Two-piece cryopreserved tracheal allotransplantation: an experimental study

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Abstract

Objectives: For successful reconstruction with tracheal allotransplants following long tracheal resections, problems related to the preservation and vascularisation of the tracheal graft have to be solved. In this study, instead of using a long-segment single-piece graft, we used a graft that has been split into two. The aim was to use this graft after cryopreservation in order to ease neo-vascularisation and to maintain tracheal integrity by transplanting it to two separate regions of the dog cervical trachea.

Methods: This experimental study was conducted in animal laboratories of the medical school on 11 half-blood dogs. The trachea obtained from the first dog was 8 cm in length; it was split into two pieces of 4 cm each and stored in the preservation solution at −80 °C for 4 weeks. Following this, the dog was sacrificed. Two 2 cm portions of cervical trachea were excised from the second dog. These parts were then reconstructed with two tracheal grafts of the same length as the cryopreserved ones. Ten dogs that were grouped into five groups of two dogs each underwent the same procedure. The subjects had a bronchoscopic evaluation on the third postoperative week. Anastomosis regions of the test tracheas were resected to be examined histopathologically.

Results: Seven subjects were found to have third-degree obstructions during bronchoscopy; two had close to fourth-degree obstructions. In the histopathological examination, contrary to the findings of the bronchoscopies, 75% of the anastomoses had intact epithelium. The cartilage was seen to have well-preserved structural characteristics in all the anastomoses. Twelve anastomoses had moderate, seven mild and one had severe inflammation. All anastomoses had either good or very good level of vascularisation.

Conclusions: The integrity of the tracheal epithelium can be maintained with cryopreservation and split anastomosis technique. The cartilage preserves its structural characteristics despite losing its viability, thereby offering an advantage to maintain airway patency.

Keywords: Trachea; Allotransplantation; Cryopreservation; Anastomosis

1. Introduction

The resection and reconstruction of the trachea necessitates a complicated surgical technique and comprehensive anaesthesiology support. Freeing manoeuvres make it possible to resect nearly half of the trachea in adults and then anastomose it end-to-end. For those lesions requiring longer resections, strategies for resection and reconstruction have not been completely delineated yet [1,2].

Tracheal allotransplantation studies are sources of hope for the future for long-segment tracheal resections. Several studies have been performed on tracheal allotransplantation. For the clinical success of this procedure, problems such as immune rejection of the graft, preservation and vascularisation need to be solved [3,4]. Neo-vascularisation of the tracheal graft starts from the anastomotic line together with wound healing [4—6]. When long-segment transplants are used, necrosis develops at the middle part of the graft due to vascular insufficiency. Short-segment tracheal allografts are successful for tracheal reconstructions [6].

The aim of this study was to use two-piece tracheal split graft instead of a long-segment single-piece graft in the treatment of diseases requiring long-segment resections in order to ease neo-vascularisation and achieve tracheal continuity by transplanting these to two different regions of dog cervical trachea.

2. Materials and methods

This experimental study was performed on 11 dogs at Gaziantep University School of Medicine in the Department of Physiology’s animal laboratories and lasted nearly 6 months. Consent of the local ethical committee was obtained prior to
the study. All the animals received humane care in compliance with the European Convention on Animal Care.

Laboratory animals used in the study were half-blood dogs between 1 and 3 years of age weighing around 15—25 kg. The first subject was sacrificed to obtain the tracheal segments that would constitute the tissue bank. The remaining 10 subjects were recruited in the study by forming groups of two animals each.

2.1. Anaesthesia technique

Anaesthesia was performed with 20 mg kg\(^{-1}\) ketamine hydrochloride (Ketalar, Pfizer, Istanbul, Turkey) and 5 mg kg\(^{-1}\) Xylazine HCL (Rompun, Bayer, Toronto, Canada). During orotracheal intubation, spontaneous respiration was allowed with 2 l min\(^{-1}\) oxygen supply. All the subjects received four doses of 1 g cephazoline to be injected intramuscularly as a pre-operative dose and one dose each on the three consecutive days after the surgery.

2.2. Surgical technique

The first subject was put under anaesthesia and was put in a supine position. Trachea was explored through an 8 cm long incision extending from the cervical region to 2 cm above the sternal ridge. Starting from the second ring of the trachea, an 8 cm (16 rings long) segment was resected. The resected graft was split into four pieces to form a tissue bank. The lumens of these segments were washed with 0.9% physiological saline and soaked into the cryopreservation solution to be stored until they were used for the second experiment. The first subject was sacrificed with a high dose potassium hydrochloride (KCl) infusion. Each piece from the donor trachea was placed in separate plastic tubes to be stored at \(-80^\circ\)C for 4 weeks.

At the end of the storage period, each stage of the experiment was conducted on two subjects. Tracheas of both subjects were explored and four cartilage rings resected, starting from the second ring. After leaving the four consecutive rings intact, four more tracheal rings were resected (Fig. 1A). During the dissection, an effort was made not to hamper the circulation in areas proximal and distal to the resected segment and avoid harm to the oesophagus. The subject was re-intubated from the distal trachea with a sterile intubation tube (Fig. 1B). The resected tracheal segments were placed into tubes containing cryopreservation solution and kept in the freezer to be used in the next experiment. The grafts that were previously kept frozen were thawed with compresses soaked with physiological saline at 30 °C. Transplantation started from the proximal anastomoses. 3/0 double needle Polydioxanone (PDS, Ethicon, New Jersey, USA) continuous sutures were used. Care was taken to ensure the sutures passed through the cartilage ring. After completing two-thirds of the second-segment distal anastomosis, ventilation from the surgical field was stopped, the orotracheal tube pushed to pass the most distal anastomosis line and ventilation was resumed. During the anastomosis an attempt was made to preserve the mucosa and have both ends in complete contact with each other. All the subjects underwent rigid bronchoscopy to have the anastomosis lines evaluated (Fig. 2). Bronchoscopic evaluation criteria are listed in Table 1.

2.3. Preparation of the cryopreservation solution

Ceftizoxime sodium 140 μm l\(^{-1}\), lincomycin 120 μm l\(^{-1}\), vancomycin 50 μm l\(^{-1}\) and amphotericin B 25 μm l\(^{-1}\) were added to Dulbecco’s modified Eagle Medium (DMSO, Sigma–Aldrich Co, Germany). There was further addition of 10% dimethyl sulfoxide (DMSO) as a cellular preservative.

2.4. Histopathological examination

During postmortem autopsies, both grafts were excised from the tracheas of the subjects (including the anastomotic sites). After all specimens were macroscopically examined (Fig. 3A), they were fixed in 10% formaldehyde solution. All the grafts were evaluated separately for pathological examination.

Table 1

<table>
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<tr>
<th>Scoring of tracheal obstruction found during bronchoscopy.</th>
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<tr>
<td>No obstruction</td>
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<tr>
<td>Obstruction between 0% and 25%</td>
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<td>Obstruction between 25% and 50%</td>
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<td>Obstruction between 50% and 75%</td>
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Sections were separately taken from all anastomosis lines and stained with hematoxyline eosine. First graft from the subject was marked with an A and the second with a B that was written next to its number. Histopathological examination evaluated the status of the epithelium, viability of the cartilage, vascularisation (by looking at the number of submucosal capillaries filled with erythrocytes) and the degree of inflammation (by the amount of lymphocytes observed on the submucosa of the transplant). Scoring of the evaluated histopathological parameters (from 0 to 3) was based on the method by Nakanishi et al. [7]

### 3. Results

One of the subjects died on day 12 due to tracheal fistula and ensuing infection from separation of the anastomosis. All others died after 3 weeks (during days 22—30). Except for the subject with premature death, all others had postoperative bronchoscopy at 3 weeks. During this procedure, seven subjects had third-degree and two almost fourth-degree obstructions.

All the subjects underwent an autopsy following their death. The cause of death was identified as tracheal stenosis in all. In macroscopic examination the initial 2 cm length of the grafts was seen to have shrunk to 8 mm on average. In microscopic examination, the cartilage tissue was observed to be piling on top of each other while healing (Fig. 3B).

Bronchoscopic findings and survival figures are presented in Table 2; the epithelisation status of both anastomoses of the subjects, the viability of their cartilages, the degree of the inflammation and the related fibrosis and vascularisation are presented in Table 3.

### 4. Discussion

Based on the results obtained, we can say that we succeeded in graft neo-vascularisation with the two-piece tracheal transplant technique. An important evidence to support this observation was the increased circulation seen during histopathological examination in nearly all the anastomoses from the specimens. This can be regarded as an evidence of hyperinflammation, but having 75% of the favourable epithelisation in the anastomoses makes us think that this technique was successful for neo-vascularisation.

Likewise, a sustainable epithelisation is a crucial step for preventing infections and removing secretions.

As a result of experimental studies during 1950s and 1960s, anatomic mobilisation manoeuvre succeeded in resecting nearly half of the adult tracheas and managing an end-to-end anastomosis without the need of a prosthesis [8]. Of note are the malignant tumoural lesions involving large segments of the trachea and requiring long-segment resections. Such lesions resulted in attempts at reconstruction with diverse prosthetic materials without leading to desired success.

Studies with tracheal allotransplants during recent years have promised new hope. The most important obstacles in successful tracheal transplantations are the neo-vascularisation of the graft as well as the problem of immune rejection [4,9—11].
According to the available information in the field, there are three possible ways to have successful neo-vascularisation in tracheal allografts [12]:

1. Primary vascularisation
2. Secondary vascularisation and
3. Vascularisation from the ends of the anastomosis.

Blood flow to the graft can be attained by attempting an anastomosis between the superior thyroid artery of the graft and the common carotid artery of the recipient via a microvascular technique. However, direct re-vascularisation technique is not routinely used in humans [11—13].

Another alternative re-vascularisation technique is the secondary vascularisation by wrapping the tracheal graft with live tissue. Omentum is the most used tissue for secondary vascularisation because of having easy surgery and wide vascular network [14]. Following a study on dogs, Park et al. [14] concluded that omentopexy is not a major option for the neo-vascularisation of the tracheal graft.

If no further steps are taken for neo-vascularisation, the vascularisation starts from both ends of the anastomosis by using the submucosal vascular network of the recipient trachea. Four days after the transplantation, vascularisation starts from both ends of the recipient trachea and is complete by day 10 [9,11,14]. In vascularisation that starts from the ends of the anastomosis, there is a limit to the length of the tracheal graft used. If the tracheal autograft is longer than 4 cm in length, necrosis develops in the middle due to insufficient vascularisation [6,14].

We focussed our efforts on a new procedure with the aim of eliminating these problems encountered in the neo-vascularisations of long-segment tracheal transplants. We had a cryopreserved tracheal graft with eight rings and divided this specimen into two and transplanted it onto two different sections of the cervical trachea with four anastomoses. We did not use omentopexy or any other neo-vascularisation technique in our study. With this two-piece graft, in addition to achieving a long-segment trachea transplantation, we obtained a short graft length and more anastomotic surface providing an advantage for neo-vascularisation.

Another major problem in tracheal allotransplantation is the issue of immunity. Immune rejection confronts us as a general problem in all allogeneic transplants. The study by Shaari and coworkers [15] demonstrated that antigenic characteristics of the trachea have their roots in different compartments. According to this study, perichondrium surrounding the cartilage tissues has a relatively lower immunity while the tracheal epithelium has high antigenicity. Due to mismatches between donors and recipients, HLA class II antigens of the graft epithelium results in a rejection reaction on the transplanted graft. Following 30 days of cryopreservation, the epithelium on the graft is completely displaced resulting in the loss of graft antigenicity. Recipient epithelium starts to cover the anastomosis by starting from the ends. Nearly 50 days later, the recipient epithelium covers the anastomosis completely creating an epithelial continuum. The studies by Nakanishi and Yasumoto [16] showed that there is destruction of the graft epithelium when immunosuppression is not used and that neo-vascularisation is hindered significantly.

Several immunosuppressive agents either suppress or minimise allogenicity. They increase tumour recurrences and the risk of infections when used in patients with tracheal resections during the course of malignant diseases. Therefore studies on tracheal allotransplantation have to be performed with alternative immunosuppressants eliminating the adverse affects [8,17]. Cryopreservation is one of the safest ways to be used for immune response suppression in allotransplantations performed in malignant diseases [18].

In tracheal transplant study on dogs by Yokomise and coworkers [19], the dogs on the cryopreservation-free arm died of tracheal stenosis while those on the cryopreservation arm lived for more than 2 months (except for one). Cryopreservation of the tracheal graft prevented rejection and eliminated the need for immunosuppressive agents. Tojo et al. [20] conducted a similar study on dogs. They divided the dogs into three groups as cryopreserved allograft, fresh autograft and fresh allograft groups. The dogs in the first and second groups survived whereas those in the third one died of tracheal stenosis. They concluded that cryopreservation suppressed immune response by diminishing the antigenicity of the graft.

We also used cryopreservation in the animal study we performed for immunosuppression. In this study, we limited the period of cryopreservation in the preservation solution to 30 days at −80°C. This period was long enough for the graft epithelium to disappear by shedding. Epithelisation was found to be normal in 85% of the anastomoses in our study; therefore we concluded they were reshaped with the help of the recipient epithelium. On histopathological examination, inflammation was found with increased lymphocytic infiltration at the anastomotic site. We thought that this was related to an inflammation during the healing process rather than being an acute rejection. Cryopreservation could only prevent the acute rejection that developed within the first 10 days; it could not handle the inflammation during the subacute phase, the generation of the granulation tissue and the resulting fibrosis. Inflammation and the ensuing fibrosis can be regarded as components of natural healing process as long as the patency of the trachea is preserved. The effects of inflammation can further be eliminated by current treatment modalities like stenting and T-tube placement [20]. Despite all the changes in the cartilage and the complicating cellular death, tracheal continuity and airway patency could be maintained in all subjects but one. Healing of the tracheal cartilage by contraction did not create a problem as the native trachea kept its integrity. Airway patency was successfully maintained. Overall, the aim in allotransplantation studies is to preserve tracheal integrity and maintain airway patency.

In order for tracheal transplantation to be routinely performed in reconstructions of long-segment tracheal lesions, transplants should be available when needed. As fresh tracheal grafts are short-lived, cryopreserving them with appropriate solutions can also contribute to the formation of tissue banks in future. In our experimental study, we used Dulbecco’s modified Eagle Medium as the storage medium. The solution contained antibiotics to prevent infections to the graft and 10% DMSO that penetrates to the cell membrane to offer protection from cold injuries [7]. The duration of cryopreservation was 4 weeks as this was
the shortest time mentioned in the literature for the disappearance of the tracheal epithelium.

The most important technical problem that could affect our results was the lack of a computer-controlled freezer.

In conclusion, the neo-vascularisation of the short-segment tracheal graft could start from the anastomotic ends eliminating the need for further procedures. Cryopreservation can, on the one hand, minimise the allogenicity of the graft while preserving them, on the other hand, without harming the structure and the viability. Therefore it can be possible to maintain tissue banks and have tracheal grafts available when needed. Stenosis in the tracheal lumen resulting from fibrosis and problems in tissue-healing are important in animal studies; however, they can be practically solved with the help of other treatment methods (such as stenting, t-tube and lasers). Tracheal allotransplantation studies with cryopreserved specimens seem to be promising for the future in the reconstruction in malignant tracheal diseases involving large segments.

References