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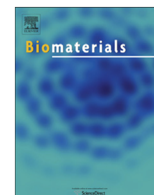

Automated procedure for biomimetic de-cellularized lung scaffold supporting alveolar epithelial transdifferentiation

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The optimal method for creating a de-cellularized lung scaffold that is devoid of cells and cell debris, immunologically inert, and retains necessary extracellular matrix (ECM) has yet to be identified. Herein, we compare automated detergent-based de-cellularization approaches utilizing either constant pressure (CP) or constant flow (CF), to previously published protocols utilizing manual pressure (MP) to instill and rinse out the de-cellularization agents. De-cellularized lungs resulting from each method were evaluated for presence of remaining ECM proteins and immunostimulatory material such as nucleic acids and intracellular material. Our results demonstrate that the CP and MP approaches more effectively remove cellular materials but differentially retain ECM proteins. The CP method has the added benefit of being a faster, reproducible de-cellularization process. To assess the functional ability of the de-cellularized scaffolds to maintain epithelial cells, intra-tracheal inoculation with GFP expressing C10 alveolar epithelial cells (AEC) was performed. Notably, the CP de-cellularized lungs were able to support growth and spontaneous differentiation of C10-GFP cells from a type II-like phenotype to a type I-like phenotype.

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1. Introduction

Lung disease, the third highest cause of death in the United States, is a major public health concern. Almost 400,000 Americans die yearly from pulmonary disease and more than 35 million people are afflicted [1]. Lung transplantation remains a final option but is significantly limited by supply of suitable donor lungs along with the accompanying need for lifelong immunosuppression and the high mortality [2,3]. Though lung transplants are life saving measures, alternative therapeutic options are desperately needed. In an effort to address these issues and improve the therapeutic use of lung transplantation, there has been rapid growth in the development of *ex vivo* tissue engineering techniques with the goal of creating functional transplantable lungs [4].

In tissue engineering, it is essential for the optimal scaffold to maintain an appropriate three dimensional configuration and retain important extracellular matrix (ECM) proteins. ECM proteins such as laminin, fibronectin, elastin, collagen I and IV have been found to play roles in trans-membrane cell signaling, cellular differentiation, respiratory mechanics and other pulmonary-specific functions [5–8]. The ability of cells to receive organotypic signals from native ECM provides a potential system for functional re-cellularization compared with synthetic constructs [9]. Therefore, the retention of key ECM proteins is critical for a de-cellularized matrix to provide pulmonary-specific cellular signals.

However, the optimal method for de-cellularization of pulmonary tissue while maintaining critical ECM proteins is unclear. The de-cellularization procedure can be performed by different processes, such as physical methods, chemical agents, and enzymatic degradations [3,9,10]. Cellular materials, particularly nucleic acids, proteins, and glycosaminoglycans, are known to be immunostimulatory and should ideally be eliminated by the de-cellularization process [11–15]. Several methods of de-cellularization have been published, including previous work from our laboratory, utilizing detergent and enzymatic washes, as well as physical methods (freeze–thaw) to de-cellularize rodent lung matrices with subsequent maintenance of fetal pulmonary cells within the scaffold

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Table 1
Summary of Rat Lung De-cellularization Methods De-cellularization reagent, duration of protocol, levels of nuclear material still present after de-cellularization and proliferation after re-seeding is summarized above. CP de-cellularization appears to remove nucleic acids as well as the MP protocol in a shorter period of time while using a single reagent. CF de-cellularization appears to be ineffective in removing all nuclear material from the matrix during this time point. Following re-seeding, cell proliferation was observed only when utilizing the CP de-cellularized scaffold following 3 days in a bioreactor. Abbreviations: 4′,6-diamidino-2-phenylindole (DAPI), Propidium Iodide (PI), Sodium Deoxycholate (SDC), Sodium Chloride (NaCl), Sodium Dodecyl Sulfate (SDS).

Decell method	Decell reagent(s)	Airway rinse volume	Vasculature rinse volume	Duration of protocol	Nuclei present	DNA/RNA present (PI)	Nucleic acids present (TOPRO-3)	Proliferation following Re-seeding
3 Day method	Triton-X, SDC, NaCl, DNAse via trachea & vasculature	150 ml	150 ml	3 Days	None	Minimal	Minimal	No
Perfusion/flow-base	0.1% SDS via trachea & vasculature	50 ml	3,456 ml	20.5 H	Scarce	Significant retention	Significant retention	No
Pressure-base	0.1% SDS via vasculature	None	30–40 L	20.5 H	None	Minimal	Minimal	Yes

[16,17]. However, there is no consensus. One particular issue that remains unclear is whether manual versus mechanized de-cellularization will yield more optimal results. Several papers have described use of manual pressure (MP) de-cellularization approach with generally good results [16,18,19]. However, this process is subject to user variability and no consensus has been reached regarding the optimal de-cellularization procedure.

As automated de-cellularization techniques may provide faster, more reliable scaffolds, we sought to determine whether varying flow or pressure with automation would affect the quality of the scaffold. As such, we compare three different detergent-based de-cellularization approaches utilizing either constant pressure (CP), constant flow (CF) or a previously published method utilizing manual pressure (MP) to instill and rinse out the cellular material and de-cellularization agents. In addition, we compared vascular de-cellularization alone or in combination with airway de-cellularization. Comprehensive assessment of the de-cellularized lungs will include analysis of the matrix composition as well as loss of immunostimulatory material. We will compare the CF and CP techniques to the previously published MP approach in order to assess whether these differences in technique affect cell adherence and viability. We will utilize an alveolar epithelial cell line to evaluate the ability of all of these scaffolds to support epithelial repopulation. This will be compared following 3 days in a physiologic bioreactor system. The data we obtain should help to define and refine optimal approaches to lung de-cellularization.

2. Materials and methods

2.1. Methods for de-cellularization of rat lungs

(Table 1/Fig. 1) 20 week-old Sprague–Dawley rats (Charles River, Wilmington, MA) were euthanized in accordance with University of Connecticut Health Center IACUC approved protocols. A midline incision was made from the upper abdomen to the throat and a median sternotomy was performed. The trachea and heart-lung block were carefully dissected and exposed for cannulation.

2.1.1. MP de-cellularization

(Fig. 1A) The following procedure was adapted from a previously described protocol [16] and was followed as a comparison to our proposed automated techniques. The heart-lung block of the rat was carefully removed and the trachea was cannulated with an 18 gauge angiocatheter and secured with sterile 2-0 silk ties (Braun Medical, Bethlehem, PA). An 18 gauge needle and a 10cc syringe were used to manually inject 10 ml of phosphate buffered saline (PBS) into the right ventricle of the heart-lung block. The trachea was manually injected with 10 ml PBS to inflate the lungs. This cycle was repeated a total of five times. Triton X-100 (0.1%) in a deionized water (DI) (Sigma, St. Louis, MO) solution was manually injected into the scaffold in the same manner and volume as described above followed by complete submersion in the solution for 24 h at 4°C. The scaffold was rinsed five times through the right ventricle and trachea with PBS as described above. 2% sodium deoxycholate (SDC) in DI (Sigma, St. Louis, MO) was injected as above followed by submersion in the solution for 24 h at 4°C. The scaffold was then rinsed five times as above with

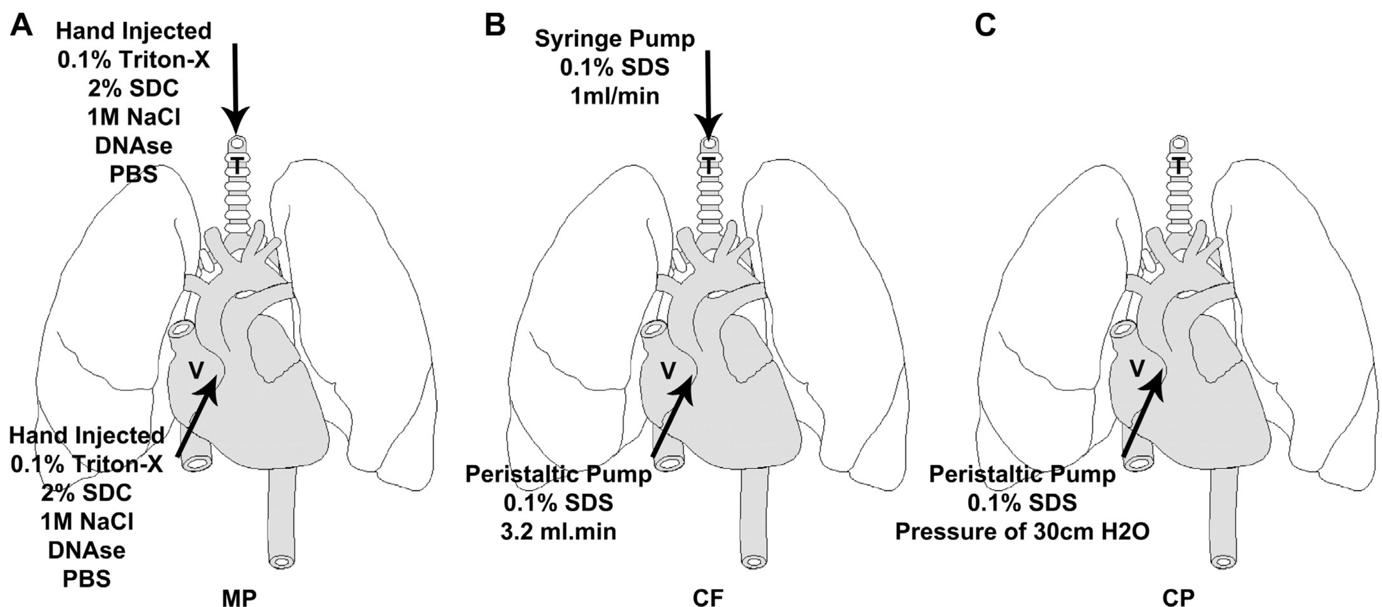

Fig. 1. Schematic of De-cellularization Process MP de-cellularization (A) and CF de-cellularization (B) are achieved by infusing detergent through the trachea (T) and vasculature (V), while CP de-cellularization (C) infuses the detergent only through the vasculature (V).

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