Evaluation of Antigenicity of Components of Tracheal Allotransplant and Effect of Immunosuppressant Regime in a Rodent Model

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Indian J Plast Surg:2020;53:357–362

Abstract

Background Tracheal transplantation seems to be the logical step in the process of reconstruction of the trachea following a long-segment resection, which is usually done to treat malignant disease or benign stenosis of the airway caused by a traumatic, congenital, inflammatory, or iatrogenic lesion. Immunosuppression following transplant is essential but not ideal after oncoresection.

Methods The tracheal allografts, harvested from Sprague Dawley rats, were implanted in the Wistar strain rat. The harvested tracheal grafts were divided into groups and subgroups, based on the layers of trachea, method of decellularization, and immunosuppression. The antigenicity of different layers of trachea and the effect of various decellularization methods were studied within three time frames, that is, day 3, 9, and 15.

Result On structural analysis, the day 3 and day 15 samples showed no meaningful comparison could be made, due to extensive neutrophil infiltration in all three layers. The day 9 tracheal grafts showed loss of epithelium, with no signs of regeneration in most of the allografts. The subepithelial lymphoid infiltration was found to be severe in nonimmunosuppressed allografts. The group in which both inner and outer layers were removed showed moderate-to-severe infiltrate of lymphoid cells in all the allografts, but there was no cartilage loss, irrespective of the method of decellularization. The irradiated specimens retained the cartilage but showed extensive ischemic damage.

Conclusion Rat trachea is a good model for tracheal transplant research but not adequately sturdy to sustain mechanical debridement. Irradiation and chemical decellularization eliminates the immune response but causes intense ischemic damage. Out of the three time frames, day 9 seemed to be the best to study the immune response. To substantiate the results obtained in this study, the immunohistochemical study of the allografts is needed to be performed among a larger group of animals.

Keywords
- decellularized tracheal allograft
- immune-mediated rejection
- rodent model
- tracheal allograft antigenicity
- tracheal transplantation

published online
December 21, 2020

DOI https://doi.org/10.1055/s-0040-1721860
ISSN 0970-0358.

Thieme Medical and Scientific Publishers Pvt. Ltd. A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Introduction

Tracheal reconstruction is a challenge when the length of the segment resected exceeds the limit, which allows direct end-to-end suturing. Long-segment trachea is usually affected by malignant diseases or by benign stenosis of the airway caused by a traumatic, congenital, inflammatory, or iatrogenic lesion. The need for an ideal tracheal replacement has transformed research into tracheal transplantation a matter of great importance.

When the resected segment is small, the continuity can be restored by end-to-end anastomosis. However, when more than 50% or one-third of the tracheal length in adults or children, respectively, is excised due to any pathology, the closure of the resultant gap requires an implant or reconstruction method.[1] When this is not possible, the inferior end of the trachea is fixed to the skin of the neck, and the patient has to depend on this tracheostoma lifelong.[2]

Various approaches have been attempted for the purpose of tracheal replacement,[3-7] including the single or combined use of autologous tissues, autografts, allografts, prosthetic materials, and tissue-engineered tracheae. Autogenous tissues have been tried experimentally, such as fascia lata, pericardium, tracheal wall, buccal mucosa with auricular cartilage as well as bronchial patches, dermal grafts, pericardium, and aortic grafts. But all have failed due to collapse or absorption in the long term.[3] While artificial materials have been added to these tissues to maintain rigidity and support, they have led to local infection, anastomotic dehiscence, vascular erosion, granulation tissue formation, and eventual stenosis.[4] Tissue engineering of trachea has attracted a lot of attention and produced remarkable results in a few cases.[8-10] But still the optimum method in cancer reconstruction is yet to emerge. Local, regional, and distant-free flaps combined with various sturdy materials require lengthy, multistage procedures and are, therefore, not feasible in cases of cancer.[3] They are also not foolproof from failure.

Transplantation seems to be a logical step in the process of reconstruction of the trachea. But, unlike other organs, trachea is difficult to transplant, because it does not have a distinct blood vessel supplying it. This necessitates remote revascularization of the transplanted trachea. The tracheal allotransplant has the main disadvantage that it will need immunosuppression to prevent rejection. Immunosuppression in cases of cancer is not ideal, as its safety and its effect on tumor progression is not clear. Also, its long-term administration leads to many other systemic complications. The trachea is a three-layered structure and there might be a possibility of variability in the antigenic properties of each layer. The cellular elements are more expressed in the mucosa and the connective tissue. Hence, removal of these by decellularization may help in reducing the antigenicity of the transplanted trachea. The best method to achieve decellularization is not certain. This study intends to look at the antigenicity of different layers of the trachea and the effect of varying methods of decellularization in reducing the antigenicity. This, in turn, may help to provide insight into the possibility of using decellularized tracheal allotransplant, needing lessened immunosuppression.

The primary aim was to study the differential antigenicity of the three layers of tracheal allotransplant. The secondary aim was to study the effect of immunosuppressants in the tracheal allotransplant models, decellularized using different methods (mechanical, chemical, and irradiation)

Material and Methods

This study protocol was approved by the Institutional Scientific Review Board and the Institutional Animal Ethics Committee. The study was conducted in the Central Laboratory Animal Facility, AIMS, Kochi, with the help of veterinary personnel trained in conducting research in small animals.

Animal Model

The small animal models, like rats, provide useful tools for studying the pathology and processes involved in the development of rejection of tracheal allograft.[2] We used Sprague Dawley rats as the donor and Wistar rats as recipient to mismatch in terms of histocompatibility. A total of 24 rats were used in this study (12 donors and 12 recipients). All the rats included were of either sex, adult, healthy and approximately 250 to 350 gm in weight.

Study Design

The tracheal allografts, harvested from Sprague Dawley rats, were divided into four groups, each having a different set of layers. In group I, all the layers were kept intact. In group II and group III, the outer adventitial layer and the inner mucosal layer was removed, respectively, by scraping with a no. 15 scalpel blade. Group IV had both inner and outer layers removed using the following methods: mechanical (IV-A), chemical (IV-B), and irradiation (IV-C). Each group was further subdivided, based on whether they received immunosuppression (►**Fig. 1**).

Tracheal Allograft Harvest and Processing

The donor rats were euthanized by an overdose of xylazine-ketamine cocktail injected intramuscularly and then

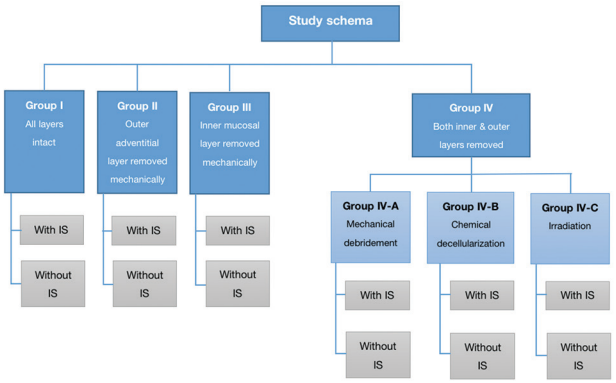

Fig. 1 The study design.

placed in CO2 chamber. Following this, the tracheal harvest was done. Midline incision was placed over the ventral aspect of neck, the strap muscles retracted laterally, and the sternum divided to expose the trachea. The trachea was dissected and divided proximally just below the larynx and distally at the level of carina (►**Fig. 2 A,B**). After harvest, the donor rats were disposed as per institutional disposal protocol. To have more number of samples with limited number of rats, each of the procured trachea was divided into three segments, each containing three to four tracheal rings. The tracheal allografts were processed according to their respective groups. Mechanical removal of the outer and inner layers of the tracheal grafts of groups II, III, and IV-A was done by scraping with No. 15 blade, taking care not to damage the underlying cartilage. Group IV-B underwent chemical decellularization as per the protocol (►**Fig. 3**). The allografts of group IV-C were exposed to a single fraction dose of 10Gy, delivered with LA machine, and stored in distilled water.

Tracheal Allograft Implantation and Retrieval

The tracheal allografts were implanted in a subcutaneous pouch over the dorsum (back) of the Wistar rats and retrieved at three time intervals–3rd, 9th, and 15th day. The role of each layer in the process of rejection was studied by examining the gross and histologic changes. All the recipient rats were rehabilitated after the retrieval.

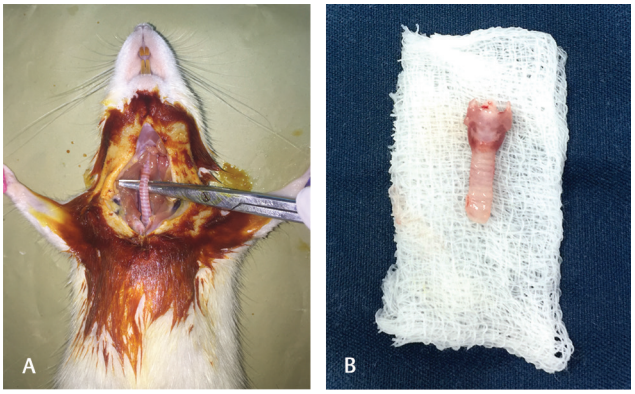

Fig. 2 (**A**) Tracheal dissection and harvest in Sprague Dawley rat. (**B**) Harvested tracheal graft.

Immunosuppression

Postoperatively, cyclosporine 10 mg/kg/day[11] was administered subcutaneously to the immunosuppressant receiving groups.

Histopathological Evaluation

It was done by a pathologist who was blinded to the procedure used. All tissue specimens were fixed in 10% formalin, the slides were stained with hematoxylin and eosin (H&E) and examined by light microscopy. The epithelial layer was assessed and graded for viability and regeneration (►**Table 1**). Subepithelium and cartilage layers were assessed and graded for lymphoid cell infiltration. Mild infiltrate was defined as < 30% of a microscopically visual field, moderate as 30 to 70% of visual field, and severe as > 70% of visual field (►**Table 1**).

Statistical Analysis

It was done using IBM SPSS 20. (SPSS Inc, Chicago, USA). For all the continuous variables, the results are either given in mean ± SD, and for categorical variables as percentage. To obtain the association of categorical variables, Chi-square test was applied. A p value < 0.05 was considered as statistically significant.

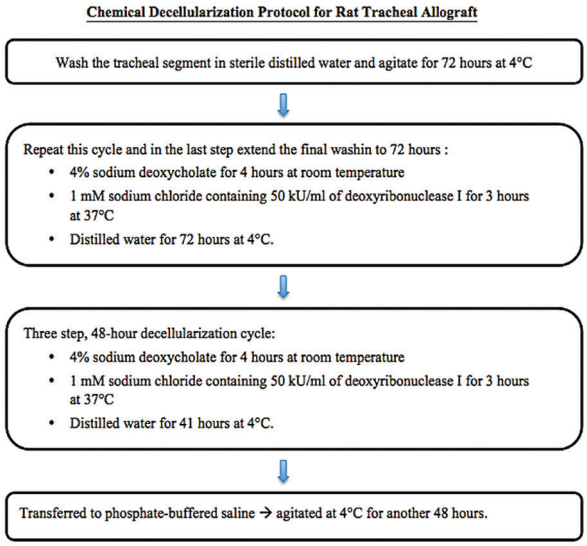

Fig. 3 Chemical decellularization protocol.

Table 1 Microscopic grading parameters for histological assessment of the tracheal allograft

Grades	Epithelial viability	Cartilage viability	Subepithelium
0	Normal mucociliary epithelium	Normal structure	Absence of any abnormality
1	Multilayer nonciliated epithelium/ regeneration	Mild/occasional infiltrate of lymphoid cells into the cartilage	Mild (infiltration area < 30% of a microscopically visual field)
2	Single layer nonciliated epithelium	Moderate-to-severe infiltrate of lymphoid cells into the cartilage	Moderate (infiltration area 30–70% of a microscopically visual field)
3	No epithelium/ulceration	Cartilage loss	Severe (infiltration area > 70% of a microscopically visual field)
NR	No re-epithelialization	–	–

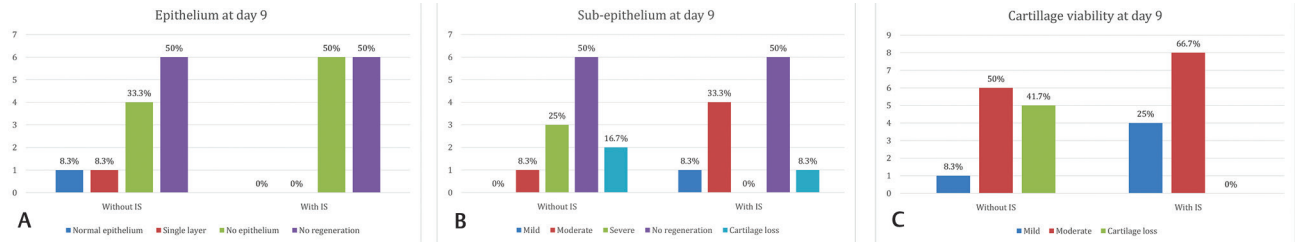

Fig. 4 (**A**) Comparison between the epithelial layer of immunosuppressed and nonimmunosuppressed groups after retrieving the allograft on day 9. (**B**) Comparison between the subepithelial layer of immunosuppressed and nonimmunosuppressed groups after retrieving the allograft on day 9. (**C**) Comparison between the cartilage viability in immunosuppressed and nonimmunosuppressed groups on day 9.

Result

The groups in which inner or outer layer was removed (Group II and III), the results were inconclusive and difficult to interpret because of extensive necrosis and damage. Hence, the main comparison could be done only between the group I (all layers intact) and group IV (both inner and outer removed). Similarly, out of the three time frames, the day 9 allograft samples showed significant identifiable findings, whereas in the day 3 and day 15 samples, no meaningful comparison could be made, due to extensive neutrophil infiltration in all three layers. Additionally, extensive destruction of the cartilage layer, with no regeneration of the epithelium or subepithelium, was seen in the day 15 allografts, irrespective of the status of the immunosuppression.

On comparing the day 9 allografts, both the groups (immunosuppressed and nonimmunosuppressed groups) showed loss of epithelial layer in most of the allografts (►**Fig. 4A**). The high percentage of absence of regeneration was equally seen in the epithelium as well as subepithelium of both the groups, irrespective of the immunosuppression. In the subepithelial layer, most of the immunosuppressed allografts had moderate lymphoid infiltration as compared with nonimmunosuppressed allografts, which had more of severe infiltration (►**Fig. 4B**). On observing the cartilage layer, moderate-to-severe lymphoid cell infiltration was seen in 50% of the nonimmunosuppressed allografts, in contrast to 66.7% in the immunosuppressed allografts. The lymphoid infiltration was restricted to the perichondrial layer in the immunosuppressed group (►**Fig. 5**), whereas in the nonimmunosuppressed group, they were seen entering the matrix of the cartilage (►**Fig. 6**). The cartilage loss seen in nonimmunosuppressed allografts was 41.7%, while the immunosuppressed group had no cartilage loss at all (►**Fig. 4C**).

As the cartilage was the layer preserved in all the groups, the changes in this layer was mainly studied and compared. The all-layers intact group at day 9 showed mild infiltrate in 75% of allografts and cartilage loss in 25% of allografts, whereas the group in which both inner and outer layers were removed showed moderate-to-severe infiltrate of lymphoid cells in 100% allografts, but there was no cartilage loss, irrespective of the method of decellularization (►**Table 2**). This was suggestive of reduced immune reaction, as the cellular load was reduced by decellularization.

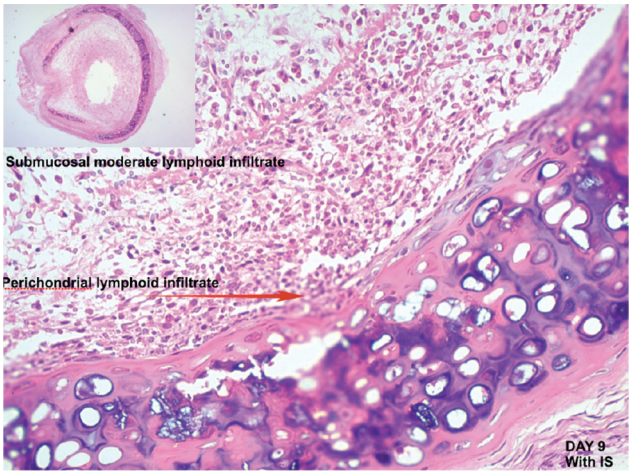

Fig. 5 Hematoxylin and eosin (H&E)-stained cross-section of trachea of immunosuppressed rat, with all layers intact, showing moderate lymphoid infiltrate in submucosa and perichondrium. The lymphoid cells have not yet entered the cartilage.

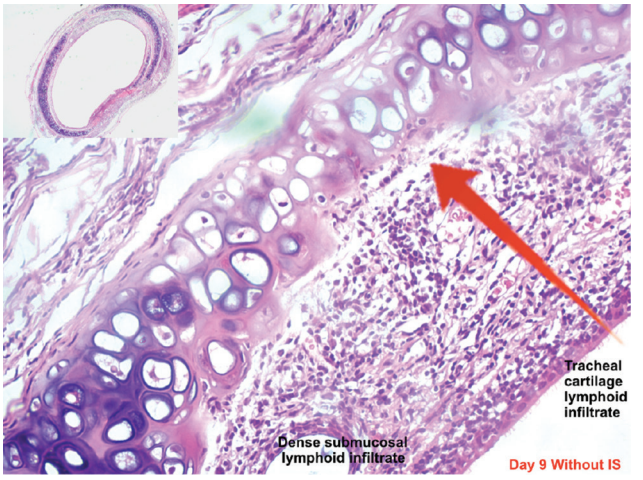

Fig. 6 Hematoxylin and eosin (H&E)-stained cross-section of trachea of non-immunosuppressed rat, with all layers intact, showing dense submucosal lymphoid infiltrate and the lymphoid cells infiltrating the cartilage substance.

Discussion

Tracheal transplant is a necessity for some patients, especially with long tracheal defects, which defy conventional reconstruction methods. The immunogenicity of the trachea

Table 2 Comparison between cartilage viability in all-layers intact group and the various method of decellularization group after retrieval on day 9

Variable	Category	All layers intact	Mechanical debridement	Chemical decellularization	Irradiation	*p*value
		n(%)	*n*(%)	*n*(%)	*n*(%)	
Cartilage viability	Mild	3 (75)	0 (0)	0 (0)	0 (0)	0.014
	Moderate-to-severe	0 (0)	4 (100)	4 (100)	4 (100)	
	Cartilage loss	1 (25)	0 (0)	0 (0)	0 (0)	

had been proved long time back by Beigel et al.[12] Initially, the mucosa was considered as the most immunogenic part of the trachea. However, with further studies, it was established that changes due to rejection occurs on both sides of the cartilage. It was also observed that even chondrocytes are weakly antigenic.[13] In this study, we have tried to assess the immunogenic potential of each layer individually and to know which layer of the tracheal allograft has the most immunogenic potential, so that the specific layer can be removed prior to the transplant and the dosage of immunosuppressive drugs can be reduced. However, no definite results could be obtained in our study, due to the damage caused by the mechanical debridement of the layers. The better method for removal of these layers needs to be investigated further.

Considering that there is no named vessel supplying the trachea, the revascularization of the tracheal allograft poses a challenge for the surgeons. Many attempts have been made to reduce the magnitude of rejection of the transplanted trachea and revascularize the tracheal allografts. A previous animal study by Delaere et al[14] on tracheal autograft revascularization showed that a large arteriovenous bundle and its surrounding vascularized island of fascia can induce perfusion of the heterotopic transplanted trachea and can therefore allow its orthotopic transfer as a prefabricated flap. For optimal revascularization, it is very important to have a close and immobile contact between the allograft and the vascular bed. Therefore, heterotopic site is more preferable for tracheal allotransplantation. The most commonly used sites for heterotopic transplantation are omentum and dorsal subcutaneous pouch. A subcutaneous pouch over the dorsum (back) of the rat was preferred as heterotopic site for the tracheal allografts, in our study.

In a study by Delaere et al, the feasibility of tracheal allotransplantation with a fascial vascular carrier was examined in the three groups with varied dose sequences of immunosuppression. It was observed that 10 mg/kg of cyclosporin A per day was effective to suppress the immune response after transplantation of vascularized tracheal allografts.[11] In the present study, cyclosporine A in a dosage of 10 mg/kg was administered to in the immunosuppressant-receiving groups. In our study, it was presumed that removing mucosal and/or adventitial lining of the allografts will reduce the immune rejection.

We selected the three time frames, that is, day 3, 9 and 15, to study the immediate, midrange, and delayed changes. These changes could be attributed to inflammation following trauma, immune-mediated rejection as well as ischemia-related changes. Out of the three time periods, day 9 was the one which gave us some decipherable findings. The epithelium, subepithelium, and cartilage were assessed for the features of rejection (►**Table 1**). The gross evaluation showed an intact structure being retained in most of the tracheal grafts retrieved on days 3 and 9. However, by day 15, there was evidence of necrosis in all of them.

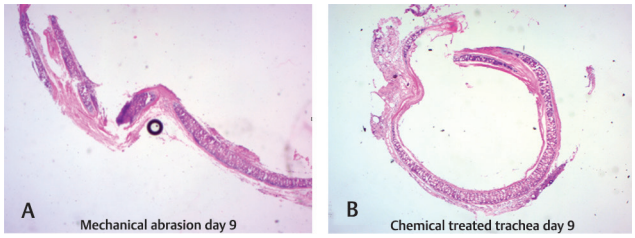

Fig. 7 (**A**) Hematoxylin and eosin (H&E)-stained cross-section of tracheal allograft after mechanical removal of both inner and outer layer, showing destruction of the cartilaginous component. (**B**) H&E-stained cross-section of tracheal allograft in which chemical decellularization has been done.

The day 9 allografts showed copious luminal exudate and marked reduction in the size of the lumen because of subepithelial edema, although there was moderate-to-severe submucosal and perichondrial lymphoid infiltrates, which was seen almost equally in the immunosuppressed and nonimmunosuppressed groups. But the contrasting feature in the nonimmunosuppressed group was the infiltration of lymphoid cells into the substance of the cartilage. This could be the reason for the increased cartilage destruction seen in this group, thereby indicating that immunosuppression helped to protect the cartilage to some extent.

Comparison of the different methods of decellularization showed that total mechanical decellularization destroyed the structure of the cartilage but noticeably with low lymphocytic infiltration (►**Fig. 7A**). In the enzymatic decellularization group, the structural integrity was maintained, but there was evidence of early avascular necrosis (►**Fig. 7B**). The group which underwent radiotherapy for reducing the immunogenicity of the transplant showed no evidence of lymphocytic infiltration. However, there was extensive necrosis, probably due to vascular or radiation induced damage, making most of the tissue unviable. Further studies will be required to get more conclusive information on this, with a larger number and varying the parameters in the process adopted for decellularization process. The interplay between

ischemia, traumatic inflammation, and immune reaction is difficult to differentiate in gross and histological evaluation. These need to be addressed by adding evaluation using immunohistochemical markers in a larger group of animals.

Limitations of the Study

Small sample size may be a limitation in drawing statistically significant conclusions for certain variables. The outcome measures were not powered enough to assess the role of the three factors that could influence the findings, that is, ischemia, inflammation, and immune-based rejections. The mechanical debridement leads to extensive physical damage in delicate rat trachea. Even though it might be possible in the human trachea, the rat model does not seem good to test this method.

Conclusion

Rat trachea is a good model for tracheal transplant research. Although mechanical debridement was the key in this study to isolate the three layers for their differential immunogenicity, the rat trachea is not sturdy enough to sustain mechanical debridement, making the interpretation of this difficult. The structural integrity of the cartilage was maintained following chemical decellularization. Irradiation markedly reduces immune response, but causes extensive radiation-induced necrosis. Lymphocytic infiltration into the cartilage is a good pointer to immunosuppression. The immunosuppression seems to benefit the retention of cartilage viability.

Note

This study was conducted in year 2017-2018 at the Department of Plastic & Reconstructive Surgery, Amrita Institute of Medical Sciences and Research Centre, Kochi. It received the APSI Best Research Paper Award and APSI–Goa Research Award Grant in the year 2016.

Ethics statement

This study protocol was approved by the Institutional Scientific Review Board and the Institutional Animal Ethics Committee. The study was conducted in the Central Laboratory Animal Facility, AIMS, Kochi, with the help of veterinary personnel trained in conducting research in small animals.

Funding sources

Partly funded by APSI–Goa Research Award Grant.

Conflicts of interest

None declared.

Acknowledgment

This study was supported by the APSI–Goa Research Award Grant 2016.

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