Automated Decellularization of Intact, Human-Sized Lungs for Tissue Engineering

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We developed an automated system that can be used to decellularize whole human-sized organs and have shown lung as an example. Lungs from 20 to 30 kg pigs were excised en bloc with the trachea and decellularized with our established protocol of deionized water, detergents, sodium chloride, and porcine pancreatic DNase. A software program was written to control a valve manifold assembly that we built for selection and timing of decellularization fluid perfusion through the airway and the vasculature. This system was interfaced with a prototypic bioreactor chamber that was connected to another program, from a commercial source, which controlled the volume and flow pressure of fluids. Lung matrix that was decellularized by the automated method was compared to a manual method previously used by us and others. Automation resulted in more consistent acellular matrix preparations as demonstrated by measuring levels of DNA, hydroxyproline (collagen), elastin, laminin, and glycosaminoglycans. It also proved highly beneficial in saving time as the decellularization procedure was reduced from days down to just 24 h. Developing a rapid, controllable, automated system for production of reproducible matrices in a closed system is a major step forward in whole-organ tissue engineering.

Introduction

TISSUE ENGINEERING using decellularized whole organs as scaffolds has become an area of intense investigation by many groups (reviewed in Refs.^{1–4}). Since the first report of using perfusion for decellularization and reseeded rat heart,⁵ similar perfusion strategies have been reported for lung,^{6–10} liver,¹¹ kidney,¹² and intestine.¹³ This approach is also being used to study pathologic extracellular matrix (ECM).^{14,15} The purpose of using decellularized whole organs is to create autologous organs for transplant by reseeded with autologous cells, thus avoiding chronic rejection (due to nonhistocompatibility), the requirement for lifelong immunosuppression and high mortality.^{16–19} Indeed, this concept has successfully been applied for the trachea and has reached clinical utility.²⁰

There is significant effort by several investigators^{6–10} to tissue-engineer autologous lungs to be comprised of induced pluripotent stem cell (iPSC)-derived endoderm, MSCs, and endothelial cells, all seeded onto decellularized whole lungs that would be sourced from human cadavers (whose lungs fail requirements for transplant) or from pigs (whose lung

structure is similar to humans). In fact, lung progenitors have been successfully derived from patient-specific iPSCs²¹ and human iPS cells seeded in decellularized rodent lungs can survive and give rise to cells expressing lung markers.²² The decellularization protocol used does not appear to affect reseeded efficiency of acellular lung tissue with MSCs²³ but whether it will affect the seeding of other cell types is not yet known.

Several decellularization protocols have been studied and compared (nicely reviewed in Refs.^{2,24}). Some reports suggest that the use of a Triton/sodium deoxycholate detergent-based protocol results in better preservation of ECM components^{25,26} while others have found that use of sodium dodecyl sulfate may result in scaffolds with less immunogenicity.¹⁰ Yet others have found that some forms of inflammation into decellularized tissue scaffolds may be beneficial for appropriate tissue remodeling.²⁷

A major challenge with preparation of decellularized organs is to achieve consistency of the final product in terms of composition, sterility, and mechanical properties. Variability and the potential for breeches in sterility are unavoidably

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introduced in a manual decellularization procedure (i.e., fluids perfused manually by pipette, and part gravity, with the normal elastic recoil of the lungs relied on to expel the fluids from the airways); This somewhat impractical procedure results in unnecessary prolongation of steps to accommodate worker hours. Although manual decellularization of small rodent lungs is usually sufficiently effective, several recent articles entailed the use of manually decellularized lungs from larger animals such as humans and nonhuman primates.^{9,14} A user-friendly, automated system would be of benefit to the field by standardizing the process resulting in consistent scaffold material. Our goal was to automate the process by incorporating a valve assembly controlled by a user-friendly software program to enable seamless transitions from one decellularization reagent to another. Our automated decellularization process also allows for independent control of airway and vascular perfusion pressures. Optimization of airway and vascular pressures allow for full perfusion of the airway system, while maintaining a high enough vascular perfusion pressure to prevent collapse during airway perfusion. Using pig lung as an example, our findings show that automated

decellularization results in more consistent product and a reduction in time while freeing up personnel hands-on effort.

Materials and Methods

Decellularization setup

General automated decellularization system design. The automated decellularization system (ADS; Fig. 1 and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec) consists of a series of fluid reservoirs connected to a valve manifold assembly operated by a program written specifically to control the valves., Peristaltic pumps are controlled by commercial software (Harvard Apparatus, Holliston, MA). All units are connected to the decellularization chamber with tubing.

Valve control program. The software design can be broken up into two components. The first component is the graphic user interface (GUI) and the second component is the communication protocol. The GUI allows the user to create a “step.” A step directs the communications protocol

FIG. 1. Decellularization system setup. The entirety of the decellularization system setup shows the different carboys holding the solutions needed for decellularization (water, Triton X, sodium deoxycholate, NaCl, DNase, and phosphate-buffered saline [PBS]), computer control of the valve system, pumps, and the decellularization chamber that has multiple inputs and outputs for tubing, air bubble traps, and pressure transducers.

to which valve (fluid) of the ADS to use and the duration of use. The GUI allows the user to create as many steps as needed for the application (Supplementary Fig. S1A).

The software protocol is a one way communication between the “master” and the “slave” (conventional terms). The type of solenoid valves used requires a 50 ms burst of power to either open or close the valve. When the user presses run, the “master” sends a control signal to open the appropriate valve. The “slave” is then responsible for shutting off the power to the valve after 50 ms. After the program completes a user step, it sends a shutoff command to close the valve. It is the “slave’s” job to interpret what valve is open and to allow a 50 ms burst of power to shut the valve. The software is open sourced to academic investigators and is available at <http://z.umn.edu/ValveControlDecell>.

Valve manifold assembly (Supplementary Fig. S1D). Six solenoid valves (two-way, NC, 1/8" pipe size; Alcon ECI, San Dimas, CA) were mounted on a stainless steel plate. The valves (coefficient of variation [CV] flow factor 0.08, psi differential 5–150) were chosen to accommodate physiological pressures and flow rates (airway flow rates of 25 mL/min for 10 min inflation and 12.5 mL/min for a 20 min deflation; pulmonary vascular pressure-regulated flow rate of 15 mmHg). The fluid flows through the valves and enters a system of stainless steel tubes connected by brass compression fittings to exit through a brass barbed hose connector that leads to the pumps. The valve assembly is interfaced with the control program (Supplementary Fig. S1A) via the control box. The control box houses our specifically designed circuit board (Supplementary Fig. S1B, C).

Reservoirs. Twenty liters carboys (Fisher, Hampton, NH) each containing one of the sterile filtered decellularization solutions were arranged in sequence (Fig. 1). The decellularization solutions were DI H₂O (deionized water), Triton (0.1% Triton X-100; Sigma-Aldrich, St. Louis MO), deoxycholate (2% sodium deoxycholate; Sigma-Aldrich), NaCl (1 M NaCl), DNase (10 L carboy with 30 µg/mL porcine pancreatic DNase; Sigma-Aldrich, in 1.3 mM MgSO₄ and 2 mM CaCl₂), and phosphate-buffered saline (PBS without calcium or magnesium, with penicillin/streptomycin (pen/strep) and gentamycin; Life Technologies, Grand Island, NY). Each carboy is connected in sequence to the valve manifold assembly with 1/4" inner diameter tubing (Fisher).

Integration with decellularization chamber (Fig. 1). The outflow tube from the manifold goes to a set of peristaltic pumps with drive motors (Harvard Apparatus) with double Masterflex Easy Load II L/S heads (Cole-Parmer, Vernon Hills, IL) and masterflex 17 tubing (Saint Gobain, Courbevoie, France). Two pumps are required for lungs because the solutions are perfused through the vasculature and infused through the airways. The pumps are controlled by an interface unit and software (Bio_1; Harvard Apparatus) as recently utilized by others.¹⁰ The tubing from the pump perfusing the vasculature leads directly to the bioreactor/decell chamber. The tubing leading to the pump inflating the airways contains a system of two check valves (Water Source, Mansfield, OH). These one way valves allow either fresh fluid from the carboys to be pumped into the lungs or effluent from the lungs to be removed from the system as the

lung deflates (Supplementary Fig. S2). The tubing from the check valves leads to the bioreactor chamber. The pump reverses direction after inflation to remove the fluid from the lungs; the reversal causes the check valve from the carboy to close and the other to open allowing fluid drainage.

Experimental protocol

Pigs. Male and female Yorkshire Cross pigs were purchased from Manthi Farms (Elk River, MN), housed in the University of Minnesota large animal facility, and cared for according to the Research Animal Resources guidelines of our institution. Protocols involving pigs were approved by the University of Minnesota Institutional Animal Care and Use Committee. Pigs weighing 18–22 kg were used as a source of lungs for decellularization.

Animal preparation. Pigs were tranquilized using 3 mg/kg injected intramuscularly of Telazol (Fort Dodge Animal Health, New York, NY) in Xylosine (Lloyd Laboratories, Shenandoah, IA). A 20-gauge I.V. catheter was then placed in an ear vein for an injection of 300 U/kg of heparin (Sagent Pharmaceuticals, Schaumburg, IL), which was allowed to circulate for 10 min. The pig was then euthanized with 1 mL/5 kg of Beuthanasia (Schering-Plough Animal Health, Union, NJ). The skin was cut below the sternum, pulled back, and then cut up to the chin. The peritoneum and the diaphragm were dissected to expose the lung, and the salivary glands and membrane around the trachea removed. Both sides of the rib cage were cut to expose the thoracic cavity.

Preparation of lungs. Lungs were removed from the pig *en bloc*. The heart and any excess tissue were removed from the lungs with care being paid to not puncture the pleura. The trachea and pulmonary artery were then cannulated using appropriately sized barbed connectors (1/4" for the current study; Fisher) fastened with zip ties (Home Depot, Clifton, NJ).

Lung decellularization. Lungs were decellularized either manually or via the automated technique using our established protocol.⁶ Briefly, for both methods, the lungs were inflated/deflated through tracheal infusions and perfused through the pulmonary artery with sequential solutions of deionized water (DI H₂O) and detergents (Triton, deoxycholate) to lyse cells and remove insoluble cellular material. Lungs were then incubated with NaCl and porcine pancreatic DNase to lyse residual nuclei and DNA, respectively, followed by final rinses with PBS containing pen/strep/gentamycin. The volume of each solution was adjusted depending upon the animal size (250 mL of solution per inflation cycle for a 20 kg pig, as was standard in the manual decellularization protocol). Figure 2 shows a comparison between the two protocols.

Manual decellularization protocol. For the manual decellularization protocol, the lungs were prepared as described previously. The lungs were inflated with DI H₂O and the trachea clamped off with a hemostat for 2 h. During the 2 h incubation, 1 L of DI H₂O was perfused through the pulmonary artery. The tracheal clamp was then removed to allow the lungs to deflate for 1 h. This was repeated

FIG. 2. Comparison of automated versus manual decellularization methods.

continuously for 1 day. For Triton, this cycle was repeated and then left overnight and repeated again the next day. This 2-day cycle was repeated for the deoxycholate. One 3-h cycle was used for the NaCl and DNase. Perfusions of PBS with 1× pen/strep, 1× gentamycin were then done for another 24 h with 2-h cycles. The total time was 6–7 days.

Automated decellularization. Lungs were prepared for decellularization as described previously. Individual carboys were filled with each of the decellularization solutions. The bioreactor tank was filled with DI H₂O, air was bled from the lines and the lung cannulae were connected to the appropriate tubing in the tank (i.e., the tracheal cannula was attached on the underside of the bioreactor chamber lid to the barbed connector leading to the check-valve system,

while the cannula in the pulmonary artery was attached to the barbed connector leading directly to the valve manifold via a pump). The pulmonary vein was not cannulated and effluent was allowed to drain into the decellularization chamber. The flow rates and inflation/deflation times were set appropriate for animal size (25 mL/min for 10 min inflation and 12.5 mL/min for a 20 min deflation), and the pump feeding into the vasculature was set to a pressure-regulated flow rate of 15 mmHg (mean physiological pulmonary arterial pressure). The order of decellularization solutions and solution treatment times were set in the valve control program (2 h for DI H₂O, 5 h Triton, 5 h deoxycholate, 5 h NaCl, 2 h DNase, and 5 h PBS with 1× pen/strep, 1× gentamycin) and started. To remove effluent from vascular perfusion, the fluid in the decellularization chamber was

changed using a pump and check valve system identical to the one previously described for perfusing the airways (Supplementary Fig. S2). This pump was set to perform a fluid change equal to 50% of the fluid volume of the bioreactor, in this case 10 L, coinciding with each change of solution type, refilling the chamber with PBS containing antibiotics.

Compliance measurements. Decellularized lungs from our automated system were attached to the IL16 system (Harvard Apparatus) using the tracheal cannula that was already in place. Normal lungs and manually decellularized lungs were cannulated and attached in an identical fashion to the lungs from our automated system. The lungs were then ventilated with a Servo 300A ventilator (Siemens, Munich, Germany) in pressure control mode with PEEP set at 5 cm H₂O and ventilation set at 20 cm H₂O above PEEP. Pressure and volume measurements were taken with the IL16 and recorded using Labchart 7 (ADInstruments, Colorado Springs, CO). Compliance measurements were calculated by averaging the values of three breaths using the following equations: Dynamic compliance = $V_T / (PIP - PEEP)$, Static compliance = $V_T / (P_{plat} - PEEP)$, Specific compliance = $V_T / (PEEP + 5 \text{ cm H}_2\text{O})$ where V_T , tidal volume; PIP, peak inspiratory pressure; PEEP, positive end expiratory pressure; and P_{plat} , plateau pressure.

Sample preparation. Intact lungs (normal and decellularized) were stored at 4°C in PBS with antibiotics (1× pen/strep, 1× gentamycin). Normal and decellularized lung samples were prepared from three areas: bronchus, bronchioles, and distal alveolar region. For the OH-proline, elastin, and DNA assays, tissues were stored at 4°C. For the glycosaminoglycans (GAGs) assay, tissues were homogenized and stored at -80°C. Biochemical assays were done within 2 weeks of decellularization. Tissues for immunofluorescence studies were stored as frozen blocks embedded in optimal cutting temperature (OCT) compound and PBS (3:1 ratio, OCT:PBS), snap-frozen in liquid nitrogen, and stored at -80°C.

Quantification of ECM components. As a measure of collagen, hydroxyproline (OH-proline) content in decellularized and nondecellularized lungs was quantitatively measured by oxidation of 4-OH-L-proline to pyrrole and reaction with *p*-dimethylaminobenzaldehyde (absorbance read at 560 nm). Elastin levels were measured using the Fastin Elastin quantitative dye-binding kit (Biocolor Ltd, Carrickfergus, United Kingdom) according to manufacturer's directions. GAGs were measured using a previously described dimethylmethylene blue assay.²⁸ Total protein was determined by Bradford assay per manufacturer's instructions (Sigma-Aldrich). Means, standard errors, and %CV were determined for each decellularization technique and region of the lung.

DNA assay. Measurements to determine the presence of any residual DNA were done using the Quant-iT PicoGreen kit (Life Technologies) according to manufacturer's directions.

Sterility test. Effluent was collected from the final PBS rinses in the decellularization process and from lung tissue homogenized in PBS. One milliliter of effluent was mixed

with 3 mL of Luria broth (LB) (Sigma-Aldrich) and incubated at 37°C overnight. Absorbance was read at optical density 595 nm on an Implen P330 Nanophotometer. Measurements were taken once per day for 3 days.

Sudan stain. Staining for residual detergent was performed with Sudan III dye (Sigma-Aldrich), using a modification of the protocol provided by the manufacturer. Six milliliters of stock solution of Sudan III in 99% isopropanol was diluted with 4 mL of water and incubated for 5 min. Frozen sections were cut at thickness of 6 µm and placed in the working Sudan III solution for 10 min. The slides were then rinsed in water and mounted with Crystal Mount (Sigma-Aldrich).

Scanning electron microscopy. Samples were set in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) for 2–4 h, rinsed, and then immersed in 1% osmium tetroxide and 0.1 M sodium cacodylate buffer for 2 h. Samples were then rinsed in ultrapure water and dehydrated through an ethanol series. While the samples were in the final 100% ethanol solution, the sample preparation was immersed in liquid nitrogen, placed on a brass surface immersed in liquid nitrogen, and then fractured into smaller pieces using a wooden dowel. The pieces were reimmersed in 100% ethanol and processed in a critical point dryer. Scanning electron microscopy (SEM) sample preparation and imaging were performed at the University of Minnesota Imaging Center; imaging was performed on a Hitachi S3500N at an accelerating voltage of 5 kV and 700× magnification.

Immunofluorescence. Samples were sectioned on a cryostat at a 6 µm thickness, fixed in acetone for 5 min, and then immunofluorescently stained for ECM proteins. Laminin and α-galactose (GAL) were co-stained to identify location of α-GAL relative to the ECM. After blocking with 10% horse serum, samples were incubated with primary antibodies to laminin, collagen I, IV (Abcam, Cambridge, MA), elastin, fibronectin (Thermo Scientific, Waltham, MA), or vitronectin (Innovative Research, Novi, MI) at a 1:250 dilution. Cy3 anti-mouse (collagen I), fluorescein isothiocyanate (FITC) anti-rabbit (collagen IV and laminin), FITC anti-mouse (elastin and vitronectin), and FITC anti-sheep (fibronectin) secondary antibodies (Jackson ImmunoResearch, West Grove, PA) staining followed at a 1:1000 dilution for 30 min. A mouse IgM α-GAL primary antibody (Enzo Life Sciences, Farmingdale, NY) was used at a 1:5 dilution, followed by a biotinylated anti-mouse IgM secondary (1:750; Jackson ImmunoResearch, West Grove, PA), with a Cy3 streptavidin tertiary used at 1:1000 (Jackson ImmunoResearch). Images were taken at 200× magnification using an Olympus Fluoview500 confocal microscope with Fluoview 3.2 software.

Statistics

Means and standard errors were provided for lung matrix protein assays, DNA quantification, and compliance. Student's *t*-test was used to compare differences between samples decelled manually or via the ADS.

TABLE 1. COMPARISON OF EXTRACELLULAR MATRIX PROTEIN AND DNA CONTENT USING MANUAL AND AUTOMATED DECELLULARIZATION

Component	Bronchus (n)	%CV	Bronchiole (n)	%CV	Distal (n)	%CV
OH-proline $\mu\text{g}/\text{mg}$ tissue						
Manual	297.24 $\pm$ 54.94 (5)	41.3	413.54 $\pm$ 104.33 (5)	56.4	142.35 $\pm$ 26.48 (5)	41.5
ADS	454.24 $\pm$ 56.00 (5) ^a	27.5	693.17 $\pm$ 52.05 (4) ^a	15.0	322.50 $\pm$ 9.38 (4) ^a	5.8
Elastin $\mu\text{g}/\text{mg}$ tissue						
Manual	8.71 $\pm$ 1.51 (5)	38.7	12.72 $\pm$ 2.99 (5)	52.5	9.66 $\pm$ 1.52 (5)	38.7
ADS	9.22 $\pm$ 1.25 (5)	30.3	44.52 $\pm$ 5.97 (5) ^a	29.9	25.45 $\pm$ 4.13 (5) ^a	36.3
GAGs $\mu\text{g}/\mu\text{g}$ protein						
Manual	0.177 $\pm$ 0.015 (3)	79.1	0.149 $\pm$ 0.081 (3)	94.0	0.088 $\pm$ 0.042 (3)	83.0
ADS	0.095 $\pm$ 0.015 (5)	34.3	0.102 $\pm$ 0.045 (4)	89.0	0.030 $\pm$ 0.004 (5)	32.4
DNA ^b ng/mL						
Manual	24.62 $\pm$ 2.70 (5)	11.0	21.99 $\pm$ 5.25 (5)	23.9	17.23 $\pm$ 2.10 (5)	12.2
ADS	-0.33 $\pm$ 0.44 (3)	N/A	0.33 $\pm$ 0.44 (3)	N/A	0 $\pm$ 0.29 (3)	N/A

OH-proline, elastin, GAGs, total protein, and DNA levels were quantified for bronchus, bronchiole, and distal regions of whole, intact lungs decellularized via the manual or automated decellularization technique. The %CV for OH-proline, elastin, GAGs, and total protein measurements was determined for each region and decellularization technique.

^a $p < 0.05$ Manual versus ADS.

^b $p < 0.03$ Manual versus ADS for all areas. N/A, not applicable since many samples did not have detectable DNA.

ADS, automated decellularization system; CV, coefficient of variation; GAG, glycosaminoglycans.

Results

Automated decellularization results in more consistent acellular matrix preparations

Bronchus, bronchiole, and distal regions of three to five different lungs were harvested and analyzed for OH-proline (collagen), elastin, GAGs, and total protein to determine the variability of the two decellularization processes through determination of matrix protein levels. OH-proline, elastin, and GAGs measurements were more consistent in lungs decellularized via the automated process as shown by decreased CVs (Table 1).

The automated decellularization protocol is more effective at producing acellular matrix

The automated decellularization protocol significantly shortens the time required for decellularization while consistently removing more of the cellular material. The automated method was also more efficient in removing detergents and any cellular material that could create micelles as shown by staining with Sudan (Fig. 3). The staining of a mock-decellularized lung shows staining of residual cellular material that has a staining pattern distinct from the decellularized lungs and also clearly demonstrates the different appearance of Sudan precipitate (Fig. 3, black arrows).

DNA was measureable in lungs decellularized manually, while lungs decellularized by the automated method had minimal to no DNA measured via the PicoGreen technique (Table 1). When effluent and homogenized lung matrix samples from the end of the procedure were tested for sterility by incubation in LB, none of the ADS samples exhibited any bacterial growth, whereas most of the manually decellularized samples did.

FIG. 3. Sudan staining was used to highlight residual detergent micelles within lung tissue decellularized via the manual or automated decellularization technique to check the efficiency of the protocol to remove and wash out the detergents. The right panels show zoomed images. The red staining indicates areas of positive Sudan staining; the red spheres highlight residual detergent micelles or micelles of cellular material. The staining of a mock-decellularized lung shows staining of residual cellular material. Black arrows indicate Sudan precipitate. Magnification 200 $\times$.

FIG. 4. Normal dynamic and static compliance of automated decellularization system (ADS) decellularized pig lung tissue. Whole nondecellularized and lungs decellularized by the automated system or manually were cannulated through the trachea and placed into a humidified chamber and ventilated as described in the text. Pressure versus volume was measured for each lung. Dynamic, static, and specific compliances are shown (mean $\pm$ SD). * $p < 0.05$ versus normal lungs, $n = 6$ /group.

Decellularization-induced changes in lung compliance

Pressure–volume curves from nondecellularized and decellularized lungs were generated using a Servo 300A Ventilator, and dynamic, static, and specific compliances were calculated. The decrease in specific compliance (Fig. 4) in both groups of decellularized lungs is consistent with the effect of complete removal of surfactants and lung

collapse. ADS lungs had normal static and dynamic compliance, whereas those decellularized manually had increases in these compliance measurements consistent with the lower amounts of the structural matrix proteins collagen and elastin, especially in the central airways in the manual group shown in Table 1.

Automated decellularization maintains lung matrix architecture and protein distribution

SEM images of bronchus, bronchiole, and distal/alveolar regions of nondecellularized and decellularized porcine lung are shown in Figure 5. The SEM images show that the decellularization process completely removed the resident lung cells and exposed the underlying matrix fibers in the bronchus, bronchiole, and distal regions. The images also demonstrate that the three-dimensional structure of the three regions was maintained after automated decellularization but was adversely affected in the manually decellularized lungs.

As seen in Figure 6, matrix protein distribution was maintained in the decellularized lungs. Collagen I/IV, elastin, fibronectin, vitronectin, and laminin stained strongly in the nondecellularized matrix. Staining was still evident in all three regions after decellularization, with differential staining between the three regions. In comparing the ADS versus the manually decellularized lungs, it is difficult to draw any conclusions regarding the levels of these components based on staining intensity but it is evident that the structure of the manually decellularized lungs has been adversely affected consistent with the SEM data of Figure 5. Staining for the α -GAL immunogenic epitope was evident in all three regions of the nondecellularized pig lung, but the staining of the decellularized bronchus, bronchiole, and

FIG. 5. Scanning electron microscopy images of control (upper row), automated (middle row), and manually decellularized (lower row) porcine lung. Samples were prepared and imaged at the University of Minnesota Imaging Facility as described in the text. Images show that lungs decellularized with the ADS maintain good structural integrity, whereas those decellularized manually have areas that appear to have been damaged. Representative images from three lungs/group shown. Magnification 700 $\times$.

FIG. 6. Maintenance of key matrix proteins and removal of α -galactose (GAL) after decellularization by both methods, with better maintenance of tissue integrity in the ADS lungs. Representative immunofluorescence confocal images of normal and decellularized porcine lung samples from the bronchus, bronchiole, and distal/alveolar region of the lung were prepared as described in the text. ADS, lungs from automated decellularized system; MD, lungs that were manually decellularized. Magnification 200 $\times$. Scale bars indicate 100 μ m.

distal regions of the pig lung indicate that both decellularization procedures were effective in removing α -GAL.

Discussion

We have developed the first fully ADS for whole, human-sized organs. In a controlled manner, the ADS can consistently decellularize whole porcine lungs with minimal requirements for user setup and programming. The ADS fully decellularizes a lung within 24 h. Beyond normal porcine lung, we have been able to decellularize liver and kidney (running the ADS protocol at physiologic pressures dependent upon organ type, for example, 13 mmHg for liver²⁹ and 70 mmHg for kidney³⁰). The ADS can be easily adapted to decellularize the organ of interest. However, the lung represents an organ with two possible routes of fluid administration, the vasculature and the airway. We have previously shown that using both routes results in better decellularization⁶ and until now, this was difficult to automate.

Full removal of all cellular debris and decellularization solutions is essential in producing a lung scaffold that does not contain components that could be detrimental to the process of recellularization. Doing so consistently with minimal perturbation of the tissue is a major development goal. As expected, the ADS is superior over manual decellularization in removing DNA, and ECM protein measurements were more reproducible from lungs decellularized by the automated process. The OH-proline and elastin measurements were higher in the lung decellularized by the automated technique. These differences could be directly related to the proportions of OH-proline and elastin that constitute the dry weights. Since the ADS is more efficient in decellularizing the lung, the proportion of OH-proline and elastin may be higher per unit of dry weight resulting in a higher amount of OH-proline and elastin in the samples from the lung decellularized via the ADS. However, static and dynamic compliances were significantly higher in the manually decellularized lungs indicating a loss of these major structural matrix proteins. Importantly, the ADS consistently removes the immunoreactive α -GAL from the porcine lung matrix, which is an essential capability that could otherwise limit the effective use of porcine matrix as an acellular scaffold for engineering new lung tissue. The ADS was also very effective at removing the detergent solutions from the lung.

Retaining organ macro- and microstructure are critical if a decellularized matrix is to serve as an effective recellularization scaffold. SEM and IF images of nondecellularized and decellularized matrix demonstrate that lung matrix produced with the ADS protocol maintains its native structure and protein distribution. With our automated protocol, the decellularized lungs had lower specific compliance after decellularization as expected due to removal of surfactant. This sets the baseline for observing the effect on increasing compliance by recellularization of the acellular matrix with the appropriate cell types to normalize specific compliance with the production of surfactant proteins.

The use of decellularized organs as acellular scaffolds for engineering new organs has become an intense area of research (reviewed in Refs.¹⁻⁴). Standardization of acellular scaffold preparations is needed for this field to advance. Our ADS is fully automated, maintaining sterility in this closed

system, with minimal manipulation of the lung during the decellularization process with the added benefit of saving a substantial amount of time. We believe the efficiency of the ADS is due to the speed of the process in addition to the more gentle nature of the ADS system in avoiding manual manipulation. Automation also avoids the variability in infusion/perfusion pressures generated when done manually in addition to considering that different personnel need to be involved to cover the time commitment required. Achieving greater consistency combined with further validation of organ decellularization protocols will be critical in providing consistent organ scaffolds suitable for recellularization with the long-term goal of engineering transplantable organs.

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Disclosure Statement

No competing financial interests exist.

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