



Mechanical properties of de-epithelialized tracheal allografts

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Background: Many studies have shown that mucosa-treated tracheas can be used for allotransplantation without the use of immunosuppressants. However, no literature has reported whether the transplanted trachea has normal biomechanics. In this study, fresh dog trachea was used as a control to determine the mechanical properties of allograft trachea after mucous membrane removal.

Methods: Cervical tracheas (~10 cm in length, consisting of the cartilage rings down to the tracheal carina) and the surrounding connective tissues were removed from 20 mixed-breed dogs and then divided into 4 groups with 5 samples in each group. The groups were defined as follows: group A, fresh canine tracheas without any treatment; group B, canine tracheas after sodium dodecyl sulphate (SDS) de-epithelialization; group C, canine tracheas implanted into the dorsal fascia of beagle dogs for 1 month after SDS de-epithelialization; group D, canine tracheas implanted into the dorsal fascia of beagle dogs for 6 months after SDS de-epithelialization. All heterotopically implanted beagle dogs were free of any immunosuppressive treatment. Uniaxial tensile (n=20) and compressive (n=20) experiments were performed on the 4 groups of trachea using a universal testing machine; the tensile load was recorded at 150% of the original tracheal length (50% displacement), and the compressive load was recorded at 50% of the initial anteroposterior diameter (50% displacement). The average difference in tensile and compressive loads between groups was compared by analysis of variance.

Results: No significant difference was found in the mean compressive strength among groups (group A =2.38±0.87 N, group B =3.33±1.03 N, group C =5.39±2.44 N, group D =3.04±2.12 N; P=0.07 for all groups). The mean tensile strength of group C was 9.68±0.86 N, resulting in a significant difference in tensile strength compared to group A (22.51±3.91 N) and B (24.24±7.46 N) (P<0.001). No significant difference in tensile strength was observed among groups A, B, and D.

Conclusions: The de-epithelialized trachea showed no significant change in mechanical properties after 6 months of implantation.

Keywords: De-epithelialized; allograft; tensile strength; compression; biomechanics

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Introduction

Tracheal defect is a common, life-threatening, clinical disease that is mainly caused by tumor, trauma, long-time intubation, or congenital disease. Tracheal defects within 6 cm in size can be repaired by anastomosis, while tracheal defects larger than 6 cm are difficult to repair. Tracheal transplantation is considered a relatively ideal method to repair large area tracheal defects. Clinical cases of successful allograft tracheal transplantation have been reported (1). However, immunosuppressive agents are required after transplantation, which increase the probability of tumor recurrence and opportunistic infection. The trachea has very low antigenicity compared with other organs (2,3). Furthermore, its antigenicity has been found to be mainly located in the mucous layer of the trachea, with only slight immunogenicity being present in the cartilage (4). Antigenicity of tracheal grafts can be significantly reduced by removing epithelial cells and mixed glands (5). Research indicates that there is no need to use immunosuppressive agents in allogeneic tracheal transplantation performed through de-epithelialization (5,6). Another requirement for successful tracheal transplantation is the maintenance of patency; thus, maintaining the supporting role of tracheal cartilage must be considered while removing immunogenicity. There are many methods (physical, chemical, or enzymatic) used for de-epithelialization, and different methods can mechanically affect tissue in several ways. For example, the sodium dodecyl sulfate (SDS), Triton X-100, and enzymatic agents all have different effects on the mechanical properties of the porcine aortic valve (7). However, Triton X-100 only exerts a mild effect and cannot effectively remove cell components after a short period of treatment (8,9). Meanwhile, SDS is powerful decellularizing agent, which can rapidly remove cellular components (10). Tracheal reconstruction should be performed promptly for patients with diseases such as cancer or traumatic injury. Therefore, a simple and fast method of decellularization is needed to reduce the wait time for treatment. SDS may be an ideal candidate for this, by virtue of its decellularization power and its ability to tighten collagen (11) and generate a more fibrous substrate (12); however, it is not clear whether or not the application of SDS will affect the mechanical properties of the trachea. The purpose of this study was thus to determine whether or not the tensile and compressive mechanical properties of trachea are altered after SDS de-epithelialization and allograft implantation. We present the following article in accordance with the ARRIVE reporting

checklist (available at <http://dx.doi.org/10.21037/jtd-20-2739>).

Methods

Trachea collection

This study was conducted at the Center for Laryngotracheal Reconstruction at Tangdu Hospital, affiliated with the Air Force Military Medical University. The study was approved by the Laboratory Animal Welfare and Ethics Committee of the Air Force Medical University (IACUC-20191009), and conducted in compliance with Air Force Medical University guidelines for the care and use of animals. The donor mixed-breed dogs were anesthetized with an intramuscular injection of xylazine hydrochloride (2 mg/kg) and diazepam (0.4 mg/kg). After anesthesia, the dogs were laid on the operating table, the hair of the middle neck was removed, the skin was incised, and the anterior cervical muscles were separated to expose the cervical trachea. The cervical tracheas of 20 mongrel dogs (~10 cm in length) were obtained, and then all animals were euthanized. The surrounding connective tissues were removed and divided into 4 groups with 5 samples in each group. Group A (the control group), consisted of fresh canine tracheas; group B, consisted of tracheas de-epithelialized with SDS; group C, consisted of tracheas de-epithelialized with SDS and then heterotopically implanted (into the latissimus dorsi of beagle dogs) for 1 month; group D, consisted of tracheas de-epithelialized with SDS and then heterotopically implanted for 6 months. Between collection and testing, the 4 groups of collected tracheas were immersed in phosphate buffered saline (PBS) to prevent drying. All mechanical tests were completed within 6 hours of collection.

De-epithelialized tracheas

First, 5 g SDS powder was dissolved in 500 mL deionized water to create 1% SDS solution, and then a 0.22- μ m fiber membrane was used to filter and sterilize the solution. With the exception of group A (the control group), all tracheas were first de-epithelialized with 1% SDS in the following fashion (10): The proximal end of the trachea was blocked with a silicone plug. The tracheas were placed in test tubes containing SDS solution on the luminal side and hypertonic citrate adenine solution (Shanghai Blood Center, Shanghai, China) on the exterior side. Then, all tubes were placed in a 4 °C refrigerator for 72 hours for de-epithelialization,

and the SDS solution was changed every day into the incubation. The tracheas in group B (n=5) were removed and immersed in PBS, and the compressive and tensile tests were performed within 6 hours of removal. One cartilage ring per trachea was taken before the test for hematoxylin and eosin (HE) staining. The remaining 10 tracheas, which were those of groups C and D, were heterotopically implanted.

Allografting of de-epithelialized tracheas

After de-epithelialization, the tracheas of group C and group D were disinfected with 0.5% iodophor and repeatedly rinsed with approximately 2,000 mL of normal saline. The cartilage rings at both ends were then removed and the tracheas were cut to a length of approximately 6 cm, and a 1–1.5 cm incision was made between 2 of the rings in each trachea. Each processed trachea was fixed to a silicone tube of the same length; the tubes had small holes in their walls, and their ends were sutured together with absorbable threads. This treatment was performed to prevent the tracheas from becoming deformed under pressure after implantation.

We selected 10 healthy adult beagles weighing about 12–15 kg to be randomly assigned as ectopic transplanted animals, and each trachea was implanted on a beagle's back. The specific operation procedure was as follows: an L-shaped incision was made in the latissimus dorsi of a beagle; the skin was cut, the fascia layer was exposed, and the treated trachea was embedded into the fascia layer and sutured in place. All the animals were housed and fed by a specialist breeder in the Animal Feeding Center. The group C tracheas were removed 1 month after implantation, and the group D tracheas were removed 6 months after implantation. The excised tracheas were immediately immersed in PBS, and compressive and tensile testing were performed within 6 hours. Before the tests, a ring was taken from the end of each trachea for HE staining.

Tracheal histology

Before mechanically testing each group of tracheas, we took a ring from one end of each trachea for routine preparation and histological examination. The tracheal rings were placed in 10% neutral formalin buffer and fixed at room temperature for 24 hours. After fixation, the rings were washed with distilled water, dehydrated with a graded ethanol series, embedded in paraffin, and sliced at a

thickness of 4 μm . The sections were stained with HE.

Mechanical testing equipment

All biomechanical tests were completed at the State Key Laboratory of Mechanical Structural Strength and Vibration, Xi'an Jiaotong University. The tests were performed on a single-column benchtop universal testing machine (UTM) (model 5848, Instron, Norwood, MA, USA) equipped with 2 kN load cells. All experimental tests were carried out at room temperature and 50% humidity. Software (Bluehill, version 5.53.00, Instron) was used to control test execution and data collection. The load (N) and displacement (%) data of each experiment were obtained and exported to a spreadsheet program (Excel, version 14.2.5, Microsoft, Redmond, WA, USA). The same instrument was used for all biomechanical tests.

Compressive strength test

The specimens that were used for the compressive strength test (n=5) contained 3 tracheal rings each. Electronic digital display calipers (LINKS 0–150 mm, HMCT Group, Harbin, China) were used to measure the initial anterior-posterior diameter of the trachea. All mechanical testing procedures were completed within 6 hours of tissue collection. Each specimen was placed between 2 smooth, parallel, disc-shaped plates to distribute the load evenly along the trachea. The 2 discs were fixed on the UTM load arm (*Figure 1A*), and the specimen was compressed at a speed of 5 mm/min. When the anterior-posterior diameter was reduced to 50% of the initial diameter, the measurement was stopped (*Figure 1B*). The compression radial load (N) of each tracheal section at 50% displacement was calculated using a spreadsheet.

Tensile strength test

Each specimen for the tensile strength test was composed of 6 tracheal rings (n=5), and all were subjected to an axial elongation test. A fixture was placed in parallel with the load cell and was used to actively pull the specimen. The other end, which was at the bottom, pulled the other end of the specimen. The outermost membrane regions on both ends of the trachea were sutured to the fixture with a 4-0 Mersilk suture (braided silk, Ethicon, Somerville, NJ, USA) (*Figure 1C*). The original length before stretching was measured using electronic digital display calipers (LINKS

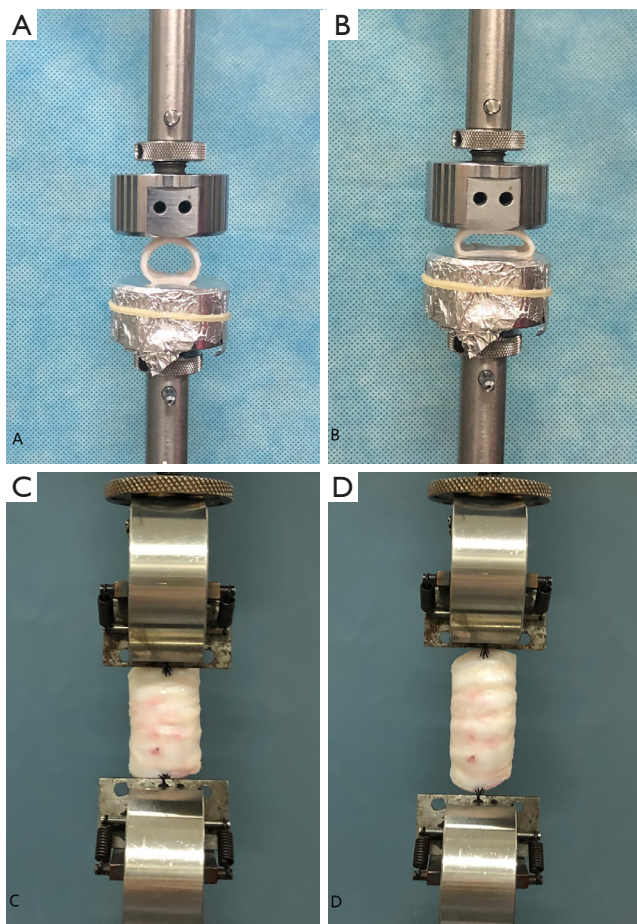


Figure 1 Mounting apparatus for force testing. (A) Each specimen was placed between 2 metal plates attached to the load arms of the UTM, as depicted. (B) Specimens were compressed until the luminal diameter was 50% of its baseline value. (C) During the axial elongation test of intact tracheal specimens, each specimen was sutured to the shaped fixtures at both ends. (D) Specimens were axially elongated to 150% of their original length to test the resilience of an intact trachea. UTM, universal testing machine.

0–150 mm, HMCT Group, Harbin, China), and the specimen was stretched at a speed of 5 mm/min. When the trachea was stretched to 150% of the original length (50% displacement), the measurement was stopped (Figure 1D). The tensile load (N) of each tracheal specimen at 50% displacement was calculated using a spreadsheet.

Statistical analysis

All statistical analyses were performed using SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA), with

an α of 0.10 as the threshold for statistical significance. One-way analysis of variance (ANOVA) was used to test the differences in compression and tension results among groups. The Tukey-Kramer multiple comparison method was used to examine the pairwise differences between specific groups in each mechanical test.

Results

Macroscopic evaluation of allografts

After 1 and 6 months of implantation, the tracheal surface was covered with a thin layer of fascia, and abundant capillaries could be seen on the surface. With the extension of implantation time, small columnar granulation tissue could be seen growing in the lumen center, but there was no obvious stenosis in the lumen.

Results of histological examination of the trachea

The fresh canine tracheas had a pseudostratified columnar epithelium visible under the microscope (Figure 2A). After de-epithelialization, the tracheal epithelium was almost absent, leaving only the extracellular matrix, and the chondrocytes were still alive (Figure 2B). On microscopic examination at 1 and 6 months post-implantation, the epithelial layer was filled with fibroblasts (Figure 2C,D).

Compressive strength test results

The compression curves of each group conformed to the typical compression displacement curve (Figure 3A). The mean compressive strength of each group is presented in Figure 3B. There was no significant difference in compressive strength among the groups (Table 1). During the test, the tracheas returned to its original state after the pressure was removed.

Tensile strength test results

The representative load-displacement curve of tracheas during elongation testing, in which rupture occurred at 61% of displacement, is shown in Figure 4A. The rupture point of all specimens happened after 50% elongation and each load-displacement curve showed an obvious deviation after 50% of displacement. The shape of the tensile load-displacement curve was similar with one trachea to the next; therefore, in this experiment, we used the load at

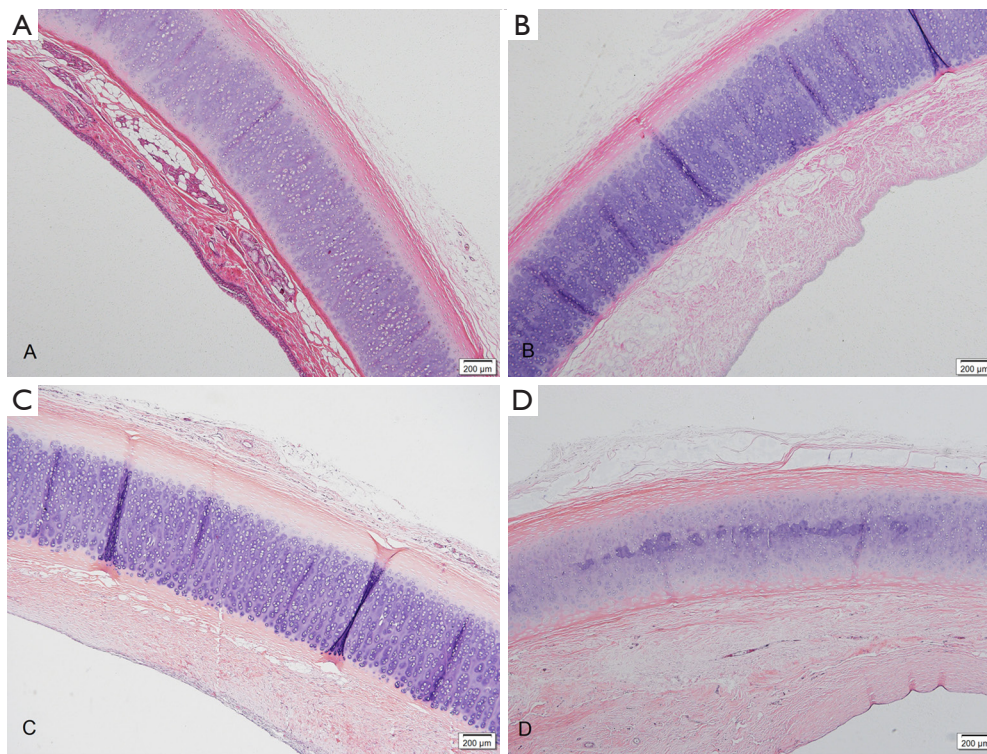


Figure 2 Microscopic sections. (A) Microscopic view of the ciliated pseudostratified columnar epithelium in a fresh trachea with HE staining. (B) After de-epithelialization treatment, the trachea is visibly denuded of its epithelium, with no remaining ciliated (or other) cellular structures present. The gross architecture and ECM are preserved. The chondrocytes are visible within the lacunae. (C) One month after implantation. (D) Six months after implantation. (C) and (D) show microscopic views of HE-stained tissue; the epithelial layer is visibly filled with fibroblasts. HE, hematoxylin and eosin; ECM, extracellular matrix.

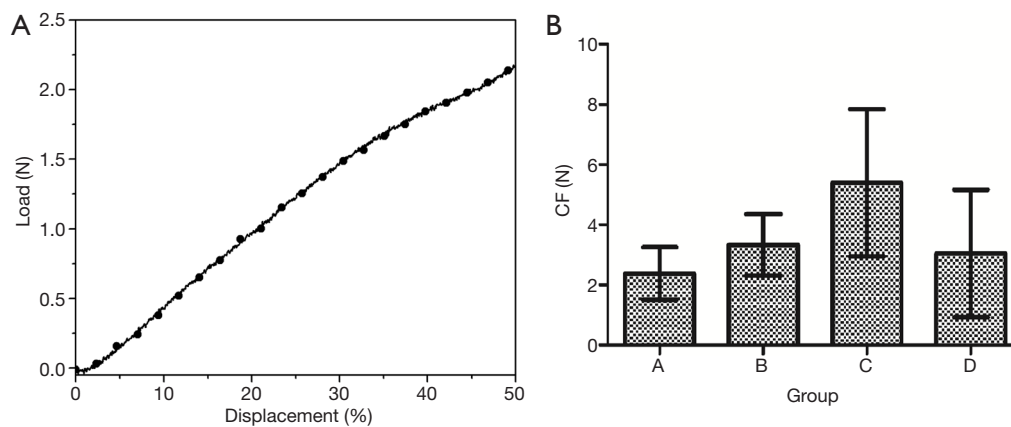


Figure 3 Radial compression force testing. Panel A shows a representative force (N) versus displacement (%) profile obtained from compression testing. As inward displacement (lumen occlusion) proceeds, the compressive force required increases in an exponential manner. When the lumen was blocked by 50%, the test was stopped, and the mechanical value was recorded. Panel B summarizes these data, which show no significant difference in compression strength among the groups ($P=0.077$ for all groups).

Table 1 Results of ANOVA performed among test groups for the compression and tension tests

Outcome	Group	Mean (N)	SD	F	P value	Tukey-Kramer post-hoc test P values					
						A vs. B	A vs. C	A vs. D	B vs. C	B vs. D	C vs. D
CF _{50D}	A	2.38	0.87	8.456	0.077	0.827	0.066	0.931	0.282	0.994	0.189
	B	3.33	1.03								
	C	5.39	2.44								
	D	3.04	2.12								
Yield load	A	22.51	3.92	10.91	<0.001	0.827	0.002	0.148	0.001	0.049	0.189
	B	24.24	7.46								
	C	9.68	0.86								
	D	16.07	3.00								

ANOVA, analysis of variance; CF_{50D}, compressive force at 50% displacement of lumen occlusion; F, F statistic; SD, standard deviation.

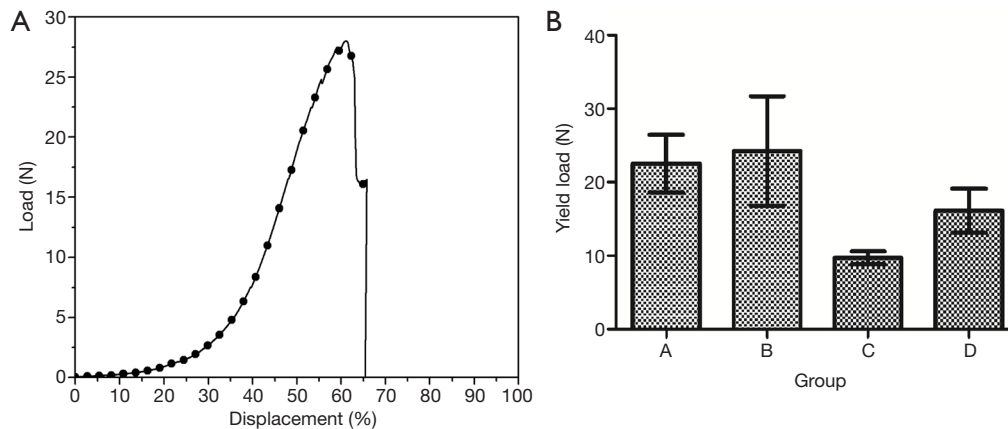


Figure 4 Yield load force testing. Panel A shows a representative force (N) versus displacement (%) profile obtained from yield load testing. The entire trachea fractured after 50% displacement, and the force suddenly decreased. Panel B summarizes these data, which show no difference among groups A, B, and D in yield load ($P>0.05$). The results of group C were significantly different from those of group A and B ($P<0.001$).

50% tensile displacement to calculate the tensile strength values that we compared among groups. The mean tensile strength was calculated for each group (Figure 4B). We found that tensile strength was significantly reduced in group C compared to the other 3 groups, whereas there was no significant difference among groups A, B, and D (Table 1).

Discussion

Tracheal defects less than 6 cm can be repaired by resection and end-to-end anastomosis. However, long-segment lesions require tracheal replacement (13), which is difficult

to perform. Transplantation is the currently used and more consistent repair method is. Liu *et al.* (5) performed heterotopic transplantation of tracheas from dogs in which the tracheal mucosa was treated with a detergent (1% Triton X-100 ϵ -octylphenoxypolyethoxyethanol; T-9284) without using any immunosuppressants, and 5 of the 6 grafts were confirmed to have been successfully transplanted. Hung *et al.* (14) also reported significant airway epithelial regeneration on the surface of the rabbit tracheal lumen after reconstruction with an acellular stent, but the tracheal stent eventually collapsed, leading to death. Therefore, ensuring the mechanical properties of the trachea and the

lack of airway obstruction is one of the main obstacles that must be overcome in tracheal reconstruction. For the de-epithelialized trachea to withstand the intrachamber airway pressure fluctuations associated with breathing, the elasticity and lateral strength of the trachea must be very close to those of the natural trachea (15,16). At present, there is no consensus on the biomechanical properties of the trachea in animal models (17-21). Therefore, we used fresh dog tracheas as a control to determine whether there were any changes in the mechanical properties of the de-epithelialized trachea 1 and 6 months after allografting and when, if at all, these changes occurred. We determined whether the biomechanical properties of the dog tracheas were greatly affected when allografts were successfully completed. We also tried to determine whether de-epithelialized trachea could be applied for allografting.

The compressive load-displacement curve of the canine trachea was a typical compression test curve (22). With increasing displacement, the load also increases exponentially. When the external pressure was removed, all samples returned to their original shape, indicating that the tracheas of each group were highly elastic. The average strength of the canine trachea under 50% radial compression in each group confirmed that the tracheas of all groups had good radial support capacity and elasticity. There was no statistically significant difference in compression load between the groups.

The tensile curve in this experiment was similar to the load-displacement curve of biological tissue subjected to tensile loading (22). The curve started with a steady and gradual rise, followed by a linear rise after 20–35% displacement, and a sharp inflection point at the time of tissue rupture. In this experiment, the fracture point appeared in all the samples after 50% elongation, and the load-displacement curve deviated greatly after this point. No fracture was found in any of the samples before 50% displacement, and the curves of the samples were consistent. Therefore, in this experiment, we adopted the load value at 50% displacement for the intergroup comparisons.

The tensile load had decreased as of 1 month after heterotopic implantation, and there was a significant difference compared with the fresh dog tracheas. The difference may be attributable to the inter-ring incisions made before implantation (the purpose of the incisions was to provide a better nutrient supply to the implanted trachea from the fascia to improve its survival), which reduced the connection between rings, making the trachea easier to pull apart. At 6 months after allografting, the tensile loading

capacity had increased. This increase may be related to the interannular parenchyma and the occurrence of gradual fibrosis, which could affect the tensile strength of the trachea and increase the maximum tensile load. There was no statistically significant difference among the newly de-epithelialized group, the fresh dog trachea group, and the 6-month allograft group. Moreover, our results were similar to the biomechanical values measured by Lee *et al.* (23).

The biomechanical properties of the trachea arise mainly from the tracheal cartilage rather than the mucosa (24). This is also consistent with the fact that the scaffolding effect of the cartilage was not impacted, since only the epithelial cells of the tracheal mucosa were removed during the process of de-epithelialization.

The present experiments had certain limitations. It would have been ideal to test and retest the same tracheas from the same mixed-breed dogs, from collection to de-epithelialization to 1 and 6 months after implantation, as this would have controlled for the individual differences between the different mixed-breed dogs' tracheas and increased the precision of the data (due to individual differences between the dogs, some tracheas were thicker and had harder cartilage, while other tracheas were thinner and had softer cartilage). However, the tracheas we implanted were all approximately 6 cm in length, and due to the limited length of the cervical trachea, it was difficult to reduce the presence of individual differences.

Conclusions

Our data suggest that during the entire treatment, the compression strength of the trachea was not affected. An interloop incision was made between the trachea rings before performing the allograft; the tensile force of the trachea after performing the allograft was affected for a short time, but was not affected 6 months after the procedure. Moreover, the tracheal cartilage of dogs was not rejected after 1% SDS de-epithelialization and observation of long-term implantation for 6 months, and the mechanical properties of the trachea were not significantly changed compared with fresh trachea. This provides the basic data for the repair of long-segment tracheal defects without immunosuppression and lays the experimental foundation for further orthotopic tracheal transplantation and re-epithelialization.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/jtd-20-2739>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/jtd-20-2739>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Laboratory Animal Welfare and Ethics Committee of the Air Force Medical University (IACUC-20191009), and conducted in compliance with Air Force Medical University guidelines for the care and use of animals.

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