

# A Comparative Study of Rat Lung Decellularization by Chemical Detergents for Lung Tissue Engineering

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## Abstract

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**BACKGROUND:** Lung disease is the most common cause of death in the world. The last stage of pulmonary diseases is lung transplantation. Limitation and shortage of donor organs cause to appear tissue engineering field. Decellularization is a hope for producing intact ECM in the development of engineered organs.

**AIM:** The goal of the decellularization process is to remove cellular and nuclear material while retaining lung three-dimensional and molecular proteins. Different concentration of detergents was used for finding the best approach in lung decellularization.

**MATERIAL AND METHODS:** In this study, three-time approaches (24, 48 and 96 h) with four detergents (CHAPS, SDS, SDC and Triton X-100) were used for decellularizing rat lungs for maintaining of three-dimensional lung architecture and ECM protein composition which have significant roles in differentiation and migration of stem cells. This comparative study determined that variable decellularization approaches can cause significantly different effects on decellularized lungs.

**RESULTS:** Results showed that destruction was increased with increasing the detergent concentration. Single detergent showed a significant reduction in maintaining of three-dimensional of lung and ECM proteins (Collagen and Elastin). But, the best methods were mixed detergents of SDC and CHAPS in low concentration in 48 and 96 h decellularization.

**CONCLUSION:** Decellularized lung tissue can be used in the laboratory to study various aspects of pulmonary biology and physiology and also, these results can be used in the continued improvement of engineered lung tissue.

## Introduction

The mortality of pulmonary diseases is increasing but mortality of other diseases such as heart disease, cancer and so on is decreasing for example 400 thousand of American people die every year [1, 2]. Lung transplantation is the only definitive treatment for some lung diseases such as CF, COPD and IPF. But, patient survival is only 50% during 5 years and 24% during 10 years after lung

transplantation [3]. On the other hand, unfortunately, many patients on the waiting list for lung transplant pass out before suitable donor organ get found because of the critical shortage of transplantable donor lungs. Moreover, immunosuppression is another lifelong drawback for transplant recipients [4]. Due to this problem in transplantation, lung tissue engineering research is increasing and demand for engineered lung tissue has been increased for transplantation. There are advantages of engineered lung tissue such as using a patient's own cells, so

avoiding the need for immunosuppression which causes many problems including neurologic disorders and infection and so on [5, 6]. Tissue engineering is a multidisciplinary field which tries to combine cellular, molecular, technological and medical advances to make replacement tissues for implantation [7-10]. However, the lung is a complex tissue and ECM scaffolds are complex bio-structures constructed of a variety of structural and functional compositions that are specially organized and suitable for the intended tissue [11]. One of lung tissue engineering strategies is that to make appropriate scaffolds for lung tissue engineering [12]. Decellularization is a new technique for removing all cellular materials and produces an intact whole lung, which is an important characteristic of a three-dimensional matrix for cellular differentiation and migration [13, 14]. Decellularization methods have been used for producing scaffolds for a variety of tissue engineering applications such as heart valves, trachea, liver and heart and so on [7, 15-19]. Decellularized organs have several advantages as a tissue engineering scaffold. The decellularized scaffold maintains the suitable 3D organization essential for tissue function, such as a vascular system and airway network in the complex structure of lung [20]. Native ECM has the optimal substrate for cell attachment, spreading, growth and differentiation. The aim of the decellularization method is to remove cellular and nuclear material while maintaining 3D and ECM protein compositions and also, diminishing any damage to lung ECM. There are various types of decellularization approaches such as physical, chemical and enzymatic methods [16, 17]. Physical approaches can cause the destruction of the delicate lung ECM which this method includes freezing, pressure, sonication and agitation, while enzymatic approaches cannot be cost-effective due to large volumes of liquid would be needed for decellularization of a whole organ such as proteases, nucleases, and calcium chelators [21]. Trypsin has been used in the enzymatic method which it can remove fibronectin, laminin, and elastin. Because of the importance of these compositions, enzymatic approaches have not been mostly used in decellularization. But, chemical approaches are cost-effective and have several of techniques such as alkaline or acidic solutions, detergents (SDS, SDC, CHAPS and ...) hypotonic or hypertonic solutions, and chelators [16, 17, 21]. A hypertonic NaCl solution can efficiently lyse cells, while it cannot help in removing cellular components from the tissue. CHAPS are a zwitterionic detergent which can be an effective solubilization for removing of cellular material [21]. The objectives of this research are to demonstrate that decellularized lung is promising substrates for producing an engineered lung scaffold and as the noted 3D and ECM protein compositions are important characteristics for cellular differentiation and migration.

## Material and Methods

### Animals and lung extraction

Fourteen male healthy rats (250–300 gr) were purchased from animal researcher centre of Baqiyatallah University of Medical Sciences and housed in this centre. Rats were controlled in a standard environment (humidity and temperature) on a 12/12 hours light/dark cycle with standard food and water, following experimental procedures approved by the Ethical Committee for Animal Research of Baqiyatallah University of Medical Sciences. The rats were anaesthetized with intraperitoneal with Ketamine and Xylazine (100 mg/kg and 10 mg/kg). A midline incision was made in the throat, and the trachea was exposed. Right ventricle perfusion was perfused with 250cc phosphate buffered saline (PBS) containing Heparin 5000 u/ml and 1% penicillin and streptomycin (P/S) (Figure 1). Lung was sliced to use in decellularization processes.

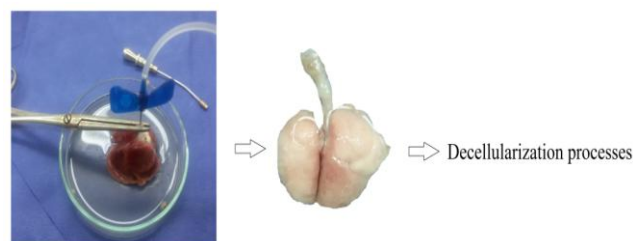


Figure 1: Perfusion of PBS, Heparin and P/S

### Twenty four hour decellularization approaches

Sliced lungs were similarly rinsed and treated with SDS with different concentrations at 24 which is shown in Table 1. Lastly, sliced lungs were rinsed and treated with 1M sodium chloride for 1 h at room temperature for single detergent. Sliced lungs were flushed with PBS before they were used for experiments.

Table 1: 24h decellularization approaches for rat lung decellularization

Approach	Methods	Decellularization approaches
24 h	1	SDC 0.5% 23h, NaCl 1 M 1h
	2	SDC 1% 23h, NaCl 1 M 1h
	3	SDC 1.5% 23h, NaCl 1 M 1h
	4	SDC 2% 23h, NaCl 1 M 1h

### Forty-eight-hour decellularization approaches

In this time, sliced lungs were rinsed and treated by mixed detergents and single detergent of SDC. These detergents were used at 48 h which is shown in Table 2. Sliced lungs were flushed with PBS before they were used for experiments.

**Table 2: 48h decellularization approaches for rat lung decellularization**

Approach	Methods	Decellularization approaches		
		24 h	24 h	
48 h	5	SDC 0. 5% 47h, NaCl 1 M 1h		
	6	SDC 1% 47h, NaCl 1 M 1h		
	7	SDC 1.5% 47h, NaCl 1 M 1h		
	8	SDC 2% 47h, NaCl 1 M 1h		
	9	SDC 0. 5%	+	SDS 0.05%
	10	SDC 1%	+	SDS 0.1%
	11	SDC 1.5%	+	SDS 0.15%
	12	SDC 2%	+	SDS 0.2%
	13	SDC 1%	+	SDS 0.05%
	14	SDC 1.5%	+	SDS 0.1%
	15	SDC 0. 5%	+	Triton X-100 0.05%
	16	SDC 1%	+	Triton X-100 0.1%
	17	SDC 1.5%	+	Triton X-100 0.15%
	18	SDC 2%	+	Triton X-100 0.2%
	19	SDC 1%	+	Triton X-100 0.05%
	20	SDC 1.5%	+	Triton X-100 0.1%
	21	SDC 0. 5%	+	CHAPS 2mM
	22	SDC 1%	+	CHAPS 4mM
	23	SDC 1.5%	+	CHAPS 6mM
	24	SDC 2%	+	CHAPS 8mM
	25	SDC 1%	+	CHAPS 2mM
	26	SDC 1.5%	+	CHAPS 4mM

**Ninety-six-hour decellularization approaches**

In this time, sliced lungs were rinsed and treated by mixed detergents and single detergent of SDC. These detergents were used at 96 h which is shown in Table 3. Sliced lungs were flushed with PBS before they were used for experiments.

**Table 3: 96h decellularization approaches for rat lung decellularization**

Approach	Methods	Decellularization approaches		
		48 h	48 h	
96 h	27	SDC 0. 5%	+	SDS 0.05%
	28	SDC 1%	+	SDS 0.1%
	29	SDC 1.5%	+	SDS 0.15%
	30	SDC 2%	+	SDS 0.2%
	31	SDC 1%	+	SDS 0.05%
	32	SDC 1.5%	+	SDS 0.1%
	33	SDC 0. 5%	+	Triton X-100 0.05%
	34	SDC 1%	+	Triton X-100 0.1%
	35	SDC 1.5%	+	Triton X-100 0.15%
	36	SDC 2%	+	Triton X-100 0.2%
	37	SDC 1%	+	Triton X-100 0.05%
	38	SDC 1.5%	+	Triton X-100 0.1%
	39	SDC 0. 5%	+	CHAPS 2mM
	40	SDC 1%	+	CHAPS 4mM
	41	SDC 1.5%	+	CHAPS 6mM
	42	SDC 2%	+	CHAPS 8mM
	43	SDC 1%	+	CHAPS 2mM
	44	SDC 1.5%	+	CHAPS 4mM

**Lung histology**

H&E staining protocol was utilized to tissue assay. Sliced lungs tissue (before and after decellularization) were fixed in paraformaldehyde 10% after fixation transferred to tissue processor device to dehydration and clearing. Finally, they were embedded in paraffin and sectioned in 5-micron thickness. Sections were stained with H&E, Trichrome- Masson staining and Elastin staining and then, they were observed under a light microscope [22-26]. H&E staining was utilized to visually quantify the nuclei loss and three-dimensional lung architecture by comparing nuclei counts in paraffin

sections of normal and decellularized sliced lungs. Nuclei were counted from three random images at 100 × magnification using a counting tool. Trichrome-Masson staining and Elastin staining were done for evaluating the level of collagen and elastin, respectively.

**Statistical analysis**

All studies were conducted at least in biological triplicate. Data were statistically analyzed by t-test. P values less than 0.05 were considered significant.

**Results**

Analysis of a 24-h decellularized rat lung by Hematoxylin and Eosin, Trichrome- Masson and Elastin staining

This 24-h decellularization process could not completely preserve the architecture of the lung and also, could not completely eliminate the nuclei utilizing SDC detergent. But, method 1 was better than other methods in 24 h decellularization which this method maintained the three-dimensional structure at very low levels and also. This method could not preserve the proteins ECM composition such as Collagen and Elastin (Figure 2).

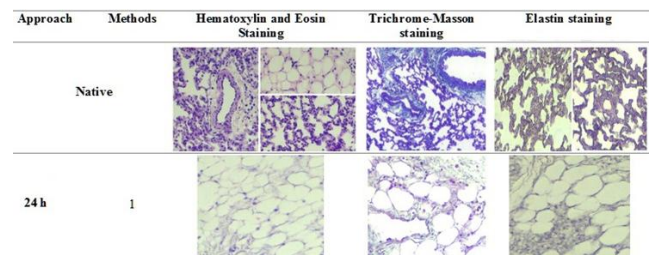


Figure 2: Hematoxylin and Eosin Staining showed structures and nuclei present in native and decellularized lungs. Trichrome-Masson staining showed collagen levels in native and decellularized lungs (Blue colour). Elastin staining showed elastin levels in native and decellularized lungs (Blue colour to black colour). Representative images for all conditions are shown (100 × magnification)

**Analysis of a 48-h decellularized rat lung by Hematoxylin and Eosin, Trichrome-Masson and Elastin staining**

**SDC detergent**

In this approach, SDC detergent was not able to maintain the three-dimensional, Collagen and Elastin. The architecture of the lung was destroyed by

increasing the concentration of SDC from 0.5% to 2%. In addition, a significant reduction was seen in Collagen and Elastin. Method 5 was better than other methods in single SDC detergent in 48 h decellularization (Figure 3) and it could relatively preserve the three-dimensional but could not maintain Collagen and Elastin.

**Mixed detergents of SDC with SDS, Triton X-100 or CHAPS**

Results showed that methods 9, 15, 21, 22 and 25 were considered as the best methods in maintaining of 3D and protein ECM composition (Collagen and Elastin) (Figure 3). Methods 9 and 15 could relatively preserve the three-dimensional and remove nuclei from lung tissue but Collagen and elastin were not seen in SDC+SDS and SDC+Triton X-100 detergent, respectively.

Methods 21, 22 and 25 relatively maintained proteins ECM (Collagen and Elastin) and also, a significant reduction in the nuclei was seen in 48 h decellularization of SDC+ CHAPS. In addition, Method 22 maintained the 3D, Collagen and elastin better than methods 21 and 25 (Figure 3).

Approach	Methods	Hematoxylin and Eosin Staining	Trichrome-Masson staining	Elastin stainin
48 h	Native			
	5			
	9			
	15			
	21			
	22			
96 h	25			

Figure 3: Hematoxylin and Eosin Staining showed structures and nuclei present in native and decellularized lungs. Trichrome-Masson staining showed collagen levels in native and decellularized lungs (Blue colour). Elastin staining showed elastin levels in native and decellularized lungs (Blue colour to black colour). Representative images for all conditions are shown (100 × magnification)

**Analysis of a 96-h decellularized rat lung by Hematoxylin and Eosin, Trichrome - Masson and Elastin staining**

In Ninety-six hour decellularization, methods 27, 33, 39 and 43 were the best decellularization approaches which methods 27 and 33 maintained the three-dimensional structure at very low levels and a significant reduction were not seen in the nuclei in SDC+SDS and SDC+Triton X-100 approaches, respectively. Methods 39 and 43 preserved the architecture of the lung while eliminating the nuclei in SDC+CHAPS approaches and also, the portions ECM composition (Collagen and Elastin) were decreased with increasing the time from 48 h to 96 h decellularization.

Approach	Methods	Hematoxylin and Eosin Staining	Trichrome-Masson staining	Elastin stainin
96 h	Native			
	27			
	33			
	39			
	43			

Figure 3: Hematoxylin and Eosin Staining showed structures and nuclei present in native and decellularized lungs. Trichrome-Masson staining showed collagen levels in native and decellularized lungs (Blue colour). Elastin staining showed elastin levels in native and decellularized lungs (Blue colour to black colour). Representative images for all conditions are shown (100 × magnification)

**Discussion**

In the percent century, pulmonary diseases are becoming world healthcare problems [27]. Chronic obstructive pulmonary diseases (COPD) such as emphysema and chronic obstructive bronchitis have caused more than 120,000 deaths in adults in the US in one year [28]. The last stage of pulmonary diseases is lung transplantation which has many challenges such as transplant rejection, immunosuppression, and

a shortage of donor organ. Waitlist of lung transplantation is increasing and many patients on the waiting list for lung transplant pass out before suitable donor organ get found because of the critical shortage of transplantable donor lungs [2, 4]. Lung tissue has a complex bio-structure which has various structural, functional compositions [11] and proteins ECM composition including collagen, laminin, elastin, fibronectin and so on [4]. Many types of research have been done for producing a lung scaffold from biomaterial but it has a lot of limitations. Recently, decellularization is appearing as decellularized biomimetic scaffold to support differentiated stem cells which it can be a significant treatment for lung diseases [13, 22]. In this regard, lung organ is decellularized and lung ECM's own cells are removed from the extracellular matrix and also, proteins ECM composition and growth factor are maintained which they are necessary for proliferation, differentiation and migration of stem cells but decreasing them during decellularization can be a major problem [4]. Native extracellular matrix has the optimum substrate for cell attachment, spreading, growth and differentiation.

The goal of the decellularization approach is to remove cellular and nuclear material while maintaining three dimensional and ECM protein compositions and also, reducing any damage to lung ECM. There are several types of decellularization methods such as physical, chemical and enzymatic methods [16, 17]. Physical approaches can destruct the complex lung ECM which this method includes freezing, pressure, sonication and agitation, while enzymatic approaches cannot be cost-effective due to large volumes of liquid would be needed for decellularization of a whole organ such as proteases, nucleases, and calcium chelators [21].

Trypsin has been used in the enzymatic method which it can remove fibronectin, laminin, and elastin. Because of the importance of these compositions, enzymatic approaches have not been mostly used in decellularization. But, chemical approaches are cost-effective and have several of techniques such as alkaline or acidic solutions, detergents (SDS, SDC, CHAPS and ...) hypotonic or hypertonic solutions, and chelators [16, 17, 21]. Jensen et al (2012) studied on the evaluation of 24h decellularization process (Triton-X100 0.1% and sodium deoxycholate 2%) which they showed lung scaffolds with preservation of key ECM proteins collagen I, collagen IV, fibronectin, and laminin, but with the loss of nuclear proteins [13]. Price et al (2015) used pig lung for decellularization by an automated method and compared to manual method and they found that an automated method offered more consistent matrix and reduced the decellularization procedure required time [29]. Neill et al (2013) used CHAPS 8 mM and SDS 1.8 mM for human and porcine decellularization and they found that Porcine lungs could be decellularized using CHAPS to produce lung ECM scaffolds with properties resembling those of human lungs, for

pulmonary tissue engineering and they proposed that porcine lung ECM can be an excellent screening platform for the envisioned human tissue engineering applications of decellularized lungs [30].

In this study, three-time approaches were used for decellularization (24, 48 and 96 h). In 24 h decellularization, method 1 could relatively maintain 3D and remove nuclei but all methods could not preserve Collagen and Elastin and also, there was significant of destruction with increasing the concentration of SDC from 0.5% to 2%. In 48 h decellularization, methods 5, 9, 15 could not preserve the integrity of the 3D and ECM proteins but they were better than other approaches in SDC, SDC+SDS and SDC+Triton X-100, respectively. On the other hand, methods 21, 22 and 25 were the best decellularization approaches which preserved the architecture of the lung while eliminating the nuclei and maintained a considerable of Collagen and Elastin.

In addition, among these approaches, method 22 was remarkable in maintaining of 3D and ECM proteins (Collagen and Elastin). In 96 h decellularization, methods 27 and 33 could not maintain the 3D, Collagen and elastin but they were better than other approaches in SDC+SDS and SDC+Triton X-100, respectively. Methods 39 and 43 preserved the architecture of the lung while eliminating the nuclei and relatively maintained Collagen and Elastin. Maghsoudlou et al (2013) used rat lung for decellularization using the intermittent intra-tracheal flow of detergent-enzymatic treatment and they presented a methodology which was applicable to rat and sheep lungs, preserved lung architecture and ECM and reduced the generating time [31]. Petersen et al (2012), two different techniques were examined using either the detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) or sodium dodecyl sulphate (SDS). CHAPS-based decellularization resulted in the elimination of the majority of cellular components (including DNA) and maintenance of collagen and elastin, whereas SDS-based decellularization caused collagen depletion and less mechanical stability [16].

Cortiella et al (2010) demonstrated that the first attempt to produce and use whole decellularized lung and they compared the influence of decellularized lung by SDS 0.1%, Gelfoam, Matrigel, and a collagen I hydrogel matrix on the mESC attachment, differentiation and subsequent formation of complex tissue and they found that decellularized lung allowed for better retention of cells with more differentiation of mESCs into epithelial and endothelial lineages [32]. In this research, we used single and mixed detergents for decellularization and results showed that destruction was increased with increasing the concentration of detergent. Single detergent showed a significant reduction in maintaining of three-dimensional of lung and ECM proteins (Collagen and Elastin). But, the best methods

were mixed detergents of SDC and CHAPS in low concentration in 48 and 96 h decellularization. On the other hand, there was a considerable loss of Collagen and elastin and also, damage in 3D with increasing time. We conclude that the best method for decellularization was approach 22 which related to SDC 1%+CHAPS 4mM in 48 h decellularization. Finally, engineered scaffold of lung tissue can be used in the laboratory to study a wide variety of important aspects of pulmonary biology and physiology.

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