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Dynamic decellularization and cross-linking of rat tracheal matrix

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ABSTRACT

Decellularized tissues and organs represent a suitable option for tissue engineering when specific scaffolds are needed. However, the optimal conditions to completely remove all the cellular components and minimally affect the biochemical and structural properties of the extracellular matrix are still to be found. For this aim, bioreactors could be an alternative means to dynamically treat the biological samples, automatically controlling all the variables involved in the process and speeding up the entire procedure in order to deal with a suitable scaffold within a limited time period. This paper presents the characterization of rat tracheae decellularized in dynamic conditions, implementing a detergent-enzymatic method, previously considered. Only 6 cycles were enough to generate a tracheal matrix that was histologically and structurally similar to the native one. The network of collagen, reticular and elastic fibers was well preserved, such as the epithelial cilia, the luminal basement membrane and the main matrix components. The elastin content decreased, even if not significantly, after the decellularization protocol. Mechanical properties of the treated tissues were slightly affected by the procedure, and were partially recovered after crosslinking with genipin, a naturally-derived agent. The use of bioreactors could enhance the decellularization procedure of tissues/organs, but a careful selection of the processing parameters is needed in order to prevent large modifications compared to the native condition.

1. Introduction

The optimum bioengineered substitute should have anatomical and morphological structure and chemical and biological cues similar to the native tissue. Biological-derived scaffolds, obtained from decellularized tissues, can be attractive substrates for cellular repopulation as the extracellular matrix (ECM) template should contain appropriate three-dimensional architecture and regional-specific cues for cellular adhesion [1–6]. Therefore, their use has been recently emerged as a promising approach for the development of engineered tissue/organ replacement. Functional decellularized scaffolds preserve the natural ECM composition, do not produce toxic biodegradable products, do not induce inflammation, and may release growth factors and peptides stimulating constructive tissue remodeling. Suitable acellular matrices have been developed from different tissues, such as liver, dermis, bladder, small intestinal submucosa, blood vessels, heart valves, pericardium, and musculoskeletal regions (the temporomandibular joint) [1,7–11].

Tissue engineered functional airway has been obtained starting from both animal and human decellularized matrices [9,12,13]: applying the detergent–enzymatic method (DEM) [14], tracheal acellular matrices, structurally and mechanically similar to native trachea with chemotactic and pro-angiogenic properties, have been developed [15]. Bioengineered human matrices have been implanted in patients with benign and malignant tracheal disease allowing the clinically successful obtainment of a cellular, functional tissue engineered airway [9,16,17]. Similar to other decellularization approaches, DEM is based on the dissolution of cellular membranes and cellular nuclei by an ionic detergent solution (sodium deoxycholate), and on the destruction of nuclear materials by an enzymatic solution (DNase-I). Each DEM step (detergent,

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enzymatic or washing step) is performed by submerging the tissue in the treating solutions and incubating it under agitation provided by an orbital shaker. Therefore, the efficiency of the procedure is dependent on the passive diffusion of solutions into the tissues and on tissue thickness and architecture [18]. To obtain an effective decellularization, long incubation, repeated treatment cycles (e.g., 9 for rats and 25 for humans) and extensive washing steps to not only strip the cellular fragments from the ECM, but also to remove solvent residues are commonly required.

Even if sodium deoxycholate is one of the detergent with less effects on ECM [19,20], its use should be minimized, whenever possible, to avoid damage to the ultrastructure and composition of the native matrix and improve the long-term in vivo outcome of bioengineered grafts. Moreover, the reduction of the time needed to complete the decellularization process can have a significant clinical (and commercial) impact, decreasing production and, hence, patient costs.

Performing a dynamic decellularization process, by using a perfusion bioreactor, could be a suitable approach to reduce the number of cycles (decreasing the period of incubation with the detergent solution) and the time period necessary to produce an acellular biological matrix for tissue engineering applications.

The objective of the present study was to evaluate the efficacy of dynamic DEM decellularization, by using a modified version of a bioreactor, specifically designed for reseeding a matrix before clinical implantation [9], to obtain rat decellularized tracheal grafts.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS), deoxycholate, DNAsi, antibiotic and antimycotic solution, papain, sodium acetate, N-acetyl cysteine and EDTA were supplied by Sigma–Aldrich (Milan, Italy), while paraffin, glutaraldehyde, hematoxylin and eosin (H&E) by Merck (Darmstadt, Germany). Movat pentachromic stain kit was supplied by Diapath (Bergamo, Italy), 4′-6-diamidino-2-phenylindole (DAPI) by Vector Laboratories (CA, USA) and sodium cacodylate buffer (pH 7.2) by Prolabo (Paris, France). Dulbecco's modified Eagle's medium with low glucose (DMEM-LG) was supplied by Gibco-Invitrogen (Milan, Italy), fetal bovine serum (FBS) by Hyclone (SouthLogan, Utah, USA), trypsin/EDTA solution by Eurobio (Courtaboeuf Cedex B, France), while Hank's Balanced Salt Solution (HBSS) by EuroClone (Milan, Italy). Genipin was supplied by Wako Chemicals GmbH (Neuss, Germany) and Lympholyte-H by Cedarlane (Burlington, Ontario, Canada). Nucleic Acid Purification Lysis Solution was supplied by Applied Biosystems (Foster City, CA), PCR Tissue Homogenizing kit from PBI International (Milan, Italy) and Master Pure™ DNA Purification kit from Epicentre Biotechnologies (Madison, WI). Fastin™ elastin assay kit and Blyscan Glycosaminoglycan Assay kit were provided from Biocolor (Carrickfergus, UK), while Vicryl 4-0 sutures by Ethicon (Johnson & Johnson Medical, Rome, Italy).

All materials and reagents were used as received.

2.2. Study design

Male Brown Norway rats ($n = 25$) (Charles River Laboratories Italia S.r.l., Calco, Italy), weighing 230–320 g, were used as donors of trachea and bone marrow. All animals received care in compliance with the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care and Use Committee and the Bioethics Committee of the University of Florence (Italy). Rats were individually housed and maintained at an environmental temperature of 25 ± 2 °C and on a 12/12 h light/dark cycle. Animals were acclimated for 7 days before experiments.

Tracheae were harvested from donor rats. Tracheae ($n = 20$) were used for structural (H&E and Movat staining), morphologic (scanning electron microscopy, SEM), matrix content (elastin and glycosaminoglycan quantification), effectiveness of decellularization (DAPI staining, nuclear counting, DNA quantification), and mechanical property evaluation. Decellularized tracheae ($n = 5$) were crosslinked with genipin (1%) and used for *in vitro* cytocompatibility studies.

Femurs and tibiae were harvested from donor rats ($n = 10$) for bone marrow harvest and used for MSC ($n = 5$) isolation.

2.3. Trachea harvesting

Tracheae were harvested in a sterile fashion, using standard surgical procedures, as previously reported [21]. Briefly, a midline sternotomy was performed to expose the entire trachea from the larynx to the origin of the main bronchi. The trachea was then separated just below the main carina and gently dissected from the esophagus and mediastinal tissue, cut below the larynx and subsequently stored in cold PBS containing 1% antibiotic and antimycotic solution.

2.4. Bioreactor

A modified version of the Bioreactor "InBreath" (BioReactor "InBreath" Low Volume Extension, Harvard Apparatus, Massachusetts, USA), especially designed for reseeding a decellularized human trachea before clinical implantation [9], was used for the dynamic decellularization. The bioreactor system consisted of a polysulphone chamber (about 150 mL) to house the solutions and the sample, a peristaltic pump (REGLO Digital Programmable Peristaltic Pump: REGLO Digital 4 Channel Roller Pump, Harvard Apparatus, Massachusetts, USA) to assure perfusion through the tracheal samples, and a subsystem-controlled DC motor (Easy-to-use Controller, Harvard Apparatus, Massachusetts, USA) to set the rotating speed of the airway along its longitudinal axis.

2.5. Decellularization process

Tracheae were processed by means of the detergent–enzymatic method (DEM) already used for airway decellularization [14,15,21,22]: osmotic lysis (distilled water) for 20 min, detergent cell-extraction (sodium deoxycholate, *Sigma Chemicals, Milan, Italy*) for 2 h, DNA digestion (DNAsi, *Sigma Chemicals, Milan, Italy*) for 3 h. Technically, before loading scaffolds into the bioreactor, tubular stainless steel adapters were inserted 3 mm into each tracheal end and secured with Vicryl 4-0 (Ethicon). The rotating speed of the sample was set at 2.5 rpm, while the decellularization solvent was perfused at an average flow rate of 25 ml/min. 100 mL of the decellularization solution were continuously circulated.

After washing steps, trachea samples were stored in PBS containing 1% antibiotic and antimycotic solution (*Sigma Chemicals, Milan, Italy*) at 4 °C. The protocol was repeated until a complete decellularization was obtained (6 DEM cycles). In order to evaluate the decellularization process, aliquots after each DEM cycle were retrieved and analyzed by H&E staining. Based on histological analysis and cellular content assessment, only the decellularized matrices after 6 cycles were further characterized.

2.6. Genipin treatment

The decellularized tracheae were cross-linked with aqueous genipin solution (1% w/v) buffered with PBS for 1 h at room temperature under constant agitation. The treated tissues were then thoroughly washed with sterile PBS (three changes at least) containing 1% antibiotic and antimycotic solution, one washing cycle lasted for 10 min. The final tissues were stored in PBS containing 1% antibiotic and antimycotic solution at 4 °C.

2.7. Decellularized matrix characterization

2.7.1. Histological analysis

Parts of tracheal samples (native, decellularized and genipin treated) were fixed for 24 h in 10% neutral buffered formalin solution in PBS (pH 7.4) at room temperature. They were washed in distilled water, dehydrated in graded alcohol, embedded in paraffin, and sectioned at 5 μm thickness. Adjacent sections were deparaffinized, rehydrated and stained with H&E to evaluate tissue decellularization and morphology. Each sample was also stained with the Movat pentachromic stain kit, according to the manufacturer's protocols, to evaluate the connective tissues, including cartilage, elastic fibers, collagen, reticulum fibers and muscle.

2.7.2. Assessment of cellular content

To evaluate the remaining cells after DEM, adjacent sections (5 μm thickness) were deparaffinized, rehydrated and stained with DAPI, a fluorescent nucleic acid stain (VECTASHIELD Mounting Medium with DAPI; excitation wavelength 350 nm, emission wavelength 460 nm) for 30 min at room temperature in darkness, and analyzed by fluorescence microscopy.

2.7.3. DNA quantification

To assess DNA quantification within native and decellularized tracheal matrices, samples ($n = 3$ for each condition) were resuspended in 200 μl Nucleic Acid Purification Lysis Solution and homogenized using PCR Tissue Homogenizing kit in microcentrifuge tubes. DNA was isolated using Master Pure™ DNA Purification kit, which is based on a gentle salt-precipitation protocol to allow a rapid purification of nucleic acids, and successively stored at −80 °C. DNA (diluted 1:50) was quantified by measuring the absorbance in a BIORAD spectrophotometer (*SmartSpec™ Plus spectrophotometer, BIORAD, Milan, Italy*).

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2.7.4. Scanning electron microscopy

To qualitatively evaluate decellularized matrix structure, tracheal (native and decellularized) matrices were fixed with 3% (v/v) glutaraldehyde in a buffered solution of 0.1 M sodium cacodylate buffer (pH 7.2). After rinsing in cacodylate buffer, specimens were dehydrated through an ethanol gradient, critical point dried, sputter coated with gold and observed by means of scanning electron microscopy (SEM; JCM-5000 NeoScope, Nikon).

2.7.5. Elastin content measurement

Insoluble elastin was extracted from native and decellularized samples ($n = 4$ for each condition) as soluble cross-linked polypeptide elastin fragments, using the hot oxalic acid extraction technique. Wet samples (mean weight 8.6 ± 2.5 mg and 12.6 ± 2.5 mg for native and decellularized samples, respectively) were mixed with oxalic acid (0.25 M) and boiled in a water bath for 1 h. The supernatant was collected by centrifugation, and the sediment was submitted to a second and third extraction under the same conditions. Soluble elastin content in the oxalic extracts was determined using the colorimetric Fastin™ elastin assay kit, based on a fastin dye reagent (5,10,15,20-tetraphenyl-21,23-pophrine tetrasulfonate), following the manufacturer's instructions. Briefly, samples were added with elastin precipitating reagent, incubated for 15 min and centrifuged. The Dye Reagent was then added to allow the formation of elastin-dye complex. After incubation (90 min) and centrifugation, the elastin-dye complex was dissolved by incubation with the dye dissociation reagent for 10 min. Absorbance was measured at 513 nm on an Epoch Microplate Spectrophotometer (*BioTek*, *VT*, *USA*). Replicate samples were averaged and corrected by subtracting the blank average, and elastin content was determined from a standard curve obtained using five concentrations (5–25 mg) of α-elastin. Final values were expressed as mg of elastin per wet weight.

2.7.6. Sulfated glycosaminoglycan content measurement

Native and decellularized samples ($n = 4$ for each condition) were digested with 0.2 mg/mL papain in 0.2 M phosphate buffer (pH 6.4) containing 0.1 M sodium acetate, 5 mM N-acetyl cysteine and 10 mM EDTA at 65 °C overnight. Total sulfated glycosaminoglycans (GAG) were quantified using the Blyscan Glycosaminoglycan Assay kit, based on 1,9-dimethyl-methylene blue binding, following the manufacturer's instructions. Briefly, samples were added with Blyscan dye reagent, incubated for 30 min and centrifuged. The insoluble GAG-dye complex was then dissolved by adding the dissociation reagent and incubated for 10 min. Absorbance was measured at 656 nm on an Epoch Microplate Spectrophotometer (*BioTek*, *VT*, *USA*). Replicate samples were averaged and corrected by subtracting the blank average, and GAG content was determined from a standard curve obtained using five concentrations (1–5 μg) of GAG. Final values were expressed as μg of GAG per dry weight.

2.7.7. Mechanical tests

Mechanical properties of decellularized and genipin-treated tracheal matrices ($n = 5$ for each condition) were evaluated by means of uniaxial tensile tests. Excised samples were stored in PBS +10% ethanol at 4 °C until evaluation. Tracheae were tested along the long axis up to rupture at 1 mm/min by using a universal testing machine (UTM; Lloyd LRX). All the measurements were carried out directly after withdrawal of the samples out of the storage solution at ambient conditions. In order to prevent sliding during the test, samples were clamped to the UTM grips by means of two strips of emery paper. Because of the complex geometrical structure and the non-homogeneous thickness of the tracheae, engineering stress could not be directly calculated. Therefore, the tensile force acquired during the test was here considered. The strain was defined as the ratio between the grip displacement and the initial gripping distance. Secant modulus, here defined as the ratio force-to-strain at 50%, force at break and strain at break were evaluated. Due to the limitation to properly define the engineering stress and in order to assess the possible consistency of a comparison for the computed mechanical parameters, each sample was measured on the sagittal and frontal planes.

2.8. Biocompatibility analysis

2.8.1. Cell isolation

Rats were euthanized, by an intraperitoneal barbiturate overdose of 150 mg/kg, and the hind limbs harvested. The femur and tibia were cleared of soft tissue and cut at the metaphyses. Bone marrow was flushed out with HBSS. Mesenchymal stromal cells (MSCs) were isolated from rat bone marrow by means of adhesion to cell culture flasks. Bone marrow was centrifuged, and the cell pellets seeded in 75 cm^2 flasks in complete medium (DMEM-LG, supplemented with 20% FBS and 1% Penicillin–Streptomycin). Flasks were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Half of the complete medium was changed after 1 week, and thereafter, the entire medium was changed every 3–4 days. After 14 days in culture, the cells that adhered to the flask were defined as MSCs passage 0. MSC P0 were incubated for 5–10 min at 37 °C with 0.05% trypsin–0.02% EDTA, harvested, washed, and resuspended in complete medium. Expansion of the cells was obtained with successive cycles of trypsinization and reseeding. MSCs, previously characterized [23], cultured above passage 4 were used for the experiments.

Table 1
Descriptive characteristics of rat donors.

Characteristics	Means ± SD
Breed	Brown Norway
Sex	Male
Number	25
Weight (g)	180.5 ± 21.2
Length of retrieved tracheae (cm)	1.66 ± 0.17
Tracheal diameter (cm)	0.36 ± 0.05

2.8.2. Cell culture on evaluated samples

Genipin cross-linked tracheal samples (5 × 5 mm) were sterilized by immersion in 100% v/v ethanol solution for 1 h, dried in laminar hood at room temperature and incubated overnight with HBSS at 37 °C in a humidified atmosphere with 5% CO_2. MSCs (0.5×10^6 cells/cm^2) were seeded on samples, in a 24-well microtiter plate, and cultured under standard conditions for 3 and 7 days.

2.8.3. Construct examination

In order to investigate the cellular adhesion and morphology, after 3 and 7 days incubation period, cellular constructs were washed with PBS, fixed for 24 h in 10% neutral buffered formalin solution (pH 7.4), washed with HBSS and embedded in paraffin. Sections (5 μm thickness) were stained with H&E. Some constructs were fixed with glutaraldehyde (3% (v/v) in a buffered solution of 0.1 M sodium cacodylate buffer), rinsed in cacodylate buffer, dehydrated, critical point dried, sputter coated with gold and observed by means of SEM (JCM-5000 NeoScope, Nikon).

2.9. Statistics

Results are expressed as mean ± standard deviation. Significant differences were estimated by Mann–Whitney *U* test. *p* values less than 0.05 were considered significant.

3. Results

3.1. Matrix characterization

Table 1 reports the characteristics of the animal donors and harvested tracheae.

Rat native trachea is characterized by ciliated epithelium covering the basal membrane, cartilage and muscular tissue (Fig. 1A,B) [24]. Some cellular elements were still visible after 5 DEM cycles (data not shown), while, after 6 DEM cycles, no epithelial, glandular and muscular cells were detected, and only few nucleated cells, with indistinct cell borders, were still visible in the cartilaginous tracheal part (Fig. 1D,E). Moreover, H&E staining suggested that the luminal basement membrane was preserved (Fig. 1D). No nuclear material was detected by DAPI staining (Fig. 1F). Furthermore, DNA quantification showed that approximately 90% of the nuclear material was removed by the decellularization process (1.4 ± 0.1 ng/μl for native and 0.2 ± 0.01 ng/μl for decellularized samples), suggesting that decellularized tracheal matrices were significantly ($p < 0.05$) depleted of DNA contents. Moreover, Movat pentachromic staining showed that the matrix three-dimensional architecture and composition remained intact and almost unaltered, with the preservation of the basement membrane and the epithelial cilia (Fig. 2A,D). SEM analysis showed that acellular tracheal matrix was characterized by intact collagen fibers both on the external and the internal surface (Fig. 2E,F). Additionally, no differences in the quantity of elastin (per wet weight) and sulfated glycosaminoglycan (per dry weight) present in the decellularized samples versus native tissues were evaluated (Fig. 3). In particular, after decellularization, elastin content appeared to decrease, while GAG content to increase, but the differences were not statistically significant (Fig. 3). Taken together, the results suggested that using a dynamic system (perfusion bioreactor) only 6 DEM cycles, instead of 9 cycles using static decellularization approach [21], are sufficient to remove cellular components without damaging the

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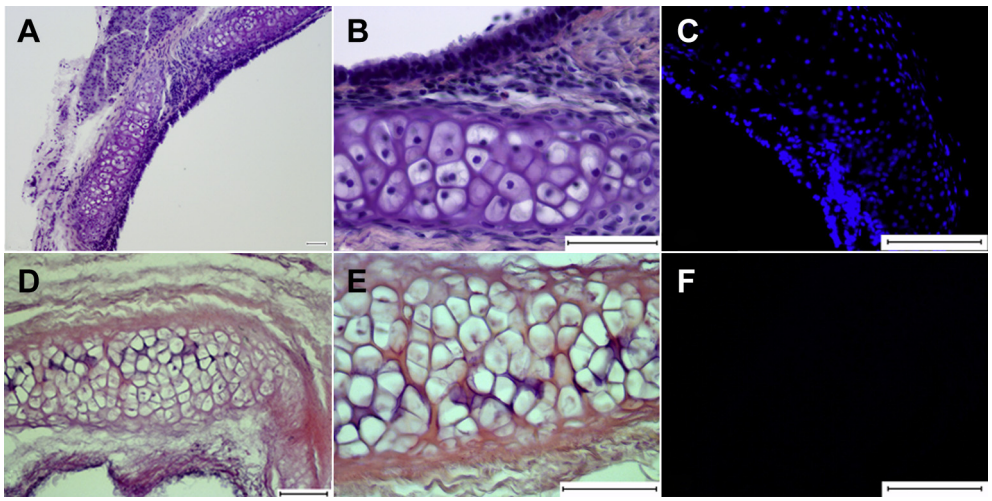

Fig. 1. Characterization of decellularized tracheal matrix. H&E (A, B, D, E), and DAPI (C, F) staining of native (A–C) and decellularized (D–F) tracheas. After 6 DEM cycles, the tracheal matrix resulted to be completely decellularized and no cells and nuclear material were detected (Scale bar = 100 μm).

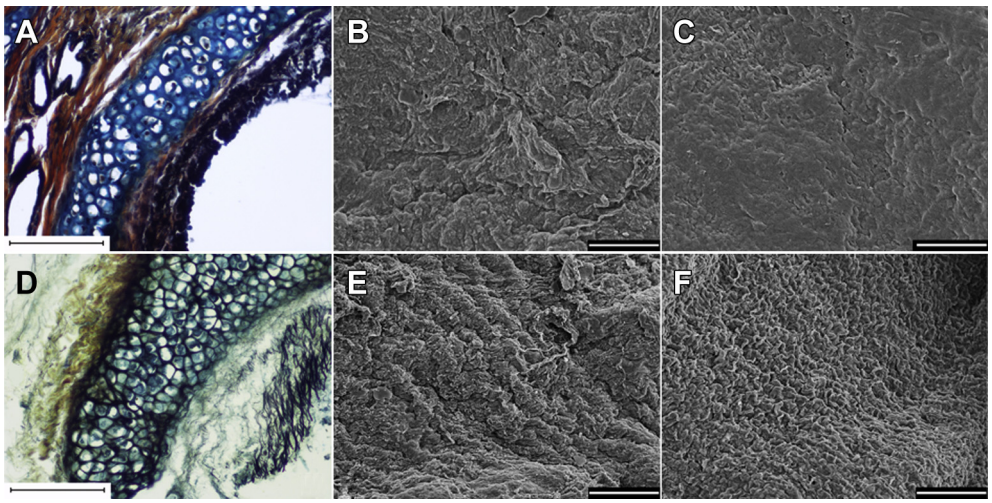

Fig. 2. Movat pentachromic (A, D) staining and SEM micrographs (B,C,E,F) of native (A–C) and decellularized (D–F) tracheas. Black indicates nuclei and elastic fibers; yellow indicates collagen and reticulum fibers. The three-dimensional architecture of tracheal matrix remained intact and unaltered (Panels A, D: scale bar = 100 μm; Panels B,C,E,F: scale bar = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

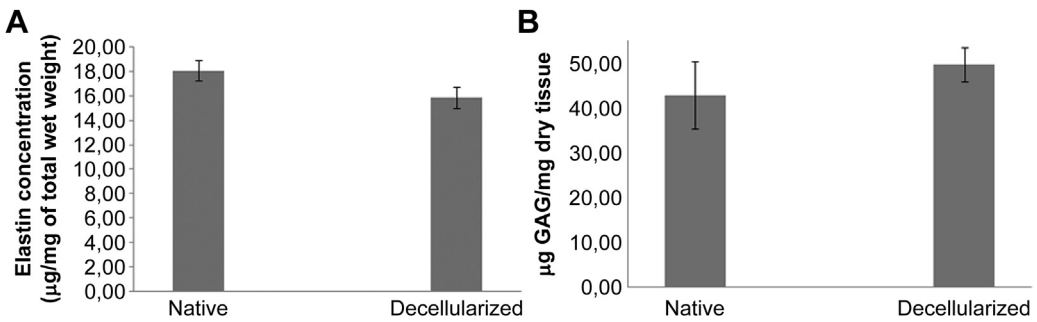

Fig. 3. (A) Elastin content measurement: elastin contents (μg/mg of total wet weight) of native and decellularized trachea. (B) Sulfated glycosaminoglycan (GAG) measurement: GAG contents (μg/mg of total dry weight) of native and decellularized trachea.

original structural components of the tracheal tissue. The network of collagen, reticular fibers and elastic fibers was well preserved, such as the epithelial cilia, the luminal basement membrane and the main matrix components.

3.2. Mechanical properties

The average size of decellularized and cross-linked tracheae was 2.55 ± 0.07 and 2.40 ± 0.36 mm along the sagittal plane, and

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3.75 ± 0.35 and 3.66 ± 0.29 along the frontal plane. The dimensions of tested tracheae were not significantly different ($p > 0.1$), and this allowed to assess the influence of the proposed approach on the mechanical response of decellularized and cross-linked matrices [21].

For this aim, uniaxial tensile tests were carried out on decellularized and genipin-treated tracheal matrices. Fig. 4 shows the representative force–strain curves for the investigated samples, while the calculated mechanical parameters were summarized in Table 2. Tensile curves were characterized by a non-linear, regular behavior up to break. Cross-linked samples showed an improved mechanical response, as average trend, with comparable strain at break values for all the investigated cases. The latter occurrence might suggest that the deformation characteristics of the tissue were not affected by the cross-linking process.

3.3. Biocompatibility analysis

The genipin-treated acellular tracheal matrices revealed an intact total ECM framework, which appeared as a more dense and compact structure compared to decellularized tracheal matrices. Cross-linked matrices resulted cytocompatible for *in vitro* rat MSC cultures (Fig. 5). H&E staining revealed that, after 3 incubation days, cells were mostly present on the luminal side of 1% cross-linked matrices (Fig. 5A). SEM analysis confirmed the results, showing that MSCs were able to adhere to decellularized genipin cross-linked matrices (Fig 5C and E). After 7 incubation days, cells were detected on both sides of tracheal matrices (Fig. 5B, D and F), suggesting that decellularized cross-linked tracheal matrices allowed MSC adhesion, growth and proliferation. Moreover, H&E staining showed the presence of MSCs not only on the external surfaces, where cells have been seeded, but also in the inner part of the cross-linked decellularized samples (Fig. 6), demonstrating that MSCs were able to migrate into tracheal matrix and suggesting an initial tracheal recellularization.

4. Discussion

The goal of decellularizing an allograft is to remove almost all of the cellular components causing immunogenicity, while preserving the structural, biochemical, and mechanical property of the tissue.

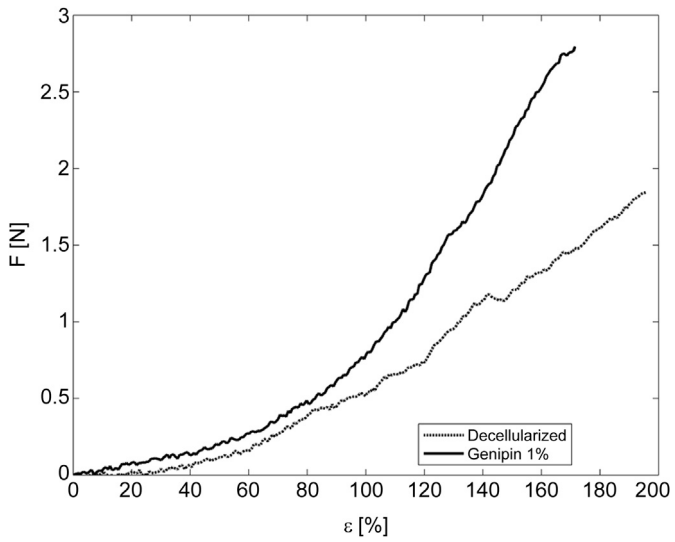

Fig. 4. Force-strain curves representative of dynamically decellularized and genipin-treated rat tracheal matrices.

Table 2
Mechanical properties of dynamically decellularized and genipin-treated rat tracheas.

	Decellularized	Gen 1%
Secant modulus [N]	0.35 ± 0.12	0.44 ± 0.05
F_{max} [N]	1.60 ± 0.24	2.21 ± 0.55
Strain at break [%]	155 ± 40	158 ± 22

We have previously demonstrated that 9 DEM cycles are able to generate decellularized rat tracheal matrices structurally and mechanically similar to native trachea, which exert pro-angiogenic properties and that could represent valid animal models for regenerative airway studies [21].

A standardized "off-the-shelf" product for clinical use should not only be characterized by the correct anatomical, functional and biomechanical characteristics but should also be prepared in a proper time frame: a faster and more effective processing reduces production, and hence patient, costs and increases commercialization prospects. Till now, DEM, as most of the decellularization approaches, has been performed by submerging the tracheal tissues in the treatment solutions followed by incubation under agitation. However, chemical solutions could be perfused through the sample in a quicker way with respect to conventional methods, thus enhancing and fastening the decellularization process. We hypothesized that the use of a bioreactor could improve the delivery efficiency of the treatment solutions into tracheal tissue, with possible reduction of the DEM cycle number and, consequently, the time period necessary to produce acellular biological matrix. The aim of this study was then to assess the effectiveness of combining dynamic decellularization together with the DEM approach for preparing tracheal matrices for tissue engineering applications.

Dynamic approaches have been recently tested to produce acellular matrices: complete cell removal, with concomitant ECM component preservation, has been obtained both in porcine heart valve and in whole rat hearts by perfusion bioreactor [25–27]. A convective flow method (pressure-based system), used for human umbilical veins, resulted to be significantly more effective at removing cells, cellular components, phospholipid and total protein than the traditional agitation model [28]. More recently, a perfusion setup allowed to completely decellularize, in a short period (4 h 20 min), rat aortic roots, preserving the organ's architecture, ECM composition, biomechanics, and obtaining a non-immunogenic scaffold [29]. A bioreactor system was also used to obtain decellularized murine lung matrices, which maintained architecture, spatial structure geometry, and critical ECM components [30]. Moreover, a better outcome was obtained using a perfusion technique, instead of diffusion, in decellularizing sheep livers [31].

To our knowledge, the work described in this article is the first to show that a dynamic system, in association with a detergent–enzymatic treatment, can be used to decellurize rat trachea. Results demonstrated that 6 DEM cycles, instead of 9 necessary when using the shaking bath method [21], were enough to obtain suitable rat acellular tracheal matrices. The tracheal cartilage has an important structural role since rings must maintain an open airway. The mechanical strength to resist compressive forces is the result of functional coupling between GAGs, providing high osmotic pressure, and fibrillar collagen network, providing tensile strength [32,33]. Moreover, GAGs provide the trachea with important binding sites for growth factors [32,33]. Altering the matrix constituents could consequently alter the functional and structural properties. Our results showed that no evident changes in the cartilage and collagen structure were evident after decellularization, demonstrating that the dynamic approach did not

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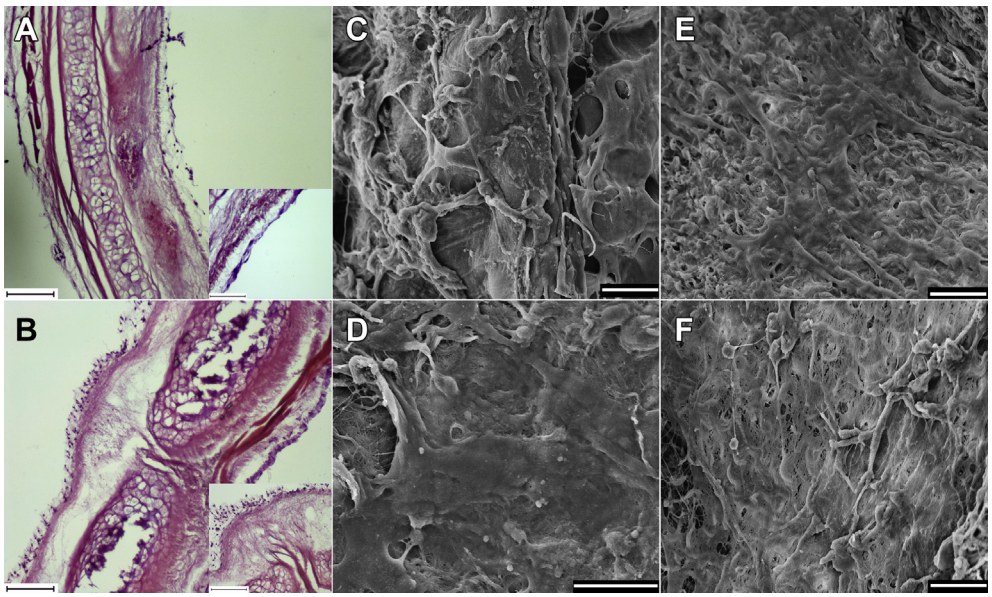

Fig. 5. 1% genipin treated tracheae seeded with rat MSCs for 3 (A,C,E) and 7 (B,D,F) days: H&E staining (A,B) and SEM micrographs (C–F; external (C,D) and internal (E,F) side) revealed the presence of cells on both sides of the tracheal matrix (Scale bar = 20 μm).

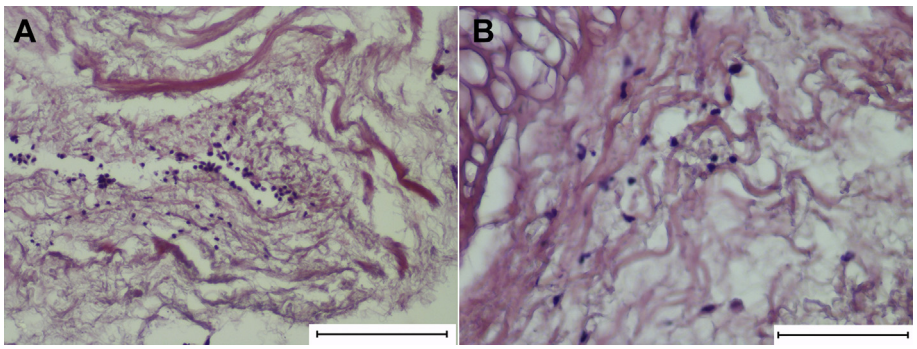

Fig. 6. H&E staining of 1% cross-linked decellularized tracheal matrices seeded with rat MSCs, after 7 incubation days (Scale bar *A* = 100 μm; *B* = 200 μm).

significantly alter the morphology and histoarchitecture of tracheal matrix and that the three-dimensional structure of tracheal ECM was well preserved. Collagen fibers appeared to be slightly loosely packed after the dynamic treatment, possibly due to the solution flow through the tracheal tissues. However, matrix content evaluation revealed that decellularized matrices were characterized by the same concentration of ECM components found in the native trachea, suggesting that dynamic DEM process was able to retain elastin and sulfated GAGs, which have been shown to have an effect on cell behavior [32,33].

Starting from these results and based on previously reported data [21], we have then evaluated the possibility to improve decellularized tracheal matrix performances using genipin, a naturally derived cross-linking agent. We compared the mechanical data herein obtained to those previously acquired, related to native, "statically" decellularized and genipin cross-linked rat tracheae [21]. Matrices decellularized using the dynamic approach resulted to be characterized by increased secant modulus and decreased force and strain at break. This result might suggest that the dynamic procedure used to decellularize the trachea influenced the mechanical performance, especially referring to the ultimate stage before break. Interestingly, the initial behavior, that might lie within the physiologic range, was characterized by an enhancement after the dynamic decellularization with respect to the static approach (0.15 ± 0.01 N), that was further improved after the genipin treatment, resulting comparable to the analogous case previously reported (i.e., the statically decellularized rat tracheae) (0.47 ± 0.06 N) [21]. A decrease in the mechanical performance after decellularization by using perfusion protocols was previously assessed investigating different organs. Sheep livers were generally characterized by lower mechanical properties, depending also on the different decellularization protocols evaluated [31]. Even if not significant, both the maximum force and the extension at maximum force, assessed by ball burst mechanical testing, decreased for cardiac ECM ventricle porcine hearts decellularized by means of pulsatile retrograde aortic perfusion [27]. The mechanical characterization, here presented, demonstrated that the dynamic decellularization might affect the behavior of the microstructure of the biological samples, in particular influencing the contribution of the elastin, which is generally exerted in the initial portion of the stress (force)–strain curve (toe region). This occurrence can therefore explain the overall limited deformation of the biological samples, suggesting the main role of collagen on the final mechanical response.

To determine whether decellularized and genipin-treated tracheal matrices were cell compatible and capable of supporting

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cell growth, rat MSCs were cultured for 3 and 7 days. The cross-linked decellularized tracheal matrices showed no toxicity to cells: cells adhered, grown and proliferated extensively on both sides of tracheal matrices. Moreover, the presence of MSCs also in the inner part of seeded tracheal matrices, after *in vitro* culturing, suggested that dynamic DEM approach and the subsequent cross-linking treatment did not remove and/or damage matrix binding sites/proteins necessary for tracheal recellularization.

5. Conclusions

The here reported data suggest that a dynamic system can be used to reduce the number of DEM cycles, and consequently the processing time, necessary to obtain decellularized tracheal matrices. Moreover, this approach, in association with a cross-linking process, can be sufficient to provide a scaffold that retains an adequate microstructure and a mechanical response for tissue engineering applications. Further studies will be necessary to develop multistep processes that can be housed within a single bioreactor to decellularize, seed, and culture recellularized constructs.

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