TITLE: Tracheal in vitro reconstruction using a decellularized bio-scaffold in combination with a rotating bioreactor

Running Title: Reconstruction of a bioprosthetic trachea

Georgia Pennarossa[1], Matteo Ghiringhelli[2], Fulvio Gandolfi[3], Tiziana A.L. Brevini[1]*

1. Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety and Center for Stem Cell Research, Università degli Studi di Milano, Milan, 20133, Italy

2. Sohnis Research laboratory for Cardiac Electrophysiology and Regenerative Medicine, the Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa, Israel, POB 9649, Haifa 3109601, Israel

3. Laboratory of Biomedical Embryology, Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, 20133, Italy

*Corresponding Author: Tiziana A.L. Brevini, Laboratory of Biomedical Embryology, Department of Health, Animal Science and Food Safety and Center for Stem Cell Research, Università degli Studi di Milano, Milan, 20133, Italy; tiziana.brevini@unimi.it

Abstract

Long-segment airway stenosis as well as their neoplastic transformation is life-threatening and still currently represent unsolved clinical problems. Indeed, despite several attempts, definitive surgical procedures are not presently available, and a suitable tracheal reconstruction or replacement remains an urgent clinical need. A possible innovative strategic solution to restore upper airway function may be represented by the creation of a bioprosthetic trachea, obtained through the combination of tissue engineering and regenerative medicine.

Here we describe a two-step protocol for the ex vivo generation of tracheal segments. The first step involves the application of a decellularization technique that allows for the production of a naturally derived extracellular matrix (ECM)-based bio-scaffold, that maintains the macro- and micro-architecture as well as 9 the matrix-related signals distinctive of the original tissue. In the second step chondrocytes are seeded onto decellularized trachea, using a rotating bioreactor to ensure a correct scaffold repopulation.

This multi-step approach represents a powerful tool for in vitro reconstruction of a bioengineered trachea that may constitute a promising solution to restore upper airway function. In addition, the procedures here described allow for the creation of a suitable 3D platform that may find useful applications, both for toxicological studies as well as organ transplantation strategies.

Key Words: Bioprosthetic trachea, Chondrocytes, Decellularization, ECM-based bio-scaffold, Rotating Bioreactor, Tissue engineering.

1. Introduction

Upper airway dysfunctions and diseases, such as stenosis and neoplastic transformation, represent life-threatening conditions and seriously affect the duration and quality of life due to altered breathing, speech and swallowing ***(1)***. Several studies have proposed different techniques to help cartilage repairing, however definitive surgical procedures are not presently available and a suitable tracheal reconstruction or replacement remains an unmet clinical need ***(2)***. In this context, tissue engineering represents one of the most promising approach to generate bioprosthetic tracheas, suitable for organ transplantation. During the past 50 years, several attempts have been made to cope with the challenging problem of reconstructing long segmental tracheal defects and diverse synthetic materials have been evaluated for the production of tracheal scaffolds ***(3)***. Although promising, the results obtained met limited success because of immunological complications and bacterial infections. In addition, the materials tested lacked many of the organ-specific biomechanical properties, namely flexibility, strength to avoid collapse, and the formation of airtight seals ***(4, 5)***.

We here describe a two-step protocol for the ex vivo creation of a bioprosthetic trachea. The first step involves a decellularization technique that allows for the production of a naturally derived extracellular matrix (ECM)-based porcine bio-scaffold (Figure 1). In the second step, human chondrocytes are seeded onto the decellularized trachea, using a rotating bioreactor to ensure a correct scaffold repopulation (Figure 2).

Nonimmunogenic tracheal bio-scaffolds were derived by using a physical-chemical method to successfully eliminate the cell compartment, while preserving the macro- and micro-architecture and maintaining an intact ECM protein composition ***(6, 7)***. The bio-scaffold was obtained from the pig which is an ideal source of organs for xenotransplantation because of its anatomical and physiological similarities to humans ***(8, 9)***. In addition, the adult porcine trachea has been recently demonstrated to match the biomechanical properties of the human organ, including bending stiffness, radial supporting force, longitudinal elongation, residual stress, and bursting strength ***(10)***. In the second step, human chondrocytes were used to repopulate the porcine bio-scaffold to generate “semi-

xenografts", where the ECM-based scaffold is animal-derived, and the repopulating cells have human origin, thus combining the advantages of both xenotransplantation and tissue bioengineering.

A key aspect of the protocol described is represented by the use of a rotating bioreactor that ensures a dynamic repopulation system with several important advantages compared to the static culture approach. Indeed, the adoption of a bioreactor favours and positively impacts on ex vivo cell and tissue re-organization, ensuring the physiologically relevant physical signals, such as shear stress, compression, pressure, and stretch. In our opinion, this is an important aspect because the strategy adopted tries to mimic as closely as possible the mechanically dynamic environment experimented by chondrocytes in vivo, thus providing the ideal milieu for tracheal segment reconstruction ***(11)***. Furthermore, the rotational bioreactor used in the present protocol is functionally superior to static or spinner flask culture, since it is able to create optimal laminar flow conditions and lower shear stress ***(11, 12)***.

They method here reported is simple and highly efficient. It paves the way for in vitro trachea re-construction and organ transplantation. At the same time, it allows for the bio-fabrication of patient specific *in vitro* platforms that provide increased similarity to the in vivo physiology and pathology. The 3D semi-xenografts may be used for the development of new therapies against upper airway diseases and inflammation and can contribute to advances in the prevention and treatments of life-threatening infections such as COVID-19.

2. Materials

Prepare all solutions immediately before use (unless indicated otherwise).

2.1 Porcine trachea collection

1. Tracheas collected from a slaughterhouse.
2. Sterile plastic containers.

3. Ice container.
4. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
5. Antibiotic/Antimycotic Solution.

2.2 Generation of the decellularized tracheal ECM-based porcine bio-scaffold

1. 50 mL centrifuge polypropylene tubes.
2. Water bath.
3. Orbital shaker.
4. 500 mL plastic or glass bottles.
5. Deionized water (DI-H_2O).
6. Sterile water.
7. Ethanol.
8. Antibiotic/Antimycotic Solution.
9. 1% sodium dodecyl sulfate (SDS): dissolve 5 gr of SDS in 500 mL of DI-H_2O.
10. 1% Triton X-100: add 5 mL in 495 mL of DI-H_2O.
11. 2% deoxycholate: dissolve 10 gr of deoxycholate in 500 mL of DI-H_2O.
12. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
13. Chondrocyte culture medium: Ham's F-12 Nutrient Mixture, 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 50 µg/mL ascorbic acid-2-phosphate (ASC), 5.0 ng/mL basic fibroblast growth factor (bFGF), 10.000 U/mL penicillin G; 2.5µg/mL Amphotericin B.

2.3 Human chondrocyte propagation and maintenance

1. Human chondrocyte cell line (see Note 1).
2. T75 cell culture flasks.
3. 15 mL centrifuge polystyrene tubes.
4. Centrifuge.
5. CO_2 incubator.
6. Inverted microscope.
7. Antibiotic/Antimycotic Solution.
8. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
9. Trypsin-EDTA solution: dissolve 0.5 gr of porcine trypsin and 0.2 gr of EDTA 4Na in 1 L of HBSS with phenol red.
10. Chondrocyte culture medium: Ham's F-12 Nutrient Mixture, 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 50 µg/mL ascorbic acid-2-phosphate (ASC), 5.0 ng/mL basic fibroblast growth factor (bFGF), 10.000 U/mL penicillin G; 2.5µg/mL Amphotericin B.

2.4 Repopulation of the decellularized tracheal ECM-based porcine bio-scaffold with human chondrocytes and bioreactor setup

1. Autoclave
2. 15 mL centrifuge polystyrene tubes.
3. Centrifuge.
4. CO_2 incubator.
5. Cell counting chambers.

6. Inverted microscope.
7. Sterile decellularized tracheal ECM-based bio-scaffold.
8. Sterile rotating bioreactor.
9. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na_2HPO_4 (8 mM) and 240 mg of KH_2PO_4 (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
10. Trypsin-EDTA solution: dissolve 0.5 gr of porcine trypsin and 0.2 gr of EDTA 4Na in 1 L of HBSS with phenol red.
11. Chondrocyte culture medium: Ham's F-12 Nutrient Mixture, 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 50 µg/mL ascorbic acid-2-phosphate (ASC), 5.0 ng/mL basic fibroblast growth factor (bFGF), 10.000 U/mL penicillin G; 2.5µg/mL Amphotericin B.

3. Methods

All the procedures described below must be performed under sterile conditions. All instruments touching or in connection to tissues, cells, scaffolds and bioreactors have to be sterilized. Cell manipulation must be carried out under laminar a flow hood and cell cultures have to be maintained at 37 °C during their handling using thermostatically controlled stages.

All studies were reviewed and approved by the Ethical Committee of the University of Milan. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH).

3.1 Porcine trachea collection

1. Collect tracheal segments from gilts weighing approximately 120 kg.
2. Transfer tracheas in cold sterile PBS containing antibiotic/antimycotic solution (5mL/500 mL) and transport them to the laboratory using ice container.

3.2 Generation of the decellularized tracheal ECM-based porcine bio-scaffold

1. Wash extensively tracheal graft in fresh PBS.
2. Completely remove the PBS, place the trachea in an empty 50 mL tube and store organ at -80°C for at least 24 hours (see Note 2).
3. Thaw trachea at 37°C for 30 min using a water bath.
4. Transfer trachea in a bottle containing 500 mL of 1% SDS. Place the bottle onto an orbital shaker at 200 rpm and incubate for 3 hours at room temperature.
5. Remove SDS solution from the bottle containing the trachea and wash it with 500 mL of DI-H_2O for 40 min using an orbital shaker at 200 rpm.
6. Remove DI-H_2O from the bottle and add 500 mL of 1% Triton X-100. Incubate trachea for 12 hours at room temperature in 1% Triton X-100, using an orbital shaker at 200 rpm.
7. Remove Triton X-10 solution from the bottle containing the trachea and add 500 mL of DI-H_2O twice.
8. Remove the last washing DI-H_2O from the bottle and incubate trachea in 500 mL of 2% deoxycholate for 12 hours at room temperature, using an orbital shaker at 200 rpm.
9. Remove deoxycholate and wash trachea with DI-H_2O for 6 hours at room temperature, using an orbital shaker at 200 rpm. Changes DI-H_2O every 2 hours (see Note 3).
10. Sterilize the tracheal ECM-based bio-scaffold in a water solution containing 70% Ethanol and 2% antibiotic/antimycotic solution for 30 min at room temperature, using an orbital shaker at 200 rpm.
11. Extensively wash the ECM-based bio-scaffold with PBS supplemented with 4% antibiotic/antimycotic solution at room temperature using an orbital shaker at 200 rpm.
12. Before chondrocyte repopulation, immerse the tracheal ECM-based bio-scaffold in chondrocyte culture medium for at least 1 hour at room temperature.

3.3 Human chondrocyte propagation and maintenance

1. Maintain human chondrocyte cell line in 5% CO_2 incubator at 37°C.
2. Monitor cells daily.
12. Once cell culture has reached 80% confluency, carefully aspirate the culture medium from T75 flask (see Note 4).
3. Wash cells 3 times with 7 mL of PBS supplemented with 1% antibiotic antimycotic solution.
4. Add 2 mL of trypsin-EDTA solution (see Note 5) and incubate at 37 °C until cell monolayer begins to detach from the bottom of the tissue culture dish and cells dissociate (see Note 6).
5. Dilute cell suspension in 8 mL of chondrocyte culture medium to neutralize trypsin action.
6. Dislodge cells by repeatedly and gently pipetting.
7. Collect cell suspension in a 15 mL centrifuge polystyrene tube and centrifuge at 300 g for 5 min.
8. Remove supernatant and resuspended chondrocytes in 10 mL of fresh culture medium.
9. Plate cells in a new culture dish and culture at 37 °C in 5% CO_2 incubator. Keep the passage ratio between 1:2 and 1:4, depending on growth rate (see Note 7).
10. Change medium every 2-3 days.
11. Maintain cells in culture until they have reached 80% confluency and passage them.

3.4 Repopulation of the decellularized tracheal ECM-based porcine bio-scaffold with human chondrocytes and bioreactor setup

1. Carefully remove culture medium from culture dish, wash cells 3 times with PBS and incubate in 2mL of trypsin-EDTA solution at 37 °C until cell monolayer begins to detach (for detailed procedure see Sec. 3.3, steps 3-4).
2. Add 8 mL of chondrocyte culture medium to cell suspension and collect chondrocytes in a 15 mL centrifuge polystyrene tube.
3. Count cells using a counting chamber under an inverted optical microscope at room temperature.

4. Calculate the volume of medium needed to re-suspend chondrocytes to obtain a concentration of 1 X 10^6 cells/cm^2 of tracheal lumen bio-scaffold (see Note 8).
5. Centrifuge cell suspension at 300 g for 5 min. Carefully aspirate supernatant and resuspend cell pellet in the previously calculated volume (see Sect. 3.2, step 4) of chondrocyte culture medium.
6. Repopulate the tracheal ECM-based bio-scaffold by injecting chondrocytes into the tracheal lumen and carefully transfer it into CO_2 incubator.
7. Allow chondrocyte adhesion for 2 hours under static conditions.
8. Connect the repopulating tracheal ECM-based bio-scaffold to an autoclaved bioreactor, set a rotation of 5 rpm (see Note 9) and culture at 37 °C in 5% CO2 incubator.
9. Change culture medium every 48 hours.

4. Notes

1. It is possible to apply the protocol here described to other cell types, such as tracheal epithelial cells (HTEpCs).
2. Tracheal segments can be stored at -80°C for longer periods without causing ECM alteration.
3. At the end of decellularization protocol, it is a good practice to verify the efficiency of the process, by confirming cellular compartment removal (e.g., DNA quantification, DAPI and/or hematoxylin and eosin staining) and the retention of intact ECM components (e.g., collagen, elastin, glycosaminoglycans, etc.).
4. Confluency normally takes between 7-10 days. If cells are not confluent after 10 days, they are not successfully growing.
5. The trypsin volume here reported is necessary for detaching cell cultured in a T75 flask. When working with smaller flask or dish, scale down the volumes accordingly.
6. It usually takes 3-5 minutes.
7. The recommended seeding density is 5,000 - 20,000 cells per cm^2.

8. The formula to calculate the final volume depends on the specific type of chamber used. Cells/µL = Average number of cells per small grid x chamber multiplication factor x dilution.
9. The bioreactor provides continuous rotation of the tracheal bio-scaffold, exposing cells to a covering film of alternating gas and liquid phases.

ACKNOWLEDGEMENTS

This research was funded by Carraresi Foundation. The Authors are members of the COST Action CA16119 In vitro 3-D total cell guidance and fitness (CellFit) and of the Trans-COST Actions Task-Force on Covid-19.

References

1. Go T, Jungebluth P, Baiguero S, et al (2010) Both epithelial cells and mesenchymal stem cell-derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. J Thorac Cardiovasc Surg 139:437–443
2. Grillo HC (2002) Tracheal replacement: A critical review. Ann Thorac Surg 73:1995–2004
3. Aoki FG, Varma R, Marin-Araujo AE, et al (2019) De-epithelialization of porcine tracheal allografts as an approach for tracheal tissue engineering. Sci Rep 9
4. Birchall M and Macchiarini P (2008), Airway transplantation: A debate worth having?, https://pubmed.ncbi.nlm.nih.gov/18431223/
5. Macchiarini P (2004), Trachea-guided generation: Déjà vu all over again?, https://pubmed.ncbi.nlm.nih.gov/15224015/
6. Pennarossa G, Ghiringhelli M, Gandolfi F, et al (2020) Whole-ovary decellularization generates an effective 3D bioscaffold for ovarian bioengineering. J Assist Reprod Genet 1–11
7. Pennarossa G, Ghiringhelli M, Gandolfi F, et al (2021) Creation of a Bioengineered Ovary: Isolation of Female Germline Stem Cells for the Repopulation of a Decellularized Ovarian

Bioscaffold. Methods Mol Biol 2273:139–149

8. Ekser B, Cooper DKC, and Tector AJ (2015), The need for xenotransplantation as a source of organs and cells for clinical transplantation

9. Michel SG, Madariaga MLL, Villani V, et al (2015), Current progress in xenotransplantation and organ bioengineering, https://pubmed.ncbi.nlm.nih.gov/25496853/

10. Shi HC, Deng WJ, Pei C, et al (2009) Biomechanical properties of adult-excised porcine trachea for tracheal xenotransplantation. Xenotransplantation 16:181–186

11. Lin CH, Hsu S hui, Huang CE, et al (2009) A scaffold-bioreactor system for a tissue-engineered trachea. Biomaterials 30:4117–4126

12. Kojima K, Bonassar LJ, Roy AK, et al (2003) A composite tissue-engineered trachea using sheep nasal chondrocyte and epithelial cells. FASEB J 17:823–828

Figure legends

Figure 1. Decellularization protocol and macroscopic images illustrating changes in trachea colour, turning from red to white, while maintaining original shape.

Figure 2. Preparation of the repopulating cells, injection into the lumen of the ECM-based trachea bio-scaffold and its placement into the rotating bio-reactor for long-term culture.