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Preservation of Capillary-beds in Rat Lung Tissue Using Optimized Chemical Decellularization

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Abstract

Promoting regeneration using scaffolds created by decellularizing native tissue is becoming a popular technique applied to a variety of tissues. We demonstrate a method to decellularize highly vascular tissue keeping the vascular structure intact down to the capillary scale. Using vascular corrosion casting (VCC), we created a method for quantitatively assessing the functionality of vascular extracellular matrix (ECM) following decellularization. Murine lung tissue was decellularized using a number of techniques, then characterized using standard histological methods, as well as our quantitative VCC (qVCC) technique. Using an optimized acellular method, we successfully decellularized lung tissue while leaving behind a patent vascular network based on qualitative and quantitative histological methods.

Introduction

As the organ wait list continues to exceed the number of donors each year (over 100,000 in need, under 15,000 donors),¹ the need for alternative therapies to transplantation, such as tissue engineered constructs, is becoming increasingly important. One of the challenges to sustaining cellular viability in a large, complex tissue construct remains to be providing a complex nutrient delivery and waste removal system to the entirety of the construct. The spontaneous vascular growth in the human body is merely several tenths of microns per day,² far too slow to traverse implanted constructs that are hundreds of microns thick in a biologically-relevant time scale. This limited vascular ingrowth results in poor nutrient and oxygen delivery to the cells comprising the construct. Nutrient delivery is a critical component for any type of organ transplantation,³ and the failure to address the metabolic needs of cells often leads to the formation of necrotic cores in engineered substitutes.^{4–8} A hindered oxygen supply is not only responsible for cell death and the failure of most grafts,⁹ but can also increase vascular endothelial growth factor (VEGF) and mRNA transcription, which can have detrimental effects on tissues.^{10, 11}

Although efforts have been made in promoting enhanced vascularization utilizing angiogenic factors and permissive scaffolds, these techniques often create vessels which are erratic, hemorrhagic, and leaky.^{11, 12} Further research to improve vessel stability can increase vascularization and the rate of vascular ingrowth when combined with a tissue construct, but they still require the host vascular system to initiate vascularization on the exterior of the tissue construct, leading to nutrient deficiency and hypoxia in the center of the construct.¹³ To overcome these potential problems, researchers have attempted to create *de novo* vascular constructs prior to implantation; however, many of these techniques fail to create mature, three-dimensional networks. Microfabrication has ideal geometries for vascular reconstruction; however, the PDMS substrate used still inhibits the practicality of this method. Another problem with microfabrication is that most systems are simply thin films that contain micro-channels. To achieve 3D constructs, researchers have resorted to stacking thin films together or rolling them into 3D tubes.¹⁴ Other methods like 3D printing have been utilized successfully in tissue engineering to create 3D constructs;^{15, 16} however, issues regarding the pore size and mechanical properties of the substrates used in this method detract from the efficacy of this technology.¹⁷ Prevascularization *in vivo* utilizes the host vascular system to perfuse constructs in an inert region prior to transplantation to the region of interest. This technique has subsequently been implemented with a variety of constructs to promote vascularization and regeneration of various tissues^{18–21} and large animal models.²² In this method, an AV loop is either wrapped around the tissue construct, or implanted directly into the graft. This method allows for rapid anastomosis to occur throughout the construct in a fully functional system. Despite these benefits, the necessity for multiple surgeries and the loss of a functional vascular axis are significant drawbacks. In addition, some cell death, and unwanted tissue formation in the graft during the vascularization period *in vivo* is likely to occur. In contrast, *in vitro* prevascularization is a co-culture method used to develop lumen in tissue constructs prior to implantation. Endothelial cells are co-cultured with mural cells and tissue-specific cell lineages to achieve an implantable construct. Once implanted, these constructs can anastomose with the host after only four days.²³ Skin,²⁴ muscle,²⁵ cardiac muscle,²⁶ and bone²⁷ have all been therapeutically tested using this method with differing levels of success. One reason for the dissonance of results, even in the same tissue type is the variability of the culture process.¹³ The nature of co-culturing cells makes optimization for each cell line used nearly impossible. Furthermore, although these constructs are fully penetrated with *de novo* lumen structures, the lumina formed might not be patent or interconnected with each other, causing failure of the construct once implanted.

Decellularization of tissue exploits the biochemical and mechanical properties, as well as architecture of native ECM. In general, decellularization methods aim to remove the cellular components from harvested tissue, including surface markers, while leaving the ECM proteins intact. This results in a biomimetic construct composed of a variety of ECM components that synergistically provide an ideal environment for cellular homeostasis that is devoid of immunogenic markers, which in turn enables tissue source material to include xenogeneic and allogeneic sources. A wide number of techniques, including thermal, chemical, and enzymatic, have been used to decellularize a variety of tissues for regeneration in muscle,^{28, 29} vasculature,³⁰ nerve,^{31–34} cardiac muscle,³⁵ and bladder.^{36, 37}

Although different processes have been used to decellularize tissue, each has its own caveats which dictate its efficacy. This behavior has been demonstrated through a comparison of decellularized nerve constructs for peripheral nerve injury.^{31–34} Each tissue decellularized needs to be assessed based on the important criteria for which the tissue is being utilized. A vascular construct that includes a working vascular network prior to implantation could be micro surgically connected to the host vasculature, which will ensure rapid anastomosis and the likely survival of cells within the construct. We assess a number of decellularization methods on their ability to preserve vascular patency down to the capillary scale.

Results and discussion

Histology

H&E demonstrated that nucleic material was removed and ECM persisted in each of the decellularized lungs (Fig. 1). An organized structure similar to native tissue was visualized following tissue processing using all the decell methods. Ultrastructural preservation of basal ECM was found following each decellularization method. Some erratic hematoxylin staining was seen in following the PSDS method and to a lesser extent, the POA method; however this was likely due to anionic SDS detergent binding to the basal proteins, as opposed to genetic material.

Immunohistochemistry

In all immunofluorescence procedures, unprocessed lung was used as a control. Immunofluorescence evaluation reveals the removal of nucleic material (Fig. 2). Some positive DAPI staining was seen in perfusion decell groups; however, this was likely a result of residual anionic detergents remaining in the tissue. ECM bioactivity was seen through the positive staining for fibronectin along with basal lamina through positive staining for laminin even after decellularization (Figs. 3, 4). Signal for the endothelial glycoprotein CD-31 and the microfilament α -SMA was diminished following decellularization using the OA and PSDS methods, whereas some signal was seen in the POA group (Figs 4, 5).

DNA quantitation

Fresh lung DNA isolation was compared to DNA isolated from segments of lung following decellularization (Fig. 6). Spectral analysis and quantification confirmed the removal of DNA observed in qualitative histological methods. DNA concentration from each decell group was significantly lower than fresh tissue. OA, PSDS, and POA lungs resulted in 85%, 91%, and 87% less ds-DNA than fresh tissue respectively. Each decellularized DNA concentration was significant to fresh tissue ($p < 0.0000001$), and significance was observed between the OA and PSDS group ($p < 0.0001$). Importantly, the 260/280 nm wavelength ratios of OA, PSDS, and POA lungs were 1.75 ± 0.06 , 2.07 ± 0.15 , and 1.87 ± 0.09 respectively, compared to 1.74 ± 0.04 for fresh tissue and close to 1.8, signifying a pure sample of DNA without protein interference.

Vascular corrosion casting

During VCC perfusion of each decellularization method, all of the Mercor resin was seen to go into the pulmonary artery and exit through the pulmonary vein. The resulting casts all

retained the shape and configuration of the lung, with a visible vascular axis intact. Macroscopically, the color of the casts appeared different. The fresh lung control was the most white in color, whereas perfusion decell methods had a pinkish tinge to them (Fig. 7). Electron micrographs enabled capillary formations to be seen (Fig. 8). The perfusion-decell groups failed to maintain vascular integrity at the capillary scale; whereas the OA decell group preserved capillary-like networks.

Quantitative VCC

Whereas differences were difficult to elucidate between the decell methods from histology, analysis of extravasations gave rise to distinct trends depending on tissue processing (Table 1). The fresh control had very few extravasations across all sizes. The OA method had a large number of extravasations less than 10 μm in diameter, but less extravasations of 10–20 μm , and significantly less 20–30 μm and over 30 μm extravasations when compared to the other decell methods. In addition, the total volume of resin that escaped OA tissue ($5.97 \times 10^5 \mu\text{m}^3$ per mm^2) was substantially less than the perfusion decell methods (4.76×10^6 and $1.83 \times 10^6 \mu\text{m}^3$ per mm^2 for PSDS and POA groups respectively) (Fig. 9). These results suggest that greater damage was done to the lumina of tissue processed through perfusion-based decellularization methods, than the OA method.

Detergent selection

The decell methods selected were chosen based on successful decellularization of other tissues in rat. Hudson *et al.* successfully used the amphoteric sulfobetaine detergents SB-10 and SB-16, along with the anionic detergent Triton X-200 to preserve basal lamina in nerve—the dimensions of which are on similar scale to capillary networks.^{31–34} These detergents were optimized by testing the efficacy of the preservation of basal lamina and structural membranes of nerve along with the removal of lipids and cellular components. Immunohistochemistry demonstrated OA detergents were superior when evaluating the composition of basal lamina in decellularized nerve cross-sections compared to existing methods utilizing more powerful anionic detergents. Ott *et al.* successfully used the anionic detergent SDS along with the nonionic detergent Triton X-100 to decellularize heart tissue, subsequently demonstrating functionality of the tissue using mechanical testing.³⁵ This method utilized the existing vascular architecture within heart tissue to rapidly deliver detergents throughout the tissue. Following decellularization, the 1% SDS solution was chosen due to improved nuclei clearance observed through H&E staining, compared to using a 1% polyethylene glycol solution or 1% Triton X-100 solution. The 1% SDS perfusion also demonstrated clearance of cardiac myosin and actin following decellularization, while maintaining the presence of ECM protein—as seen using immunohistochemistry. Although ECM proteins were verified using immunohistochemistry, the general architecture appears different compared to cadaveric tissue, again suggesting a more quantitative method would provide greater insight into the efficacy of the decellularization method. The researchers subsequently analysed the mechanical integrity of the heart tissue to demonstrate decellularization efficacy. The goal of our study was to preserve the vascular structure of the decellularized tissue down to the capillary-scale. Based on the results from the papers corresponding to each decellularization method, we hypothesized the OA method would allow for greater preservation of the basal lamina in the capillary-scale. In this aim, we

focused on optimizing the decellularization protocol from the Hudson paper on the thicker, yet less dense lung tissue. Finally, the POA method was used as another comparison method. This method would utilize the rapid delivery seen in the PSDS method to deliver the less denaturing detergents from the OA protocol.

Standard histology fails to elucidate differences in decellularization efficacy

H&E staining demonstrated that the general architecture was maintained following decellularization in each of the decell methods tested. Erratic hematoxylin was seen in some of the PSDS tissue samples; however the staining was not specific to nuclei, and was likely binding to the residual anionic detergent that failed to rinse away. This method utilized SDS, a powerful anionic detergent that commonly binds to basal proteins and is difficult to rinse away.^{38–40} Although evident when looking macroscopically at the decellularized tissues—the decellularized lung from perfusion methods were more translucent than from the OA method, suggesting lack of cellular material—further evidence that residual detergents were responsible for cationic staining was confirmed in the quantitative DNA analysis of the tissues following decellularization. Each decellularized scaffold analyzed for DNA content demonstrated significant removal of DNA compared to fresh lung, and in fact, the perfusion methods resulted in a larger decrease in DNA content than with the OA method. Thus the cationic agents used to detect anionic residues could only be from the bound anionic detergents. The immunohistochemistry analysis performed demonstrated a conservation of bioactivity in the ECM proteins stained for in the decell groups comparable to the fresh control tissue. Staining for cellular markers showed an attenuation of signal following decellularization compared to the fresh control. The POA method seemed to perform the poorest in this respect, although the technique effectively eliminated nucleic material. From these data, decellularization efficacy was not immediately evident through standard histological methods.

The paramount objective of our decellularization methods was to remove the immunogenic component of the tissues while keeping the patency of the vascular network intact down to the capillary scale. Such a construct could potentially be directly anastomosed to the host vascular system upon implantation—allowing for immediate perfusion to the entirety of the graft, and prevent necrotic core formation. If damage existed to the vascular network, the ability to properly perfuse the whole graft would be compromised, essentially rendering it a construct with a leaky hemorrhagic vascular network. By utilizing qVCC we would be able to more accurately assess the chief function of our construct and appropriately award merit to our decellularization methods.

Vascular corrosion casts demonstrate differences between decellularization methods

Vascular Casts (VC) have been used to image and explore the morphology of vascular networks and structures since the 1980s.^{41, 42} These permanent representations of the vascular luminal space are obtained after the process of perfusing the vascular system to remove blood, injecting the vasculature with resin, allowing the resin to polymerize, and finally removing the surrounding tissue (Vascular Corrosion Casts or VCC), or optically clearing the surrounding tissue. These polymer casts can then be used to accurately visualize vascular structures down to the capillary scale. Scanning Electron Microscopy (SEM) and

Confocal Microscopy techniques are the most commonly used to investigate the vascular structure preserved by the cast due to the high resolution images they are able to produce.^{43, 44} Ohtake *et al.* demonstrated the use of VCs to elucidate differences in damaged tissues. After evaluating vessel defects, truncations, and extravasation patterns, they found larger extravasations correlated with increased microvascular injury.⁴⁵ Our technique aimed to quantitatively describe the similarities and differences between the microvascular networks present in fresh tissue and following decellularization.

In characterizing the qVCC data we correlated larger extravasations with greater luminal damage. Given small leaks in the lumina of our vessels, we would expect to see smaller extravasations; whereas a larger leak would result in more rapid diffusion out of the luminal space and henceforth larger extravasations. These sentiments are in agreement with previous studies characterizing extravasations in relation to increased, manually-induced vascular damage in the brain.⁴⁵ From our results the OA method resulted in the most small (less than 10 micron) extravasations; however, perfusion-based methods medium consisted of more (10-30 micron) and large (over 30 microns) extravasations. When analyzing the total volume of extravasations, we gain a clearer insight to the amount of damage present in the vascular network. Of the decell methods tested, the perfusion-based methods had a greater total extravasational volume when compared to the diffusion-based OA method, and fresh tissue. When performing the qVCC analysis, very little variation in extravasation number and type was seen throughout the construct of a particular decell method as opposed to comparing techniques to each other. Although unclear at the moment as to the correlation between increased vascular ECM damage and subsequent scaffold performance both *in vitro* and *in vivo*, the formation of vascular networks that have a decreased potential for hemorrhages is a strong starting point for improving decellularized vascular tissue. The importance of a continuous vascular ECM is linked to the role of the ECM in vascularization. Following injury, the basal membrane is first degraded, after which endothelial cells (ECs) proliferate and migrate within tissues containing interstitial collagen.^{46, 47} Interactions with collagen cause a spindle-like morphology that will eventually reorganize into lumina over time.^{48, 49} The transduction of mechanical forces through the ECM allows ECs to establish tension-based guidance pathways, leading to interconnected cords. Another important function of the ECM is to sequester cells. Research has demonstrated that the conformation and orientation of fibronectin are key components in EC cell adhesion.⁵⁰ This important finding, intimates at the importance of ECM that is biomimetic not only in biochemical composition, but also in spatial architecture. Overall, the ability of endothelial ECM to direct EC fate makes scaffold composition a significant characteristic to define in a vascular construct.

Experimental

All materials obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Tissue harvest and isolation

Female Fischer rats (175-200g) (Harlan, Indianapolis, IN) were given intraperitoneal injections of 1000U per kg of heparin sodium injection (TW Medical, Austin, TX) 15 minutes prior to 100/10 mg per kg of ketamine/xylazine (Bioniche Teoranta Inverin, Co.,

Galway, Ireland/Akorn Inc., Decatur, IL) cocktail. The animals were perfused with phosphate buffer solution until lungs were blanched. Whole lung, thymus and heart were excised from the animals and placed in 4°C buffer. Next, the singular left lung lobe was isolated including its vascular axis using a stereoscope (Leica Zoom 2000). This tissue was either fixed using 4% paraformaldehyde, or subjected to decellularization then fixed for analyses.

Decellularization

Three chemical decellularization methods were selected for comparison. The first method, optimized acellular (OA), was based off of a decellularization protocol developed previously in for peripheral nerve decellularization,³² differing only by increasing the detergent incubation durations. Briefly, lung tissue was incubated in 125 mM sulfobetaine-10 (amphoteric) detergent for 24 hr followed by one rinse of 100/10 sodium/phosphate buffer for 15 min. Next tissue was incubated in a combination 0.14% Triton X-200 (anionic) and 0.6 mM sulfobetaine-16 (amphoteric) detergents for 48 hr followed by three rinses of 100/10 sodium/phosphate buffer for 15 min each. Tissue was then incubated in SB-10 solution for 15 hr followed by one rinse of 100/10 sodium/phosphate buffer for 15 min. Tissue was then incubated in Triton X-200/SB-16 solution for 24 hr followed by three rinses of 50/10 sodium/phosphate buffer. All incubation and rinse steps were performed on a vertical rotator. The next method, referred to as the PSDS method, was taken from a protocol developed by Ott, *et al.*, effective in decellularizing heart tissue.³⁵ Lung tissue was first cannulated prior to perfusion of detergents using a 24Ga cannula (TW Medical) with cured PDMS on the end. Next, 1% SDS (anionic) in deionized water was perfused through the tissue for 12 hr, followed by 15 min perfusion of deionized water, then 30 min of 1% Triton X-100 (nonionic) in deionized water. Finally, PBS was perfused through the tissue for 124 hr to ensure the remove of residual detergent. The final method (POA) combined the detergents and timings of the OA method, but delivered the detergents utilizing perfusion.

Histology

Histological methods were first used to compare the composition of the decellularization methods compared with fresh lung tissue. For all histological studies three unprocessed and processed tissues from each method were fixed in 4% paraformaldehyde for 4 hr, and then mounted and cut into 20 µm thick sections using a cryostat (Microm HM 550). Hematoxylin and eosin (H&E) staining was subsequently performed on these samples and fresh lung as control. Representative images of each tissue were then selected for evaluation.

Immunohistochemistry

Staining for the ECM proteins laminin and fibronectin was performed using standard immunofluorescence imaging procedures with antibodies against fibronectin (Sigma, F0916) and laminin (Sigma, L9393) on 20 µm thick sections of processed and unprocessed tissues. Immunofluorescence imaging against cellular components targeting the endothelial glycoprotein, CD-31 (Pharminogen, 550300), α-smooth muscle actin (Abcam, ab7817) and DAPI staining for nuclei was performed on decellularized and unprocessed tissue sections. Secondary antibodies attaching to either rabbit or mouse IgG and bound to the fluorophores,

Alexa-Fluor 488, and Alexa-Fluor 568 were subsequently bound (at 1:400). In each study a negative control using only the secondary antibodies was imaged to prevent false-positive signal interpretation. Representative images of each tissue were then visualized on an Olympus IX70 (Olympus America, Melville, NY) inverted microscope, and captured with an Optronics (Goleta, CA) MagnaFire digital color camera. For each stain, a fixed exposure time was used.

DNA quantitation

To quantitatively compare the efficacy of the different decellularization methods used to remove cellular material, the DNA content remaining from our scaffolds was compared to fresh lung tissue. Processed and unprocessed lung tissue (three lungs per technique) were rapidly frozen with liquid nitrogen and crushed using a mortar and pestle. Three 50 mg samples per lung were then incubated in 40 mM NaOH solution for 5 hours at 90°C, and then neutralized with Tris-HCl to a pH of 7.8. These samples were then centrifuged at 500g for 15 min. The supernatant was then drawn and run with a NanoDrop machine to read the optical density at 260 nm and calculate the corresponding ds-DNA concentration.

Vascular corrosion casting

Mercor II CL-2R resin mixed with a 40% benzoyl peroxide dibutyl phthalate catalyst (Ladd Research, Williston, VT) was injected via syringe pump (KD Scientific, Model 200) (0.2 g catalyst/10 ml resin) at 2 ml/min into the vascular axis of six processed and two unprocessed tissues at 2 ml/min. The resin was allowed to polymerize in water baths at 65°C overnight, after which the organic components were removed by chemical washes (25% potassium hydroxide for 48 hours), and warm water washes to remove remaining tissue. Images were taken using a standard digital camera through a stereoscope (Leica Zoom 2000).

Quantitative VCC

The presence of capillaries in the remaining plastic structure was visualized using scanning electron microscopy (SEM). The plastic molds were air dried for 24 hours in a vertical flow hood, then coated with 20 nm platinum palladium for observation with SEM (Zeiss Supra 40 VP) with an acceleration voltage of 5 kV. A total of 32 images were taken for each decellularization method, and then normalized to account for the same total surface area for the particular decellularization method. Four researchers independently analyzed the images for extravasations of the resin from the luminal space. Extravasations were counted and sorted by diameter in four categories: <10 μm , 10-20 μm , 20-30 μm , and > 30 μm . These counts were normalized with respect to the area of the image, and then averaged for each decellularization group among the four researchers. Total extravasational volume was determined by estimating the average diameter of extravasations found in each category as 5 μm , 15 μm , 25 μm , and 30 μm .

Statistics

A paired t-test was used to determine if statistical significance existed between the researchers' extravasation counting totals for each decellularization group. An F-test was used to determine variance significance followed by a Student's t-test to determine statistical

significance between each decellularization group. All error bars in figures represent plus and minus two standard error of the mean, representing a 95% confidence interval.

Conclusions

Using qVCC we were able to elucidate differences in tissue functionality following decellularization that would not have been able to be visualized using conventional histological methods. We maintain that greater damage to the vascular space caused by decellularization can be correlated with the total volume of extravasations present. In qualitative observation of VCCs, we saw the preservation of capillary-like networks in the OA method that was not present in other decell methods. Quantitatively calculating the damage to the vascular network following decellularization is a technique that future decellularization methods that attempt to preserve vascular functionality can implement to assess the efficacy of their technique. However, the effect that the conservation on capillary-like networks and vascular patency have on the viability of a tissue construct will need to be tested through subsequent studies involving recellularization, and anastomosis *in vivo*.

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Fig. 1. Hematoxylin and eosin (H&E) staining following different decellularization methods on lung tissue. Fresh (A), diffusion OA (B), PSDS (C), POA (D) lung were examined. No presence of intact nuclei remained, however, in PSDS processing, basophilic staining from hematoxylin was observed. ECM preservation was most continuous in the OA groups (B, D) compared to the PSDS method (C). Scale bar = 500 μ m.

Fig. 2. Staining for DAPI reveals removal of nuclei following decellularization of lung tissue using different methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method). Residual anionic detergents from the perfusion based methods (C, D) stained positive in erratic fashion. Scale bar = 200 μ m

Fig. 3. Immunostaining against fibronectin reveals preservation of fibronectin following decellularization of lung tissue using different methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method) Scale bar = 500 μ m.

Fig. 4. Immunostaining against laminin (red), and the endothelial marker CD-31 (green) demonstrates basal lamina remains whereas cellular signal is diminished following decellularization of lung tissue using different methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method) Scale bar = 200 μ m

Fig. 5.

Immunostaining for α -smooth muscle actin following different decellularization methods on lung tissue. Staining against α -smooth muscle actin demonstrates attenuation of the cellular component following decellularization methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method) when compared to fresh tissue (A). Scale bar = 500 μ m.

Fig. 6.

DNA quantitation of fresh and decellularized lung tissue. DNA concentration was determined using a NanoDrop spectrophotometer. Lung tissue decellularized using OA, POA, and PSDS methods demonstrated a significant decrease in concentration when compared to fresh tissue. The 91% decrease in DNA concentration following the PSDS method was also significant against 85% decrease from the OA method. Error bars represent $\pm 2\text{SEM}$, a 95% confidence interval.

Fig. 7.

VCC of lungs following decellularization using different methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method) reveals a defined geometry of mimicking the original tissue structure. The color of the casts appears to range from white to pink in order from A–D.

Fig. 8. SEM images of VCC of lungs following decellularization using different methods (A, fresh lung; B, OA method; C, PSDS method; D, POA method). Fresh tissue displays a highly organized structure with intact vessels branching to capillary networks. OA method has some disorganized features to its capillary structure. The perfusion methods (PSDS and POA) are highly disorganized. Extravasations (asterisks) are most prevalent in the perfusion methods with the PSDS method containing the largest extravasations. Scale bar = 40 μ m

Fig. 9.

Total extravasation volume was calculated per mm^2 by cubic micron for each decellularization method investigated. The amount of vascular damage correlated with larger extravasations was apparent when assessing the volume of resin that escaped the vascular space of the tissue. Although damage was found following each decellularization method, the diffusion based OA method was by far the most effective at preventing the escape of

resin from the remaining vascular network of ECM. Error bars represent $\pm 2\text{SEM}$, a 95% confidence interval

Table 1

Extravasation counts per mm² following vascular corrosion casting of fresh and decellularized lung tissue plus or minus one standard deviation. A greater number of extravasations less than 10 microns in diameter was seen in OA decellularized lung casts compared to fresh and PSDS casts; however, larger extravasation counts for OA casts were not significantly different from fresh tissue, whereas PSDS and POA casts displayed significance versus fresh tissue.

Extravasation Diameter	Decellularization Method			
	Fresh	OA	PSDS	POA
10	95.26 ± 113.8	4125.19 ± 4978.53 [*]	418.76 ± 541.80 [†]	4494.30 ± 2691.37 ^{*‡}
10 to 20	24.01 ± 40.96	119.44 ± 11.700 [*]	374.99 ± 474.49 [*]	464.08 ± 425.11 ^{*‡}
20 to 30	3.37 ± 9.80	9.80 ± 19.5	71.78 ± 87.78 ^{*‡}	55.55 ± 65.25 ^{*‡}
< 30	3.80 ± 5.88	2.56 ± 10.2	246.28 ± 148.39 ^{*‡}	18.15 ± 23.84 ^{*‡‡}

^{*}, [†], and [‡] denote significance (p<0.05) versus fresh, OA, and PSDS groups respectively