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# Tissue engineered human tracheas for *in vivo* implantation

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ABSTRACT

Two years ago we performed the first clinical successful transplantation of a fully tissue engineered trachea. Despite the clinically positive outcome, the graft production took almost 3 months, a not feasible period of time for patients with the need of an urgent transplantation. We have then improved decellularization process and herein, for the first time, we completely describe and characterize the obtainment of human tracheal bioactive supports. Histological and molecular biology analysis demonstrated that all cellular components and nuclear material were removed and quantitative PCR confirmed it. SEM analysis revealed that the decellularized matrices retained the hierarchical structures of native trachea, and biomechanical tests showed that decellularization approach did not led to any influence on tracheal morphological and mechanical properties. Moreover immunohistological staining showed the preservation of angiogenic factors and angiogenic assays demonstrated that acellular human tracheal scaffolds exert an *in vitro* chemoactive action and induce strong *in vivo* angiogenic response (CAM analysis). We are now able to obtained, in a short and clinically useful time (approximately 3 weeks), a bioengineered trachea that is structurally and mechanically similar to native trachea, which exert chemotactive and pro-angiogenic properties and which could be successfully used for clinical tissue engineered airway clinical replacements.



## 1. Introduction

Extensive damage to the trachea (windpipe) can result in considerable reduction in quality of life with problems of breathing, speaking and swallowing. Unfortunately, there is no good conventional solution to advanced disorders of the windpipe. There has been considerable progress in the translation of tissue engineered organs, repopulated with cells or stem cells, into the clinic in the last few years. The *in vivo* tissue engineering concept, a very promising and simple strategy, that conforms to global regulatory issues, represents a real promise for performing bedside, elective or urgent, tracheal engineering replacements [1,2]. Such an approach is based on the intraoperatively replacement of the native airway using a scaffold (which plays the role of a biological incubator) and specific environmental signals: boosting host (stem) cells mobilization allows tissue regeneration in the patient (which plays the role of a natural bioreactor) with cell proliferation, migration and commitment within the implanted polymer. Along with suitable co-stimulation, the use of an appropriate scaffold plays a major role in achieving strong, high quality remodelling activity. The optimum bioengineered substitute should have anatomical and morphological structure and chemical and biological cues similar to the native tissue. Moreover, the viable "new" tissue should have the capacity to self-repair, remodel, revascularise and regenerate, along with the ability to grow without the risk of rejection. Recently, regenerative studies have focused attention on biological scaffolds derived from decellularized tissues and organs which, maintaining the natural extracellular matrix (ECM) composition without releasing toxic biodegradable products or inducing inflammation, can provide attractive scaffolds for cellular repopulation as the extracellular matrix template should contain appropriate three-dimensional architecture and regional-specific cues for cellular adhesion [3–8]. The method used to decellularize tissues is crucial as all cells must be removed to avoid any immune response post-implantation, but structural components and ultrastructure of the remaining ECM tissue must be preserved to provide a scaffold with good biomechanical characteristic and to foster efficient reseeding [9]. Tissues may respond variably to processing because of differences in ECM

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structure, which sometimes makes it difficult to expose cells and to remove the cellular material with minimal tissue disruption. Starting with these considerations in mind, decellularized matrices obtained from animal tissues have been studied as scaffolds for a tissue engineered functional trachea [10–12] and resulted in an ideal environment for cells, without any induction of an immunological response when implanted as allograft and xenograft constructs [13]. Based on these results, a bioengineered human tracheal matrix has been created allowing the clinically successful obtainment of a cellular, functional tissue engineered airway [14]. Herein, we demonstrated that the bioengineered matrix results to have native biological and biomechanical characteristics. Moreover, starting from previous studies which reported that acellular matrices maintained the expression of angiogenic proteins (such as bFGF and TGF-β) and exerted strong angiogenic responses [5,7,15,16] the expression of such proteins and the *in vitro* and *in vivo* angiogenic response induced by human bioengineered trachea were investigated.

## 2. Materials and methods

### 2.1. Matrix obtainment and characterization

#### 2.1.1. Preparation of bioengineered human airway matrices

Cadaveric tracheas and adjacent cricoid cartilage were retrieved from 9 donors (donor characteristics are reported in Table 1) and treated according to the detergent-enzymatic method (DEM) [17]. All connective tissue was stripped off, and the trachea rinsed in phosphate buffered saline (PBS), containing 1% povidone-iodine (PVPI) (*Sigma Chemicals, Barcelona, Spain*). Tissue was rinsed twice in distilled water (containing 1% PVPI), twice in Milli-Q water containing 1% antibiotic and antimycotic (*Sigma Chemicals, Barcelona, Spain*), then incubated in 4% sodium deoxycholate (*Sigma Chemicals, Barcelona, Spain*) solution diluted in distilled water and continuously shaken for 4 h at room temperature. After two wash steps with distilled water, to remove residual cell detritus, trachea was incubated in 2000 KU (Kunitz Units) DNase-I in 1 M NaCl (*Sigma Chemicals, Barcelona, Spain*) and gently shaken for 3 h at room temperature, to solubilize nuclear contents and degrade DNA. After two further wash steps with distilled water, trachea samples were stored in PBS containing 1% antibiotic and antimycotic solution at 4 °C. The decellularization protocol was repeated for the number of cycles necessary to obtain complete organ decellularization (25 DEM cycles).

#### 2.1.2. Histological analysis

Samples 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 (Merck, Darmstadt, Germany), and sectioned at 5 μm. Adjacent sections were stained with haematoxylin and eosin stain (H&E) (Merck, Darmstadt, Germany).

#### 2.1.3. DNA analysis

To assess total DNA content within native tracheas and bioengineered matrices, specimens (90 mg) were disintegrated and homogenized in 1 mL of denaturing solution using TISSUE LYSER (Qiagen Inc.) (4 cycles at max speed for 2 min). The lysate was mixed with chloroform and centrifuged to 15,000 rpm for 15 min. After phenol/chloroform extraction, the DNA was precipitated from the acqueous phase with isopropanol, washed with 75% ethanol and air dried. The pellet was then dissolved in ribonuclease-free water and stored +4 °C. DNA content was qualitatively evaluated by amplifying genomic DNA (1–5 μL) using β-actin primers. The PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed using 10 μM of each primer, 200 μM each dNTP and 2.5 U of puRe*Taq*™ DNA-polymerase (Amersham Biosciences Corp) in a final volume of 25 μL. Parallel positive (using a known genomic DNA) and negative (without added DNA) samples were performed. The PCRs were performed with a standard thermal profile and appropriate annealing temperature (60 °C) for 30 cycles. The PCR products (5 μL) were electrophoresed on agarose gels, stained with ethidium bromure at 100 V and visualized with ultraviolet transillumination.

**Table 1**
Descriptive characteristics of donors ($n = 9$) of trachea.

| Characteristics | Means ± SD |
|---|---|
| *Donor* | |
| Sex | Female ($n = 6$); Male ($n = 3$) |
| Age (years) | 45.78 ± 8.93 |
| Height (cm) | 159.80 ± 5.26 |
| Weight (kg) | 61.60 ± 5.22 |
| Cause of death | Cerebral haemorrhage ($n = 5$); Heart attack ($n = 2$); Cranial trauma ($n = 2$) |

#### 2.1.4. DNA quantification

Genomic DNA quantification was performed by the measurement of the amplicon copy number using a quantitative PCR approach combined with the flexibility of SYBR Green I detection. The analysis was performed by using the Roto-Gene 6000 (Cobett Bobotics Inc. San Francisco USA). Reaction mixture contained 10 μM of each β-actin primer and 5 μL of 2X QuantiTect™ SYBR® Green PCR Master Mix (Qiagen Inc.), which includes the HotStarTaq™ DNA-Polymerase in an optimized buffer, the dNTP mix and the SYBR® Green I fluorescent dye. Each assay included dilutions of control genomic DNA in a final concentration of $10^8$, $10^6$, $10^4$, $10^2$, $10^1$ number of DNA copies, a no-template control (NTC), or the DNA (2 μL) of native or bioengineered tracheas in duplicate ($n = 2$) in a total volume of 25 μL. Thermal cycling conditions used were an initial DNA denaturing step at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 25 s. Finally, melt-curve analysis was performed by slowly heating the PCRs to 95 °C (0–3 °C per cycle) with simultaneous measurement of the SYBR Green I signal intensity. Product amount was measured by interpolation from standard curve of genomic DNA.

#### 2.1.5. Scanning electron microscopy

To qualitatively evaluate decellularized matrix structure, matrices were fixed with 3% (v/v) gluteraldehyde (*Merck, Darmstadt, Germany*) in a buffered solution of 0.1 M sodium cacodylate buffer (pH 7.2) (*Prolabo, Paris, France*). After rinsing in cacodylate buffer, specimens were dehydrated through an ethanol gradient, critical point dried, sputter coated with gold and analyzed using Leo Supra 35 microscope.

### 2.2. Biomechanical tests

Following our previously published methods [13], both native tracheas and bioengineered matrices were tested *via* a tensile-test device (Zwick/Roell®, version Z0.5TS, Barcelona, Spain) for its strain characteristics. Tissue was subjected to increasing uniaxial tensile testing until rupture, confirmed by the loss of load and the appearance of tears in the tissue. The specimens were clamped into sample holders, a pre-load (preliminary force) of 2 N was applied and the trial started at a constant elongation rate of 1 mm/s at room temperature. The tensile tester recorded in real-time the load and the elongation to which the tissue was subjected. Parameters such as maximum force (N), rupture force (N), tracheal rupture point (cm) and tissue deformation (%) were recorded.

### 2.3. Pro-angiogenic properties of bioengineered tracheal matrices

#### 2.3.1. bFGF immunohistochemical analysis

Tissue sections (5 μm) were deparaffinised, rehydrated, and rinsed in PBS with 0.1% Triton X-100 for 10 min. Sections were then blocked with 10% (v/v) fetal calf serum (FCS) in PBS for 1 h at room temperature and incubated with primary polyclonal antibody anti-FGF2 (*Santa Cruz, Ca*) at a 1:200 dilution in blocking buffer for 1 h at room temperature. The samples were then labelled using the avidin-biotin amplified immunoperoxidase method, using the Large Volume Dako LSAB Peroxidase kit (*Glostrup, Denmark*), counterstained with haematoxylin, mounted and examined under an optical Laborlux S microscope (*Leitz, Seattle, WA*). Negative controls were carried out by similarly treating cultures omitting the primary antibody.

#### 2.3.2. Endothelial cell migration assay (in vitro)

To evaluate *in vitro* chemoattractant properties of bioengineered tracheal matrices, the human umbilical vein endothelial cells (HUVEC) migration assay was performed using Transwell polycarbonate filters of 5.0 μm pore size (*Corning Life Sciences, Amsterdam, Netherlands*). "Non-cartilaginous" and "cartilaginous" samples of acellular tracheas were cut, weighed and placed in the lower compartment of the chamber with 0.5 ml endothelial cell basal medium MV (*PromoCell, Heidelberg, Germany*) containing 1% FCS. Endothelial cell basal medium MV containing 1% FCS and endothelial cell basal medium MV2 (*PromoCell, Heidelberg, Germany*) were used respectively as negative and positive controls. HUVEC were then seeded ($5 \times 10^4$ cells/cm$^2$) in the upper compartment in 1% FCS basal medium. The plates were incubated for 4 h at 37 °C. The filters were then removed, the upper surface was scraped, and cells that migrated towards the chemoattractant, remaining on the underside of the membrane, were stained with 4'-6-diamidino-2-phenylindole (DAPI). Five fields at 5× magnification were observed by fluorescence microscopy. The results were normalized according to equivalent tracheal samples weight in mg. Each sample was tested in triplicate.

#### 2.3.3. Chicken chorioallantoic membrane (CAM) angiogenic assay (in vivo)

The chicken embryo chorioallantoic membrane (CAM) assay was used as an *in vivo* model for evaluating the angiogenic properties of human bioengineered tracheas. As previously reported [18,19], fertilised chicken eggs (Henry Stewart and Co., UK) were incubated at 37 °C at constant humidity. At day 3 of incubation, a false