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An overview of tissue and whole organ decellularization processes

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Abstract

Biologic scaffold materials composed of extracellular matrix (ECM) are typically derived by processes that involve decellularization of tissues or organs. Preservation of the complex composition and three-dimensional ultrastructure of the ECM is highly desirable but it is recognized that all methods of decellularization result in disruption of the architecture and potential loss of surface structure and composition. Physical methods and chemical and biologic agents are used in combination to lyse cells, followed by rinsing to remove cell remnants. Effective decellularization methodology is dictated by factors such as tissue density and organization, geometric and biologic properties desired for the end product, and the targeted clinical application. Tissue decellularization with preservation of ECM integrity and bioactivity can be optimized by making educated decisions regarding the agents and techniques utilized during processing. An overview of decellularization methods, their effect upon resulting ECM structure and composition, and recently described perfusion techniques for whole organ decellularization techniques are presented herein.

Keywords

extracellular matrix; biomaterials; scaffolds; decellularization; regenerative medicine

1. Introduction

Biologic scaffolds composed of extracellular matrix (ECM) are commonly used for a variety of reconstructive surgical applications and are increasingly used in regenerative medicine strategies for tissue and organ replacement. The ECM represents the secreted products of resident cells of each tissue and organ, is in a state of dynamic reciprocity with these cells in response to changes in the microenvironment, and has been shown to provide cues that affect cell migration, proliferation, and differentiation [1–7]. Preservation of the native ultrastructure and composition of ECM during the process of tissue decellularization is highly desirable [8–14]. A review of tissue decellularization techniques and their effect upon ECM properties was published in 2006 [15]. However, the development of new decellularization techniques and the advent of three-dimensional whole organ decellularization have since emerged. In addition, the deleterious *in vivo* effects of residual

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cellular material are becoming more recognized [16–19]. The rapid diversification of both decellularization methods and source tissues, and the expanding list of clinical applications suggest that cell residues in ECM should be evaluated objectively against a quantitative definition. The objectives of the present manuscript are (1) to provide an updated overview of tissue and organ decellularization techniques and their expected effects on the mechanical and biological properties of the remaining ECM as determined by systematic investigations [20–45] and (2) to define a decellularization standard that has been shown to avoid adverse host responses following implantation and is associated with constructive host responses.

2. Clinical relevance and rationale for the use of ECM as a biologic scaffold

The use of ECM derived from decellularized tissue is increasingly frequent in regenerative medicine and tissue engineering strategies, with recent applications including the use of three-dimensional ECM scaffolds prepared by whole organ decellularization [8–10, 46, 47]. Clinical products such as surgical mesh materials composed of ECM are harvested from a variety of allogeneic or xenogeneic tissue sources, including dermis, urinary bladder, small intestine, mesothelium, pericardium, and heart valves, and from several different species (Table 1). The potential advantage of tissue specificity for maintaining selected cell functions and phenotype has been suggested by studies of cells and ECM isolated from tissues and organs such as the liver [9, 48], respiratory tract [10, 30], nerve [49], adipose [50], and mammary gland [51].

The ECM has been shown to influence cell mitogenesis and chemotaxis [1, 7], direct cell differentiation [2, 12, 47, 48, 52–54], and induce constructive host tissue remodeling responses [55–57]. It is likely that the three-dimensional ultrastructure, surface topology, and composition of the ECM all contribute to these effects. There is also evidence that residual cellular material attenuates or fully negates the constructive tissue remodeling advantages of biologic scaffold materials *in vivo* [18, 19, 58]. Therefore, tissue processing methods, including decellularization, are critical determinants of clinical success [59–61].

3. Decellularization agents

The most effective agents for decellularization of each tissue and organ will depend upon many factors, including the tissue's cellularity (e.g. liver vs. tendon), density (e.g. dermis vs. adipose tissue), lipid content (e.g. brain vs. urinary bladder), and thickness (e.g. dermis vs. pericardium). It should be understood that every cell removal agent and method will alter ECM composition and cause some degree of ultrastructure disruption. Minimization of these undesirable effects rather than complete avoidance is the objective of decellularization. An overview of some commonly used agents (e.g. chemical, enzymatic, and physical) and their effects on cellular and extracellular tissue constituents is provided in the text below and in Table 2.

3.1. Chemical agents

3.1.1. Acids and bases—Acids and bases cause or catalyze hydrolytic degradation of biomolecules. Peracetic acid is a common disinfection agent that doubles as a decellularization agent by removing residual nucleic acids with minimal effect on the ECM composition and structure [62–64]. Acetic acid damages and removes collagens with a corresponding reduction in ECM strength, but it does not affect sulfated glycosaminoglycans (sGAG) [20]. Bases (e.g. calcium hydroxide, sodium sulphide, and sodium hydroxide) are harsh enough and commonly used to remove hair from dermis samples during the early stages of decellularization [21, 22]. However, bases can completely eliminate growth factors from the matrix and decrease ECM mechanical properties more significantly than chemical and enzymatic agents [22]. The primary mechanism by which

bases such as sodium hydroxide reduce mechanical properties is the cleavage of collagen fibrils and disruption of collagen crosslinks [65].

3.1.2. Hypotonic and hypertonic solutions—Hypertonic saline dissociates DNA from proteins [66]. Hypotonic solutions can readily cause cell lysis by simple osmotic effects with minimal changes in matrix molecules and architecture [67]. For maximum osmotic effect, it is common for the tissues to be immersed alternately in hyper- and hypotonic solutions through several cycles. Hypertonic and hypotonic solutions also help rinse cell residue from within tissue following lysis.

3.1.3. Detergents—Ionic, non-ionic, and zwitterionic detergents solubilize cell membranes and dissociate DNA from proteins, and they are therefore effective in removing cellular material from tissue [66, 68]. However, these agents also disrupt and dissociate proteins in the ECM as evidenced by their use in protein extraction procedures in tissue proteomics [69, 70]. The removal of ECM proteins and DNA by detergents increases with exposure time [23–25] and varies with organ subunit, tissue type, and donor age [11, 69, 70]. Combining multiple detergents increases ECM protein loss [69] but also allows for more complete detergent removal from ECM after decellularization [26, 71]. Triton X-100 can effectively remove cell residues from thicker tissues such as valve conduits where enzymatic and osmotic methods are insufficient, with concomitant ECM protein loss accompanied by decreased adverse immune response *in vivo* [27]. Sodium dodecyl sulfate (SDS) appears more effective than Triton X-100 for removing nuclei from dense tissues and organs such as the kidney and temporomandibular joint while preserving tissue mechanics [11, 28]. The addition of a detergent such as SDS to a decellularization protocol can make the difference between complete and incomplete cell nuclei removal [29] but has the associated drawback of ultrastructure disruption [30–32] and growth factor elimination [22]. SDS is typically more effective for removing cell residues from tissue compared to other detergents but is also more disruptive to ECM [25, 32–35]. For tissue delipidation, non-ionic detergents such as Triton X-100 are more effective than ionic detergents such as deoxycholate [71, 72]. The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is most effective for cell removal from thinner tissues such as lung [10] and may be ineffective for decellularization of thicker tissues, even when used in combination with SDS [73] or alone for relatively acellular tissues [25]. A blinded categorical comparison of detergents for peripheral nerve decellularization showed better preservation of ultrastructure by non-ionic and zwitterionic detergents but better cell removal by ionic detergents [74]. However, the low concentration of cell bodies in peripheral nerve and the size of the tissue may limit the translation of these results to other tissues.

Care must be taken to flush residual chemicals from ECM after decellularization, particularly detergents such as SDS that penetrate into thick or dense tissues. Cytotoxicity is possible even at reduced agent concentrations and will inhibit or completely negate the beneficial properties of a cell-free ECM scaffold [71, 72]. Even thin tissues such as valve leaflets require multiple (more than six) agitated washes to completely remove detergents [71].

3.1.4. Alcohols—Alcohols such as glycerol aid in tissue decellularization by dehydrating and lysing cells [21]. Phospholipids in valve leaflets and conduits contribute to prosthesis calcification and failure and can be extracted using alcohols [75, 76]. In fact, alcohols such as isopropanol, ethanol, and methanol are more effective than lipase in removing lipids from tissue and are capable of rendering adipose tissue lipid-free in a relatively brief period [50, 77]. Methanol in combination with chloroform has been used during delipidation of tissues [21]. Caution should be used in treating tissues with alcohols such as ethanol and methanol

due to their use as tissue fixatives in histology, their ability to precipitate proteins [78], and the damage they cause to ECM ultrastructure [60, 65, 79].

3.1.5. Other solvents—Acetone can also be used to remove lipids during decellularization [28, 80]. However, like alcohols, the use of acetone as a tissue fixative [78] and its damage of ECM ultrastructure [79] warrant conservative use, especially for biological scaffolds used in load-bearing clinical applications [60, 65]. In comparison to detergent treatments, acetone crosslinks ECM to produce stiffer scaffolds with mechanical properties further removed from those of native tissue [28].

Tributyl phosphate (TBP) is an organic solvent with viricidal properties [81]. For decellularization of dense tissues such as tendon, TBP appears to be more effective than detergents such as Triton X-100 and SDS, with varying effects on retention of ECM constituent and native mechanical properties [35, 36].

3.2. Biologic agents

3.2.1. Enzymes—Enzymes reported in tissue decellularization protocols include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase. Enzymes can provide high specificity for removal of cell residues or undesirable ECM constituents. However, complete cell removal by enzymatic treatment alone is difficult and enzyme residues may impair recellularization or evoke an adverse immune response.

Nucleases (e.g. DNases and RNases) cleave nucleic acid sequences and can therefore aid in removal of nucleotides after cell lysis in tissues [10, 23, 29, 33]. Endonucleases such as benzonase [10] may be more effective than exonucleases because they cleave nucleotides mid-sequence and thereby more effectively fragment DNA in preparation for its removal. Likewise, non-restriction endonucleases will more effectively fragment DNA compared to their sequence-dependent counterparts.

Trypsin is a serine protease commonly used as an enzymatic decellularization agent. However, ECM proteins such as collagens have limited resistance to trypsin cleavage [82] and tissue exposure to trypsin should therefore be used with caution. In comparison to detergents, trypsin is more disruptive to elastin and collagen and slower to remove cells but shows better preservation of GAG content [26, 32, 37, 83]. Trypsin disruption of ECM can be correlated to changes in mechanical properties [83]. Removal of cells and ECM constituents by trypsin is time-dependent, and complete decellularization by trypsin alone may require lengthy incubation even for thinner tissues such as valve leaflets [38]. Trypsin can be used effectively to disrupt tissue ultrastructure and improve penetration of subsequent decellularization agents; therefore, exposure to trypsin as the initial step in a tissue decellularization protocol may be desirable or even necessary, particularly for complete removal of cell nuclei from dense tissues [29].

Collagenase may be used during decellularization, but only when ultrastructure preservation and maximum collagen retention are not critical to the intended clinical application of the resultant ECM. Lipase aids in delipidation but is typically insufficient to remove all lipids when used alone [50, 77].

After delipidation of dermis, direct comparison of trypsin and dispase treatments showed superior decellularization by dispase accompanied by increased ECM disruption [21]. The same study also showed greater cell infiltration in dispase-treated tissue after four weeks of subcutaneous implantation. Dispase and trypsin can be used successively to improve cell removal from thicker tissues such as dermis if used in combination with detergents, and repeated treatments with dispase may further improve decellularization [24]. Using an

enzyme such as dispase or thermolysin as the sole decellularization agent is only effective for removing cells on the surface of a tissue and is likely to require mechanical abrasion for complete cell removal [39]. With regard to the underlying basement membrane and ECM, thermolysin is less disruptive compared to dispase [39].

Decellularized xenogeneic tissues can be treated with α-galactosidase to reduce the immunogenic cell surface antigen galactose-α-(1,3)-galactose (Gal epitope) [58], although the immunomodulatory effect of Gal epitope does not adversely affect *in vivo* remodeling of xenogeneic ECM [84]. A more complete review of host immune responses to Gal epitope is available elsewhere [85].

3.2.2. Non-enzymatic agents—Chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) aid in cell dissociation from ECM proteins by sequestering metal ions [86, 87]. It is likely that chelating agents contribute to subtle disruptions in protein-protein interactions by the same mechanism [88]. Chelating agents alone are insufficient for superficial cell removal even with agitation [39], and they are therefore typically used in combination with enzymes such as trypsin [27, 29, 33, 40, 41, 46] or detergents [22, 27, 29, 33, 35, 42, 46, 73, 89]. The combined efficacy of chelating agents and simple hyper- or hypotonic solutions for decellularization is unknown [27, 29, 34, 89].

Toxins such as latrunculin allow investigators to take advantage of naturally occurring cytotoxic agents for the purpose of decellularization. Gillies et al. demonstrated removal of DNA and intracellular proteins from a dense tissue, tibialis anterior, using only latrunculin B, hyper- and hypotonic solutions, and DNase treatments [90]. This method was superior in its removal of DNA and retention of GAG compared to an enzymatic and detergent decellularization method, and passive mechanical testing of the ECM scaffold and native tissue showed similar properties.

Serum associates with nucleic acid fragments to aid in their removal from tissue, but it does not remove some immunogenic constituents such as phospholipids [73]. Additionally, xenogeneic serum has the disadvantage of introducing immunogenic constituents that may associate with the ECM, thus potentiating a downstream adverse host response. The use of serum in preparing ECM for clinical applications is therefore limited.

Serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) [27, 31, 34–36, 50, 89], aprotonin, and leupeptin [51] prevent undesirable damage to ECM that might otherwise result from release of intracellular proteases during cell lysis. Antibiotics and antimycotics such as penicillin, streptomycin, amphotericin B, and sodium azide may be used to minimize microbial contamination during decellularization [8, 9, 11, 34–36, 42, 46, 47] but also present a potential regulatory hurdle for clinical biologic scaffolds.

3.3. Physical and miscellaneous agents

3.3.1. Temperature—Freeze-thaw processing effectively lyses cells within tissues and organs, but the resulting membranous and intracellular contents remain unless removed by subsequent processing. A single freeze-thaw cycle can reduce adverse immune responses such as leukocyte infiltration in vascular ECM scaffolds [89]. Multiple freeze-thaw cycles may be used during decellularization [47, 50, 91] and do not significantly increase the loss of ECM proteins from tissue [70]. Freeze-thaw processing does produce minor disruptions of the ECM ultrastructure [21, 39] and should therefore be used only when such effects are acceptable in the final ECM product. The effect of freeze-thaw processing on mechanical properties is minimal for load-bearing, mechanically robust tissues [23, 92].

3.3.2. Force and pressure—Cells on the surface of a tissue or organ (e.g., urinary bladder, small intestine, skin, amnion) can be effectively removed by mechanical abrasion in combination with enzymes [39], hypertonic saline, or chelating agents, all of which facilitate dissociation of cells from their subjacent basement membrane. However, underlying ultrastructure and basement membrane integrity are invariably damaged by any direct application of mechanical force [39].

Hydrostatic pressure requires relatively little time and can be more effective than detergents or enzymes for removing cells from blood vessel and corneal tissues, although the baric formation of ice crystals may disrupt ECM ultrastructure [33, 43]. Increased temperature during pressure decellularization prevents ice crystal formation [33] but may disrupt ECM due to the associated increase in entropy, which can be mitigated by colloids such as dextran [44].

3.3.3. Non-thermal irreversible electroporation—Non-thermal irreversible electroporation (NTIRE) has been investigated as an approach to tissue decellularization. Microsecond electrical pulses are applied across a tissue and the resident cells of that tissue, inducing the formation of micropores in the cell membrane due to the destabilization of the electrical potential across the membrane [93, 94]. The presence of the micropores causes loss of cell homeostasis and leads to cell death. When NTIRE was applied to rat carotid arteries *in vivo*, it was shown that the cellular remnants were slowly removed from the tissue over a period of days, leaving an acellular tissue approximately three days after NTIRE treatment [95]. The mechanisms for the cellular removal are not clear. By selecting appropriate parameters to reduce heat generation, the integrity and morphology of the remaining ECM appears to be retained, which allows for recellularization by host cells.

NTIRE has some obvious limitations. The probes are relatively small, thereby limiting the size of the tissue that can be decellularized. More importantly, since the mechanism of cellular removal has been suggested to be immune mediated, the decellularization process would need to occur *in vivo*, thereby significantly limiting potential applications. However, one recent study has investigated the *in vitro* use of NTIRE combined with vascular perfusion to decellularize porcine liver. The results showed that perfusion with lactated ringer's solution facilitated removal of cellular debris resulting from electroporation, but also showed the limited effective geometric distribution of this approach [96]. The amount of cellular remnants retained within the tissue remains to be determined. As discussed previously, ECM scaffolds that have not been thoroughly decellularized promote a very different, less desirable host response than those that have been cleared of cell remnants as determined by residual DNA content.

4. Techniques to apply decellularization agents

The optimal application of decellularization agents is dependent upon tissue characteristics such as thickness and density, the agents being used, and the intended clinical application of the decellularized tissue. Prior to applying decellularization agents, undesirable excess tissue may be removed to simplify the cell removal process [29, 97]. Tissue removal may focus on retention of key ECM components such as basement membrane. Direct force may also be applied to tissue to aid in decellularization [48, 51, 77]. For thin tissue laminates such as urinary bladder, intestine, pericardium, and amnion, the most commonly used decellularization techniques are freezing and thawing, mechanical removal of undesirable layers such as muscle or submucosa, and relatively brief exposure to easily-removed detergents or acids followed by rinsing (Fig. 1A). Thicker tissue laminates such as dermis may require more extensive biochemical exposure and longer rinse times (Fig. 1B). Fatty, amorphous organs and tissues such as adipose tissue, brain, and pancreas typically require

the addition of lipid solvents such as alcohols (Fig. 1C). The complexity and length of the decellularization protocol is usually proportional to the degree of geometric and biologic conservation desired for the post-processed tissue (e.g. macrostructure, ultrastructure, matrix and basement membrane proteins, growth factors, etc.), especially for composite tissues and whole organs (Fig. 1D–G).

4.1. Whole organ perfusion

Antegrade or retrograde perfusion has been used as a technique for decellularization of organs while largely preserving the three-dimensional architecture of the organ from which the ECM has been isolated. Vascular networks exist within organs that minimize the diffusion distance for oxygen to cells. Therefore, perfusion through the vasculature is a particularly efficient method to deliver decellularizing agents to cells and for transport of cellular material from the tissue. It should be noted that perfusion decellularization is not necessary to produce an intact, perfusable vascular network within the ECM [98].

The heart has been decellularized by perfusion to generate a 3-dimensional scaffold that preserves the native organ geometry [8]. The aorta of a rat heart was cannulated for retrograde coronary perfusion with heparizined PBS with adenosine for 15 minutes, 1% SDS for 12 hours, and 1% Triton X-100 for 30 minutes with each step followed by rinsing with deionized water. Decellularization was followed by perfusion with antibiotic containing PBS for 124 h. The result was a decellularized tissue that appeared similar to the heart with the exception of a translucent white appearance. Subsequent perfusion of the decellularized organ suggested that the vascular network was intact despite the use of detergents. Recellularization showed that the ECM was amenable to cell seeding with cardiomyocytes that formed small foci of contractile muscle.

A subsequent report described methodology for decellularizing larger hearts from a porcine origin [46]. This methodology also utilized retrograde coronary perfusion with successive perfusates of 0.02% trypsin/0.05% EDTA, 3% Triton X-100, and 4% deoxycholate with PBS rinses between reagents. The process for decellularization of porcine hearts was reduced to a time span of less than 10 hours by progressively increasing the pressure at which the perfusate was pumped through the vasculature. It was hypothesized that progressively increasing the pressure allowed for dilation of the vessels, effectively decreasing the diffusion distance to the cells, and increased flow, facilitating removal of cellular material. There was no evidence that the increased pressure or flow had deleterious effects on the vascular structures as perfusion was maintained throughout the process and decellularization was consistent throughout the organ. The decellularized heart tissue showed passive mechanical behavior similar to native myocardium and was also capable of supporting cardiac cells.

Recently, a number of groups have investigated perfusion decellularization for lung tissues [10, 30, 47, 99]. The function of the lung requires two accessible compartments with a short diffusion distance, the vascular network and the airway compartment with alveolar structures. The published work has included a variety of combinations of perfusion into and through these two compartments. Ott et al. used vascular perfusion alone in an approach similar to that used for earlier work with the heart [30]. It was found that perfusion with 0.1% SDS for 2 h at physiologic pressure was sufficient to decellularize rat lungs. In contrast, work by Petersen et al. delivered CHAPS in PBS into the airway compartment by intracheal instillation combined with perfusion of the vascular compartment, with an effort made to maintain pressure below 20 mmHg [10]. Price et al. also took advantage of both the airway and vascular compartments for delivery of decellularization agents, but the reagents, which included Triton X-100, deoxycholate, DNase solutions, and bleach, were perfused and then repeatedly incubated under static conditions [99]. Cortiella et al. perfused lungs

with 1% SDS into the airway compartment while the tissue was circulated in a bioreactor system [47]. In contrast to the previous three protocols described for lung decellularization that had durations of hours to days, the Cortiella protocol lasted approximately five weeks. All four groups showed that major components of the ECM and general microstructure of the lung was preserved, but further comparisons are needed to determine the full effects of each protocol on the ECM and the implications for the resulting host response after implantation. All four groups showed that the lung ECM could be repopulated with cells. Three of the groups used mixtures of whole lung cell isolates for the recellularization process; therefore the effect of the ECM on cell differentiation was unclear. In the work by Cortiella et al., a homogenous mouse embryonic stem cell population was used, and the results showed that the matrix was capable of promoting site-appropriate differentiation without any other specific differentiation cues. Ott et al. and Peterson et al. also performed orthotopic implantation of the lung constructs in rats for a period of a few hours. The results in both studies showed vascular leakage, evidence that the decellularization process caused some damage to the microvasculature that was not detected by histologic examination.

Liver decellularization has been performed by perfusion through the portal vein (e.g., antegrade perfusion) with combinations of SDS and Triton X-100 [9, 100]. Uygun et al. perfused the liver with 0.1% SDS alone [9], whereas Shupe et al. perfused the liver with increasing concentrations of Triton X-100 followed by 0.1% SDS and serum [100]. As with other decellularized whole organs, the liver took on a translucent, white appearance during perfusion in both studies. The investigation by Shupe et al. showed an absence of DNA by hematoxylin and eosin (H&E) and retention of collagen IV and laminin within the ECM. Uygun et al. also showed evidence that DNA was removed and that microvasculature, ECM ultrastructure, and constituents such as collagens I and IV, fibronectin, and laminin were preserved. Hepatocytes were reintroduced into decellularized livers by portal vein perfusion in both studies. Uygun et al. additionally showed preservation or restoration of hepatocyte functions such as synthesis of lactate dehydrogenase and albumin and production of urea persisting up to eight hours after heterotopic implantation.

Multiple groups have decellularized whole kidneys by perfusion with reports of intact vasculature and complete cell removal [53, 101, 102]. Ross et al. reported kidney decellularization by perfusing with 3% Triton X-100, 0.0025% DNase in hypertonic solution, 3% Triton X-100 a second time, and 4% SDS with additional rinses [53]. The murine renal ECM retained laminin and collagen IV and supported renal differentiation of murine embryonic stem cells. Modification of the decellularization protocol, mainly by substituting deoxycholate for the second Triton X-100 step, also produced positive results. It is noteworthy that a similar study by Nakayama et al. [11] on transverse sections from rhesus monkey kidney did not employ perfusion but also demonstrated retention of laminin and collagen IV as well as collagen I, fibronectin, and heparin sulfate. The organization of ECM constituents showed intact glomerular basement membrane and supported developmental renal phenotypes in fetal cells after they migrated into the ECM from juxtaposed fresh explants. The second study used the same detergents (1% SDS or 1% Triton X-100 for 7–10 days), which rendered the tissue semi-transparent and decreased its compressive modulus.

Together, the above studies demonstrate the initial development of decellularization strategies to create whole organ ECM structures that support cell infiltration and normal phenotypic function. A more complete review of whole organ decellularization and recellularization is available elsewhere [Badylak and Taylor, Ann Rev Biomed Eng, submitted].

4.2. Pressure gradient

Inducing a pressure gradient across tissue during decellularization can be used to supplement enzyme treatment, resulting in superior preservation of ultrastructure [21]. Luminal perfusion of hollow tissues with a transmural pressure gradient, designated as convective flow, can effectively force decellularization agents through dense tissues and drive cell residues out of the ECM. Montoya et al. showed qualitative elimination of DNA and 150% greater phospholipid reduction after convective flow decellularization of umbilical veins compared to conventional agitation (which stained positive for nucleic acids), using the same volume of a 20% acetone, 60% ethanol solution for both methods [80]. Convective flow degraded collagen to a lesser extent than agitation as assessed by hydroxyproline quantification. The lowest transmural pressure tested, 5 mmHg, yielded cumulative protein extraction and an ECM modulus that did not differ significantly from the native tissue or the agitation decellularized ECM. Bolland et al. have also reported using a pressure gradient to decellularize bladder tissue by combining submersion and cyclic intravesical pressurization [103]. Collagens I and IV, laminin, and GAG were retained along with some intracellular proteins, and mechanical properties were unaffected by the decellularization process.

4.3. Supercritical fluid

Supercritical carbon dioxide removes cell residues when passed through tissues at a controlled rate similar to critical point drying. Sawada et al. showed qualitative elimination of DNA from aortic tissue after only 15 minutes of treatment with supercritical carbon dioxide and an ethanol entrainer [104]. Phospholipids were reduced by about 80% in the same period, although additional exposure time did not yield further reduction. While ethanol was necessary for DNA and phospholipid removal in this study, multiple entrainers would solubilize cell residues and yield similar results. The advantages of supercritical gas decellularization include use of an inert substance (e.g. carbon dioxide) for cell removal, minimal alteration of ECM mechanical properties, and elimination of lyophilization as a preparatory step for ECM processing and storage.

4.4. Immersion and agitation

The difference in the duration of lung decellularization protocols by perfusion and agitation shows the efficiency gained by perfusing a tissue's vascular network as a decellularization method when possible. However, not all tissues have vasculature that allows for straightforward, discrete, complete access [98], and such tissues require other strategies. The most common approach for such tissues is immersion in decellularization agents while being subjected to agitation. Immersion and agitation methods of tissue decellularization have been described for a wide variety of tissues, including heart valves [27, 40, 71], blood vessel [73, 80, 89], skeletal muscle/tendon [34–36, 54, 90, 105–108], peripheral nerve [49, 74], spinal cord [109], cartilage/meniscus [23, 52], trachea [110–113], esophagus [42], dermis [22, 58], and urinary bladder [29, 97]. The duration of the protocol using this approach is a function of the tissue thickness and density, detergent used, and intensity of agitation.

Particularly thin tissues such as the submucosa of small intestine and urinary bladder can be effectively decellularized after a relatively short exposure to peracetic acid with agitation. For these materials and narrow pieces of other tissues, the removal of DNA and loss of ECM constituents are a function of the agitation speed during tissue decellularization (Fig. 2). Denser tissues such as dermis, tendon, and trachea require prolonged agitation protocols lasting days to months, often with exposure to combinations of detergents, enzymatic solutions, and alcohols.

A number of protocols that utilize immersion and agitation have also been proposed for decellularization of intact tracheal tissue. Baiguera et al. [110] recently published a protocol for decellularization of human tracheas that improved upon a previous protocol ultimately used to generate scaffolds that served as a substrate for seeding of autologous airway epithelial cells and bone marrow cells for clinical airway reconstruction [111]. The protocol most recently described involves 25 cycles of exposure to deoxycholate followed by treatment with DNase, with each cycle lasting approximately 8 hours. In contrast, Remlinger et al. has described a protocol to decellularize porcine tracheas that involves exposure to Triton X-100, 3M NaCl, and isopropanol in a protocol that lasts approximately 4 days [112]. Both protocols showed effective removal of cellular material from the connective tissue surrounding the cartilage structures, but there was evidence of residual chondrocyte material within the cartilage tissue due to the density of the native tissue. Several decellularization methods have been compared directly [113], but systematic studies have not been performed to compare the effects of each detergent, the length of exposure to the detergent, or the agitation speed on removal of DNA, loss of ECM constituents and ultrastructure, or, most importantly, *in vivo* function of the scaffold.

5. Sterilization of decellularized ECM

It is necessary to sterilize biologic scaffolds composed of ECM prior to implantation or *in vitro* use, including depyrogenation to eliminate endotoxins and intact viral and bacterial DNA that may be present. Biological scaffolds may be sterilized by simple treatments such as incubation in acids [64] or solvents [60], but such methods may not provide sufficient penetration or may damage key ECM constituents [65]. However, sterilization methods such as ethylene oxide exposure, gamma irradiation, and electron beam irradiation are known to alter ECM ultrastructure and mechanical properties [97, 114], including properties of clinical products composed of ECM [115, 116]. Of these, ethylene oxide may substantially change ECM mechanical properties [45] or leave them unaltered [92, 97], and ethylene oxide treatment can cause undesirable host immune responses that impair proper function of the biologic scaffold after implantation [117]. ECM degradation during irradiation is at least partially attributed to denaturation of key structural proteins such as collagen, a process which cannot be mitigated by exposure rate and occurs even at relatively low doses [114]. Gamma irradiation causes residual lipids to become cytotoxic [115] and accelerates enzymatic degradation of ECM [116]. Supercritical carbon dioxide has recently been investigated as an alternative method for sterilizing ECM, with multi-log reductions in bacterial and viral loads within porcine dermal ECM accompanied by minor changes in mechanical properties relative to other sterilization methods [118]. However, this approach is relatively new and requires further investigation.

6. Evaluation of decellularized ECM

Residual cellular material within ECM may contribute to cytocompatibility problems *in vitro* and adverse host responses *in vivo* upon reintroduction of cells [16–19]. Although decellularization techniques cannot remove 100% of cell material, it is possible to quantitatively assay cell components such as double-stranded DNA (dsDNA), mitochondria, or membrane-associated molecules such as phospholipids. The threshold concentration of residual cellular material within ECM sufficient to elicit a negative remodeling response has not been investigated in detail and may vary depending upon ECM source, tissue type into which the ECM is implanted, and host immune function.

The term decellularization has not been defined by quantitative metrics. Based upon the findings of studies in which an *in vivo* constructive remodeling response has been observed

and adverse cell and host responses have been avoided, the following minimal criteria suffice to satisfy the intent of decellularization:

- <50 ng dsDNA per mg ECM dry weight
- <200 bp DNA fragment length [16, 119]
- lack of visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) or H&E

The focus upon nucleic material is justified because DNA is directly correlated to adverse host reactions [16, 119] [Keane et al., in preparation], is ubiquitous across tissue and cell types, is readily assayed, and provides a general index for other cell residues within ECM. The first and second criteria are easily quantified using commercially available dsDNA intercalators such as PicoGreen [120], propidium iodide, or bisbenzimide and by gel electrophoresis, respectively. The third criterion is easily evaluated by routine histologic staining or immunofluorescent methods and serves as a qualitative verification of the first two criteria. It should be noted that histologic stains such as H&E or trichrome provide relatively insensitive methods for identifying and qualitatively analyzing DNA within ECM.

A standard for tissue decellularization provides numerous benefits, including: (1) allowing investigators and ECM product manufacturers to evaluate the effectiveness of a protocol when reporting new decellularization techniques or describing products composed of ECM derived from a decellularized tissue; (2) enabling congruous comparison of different ECM products; (3) eliminating variations in cell and host responses to ECM products caused by variations in residual DNA, thereby facilitating interpretation and comparison of *in vitro* and *in vivo* results; and (4) promoting the rapid and effective development of additional clinical applications for ECM products within the field of regenerative medicine and tissue engineering.

As evidence and techniques become available that show an association between cytoplasmic and membrane-associated constituents and adverse host responses, the above criteria should be modified or supplemented to maintain quality and consistency of decellularization methods and *in vivo* outcomes. For example, residual phospholipids are associated with valve and conduit calcification [75, 121, 122] and can be quantified using enzyme-based assays [123]. A systematic investigation of the relationship between phospholipid concentration in decellularized valves and constructive remodeling in valve prostheses would allow the establishment of a criterion for acceptable phospholipid content to avoid adverse remodeling events.

With verification of cellular removal completed, the effects of decellularization on the mechanical and material properties of the remaining ECM scaffold are of interest. Currently, there is no consensus regarding the effects of any individual decellularization agent upon mechanical properties. There are conflicting reports regarding the effects of SDS and Triton X-100 upon the properties of various decellularized tissues. Studies have shown that detergents disrupt collagen in certain tissues, thereby decreasing the mechanical strength of the tissue, while the same detergent may have no apparent effect on the collagen even in fairly similar tissues (e.g., tendon vs. ligament) [34, 36]. Studies have also shown that most detergents cause at least some removal of GAG from the scaffold, an effect which has varying degrees of negative impact on the viscoelastic behavior of the scaffold [124, 125]. Another variable that can affect the mechanical properties of a graft is the duration of exposure to decellularization agents. For example, a protocol established to decellularize tracheal tissue that involved repeated cycles of deoxycholate and DNase showed profound mechanical changes between cycles 18 and 22 that rendered it mechanically inadequate

[113]. The specific mechanical testing that should be performed is dependent on the intended clinical application [14].

7. Conclusion

The preparation of biologic scaffold materials composed of mammalian ECM requires decellularization of source tissues. Such decellularization typically involves exposure to selected non-physiologic chemical and biologic agents such as detergents and enzymes and physical forces that unavoidably cause disruption of the associated ECM. Since the source tissues for biologic scaffolds are typically allogeneic or xenogeneic in origin, maximal decellularization is desirable. The choice of decellularization methods can be rationally selected if a thorough knowledge of the mechanism of disruptive action is contemplated and understood. The host tissue response following *in vivo* implantation of these scaffold materials is dependent upon the efficacy of decellularization and removal of cell remnants, and until further data is available regarding the effects of cell remnants upon the host response it is reasonable to establish standards of decellularization based upon readily determined quantitative criteria of remaining nuclear material within the biologic scaffold. The potential beneficial effects of biologic scaffolds in the field of tissue engineering and regenerative medicine can be realized if optimal methods of decellularization are employed.

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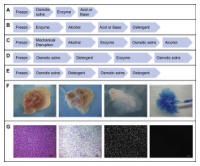

Figure 1.

Example decellularization protocols for (**A**) thin laminates such as pericardium, (**B**) thicker laminates such as dermis, (**C**) fatty, amorphous tissues such as adipose, (**D**) composite tissues or whole simple organs such as trachea, and (**E**) whole vital organs such as liver. Arrow lengths represent relative exposure times for each processing step. Rinse steps for agent removal and sterilization methods are not shown to simplify comparison. (**F**) Representative images of the gross appearance of intact rat liver subjected to decellularization: (left to right) before, during, and after decellularization; decellularized liver perfused with blue dye. (**G**) Representative photomicrographs showing no nuclear staining after whole organ decellularization: (left to right) native rat liver H&E; decellularized liver ECM H&E; native rat liver DAPI; liver-ECM DAPI. Scale bars are 50 μm.

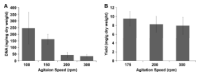

Figure 2.
Quantification of (**A**) residual DNA and (**B**) ECM yield after decellularization of porcine spinal cord. DNA was quantified by PicoGreen assay (Invitrogen, Carlsbad, CA, USA). Yield is per unit mass of lyophilized ECM. Sample size is 3 for all groups.

Table 1

Examples of clinical products composed of decellularized tissues.

Product (Manufacturer)	Tissue Source	Application Focus
AlloDerm® (Lifecell Corp.)	Human dermis	Soft tissue
AlloPatch HD™, FlexHD® (Musculoskeletal Transplant Foundation)	Human dermis	Tendon, breast
NeoForm™ (Mentor Worldwide LLC)	Human dermis	Breast
GraftJacket® (Wright Medical Technology Inc.)	Human dermis	Soft tissue, chronic wounds
Strattice™ (Lifecell Corp.)	Porcine dermis	Soft tissue
Zimmer Collagen Repair Patch™ (Zimmer Inc.)	Porcine dermis	Soft tissue
TissueMend® (Stryker Corp.)	Bovine dermis	Soft tissue
MatriStem®, Acell Vet (Acell Inc.)	Porcine urinary bladder	Soft tissue
Oasis®, Surgisis® (Cook Biotech Inc.)	Porcine small intestine	Soft tissue
Restore™ (DePuy Orthopaedics)	Porcine small intestine	Soft tissue
FortaFlex® (Organogenesis Inc.)	Porcine small intestine	Soft tissue
CorMatrix ECM™ (CorMatrix® Cardiovascular Inc.)	Porcine small intestine	Pericardium, cardiac tissue
Meso BioMatrix™ (Kensey Nash Corp.)	Porcine mesothelium	Soft tissue
IOPatch™ (IOP Inc.)	Human pericardium	Opthalmology
OrthAdapt®, Unite® (Synovis Orthopedic and Woundcare Inc.)	Equine pericardium	Soft tissue, chronic wounds
CopiOs® (Zimmer Inc.)	Bovine pericardium	Dentistry
Lyoplant® (B. Braun Melsungen AG)	Bovine pericardium	Dura mater
Perimount® (Edwards Lifesciences LLC)	Bovine pericardium	Valve replacement
Hancock® II, Mosaic®, Freestyle® (Medtronic Inc.)	Porcine heart valve	Valve replacement
Prima™ Plus (Edwards Lifesciences LLC)	Porcine heart valve	Valve replacement
Epic™, SJM Biocor® (St. Jude Medical Inc.)	Porcine heart valve	Valve replacement

Table 2

Selected agents and techniques for decellularizing tissue.

Agent/Technique	Mode of action	Effects on ECM	References
Chemical Agents			
Acids and bases	Solubilizes cytoplasmic components of cells, disrupts nucleic acids, tend to denature proteins	May damage collagen, GAG, and growth factors	[18, 20–22, 35, 45, 46, 64, 65, 77, 97, 99, 103, 112]
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock, disrupt DNA-protein interactions	Effectively lyses cells, but does not effectively remove cellular residues	[20, 27, 29, 31, 41, 42, 45, 50, 51, 53, 54, 60, 67, 83, 97, 103, 108, 109]
Non-ionic detergents	Disrupt DNA-protein interactions, disrupt lipid-lipid and lipid-protein interactions and to a lesser degree protein-protein interactions		
- Triton X-100		Mixed results with efficacy dependent on tissue, more effective cell removal from thin tissues, some disruption of ultrastructure and removal of GAG, less effective than SDS	[8, 9, 11, 18, 20, 22, 25–29, 31–37, 41–43, 46, 48, 53, 54, 74, 77, 83, 99, 100, 109, 112]
Ionic detergents	Solubilize cell and nucleic membranes, tend to denature proteins		
- Sodium dodecyl sulfate (SDS)		Effectively removes nuclear remnants and cytoplasmic proteins from dense tissues, tends to disrupt ultrastructure, removes GAG and growth factors and damages collagen	[8–11, 22–25, 28–36, 40, 41, 43, 45, 47, 53, 71, 73, 77, 100, 103, 107, 108]
- Sodium deoxycholate		Mixed results with efficacy dependent on tissue thickness, some disruption of ultrastructure and removal of GAG	[18, 25, 26, 32–34, 40–42, 46, 48, 71, 74, 77, 83, 98, 99, 106, 109, 113]
- Triton X-200		More effectively removes cells from thin tissues but with greater disruption of ultrastructure compared to other detergents	[74]
Zwitterionic detergents	Exhibit properties of non-ionic and ionic detergents		
- CHAPS		Effectively removes cells with mild disruption of ultrastructure in thin tissues	[10, 25, 73, 74]
- Sulfobetaine-10 and -16 (SB-10, SB-16)		Effectively removes cells with mild disruption of ultrastructure in thin tissues	[74]
Solvents			
- Alcohols	Cell lysis by dehydration, solubilize and remove lipids	Effectively removes cells from dense dense tissues and inactivates pyrogens, but crosslinks and precipitates proteins, including collagen	[21, 22, 28, 50, 51, 60, 65, 67, 75, 77, 80, 104]
- Acetone	Cell lysis by dehydration, solubilizes and removes lipids	Effectively removes cells from dense dense tissues and inactivates pyrogens, but crosslinks and precipitates proteins, including collagen	[28, 60, 65, 80]
- Tributyl phosphate (TBP)	Forms stable complexes with metals, disrupts protein-protein interactions	Mixed results with efficacy dependent on tissue, dense tissues lost collagen but impact on mechanical properties was minimal	[34–36]

Agent/Technique	Mode of action	Effects on ECM	References
Biologic Agents			
Enzymes			
- Nucleases	Catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue, could invoke an immune response	[10, 20, 23, 26, 29, 31, 33, 42, 44, 47, 50, 53, 67, 77, 83, 98, 99, 103, 106, 113]
- Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM ultrastructure, removes ECM constituents such as collagen, laminin, fibronectin, elastin, and GAG, slower removal of GAG compared to detergents	[18, 21, 22, 24, 26, 27, 29, 32, 33, 37, 38, 40, 41, 46, 48, 54, 77, 83]
- Dispase	Cleaves specific peptides, mainly fibronectin and collagen IV	Prolonged exposure can disrupt ECM ultrastructure, removes ECM components such as fibronectin and collagen IV	[21, 24, 39]
Chelating Agents (EDTA, EGTA)	Chelating agents bind metallic ions, thereby disrupting cell adhesion to ECM	Typically used with enzymatic methods (e.g. trypsin) but can be used with other agents, ineffective when used alone	[18, 21–24, 26, 27, 29, 32, 33, 37–42, 45, 46, 48, 54, 73, 77, 83, 103]
Physical and Miscellaneous Agents			
Temperature (freezing and thawing)	Intracellular ice crystals disrupt cell membrane	Ice crystal formation can disrupt or fracture ECM	[18, 21, 23, 39, 46–48, 50, 89, 91, 92, 112, 117]
Direct application of force	Removal of tissue eliminates cells and force can burst remaining cells	Force can directly damage ECM	[29, 39, 48, 51, 77, 97]
Pressure	Pressure can burst cells and aid in removal of cellular material	Pressure can disrupt ECM	[33, 43, 44, 104]
Electroporation	Pulsed electrical fields disrupt cell membranes	Electrical field oscillation can disrupt ECM	[95, 96]
Techniques to Apply Agents			
Perfusion	Facilitates chemical exposure and removal of cellular material	Pressure associated with perfusion can disrupt ECM	[8–10, 30, 46, 47, 53, 96, 99, 100]
Pressure gradient across tissue	Facilitates chemical exposure and removal of cellular material	Pressure gradient can disrupt ECM	[21, 80, 103]
Supercritical fluid	Pressure can burst cells, supercritical fluid facilitates chemical exposure and removal of cellular material	Pressure necessary for supercritical phase can disrupt ECM	[104]
Agitation	Can lyse cells, but more commonly used to facilitate chemical exposure and removal of cellular material	Aggressive agitation or sonication can disrupt ECM	[11, 18, 22, 23, 25–29, 31, 32, 34–40, 42, 44, 48, 49, 52, 54, 58, 60, 71, 73, 74, 77, 80, 83, 89, 90, 97, 98, 103–113]