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Development of bioengineered human larynx

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ABSTRACT

To date, only two human laryngeal allotransplants have been reported and, although they were successful, both patients required life-long immunosuppression. A bioengineered human larynx could represent a possible alternative to allotransplantation. Human larynxes were decellularized enzymatically to obtain acellular matrices. Histological and molecular analysis demonstrated that all cellular components and nuclear material were removed. SEM showed that decellularized matrices retained the hierarchical structures of the native larynx, and mechanical tests demonstrated that the decellularization did not significantly impaired the biomechanically properties of the obtained matrices. Immunohistochemical staining found residual angiogenic factors after decellularization, and CAM analysis demonstrated that acellular laryngeal scaffolds induce a strong *in vivo* angiogenic response. Using a decellularization method, we are now able to obtain, in a short and clinically useful time, natural bioengineered laryngeal scaffolds which could be use for partial or total implantation in humans.

1. Introduction

The larynx is a finely tuned sphincter that orchestrates swallowing, breathing, coughing and voice. In 2006, there were 11,826 new cases of laryngeal cancer in EU [1], and a smaller, but severely impaired, group of patients with non-functioning larynxes due to benign disease and trauma [2]. Since conventional reconstruction and prosthetics have failed to replace the complex functions of the human larynx, laryngectomy is, at the moment, the only possible therapeutical option for patients with laryngeal advanced trauma.

An attractive therapeutical alternative to laryngectomy, in patients with irreversible laryngeal disease, would be either total or partial laryngeal transplantation. To date, only two documented laryngeal transplants have been performed on humans [3,4]. Both required life-long immunosuppression, and this may explain why this procedure has not gained worldwide acceptance [5]. More recently, it has been demonstrated that most of the larynx can be removed with the preservation of one muscle-nerve-joint unit and, as a consequence, without the need of neuromuscular activity regeneration [6,7]. However, besides the good breathing outcome, voice and swallowing remain sub-optimal, due to the lack of a truly laryngeal architecture. It is not unrealistic to postulate, therefore, that the availability of natural or synthetic substitutes displaying equivalent anatomical, physiological and biomechanical properties of normal human larynxes would provide the right, complex architecture and dynamics for normal voice production and sphincter action.

In recent years, there has been considerable progress in the translation of tissue engineered organs into the clinic [8,9], and decellularized tissues and organs have been successfully used as scaffolds for engineering a variety of tissues, including heart, liver and trachea [10–15]. Unlike scaffolds made of synthetic materials, natural matrices, consisting mainly of extracellular matrix (ECM), are degraded by cellular enzymatic activity, releasing growth factors and peptides that could stimulate constructive tissue remodeling. Decellularized matrices have been recently considered

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for laryngeal regeneration [16–18]. Partial hemilaryngectomies in a canine model were reconstructed using porcine decellularized urinary bladder matrix [16], obtaining the regeneration of thyroid cartilage, epithelium, connective tissue, glandular structures and some skeletal muscles. The ECM-based repair resulted superior to that observed using control standard procedure [17], suggesting that ECM scaffolds could be promising templates also for constructive remodeling of laryngeal tissue [16,17].

Although the ECM scaffold tends to facilitate tissue repair, the method of its preparation (decellularization approach) can dramatically alter the biomechanical properties of the resulting scaffold, compromising its ability to provide mechanical support during the remodeling process as well as altering the host remodeling response [10]. Using a detergent-enzymatic approach, we have successfully obtained a bioengineered human tracheal matrix, structurally and mechanically similar to native trachea and containing angiogenic factors which exert chemotactive and pro-angiogenic properties [19]. This human tracheal bioactive support provided a natural environment for cellular growth and differentiation [20,21] and allowed us to perform the first-in-man transplantation of fully tissue-engineered organ (windpipe) [14].

The success of our study led us to hypothesize that the best regenerative substitute would be the natural laryngeal ECM, which contain all the cytoskeleton components necessary to provide structural, biochemical and biophysical requirements for suitable laryngeal remodeling. We have therefore applied a decellularization approach to human larynges to obtain bioengineered scaffold for *in vivo* partial or sub-optimal larynx regeneration. Herein we describe the complete biological, mechanical and pro-angiogenic characterization of decellularized human laryngeal matrices with the ultimate goal to explore the functional solution for larynx regeneration without the risk of rejection.

2. Materials and methods

2.1. Matrix obtainment and characterization

Cadaveric larynxes, upon signed informed consent by the relatives and protocol approval from the Ethic Commission of University Hospital Careggi (Italy) and Italian National Transplant Service, were retrieved from 5 donors (Table 1). The eligibility criteria for harvesting were: donor age <40 years, absence of structural abnormalities, neck trauma, previous neck surgery (tracheotomy) and no presence of infection.

2.1.1. Preparation of decellularized human laryngeal matrices

Larynxes were treated according to our modified version of the detergent-enzymatic method (DEM) to obtain bioengineered human tracheas [19,22]. All connective tissue was stripped off, and the larynxes rinsed in phosphate buffered saline (PBS), containing 1% povidone-iodine (PVPI) (*Sigma Chemicals, Barcelona, Spain*). Organs were 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, larynxes were incubated in 2000 KU (Kunitz Units) DNase-I in 1 M NaCl (*Sigma Chemicals, Barcelona, Spain*) to remove residual cell detritus, and gently shaken for 3 h at room temperature, to solubilize nuclear contents and degrade DNA. After two further wash steps with distilled water, larynx samples were stored in PBS containing 1% antibiotic and antimycotic solution at 4 °C. The decellularization protocol was repeated for 25 number of cycles. In order to carefully evaluate the decellularization process, aliquots of different parts of the larynx (epiglottis, false vocal cords, vocal cords, cricoid and thyroid cartilage) were retrieved and characterized.

Table 1
Descriptive characteristics of donors ($n = 5$) of larynx.

Characteristics	Means ± SD
Donor	
Sex	Female ($n = 1$); Male ($n = 4$)
Age (years)	45,78 ± 8,93
Height (cm)	159,80 ± 5,26
Weight (kg)	61,60 ± 5,22
Cause of death	Heart attack ($n = 2$); Cranial trauma ($n = 2$); Road accident ($n = 1$)

2.1.2. Histological and immunohistochemical analysis

Aliquots of the larynx were fixed for 24 h in 10% buffered formalin at room temperature. They were washed in distilled water, dehydrated in graded alcohol, embedded in paraffin (*Merck, Darmstadt, Germany*), and sectioned at 5 μm thickness. Adjacent sections were cut and stained with hematoxylin and eosin (H&E) (*Merck, Darmstadt, Germany*) to evaluate tissue decellularization and morphologic changes. The presence of MHC markers was evaluated by immunohistochemical analysis. The sections were deparaffinized in Bio-Clear (*Bio-Optica, Milano, Italy*) and hydrated with grade ethanol concentrations until distilled water. Antigen retrieval was routinely performed by immersing the slides in thermostatic bath containing pre-heated EDTA Buffer (1 mM EDTA, 0.05% Tween 20, pH 8.0) for 30 min at 98 °C and cooling down at room temperature for 20 min. To block endogenous peroxidase activity, the slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 min. After blocking non specific antigen with normal horse serum (*UltraVision, Lab Vision, Fremont, CA*), the sections were incubated for 60 min at room temperature with monoclonal anti-HLA Class 1 ABC antibody (clone EMR8-5, 1:50 dilution, Abcam Inc., Cambridge, MA) in Antibody Diluent (Ventana). Staining was achieved using EnVision™+ System Peroxidase (*Dako Denmark A/S, Glostrup, DK*) for 30 min at room temperature. Signal was detected using 3.3′diaminobenzidine (*Dako*) as chromogen. Nuclei were slightly counterstained with Mayer's hematoxylin. Negative controls were performed by substituting the primary antibody with non-immune serum immunoglobulins at the same concentration as the primary antibody. Control sections were treated at the same time as sample sections.

2.1.3. DNA analysis

To assess total DNA content within native and decellularized laryngeal 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 aqueous 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 2.5U 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.

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 (*CobettBobotics 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 decellularized larynxes 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. Mechanical tests

Mechanical characteristics of native and bioengineered epiglottis, cricoid and thyroid cartilages were evaluated by means of uniaxial tensile tests. Excised samples were stored in PBS at 4 °C until evaluation and tested to rupture at 1 mm/min by using an universal testing machine (UTM) equipped with a 100 N load cell (Lloyd LRX). All the measurements were carried out directly after withdrawal out of PBS at