown to induce the balance between apoptosis

Table I. Comparison between decellularization approaches.

Aspect	Biological approach	Chemical approach	Physical approach	Reference
Decellularization agents	DNase I, PNGase F	Ionic and anionic detergents, acid and bases	High pressure, thermal shock, supercritical fluid, ultrasonication	[4, 44, 51, 53, 54]
Removal of residual DNA	High (chemical reagents and /or freeze-thaw method applied)	Intermediate to high	High	[18, 62, 54, 55]
Residual chemicals	From chemical reagents	Higher risk	Low to no risk	[42, 53, 54, 62]
Immunogenicity	Low, in the case of removal of xenoantigens in the form of glycosylated proteins in the ECM via PNGase F treatment	High, caused by residues in the ECM and higher concentration needed for better decellularization	Low, caused by changes in ECM components	[18, 54, 65]
Effect to structure and components of ECM	Compromised ECM (e.g. ruptured vascular graft *in vivo*)	Depletion of structural proteins and growth factors	Changes in mechanical properties	[22, 51, 66, 67]

(ECM: Extracellular matrix)

Table II. Comparison between key metrics across various decellularization protocols

Key metric	SDS/Triton X100	Trypsin/ Triton X100	High Hydrostatic Pressure	Sonication	Freeze-thaw
Targeted tissue(s)	Small intestine submucosa	Pulmonary heart valves	Porcine meniscus	Porcine descending aorta	Sheep pericardium
DNA content	<30 ng/mg	< 5×10^3 ng/mg	< 50 ng/mg	0.001 ± 0.001 µg/mg	41.7 ±15.3 ng/mg
Elastic modulus	<3500 MPa	N/A	2-3 MPa	2.6 ± 0.4 N/mm	6-10 MPa
Immunogenicity/ Cytotoxicity	High cytotoxicity (metabolic activity less than 20%)	Low immunological response (Lowest number of CD45-Positive cells (20 ± 7%))	Low immunological response after transplantation (< 2 multinucleated cells)	N/A	N/A
Reference	[53]	[46]	[6]	[25]	[13]

(N/A: Data not available)

and necrosis, and high pressure (>300 MPa) induces primarily necrosis. As the pressure applied escalates, the permeability of the cells also increases, which may instigate an immunological response due to the discharge of the intracellular components.

HHP treatment parameters for optimization

In order to achieve the desired cell removal effect, treatment parameters should be taken into account. In HHP, controlling the pressure, temperature, and time is pivotal to the success of the decellularization protocol while maintaining the structure of the ECM [69, 70].

Pressure

In HHP, ultrahigh pressure in the range of 800 to 1,000 MPa is common in research regarding decellularization involving this method. The diverse types of tissues and organs that utilize HHP show that this method is versatile and can cater to the specific requirements of each tissue or organ. Uterine, aortic blood vessels, menisci, femoral head, and costa have been subjected to ultrahigh-pressure treatment for decellularization [6, 10, 14, 22]. Other than whole organs or organized tissues, cell lines such as osteoblasts and fibroblasts from human or animal models were also treated with HHP [63, 71]. In general, it is shown that higher pressure implemented indicates more damage to the cells, such as in the degeneration of the cell's membrane [72].

Despite its versatility, the effect of ultrahigh-pressure on the cells should also be taken into consideration. The pressure subjected to the cells influences the type of cell death, i.e., either necrosis or apoptosis. Previous studies found that higher pressure in the range of 300 to 500 MPa primarily caused necrosis, also defined as uncontrolled cell death. Meanwhile, both apoptosis and necrosis were induced at a pressure under 300 MPa [71]. Apoptosis is defined as programmed cell suicide, where the cells form apoptotic bodies instead of undergoing cell lysis [71]. Based on Waletzko 2020, the most effective HHP therapy is suggested to result in cells that are metabolically inactive and, preferably, have not yet experienced necrosis. In other words, apoptosis type of cell death is desirable.

The lower range of HHP has also been studied, where it is coupled with supercooling treatment [63, 72]. Nevertheless, a significant number of studies on ECM decellularization implemented ultra-high pressure at 980 MPa, compared to the lower applied pressure at 450 MPa of the aforementioned study [14, 22, 26, 42]. Hence, the effect of ultra-high pressure on the type of cell death requires more extensive studies, and its effect on the ECM and recellularization should be elucidated.

Temperature

Temperature is another vital parameter for an effective decellularization protocol. The relationship between pressure and temperature is linear, and this principle is fundamental to the HHP treatment [22]. Studies have shown that higher onset temperature can prevent the solid phase, where ice forms, as the pressure increases [22, 42]. Funamoto et al. (2010) suggested that ice formation is implied by the exposure of the biological tissue to high pressure. To be exact, lower onset temperature during HHP led to a solid phase of the tissue, where ice formed. This freezing process may cause further damage to the tissues' structural integrity. Therefore, it is suggested to have a higher onset temperature to prevent this issue. It also shows that temperature control throughout the HHP process is important to avoid damage to the ECM. Onset temperature at 30^0C is common in decellularization of ECMs such as porcine menisci, porcine aortic valve, and rat uterine [6, 14, 22].

Additionally, few studies applied either low temperature or supercooling pretreatment to the tissues before HHP. Low starting temperature at 10^oC in the decellularization of blood vessels caused water in the tissue to undergo a freezing state, i.e. ice formation at pressurization. This led to the destruction of tissue through ice formation in the tissue [22]. In another study, the temperature of the tissues was controlled before the moderate HHP (250 MPa) application instead, with the utilization of supercooling pretreatment. This pretreatment reduced the samples' temperature to -4 and -8^oC, which helped to weaken the cell membranes, which was shown to reduce the viability of cells compared to the absence of pretreatment [63]. These studies revealed the importance of temperature control in different phases of HHP, including sample preparation and pressurization, in order to increase decellularization effectivity and maintain the native ECM in the tissue.

Duration of treatment

Duration of treatment for HHP across various studies was similarly implemented, i.e., 10 minutes of maintaining the high pressure. It is, of course, paired with ultra-high pressure in the range of 980 to 1000 MPa and onset temperature, 30^0C [6, 14, 22, 42]. This is desirable to attain sudden expansion of cell membranes due to rapid depressurization [22, 51]. Since these exact conditions have been replicated across various ECMs, therefore, it is trustworthy for future experiments that involve decellularization via HHP. Interestingly, there are efforts to suggest the same duration treatment, i.e., 10 minutes, with lower pressure on the biological scaffold. Moderate HHP in the range of 150 to 300 MPa was implied [63, 71, 72]. It is important to take note that the studies that implement moderate HHP use cell suspension as samples, instead of tissues and whole organs in studies with high-pressure treatment.

Another treatment condition that has been compared is the number of cycles of HHP. There can be static or cyclic HHP, where the latter were experimented with up to 5 cycles on normal human skin fibroblast cell suspension [72]. Cyclic HHP on the cells showed more degeneration of cell membranes compared to static HHP, both at the same pressure of 200MPa. Even so, more evidence is needed to prove the efficiency of cyclic HHP. This is because although more irreversible damage was made on the cell membrane of the cyclic group, similar observations were seen in the DNA residue and a number of live cells in both static and cyclic groups. More extensive studies are required to prove this theory that changes in pressure in cyclic HHP would promote the movement of intracellular fluid in and out of the cells. Hence, adjustments to these parameters are vital to the effective destruction and removal of cells, specifically through HHP treatment.

Washing process

The washing process occurs after the cells or tissues are subjected to HHP cycles. Washing the dECM is vital to removing cell debris including cellular genetic material from the ECM thoroughly [7, 14]. Removal of cell debris should be executed as any remaining cell debris might cause an immune response by the host cells. Even so, the simplification of the host response towards dECM is still limited and poorly understood [1]. Typically, the washing duration of the decellularized tissues such as blood vessels, uterine, and menisci, took between 7 to 14 days, except for mineralized tissue such as the femur and costa were washed for 3 weeks [6, 14, 22, 42]. A saline solution such as phosphate buffered saline (PBS) was utilized washing solution in these studies, in addition to antibiotics and washing with ethanol for several days in the washing process. Therefore, this process is arguably undetachable from the HHP protocol, where the decellularization is incomplete without a proper washing process. In brief, these treatment conditions should be considered when constructing the HHP protocol. Consequentially, the effects of HHP could determine the suitability and safety of the dECM towards the host.

POST-DECELLULARIZATION ASSESSMENT

Immunogenicity

Xenogeneic dECMs are ideal for transplantation as they are abundant, though safety concerns due to host rejection arise [4]. Moreover, it is composed of vital mechanical characteristics for physiological functions and appropriate components for cell signaling in binding, growth, and differentiation. Despite this, the main advantage of the application of xenogeneic dECM for in vitro development is associated with its inherent ECM composition and structure [73]. Therefore, ensuring a very low risk of immunogenicity of the dECM is a crucial step before application to the human host. A low elicitation of immunogenicity indicates good biocompatibility of the dECM. The definition of

biocompatibility of a scaffold, i.e. dECM, explores three main components; functionality, low immunogenicity or no host rejection, and desirable outcome in cellular or tissue response. Hence, biocompatibility can be referred to as the ability of the scaffold to evoke its expected function in medical therapy, without causing any undesirable consequences on the recipient, while eliciting advantageous cell or tissue response and optimization of the therapy, in clinical settings [11].

Thus, to accomplish biocompatibility, "the undesirable consequences on the recipient" must be eliminated. This can be done by eliminating the agents that refer to such host rejection. In other words, minimizing the impact of immunogenicity by removing the factors or components that provoke the immune response in the host. The primary cause can be attributed to incomplete decellularization of the ECM, which is associated with residues, cells, or chemicals, that may lead to the development of the innate and adaptive immune reaction. These agents include DNA fragments, endogenous retroviruses, α(1,3) galactose disaccharide (α-Gal) epitope, dECM proteins and their respective binding sites, and residual chemical detergents [18, 74]. When these agents reside in the dECM scaffold during in vivo transplantation, it could lead to adverse rejection from the recipient, ranging from hyperacute to acute, and chronic rejection of the decellularized tissue or organ [73]. Hence, the removal of these immunogenicity agents should be vigorously executed during the decellularization process.

Albeit the ability of decellularization techniques to effectively achieve effective cell removal of the ECM, immunogenicity factors such as antigens persist which exacerbate host immune response, as shown in previous studies [75-77]. The assumption that decellularization of these antigens from ECM solely originated from the cells is detrimental to a safe and biocompatible tissue or organ scaffold [73]. Hence, methods to remove these factors that elicit immunogenicity have surfaced, such as using DNase treatment, targeted antigen removal, and stepwise solubilization-based techniques [18, 77, 78].

Following the removal of these immunogenicity agents, an appropriate assessment should be carried out to elucidate the effectiveness of the said method. For DNA, molecular assessment can be executed that involves DNA quantification. DNA from the HHP-decellularized bovine dorsalis pedis arteries and rat uterine was quantified, respectively [14, 23]. There is a general understanding that a complete decellularized ECM should only have residual DNA lower than 50 ng, and these residual fragments should have a length of less than 200 bp [43]. Other than that, antigens such as α-Gal were assessed through immunohistochemical staining of tissue sections, ELISA-based approach, and Western blot approach [76, 79-81]. Albeit the rigorous means of assessing immunogenicity in vitro, in vivo evaluation of the dECM scaffold in the recipient is a vital measure to elucidate the immune response towards the longevity of the scaffold, without host rejection [73]. Hence, this in vivo evaluation can serve as a criterion for the complete removal of immunogenicity-inducing factors.

Mechanical properties

On the other hand, the mechanical properties of the dECM also impose importance in the assessment of the decellularization effectiveness. ECM is composed of the molecular network that confers appropriate mechanical architecture of the tissue applied to grow the desired cell population in tissue remodelling [69]. Mechanical properties of the ECM are primarily dictated by its structural proteins including elastin, collagen, fibronectin, fibrillin, and laminin. After decellularization commences, properties of interest such as elastic modulus, tensile strength, and viscous modulus are evaluated, albeit the main properties are usually governed by the tissue or organ type and desired function [4, 82]. The main medium of mechanical testing is the elucidation of elastic modulus through the application of stress and strain onto the dECM. Elastic modulus or Young's modulus was observed in various studies as a method to evaluate the changes in mechanical properties of the ECM in the decellularization of arteries, meniscus, and pericardium [6, 7, 13, 22, 23]. This assessment is vital as the ECM's elasticity governs the specification of the stem cell lineage [69]. The distribution and composition of the ECM with structural proteins, mainly collagen and elastin, imposes an effect on the mechanical properties of the ECM, due to the contribution of each structural protein. As an example, in collagen-rich tissues such as bone and tendon,

Mechanical properties of the ECM can be affected primarily by the decellularization protocols. The changes in the ECM structure and properties caused by the decellularization process could imply adverse consequences to cell adhesion during in vitro recellularization and immunogenicity during in vivo transplantation [18, 83]. Furthermore, ECM proteins denaturation during decellularization could elicit higher immunogenicity in the host. This is due to the exposure of immunogenic domains, conversion of inert molecules into immunogens, and depletion of immunomodulatory moieties [84]. Therefore, in addition to minimizing the effect on the mechanical properties of the ECM, a compatible decellularization method should also preserve the ECM's structure and components to prevent unwanted modulation of the ECM.

Physical methods such as HHP have been shown to preserve the structure and mechanical parameters to a certain degree; however, the risk of collagen denaturation after ultra-high pressure exposure is also concerning, as seen in the decellularization of porcine aorta via HHP [22]. This highlights the importance of optimization of efforts, especially when experimenting with various tissue or organ sources. As an example,

the freeze-thaw method could preserve the mechanical criteria and ECM structural components, albeit affected by the number of treatment cycles. On the other hand, extensive exposure time when employing ultrasonication may induce damage to the ECM structure [12, 25]. Furthermore, HHP exposure to the ECM may reduce the mechanical strength in terms of elastic modulus, which highlights the importance of optimization efforts while attempting this promising method [22, 23]. In brief, the maintenance of ECM's mechanical properties could be attributed to the decellularization method primarily, hence understanding the underlying principles and mechanisms of the methods is prominent in order to reduce the undesirable effects on the ECM.

CONCLUSION

In summary, various methods for decellularizing tissues and organs aim to achieve effective cell removal, lower immunogenicity, improve biocompatibility, and maintain structural integrity. Different types of tissues and organs require tailored decellularization methods, which are generally divided into chemical, physical, and biological approaches, although biological methods are less commonly used. Effective decellularization involves removing DNA and cellular debris, as well as other factors like α-Gal epitopes and ECM alterations, while also preserving mechanical properties like elasticity and structural integrity. Chemical methods often involve detergents, which can leave residues that increase immunogenicity or damage the ECM if concentration levels are too high. Physical methods like high hydrostatic pressure (HHP) have shown promise by effectively removing cells and residues, though they can sometimes negatively affect the mechanical strength of the ECM. Thorough washing steps during HHP processes are important for removing any remaining debris.

Nonetheless, varying challenges persist in the long-term implantation of devices and grafts. Regulatory requirements include compliance with Good Manufacturing Practice (GMP) for scaffold fabrication, cell sourcing, and clinical-grade bioreactor systems. The variability in patient responses necessitates standardized protocols and multi-center clinical trials to ensure reproducibility and scalability [85]. Moreover, regulatory bodies have been focusing on sustainability and environmental impacts, as well as ethical practices, which may push research into more sustainable biomaterials for tissue engineering. Adoption of sustainable materials in this case could be a challenge; however, it can improve public trust and acceptance of medical technologies through visible commitment to sustainable practices [86]. Other than that, translating implantation or biomaterial research into safe clinical practice requires rigorous clinical trials is another challenge, where it is vital to assess the material's safety, efficacy, and functionality in long-term human hosts [87]. As an example, a previous study revealed that exertion of ultra-high pressure above 300 MPa to the target cells, i.e. human osteoblasts and chondrocytes, led to mostly necrosis [71]. This pressure-dependent cell death becomes an issue for the applicability of scaffolds derived from HHP because necrotic effect will result in inflammatory response, such as the damage-associated molecular patterns (DAMPS). Such response will further stimulate pro-inflammatory response, which can be detrimental to the host [44, 88]. Hence, this concern must be addressed in future research on HHP and scaffolds from it, as tissue replacement materials should have low to negligible immunogenicity to the host. This hurdle shed light on the importance of standardization of dECM assessment across tissues and organs, whether the standardization can occur within tissue type or generally.

Finally, future research should focus on improving the mechanical properties of ECMs after HHP treatment and more thoroughly assessing immunogenic agents like antigens to ensure scaffolds are biocompatible. Additionally, understanding how ECM structure and components affect cell viability and immune response will help optimize transplantation methods using xenogeneic tissues and organs. This knowledge could pave the way for safer and more effective approaches to using xenogeneic sources for human transplants.

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