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Trypsin as enhancement in cyclical tracheal decellularization: Morphological and biophysical characterization

D.M. Giraldo-Gomez [a,b,*], B. Leon-Mancilla [c], M.L. Del Prado-Audelo [b], A. Sotres-Vega [d], J. Villalba-Caloca [d], D. Garciadiego-Cazares [e], M.C. Piña-Barba [b]

[a] Posgrado en Ciencia e Ingeniería de Materiales, Universidad Nacional Autónoma de México (UNAM), Unidad de Posgrado Edificio "C" 1er Piso, Circuito de Posgrados, Avenida Universidad 3000, Ciudad Universitaria, Coyoacán, C.P. 04510, México D. F., México

[b] Instituto de Investigaciones en Materiales, Universidad Nacional Autónoma de México (UNAM), Circuito Exterior, Avenida Universidad 3000, Ciudad Universitaria, Coyoacán, C.P. 04510, México D.F., México

[c] Departamento de Cirugía, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), Edificio "D" Planta Baja, Circuito Interior, Avenida Universidad 3000, Ciudad Universitaria, Coyoacán, C.P. 04510, México D.F., México

[d] Unidad de Trasplante Pulmonar Experimental, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosio Villegas", Calzada de Tlalpan 4502, Sección XVI, Tlalpan, C.P. 14080, México D.F., México

[e] Unidad de Ingeniería de Tejidos Terapia Celular y Medicina Regenerativa, Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra Ibarra", Calzada México Xochimilco 289, Arenal de Guadalupe, Tlalpan, C.P. 14389, México D.F., México

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ABSTRACT

There are different types of tracheal disorders (e.g. cancer, stenosis and fractures). These can cause respiratory failure and lead to death of patients. Several attempts have been made for trachea replacement in order to restore the airway, including anastomosis and implants made from synthetic or natural materials. Tracheal allotransplantation has shown high rejection rates, and decellularization has emerged as a possible solution. Decellularization involves the removal of antigens from cells in the organ or tissue, leaving a matrix that can be used as 3D cell-scaffold. Although this process has been used for tracheal replacement, it usually takes at least two months and time is critical for patients with tracheal disorders. Therefore, there is necessary to develop a tracheal replacement process, which is not only effective, but also quick to prepare. The aim of this research was to develop a faster trachea decellularization protocol using Trypsin enzyme and Ethylenediaminetetraacetic acid (EDTA) as decellularization agents. Three protocols of cyclic trachea decellularization (Protocols A, B, and C) were compared. Following Protocol A (previously described in the literature), 15 consecutive cycles were performed over 32 days. Protocol B (a variation of Protocol A) — EDTA being added — with 15 consecutive cycles performed over 60 days. Finally, Protocol C, with the addition of Trypsin as a decellularization agent, 5 consecutive cycles being performed over 10 days. For the three protocols, hematoxylin–eosin (H&E) staining and DNA residual content quantification were performed to establish the effectiveness of the decellularization process. Scanning Electron Microscopy (SEM) was used to observe the changes in porosity and microarrays. To evaluate the structural matrices integrity, Thermogravimetric Analysis (TGA) and biomechanical test were used. None of the protocols showed significant alteration or degradation in the components of the extracellular matrix (ECM). However, in Protocol C, more cellular components were removed in less time, making it the most efficient process. In addition, the cell tracking and viability was evaluated with chondrocytes seeding on the scaffold obtained by Protocol C, which showed an adequate cell scaffolding ability of this matrix.

1. Introduction

There are different types of tracheal disorders (e.g. cancer, stenosis and fractures), all of which can cause a dynamic collapse with airway obstruction and respiratory failure in the patient [1]. The common treatment for these patients is to remove the damaged tracheal segment. Other palliative solutions include tracheostomy; laser application, tracheal dilation or stent implantation [2,3]. Due to palliative character of these treatments, decellularization has emerged as a complete treatment for tracheal disorders including tumors and malacias [1].

Decellularization is a process to obtain scaffolds of natural origin to replace organs and tissues. The process removes the allogeneic or xenogeneic antigens from cells in the tissue or the organ that could lead to an immune response in the host body. This generates a natural three-

* Corresponding author at: Instituto de Investigaciones en Materiales, Universidad Nacional Autónoma de México (UNAM), Circuito Exterior, Avenida Universidad 3000, Ciudad Universitaria, Coyoacán, C.P. 04510, México D.F., México.
E-mail address: davidmauro2008@gmail.com (D.M. Giraldo-Gomez).

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dimensional scaffold containing all the elements of the extracellular matrix, which can be repopulated with host cells [4].

The first transplant of a partially decellularized human trachea was conducted in 2008, using a trachea from a cadaveric donor. Decellularization was carried out using an enzymatic method [1]. In 2010, Baiguera et al. published a cyclic decellularization protocol for human trachea, where a complete decellularization of the organ was reported after completing 25 cycles. However, this protocol takes around 51 days to perform and time is critical for the patients due to the abovementioned consequences of trachea disorders. It is therefore important to develop a faster decellularization protocol.

The objective of this research was to determine the effect of Ethylenediaminetetraacetic acid (EDTA) and Trypsin on the speed and effectiveness of cyclical decellularization protocols for tracheas. We compared three protocols. First, we reproduced the protocol reported by Baiguera et al. [5] for only 15 cycles. Then, we added EDTA as a step in a second decellularization protocol and carried it out for 15 cycles as comparison [5]. In a third protocol, we added Trypsin and it was performed by 5 cycles. The results of the first two protocols were similar and no degradation was observed in any of the protocols. The addition of Trypsin, however, showed an increase in the speed and effectiveness of decellularization, further the cell tracking marking and the cell viability assay showed a good performance of this matrix as a 3D cell scaffold.

2. Materials and methods

2.1. Trachea harvest

We obtained 6 tracheas from pigs according to the Standard NOM-062-ZOO-1999 [6]. The Ethics Committee of the Medicine Faculty from UNAM authorized these studies.

The tracheas were harvested from 6-month-old (40–50 kg) pigs, and later, the tracheas were cut in four cylinders of 3 to 5 cm in length. Four groups of six trachea segments each were prepared ($n = 6$), one group was used as a control group, and the rest were the experimental groups.

2.2. Decellularization protocols

We carried out three different decellularization protocols: A, B and C. Protocol A corresponds to a protocol described by Baiguera et al. [5], but we changed the total number of cycles from 25 to 15. Protocol B is a variation of Protocol A, where we added incubation in EDTA solution as an extra step, also for 15 cycles. Finally, we designed a third protocol (C), where we added a Trypsin incubation step, but only for 5 cycles to prevent possible adverse effects of the Trypsin on the ECM.

2.2.1. Protocol A

We followed the protocol proposed by Baiguera et al. briefly described as follows: the connective tissue was removed and rinsed with a solution of 1% povidone–iodine in PBS. Then, two rinses were done with a solution of 1% antibiotic–antimycotic (Gibco). Later on, the trachea segment was incubated for 4 h with a solution of 4% sodium deoxycholate (Sigma) at room temperature with continuous stirring. The tissue was rinsed twice with distilled water, and subsequently incubated in 2000 KU DNase-I (Sigma) dissolved in 1 M NaCl for 3 h at room temperature with continuous stirring. Finally, another two more rinses with distilled water were carried out, and later the trachea segments were stored in 1% antibiotic–antimycotic (Gibco) in PBS at 4 °C for 15 cycles.

2.2.2. Protocol B

This protocol is a modification of Protocol A, with an added incubation step in a 10 mM EDTA (Sigma) for 3 h at room temperature. This step was carried out between the first rinse with 1% antibiotic–antimycotic (Gibco) and the incubation in 4% deoxycholate (Sigma). The rest of the process followed as Protocol A for 15 cycles.

2.2.3. Protocol C

We started by removing the connective tissue around the trachea, soaking it in 1% povidone–iodine in Milli-Q water at 4 °C and rinsing it twice with deionized water. We then rinsed the trachea with deionized water, and incubated it in 1% Trypsin (Gibco) in PBS for 3 h at 4 °C. We continued with two rinses in deionized water and then we incubated the tissue in 4% sodium deoxycholate (Sigma) for 4 h at room temperature. We followed with an incubation of trachea with 2000 KU DNase-I (Sigma) in 1 M NaCl, stirring for 3 h at room temperature. Finally, after two rinses with deionized water, we stored the trachea in 1% antibiotic–antimycotic (Gibco) in PBS at 4 °C. We repeated the process 5 times.

2.3. Verification of decellularization trachea protocols

We evaluated the effectiveness of the decellularization protocols through hematoxylin–eosin (H&E) staining and DNA residual content quantification in each sample. We compared the mean measures in decellularized samples with native sample to complete the quantitative evaluation of the decellularization protocols.

To carry out the H&E staining, we performed a gradual dehydration from 70% ethyl alcohol to absolute alcohol in the tissues. Then, we fixed the tissues in Xylene (Merck) and embedded them in paraffin (Sigma). Afterwards, we obtained 5 μm sections of each sample using a rotary microtome (Leica). Finally, we stained the samples with hematoxylin and eosin (Merck).

To quantify the DNA present in the samples, we isolated the DNA of the cells with TRIzol (Invitrogen), and then measured the concentration with an absorbance spectrophotometer (GeneQuant). We used 260 nm wavelength to measure and 260/280 nm wavelength ratio to determine sample purity.

2.4. Biophysical characterization of the decellularized matrices

We used Scanning Electron Microscopy (SEM) for the qualitative assessment of topography samples. To carry out this observation, we fixed the samples using a buffer of 3% (V/V) glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2). Then, we dehydrated samples with ethanol (from 30% to 100%), and, finally, we dried the samples using a critical point dryer CO_2 chamber as previously reported by Rodriguez-Fuentes et al. and Rivera et al. [7,8]. We observed the samples in a SEM DSM-950 (Zeiss) at 15 kV.

We carried out Thermogravimetric Analysis (TGA) to characterize the thermal behavior of the samples and to find any possible degradation in the matrices due to decellularization protocols. To carry out the TGA processes, we freeze-dried all samples at 3.6 Pa and −47 °C in a Freeze Dryer (Labconco FreeZone 1). We used a Thermogravimetric Analyzer (TA Instruments Q500) with a heating rate of 10 °C/min under an argon atmosphere with a temperature range from 35 °C to 500 °C.

We used uniaxial tension test to assess the no alteration on the mechanical properties on trachea ring cartilage due to Trypsin used in Protocol C. We compared the Young's modulus of the decellularized cartilage trachea trough the Protocol C with the native trachea cartilage; we performed the test in a Dynamic Mechanical Analyzer (TA Instruments Q800) in displacement ramp mode with tension clamps mounted. We used 1 mm/min deformation rate and 37 °C constant temperature. We conserved the samples of native cartilage tracheas in a 10% antibiotic–antimitotic in PBS solution and stored at 4 °C for a week changing the solution even 2 days until the test performing for native trachea and 3 days for decellularized samples. We bound the cartilage pieces of sandpaper to facilitate a predefined clamping and to avoid sliding between the tips of the samples and the clamps (Fig. 1). The specimens of the cartilage measured 1.6 ± 0.2 mm in thickness, 5.3 ± 0.9 mm in width and 25 ± 1.5 mm in length. The distance between

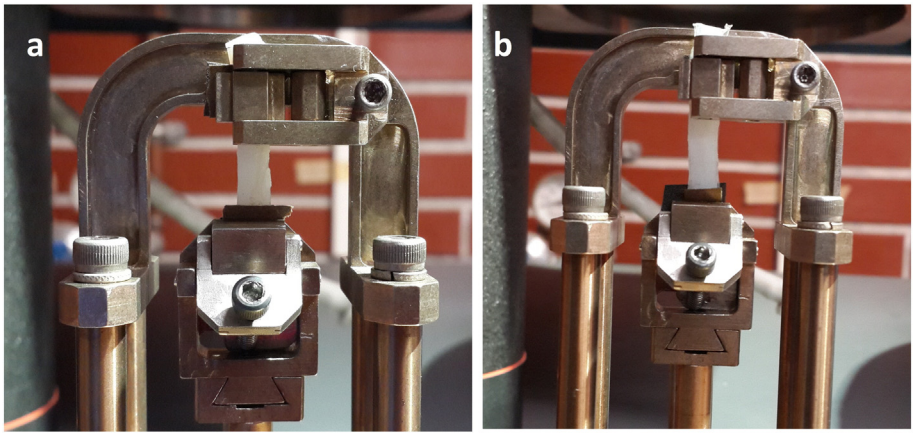

Fig. 1. Experimental setting used to perform the biomechanical test. Tracheal native cartilage ring (a). Traqueal ring decellularized by protocol C (b).

the two reference points was about 18.1 ± 1.7 mm. At least four measurements were performed per each condition.

2.5. Cell-scaffolding ability of the decellularized matrix

We evaluated the answer of the cells to the decellularized matrix with cell tracking and cell viability assays in chondrocytes. We used three samples of decellularized matrices trough the Protocol C to carry out the assay.

2.5.1. Isolation of chondrocytes

We isolated porcine articular chondrocytes from the femoral condyles of 4–6 months old. We isolated cartilage, minced to 3 × 3 × 3 mm pieces and digested with 3 mg/ml collagenase-I. We passed the isolated chondrocytes through a 100-μm nylon cell strainer and centrifuged to obtain a pellet. We plated the chondrocytes at 2×10^4 cells/cm^2 for cell expansion in DMEM-F12 (Gibco) with 10% FBS and 1% penicillin–streptomycin, and incubated at 37 °C with 5% CO_2. We changed culture medium every 36 h. We cultured these cells until the 90% confluence. Then, we lifted the cells using 0.05% Trypsin-EDTA (Gibco), after first passage, we seeded the chondrocytes in the decellularized matrix a second passage.

2.5.2. Cell seeding

We sterilized nine decellularized matrices of 2 rings trachea segments trough UV cross linker during 30 min, then we impregnated the matrices in DMEM-F12 (Gibco) with 10% FBS and 1% penicillin–streptomycin during 30 min, then, we seeding 100,000 cell/cm^2 in the matrices (Fig. 2).

2.5.3. Cell tracking

We selected three matrices to follow the cells trough the matrix. Before the cell seeding, we marked these chondrocytes with Cell Tracker (Invitrogen). We followed the cells during 8 days.

2.5.4. Viability assay

We evaluated viability of monolayer culture as well as constructs of articular chondrocytes and matrices with a Live/Dead viability/cytotoxicity kit for mammalian cells (Molecular Probes), according to the manufacturer's instructions. Fluorescent calcein green signal is positive for viable cells and EthD-1 red signal is positive for dead cells. We captured and analyzed the images with fluorescence microscopy (Axiovision Observer A.1 microscope, Zeiss). We evaluated the constructs (matrix plus cell seeding) after 4 days and 8 days. Three samples each day.

2.6. Statistical analysis

We performed statistical analysis using GraphPad Prism 6.05 statistical software (San Diego). The decellularized groups were compared with a native sample as a control using Dunnett's multiple comparison test analysis of variance. We considered a p-value of <0.05 significant.

3. Results

3.1. Effectiveness of decellularization protocols

The H&E staining results (Fig. 3) showed differences between the native and the decellularized samples. In the native trachea (Fig. 3a), chondrocytes with nuclei were observed inside the lacunae. In Protocol A and Protocol B (Fig. 3b and c, respectively), the presence of some cellular elements was observed in the cartilage matrices, but no nucleus was distinguished. In Protocol C (Fig. 3d), there were no cellular elements inside the lacunae.

The result of DNA residual content quantification (Fig. 4) showed a significant difference in decrease of DNA content between the native trachea and each sample after the decellularization protocols.

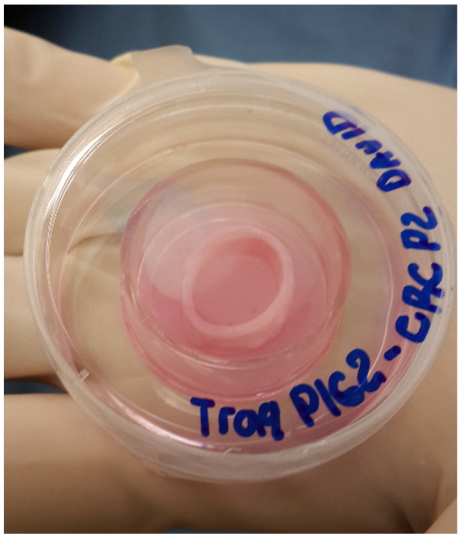

Fig. 2. Decellularized trachea rings seeded with chondrocytes (constructs).

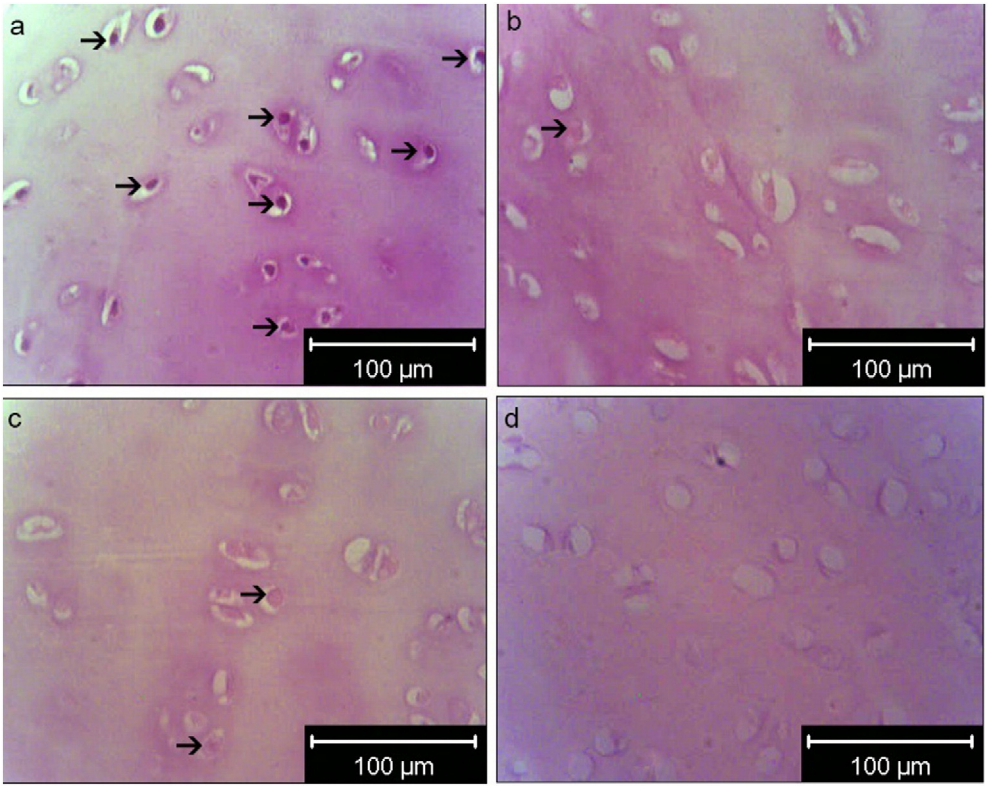

Fig. 3. The micrographs show the tracheal cartilage stained with hematoxylin–eosin (H&E). Chondrocytes with nuclei inside the lacunae of the cartilage matrix in the native sample (a), tracheal cartilage showing some cell structures without nuclei after being subjected to 15 cycles of the decellularization Protocol A (b) and Protocol B (c), sample of tracheal cartilage after 5 cycles of Protocol C, where there is no evidence of cellular material (d). Arrows indicate the presence of chondrocytes embedded in the matrix.

3.2. Morphological and structural integrity of decellularized matrices

The SEM micrographs (Fig. 5) showed the tracheal cartilage of the native and decellularized samples. The native cartilage cross section (Fig. 5a) showed a typical arrangement of this type of matrix, this is, an ECM with chondrocyte cells inside the lacunae. No alteration in the matrices was detected after the protocols and there were no cells present inside the lacunae (Lc) after completion of decellularization protocols (Fig. 5b, c, d, Protocol A, B and C respectively). However, in the Protocol B sample (Fig. 5c) there was some cellular material on the matrix outside the lacunae. The Protocol C micrograph (Fig. 5d) showed that the Collagen fibers (Fr) were preserved from the surrounding tissue of the cartilage.

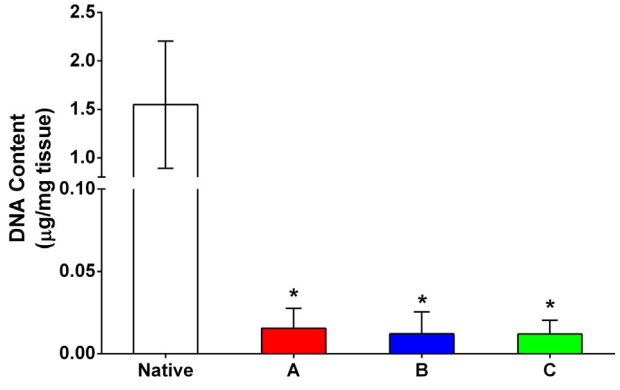

Fig. 4. Quantification of residual DNA. Decellularized groups are compared with a native control using Dunnett's multiple comparison test analysis of variance. A p-value of <0.05 is considered significant. Mean results are shown. Error bars: standard deviation (SD).

The TGA profiles (Fig. 6) obtained from the native and decellularized samples were typical of Collagen (the main component of the matrix) and showed no significant difference between the native trachea and the decellularized samples. These curves (Fig. 6) show a weight loss of all samples of 5% to 10% up to 105 °C. After this temperature, the weight remained virtually constant up until 230 °C. The largest weight loss rate occurred between 230 °C and 380 °C. After this temperature, the weight loss rate decreased again until 500 °C.

The biomechanical result (Fig. 7) obtained from the uniaxial tension tests from native and decellularized samples (only for Protocol C) shown the elastic behavior of the cartilage ring (Fig. 7a) with these plots Young's modulus were calculated from all samples and there was not significance difference after decellularized protocol.

3.3. Cell scaffolding ability

With the cell-tracking assay, there was possible to follow the cell seeding into the scaffold. After 4 days in the matrix (Fig. 8a), the cells retain the characteristic morphology for chondrocytes, furthermore, after 8 days the cell tracking results showed increase in the cell number (Fig. 8b).

In the viability assay, the calcein AM is a cell-permeable molecule, which was turned into fluorescent calcein by esterase activity within live cells and the EthD-1 red signal was positive for dead cells after 4 and 8 days (Fig. 9a and b respectively). After counting individual cells in different fields, viability of chondrocytes after 4 days was found to be about 83%, and 87% after 8 days (Fig. 9c). There are not significant differences between both days.

4. Discussion

EDTA and Trypsin were evaluated as separate decellularization agents in cyclical decellularization protocols for trachea in order to

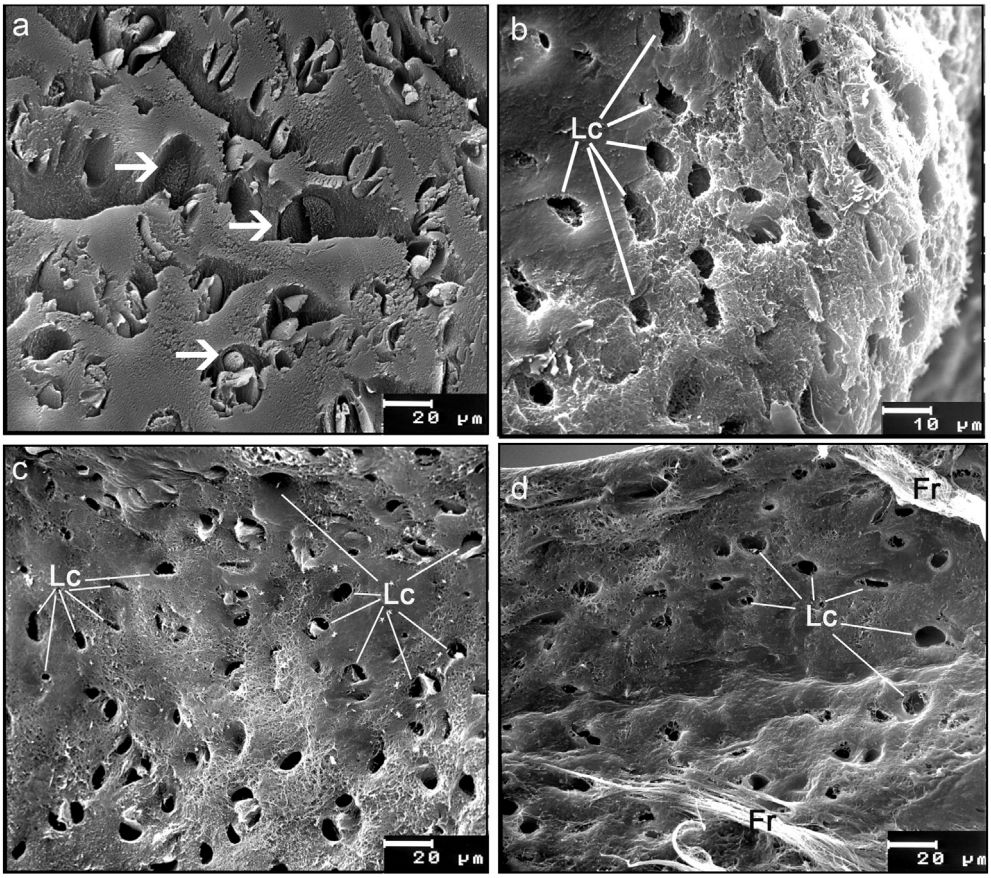

Fig. 5. SEM micrographs of trachea cartilage from native and decellularized samples. Cross section of native trachea where arrows show the chondrocyte cells inside the lacunae (a). Cartilage end section after Protocol A, where lacunae (Lc) are shown without cells (b). It shows the trachea cartilage after following Protocol B, shown presence of some cells in the matrix but not in the lacunae (Lc) (c). Trachea cartilage post-Protocol C, with visible collagen fibers (Fr); there are no cells in the matrix or inside the lacunae (Lc) (d).

determine their effect on the speed and effectiveness of this process. The H&E staining (Fig. 3) showed a decrease in the number of cells after performing each protocol. This histological result was confirmed by residual DNA quantification (Fig. 4), as a significant difference was observed between the native sample and the decellularized samples. This result can be attributed to the synergy generated between the agents used in each decellularization protocol. Sodium deoxycholate, used in three protocols, produced a rupture of the phospholipid cell membrane [9]. DNase-I enzyme, also used in all three protocols, hydrolyzed the DNA chain present in the cell tissue. However, when we used EDTA, no significant difference in the decellularization process was observed between Protocols A and B. This confirms the reports of other authors that this acid is insufficient when used alone [9,10].

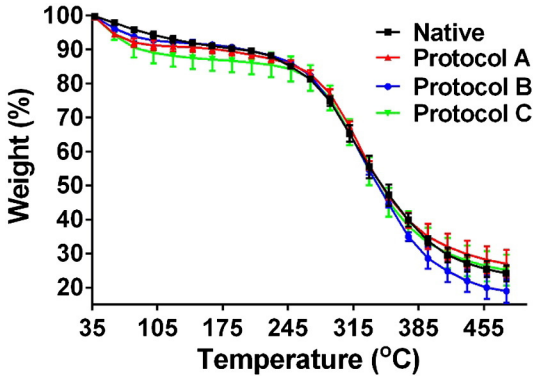

Fig. 6. Analysis of the thermal changes associated with structural changes in the ECMs. The TGA profile shows the evaluation of thermal behavior of native trachea and three decellularization protocols. Mean profile of the all samples is shown. Error bars: standard deviation (SD).

The effectiveness of decellularization process was improved with Trypsin, which was used only in Protocol C. This can be attributed to the loss of attachment activity in Chondronectin [11], because the Chondronectin play an important role in the binding of cells to the extracellular matrix [11,12]. It has been reported that Trypsin can break the bonds of the extracellular matrix and this promotes the degradation of Collagen in the ECM [9,13,14]. However, the observations of porosity and microarchitecture made through SEM (Fig. 5) did not show any alteration in the decellularized samples, even in Protocol C.

The TGA of the ECM's structural integrity (Fig. 6) of all evaluated samples showed a typical curve for Collagen in accordance with previous studies [15,16]. These curves showed two important weight losses. The first one occurred up until 105 °C due to the elimination of absorbed water (water from the environment that a molecule can absorb due to its hygroscopic nature) [17]. The decellularized samples showed more water loss than the native sample, but this difference was not a statistical one. The second significant weight loss occurred between the temperatures of 105 °C and 230 °C due to the decomposition of collagen chains [17,18]. The superposition of the four curves shows that there was no alteration of the thermal stability. This suggests that there was no degradation of decellularized matrices with respect to the native trachea.

Biomechanical evaluation of decellularized scaffolds is an important assessment for the rate of preservation of functional integrity. Numerous studies have shown that biomechanical properties change after decellularization of tissues [19,20,21]. Although the morphology and the thermal behavior of the decellularized matrices did not show any appreciable alteration or modification, there is important to assess the

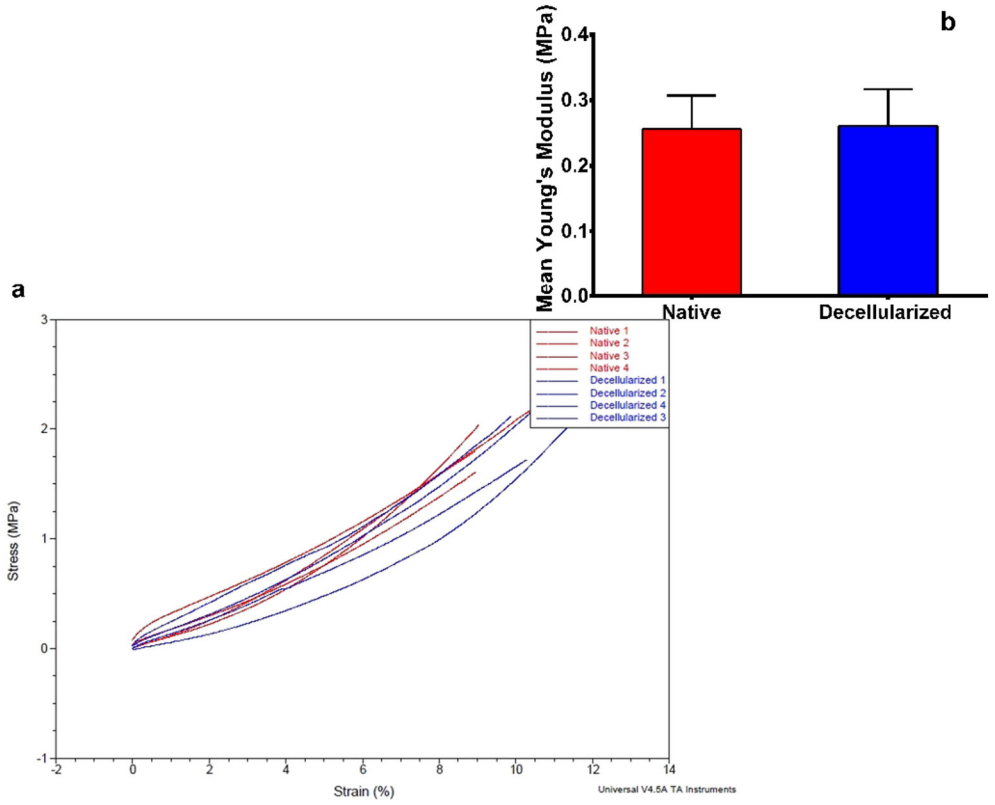

Fig. 7. Biomechanical assessment of the native and Protocol C decellularized trachea rings. Stress–strain curves from the uniaxial tension tests (a). Mean Yung's modulus obtained from the curves of the all samples is shown (b). Error bars: standard deviation (SD). No significant difference. A p-value of <0.05 is considered significant.

biomechanical properties of the decellularized matrix, this is critical in the Protocol C where the Trypsin was used as decellularization agent. By using uniaxial tension test, we measured the elasticity of the decellularized trachea cartilage ECM. Our results demonstrated that in Young's modulus of decellularized trachea cartilage (Fig. 7) there was no modification of the elastic properties of the trachea cartilage after decellularization. Young's modulus as determined from the stress–strain curves was found to be 0.25 ± 0.04 MPa for native trachea cartilage, while the Young's modulus for the decellularized samples (Protocol C) was 0.26 ± 0.05 MPa. This result confirmed the SEM and TGA results, since this showed no significant differences between the tested groups. The fact that no degradation was observed in Protocol C can be attributed to the short periods of incubation in Trypsin.

The ECM of organs is a complex system with fibrous networks of structural and functional molecules including proteins, glycoproteins, and growth factors. The advantage of using decellularized organs as scaffolds is that it is possible to use ECM for not only structural proposes but also for signals that can be exchanged with cells to induce their migration and normal development.

To assess the potential scaffold of acellular trachea matrix for airway regeneration, we performed the cell tracking and viability assays (Figs. 8 and 9 respectively). The decellularized matrix trough Protocol C, which

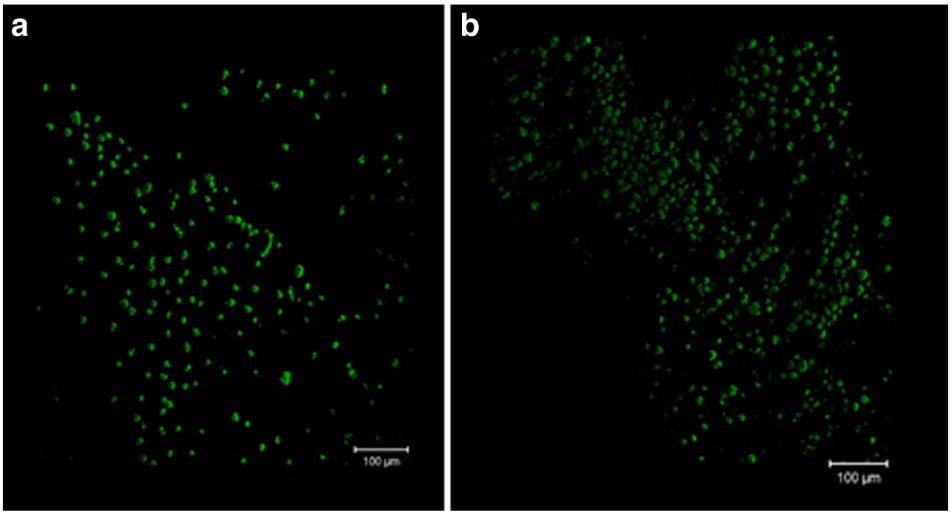

Fig. 8. Chondrocytes presteined with cell tracker and seeded in the decellularized trachea trough Protocol C. After 4 days (a), and after 8 days (b).

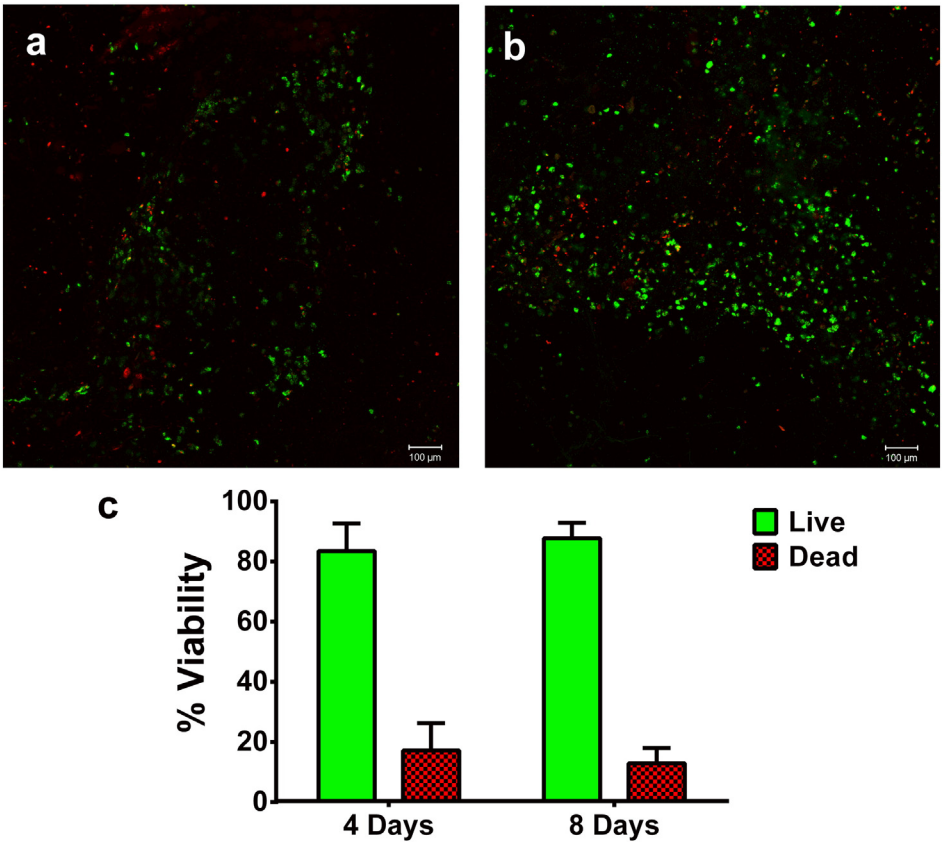

Fig. 9. Cell viability assay in the decellularized trachea trough Protocol C. Chondrocytes after 4 days seeding (a), chondrocytes after 8 days seeding (b), and cell viability was determined to be around 87% for both days evaluated without significant statistical differences between them (c).

were decellularized with Trypsin aid, retained almost the same features as the native trachea. This provides support and biochemical cues for cellular attachment, proliferation, and migration.

Cytocompatibility is one of the main features of a successful in any biomaterial and it is closely related to its chemical composition and structure. The biomaterial surface will interact with chondrocyte promoting cell attachment, as shown by cell tracking analysis (Fig. 8). Furthermore, this result revealed that the chondrocytes remained their characteristic morphology (Fig. 8) and were able to survive, as demonstrated through calcein assay (Fig. 9).

The present study provides an alternative and fast decellularization protocol as a possible platform for trachea regeneration. This study suggests that the basic structural characteristics of decellularized trachea matrix were preserved. The features of the matrix, which are benefit for the reseeded cells to attach and remain viable, were well conserved. In our future studies, we will focus on differentiation of stem cells on decellularized long trachea segments.

5. Conclusions

In summary, the thermal analysis showed that none of the three compared processes had a significantly adverse effect on the structure of ECMs. We confirmed that the use of EDTA has no relevance in decellularization protocols when used alone. The results of H&E staining and DNA residual content quantification showed that Protocol C, where Trypsin was used, was the fastest, requiring only 5 cycles for a complete decellularization process, the Protocol C furthermore remain the biomechanical properties of the ECM, and the chondrocytes assays demonstrated well performance as a cell scaffold.

This study showed that under specific conditions the use of Trypsin as a decellularization agent can allow for a faster complete cyclical tracheal decellularization. These results may aid in the development of a fast working and effective trachea replacement method, which is important because tracheal disorders can swiftly cause respiratory failure and lead to the death of patients. The findings of this study are limited to the effect of EDTA and Trypsin as decellularization agents. Furthermore, they are a starting point and should be verified with longer trachea segments.

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