Design and use of a rotating bioreactor for the culture of laryngeal transplants

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Abstract

Background: Patients requiring laryngeal transplants are faced with possible immune rejection and difficulty with donor matching, resulting in a procedure with very low efficacy. It has been shown that organs can be decellularized, leaving behind the extracellular matrix, and then recellularized with a patient's stem cells to avoid immune rejection upon transplantation. This presents an opportunity for a larynx to be decellularized and recellularized for use in transplants.

Methods: A bioreactor was fabricated with polycarbonate plastic using a CNC mill. To decellularize the tissue, a canine larynx is perfused with sodium dodecyl sulfate (SDS) through the vasculature and inner lumen. Progressive biopsies and hematoxylin and eosin stains are used to analyze the tissue. These stains are used to look at percent viability as well as give a gross overview of tissue condition and makeup after decellularization. Proteomic analysis by use of mass spectrometry give quantitative insight into the tissue condition post-decellularization, specifically in terms of protein numbers and percent makeup of the decellularized tissue.

Results: During initial decellularization trials, gross visualization of decellularized tissue as well as H&E stains revealed translucent tissue free of cell nuclei, indicating successful decellularization. This was confirmed by proteomic analysis, which demonstrated that 97.5% of total protein mass was eliminated from the tissue during decellularization trials. Therefore, a 1% SDS perfusion of laryngeal tissue for 72 hours is capable of decellularization of laryngeal tissue.

Conclusions: The novel bioreactor presented successfully facilitates decellularization of a canine larynx. Next, research into recellularization of decellularized tissue will occur.

Keywords: Laryngeal, bioreactor, recellularization, decellularization, perfusion, fluid dynamics modeling

Background

The need for organ replacement has grown in prominence over the last fifteen years. Wait lists for organs often exceed the donor rate ten-fold (1). Laryngeal cancer affects 136,000 individuals worldwide each year, and a majority of these patients require a partial or total removal of this organ to

stop the spread of cancer (2). After surgery, patients may be mute or may have to breathe through a stoma which can be problematic as it requires changes in daily activities such as speaking, showering, and sleeping (3). Laryngeal transplants can eliminate the need for a stoma, but at present there have only been two successful cases of full laryngeal transplant due to a complicated surgery and anatomical complexities making donor matching difficult (2, 4, 5). Additionally, patients that receive an allograft larynx transplant must live with the risk of tumor recurrence, metastasis, and multi-infection as well as face lifelong immunosuppression. Researchers have suggested that decreasing the immune response of the implantation by using the patient's own mesenchymal stem cells (MSC) to generate an organ could alleviate many of the problems currently associated with laryngeal allografts (2). Decellularizing the donor organ using detergents leaves behind a viable extracellular matrix (ECM) that can then be repopulated with the patient's MSCs. This process cannot be controlled *in vivo*, so a bioreactor must be available to construct an *ex vivo* environment that encourages cell proliferation and differentiation.

This method has been applied to a variety of whole organs, including the heart (6), lungs (7), and liver (8). Additionally, isolated structures of the larynx, such as laryngeal vocal folds, have been grown in a bioreactor and have been shown to withstand stresses similar to those seen in a biological environment (9). Finally, a trachea, which shares similar anatomical features to the larynx, has been grown *in vitro* using this method and implanted in a patient (10). As of March 2014, this surgery has been performed over a dozen times, and, while at least 3 patients died from complications unrelated to this transplant surgery, many have resumed normal functioning, including the first recipient of a bioengineered trachea, who has benefited from restored function to the present (11). Here, we present a novel bioreactor that facilitates the decellularization of a donor larynx and recellularization of the extracellular matrix with MSCs by rotation of the larynx in media and perfusion of detergents or media through the inner lumen and vasculature. The bioreactor minimizes the amount of media needed, is autoclavable, and allows automation of pump flow and larynx rotation.

Methods

Bioreactor Materials and Fabrication

The following descriptions of the bioreactor can be seen pictorially in figures 1A-D below. The bioreactor walls were cut out of polycarbonate (Grainger, Lake Forest IL) using a CNC mill to the external dimensions of 15x15x25cm. The 4x4x4cm stepper motor housing was cut in a similar fashion. A 17cm long cage was fabricated using a CNC mill. This cage is composed of a semi-circular polycarbonate piece and five 17cm long polycarbonate bars, which were cut to size using a band saw. Polycarbonate was chosen because it is one of the strongest thermoplastic polymers, can be machined easily, and is inert in contact with media (12). The holes for the cage and the bars connecting the cage to the walls were drilled with a mill. The stepper motor is connected to the cage via a polycarbonate rod and a set-screw. To add support and strength to the bioreactor walls, number 8 machine screws (Dorn True Value, Madison WI) are used to secure the base of the bioreactor to the sides. The hole for the drain was cut on the bottom of the bioreactor, 5mm from the edge. An adjustable 1.27cm rubber stopper (Dorn True Value, Madison WI) is used to allow draining of the device. Two rubber O-rings (Parker, Cleveland OH) are mounted on the cage supports to prevent leaking. The bioreactor is assembled with silicone sealant (DAP, Baltimore MD).

Pumps and Electronics

The system includes two peristaltic pumps (Langer Instruments, Boonton NJ). The bioreactor cage is rotated using a stepper motor that is controlled by an Arduino Uno (SparkFun, Boulder CO) and an EasyDriver (SparkFun, Boulder CO). Open source Arduino IDE software is used to control the cage rotation. The electronics were soldered together, and the system was tested to verify that the electronics and pumps functioned according to the manufacturers' specifications. The resulting circuitry is shown in figure 1C below. Testing of the system can be found in the supplementary information.

Setup of the System

During normal system operation, the larynx is secured to the cage using four sutures. The Model BT100-

1F pump tubing is attached to endotracheal tubes which are inserted into the superior and inferior orifices of the larynx. This is used to perfuse media through the interior of the larynx, inferiorly to superiorly, during the trial. The Model BT100-2 pump tubing is cannulated into the right and left superior laryngeal arteries in order to pump media or other fluids through the vasculature. The desired program for cage rotation frequency and time is uploaded from the Arduino IDE to the Arduino microcontroller and the system runs. When the trial is finished the drain at the bottom can be removed to drain the media from the bioreactor. For cleaning and gross adjustments, the cage can be removed from the support rods and the electronics can be removed from the device. For further information about the setup and operation of this device, see the user guide in the supplementary materials.

Decellularization

A fresh canine larynx is secured with four sutures (two superiorly, two inferiorly) to the cage portion of the device. Sodium dodecyl sulfate (SDS) solution of concentrations 0.1% and 1% is perfused through the interior of the tissue, as well as through the vasculature for periods of 72 and 96 hours. The void volume of the bioreactor is also filled with SDS solution, fully submerging the organ. The larynx, attached to the device, is then rotated 120 degrees back and forth for the duration of the testing. The specific conditions of each test are outlined in the results section below. After completion of the trial, immunohistochemistry is used to verify the presence of extracellular elements. Additionally, DAPI stains are used to detect residual nuclei. Furthermore, proteomic analysis is conducted on the decellularized tissue using 2D electrophoresis and high-resolution mass spectrometry. In this procedure, samples are minced and immersed in protein solubilization buffer before 2D SDS-PAGE is performed. 2D gel spots of interest are excised and run through a mass spectrometer. Samples are compared against a database to determine peptide sequences. For a more detailed report, see Welham, et al. (13).

Modeling

Computational fluid dynamics (CFD) modeling can be used to provide a wealth of information for tissue

engineers before they conduct experiments. By using a good CFD model, engineers and researchers can predict suitable conditions for experimentation and better streamline their experimental design. Therefore, a CFD model of the fluid flows within the inner lumen of the larynx within the laryngeal bioreactor was developed using ANSYS-Fluent (ANSYS, Canonsburg PA). A mesh describing the geometry of the larynx was designed using 36 progressive 0.5 mm thick coronal MRI image sections of a 10 year-olds' larynx. These images were uploaded into ImageJ, and their cross sectional area was analyzed. The geometry of each of these images was then duplicated in Solidworks (Dassault Systèmes, Waltham MA), and the images were joined together using the lofted base function within this program. The resulting mesh contains 109,000 nodes and 336,000 elements that are analyzed in each simulation, and is pictured in figure 2A below. The mesh was analyzed using a k-ε model, which is the most common turbulent fluid dynamics model, and takes into account turbulent energy and turbulent kinetic dissipation. The model was analyzed three times, with fluid flow rates of 1, 15, and 50 mL/min respectively. In each simulation, the parameters for aqueous media with 1% SDS were used, that is, the fluid passing through the inner lumen of the mesh was set to have a dynamic viscosity of 0.00078 Pa*s (0.75 centipoise) and a density of 1 g/mL.

Results and Discussion

Decellularization

The decellularized larynx was first examined grossly to qualitatively assess the state of the tissue. Figure 3 compares a complete human larynx with a decellularized canine larynx, exemplifying the difference in color between normal and decellularized tissue. Figures 4A-D compares trials 1-4 respectively, focusing attention on the vocal fold region. Trials 1 and 4 (2A, 2D) show loss of color in the vocal fold region, but some pink hues remain. The absence of pink tissue in trial 2 (2B) shows the complete loss of cells in that region, whereas trial 3 (2C) displays portions of the organ that were not successfully decellularized. The tissue was then evaluated using histological stains. Figure 5 displays a comparison of H&E stained sections of a normal larynx and a decellularized larynx. The lack of basophilic structures (stained in

purple) shows the absence of nuclei while the ECM proteins remain. Next, the tissue was examined using proteomic analysis. The results as well as conditions for each test are summarized in Table 1. The first trial had an ECM to cytoplasmic protein ratio of 0.88, indicating that there are more cytoplasmic proteins than ECM proteins in the decellularized matrix. The second trial aimed to improve this ratio by increasing the amount of time the tissue was in contact with the SDS solution. In this case, the ratio was even smaller. This could be due to the cytoplasmic proteins adhering to the matrix as the cells lyse accompanied by the ECM being washed away by the increased time in contact with the SDS. The third trial added a wash step of Triton X-100 to remove the proteins that may be adhering to the ECM. This trial had a ratio of approximately 1, showing an even amount of cytoplasmic to ECM proteins. Although this trial had a better ratio, the amount of cytoplasmic protein left over could prove immunogenic when eventually implanted, and may again be due to the SDS detergent degrading the ECM. Of note however, is the amount of total protein remaining in the third trial; a comparable amount to other decellularized tissues such as the lungs and liver. The fourth trial will use a less concentrated SDS solution of 0.1% in order to see if the SDS is removing the desired ECM matrix along with the cytoplasmic proteins. This data was not available upon submission, but visual inspection of the tissue looked promising. The successful decellularization of the larynx is a vital step before progressing to the recellularization stage.

Modeling

The resulting CFD model provides researchers with fluid velocities, shear stresses on tissue inner walls, locations of turbulence, pressures within the larynx, and heat fluxes within the part with accuracy of more than 0.1mm, and at 316,000 different points within the larynx. Because it would be impossible to present all of the results in this discussion, the resulting models are available, by request, from the authors. Two representative images from the final model, demonstrating shear stresses and fluid velocities under 50mL/min flow rate conditions, are shown in figure 2B and 2C respectively. Of note, at 1mL/min of flow, the maximum shear stress within the model is 0.4kPa and the maximum fluid velocity is 1.37 m/s, which corresponds to a Reynolds number of 126.88 near the vocal folds. All of these values

are well below normal physiological conditions, and it should be noted that this Reynolds number represents laminar flow. Using a flow rate of 15 mL/min, the model shows that the Reynolds number at the vocal folds reaches 1903. This demonstrates that the fluid flow transitions from laminar to turbulent flow. When the flow rate is increased to 50 mL/min, the Reynolds number reaches 6344, the velocity reaches 4.7 m/s, and the maximum shear stress is 5.6kPa. It should be noted that the Reynolds numbers, shear stresses, and fluid velocities presented here are the maximum that occurs in the model under the respective conditions. Elsewhere in the model, laminar flow is maintained and shear stresses on the walls are reduced. The equations for each of these Reynolds numbers can be found in the supplemental materials. While, in most conditions normal subglottal pressures are modeled between 0.8 and 1.2 kPa, coughing or singing can cause vastly increased subglottal pressures of up to 8kPa (14, 15). Additionally, the physiological max flow rates (of air) are 7.5 m/s, and Reynolds numbers under physiological conditions can reach up to 6,400 (15).

Because of the different material properties of cartilage, tendon, epithelial tissue, and muscle tissue, it is complex to design experimentation protocols for an organ such as the larynx. However, for the purposes of this research, the vocal folds are the most important region of the tissue, and therefore experimental design should be centered on their effective decellularization and recellularization. This presents a paradox because this region of the organ is also subject to the greatest stresses and fluid velocities, both *in vivo* and *in vitro* due to the narrowing of the organ, which is confirmed in the model. Additionally, different stresses must be exerted on this tissue at various points in the experimentation to cause different behaviors in the cells. Therefore, to maintain laminar flow in all portions of the larynx, a flow rate of less than 15mL/min should be used; these speeds will be appropriate for initial recellularization (16). However, for later recellularization, *in vivo* conditions should be mimicked to acclimate cells to their future environment. Therefore, at these points of experimentation, flow rates of above 15mL/min, even reaching 50mL/min should be used.

Device Redesign

After using the initial prototype, several changes were proposed for the second prototype. The cage portion of the device was too small to accommodate a human larynx, so the length of the cage was increased from 13cm to 17cm. Similarly, the width of the entire device was too small for the researchers to easily access and manipulate the cage and larynx. The size of the bioreactor was thus increased from 25x12x15cm to 25x12x20cm. The legs of the original prototype were removed to add durability to the device. As a result the drain was moved from the bottom of the device to the side, which provides easier access to the drain. It was also found that the tubes going to the lumen and vasculature were being disturbed by the rotation of the cage. To fix this, wide semi-circular notches were cut on the sides of the bioreactor to better hold these tubes. Additionally, the O-ring was attached in a way that allows it to be removed and replaced, and the bottom portion of the motor housing was removed to prevent fluid pooling around the motor. Finally, the sealant was changed to from a silicone aquarium sealant to acrylic cement. This added durability and prevented leaking found with the silicone sealant.

Future Work

Moving forward, a number of actions need to be considered. First, the decellularization process must be optimized in order to be completed in the shortest amount of time possible. When an optimized process for decellularizing the tissue is found, the recellularization of the organ can begin. Once the tissue is devoid of cells, fibroblasts will be injected into multiple points of the larynx focusing around the vocal folds. A period of stagnant placement of the seeded organ in media for approximately six hours will likely result in the best adhesion of cells to the decellularized tissue (17, 18). The cells will be cultured and assayed for proliferation and viability using BrdU and Trypan Blue assays. This process will also be optimized by altering flow rates suggested by the computational model to induce shear stresses on the cells. The computational model will be expanded to examine stresses on the exterior of the larynx as well as the interior, and to include the dynamic properties of laryngeal tissue.

Conclusion

We present a novel bioreactor that can facilitate the decellularization and recellularization of a canine or human larynx. The system allows for separate perfusion of the inner lumen and vasculature of the larynx. We have shown that the larynx can be successfully decellularized using 1% SDS detergent, but the process has yet to be optimized. Future work entails recellularizing the tissue and further developing the computational model.

List of abbreviations

SDS – sodium dodecyl sulfate

MSC – mesenchymal stem cell

ECM – extracellular matrix

H&E – hematoxylin and eosin

CFD – computational fluid dynamics

SDS-PAGE – polyacrylamide gel electrophoresis

CNC mill – computer numerical control mill

Arduino IDE – integrated development environment

Competing interests

The authors have no competing interests to report.

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References

1. "Organ Donation: The Need Is Real." *Organdonor.gov*. US Department of Health and Human Services, n.d. Web. 06 Oct. 2013. <http://www.organdonor.gov/about/data.html>.

2. Hou, Nan, Pengcheng Cui, Jiasheng Lou, Ruina Ma, and Li Zhu. "Tissue-engineered Larynx Using Perfusion-decellularized Technique and Mesenchymal Stem Cells in a Rabbit Model." *Acta Oto-Laryngologica* 131 (2011): 645-52.

3. Song, John, and Ed Zimmey. "Is It Hard to Live With a Stoma After Laryngeal Cancer?"*EverydayHealth.com*. Everyday Health Media LLC, 23 June 2008. Web. 06 Oct. 2013.

4. Strome, Marshall, Jeannine Stein, Ramon Esclamado, Douglas Hicks, Robert R. Lorenz, William Braun, Randall Yetman, Isaac Eliachar, and James Mayes. "Laryngeal Transplantation and 40-Month Follow-up." *New England Journal of Medicine*344.22 (2001): 1676-679.

5. Varney, Sarah. "Larynx Transplant On Calif. Woman Successful." *NPR*. N.p., 20 Jan. 2011.

6. Ott, Herald, Thomas Matthiesen, Saik-Kia Goh, Lauren Black, Stefan Kren, Theoden Netoff, and Doris Taylor. "Perfusion-decellularized Matrix: Using Nature’s Platform to Engineer a Bioartificial Heart." *Nature Medicine* 14.2 (2009): 213-21.

7. Petersen, Thomas H., Elizabeth A. Calle, Maegan B. Colehour, and Laura A. Niklason. "Bioreactor for the Long-Term Culture of Lung Tissue." *Cell Transplantation* 20 (2011): 1117-126

8. Gilbert, T., T. Sellaro, and S. Badylak. "Decellularization of Tissues and Organs."*Biomaterials* 27.19 (2006): 3675-683.

9. Titze, I., R. Hitchcock, K. Broadhead, K. Webb, W. Li, S. Gray, and P. Tresco. "Design and Validation of a Bioreactor for Engineering Vocal Fold Tissues under Combined Tensile and Vibrational Stresses." *Journal of Biomechanics* 37.10 (2004): 1521-529.

10. Asnaghi, M. Adelaide, Philipp Jungebluth, Manuela T. Raimondi, Sally C. Dickinson, Louisa E.N. Rees, Tetsuhiko Go, Tristan A. Cogan, Amanda Dodson, Pier Paolo Parnigotto, and Anthony P. Hollander. "A Double-chamber Rotating Bioreactor for the Development of Tissue-engineered Hollow Organs: From Concept to Clinical Trial." *Biomaterials* 30.29 (2009): 5260-269.

11. Vogel, G. "Trachea Transplants Test the Limits." *Science* 340.6130 (2013): 266-68.

12. Osswald, T., and G. Menges. *Materials Science of Polymers for Engineers*. 3E ed. Munich: Carl Hanser, 1996

13. Nathan V. Welham, Zhen Chang, Lloyd M. Smith, Brian L. Frey. “Proteomic analysis of a decellularized human vocal fold mucosa scaffold using 2D electrophoresis and high-resolution mass spectrometry.” *Biomaterials.* Volume 34, Issue 3, January 2013, Pages 669-676

14. Gunter, Heather Elspeth. *Mechanical Stresses in Vocal Fold Tissue during Voice Production.* Thesis. Harvard University, 2003.

15. Farahani, Mehrad. *Modeling of the Human Larynx with Application to the Influence of False Vocal Folds on the Glottal Flow*. Thesis. University of Iowa, 2013. N.p.: Iowa Research Online, 2013.

16. Luo, Haoxiang, Rajat Mittal, and Steven A. Bielamowicz. "Analysis of Flow-structure Interaction in the Larynx during Phonation Using an Immersed-boundary Method." *The Journal of the Acoustical Society of America*126.2 (2009): 816-36.

17. Asnaghi, M. Adelaide, et al. "A Double-chamber Rotating Bioreactor for the Development of Tissue-engineered Hollow Organs: From Concept to Clinical Trial." *Biomaterials* 30.29 (2009): 5260-269.

18. Lin, Chen-Huan, Shan-Hui Hsu, Chi-En Huang, Wen-Tung Cheng, and Jang-Ming Su. "A Scaffold-bioreactor System for a Tissue-engineered Trachea." *Biomaterials* 30.25 (2009): 4117-126.

19. "Remote Monitoring and Control of Bioprocesses." *DASGIP*. Eppendorf, n.d. Web. 24 Feb. 2014. <http://www.dasgip.com/catalog/DW_access/>.

Tables

Table 1: This table displays the trial conditions and proteomic results of the four trials (trial 4 data unavailable). All trials resulted in decreased protein amounts and types from the initial state. The ECM protein to cytoplasmic protein ratio in the 3 trials is below 1, indicating that ECM proteins are being degraded.

Trial	**Wash**	**SDS%**	**Time (hr)**	**Rotation**	**# of Protein Types Remaining** (1640 for intact tissue)	**Grams protein remaining** (60 µg for intact)	**Results**
1	PBS	1	72	120°	1137	3.9 µg	ECM proteins = 29.8%
							Cytoplasmic proteins = 33.5%
2	PBS	1	96	120°	682	4.0 µg	ECM proteins = 19.7%
							Cytoplasmic = 44.2%
3	Triton X-100	1	72	120°	1097	1.5 µg	ECM proteins = 31.0%
							Cytoplasmic = 31.6%
4	Triton X-100	0.1	72	120°	No data	No data	No data

Figures

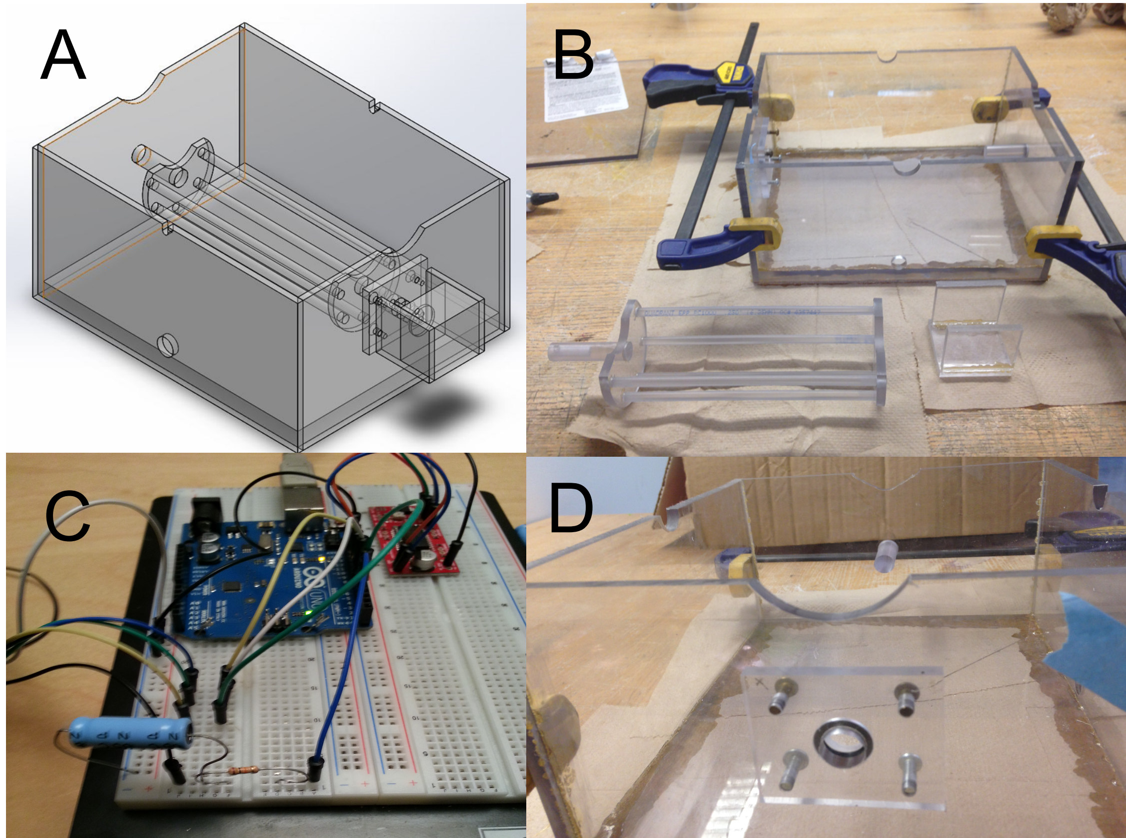

Figure 1: A: Solidworks drawing of redesigned prototype. **B:** Construction of redesigned prototype. **C:** Arduino microcontroller and EasyDriver configuration for automation of stepper motor. **D:** Focus on removable O-ring attachment and notches for tubes.

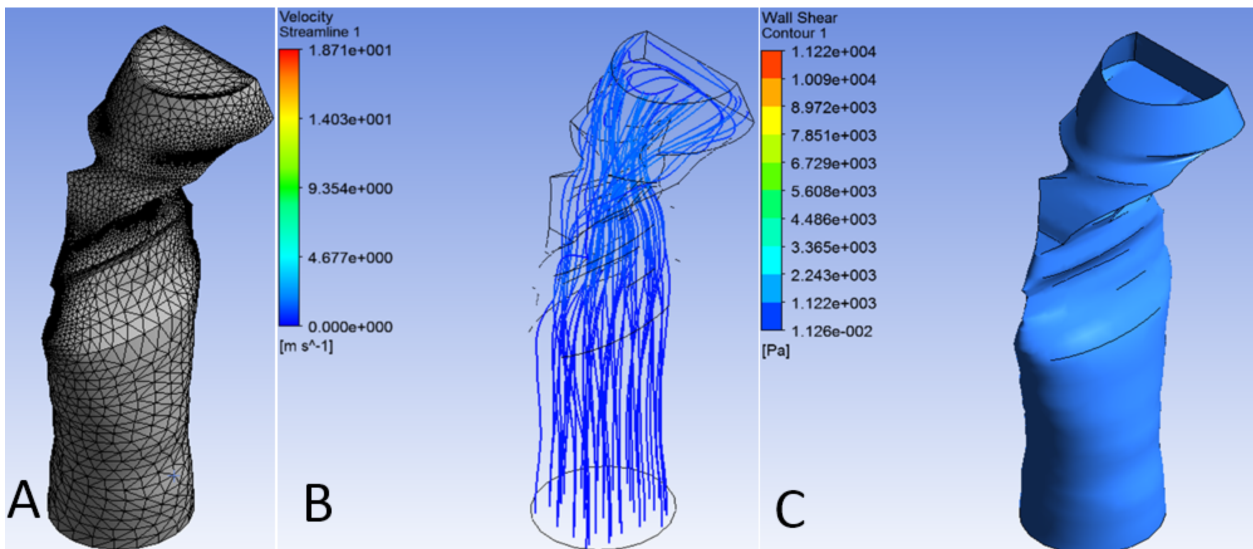

Figure 2: Pictures from ANSYS modeling **A:** Anatomical model of the mesh. **B:** Velocity profiles within the mesh, showing fastest velocities at the vocal folds. **C:** Shear stress profiles in the mesh, showing highest stresses at the vocal fold region.

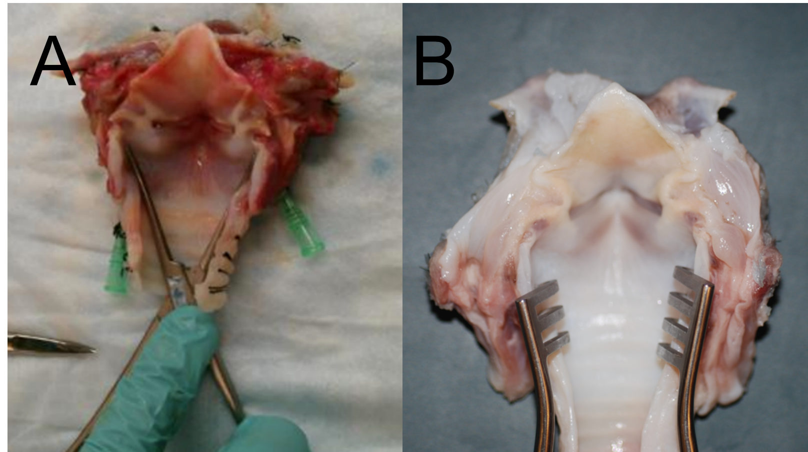

Figure 3: A: Complete larynx extracted from a human donor. **B:** Complete canine larynx partially decellularized. This larynx was placed in 1% SDS solution for 72 hours. Note the lack of redness and the whiteness of the tissue. This is indicative of decellularized tissue.

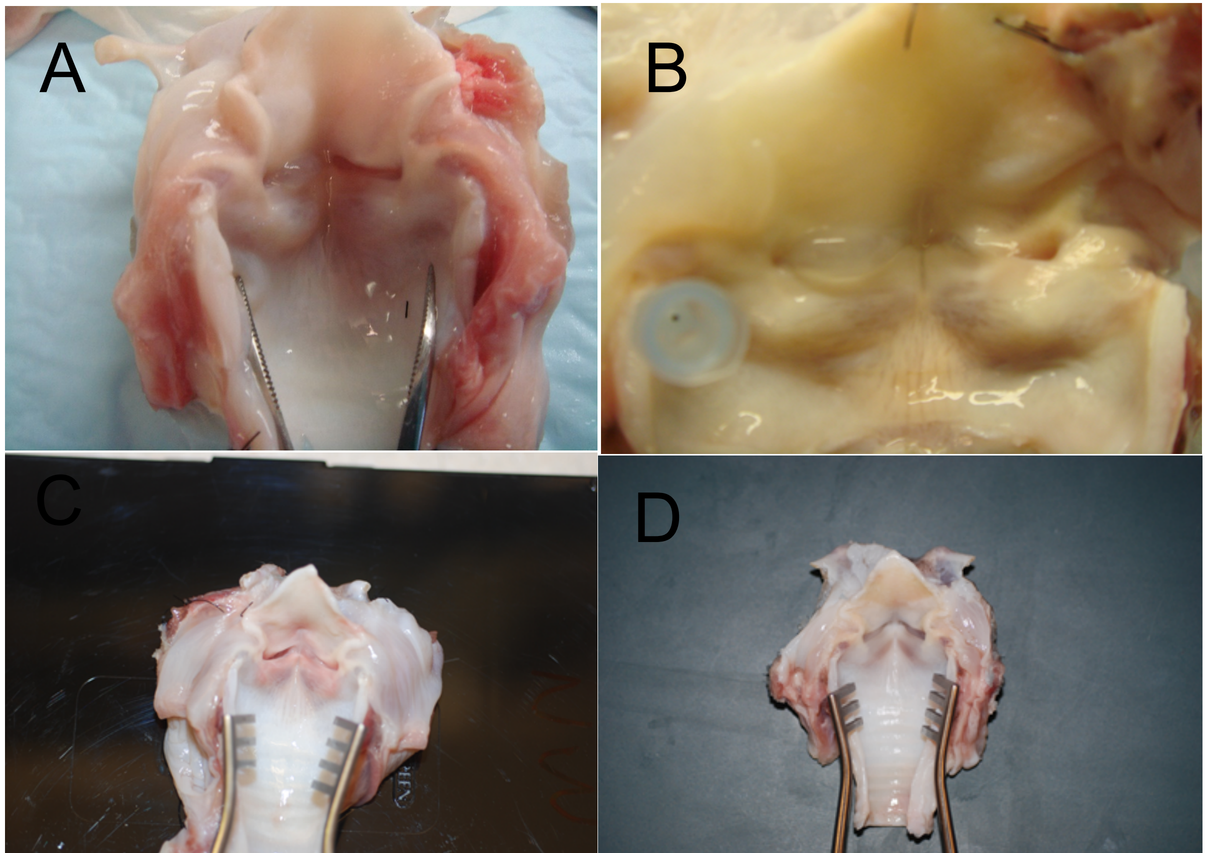

Figure 4: Gross evaluation of each trial. **A:** For trial 1, the lack of color inside the tissue indicates that most of the cells have been removed. Some color remains. **B:** Trial 2 shows complete lack of color. **C:** Trial 3 shows lack of color with slight pink in vocal fold region. **D:** Trial 4 shows lack of color in trachea and vocal fold regions.

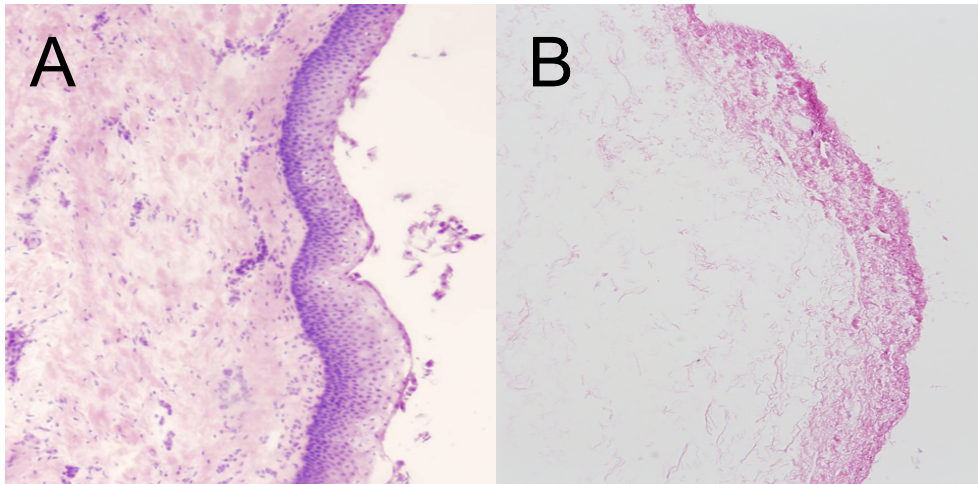

Figure 5: A: H&E stain of intact laryngeal tissue, showing concentrated regions of nuclei. **B:** Stain shows little to no evidence of residual nuclei while ECM remains.