Title: Methods of Tissue Decellularization Used for Preparation of Biologic Scaffolds and In-vivo Relevance

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Abstract	

Biologic scaffolds composed of extracellular matrix (ECM) are widely used in both preclinical animal studies and in many clinical applications to repair and reconstruct tissues. Recently, 3-dimensional ECM constructs have been investigated for use in whole organ engineering applications. ECM scaffolds are prepared by decellularization of mammalian tissues and the ECM provides natural biologic cues that facilitate the restoration of site appropriate and functional tissue. Preservation of the native ECM constituents (i.e., three-dimensional ultrastructure and biochemical composition) during the decellularization process would

theoretically result in the ideal scaffold for tissue remodeling. However, all methods of decellularization invariably disrupt the ECM to some degree. Decellularization of tissues and organs for the production of ECM bioscaffolds requires a balance between maintaining native ECM structure and the removal of cellular materials such as DNA, mitochondria, membrane lipids, and cytosolic proteins. These remnant cellular components can elicit an adverse inflammatory response and inhibit constructive remodeling if not adequately removed.

Many variables including cell density, matrix density, thickness, and morphology can affect the extent of tissue and organ decellularization and thus the integrity and physical properties of the resulting ECM scaffold. This review describes currently used decellularization techniques, and the effects of these techniques upon the host response to the material.

1. Introduction

Biologic scaffold materials composed of extracellular matrix (ECM), and derived via decellularization of source mammalian tissues, are widely used for clinical applications that involve repair and reconstruction of musculoskeletal tissues, cardiovascular structures, lower urinary tract, gastrointestinal tract, and central nervous system, among others [1-6]. Such scaffold materials have the potential to promote and facilitate “constructive remodeling” processes that form vascularized, innervated, functional tissue. Mechanisms by which these constructive remodeling events occur include the recruitment and differentiation of stem/progenitor cells [7-10] and modulation of the innate immune response [11-13]. The effector molecules responsible for these processes represent a combination of sequestered cytokines and chemokines within the matrix, and matricryptic peptides generated or exposed during the

process of ECM degradation [8, 14-16]. Thorough decellularization of source tissues to generate the ECM scaffolds is critical for realization of the full potential of these ECM mediated events. Failure to effectively decellularize the tissue results in retained cell remnants, which can act in a manner similar to damage associated molecular pattern molecules (DAMPs) following tissue injury. Ineffective decellularization has been shown to be associated with an intense inflammatory response which can mitigate or completely inhibit a constructive remodeling outcome [17].

All methods of decellularization disrupt the structure and composition of the ECM. The goal of tissue decellularization is thorough removal of cells and cell remnants while retaining the three dimensional ultrastructure and composition of the native ECM to the extent possible. Optimal methods of decellularization vary among tissues and organs due to tissue-specific factors such as cell density, matrix density, and geometric considerations including tissue thickness and shape. Complete removal of all cell remnants is not possible, and decellularization processes inevitably and invariably cause some disruption of matrix architecture, orientation, and surface ligand landscape. The specific cellular elements that elicit adverse and proinflammatory responses are only partially known. One objective of the present manuscript is to provide guidance as to the effects of various decellularization methods upon both the resulting biologic scaffold and the associated host remodeling response and outcome. Decellularization methods described include mechanical, chemical, detergent, and enzymatic techniques, or combinations thereof. Table 1 provides an overview of decellularization processes used for various tissues and organs and the effects of those processes on ECM constituents and the host response.

2. Decellularization agents and techniques

Decellularization protocols have been described for nearly every tissue in the body. The decellularization methods must be tuned to the tissue of interest and ultimately the intended use

of the decellularized tissue. It is not uncommon for vastly different protocols, with varied detergents and delivery methods, to be described for decellularization of the same tissue.

2.1. Physical methods

2.1.1. Freeze-thaw cycles

Numerous studies have used multiple freeze-thaw cycles as a method for cellular disruption. This technique can minimize the amounts of chemical agents required for effective decellularization. It has been shown that freeze-thaw cycles do not significantly alter the mechanical properties of the ECM [18, 19]. Freeze-thaw cycles produce minor disruptions in the tissue ultrastructure. To minimize adverse effects on tissue architecture without impeding cell lysis, the use of a cryoprotectant such as 5% trehalose has been suggested [20].

2.1.2. Agitation and immersion

One of the most commonly used techniques for tissue decellularization is immersion in chemical, detergent, and/or enzymatic solutions with mechanical agitation. Protocols have been described for numerous tissues, including urinary bladder [21, 22], esophagus [23, 24], trachea [25, 26], skeletal muscle [27, 28] heart valves [29, 30], peripheral nerve [31], spinal cord [32, 33], cartilage [34, 35], and dermis [36, 37]. The length of time for each protocol is dependent upon factors such as the degree of agitation and mechanical disruption, the concentration and type of chemical/detergent/enzyme used, and the source tissue thickness and density. Thin tissues such as the urinary bladder or small intestine may be readily decellularized by agitation over a short period of time (e.g., 1-2 hours) of exposure to relatively mild peracetic acid. For thin tissues in particular, the degree of cellular removal is a function of the aggressiveness of agitation [38]. More dense tissues such as dermis and trachea require much longer exposure (e.g., 12-72 hours) to combinations of solutions of enzymes, alcohols, and/or detergents with constant agitation.

A number of protocols have been described to achieve decellularization of esophageal tissue. These protocols have varied combinations of detergents and enzymes to achieve the

same goal of decellularization (Table 2). Ionic detergents are used in each of the published protocols. Bhrany et al [39] used an SDS-based solution while Marzaro et al. [24], Ozeki et al [40], Totonelli et al [41], and Keane et al [23] used a 4% sodium deoxycholate solution as the main decellularization agent. Each of the protocols also required an endonuclease (DNase and/or RNase) to remove residual nucleic acids. It is noteworthy that the measurement of decellularization efficacy used in each study was unique and common criteria were not used. Decellularization efficacy is discussed in more detail in a later section. Undoubtedly, the resultant esophageal ECM for each protocol was distinct and unique. The “optimal” technique will depend upon the intended use of the ECM scaffold and upon factors such as cytocompatibility and intended clinical application.

Immersion and agitation protocols have also been investigated for the decellularization of tracheal tissue. However, compared to the esophagus, effective decellularization of the trachea is challenging due to the presence of hyaline cartilage rings. As a result, the protocols described by Remlinger et al. [26], Jungebluth et al [42], Zang et al , [43], and Haykal et al [44] result in a tracheal matrix that contains residual chondrocyte material within the tracheal rings. These groups propose that decellularization reduces antigenicity (measured by the reduction or loss of MHC antigens) of the scaffold to a degree that allows tissue remodeling to occur. Each of the protocols also results in measureable alterations to the tissue mechanics (Table 3). Whether these effects are critical to the clinical use is debatable as Macchiarini et al [45] adapted the protocol described by Jungebluth et al [42] and showed that implantation of decellularized human tracheal ECM reseeded with autologous airway epithelial and bone marrow cells provides for mechanical properties that allow relatively normal function in the immediate post operative period and absence of evidence for classic tissue rejection. Elliott et al [46] also reported the successful use of a similar construct in a pediatric patient. At two years

post-surgery, the patient had a functioning airway with the construct exhibiting appropriate growth thereby eliminating the need for future surgical interventions [46].

2.1.3. Use of pressure as a decellularization technique

Pressure gradients can be applied to tissue during decellularization to accelerate and improve the efficiency of delivering cell-lysing agents into the tissue and forcing cellular debris out of the tissue. For hollow tissues such as blood vessels and the intestinal tract, luminal perfusion with a pressure gradient can be useful. Montoya et al [47] compared the use of luminal perfusion vs. agitation alone in the decellularization of umbilical veins. The study showed that the pressure-based decellularization process with a flow rate of 50 mL/min with pulse frequency of 2 Hz removed all cells from the tissues as determined by histologic examination. In contrast, traditional rotary agitation (100 RPM) showed retention of whole cells and cellular components. This study also showed that the use of a pressure gradient compared to passive diffusion techniques has only minor effects on the mechanical properties and molecular components of the resulting ECM construct. Bolland et al [48] have used a pressure gradient to decellularize urinary bladder tissue by combining immersion and agitation with cyclic bladder distension. The authors showed that cytoskeletal components of the smooth muscle cells are maintained but the basement membrane components are removed during the decellularization process.

High hydrostatic pressure has been reported as a method that can eliminate or reduce exposure time to harsh detergents in the decellularization of tissues. At a controlled temperature, Funamoto et al [49] decellularized porcine blood vessels by immersion in saline and subsequent exposure to increasing pressure up to 980 MPa. Normal atmospheric pressure was then reestablished by step-wise reduction of pressure. Sasaki et al [50] similarly decellularized porcine cornea using high hydrostatic pressure of over 1,000 MPa. Both studies showed that the use of high pressure led to more effective removal of cellular components when

compared to detergent-based methods under normal atmospheric conditions. DNA content in tissues decellularized by detergent-based methods ranged from 0.3-2.3 µg/mg, but after high hydrostatic pressure decellularization, corneas and blood vessels contained 0.1 µg/mg and undetectable levels of DNA, respectively [49, 50]. High hydrostatic pressure has been shown to reduce GAG content to a greater degree than immersion/agitation methods [51, 52]. It is necessary to avoid the freezing phase when using high hydrostatic pressure in order to maintain native ECM structure. Studies have shown that the pressurization effect damages both collagen and elastin fibers and alters their mechanical properties [53].

2.1.4. Use of supercritical fluids as decellularization agent

A recently described method of tissue decellularization utilizes the unique properties of a supercritical fluid. The low viscosity and high transport characteristics of a supercritical fluid allow for simple and short decellularization protocols. The advantages of supercritical decellularization include the use of an inert substance (e.g. carbon dioxide) for cell removal and minimal alteration of ECM mechanical properties. Further, tissues can be obtained in a dry condition following decellularization and thus eliminate the need for lyophilization; a commonly used technique to facilitate long term storage. Carbon dioxide forms a critical fluid under moderate conditions (at a temperature of 32°C and pressure of 7.4 MPa) and has been shown to effectively remove cells from aortic tissue after only 15 minutes when placed in an ethanol solution [54]. The widespread applicability of supercritical fluids for decellularization of other tissues remains to be determined.

2.2. Chemical and biologic methods

2.2.1. Effect of pH on tissue decellularization

Solutions at an extreme pH can increase the efficacy of cell removal but can impart substantial changes to the ECM constituents. Tomoshi et al [55] have shown that increasing the pH of an 8 mM CHAPS solution during lung decellularization increases the effectiveness of cell and cytoskeletal protein removal. However, highly alkaline CHAPS (pH 12) disrupted ECM

architecture and resulted in a more fibrotic response compared to the lungs (less effectively) decellularized in 8mM CHAPS at pH 8 and pH 10 following subcutaneous implantation. The use of acidic or alkaline solutions during decellularization therefore requires a balanced approach to achieve effective decellularization without severe detriment to the ECM constituents.

2.2.1.1. Alkalines

Alkaline bases denature chromosomal and plasmid DNA. Commonly used alkaline bases include ammonium hydroxide, sodium sulphide, sodium hydroxide, and calcium hydroxide [36, 56-59]. Such compounds have been used in the decellularization of dense tissues such as dermis [36, 56], but alkalines are likely to degrade structural component of the matrix including collagen to some degree. Sheridan et al [58] used 0.5M NaOH and sonication to controllably degrade small collagen fibrils of decellularized pig arterial tissue to increase access of cells during the recellularization process. Mendoza-Novela et al [60] showed that a calcium oxide treatment results in a dramatic reduction in GAG content and altered viscoelastic properties of pericardial tissue. The use of bases in a decellularization procedure can also eliminate growth factors from the resulting ECM and reduce mechanical properties of dermal ECM constructs [61].

2.2.1.2. Acids

Acids are used to dissociate DNA from the ECM by solubilizing cytoplasmic components and disrupting nucleic acids. Acids can also denature ECM proteins including GAGs, collagen, and growth factors. It is important to optimize the dose and exposure time when acids are used for decellularization. Peracetic acid, applied at 0.1% (v/v) in a single wash for 2 hours, and combined with appropriate mechanical methods and rinsing, can thoroughly decellularize thin tissues such as small intestinal submucosa and urinary bladder matrix (UBM). Acids commonly used for decellularization include deoxycholic acid and acetic acid [40, 62]. However, acetic acid has been shown to cause damage and removal of collagens from ECM with a corresponding reduction in construct strength [63].

2.2.2. Non-ionic detergents

Non-ionic detergents are generally considered to be gentle detergents that solubilize proteins while maintaining native protein structure and enzymatic activity. However, success in achieving effective decellularization varies with non-ionic detergents such as X-100 [59, 64-70], likely as a result of differences in source tissue composition and architecture. For example, in attempts to decellularize aortic valve tissue, Grauss et al [71] reported little to no cell removal for 1-5% Triton X-100 treated rat aortic valve, while Liao et al [72] showed effective decellularization of porcine aortic valve using 1% Triton X-100. These results highlight the fact that differences in tissue density and cellularity require a decellularization process that is adapted to the tissue of interest and there is no “one size fits all” decellularization process. While commonly considered to be preferred detergent for decellularization procedures [73] [74], like all decellularization agents, Triton X-100 alters properties of ECM, including the formation of a more open collagen network in anterior cruciate ligament ECM [75] and removal of GAGs in pericardial tissue [76].

2.2.3. Ionic detergents

Ionic detergents are strong detergents that can completely disrupt cell membranes and fully denature proteins. Sodium dodecyl sulfate (SDS), sodium deoxycholate, and Triton X-200 are among the most commonly used ionic decellularization agents because they effectively solubilize cytoplasmic membranes, lipids, and DNA [47, 77-80]. SDS is commonly used to denature and unravel proteins for polyacrylamide gel electrophoresis (SDS-PAGE) and will also disrupt covalent bonds between proteins when used in decellularization procedures. To minimize adverse effects on the remaining matrix constituents of the decellularized tissue, protocols that use SDS would ideally use multiple low-concentration washes with short exposure time, or apply SDS at a decreased temperature [64]. SDS can achieve adequate cell removal and retain collagens, glycoproteins, and fiber orientation for rat thick aortic heart muscular tissue [81], monkey kidney [64], and rat tendon [65]. Other reports suggest SDS

reduces GAGs by 50% [82], and significantly reduces growth factor content [36]. Some denaturation of collagen has been reported, but SDS also targets the cytoskeletal protein vimentin, to completely remove the tethered nuclear envelopes [66]. It should be noted that SDS can be difficult to completely remove from the remaining matrix and can adversely affect cytocompatibility.

2.2.4. Zwitterionic detergents

The net zero electrical charge on the hydrophilic groups of zwitterionic detergents protects the native state of proteins during the decellularization process. Examples of zwitterionic detergents include 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sulfobetaine-10 (SB-10) and SB-16 [77]. SB-10 and SB-16 show greater ECM preservation and better cell removal than non-ionic detergents [79], and CHAPS retains more collagen, GAGs, and elastin [83-85] than SDS ionic detergent while still removing 95% of nuclear material. However, the efficiency of cell removal of zwitterionic detergents has been disputed [86]. The use of detergents for retaining the basement membrane complex of urinary bladder was assessed using various detergents and surprisingly the zwitterionic detergent (8 mM CHAPS) were destructive to the collagen network. The basement membrane integrity of CHAPS treated bladders was disrupted to a similar extent as those treated with 1% SDS, whereas the basement membrane complex was less disrupted when other ionic detergents (4% sodium deoxycholate) and non-ionic detergents (3% Triton X-100) were used [73].

2.2.5. Alcohols

If cell membranes are permeabilized, the polar hydroxyl groups of alcohols can diffuse into the cell where alcohols replace intracellular water, and lyse the cell by dehydration [87, 88]. Ethanol or methanol may be used as a final wash to remove residual nucleic acids from tissue. Moreover, the nonpolar carbon chain of alcohols dissolves nonpolar substances such as lipids

[47]. Ethanol and isopropanol delipidize tissue and are used to remove phospholipids in liver, adipose tissue, and cornea [56, 89-91]. However, Levy et al [92] showed ethanol pretreatment of tissue alters the collagen structure by crosslinking the ECM. Lumpkins et al [80] used a 75% ethanol/-25% acetone mixture to decellularize the porcine temporomandibular joint disc. After 24 hours, the tissue had no visible cell nuclei, but was three times as stiff as the native tissue.

2.2.6. Tri (n-butyl) phosphate

Tri(n-butyl)phosphate (TnBP) is an organic solvent that disrupts protein-protein interactions. TnBP was first used to inactivate lipid-enveloped viruses without damaging blood proteins [93]. Recently reported decellularization protocols show that TnBP can match SDS in cell removal ability for tendon and ligament tissues [65-67, 94]. Moreover, collagen matrix, GAGs, and biomechanical properties (including tensile strength and elasticity) were not significantly altered. Although residual cytoskeletal proteins (e.g., vimentin) were not completely removed, TnBP is a viable option that warrants further study for other tissue types.

2.2.7. Enzymatic agents

Enzymatic agents include proteases (e.g., trypsin, dispases), esterases (phospholipase A2), and nucleases (e.g., DNase, RNase), and are advantageous because of their specificity for biologic substrate. Trypsin selectively cleaves cell adherent proteins on the carboxyl side of the amino acids arginine or lysine to detach cells from the tissue surface. Trypsin has been shown to be effective for decellularizing adjuvant, but long exposure times damage the collagen matrix [56, 68, 90]. Meyer et al [68] showed 0.5% trypsin causes extensive damage to aortic valve ECM following 48h exposure. Brown et al [90] used 0.02% trypsin for 1h with negligible change in tissue architecture for porcine adipose decellularization. Prasertsung et al [56] suggest 1% trypsin not be used longer than 24h to prevent collagen damage in porcine dermis.

Dispase II is a bacterial protease that selectively cleaves fibronectin and collagen IV in the basement membrane, and is used to separate epithelial sheets from the substratum [95, 96]. Dispase II is used in the initial steps of decellularization for many tissues, including porcine skin and corneas, but requires subsequent treatment with other agents to achieve adequate decellularization [56, 97]. The decellularization agents that are used in conjunction with Dispase depend upon tissue specific factors. For example, porcine corneas can be decellularized with 4 mg/ml Dispase II for 45 min followed exposure to hypertonic (12h in 1.5 M NaCl at 200rpm) or, more effectively, ionic detergent (12h in 0.1% SDS at 200rpm) [96]. Dispase II (0.24 mg/ml for 3h) was also used to decellularize porcine skin but required pretreatment in strongly alkaline 20% sodium sulfide, hypertonic 1M NaCl, 85% glycerol, and 2/1 (v/v) chloroform/methanol for hair, epidermis, and fat removal [56].

DNase and RNase are endonucleases that hydrolyze deoxyribonucleotide and ribonucleotide chains, respectively. Typically, these enzymatic agents are added to detergent treatments if effective decellularization is not achieved with detergents alone, to help remove residual DNA [69, 70, 98, 99]. When a 24-hour SDS treatment of rat aortic valves still had remaining nuclei, Grauss et al [98] added an additional 1 hour step of DNase (20 ug/mL) and RNase (0.2 mg/mL) to produce a completely acellular material based on hematoxylin and eosin staining. Likewise, phospholipase A2 is often added to detergent treatments. Phospholipase A2 can hydrolyze the phospholipid component of tissues such as cornea, which preserves the collagen ultrastructure and proteoglycans, but can result in reduction in GAGs [100, 101].

A special consideration when using enzymatic methods for decellularization is activity inhibition by natural protease inhibitors released from lysed cells. Prasertsung et al [56] found the activity of trypsin to decrease by 60% after 12 hours, and cell removal percentage significantly increased with more frequent enzyme refreshments. The addition of certain protease inhibitors (e.g., phenylmethylsulfonyl fluoride (PMSF), aprotonin, leupeptin) may partially ameliorate this limitation [38].

2.2.8. Chelators and toxins

Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetracetic acid (EGTA) are commonly used chelating agents that bind divalent metal cations at cell-adhesion sites of the ECM. This sequestering causes cell dissociation from the ECM. EDTA and EGTA can be used with trypsin [70, 87, 102, 103] or detergents [85] to ensure complete removal of cell nuclei while retaining the major constituents of the ECM. However, this procedure may leave some cellular remnants [70, 87]. Because the successful applications of EDTA and EGTA are typically used in combination with other decellularization agents, the direct effect of these chemicals on ECM has not been determined.

Latrunculin B, a marine toxin, offers an alternative to detergent methods as a powerful disruptor of the actin cytoskeleton. When latrunculin B was applied to skeletal muscle with hypertonic solutions and DNase, no intact nuclei remained in the tissue. GAGs were reduced by 40%, but collagen content and fibrillar ultrastructure remained largely unchanged [28].

2.3. Perfusion decellularization of whole organ constructs

Vascular perfusion is a technique that is intended to preserve the three-dimensional architecture of an organ while eliminating the parenchymal cell population. Since all cells are in close proximity to a vascular network, perfusion through the vasculature is an efficient method for delivery of decellularizing agents. For obvious reasons however, effects of these agents upon the components of the vascular basement membrane must be considered when perfusion techniques are used.

Decellularization of the heart via vascular perfusion has been used to generate a three-dimensional scaffold that preserves the geometry of the native organ. By cannulating the aorta, Ott et al [104] have shown that retrograde perfusion of decellularization agents, including 1% SDS and 1% Triton X-100, is an effective method for removal of cell material from a rat heart with the vascular network remaining intact throughout the process. Recellularization with

cardiomyocytes showed that the ECM was compatible with cell growth and the recellularized tissue had a small degree of contractility. Wainwright et al [105] have shown that the vascular perfusion can be scaled up to larger organs using a porcine heart. While still using retrograde coronary perfusion, the decellularization of the porcine heart required the successive perfusion of trypsin and detergents, and a progressive increase of perfusion pressure.

Perfusion decellularization, alone or in combination with tracheal flushing, has been applied to lung tissue by a number of investigators [106-110]. These groups have demonstrated that perfusion decellularization is effective both in small animal models as well as large animal and human lungs. However, the methodology has varied significantly among studies as outlined in Table 4. Several groups began decellularization procedures with freeze/thaw cycles. Cortiella et al demonstrated that freezing was necessary to completely remove nuclei and DNA [111]. Perfusion with detergent solutions is common in lung decellularization procedures, however the type of detergent and length of exposure time vary dramatically. Ott et al [112] used vascular perfusion of 1% SDS at physiologic pressure to decellularize rat lungs in 2 hours. Another method published by Peterson et al [85] utilized the zwitterionic detergent, CHAPS, and perfused the airway compartment via the trachea rather than the vasculature. Price et al [113] perfused both the airway and vascular compartments but did not use continuous perfusion techniques. Instead, the lungs were repeatedly incubated under static conditions in solutions of Triton X-100, sodium deoxycholate, DNase, and bleach. Cortiella et al [114] perfused lungs via the airway compartment with 1% SDS while the tissue was circulated in a bioreactor system for approximately five weeks. Nichols et al perfused lungs with 1% SDS for five days following a freeze/thaw cycle [115]. All five groups showed that the lung ECM could be repopulated with cells. Cortiella et al [114] used a homogenous mouse embryonic stem cell population for reseeded and showed that the ECM was capable of promoting site-appropriate differentiation without the addition of further growth factors. After reseeded the decellularized lungs with cells,

orthotopic transplantation was performed by Ott et al [112] and Peterson et al [85]. Vascular leakage was evident upon implantation in recipient rats in both studies, and these lung constructs did not function for more than a few hours. Despite significant differences in decellularization techniques, all groups have shown removal of nuclei and reduction in DNA content as well as preservation of lung microstructure together with the major components of the ECM such as collagen, laminin, elastin, and GAGs. Further studies are necessary, however, to compare the effects of each protocol on the ECM composition and, more importantly, on the suitability for recellularization and the host response following implantation.

The liver is another example of an organ that has been decellularized by perfusion. Antegrade perfusion via the portal vein has resulted in a decellularized tissue with maintained vascular networks. Table 5 highlights the techniques and agents that have been used for perfusion-based liver decellularization along with considerations for the use of each agent. Uygun et al [82] used increasing concentrations of SDS (0.1-1%) followed by 1% Triton X-100 to achieve decellularization of rat liver whereas the protocol published by Shupe et al [116] used increasing concentrations of Triton X-100 followed by 0.1% SDS. Shupe et al also demonstrated that perfusion with Triton X-100 alone was not sufficient for decellularization and SDS was required for complete nuclear removal. The protocol used by Uygun et al was optimized by DeKock et al [117] who decellularized rat liver with 1% Triton X-100 followed by 1% SDS in 1 hour. Soto-Gutierrez et al [118] published an effective decellularization protocol that, unlike those previously reported, excludes the use of harsh detergents, such as SDS. Using this method they report that growth factor content retained within the matrix was 30-50% of the native tissue. Kajbafzadeh et al [119] compared the protocol developed by Uygun et al to 4 other protocols for the decellularization of sheep liver. The authors determined that increasing concentrations of SDS followed by Triton X-100 achieve effective decellularization, however, the mechanical properties of the resulting ECM are poor compared to protocols that do not use

SDS. They suggest that the best protocol to completely decellularize the liver while maintaining mechanical properties is perfusion with 0.05% ammonium hydroxide followed by 0.5% Triton X-100. Following decellularization, similar to other tissues, the liver is a translucent and white acellular matrix. The vascular network is maintained and can be visualized by perfusion of a colored dye. Multiple studies have shown that integral components of the basement membrane, collagen IV and laminin, remain in the decellularized liver while intact cells are removed [117, 120-123]. Following decellularization, the matrix has been recellularized, via portal vein perfusion, with rat liver progenitor cells [121], human fetal liver cells [120], and human liver progenitor cells [124]. For up to 8 hours following heterotopic transplantation in a rat, the hepatocytes maintained functional synthesis of albumin and lactate dehydrogenase [121].

Intact renal constructs have been prepared by perfusion decellularization. Ross et al [125] developed a protocol for decellularization of mouse kidney using solutions of Triton X-100, DNase, and SDS. The ECM was supportive of tissue-specific differentiation of embryonic stem cells. Song et al [126] used SDS to perfuse cadaveric rat kidneys at a pressure of 40 mm Hg. Renal ECM components were maintained including the glomerular and tubular basement membrane, which serve essential roles in renal filtration and reabsorption. The scaffolds were recellularized with endothelial cells and a heterogeneous neonatal kidney cell population. In vitro, the recellularized construct produced urine and cleared creatine nearly 20% as well as cadaveric kidney, with urine production also demonstrated following orthotopic transplantation. For more information on perfusion decellularization of whole organ constructs refer to [127] and [128].

2.4. Host response to decellularized ECM bioscaffolds

Decellularized small intestine, urinary bladder, dermis, blood vessels, muscle, cornea, and others have been used as effective substrates to facilitate replacement of functional tissue.

This replacement of the ECM graft with functional host tissue is considered a constructive remodeling response and has been observed for the repair of multiple tissues and with different sources of ECM [129-133]. However, despite this information, no systematic analysis has been performed to assess the effects of decellularization agents on host response or on the functional outcomes of remodeling. A greater understanding of how each decellularization agent affects the host response to ECM materials will guide the optimization of decellularization protocols.

2.4.1. Consequences of ineffective decellularization

As stated previously, a careful balance must be made between the removal of cellular materials and damage to the resulting matrix. Decellularization agents that are very effective at removing cellular remnants can also cause damage to the collagen structure and remove growth factors and other critical ECM components. However, insufficient decellularization can induce a strong inflammatory response. The results of ineffective decellularization on the host response have been examined in the context of SIS-ECM. Keane et al [134] compared SIS-ECM produced by PBS washes alone, 1-hour PAA treatment, and 2-hour PAA treatment. Results showed that a greater degree of decellularization, as measured by reduced DNA content, is associated with a more favorable macroscopic host tissue response, less swelling of surrounding tissues and the absence of seroma formation. However, DNA content alone is not the sole determinant of the host response. Additional studies will be necessary to understand how residual DNA and other cellular remnants such as mitochondrial elements, membrane lipids, and other cytoplasmic constituents affect host immune response and scaffold remodeling.

2.4.2. Effects of residual detergent

It has been suggested that the presence of residual SDS within ECM biomaterials has severe cellular toxicity and may be responsible for discouraging cellular ingrowth [70, 135]. Rieder et al [70] demonstrated that scaffolds decellularized using SDS were unable to be recellularized due to the toxic effects of the ionic detergent, whereas, scaffolds prepared with

0.25% tert-octylphenyl-polyoxyethylen in combination with sodium-deoxycholate enabled host recellularization.

Cebotari et al [18] examined detergent levels remaining in the wash solution following decellularization with SDS or a combination of SDS and SDC using solid phase extraction and high performance liquid chromatography (HPLC). They determined that a detergent concentration of <50 mg/L in the wash solution did not influence the receptiveness of the matrix to reseeding with endothelial cells. The authors also showed that the combination of SDS and SDC was more easily washed out from the tissue.

Another method for quantification of residual SDS takes advantage of the fact that SDS and methylene blue produce blue compounds under acidic conditions. This colorimetric assay allows for quantification of residual SDS in the ECM scaffold. The lower cytotoxic threshold for SDS is approximately 10 ug/mg dry weight. Wang et al showed that following SDS perfusion with BSA or PBS reduces the residual SDS to well below this level and washing with Triton X-100 results in almost complete removal of residual SDS.

Sansoto et al [136] assessed the effects of the SDS compared to high hydrostatic pressure on the decellularization of uterine tissue and their subsequent effects on tissue regeneration *in vivo*. Their methods achieved effective decellularization as shown by H&E staining and quantification of residual DNA. Analysis of the *in vivo* host response showed that cells migrated into the area surrounding the grafts but only the sample that was decellularized using high hydrostatic pressure stimulated cell migration into the graft. Cells did not migrate into decellularized grafts generated using SDS treatment.

Decellularization methods can clearly affect the host response to biologic scaffolds. However, it remains possible that these effects are due to differences in structure and composition as a result of the decellularization process rather than due to residual detergent in

the material after processing with SDS. Gratzner et al [137] found that the level of repopulation and viability of cells were the same regardless of washing technique and resulting level of residual SDS in the tissue. These data suggest that alterations in tissue matrix biochemistry or structure from SDS treatment are responsible for low cell repopulation observed in SDS decellularized anterior cruciate ligament. Additional studies are necessary to determine if this holds true for other tissues.

3. Establishing metrics for effective decellularization

While it is unlikely that any decellularization protocol will completely remove all cell remnants, cell components that do remain (e.g., DNA, phospholipids) can be quantitatively assayed. Until recently, no quantitative metric has been suggested to evaluate the efficacy of a decellularization protocol [38]. It is important to note that for most studies reviewed in the present manuscript, no objective criteria were used to assess degree of decellularization. Only one study to date has related quantitative degree of decellularization to host remodeling outcome [17].

Commercially available scaffolds are regulated by the FDA and therefore are subject to sterility guidelines (see section 5) and endotoxin amounts. Endotoxin is a complex lipopolysaccharide (LPS) that is a major part of the gram-negative bacteria cell wall. Endotoxins are especially abundant in the gut, the tissue source for small intestine submucosa. The FDA standard for a biologic scaffold eluate is 0.5 EU/mL, and 0.06 EU/mL for cerebrospinal devices. While the exact level of endotoxin concentration necessary to elicit an adverse reaction is unknown, dermal matrices spiked with 20 times the FDA limit had similar immune responses to devices below the FDA standard suggesting that the current endotoxin standard may fall well below the levels required to elicit an acute proinflammatory response [138]. While strict guidelines for some metrics do exist, the FDA does not yet have guidance related to remnant cellular content in commercially available decellularized tissues.

The definition of effective decellularization varies greatly across published reports. Decellularization has been defined as a lack of positive staining for intact nuclei and cellular antigens (e.g., MHC-1). Other definitions include a “significant” reduction in dsDNA content compared to native tissue as criteria for decellularization. The increased clinical use of ECM-based scaffolds is prompting the need for established decellularization guidelines. A recent report recommended tissue decellularization criteria that utilize nucleic acid content as a basis. The three-part criteria include: 1) No visible nuclei upon histologic evaluation via hematoxylin and eosin and DAPI stains, 2) The remaining double stranded DNA (dsDNA) content should not exceed 200 base pair in length, and 3) the amount of dsDNA should not exceed 50ng per mg of dry weight of the material [38]. These criteria were shown to make a difference in the host response in a separate study [17].

The criteria referenced above may be too stringent, sufficient, or too liberal; and these criteria may not be appropriate for all source tissues from which such bioscaffolds are prepared. It is unknown but likely that the threshold level of cellular content necessary to elicit a proinflammatory host response will vary depending on anatomic location. In addition to nucleic acid content, it may be necessary to assay cellular components such as remnant mitochondria. Mitochondria are evolutionarily derived from bacteria, bear damage associated molecular patterns (DAMPs) and may elicit a proinflammatory host response if present above some threshold level [139]. As a greater understanding of the association between specific cellular components and the host response is achieved, the criteria may need to be revised or supplemented.

4. Terminal sterilization of decellularized tissues

4.1. Regulatory requirements for sterilization

There are many examples of biologic scaffold products composed of decellularized tissues. These include dermis, small intestine, urinary bladder, mesothelium, pericardium, and heart

valve. These products are used for repair applications in soft tissue, tendon, chronic wounds, breast reconstruction, ophthalmology, dentistry, valve replacement and others. Biologic scaffolds from decellularized xenogenic source tissue are typically regulated by the Food and Drug Administration (FDA) as medical devices. This classification requires that the methods used to terminally sterilize ECM products are adherent to directed guidelines regarding bacterial load (e.g., ISO/DIS 1135-1, ISO/DIS 1137-1). Incubation with peracetic acid has been used as a decellularization agent for biologic scaffolds and has also been shown to be an effective sterilization method for polymeric scaffolds [140]. However, incubation with acid may not provide sufficient penetration of ECM bioscaffolds to achieve satisfactory sterilization. Terminal sterilization methods such as ethylene oxide exposure, gamma irradiation, and electron beam irradiation achieve effective sterilization but are known to alter ECM ultrastructure and mechanical properties. The effect of terminal sterilization on ECM structure is a particular concern for hydrogel forms of ECM because sterilization methods can have significant effects on the rheologic properties of the hydrogel. While all biologic scaffolds must pass sterility testing prior to their approval for clinical use, it will be important for regulatory agencies to consider the effects of terminal sterilization on the structure and biologic activities of these materials.

4.2. Terminal sterilization methods

4.2.1. Ionizing radiation

The effects of a wide range of gamma irradiation exposure on ECM have been investigated. At low dosages (<15 kGy), gamma irradiation increases the strength and modulus of the ECM scaffold but at dosages higher than 15 kGy mechanical properties decrease in a dose-dependent manner [141]. Structural changes to the ECM can also be observed following gamma irradiation [142]. ECM degradation during irradiation is at least partially attributed to the cross-linking of key structural components such as collagen that occurs even at relatively low

doses of 5 kGy [143]. As irradiation dose increases, collagen chain scission continues to increase resulting in dose-dependent alterations in mechanical properties [144]. Gamma irradiation causes residual lipids to become cytotoxic and accelerates enzymatic degradation [145]. Gamma irradiation at a dose of ~13 kGy has also been shown to have a negative effect on the ability of cells to attach to the ECM *in vitro* [146].

4.2.2. Ethylene oxide exposure

There are conflicting results on the effect of ethylene oxide exposure on ECM. It has been shown that ethylene oxide sterilization does not affect cell attachment to ECM or the stimulation of growth factor secretion by fibroblasts *in vitro* [147]. Ethylene oxide treatment can alkylate proteins, leaving them inactive and undetectable [148]. Ethylene oxide may substantially change the mechanical properties of ECM [149] or leave them unaltered [150, 151]. Ethylene oxide treatment can leave behind harmful residues and has recently been classified as a mutagen and carcinogen. These residues have been shown to cause undesirable host immune responses that impair proper remodeling and function of biologic scaffolds after implantation [152]. For these reasons, the use of ethylene oxide for terminal sterilization of biologic scaffolds should be considered carefully.

4.2.3. Supercritical carbon dioxide

Supercritical carbon dioxide (SC-CO₂) has become a mainstay for sterilization of food and pharmaceuticals [153]. SC-CO₂ has been used for tissue decellularization [154] and more recently SC-CO₂ has been investigated as a method for sterilization of biologic scaffold materials. SC-CO₂ is compatible with biological materials and leaves no toxic residues within the treated material [153, 155, 156]. SC-CO₂ sterilization of porcine dermal ECM has been examined and causes only minor changes in mechanical properties when compared to other

sterilization methods [156]. Further studies will be necessary to determine the efficacy of SC-CO₂ as a sterilization method for ECM bioscaffolds derived from other tissues.

5. Conclusion

The potential benefits provided by biologic scaffold materials for replacement and reconstruction of damaged or missing tissues and organs are noteworthy. However, the effects of such materials upon stem/progenitor cell recruitment and the innate immune response are critically dependent upon the methods used to manufacture these materials; decellularization and terminal sterilization being among the most important. With respect to decellularization, one size does not fit all when considering different source tissues and intended clinical applications. The methods described herein, the effects of these methods upon matrix structure/composition, and the subsequent host response are intended as guidelines in the design and manufacture of effective ECM based bioscaffolds.

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7. References

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Table 1. Decellularization techniques for various tissues

Tissue/Organ Type	Decellularization Technique	Effects on ECM and Host Response	References
Laminate			
Pericardium Urinary Bladder Intestine Amnion Dermis	Freeze/thaw, mechanical removal of undesirable layers, brief exposure to acids/bases, rinse. Thicker laminates may require longer exposure to remove cellular debris.	Cellular remnants can elicit an adverse immune response. Microstructure and ultrastructure will be altered. Increased exposure times may damage collagen, reduce GAG and growth factor content.	[17-20]
Amorphous Organs			
Adipose Brain	Freeze/thaw, mechanical disruption, alcohol/solvent treatment, exposure to acid/base, rinse.	Alcohols required for lipid solubilization crosslink collagen and increase tissue stiffness.	[21-23]
Composite Tissues/Organ Fragments			
Trachea Nerve Testes Uterus	Freeze/thaw, osmotic solutions, detergent treatment, enzymatic treatment, rinse.	Detergents can reduce growth factor content, denature and loosen collagen network. Residual detergent may effect cell migration and remodeling.	[24-29]
Whole Vital Organs			
Liver Lung Kidney Pancreas	Freeze/thaw, perfusion of decellularization solutions.	Pressure associated with perfusion can disrupt ECM. Vascular basement membranes will be affected by solutions used for decellularization.	[30-35]

Table 2. Esophageal tissue decellularization protocols

Species	Rats (Fischer 344)	Pig (newborn)	Rats (Wistar)	Pig	Pig
Form	Intact full thickness	Intact full thickness	Intact full thickness	Intact full thickness esophagus	Mucosa and submucosa
Method of delivery for decellularization agent	Immersion and agitation rate = N/A	Immersion	Immersion and agitation rate = N/A	Luminal perfusion, rate = 0.6mL/min	Immersion and agitation on rotating shaker rate = 300 rpm
Summary of decellularization protocol	48h 10mM Tris 48h 1% Triton X-100 6h 400 U/ml DNase-I/ .125 mg/ml RNase-A 48h .5% SDS	5 cycles of: 72h distilled water, 4h 4% deoxycholic acid, 3h 2,000 kU DNase-I	24h 4% deoxycholic acid, 12h .2 mg/ml DNase-1	3 cycles of: 24h deionized water, 4h 4% deoxycholic acid, 3h 2,000 kU DNase-I	1h 1% trypsin/ .05% EDTA, 30min 1M sucrose, 48h 3% Triton X-100, 4h 10% deoxycholic acid 2h .1% peracetic acid/4% Ethanol 2h 100 U/ml Dnase-I
Evaluation of decellularization efficacy	<ul style="list-style-type: none"> No nuclei present on H&E-stained section 	<ul style="list-style-type: none"> No nuclei present on H&E-stained section Lack of MHC antigens 	<ul style="list-style-type: none"> No nuclei present on H&E-stained section DNA concentration: 140 ng/mg 	<ul style="list-style-type: none"> No nuclei present on H&E-stained section DNA concentration: 500 ng/mg 	<ul style="list-style-type: none"> No nuclei present on H&E- and DAPI-stained sections DNA concentration: 50 ng/mg
Reference	Bhrany, 2006	Marzaro, 2006	Ozeki, 2006	Totellini, 2012	Keane, 2012

Table 3. Effects of tracheal decellularization mechanical properties

Tissue source species	pig	pig	rat
Delivery method of decellularization agent	immersion	Immersion and agitation on rotating shaker at 300 RPM	Immersion
Summary of protocol	17 cycles of: 48h Deionized water 4h 4% deoxycholic acid 4h 2,000 kU DNase-I	48h 3% Triton X-100 2h .1% peracetic acid/4% Ethanol	2 cycles of: 4h 4% deoxycholic acid 3h 50kU/ml DNase-1 41h Deionized water
Measured effects on mechanical properties	<ul style="list-style-type: none"> Maintained strain ability similar to native trachea Decrease in tracheal volume compared to native trachea 	<ul style="list-style-type: none"> Increased tissue compliance compared to native trachea 	<ul style="list-style-type: none"> Maintained strain ability similar to native trachea Decreased elastic modulus compared to native trachea
References	Jungebluth, 2009 and Haykal, 2012	Remlinger, 2010	Zang, 2012

Table 4. Lung decellularization: many methods achieve effective cellular removal.

Publication	Source Animal	Procedure
Cortiella et al 2010	Rat	Freeze/thaw→1% SDS→PBS→DNase/RNase→PBS
Price et al 2010	Mouse	dH ₂ O→Triton X-100→SDC→Hypertonic Saline→DNase→PBS
Bonvillain et al 2012	Non-human primate	
Ott et al 2010	Rat	0.1%SDS→dH ₂ O→1% Triton X-100→PBS
Peterson et al 2010	Rat	PBS→8mM CHAPS/1M NaCl/25mM EDTA
Nagao et al 2013	Rat	1% SDS→dH ₂ O→1% Triton X-100→PBS SB-10→Triton X-200/SB-16→Buffer→Triton X-200/SB16→Rinse
Nichols et al 2013	Pig, Human	Freeze/thaw→1% SDS→dH ₂ O→1% Triton X-100→PBS
Melo et al 2014	Rat	Freeze/thaw→1% SDS→PBS Freeze/thaw→8mM CHAPS→PBS
Gilpin et al 2014	Rat, Pig, Human	PBS→SDS/Triton X-100→dH ₂ O→PBS PBS→SDC/Triton X-100→dH ₂ O→PBS PBS→CHAPS→dH ₂ O→PBS

Table 5. Methods and Results of Liver Decellularization

Method/Agent	Result	Considerations
Freeze/thaw [122, 123, 135, 157]	<ul style="list-style-type: none">• Not required for complete decellularization	<ul style="list-style-type: none">• May be useful for tissue storage• Effects of freeze/thaw vs. fresh have not been examined
SDS [117, 119-121, 123, 124, 135, 158, 159]	<ul style="list-style-type: none">• Improves removal of cytoplasmic and nuclear material• Effective for cellular removal when used alone	<ul style="list-style-type: none">• Concentration and exposure time vary widely among protocols and for different source animals• More detrimental to mechanical properties than other detergents• Residual SDS is harmful
Triton X-100 [117, 119, 121-124, 135, 157-159]	<ul style="list-style-type: none">• Only effective for cellular removal when used in conjunction with other agents	<ul style="list-style-type: none">• Concentration and exposure time vary widely among protocols• Effective for removal of residual SDS
Sodium Deoxycholate [119, 135]	<ul style="list-style-type: none">• Not sufficient for cellular removal when used without additional decellularization agents	<ul style="list-style-type: none">• Ionic detergents damage matrix components
Trypsin [119, 122]	<ul style="list-style-type: none">• Only effective for cellular removal when used in conjunction with other agents	<ul style="list-style-type: none">• May be used to reduce amount or time of exposure to harsh detergents• Can damage collagen structure
Peracetic Acid [119, 122]	<ul style="list-style-type: none">• Not effective for cellular removal when used alone	<ul style="list-style-type: none">• Can denature ECM proteins, damaging collagen and reducing growth factor content
Ethanol [122]	<ul style="list-style-type: none">• Not effective for cellular removal when used alone	<ul style="list-style-type: none">• Can alter ECM structure by crosslinking collagen

<p>Ammonium Hydroxide [119, 157]</p>	<ul style="list-style-type: none"> • Effective for cellular removal when used in conjunction with Triton X-100 	<ul style="list-style-type: none"> • The combination of ammonium hydroxide and Triton X-100 is effective for decellularization and maintains mechanical properties better than SDS/Triton X-100
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