METHODS ARTICLE

Standardization of Microcomputed Tomography for Tracheal Tissue Engineering Analysis

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Tracheal tissue engineering has become an active area of interest among clinical and scientific communities; however, methods to evaluate success of *in vivo* tissue-engineered solutions remain primarily qualitative. These evaluation methods have generally relied on the use of photographs to qualitatively demonstrate tracheal patency, endoscopy to image healing over time, and histology to determine the quality of the regenerated extracellular matrix. Although those generally qualitative methods are valuable, they alone may be insufficient. Therefore, to quantitatively assess tracheal regeneration, we recommend the inclusion of microcomputed tomography (μ CT) to quantify tracheal patency as a standard outcome analysis. To establish a standard of practice for quantitative μ CT assessment for tracheal tissue engineering, we recommend selecting a constant length to quantify airway volume. Dividing airway volumes by a constant length provides an *average* cross-sectional area for comparing groups. We caution against selecting a length that is unjustifiably large, which may result in artificially inflating the average cross-sectional area and thereby diminishing the ability to detect actual differences between a test group and a healthy control. Therefore, we recommend selecting a length for μ CT assessment that corresponds to the length of the defect region. We further recommend quantifying the *minimum* cross-sectional area, which does not depend on the length, but has functional implications for breathing. We present empirical data to elucidate the rationale for these recommendations. These empirical data may at first glance appear as expected and unsurprising. However, these standard methods for performing μ CT and presentation of results do not yet exist in the literature, and are necessary to improve reporting within the field. Quantitative analyses will better enable comparisons between future publications within the tracheal tissue engineering community and empower a more rigorous assessment of results.

Keywords: microcomputed tomography, trachea, tracheal lumen, tracheal stenosis, tracheal tissue engineering

Impact Statement

The current study argues for the standardization of microcomputed tomography (μ CT) as a quantitative method for evaluating tracheal tissue-engineered solutions *in vivo* or *ex vivo*. The field of tracheal tissue engineering has generally relied on the use of qualitative methods for determining tracheal patency. A standardized quantitative evaluation method currently does not exist. The standardization of μ CT for evaluation of *in vivo* studies would enable a more robust characterization and allow comparisons between groups within the field. The impact of standardized methods within the tracheal tissue engineering field as presented in the current study would greatly improve the quality of published work.

Introduction

THE TRACHEAL TISSUE ENGINEERING community has actively developed novel strategies for the treatment of tracheal defects culminating in a plethora of creative approaches.^{1–5} Tracheal stenoses resulting from severe tracheal abnormalities from cancer, intubation, trauma, and idio-

pathic disorders have driven the need for innovative strategies to treat afflicted patients. Leading clinical treatment paradigms to address short-segment tracheal stenosis have relied on tracheal resection with end-to-end anastomosis and/or laryngotracheal reconstruction, utilizing autograft cartilage tissue harvested from the rib cage.^{6–8} Long-segment tracheal defects have previously been treated in the clinic

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using slide-tracheoplasty, a complicated surgical procedure where the trachea is divided, incised lengthwise, then fitted together, thereby shortening the length while expanding the lumen diameter.^{9–11} Harvesting autograft tissue can be painful for the patient and manipulation of cartilage to fit the defect can be difficult.¹² In addition, complicated surgical procedures such as slide-tracheoplasty can limit the number of available surgeons who can perform the procedure. An obvious need exists for new treatment methods for both long- and short-segment defects, empowering the tracheal tissue engineering community to fill the present unmet need.

Various tracheal tissue engineering strategies include the use of decellularized tracheal allografts,^{13–15} naturally derived acellular materials such as silk fibroin scaffolds,¹⁶ shape-specific three-dimensional (3D) bioprinted materials,^{17–19} electrospun scaffolds,^{20,21} and cell-based constructs utilizing scaffold-free technology.^{22–25} Methods to evaluate success criteria have generally relied on the use of photographs to qualitatively demonstrate tracheal patency, endoscopy to image luminal healing over time, and/or histology to demonstrate regenerated extracellular matrix quality. Although the aforementioned methods are valuable and reasonable for assessment of regeneration for a tissue-engineered approach, the limitation is that these methods remain generally qualitative.

The purpose is therefore to present the use of micro-computed tomography (μ CT) to quantify tracheal patency as an addition to the standard methods already used. μ CT is a nondestructive method to image samples and create 3D models that can quantify tracheal patency. The method can be applied *in vivo* to evaluate regeneration over time or *ex vivo* as an endpoint analysis. Previous groups have used computed tomography to visualize the trachea after applying a tissue-engineered intervention; however, patency was not quantified.^{26,27} Quantifiable methods for evaluating success will allow for better comparisons among various groups within the field. μ CT testing can be easily implemented into the current testing paradigm used by many groups as it is a nondestructive imaging method that can be applied to samples designated for histology. Our group has previously used μ CT for assessment and quantification of tracheal regeneration in both sheep and rabbit animal models.^{28–30} The proceeding *ex vivo* data and associated analyses serve to illustrate methods to perform μ CT, how to determine the appropriate volume of interest for analyses, and form the basis for a recommendation for standard practice within the tracheal tissue engineering field.

Materials and Methods

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (protocol #18-008-SS-A-U).

Explanted rabbit tracheas were fixed in formalin for 48 h followed by incubation in a diluted contrast solution of 40% Optiray 320 and 60% phosphate-buffered saline for 24 h. Incubation in contrast solution enhanced μ CT image quality and improved soft tissue contrast. Immediately before μ CT imaging, explanted tracheas were removed from contrast solution and patted dry using gauze. The Quantum FX imaging system with a 50 kV X-ray source at 160 μ A was used for imaging. A square 60 mm field of view produced a voxel resolution of 118 μ m. After μ CT imaging, Avizo computa-

tional software was used to reconstruct μ CT images into a 3D model to quantify tracheal patency. To demonstrate the importance of selecting a representative tracheal length for volumetric quantification, the current study used three lengths of 15, 25, and 35 mm for μ CT analysis. The rendered 3D model was then cropped to the desired length of trachea for quantification of lumen volume and minimum cross-sectional area. The empty lumen space could then be partitioned separately from the exterior tissue using a global threshold range. Volumetric quantification of the partitioned lumen volume was then computed using the Avizo software. Other computational software could be used to conduct the analysis described in the current study if desired.

Experiment

Experimental groups

To best illustrate a focus on μ CT as an outcome assessment method, we returned to raw data from a rabbit study that we recently published.³⁰ Data from both healthy and stenosed tracheas ($n=6$) were obtained for the purpose of comparing the chosen length scale for the volume of interest (VOI). In-depth information regarding patch fabrication was described in the previous publication.³⁰ Stenosed tracheas were induced with a tracheal defect and then patched using an electrospun polycaprolactone (PCL, Mw = 80,000; Wuhan Pensieve Technology Ltd., Wuhan, China) nanofiber patch containing 3D-printed PCL rings. The baseline electrospun PCL patches induced a stenosis after implantation into the proceeding rabbit animal model.

Animal model

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (protocol #18-008-SS-A-U). The animal model and surgical method are described in detail in our previous publication.³⁰ Briefly, six skeletally mature female New Zealand white rabbits (~ 4.5 kg) were purchased from Robinson Services Incorporated (Mocksville, NC). A tracheal defect was induced by excising a diamond-shaped portion of the trachea measuring 15 $\times$ 5 mm, where the longer axis was oriented along the length of the trachea. The defects were centered approximately between the carina and cricoid cartilage. For the stenosed group, an electrospun PCL patch with an approximate length of ~ 18 mm was sutured to the trachea to cover the defect. After a 12-week surgery, the rabbits were euthanized in accordance with AVMA guidelines. Directly after euthanasia, the rabbit tracheas were harvested and fixed in 10% neutral buffered formalin before μ CT analysis. Although tissue contraction from formalin fixation was expected, the preservation of the tracheal tissue counterbalanced the negative effect. Healthy control tracheas received no surgery and were acquired from age-, breed-, and sex-matched rabbits from unrelated studies.

Microcomputed tomography

μ CT was used to quantify tracheal patency on harvested tracheas after 12 weeks of recovery ($n=6$) as previously described.^{29,30} Before imaging, explanted tracheas were removed from formalin and then incubated in a diluted solution of 40% Optiray 320 (Mallinckrodt Pharmaceuticals,

Staines-upon-Thames, UK) and 60% phosphate-buffered saline for 24 h. Soaking explanted tracheas in the diluted contrast agent was used to better image soft tissue for quantifying tracheal patency. A Quantum FX imaging system (Perkin-Elmer, Waltham, MA) with a 50 kV X-ray source at 160 μ A was used for imaging. A square 60 mm field of view was used that produced a voxel resolution of 118 μ m.

Avizo analysis

Avizo computational software (FEI Company, Hillsboro, OR) was used to reconstruct μ CT images into a 3D model to quantify tracheal patency. To illustrate the importance of tracheal length that the investigator selects for μ CT analysis, three different lengths of 15, 25, and 35 mm were analyzed, each with a corresponding VOI. We emphasize that the 15 mm VOI length corresponds to the 15 mm length of the induced defect. From this VOI, the lumen volume, average cross-sectional area, and minimum cross-sectional area were determined. A global threshold range was used for quantifying the empty space within the trachea. The average cross-sectional area (i.e., normalized volume) was calculated by dividing the lumen volume by the selected VOI length. Tracheal tissue was depicted using a gray scale and the quantified lumen space within the VOI was colored blue for delineation (Fig. 1). Due to potential tissue contraction from formalin fixation, comparisons should only be conducted among samples that have undergone formalin fixation. In addition, species/breed/age/sex-matched comparisons across

studies should be careful to avoid *in vivo* versus *ex vivo* comparisons due to tissue contraction from formalin fixation.

Statistical methods

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA) statistical software. An unpaired *t*-test (two-tailed) was used to analyze μ CT results between the two groups (i.e., healthy vs. stenosed) for each VOI. Results were considered significant at a level of $p < 0.05$ and all data were reported as mean $\pm$ standard deviation. μ CT testing had $n = 6$ samples per group.

Results

Lumen volume

Tracheal lumen volume was compared between the healthy and stenosed tracheas for length scales of 15, 25, and 35 mm (Fig. 2A). The healthy lumen volume ($276 \pm 36 \text{ mm}^3$) for the 15 mm VOI length scale was 1.45 times larger than the stenosed group ($p < 0.05$). In contrast, in analyzing the exact same specimens using the VOI corresponding to a length of 25 mm, the healthy lumen volume ($471 \pm 54 \text{ mm}^3$) was only 1.13 times larger than the stenosed group, which was *not* significant ($p > 0.05$). Likewise, for the VOI corresponding to a length of 35 mm, the healthy lumen volume ($662 \pm 78 \text{ mm}^3$) was 1.05 times larger than the stenosed lumen volume ($p > 0.05$, not significant).

Average cross-sectional area

Average tracheal cross-sectional area was compared between the healthy and stenosed tracheas at length scales of 15, 25, and 35 mm (Fig. 2B). We emphasize that because a specific length was selected, for example, 15 mm, dividing the volumes of the healthy and stenosed groups by that fixed length resulted in average cross-sectional area differences between the groups that were identical to volume differences reported in the previous section (Fig. 3). Therefore, the healthy average cross-sectional area ($18 \pm 2 \text{ mm}^2$) for the 15 mm VOI length scale was 1.45 times larger than the stenosed group ($p < 0.05$). For the 25 mm VOI length scale, the healthy average cross-sectional area ($19 \pm 2 \text{ mm}^2$) was 1.13 times larger than the stenosed group ($p > 0.05$, not significant). Finally, for the 35 mm VOI length scale, the healthy average cross-sectional area ($19 \pm 2 \text{ mm}^2$) was 1.05 times larger than the stenosed group ($p > 0.05$, not significant).

Minimum cross-sectional area

Minimum tracheal cross-sectional areas were compared between the healthy and stenosed tracheas at length scales of 15, 25, and 35 mm (Fig. 2C). The healthy minimum cross-sectional area ($18 \pm 2 \text{ mm}^2$) for the 15, 25, and 35 mm VOI length scale was 2.70 times larger than the stenosed group ($p < 0.05$). The minimum cross-sectional area is of course independent of the choice of VOI length scale.

Discussion

Determining the appropriate length for the VOI is vitally important as the volume and average cross-sectional area are both dependent on this choice. Too long a length scale can reduce or diminish the ability to detect actual differences as

FIG. 1. Representative μ CT reconstructions of a healthy control trachea and a stenosed trachea. Reconstructions are presented as sagittal cross sections to visualize the inner lumen diameter. The lumen VOI was quantified across investigator-selected lengths of 15, 25, and 35 mm as illustrated by the blue volume within the trachea. Scale bar = 10 mm. $n = 6$. μ CT, microcomputed tomography; VOI, volume of interest.

FIG. 2. Quantitative analyses from the μ CT testing across VOI lengths of 15, 25, and 35 mm. (A) Lumen volume between healthy control tracheas and stenosed tracheas. Note that increasing VOI length sacrificed the detection of a statistically significant difference between the control and stenosed group by artificially inflating the volume of the stenosed group (visualized in Fig. 1). (B) Average cross-sectional area determined across varying VOI lengths. Note that the value of the healthy cross-sectional area remains virtually unchanged regardless of the selected length, but that the stenosed values become artificially inflated to approach the healthy value with longer investigator-selected lengths, which likewise sacrifices the detection of a statistically significant difference. (C) Minimum cross-sectional area is independent of the selected length for a given group. Results can therefore be artificially manipulated based on inappropriate (i.e., too long) selection of length for lumen volume and average cross-sectional area, but not for minimum cross-sectional area. * $p < 0.05$, $n = 6$.

demonstrated by the lumen volume and average cross-sectional area data reported in Figure 2. To choose an appropriate length scale, we recommend that the decision be based on a standardized length that matches the dimension of the original tracheal defect. The smallest length scale used in the current study (i.e., 15 mm) represents the VOI length scale matching the length of the original defect, allowing the detection of a significant decrease in lumen volume and average cross-sectional area for the stenosed group compared with the healthy control. The ability to detect this difference was sacrificed when larger length scales were used because a greater length of healthy tissue was averaged into the stenosed treatment group, thereby artificially increasing its volume and average cross-sectional area. In other words, increasing length scales (i.e., 25 or 35 mm) demonstrated a “padding effect” that minimized the average difference for the lumen volume and average cross-sectional area between the healthy and stenosed groups. Therefore, the future investigator’s selection of an

unjustifiably long length may inaccurately imply that a treatment group performed better than it had in reality. To better represent findings, we therefore recommend specifically using a VOI length scale representative of the original defect length.

Generally, the most common method of quantifying tracheal patency has been the use of average cross-sectional diameter or area using an endoscope.^{31,32} Measurements acquired using an endoscope can be unintentionally skewed as the endoscopic probing can manipulate the shape of the trachea if the regenerated tissue is weak. Use of a rigid endoscope can potentially displace regenerating tracheal tissue. In addition, endoscopic imaging is dependent on the user subjectively determining the point of minimum stenosis. The addition of μ CT improves upon current methods because no manipulation is required to achieve full 3D reconstructions. In addition, the total volume can be assessed rather than just a given cross-sectional area. Using a unified length scale among all samples enables both the lumen volume and average cross-sectional area to be presented on the same graph (Fig. 3), allowing for data to be compared with previous and future studies easily.

Separately, as an entirely different outcome metric, we recommend reporting the minimum cross-sectional area in addition to the lumen volume and average cross-sectional area. As demonstrated by the various length scales, the minimum cross-sectional area is independent of the chosen length scale. In addition, the comparison between the lumen volume and minimum cross-sectional area can lend insight to whether the treatment is causing a gradual restenosis across the defect or an acute stenosis (or “pinch point”) at a single point of the defect. Furthermore, there is a functional significance with the minimum cross-sectional area, which may be the most important factor in determining whether the subject will have difficulty breathing. Future studies may additionally normalize the VOI and minimum cross-sectional area to the normal healthy control for comparing groups to enable easier comparisons among future studies.

FIG. 3. Representation of data to report for future publications. Note that both lumen volume and average cross-sectional area are able to be presented on the same chart, but only because a consistent VOI length (15 mm) is used. The 15 mm VOI length matched the length of the induced defect. * $p < 0.05$, $n = 6$.

In conclusion, we recommend the use of μ CT to quantify tracheal patency, which enables improved comparisons among treatments within the field. We recommend the use of a VOI corresponding to the defect region and a standardized length scale for evaluating all samples. Increasing the lengths to arbitrarily large values for the VOI was demonstrated to artificially inflate the average cross-sectional area of the stenosed group, thereby decreasing differences between the healthy and stenosed groups and failing to detect statistically significant differences that were evident with the more reasonable 15 mm length corresponding to the true defect region of interest.

In summary, we recommend as standard practice in tracheal tissue engineering reporting the lumen volume and average cross-sectional area together on the same chart (Fig. 3), made possible by selecting a fixed length for all samples that corresponds to the actual length of the defect. Although volumes may differ based on the selected fixed length, the average cross-sectional area for a given healthy species, breed, sex, and age should be fairly reproducible from study to study and thereby offer more rigorous comparisons of treatment groups in studies from different investigators around the world. The corresponding method can equally be applied to a full segmental defect or a defect placed at a different location on the trachea, provided that the proximal and distal anastomoses are included within the VOI. Moreover, we recommend reporting the minimum cross-sectional area as standard practice, given the functional importance with regard to breathing difficulty. Implementation of these quantitative methods will improve the field of tracheal tissue engineering by allowing more standardized, rigorous, and reproducible comparisons across studies.

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