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Preservation over time of dried acellular esophageal matrix

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Abstract

Preservation of acellular matrices represents a big challenge for the improvement of tissue engineering. In this work, a new method to preserve over time a decellularized esophageal scaffolds was explored. Dried and sterile acellular esophagi were obtained with a combined treatment of ethanol and a subsequent supercritical CO_2 drying. Preservation of the extracellular matrix architecture, collagen content, and mechanical properties up to 6 months demonstrated the efficiency of the methodology with implications in natural scaffold storage. *In vitro* support of mesenchymal stem cells showed a promising indication to the further use of the technology in pre-clinical and clinical application.

Introduction

Several congenital and acquired conditions may require surgical esophageal replacement. Esophageal atresia (EA) is the most common congenital anomaly of the esophagus [1], while malignant conditions such as leiomyosarcoma, teratoma and inflammatory pseudo tumor predominate in the adult population [2]. Esophageal cancer is the eighth most common cancer in the world, with 456 000 new diagnosed cases and 40 000 deaths in 2012 [3]. The two main histological types of esophageal cancer are adenocarcinomas and squamous cell carcinomas sub-types which have a significant heterogeneity in incidence, geographic distribution, ethic pattern and etiology [4]. Despite modern therapies overall 5-years survival rates range from 15 to 30% [5–7]. Current choices for esophageal replacement involve the use of more distal parts of the gastrointestinal tract such as stomach, jejunum and colon, with long-term problems associated to each technique [8]. Tissue engineering offers valid alternative to conventional transplantation by replacing artificial tissue or organ function with constructs that contain specific populations of living cells [9]. Specifically, patients with esophageal severe disorders would have a great advantage from tissue-engineered esophagi used in replacement therapies.

Several esophageal scaffolds have been proposed for tissue engineering: synthetic scaffolds, naturally derived scaffolds and natural acellular matrix [10]. Some experiments with synthetic scaffolds have led to good results in animal models [11–13]. Natural collagen scaffolds showed promising outcomes in the past years [14, 15]. Decellularized esophageal scaffolds proved to be a valuable resource for clinical translation [16, 17]. Natural acellular matrices are derived from human and animal organs or tissues that have been treated to remove cells and immunogenic material. They retain the architecture of the tissue of origin and also the molecular components of the native

extracellular matrix (ECM). Our group previously demonstrated how a pig esophagus can be efficiently decellularized while maintaining defined ultra-structure, which is essential for medical applications [18].

One of the major drawbacks of tissue engineered organs is the difficulty to preserve and store decellularized scaffolds in a highly sterile condition, which is essential in the perspective of clinical use. We recently published a work where we showed the possibility to cryopreserve rabbit esophagi which maintain proper ECM structure and function [19]. Considering scaffold preservation for cell repopulation and transplant, there is the necessity to move from simple cellular cryobiology to other more sophisticated techniques valid for whole bigger organs.

Not all sterilization methods can be used with tissue-engineered scaffolds. Flame, steam or heat at 121 or 136 °C cannot be applied to biological tissues. Other methods, such as chemical sterilization with molecules like paracetic acid or ethylene oxide have been found to alter mechanical properties of the tissues or leave possible toxic residuals within the ECM [20, 21]. One of the most commonly used method for sterilization in tissue engineering is the ionizing radiation. Gamma irradiation has been shown to increase stiffness and strength at dosage lower than 15 kGy, while mechanical properties decrease at higher dosages [22]. Ionizing radiation has been shown to also induce structural changes to the ECM proteins [23, 24], which can eventually alter and compromise cell adhesion capacity [25].

Supercritical carbon dioxide (SC-CO_2) has been widely applied to the sterilization of pharmaceuticals and foods. SC-CO_2 has been used as a method to decellularize tissues [26] and more recently it has been investigated as a method for sterilization of biological scaffolds in combination with additive [27]. The process works at low temperatures (30 °C–35 °C) which do not compromise biological molecules, and leaves no cytotoxic residuals, essential in perspective of cell repopulation. SC-CO_2 has been recently applied to the sterilization of soft decellularized tissues such as the aortic valve and portions of lung tissue [28, 29]. We recently developed a method that combines ethanol and SC-CO_2 to dry esophageal decellularized tissue, which allows to maintain physiological and mechanical properties and biocompatibility for cell repopulation [30].

Our aim is to explore the capacity of the ethanol/SC-CO_2 method to sterilize a decellularized swine esophagus as well as the conservation over time of this tissue for clinical application. Therefore, we present an innovative application of supercritical fluid, which has never been explored so far. The innovative key points of this work are the use of SC-CO_2 as a method to both sterilize and maintain over time a biological decellularized scaffold.

Materials and methods

Organ harvest
Esophagi were collected from white domestic piglets (*Sus scrofa domesticus*), 12 to 16 weeks-old, from 25 to 40 kg in weight. Piglets were euthanized by administration of an overdose of intravenous Sodium Pentobarbital (Sigma Aldrich). The esophagus was harvested with a midline incision, and thoroughly washed with phosphate buffer solution (PBS, Thermo Fisher) with 5% antibiotic antimycotic solution (Thermo Fisher). All surgical procedures and animal handling were carried out in accordance with UK Home Office guidelines under the Animals (Scientific Procedures) Act 1986 and the local ethics committee.

Tissue decellularization
Esophageal decellularization was performed with detergent enzymatic treatment (DET) following a previously published procedure [18]. Briefly, the lumen was continuously perfused by three subsequent cycles of DET using a variable speed roller pump (Masterflex L/S, Cole-Parmer) at 0.6 ml min^{-1}. Three subsequent solutions were used for each cycle: MilliQ water at 4 °C for 18 h, 4% sodium deoxycholate at room temperature for 4 h, and 50 Kunitz units DNase I in 1M NaCl solution (all from Sigma Aldrich) at room temperature for 3 h. Samples were washed, divided into cylindrical segments, and stored in sterile PBS at 4 °C until use.

Supercritical CO_2 drying
The carbon dioxide supercritical drying process (SC-CO_2) was performed as previously reported [30]. Briefly, the samples were dehydrated by submersion in a series of ethanol (Sigma Aldrich) solutions (50%–75%–95%–99.8%) for 20 min at each dilution. Then, samples were CO_2 treated at 35 °C and 10 MPa for 90 min. A high pressure sapphire window cell (Separex S.A.S.) was used to carry out the experiments. The internal temperature and pressure were set with a thermostatic bath and an HPLC pump (Gilson 25SC). CO_2 (purity 99.990%, Messer) was flowed into the reactor at a constant flow rate of 23 ml min^{-1} until 10 MPa, then the pressure was maintained constant by tuning on a micrometric valve. To avoid any microbial contamination during the opening of the cell, a Bunsen burner flame was placed near the reactor chamber and the treated samples were collected with sterile tweezers in sterile tubes. The glass chamber was disinfected with EtOH after each sampling. Samples were vacuum packaged for storage in sterile plastic bag at 4 °C. Throughout the text NT and T t_0 refer to the control non-treated, and treated sample at time 0; T t_1 refers to the treated sample at 3 months from the process, T t_2 refers to the treated sample after 6 months from the process.

Weight loss analysis

Samples were weighted after ethanol and supercritical CO_2 treatments with a precision scale (Ohaus Explorer). It was assumed that all weight loss observed was due to the moisture removal from the ECM. Weight reduction was expressed as the ratio of % W_{end}/W_{start}, with W_{end} and W_{start} expressing respectively the weight of the sample before and after the treatment.

Microbiological analysis

Microbial load was analyzed before and after the treatment in terms of mesophilic bacteria, mesophilic spores, yeasts and molds by means of the standard plate count techniques as previously reported [31]. Serial dilutions in PBS were done with a weight ratio of 1:10. For the enumeration of mesophilic spores, the first dilution tubes were inserted in a thermostatic bath at 80 °C for 10 min before plating. Colonies of mesophilic bacteria and spores were counted after 72 h incubation at 30 °C on Plate Count Agar (Microbiol Diagnostici), while yeasts and molds were counted after 72–120 h at 25 °C on dichloran rose-bengal chloramphenicol agar (DRBC, Biotec srl) plates. The enumeration was referred to the weight of initial product and expressed in colony forming units CFU/g. The degree of inactivation was calculated considering the $\log(N/N_0)$, where N_0 (CFU/g) and N (CFU/g) is the number of CFU per gram in the untreated and treated sample, respectively. The quantification limit was 300 CFU/g for the agar pour plates, and 2000 CFU/g for the DRBC spread plates, while the limit of detection was <10 CFU/g for the pour plate and <100 CFU/g for the spread plates.

Mechanical characterization

Tensile tests were performed with a Midi 10 electromechanical testing machine (Messphysik Materials Testing). Before testing, the dried samples were rehydrated for 24 h in PBS. Then, samples including both epithelium and muscle tissues were cut with scissors in a regular rectangular shape (with width of 4–5 mm and length of 10–20 mm, depending on the size of the esophagus sample) and fixed to the loading frame through double adhesive tape at both ends. Esophageal samples were cut both in longitudinal and transversal direction. All analyses were performed at room temperature at least in triplicates and at a speed of $0.1\ \mathrm{mm\ s^{-1}}$.

Collagen quantification

All collagen isoforms were quantified using a total collagen assay kit (QuickZyme Biosciences) according to manufacturer's instructions. Before starting the analysis, dried samples were rehydrated in PBS for 48 h, to be comparable with non-dried decellularized scaffolds. Collagen standard (1200 μg ml^{-1} in 0.02 M acetic acid) was provided by the manufacturer. Briefly,
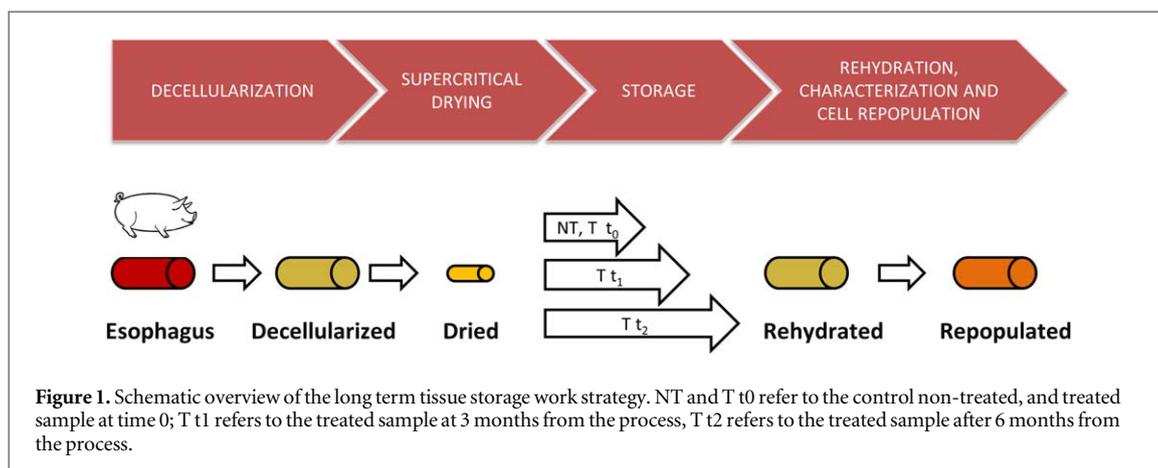
representative decellularized esophageal samples of each group (untreated and supercritical CO_2-dried) were weighed and directly hydrolyzed in 6 M HCl at 95 °C for 20 h. Samples were cooled to room temperature and centrifuged to remove debris. Supernatants were mixed with a chromogen reagent at 60 °C for 1 h to detect hydroxyproline residues. Absorbance for each sample was determined at 555 nm using a microplate reader (Infinite 200 Pro Tecan).

Cell culture and scaffold seeding

Human bone marrow mesenchymal stem cells (hBMMSCs) were provided by the Cell factory of Policlinico Hospital (Milan, IT). Cells were expanded in tissue culture T75 flask in MEM-alpha, 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin (all from Thermo Fisher). At 80%–90% confluence, cells were detached with Trypsin-EDTA 0.25% (Thermo Fisher) and seeded in 48multi-well and on the decellularized scaffold. Only early passages of cells were used for the experiments (p4–p8). The dry decellularized scaffolds were rehydrated for 24 h in PBS and mild agitation, then punched with 8 mm biopsy punches (B life) and washed twice in mem-alpha with the second wash incubated overnight at 37 °C to equilibrate the tissue and avoid osmotic shock during cell seeding. Tissue disks were transferred to 48 multiwells with the luminal side up, and incubated with 300 μl/well of complete medium for 24 h at 37 °C and 5% CO_2. For conditioned medium cytotoxicity experiments, 2×10^4 cells/well were seeded in 300 μl of each conditioned medium and cultured for 48 h. For cell adhesion experiments, 1.5×10^5 cells/disk were seeded and analyzed after 48 h of culture, and fixed after 10 days of expansion, with 1 ml fresh medium change every other day.

Histological and fluorescence analyses

Esophageal samples were fixed overnight in paraformaldehyde 4% at 4 °C, then dehydrated with sucrose (Sigma Aldrich) serial passages (10%–20%–30%, 12 h each at 4 °C). Samples were then frozen by submersion in isopentane (C_5H_{12}, Sigma Aldrich) cooled in a liquid nitrogen bath, and included in Optimal Cutting Temperature compound (OCT, Kaltech). Cryosections of 10 μm were prepared onto Superfrost Plus slides (Thermo Fisher) using a cryomicrotome (Cryostat Leica CM1860). Tissue slides were stained with Haematoxylin and Eosin (H&E), Masson's trichrome (MT) and Alcian blue (AB) stains (all from Bio-Optica), according to manufacturer's instructions. Cytochemistry analyses on tissue slices were performed using Alexa Fluor 488 Phalloidin (Thermo Fisher) 1:200 and Hoechst (Sigma Aldrich) 10 μM diluted in PBS with 0.1% Triton X-100 (Sigma Aldrich). Color and fluorescence images were obtained using a color camera mounted on fluorescence inverted microscope (Leica DMI4000).

Figure 1. Schematic overview of the long term tissue storage work strategy. NT and T t0 refer to the control non-treated, and treated sample at time 0; T t1 refers to the treated sample at 3 months from the process, T t2 refers to the treated sample after 6 months from the process.

Cell vitality assay

Cultured samples were analyzed with Live/Dead viability/cytotoxicity kit for mammalian cells (Thermo Fisher) according to manufacturer's instructions. Briefly, samples were washed with PBS twice. 2 μM calcein AM and 2 μM ethidium homodimer-1 were dissolved in basal medium (without serum), together with sterile 1 μM Hoechst (Sigma Aldrich). Samples were incubated in Live/Dead solution for 45 min at 37 °C, then washed in basal medium and analyzed at fluorescence microscope, with 360 nm filter for Hoechst, 488 nm for calcein and 594 nm for EthD-1.
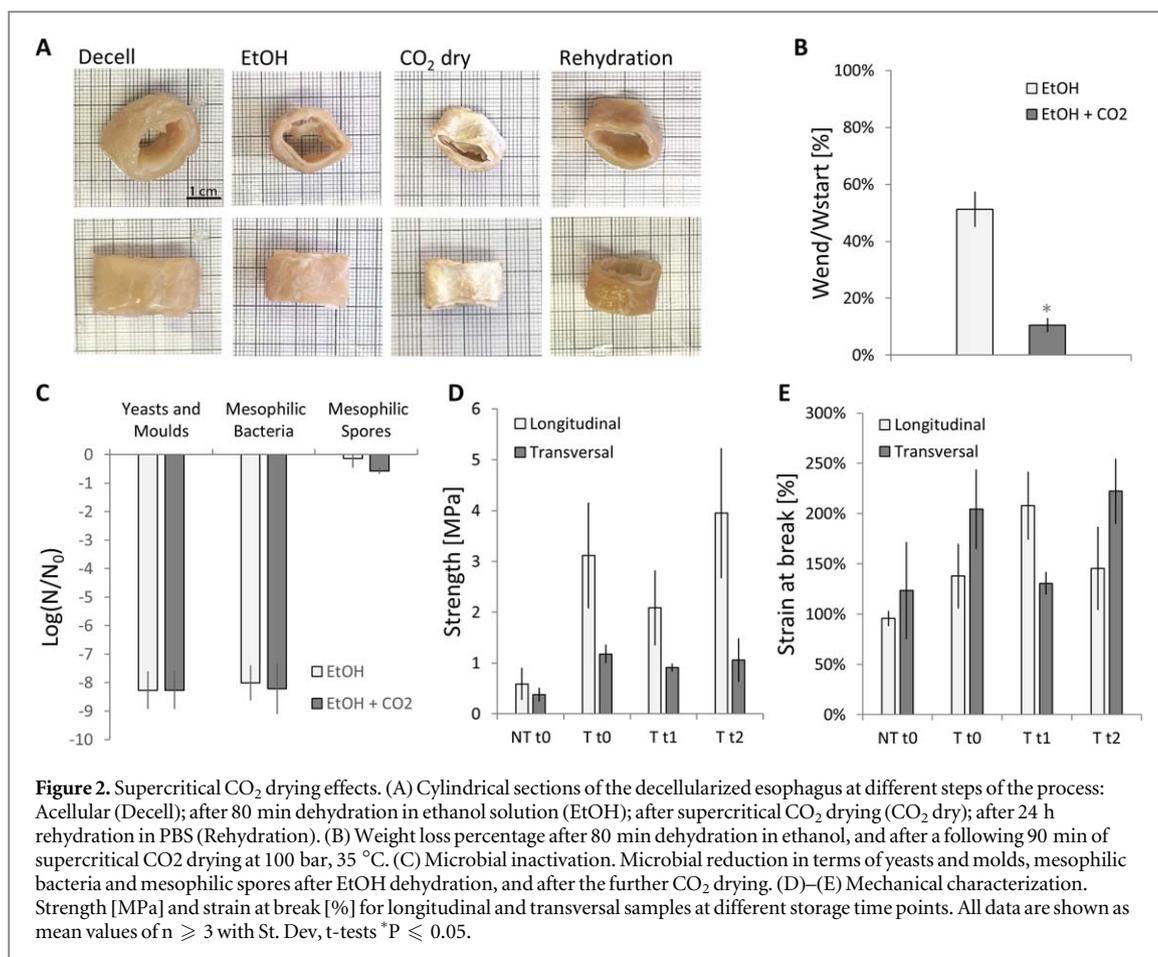
Results

The first step was the decellularization of esophageal tissues obtained from euthanized piglets. Organs were harvested, washed, and the decellularization process was started within 12 h from collection. Decellularized tissues were divided into portions to be processed with supercritical CO_2 and stored at different time points, in triplicate for each condition. Tissues were stored without need for γ-irradiation after the dehydration procedure. At specific time points, tissues were rehydrated and analyzed (figure 1).

The quality of the decellularization protocol to obtain esophageal tissue with absence of genetic material and cellular components was deeply investigated in our previous works [18, 19, 30]. The morphology of the tissue after rehydration process was comparable with freshly decellularized tissue (figure 2(A)) at all the time points (data not shown). The process of supercritical drying shows high loss in weight compared to ethanol dehydration, due to complete loss of water residual and ethanol in the ECM (figure 2(B)). We performed complete microbial inactivation test to assess whether the CO_2 drying was able to reduce bacterial load compared to non-treated sample. Results were negative in all the contaminating agent categories analyzed (figure 2(C)), meaning that supercritical CO_2 drying procedure has a high sterilization capacity.

A key feature of decellularized scaffolds for organ transplantation is the preservation of their mechanical properties, which must be comparable to those of the original tissue. We performed tensile tests in order to derive the strength and strain at break of dehydrated decellularized esophageal samples in longitudinal and transversal direction (suppl. figure 1(A) is available online at stacks.iop.org/BPEX/4/065021/mmedia). All the different storage time points were analyzed. Mechanical tests showed that treated samples have higher strength compared to control samples at t_0, especially along the longitudinal direction (figure 2(D)). Considering the strain at break, both longitudinal and transversal sections showed less variation between the non-treated samples and the dehydrated ones at different time points (figures 2(E) and suppl. 1(B)). Some differences were expected because of the biological nature of the tested samples, which can cause significant variation in the mechanical behavior of samples even taken from the same esophagus. Furthermore, we can observe that both the strength and strain at break of treated samples do not vary significantly at different time points. Overall, non-treated controls and treated samples showed comparable mechanical properties, stating that storage over time of dehydrated scaffolds does not seem to affect physical characteristics of the matrix (at these specific time points).
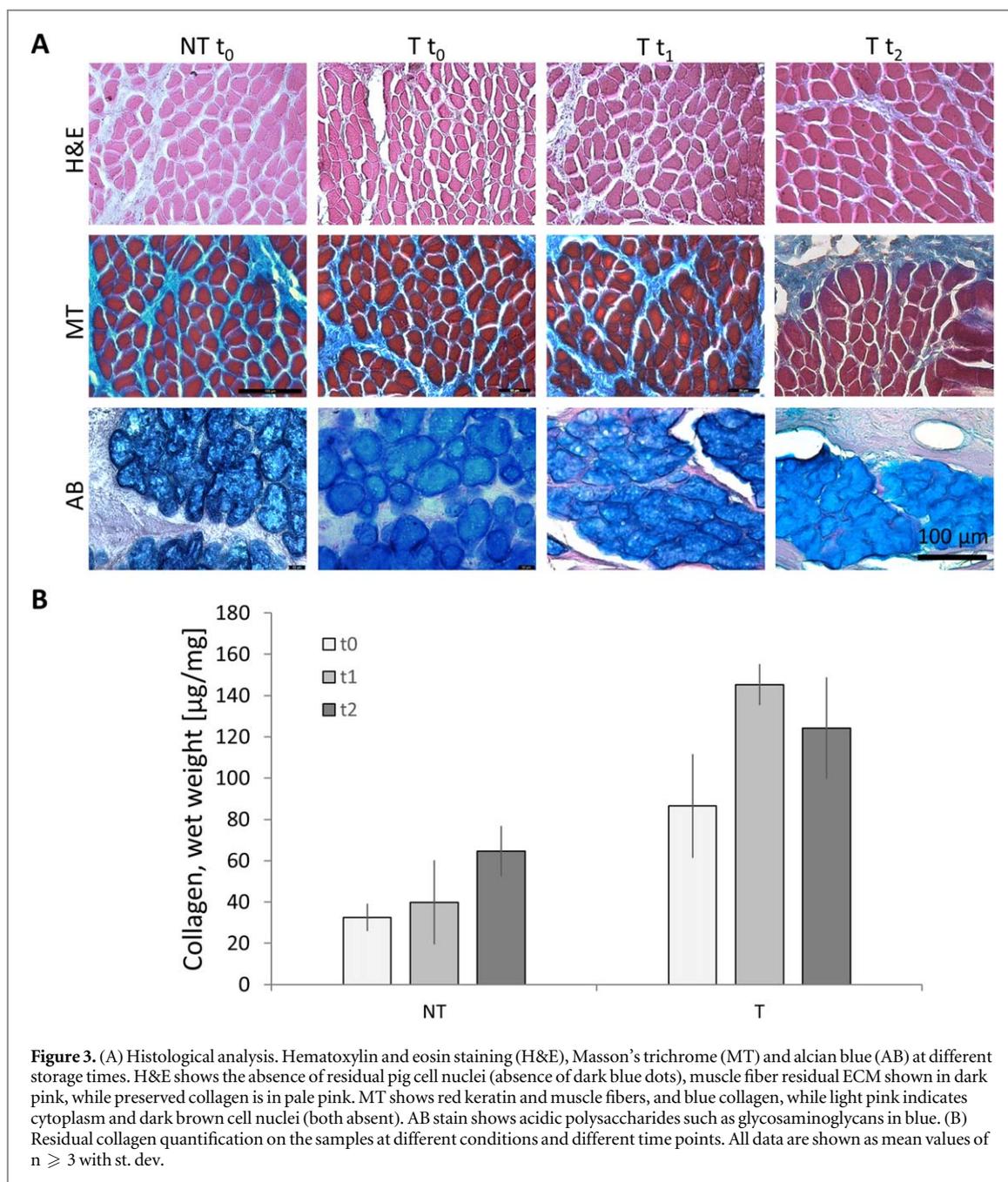
To further assess the stability of the dehydrated decellularized scaffolds after long-term storage, staining and quantification for specific ECM components were performed. The quality of the decellularization process and the preservation of the esophageal architecture, previously demonstrated with our dehydration method [30] (suppl. figures 2(A)–(B)) by the presence of the three esophageal layers, namely mucosa, submucosa and muscular layers, was also confirmed in all the analyzed samples by H&E staining (figure 3(A)). H&E staining performed on non-treated and treated tissue sections at different time points showed the quality of the decellularization process and preservation of muscle ECM fiber structure (figure 3(A)). MT staining showed how keratin,

Figure 2. Supercritical CO_2 drying effects. (A) Cylindrical sections of the decellularized esophagus at different steps of the process: Acellular (Decell); after 80 min dehydration in ethanol solution (EtOH); after supercritical CO_2 drying (CO_2 dry); after 24 h rehydration in PBS (Rehydration). (B) Weight loss percentage after 80 min dehydration in ethanol, and after a following 90 min of supercritical CO2 drying at 100 bar, 35 °C. (C) Microbial inactivation. Microbial reduction in terms of yeasts and molds, mesophilic bacteria and mesophilic spores after EtOH dehydration, and after the further CO_2 drying. (D)–(E) Mechanical characterization. Strength [MPa] and strain at break [%] for longitudinal and transversal samples at different storage time points. All data are shown as mean values of n $\geqslant$ 3 with St. Dev, t-tests *P $\leqslant$ 0.05.

muscle fiber structure and collagen were maintained in all the time points and conditions. Finally, AB staining revealed good preservation of glycosaminoglycans in the mucosa. These qualitative analyses prove that supercritical CO_2 drying does not affect the quality of the ECM in decellularized scaffolds after long-term storage. To corroborate what evidenced with the staining, we quantified collagen in randomized portions of treated and untreated esophagi. Dried samples showed high preservation of all isoforms of collagens in all the time points, compared to non-treated samples (figure 3(B)). Collagen slight increase during time could be ascribed to a small loss in weight due to loss of other proteins, with consequent concentration of collagen in the scaffold (data frequently observed in literature [32, 33]).

Following the mechanical and biological characterization of decellularized dehydrated scaffolds, we performed biocompatibility assays to prove that no cytotoxic degradation products were released during the storage. These assays were also necessary to investigate the capability of the dehydrated matrices to support cell adhesion and culture in cell repopulation experiments. At first, disks of esophagi stored at different time points were rehydrated and incubated with cell culture media for 24 h (suppl. figure 3). This medium was then used to culture hBMMSCs. Non-treated control scaffold conditioned medium did not allow

any cell survival as demonstrated by absence of cells at 48 h of culture (figure 4(A)), while dried stored samples did not affect the medium quality. Non-treated control samples could cause the release of some toxic compounds from the decellularization procedure that were not completely removed with conventional washing treatment, possibly due to the tissue thickness. Another hypothesis might be ascribed to a deterioration of the scaffold during the storage in PBS (at least 10 days from harvest/cell culture experiments). On the other hand, any potential toxic molecule was extracted and washed by the ethanol/supercritical CO_2 process, and the deterioration is inhibited in the dried state. Quantification of the live/dead assay was also performed and compared to control cells, cultured in non-conditioned media, calculating the ratio of dead cells over total cells per well, resulting in high cell survival in conditioned medium during all time points (suppl. figures 4(A)–(B)). Secondly, the disks of esophageal tissue were used as scaffolds for 3D culture to verify direct cytotoxicity, capacity of cellular adhesion in the stored ECM, and promotion of cell proliferation. No or few cells adhered and proliferated during the 10 days of expansion on non-treated control scaffolds, confirming what previously observed with conditioned media. On the other hand, cells adhered and proliferated on the scaffolds stored at the different time points (figure 4(B)). To better visualize
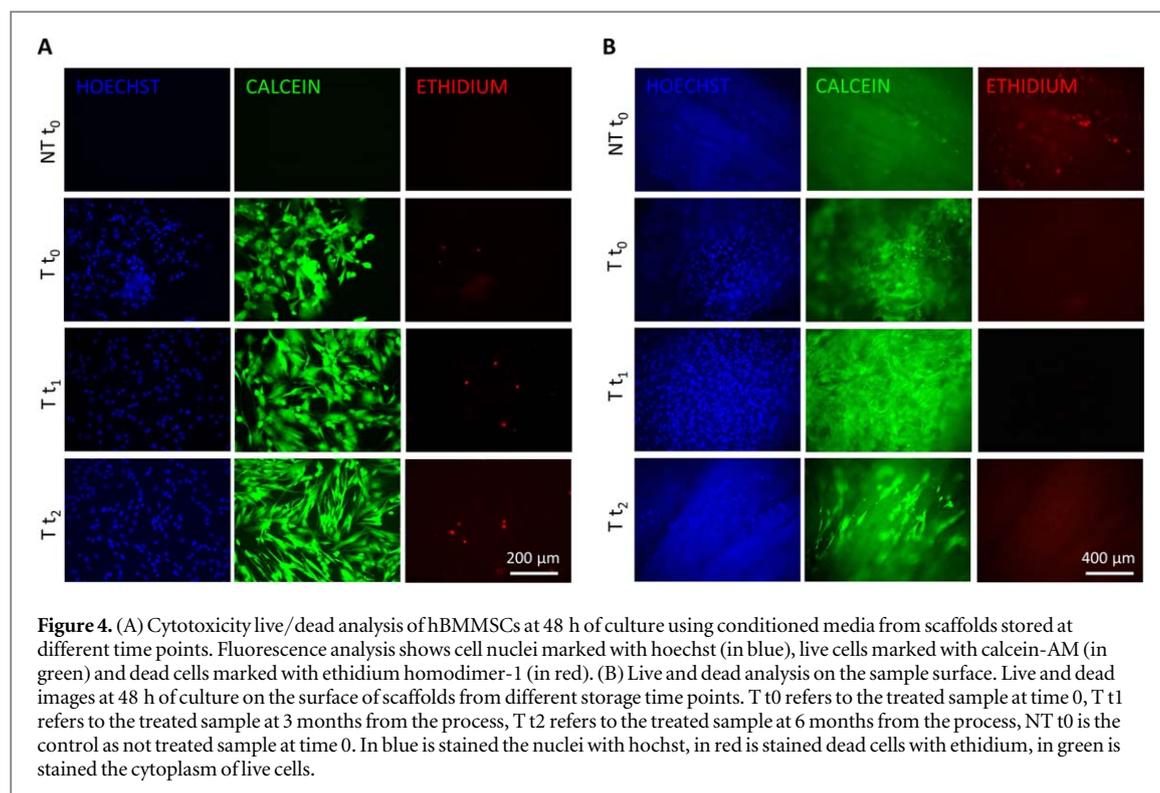
Figure 3. (A) Histological analysis. Hematoxylin and eosin staining (H&E), Masson's trichrome (MT) and alcian blue (AB) at different storage times. H&E shows the absence of residual pig cell nuclei (absence of dark blue dots), muscle fiber residual ECM shown in dark pink, while preserved collagen is in pale pink. MT shows red keratin and muscle fibers, and blue collagen, while light pink indicates cytoplasm and dark brown cell nuclei (both absent). AB stain shows acidic polysaccharides such as glycosaminoglycans in blue. (B) Residual collagen quantification on the samples at different conditions and different time points. All data are shown as mean values of $n \geqslant 3$ with st. dev.

the adhesion of the mesenchymal stem cells on the scaffolds, sections of tissue were stained for f-actin, which showed the superficial adhesion and cell spreading on the luminal side of the scaffolds (suppl. figure 4(C)).

Discussion

When a patient has an ultimate organ failure, the only option available is organ transplant. Yet, transplantable organs shortage and long waiting lists are the major problems in the field [34], together with the limited survival time of vital organs after donor explants [35]. For this reason, recellularized organs are a desirable perspective in the field of allogeneic transplants.

To this end, we developed a method for dehydrating, sterilizing and storing tissues for preservation over time. In this work we applied ethanol and supercritical carbon dioxide to dehydrate sections of porcine esophagus to be stored for 3 and 6 months after processing. Dehydration method was optimized with the combination of both EtOH/SC-CO_2 (10 MPa and 35 °C) since SC-CO_2 alone was not sufficient to obtain cytocompatible scaffolds, as previously described by our group [30]. This procedure was sufficient for complete removal of cytotoxic residues from the decellularization process, together with complete ethanol and water elimination from the decellularized esophagi [36]. Moreover, the process was able to eliminate any form of contaminating bacteria, yeasts or molds, thus to guaranty a highly sterile scaffold for

Figure 4. (A) Cytotoxicity live/dead analysis of hBMMSCs at 48 h of culture using conditioned media from scaffolds stored at different time points. Fluorescence analysis shows cell nuclei marked with hoechst (in blue), live cells marked with calcein-AM (in green) and dead cells marked with ethidium homodimer-1 (in red). (B) Live and dead analysis on the sample surface. Live and dead images at 48 h of culture on the surface of scaffolds from different storage time points. T t0 refers to the treated sample at time 0, T t1 refers to the treated sample at 3 months from the process, T t2 refers to the treated sample at 6 months from the process, NT t0 is the control as not treated sample at time 0. In blue is stained the nuclei with hochst, in red is stained dead cells with ethidium, in green is stained the cytoplasm of live cells.

clinical application. Given the absence in humidity, scaffolds can be stored at low temperatures with no risks of re-contamination, and no risk of water crystals formation, which could eventually disrupt the ECM structure.

Supercritical drying generated a scaffold with preserved mechanical properties, similar to control, over time. Being the esophagus one active organ in the food motility, robustness of the structure a critical key feature for clinical application of tissue engineered organs, as tissue fragility can lead to graft rupture or anastomotic adverse events after implantation. We observed preserved mechanical properties at 3 and 6 months' storage, namely transversal strength, a slightly increased longitudinal strength compared to non-treated samples, and similar longitudinal and transversal strain at break. Standard deviations observed in both controls and treated samples were within the extent of biological variability, given by the sex of the animals, age and weight range, which inevitably influence dimension and mechanical characteristics of the esophagi.

Mechanical properties strictly depend on the quality of the ECM, which needs to be preserved during decellularization [18, 19] and supercritical drying [30] processes. The EtOH/SC-CO$_2$ treatment generated a dried scaffold with no visible histological damages at ECM structural level. During the long-term storage at t_1 and t_2, we did not appreciate any visible change in the ECM structure. All the histological analyses confirmed what already observed at mechanical level. Good conservation of collagen, keratin and glycosaminoglycans was highlighted throughout the six months

of dry storage. Moreover, the muscle matrix structure and fiber disposition in the tissue showed a remarkable degree of preservation at the different time points. These characteristics are essential in the view of cellular re-population experiments, where cell adhesion, survival (absence of tissue cytotoxicity), differentiation, proliferation and functionality are strictly required.

To this end, we verified the degree of cytotoxicity of the dried scaffolds at different time points. It is known how even small amount of residual detergents in decellularized scaffolds compromises the re-population process [37, 38]. Infinitesimal amounts of detergent could remain in the scaffolds even after washing, due to the difficulty in cannulating and perfusing the organ, or to the intrinsic ECM complexity. The combined treatment with EtOH and continuous flow of supercritical CO$_2$ was able to remove any toxic residual derived from the decellularization process, or from the initial degradation of the ECM that begins soon after decellularization, during the first days of tissue storage before drying treatment. Delicate primary cells, such as mesenchymal stem cells, growing in scaffold-derived conditioned media indicated biocompatibility of the tissue. On the other hand, the capacity of cell adhesion and expansion, tested with a 10 days' 3D culture experiment, provided information of the quality of the ECM. Preservation of adhesion foci, integrin sites and tissue specific epitopes after long-term storage is essential to support adhesion and differentiation into tissue specific subtypes of epithelial, muscle, endothelial, mesenchymal and neuronal precursors,

when re-population experiments are required prior to transplantation.

It has to be emphasized that the tissue desiccation process that we described successfully acted as both long-term storage technique and high grade sterilization method. This outcome, previously shown for other applications [39–41], has now been proved also in tissue engineering. Previous works have shown how different sterilization methods, commonly used for clinical application, such as gamma irradiation, when applied to biological tissues at low dosage can increase tissue stiffness, while at high dosage can alter and damage the composition of the ECM [38, 42, 43]. Being decellularized scaffolds composed of defined macromolecules, their structure can be altered by radiation causing free radicals and chemical bonds breakage. These techniques compromise the sterility and long-term maintenance of scaffolds to be used in clinical application. On the other hand, our approach of using the supercritical gas for esophageal tissues was able to sterilize and allow long-term conservation for translational and clinical studies.

Conclusions

A bank of dried decellularized organs and tissues readily accessible to laboratories and medical équipes would be greatly beneficial to the development of new successful therapies. In a context of emergency operations, medical staffs would have access to a set of sterile organs ready to be rehydrated and repopulated with autologous or heterologous cells, just days before planned organ or tissue transplants.

Taking advantage of SC-CO$_2$ drying process, we obtained a dry decellularized scaffold that could be used for the development of organ/tissue substitution procedures. The method that we proposed has a double desirable effect on the decellularized porcine esophagi: high sterilization capacity, and high dehydration capacity, which lead to the possibility to store over time the organ, still maintaining its structural and functional characteristics.

Further studies on the sterilizing effect and long-term storage possibility given by supercritical gases applied on decellularized scaffolds and biological matrices could be a great advantage in the development of a clinical grade method to be applied in translational research.

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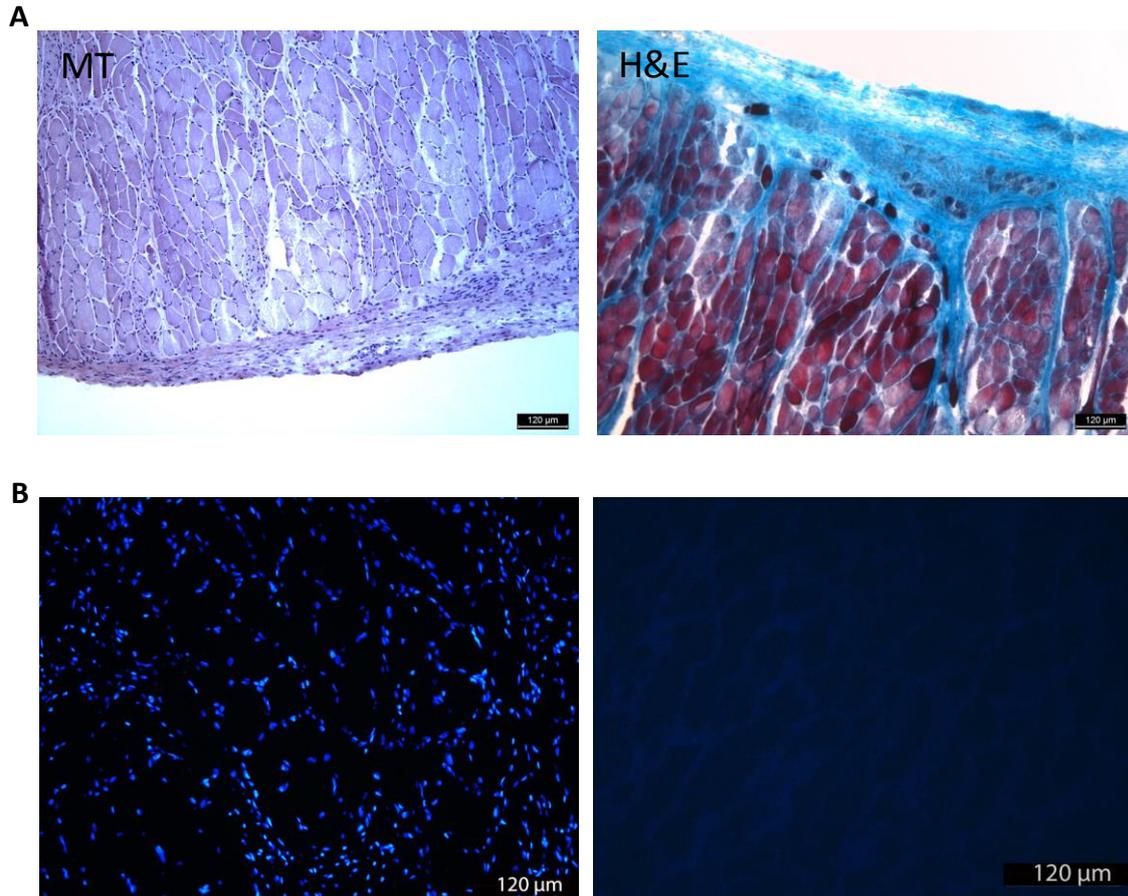
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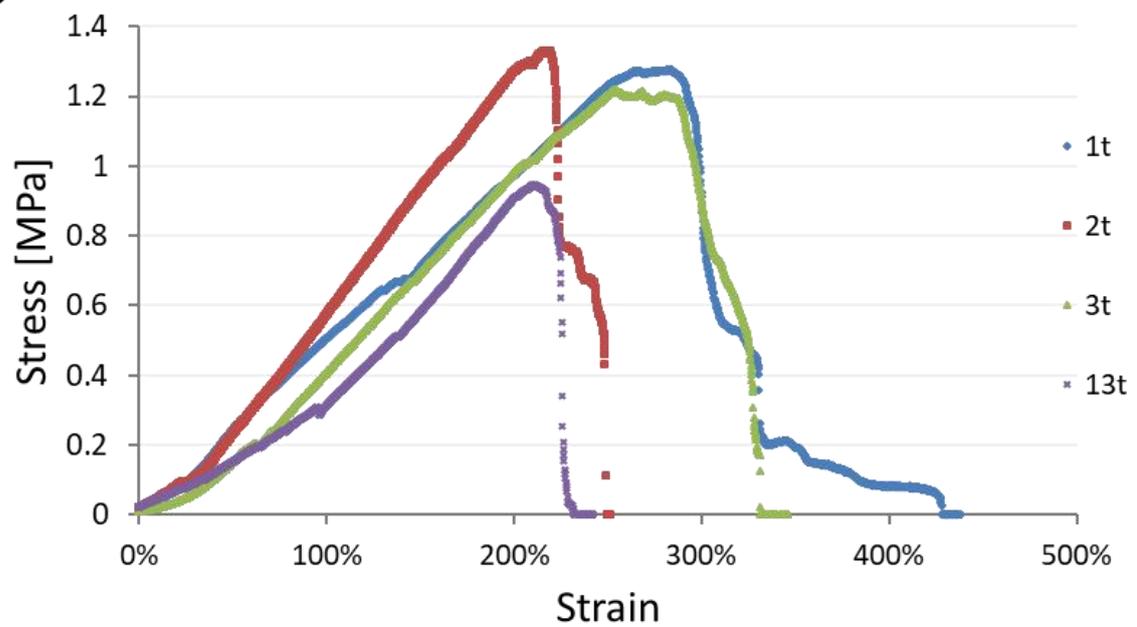
Supplementary figure 1: (A) Example of transversal sections of decellularized porcine esophagus after 24 h rehydration in PBS to be analyzed for mechanical properties. (B) Example stress-strain curves of 4 samples at the same time point.

Supplementary figure 2: (A) Histological analysis of a fresh porcine esophageal tissue before decellularization process. Hematoxylin and eosin staining (H&E) showing presence (in deep blue/violet) of cell nuclei around muscle fibers and epithelial layer. Masson's trichrome (MT) showing muscle fibers. (B) Fluorescent hoechst staining showing cell nuclei in esophageal tissue before decellularization (left) and complete nuclei removal after decellularization (right).
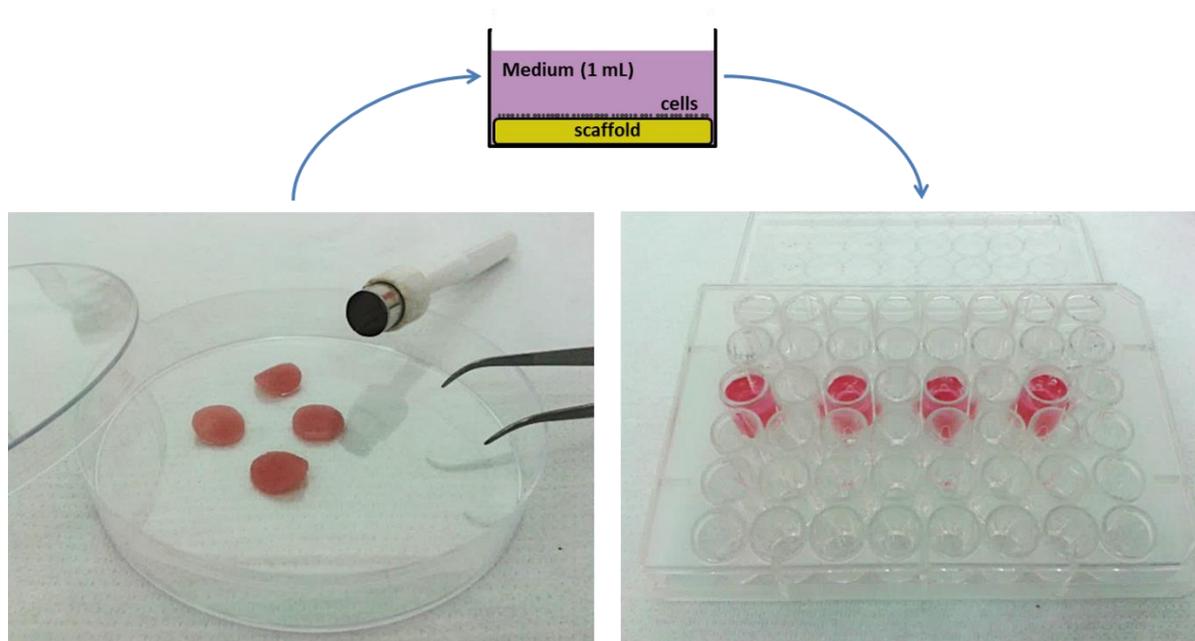
Supplementary figure 3: 1 cm Ø disks of esophageal scaffolds for conditioning media experiments, rehydrated and equilibrated with culture medium for 24 h. Disks are positioned with the luminal side of the esophagus up in 48 multiwells, to be seeded with hBMMSCs for cell adhesion and expansion experiments.

A

B

C
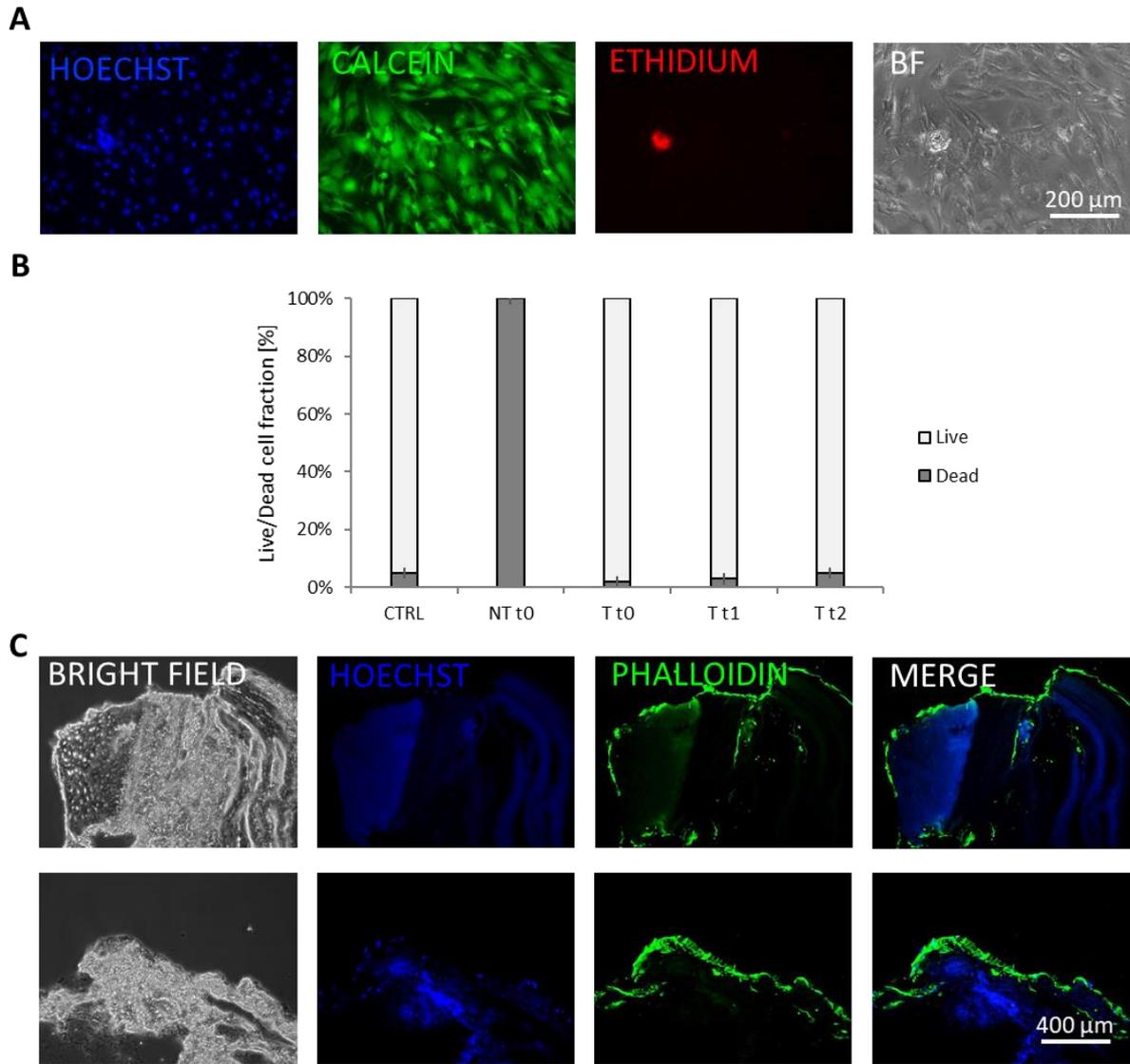
Supplementary figure 4: (A) Live/Dead assay in control hBMMSCs seeded on petri dish. Hoechst in blue shows cell nuclei, Calcein in green shows live cells, Ethidium in red shows dead cells (B) Graph showing survival rate of hBMMSCs cultured in conditioned media by the scaffolds in different conditions. (C) Cryostat 20 µm thick sections of fixed disks seeded with hBMMSCs after 10 days of culture. Hoechst in blue shows cell nuclei. Phalloidin in green shows cytoskeletal f-actin.