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Original Article

Long-term observation of airway reconstruction using decellularized tracheal allografts in micro-miniature pigs at growing stage

Michinobu Ohno [a, b], Yasushi Fuchimoto [c, d, *], Masataka Higuchi [e], Tetsuji Yamaoka [f], Makoto Komura [g], Akihiro Umezawa [h], Huai-Che Hsu [i], Shin Enosawa [i], Tatsuo Kuroda [d]

[a] Department of Pediatric Surgery, Saitama City Hospital, 2460 Mimuro, Midori-ku, Saitama-shi, Saitama 336-8522, Japan
[b] Division of Surgery, Department of Surgical Specialties, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[c] Department of Pediatric Surgery, International University of Health and Welfare School of Medicine, 2600-1 Kitakanemaru, Ohtawara-shi, Tochigi 324-8501, Japan
[d] Department of Pediatric Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan
[e] Division of Pulmonology, Department of Medical Specialties, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[f] Department of Biomedical Engineering, National Cerebral and Cardiovascular Center Research Institute, 6-1 Kishibe-Shimmachi, Suita, Osaka 564-8565, Japan
[g] Department of Pediatric Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[h] Department of Reproductive Biology, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[i] Division for Advanced Medical Sciences, National Center for Child Health and Development, 2-10-1 Okura,Setagaya-ku, Tokyo 157-8535, Japan

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ABSTRACT

Introduction: Decellularized tissue exhibits cell matrix-like properties, along with reduced antigenicity. We explored the potential of decellularized allogeneic trachea to restore the upper respiratory tract, focusing on pediatric application. This study specifically aimed at long-term observation of tissue regeneration using a micro-miniature pig model.
Methods: Artificial defects (15 × 15 mm) in the subglottis and trachea of micro-miniature pigs were repaired by transplantation of either allogeneic decellularized or fresh (control) tracheal patches. Pigs were evaluated *in situ*, by bronchoscopy, every three months, and sacrificed for histological examination at six and twelve months after transplantation.
Results: No airway symptom was observed in any pig during the observation period. Bronchoscopy revealed the tracheal lumen to be restored by fresh grafts, showing an irregular surface with remarkable longitudinal compression; these changes were mild after restoration with decellularized grafts. Histologically, while fresh graft patches were denatured and replaced by calcified tissue, decellularized patches remained unchanged throughout the observation period. There were regeneration foci of cartilage adjacent to the grafts, and some foci joined the decellularized graft uniformly, suggesting the induction of tracheal reconstitution.
Conclusion: Allogeneic decellularized tracheal tissue could serve as a promising biomaterial for tracheal restoration, especially for pediatric patients at the growing stage.

1. Introduction

Tracheal restoration is one of the various unmet medical needs in regenerative therapy [1,2]. To date, treatments have been performed using autologous rib cartilage, atelocollagen-coated polypropylene, cryopreserved trachea, and decellularized tracheas [3–5]. Cryopreservation enables long-term storage and

Abbreviations: HE, Hematoxylin and Eosin.

* Corresponding author. Department of Pediatric Surgery, International University of Health and Welfare School of Medicine, 2600-1 Kitakanemaru, Ohtawara-shi, Tochigi, 324-8501, Japan. Fax: +81-287-39-3001.
E-mail addresses: mohnomohno2000@hotmail.co.jp (M. Ohno), yfuchimoto@iuhw.ac.jp (Y. Fuchimoto).

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may attenuate alloantigenicity. Lu et al. reported that the tracheal epithelium disappeared after cryopreservation, but lethal stenosis occurred due to immunogenic inflammation probably caused by remaining epithelial alloantigens in dogs [6]. From this perspective, decellularization will be more effective on inflammation suppression because the treatment depletes almost all protein components of the tissue.

Despite failures in the use of decellularized trachea in inappropriate clinical studies [5,7], decellularized medical products have been recognized as promising biomaterials for repairing various tissues, such as heart valve, blood vessel, bone, and cerebral dura matter [8,9]. Here, we explored the potential of allogeneic decellularized tracheal tissue to restore tracheal defects, in a long-term porcine study, especially aiming at treatment for children.

Pediatric surgery has close relation to growth of infants and children. Thus, regenerative therapy plays an important role for pediatric diseases. Specifically, for diseases involving cartilage, regenerative therapy is worth exploring, since regeneration of cartilage is difficult. Tracheal restoration has been conducted for neoplastic diseases, closure of insertion sites after long-term intubation, and congenital tracheal disorders, such as subglottic stenosis and tracheomalacia [10,11]. Although pediatric surgeons frequently encounter such diseases, the existing restoration techniques are not satisfactory, and more suitable prosthetic materials, different from those used for adult patients, would be preferable. Therefore, we considered the use of allogeneic decellularized trachea in such cases.

Our decellularization technique, using hydrostatic pressurization, is a superior tissue engineering procedure that achieves both perfect sterilization and antigenicity reduction, while retaining the scaffold property [12–14]. Previously, we had reported the early changes, up to three months, after transplanting allogeneic decellularized tracheal grafts in a porcine model [15]. Decellularized graft patches were shown to be less inflammatory and more rigid than fresh patches, and interestingly, regeneration foci were seen to arise around the graft. In the present study, we set out to evaluate the biological characteristics of decellularized tracheal grafts over longer periods and found remarkable development of regeneration foci at six and twelve months, using young micro-miniature pigs, in which body weight was maintained at approximately 30 kg, even after maturation. In addition to the size advantage, the use of microminiature pigs has another scientific significance, as indicated by Tohyama and Kobayashi [16]. Growth activity, such as wound healing, is superior in young piglets and it sometimes leads to overestimation of the regenerative activities after treatments. To eliminate the effect of intrinsic physiological repairing, the maturity of test animals should be carefully considered. Because our concept is to treat tracheal defect of pediatric or juvenile patients, not babies, we chose 4-7-month-old microminiature pigs as recipients.

Under the above-mentioned experimental scheme, we report here a successful one-year observation after airway reconstruction using decellularized allogeneic tracheal tissue in microminiature pigs corresponding to putative pediatric patients.

2. Materials and methods

2.1. Ethical considerations

The institutional animal ethics committee of the National Center for Child Health and Development approved all the experimental procedures (reference no. A2000-001 and A2014-007).

2.2. Materials

Porcine tracheal tissues were purchased from Tokyo Shibaura Zouki Co., Ltd. (Tokyo, Japan) as an experimental material (Fig. 1a). The donor animals were domestic pigs (approximately 6–8 months old). Recipient pigs (micro-miniature pigs: 4–7 months old, weighing 10–13 kg; summarized in Table 1) were purchased from Fuji Micra Inc. (Shizuoka, Japan).

2.3. Decellularization of trachea

Decellularization was performed using a high hydrostatic pressure technique, as reported previously [8–10]. Briefly, tracheal tissues of adult domestic pigs were packed in plastic bags filled with saline solution and sealed. The bags were set in a cold isostatic pressing machine chamber (Dr. CHEF; Kobe Steel, Ltd., Kobe, Japan), filled with anti-freezing transmission fluid. Pressure was raised to 980 MPa at the rate of 65.3 MPa/min, kept constant for 10 min, and subsequently de-pressurized at the same rate to atmospheric pressure. Thereafter, the tissue was washed with saline, containing 40,000 U/L DNase I (AMPD1; Sigma–Aldrich, St. Louis, MO, USA) and 20 mM $MgCl_2$, for 2 weeks. Finally, the tissue was soaked in saline containing 2 mM ethylenediaminetetraacetic acid for 2 weeks, followed by washing with saline for 1 week. The decellularized tracheas (Fig. 1b) were stored in saline at 4 °C until use.

2.4. Surgical procedure

Surgical procedure of tracheal patch grafting was performed as described previously [15]. Briefly, recipient pigs were sedated

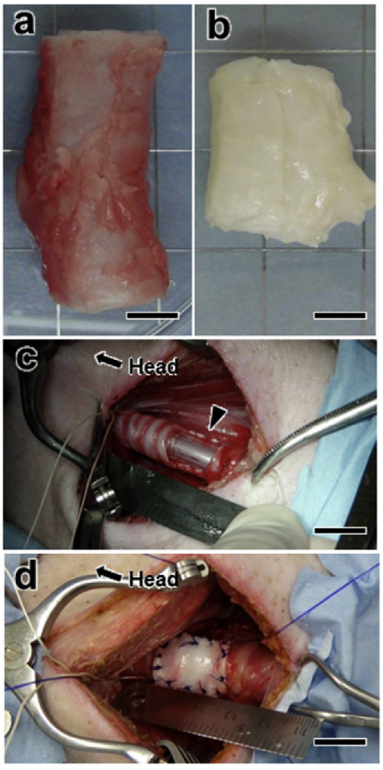

Fig. 1. Fresh (a) and decellularized (b) tracheal grafts and outline of the surgical procedure (c, d). Tracheas (a) were purchased from Tokyo Shibaura Zouki and decellularized using an ultra-high pressure protocol. The decellularized tissue has a characteristic white appearance (b). After dissection of approximately 1/2 of the tracheal trunk (c, arrowhead indicates intubation), the defect was restored with either fresh or decellularized allogeneic tracheal patch of appropriate size (d). Orientation of recipients was indicated by arrows. Bars: 10 mm.

Table 1
Summary of experimental groups, pig numbers, and individual data.

Experimental group # Pig number	Graft	Day of Transplantation		Day of Sampling	
		Age, month	B.W. kg	Age, month	B.W. kg (fold increase)
6-month group					
#3149	Fresh	4.4	12.5	10.4	24.2 (1.94)
#3112	Decellularized	6.9	11.6	12.9	22.6 (1.95)
Mean ± range		5.7 ± 1.3	12.1 ± 0.5	11.7 ± 1.3	23.4 ± 0.8 (1.95 ± 0.05)
12-month group					
#3148	Fresh	4.9	10.3	16.9	27.5 (2.67)
#3119	Decellularized	6.8	13.5	18.6	27.2 (2.01)
#3168	Decellularized	4.9	12.9	16.9	34.5 (2.67)
Mean ± SD		5.5 ± 1.1	12.2 ± 1.7	17.5 ± 1.0	29.7 ± 4.1 (2.45 ± 0.38)

B.W.: body weight.

with medetomidine (30 μg/kg, intramuscular injection; Dorbene®, Kyoritsu Seiyaku Co., Ltd., Japan) and thiopental (25 mg/kg, intravenous injection; Ravonal; Tanabe Mitsubishi, Osaka, Japan). After intubation with 4–4.5-mm endotracheal tubes, ventilation was started under routine conditions (respiration rate, 16 cycles per min; tidal volume, 120–130 mL; 2.5–3.0% isoflurane, Wako Pure Chemicals, Osaka, Japan). Butorphanol tartrate (0.3 mg/kg, intramuscular injection; Vetorphale; Meiji Seika Pharma Co., Ltd., Japan) was used as an analgesic during and after operation.

Pigs were placed in a supine position, and the cervicothoracic skin was sterilized using iodous antiseptics and 70% ethanol. A midline incision was performed, and the cervical trachea was exposed. The area under the second or third tracheal rings from the cricoid cartilage (approximately 15 × 15 mm, one-third to a near semicircle of the tracheal trunk) was dissected (Fig. 1c). Decellularized or fresh tracheal grafts, whose size was adjusted to cover the defect, were anastomosed circumferentially with interrupted 4-0 PDS-II (Z771D; Johnson & Johnson, NJ, USA) (Fig. 1d). After sealing tests, muscle and skin were closed with interrupted 3-0 VICRYL (J215H; Johnson & Johnson). The operation time was approximately 30 min. To prevent post-operative infection, pigs were injected once with enrofloxacin (5 mg/kg, intravenous; Baytril, Bayer, Japan), just after the operation, and with levofloxacin (10 mg/kg, orally; Cravit, Daiichisannkyo, Japan) for 7 days. Pigs were fed in an institutional animal room with standard pig chow and water. No immunosuppressive treatment was performed.

2.5. Estimation of tracheal grafts by bronchoscopy and histopathological study

Every three months after grafting, recipient pigs were anesthetized as described above, and bronchoscopy (ENT-30PC; Machida Endoscope Co., Japan) was performed. The animals were euthanized at a defined period (6 or 12 months after transplantation) with an intravenous injection of potassium chloride (B. Braun Melsungen, Melsungen, Germany), following which tracheas were excised. The sectional areas engrafted with decellularized and fresh tracheas were fixed with 10% formalin, embedded in paraffin, and sectioned at 5-μm thickness. The specimens were stained with hematoxylin and eosin (HE), toluidine blue, safranin-O, or alizarin red.

3. Results

3.1. Outline of post-operative growth and bronchoscopy findings

During the observation period, the recipient micro-miniature pigs grew from 12.1 to 23.4 kg (averaged across the 6-month group) or 12.2S–29.7 kg (averaged across the 12-month group; Table 1), approximately corresponding to the period from 2-years old to 6- or 11-years old in humans. Fig. 2 shows bronchoscopy images of the engrafted area of the recipient tracheal lumen at 6 (Fig. 2a, b) and 12 months (Fig. 2c, d) after the transplantation of fresh and decellularized allogeneic tracheal patches. In all cases, the inner surfaces were covered well with tracheal mucosa and there was no breathing abnormality, including in- and out-flow noises, throughout the observation period. Some wall irregularities and longitudinal compressions were slightly more severe in the fresh tracheal graft than in the decellularized graft (Fig. 2a, c vs. b, d).

3.2. Macroscopic observation of engrafted areas of the trachea

Fig. 3 shows *in situ* appearances of the engrafted area of recipient tracheas 12 months after the transplantation of fresh (Fig. 3a) or decellularized (Fig. 3b) tracheal patches. Inflammatory responses, such as synechia, granuloma, and enlarged lymph nodes, were more noticeable around the trachea engrafted with the fresh patch (arrowheads in a and e), compared to that around the decellularized graft, where virtually no inflammatory response was observed (Fig. 3b, d, f). Macroscopic observation of autopsy specimens corresponded well with bronchoscopy data (Fig. 2). Tracheal narrowing and stenosis were more severe in the fresh patch-engrafted region, at both 6 and 12 months after transplantation (Fig. 3c, e), than in the decellularized patch-engrafted region (Fig. 3d).

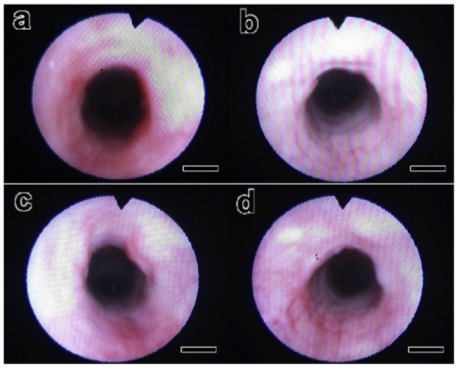

Fig. 2. Bronchoscopic observation of the engrafted section of recipient trachea. At 6 months after transplantation of fresh (a) and decellularized (b) allogeneic tracheal patches, and 12 months after the transplantation of fresh (c) and decellularized (d) allogeneic tracheal patches. In all cases, the inner surfaces were covered well with tracheal mucosa, and there was no breathing abnormality, including in- and outflow noises. Some wall irregularities and longitudinal compressions were slightly more severe in the fresh tracheal graft than in the decellularized graft. Bars: 10 mm.

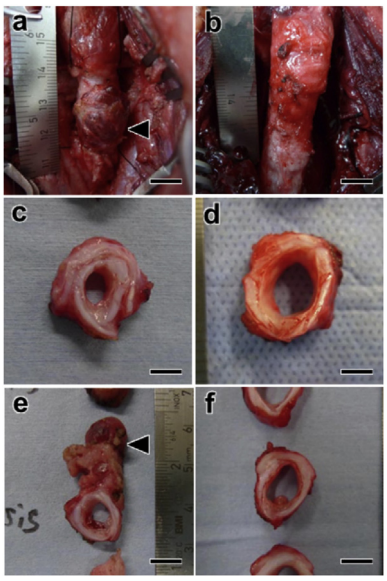

Fig. 3. Appearance of exposed trachea 12 months after the transplantation of fresh (a) and decellularized (b) tracheal patches. Macroscopic views of the cross sections of autopsy tracheas, harvested at 6 and 12 months after the transplantation of fresh (c, e) and decellularized (d, f) grafts. Arrowheads in a and e indicate inflammatory granulation and large lymph nodes. Bars: 10 mm.

3.3. Pathohistological assessment of tracheal sections

Tracheal restoration with the decellularized tracheal patch was assessed microscopically (Fig. 4). The patch graft remained solid even 12 months after the transplantation (Fig. 4a, arrowhead), although the staining properties became faint in HE, toluidine blue, and safranin-O (Fig. 4a, e, f, respectively). At present, the reasons of color change were unknown; due to high hydrostatic pressure decellularization or due to long-lasting poor blood supply. Further analysis, including immunohistochemistry, will be necessary. Interestingly, the recipient tracheal cartilage was seen to be extended toward the decellularized graft (Fig. 4b, magnified view of the framed area in Fig. 4a). The regenerated cartilage and decellularized graft were seen to be in contact with each other, forming a smooth boundary surface, as if the graft had induced cartilagenesis (Fig. 4b, c). In Fig. 4c, two different types of regenerated cartilage tissues were observed; a thin membranous structure (asterisk) and nodules (arrows). As for surrounding soft tissue, there was no sign of inflammatory responses such as cell infiltration, granulation, and vascular hyperplasia (Fig. 4d).

In contrast, the fresh patch became dark blue in color (Fig. 5a arrowhead) and positive in alizarin red staining (Fig. 5b arrowheads) 12 months after the transplantation, suggesting calcification. Definite cracks were found within the graft (Fig. 5b, arrowhead). While nascent cartilage tissue was seen (Fig. 5c), no evidence of fusion to the engrafted patch could be found.

4. Discussion

In our previous report, we established a porcine model of tracheal restoration using domestic pigs and showed the superiority of decellularized tracheal tissue to unprocessed fresh tissue [15]. However, the observation period in the study was only eleven weeks, whereas the putative clinical use would be life long. To conduct a long-term study, domestic pigs are not suitable, since they grow very rapidly, with the average body weight of a 1-year-old reaching approximately 200 kg in males and 150 kg in females [17]. Therefore, we elected micro-miniature pigs, established from the Potbelly strain, for animal experiments in Japan. In this experiment, we used 5.5-month-old micro-miniature pigs, weighing approximately 12 kg and observed them for 12 months until they were 17.5-months old, weighing 30 kg (Table 1). Based on body weight, this growth corresponded approximately to 2-year-old to 11-year-old humans.

In regards of resection size, we considered that the medical indication of decellularized tracheal patch grafting will be small

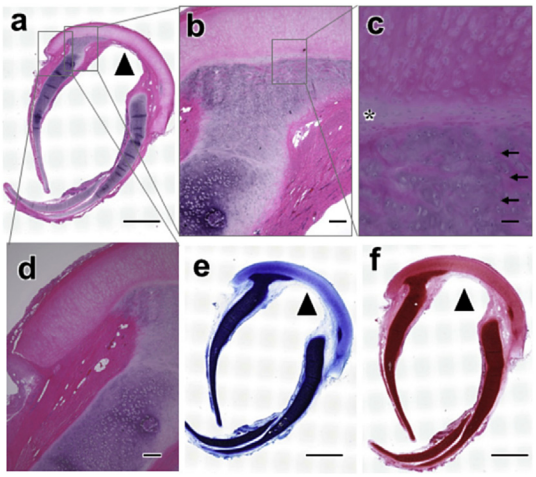

Fig. 4. Microscopic observation of tracheal restoration of decellularized tracheal patches. Microscopic observation of tracheal restoration with decellularized tracheal patches, 12 months after transplantation, using hematoxylin and eosin (a–d), toluidine blue (e), and safranin-O (f) staining. Arrowheads indicate decellularized tracheal grafts. b and d indicate magnified views of the framed area in a. c indicates a magnified view of the framed area in b. In c, asterisk and arrows indicate membranous and nodulous structure, respectively. Bars: 500 μm in a, e, f, 50 μm in b, c, d.

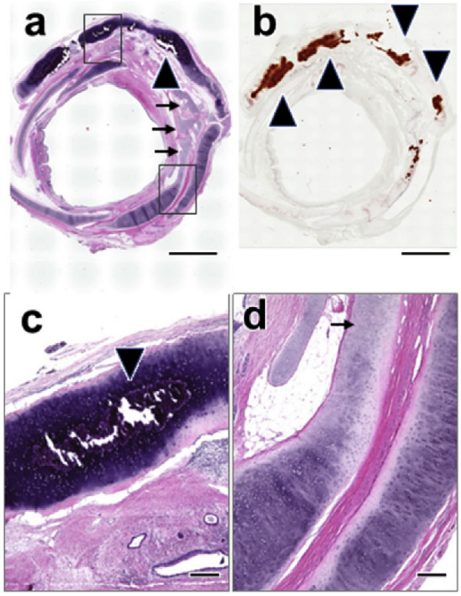

Fig. 5. Microscopic observation of tracheal restoration of fresh tracheal patch. Microscopic observation of tracheal restoration 12 months after transplantation of fresh tracheal patch (a; hematoxylin and eosin staining, b; alizarin red staining). Arrowheads indicate degradation and calcification of fresh tracheal graft. c and d show magnified views of the framed areas in a (upper; B, lower; C). Arrows in a and d indicate the cartilage that appeared to be regenerated. Bars: 500 μm in a, b and 50 μm in c, d.

regional tracheal stenosis after closing tracheostomy or congenital subglottic stenosis. Therefore, we set a defect size of 15 × 15 mm, i.e., approximately one-third of tracheal trunk.

Long-term observation clarified the superiority of decellularized graft. Based on macroscopic observations, fresh graft induced heavy granulation, lymphadenopathy, and severe stenosis, compared to the decellularized graft (Fig. 3), corresponding to the smoothness of tracheal lumen (Fig. 2). Histologically, the decellularized graft remained rigid (Fig. 4), whereas the fresh graft denatured completely and calcified 12 months after transplantation (Fig. 5). We considered this difference between decellularized trachea and fresh trachea to be the result of rejection in cartilaginous and perichondral tissues. In both cases of decellularized and fresh patch grafting, the epithelium was likely to be recipient origin. The epithelium may spread over the decellularized tissue surface without any inflammatory interference. However, the epithelium on the inflammatory fresh patch seemed to cause severe stenosis.

High-pressure decellularization treatment reduced the amount of residual DNA in cartilaginous and perichondral tissues, thus preventing rejection [15]. An interesting finding was the fusion between decellularized graft and nascent cartilage (Fig. 4a, b). The fusion area may act as a meristematic tissue site of cartilage, and therefore, contribute to tracheal growth. The decellularized graft is hoped to be a natural prosthesis to induce cartilage neogenesis.

Animals transplanted with neither decellularized nor fresh graft, showed several harmful symptoms, such as respiratory failure, until 12 months [18,19]. However, endoscopic and macroscopic findings revealed slight stenosis even in the decellularized trachea. Although slight stenosis due to the use of decellularized graft may not be a problem clinically, it might be important to consider tracheal growth accompanying overall body growth in the case of pediatric patients. The following reasons may explain the pathogenesis of stenosis. First, the stenosis may be attributable to the loss of mechanistic support due to the formation of artificial defects. The impaired tracheal framework and negative pressure associated with respiration may cause such a change [15,20,21]. A more ideal restoration procedure may be able to prevent such stenosis. Second, since tracheal tissue experiences poor blood supply, it may be induced by ischemic change. This stenosis is unlikely to be caused by rejection, since a similar stenosis was seen for patch-transplantation using an autologous graft (as reported in our preliminary observation) [15]. To support blood flow, the use of a rich vascular flap around the anastomotic site may be effective to palliate stenosis [22,23].

The present study indicated that the allogeneic patch procedure, using high hydrostatic pressure decellularized trachea, could be a useful application for pediatric tracheal restoration. High hydrostatic pressure reduces the antigenecities of epithelial and extracellular matrix and alleviates rejection compared to cryopreservation [6]. Possible indications include relatively small regional tracheal stenosis after closing tracheostomy or congenital subglottic stenosis. The ultimate objective of our study was orthotopic circumferential tracheal replacement for broad congenital tracheal stenosis. To realize this treatment, improvement of blood supply to the graft area would be crucial.

5. Conclusion

Long-term observation of tracheal restoration demonstrated that the allogeneic decellularized tracheal patches have significant advantages over fresh patches, in terms of 1) rigidity, 2) a non-inflammatory effect, and 3) the affinity for nascent cartilage. These properties lead to a smoother surface and more spacious lumen after tissue transplantation. Therefore, decellularized allogeneic tracheal tissue may serve as a promising biomaterial for tracheal restoration, especially for pediatric patients at growing age.

Declaration of Competing Interest

The authors declare no competing interests.

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