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ORIGINAL ARTICLE

Long-term outcomes of patch tracheoplasty using collagenous tissue membranes (biosheets) produced by in-body tissue architecture in a beagle model

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Abstract

Purpose Although various artificial tracheas have been developed, none have proven satisfactory for clinical use. In-body tissue architecture (IBTA) has enabled us to produce collagenous tissues with a wide range of shapes and sizes to meet the needs of individual recipients. In the present study, we investigated the long-term outcomes of patch tracheoplasty using an IBTA-induced collagenous tissue membrane ("biosheet") in a beagle model.

Methods Nine adult female beagles were used. Biosheets were prepared by embedding cylindrical molds assembled with a silicone rod and a slitting pipe into dorsal subcutaneous pouches for 2 months. The sheets were then implanted by patch tracheoplasty. An endoscopic evaluation was performed after 1, 3, or 12 months. The implanted biosheets were harvested for a histological evaluation at the same time points.

Results All animals survived the study. At 1 month after tracheoplasty, the anastomotic parts and internal surface of the biosheets were smooth with ciliated columnar epithelium, which regenerated into the internal surface of the biosheet. The chronological spread of chondrocytes into the biosheet was observed at 3 and 12 months.

Conclusions Biosheets showed excellent performance as a scaffold for trachea regeneration with complete luminal epithelium and partial chondrocytes in a 1-year beagle implantation model of patch tracheoplasty.

Keywords Regenerative medicine · Trachea · Scaffold · Patch tracheoplasty · Dog (beagle)

Introduction

Tracheal disorders in pediatric patients, such as congenital tracheal stenosis, are often life-threatening diseases that require surgical reconstruction with adequate replacement of tissue. Although current treatments utilize autologous rib cartilage grafts or pericardial patches for tracheoplasty [1], this procedure requires a specialized surgical technique and an invasive, multisite surgery. In addition, the limited size and shape of available rib cartilage grafts and pericardial patches that can be collected are important clinical problems that cannot be ignored. Therefore, new types of replacement tissues as tracheal scaffolds are urgently needed.

In-body tissue architecture (IBTA) based on the body's defense mechanisms against foreign materials has led to the development of autologous prosthetic collagenous tissues [2]. The collagenous tissue scaffolds produced by IBTA are biocompatible and have tissue-reconstruction properties similar to those of cells in the native organ. This novel approach to regenerative medicine has several advantages because prostheses can be fabricated in a range of shapes and sizes to meet the needs of individual recipients [3]. This technology has been successfully applied to the engineering of cardiovascular tissues, for vascular grafts as biotubes, or for heart valvular-like tissues as biovalves [4]. In addition,

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esophageal reconstruction using a scaffold produced by IBTA demonstrated the novel scaffold's remarkable self-organization ability [5]. Furthermore, patch tracheoplasty using connective tissue membranes as biosheets has been used to demonstrate epithelial and cartilage regeneration in a rabbit model [6]. However, to ensure the safe clinical application of biosheets for airway reconstruction, long-term observation studies in large-animal models are required.

Therefore, in this study, we aimed to investigate the long-term outcomes of patch tracheoplasty using biosheets in a beagle model.

Methods

Ethical considerations

All procedures and protocols were approved by the Committee on the Ethics of Animal Experiments of the National Cerebral and Cardiovascular Centre Research Institute, Suita, Osaka, Japan (protocol no. 17013).

Animal model

A cylindrical mold was assembled with a silicone rod (outer diameter 15 mm) and a slitting acrylate pipe (outer diameter 20 mm) (Fig. 1a). Adult female beagle dogs (weight approximately 8–10 kg; approximately 1–1.5 years old) were used to develop the biosheets and for subsequent patch tracheoplasty. Pre-anesthetic medication with 5 mg/kg ketamine, 0.02 mg/kg intravenous (IV) buprenorphine, and 0.025 mg/kg intramuscular atropine sulfate was administered, and anesthesia was performed with 15 mg/kg IV pentobarbital and maintained by bolus induction of IV pentobarbital at a quarter or half of the initial dose. The molds were embedded into subcutaneous pouches in the dogs ($n=3$; two molds per dog). At 2 months following the embedding procedure, the molds were harvested, and tubular connective tissues (internal diameter 15 mm, length 40 mm) were extracted by removing the molds. Biosheets (45 mm × 40 mm; thickness of approximately 1 mm) were obtained by longitudinally cutting the tubular tissues. The biosheets were stored in 70% ethanol and washed with physiological saline for at least 10 min immediately before implantation.

After a histological assessment, patch tracheoplasty with the biosheets was performed in the adult female beagle dogs ($n=9$). In brief, with the dogs under anesthesia as mentioned above, a midline longitudinal tracheotomy with a 10-mm-wide × 20-mm-long defect (about 30% circumference and 3 rings long) was created, and rectangular allogenic biosheets of the same size (10 × 20 mm) were implanted into the defect. An endoscopic evaluation was performed under general anesthesia at 1 and 3 months after tracheoplasty. Tracheas containing biosheets were harvested as samples for morphological observation at 1 ($n=3$), 3 ($n=4$), and 12 months ($n=2$) after tracheoplasty, and the size of the implanted biosheets was measured. The evaluation at different time points during follow-up was performed on the

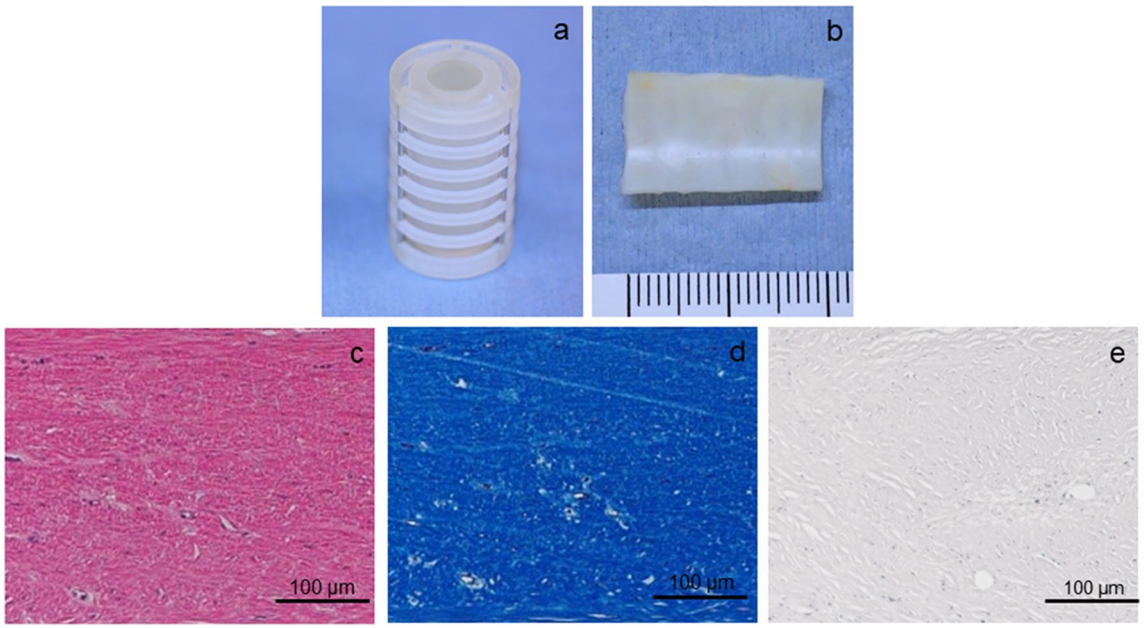

Fig. 1 The preparation and histological examination of the biosheets. **a** Mold. **b** Gross appearance of the biosheets. **c** H&E staining of the biosheets. **d** Masson's trichrome staining of the biosheets. **e** H&E staining of the biosheets immersed in 70% ethanol

same dogs. In this study, the implanted biosheets originated from different dogs. The biosheets were immersed in 70% ethanol before tracheoplasty.

Morphological observations

The samples were harvested, immersed in fixative, embedded in paraffin, and sectioned in 4-μm-thick serial sections. Sections were stained with routine hematoxylin and eosin (H&E), safranin-O stain for the cartilage matrix assessment, and Masson's trichrome stain for the collagen assessment. The sections were also stained with alcian blue (Muto Pure Chemicals Co. Ltd., Tokyo, Japan) and fast red (Muto Pure Chemicals Co. Ltd.) to identify mucin and nuclei, respectively, using routine techniques. An immunohistochemical assessment was performed using anti-α-tubulin, anti-type II collagen, and anti-von Willebrand Factor (vWF) (Abcam, Cambridge, UK). Histopathological assessments were performed with a Biorevo BZ-9000 (Keyence, Osaka, Japan). X-ray fluoroscopy of the trachea at 12 months after tracheoplasty was performed before a histological evaluation.

Results

Two months after the subcutaneous embedding of the molds, the inside of the cylindrical molds was completely filled with connective tissues. The tubular tissues obtained after removing all mold parts were cut in the longitudinal direction to form sheet-like tissues as biosheets (Fig. 1b). H&E and Masson's trichrome staining revealed enrichment of collagenous connective tissue in the biosheets (Fig. 1c, d), which had sufficient strength and flexibility to replace the tracheal defect. In addition, by immersing the biosheets in 70% ethanol, most of the cellular components detached from the biosheets, so strong rejection was not induced (Figs. 1e, 3a).

Tracheoplasty using the biosheets was carried out without surgical difficulty. No air leakage was observed after implantation. Of the nine dogs that underwent patch tracheoplasty, all survived without any respiratory symptoms during the observation period. Furthermore, of the nine dogs, two reached 1-year follow-up. Endoscopic airway observation showed complete respiratory mucosal coverage on the entire luminal surface of the biosheets, even at just 1 month after implantation (Fig. 2a–c). The

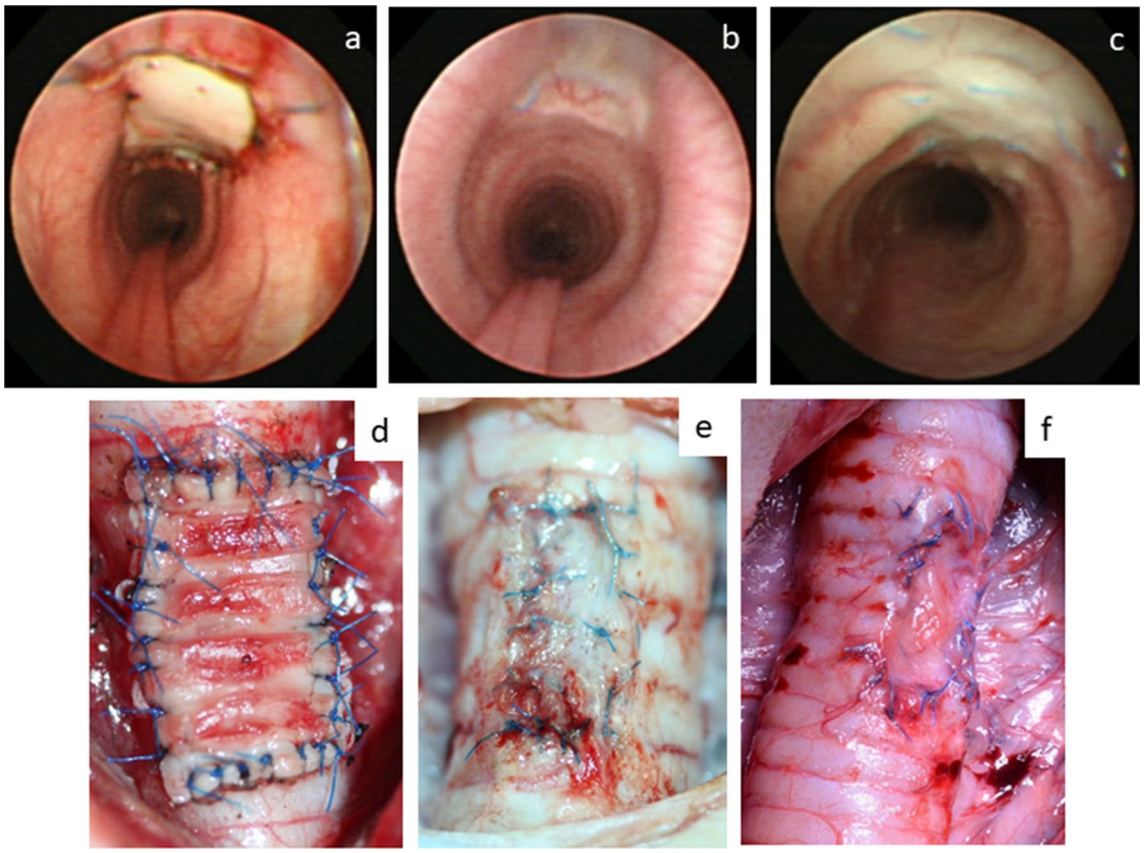

Fig. 2 Bronchoscopic findings. **a** Immediately after tracheoplasty. **b** 1 month after tracheoplasty. **c** 3 months after tracheoplasty. Macroscopic findings. **d** Immediately after tracheoplasty. **e** 1 month after tracheoplasty. **f** 3 months after tracheoplasty

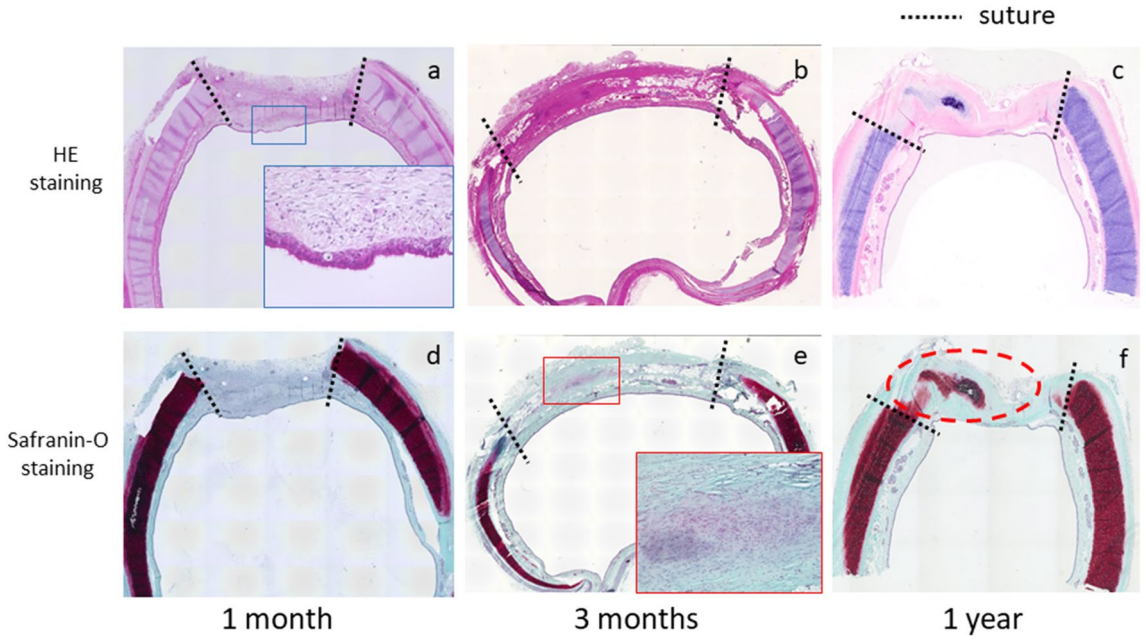

Fig. 3 The histological findings of the transplanted biosheets. Hematoxylin and eosin (HE) staining. **a** 1 month after tracheoplasty. **b** 3 months after tracheoplasty. **c** 12 months after tracheoplasty. Safranin-O staining. **d** 1 month after tracheoplasty. **e** 3 months after tracheoplasty. **f** 12 months after tracheoplasty

outermost luminal surface was lined with ciliated epithelial cells, and strong rejection was not observed (Fig. 3a). Furthermore, immunohistochemical staining of α-tubulin and alcian blue staining revealed sufficient regeneration of the tracheal epithelium, including ciliated epithelial cells and mucus-producing cells (Fig. 4c–e, g–i). During the entire observation period in all cases, mucosal regeneration was maintained without symptomatic scar contraction or deformation as a complication of tracheal reconstruction using a biosheet (Fig. 2d–f). Furthermore, the size of the implanted biosheets at different time points was not marked different from that at tracheoplasty.

Safranin-O staining at 1 month after the tracheoplasty revealed that no cartilage was present in the biosheets (Fig. 3d). At 3 months after tracheoplasty, chondrocyte migration into the biosheet was observed (Fig. 3e). At 12 months after tracheoplasty, cartilage regeneration in up to half of the scaffold was confirmed (Fig. 3f, red circle). Consistent with the results of safranin-O staining, immunohistochemical staining with anti-type II collagen antibody revealed chondrocyte migration similar to that in the native trachea (Fig. 4a–c). In addition, immunohistochemical staining with anti-vWF revealed the continuity of neovascularization from the native trachea to the biosheet at 1 month after tracheoplasty (Fig. 5a–c). However, capillaries were rarely observed in the mature cartilage matrix at 1 year after tracheoplasty (Fig. 5d–f). No stenotic or torose lesions were observed by X-ray fluoroscopy (Fig. 6a–c).

Discussion

This is the first report to investigate the long-term outcomes of patch tracheoplasty using collagenous connective tissue membranes, i.e., biosheets, in a large animal model. This novel scaffold had sufficient strength and flexibility to attach via sutures to the native trachea. No air leakage was observed, and all dogs survived without any symptoms during all observation periods. In addition, endoscopic and histological examinations revealed that mucosal regeneration occurred at 1 month after the patch tracheoplasty. Consistent with the results of a previous report using a rabbit model [6], a histological examination using safranin-O staining and immunohistochemical type II collagen staining revealed that chronologically spreading cartilage regeneration was present 3–12 months after patch tracheoplasty in this study. In addition, immunohistochemical staining of α-tubulin and alcian blue staining revealed sufficient regeneration of functional tracheal epithelium.

Various artificial materials (e.g., stainless steel, polyethylene, silicone, and Teflon) have been investigated for their potential application as a scaffold in tracheal transplantation. However, these materials can induce progressive scar tissue formation due to incomplete re-epithelialization, leading to stenosis and scaffold obstruction [7]. Previous reports have demonstrated that tracheal transplantation using artificial prostheses is not possible [8, 9]. Therefore, biocompatible

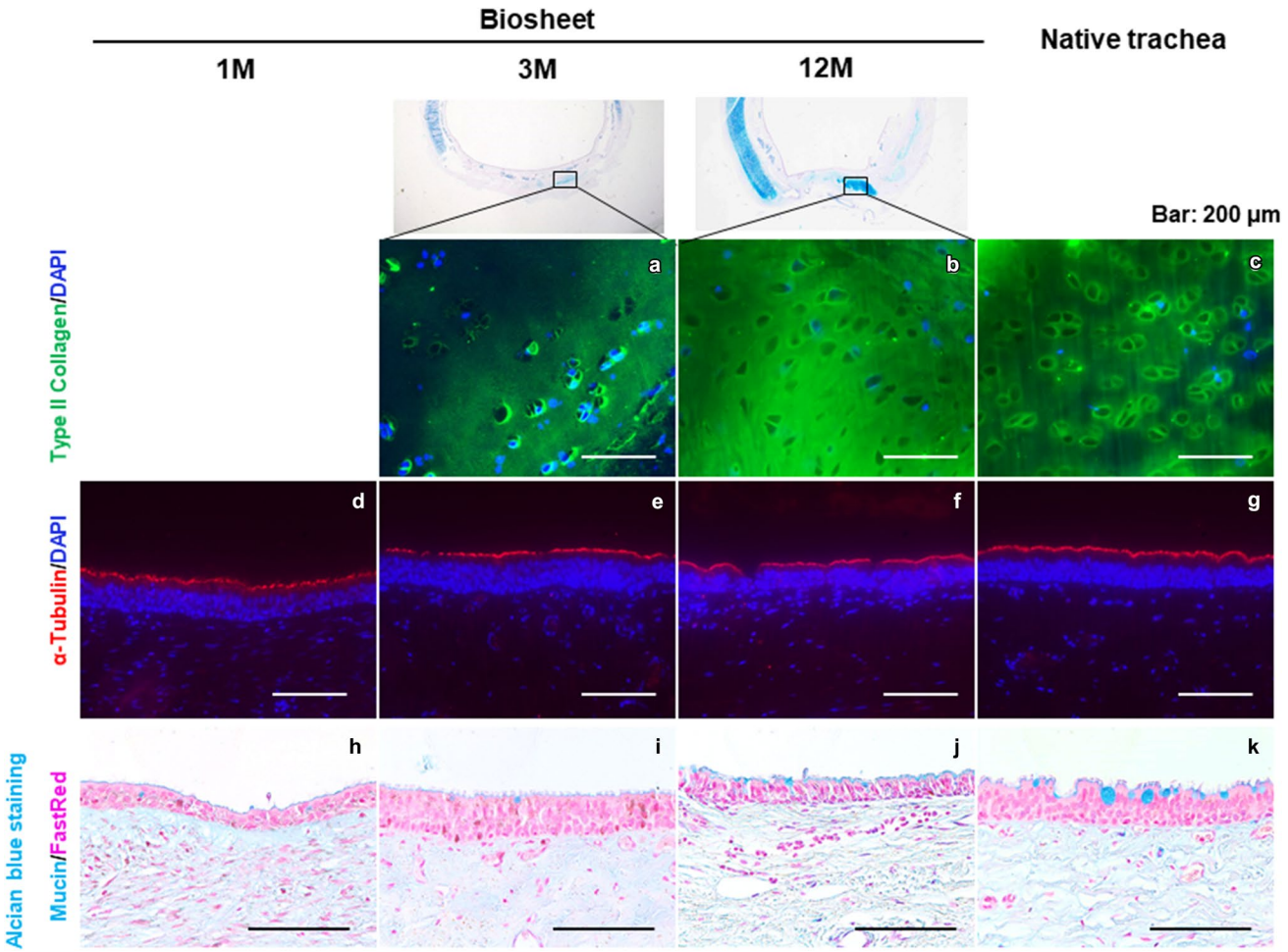

Fig. 4 Immunohistochemical findings and alcian blue staining of the transplanted biosheets. Type II collagen. **a** 3 months after tracheoplasty. **b** 12 months after tracheoplasty. **c** Native trachea. α-Tubulin. **d** 1 month after tracheoplasty. **e** 3 months after tracheoplasty. **f** 12 months after tracheoplasty. **g** Native trachea. Mucin. **h** 1 month after tracheoplasty. **i** 3 months after tracheoplasty. **j** 12 months after tracheoplasty. **k** Native trachea

materials that can prevent scar tissue formation are required for tracheal reconstruction.

Patch tracheoplasty using autologous rib cartilage grafts has been described with reasonable results [10]. However, a previous report evaluating the histopathological changes after patch tracheoplasty with rib cartilage grafts showed that fibrosis and granulomatous tissue formation were observed at the edge of the grafts where the implanted cartilage was absorbed and replaced with mature scar tissue [11]. Another technique—an autologous pericardial patch—was used to enlarge the tracheal lumen; this method may be a reasonable alternative because it easily resulted in a wide lumen. The disadvantage of this technique is the long postoperative intubation period required for stenting the trachea and avoiding collapse of the flaccid pericardium [12, 13]. These tracheoplasty procedures using autologous tissues require an additional surgical procedure. The limited size and shape of available grafts may also cause clinical problems.

In the present study, we used a collagenous connective tissue membrane that did not require a complex in vitro cell culture system to generate a tracheal scaffold. In addition, this technology requires less surgical invasion to produce the scaffold than tracheoplasty using an autologous pericardial patch and strips of rib cartilage. For example, tracheoplasty using an autologous pericardial patch and strips of rib cartilage requires the resection of a patient's native tissue. In addition, a pericardial patch often requires thoracotomy. IBTA does not necessitate the resection of autologous native organs. In fact, this technology only requires the formation of subcutaneous pouches in the body trunk. In other words, IBTA only requires surgical invasion of the body surface. This technology has been used to produce tissue scaffolds for replacement of various types of organs [14, 15] that are biocompatible and have tissue reconstruction properties, similar to cells in the native organ [16].

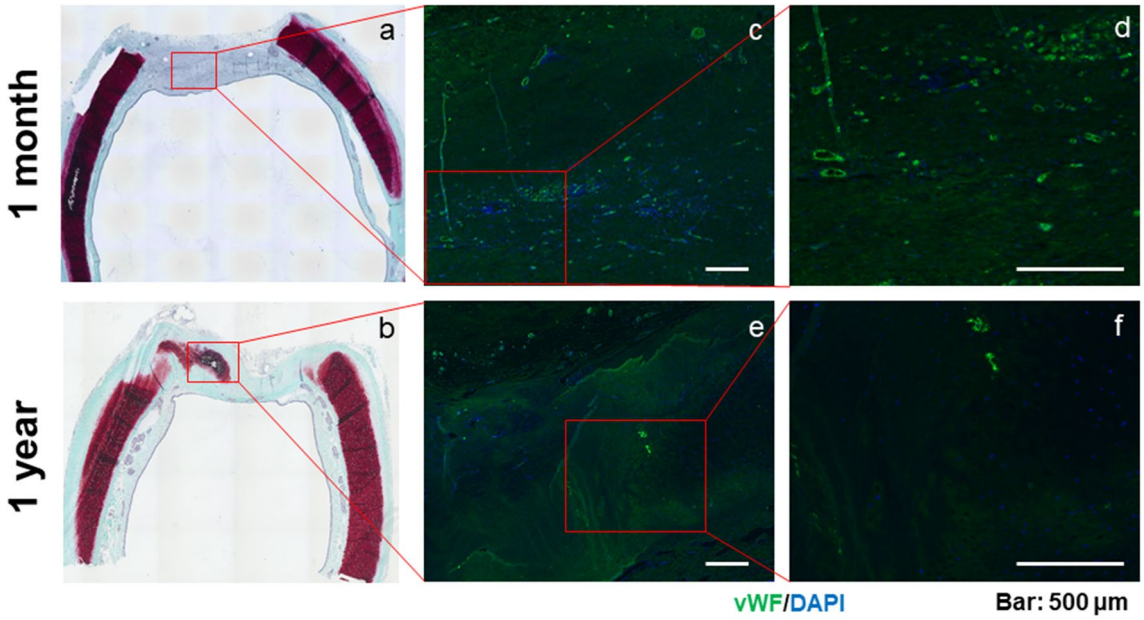

Fig. 5 Histological findings of the transplanted biosheets. Safranin-O staining. **a** 1 month after tracheoplasty. **b** 12 months after tracheoplasty. Immunohistochemical findings of von Willebrand Factor (vWF) in the transplanted biosheets. **c** 1 month after tracheoplasty (low-power field). **d** 1 month after tracheoplasty (high-power field). **e** 12 months after tracheoplasty (low-power field). **f** 12 months after tracheoplasty (high-power field)

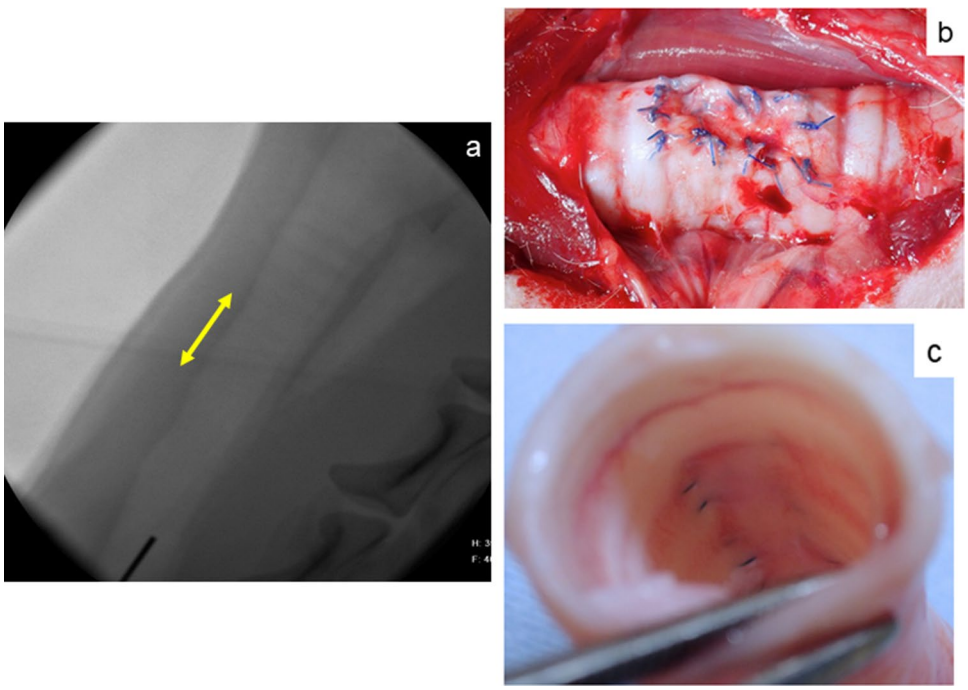

Fig. 6 No stenotic lesions or torose lesions were observed at 12 months after tracheoplasty. **a** X-ray fluoroscopy of the trachea at 12 months after tracheoplasty (yellow arrow). **b**, **c** Macroscopic findings at 12 months after tracheoplasty

Consistent with these previous reports, we observed the regeneration of ciliated epithelial cells, mucus-producing cells, and cartilage matrix. Immunohistochemical staining using anti-vWF demonstrated the continuity of neovascularization from the native trachea to the biosheet at 1 month after tracheoplasty and regression of capillaries in the mature cartilage matrix at 1 year after tracheoplasty, suggesting that the vascularity originated from the resection stump of the native trachea, contributing to cartilage regeneration. A study using a rabbit model reported that cartilage regeneration was observed at 1 month after implantation [6]. In the present study, regeneration of the ciliated columnar

epithelium was histologically confirmed at 1 month after tracheoplasty. In contrast, cartilage regeneration after tracheoplasty required more than 3 months to occur, suggesting that cartilage regeneration required a longer period than epithelial regeneration. The difference in the findings between the rabbit and beagle models may be due to differences in animal species. Cartilage regeneration in larger animals may require more time. Furthermore, although strong rejection was not observed, the allogenic model may have contributed to the delay in cartilage regeneration in this study. To elucidate the underlying cause of the difference between animal species, further investigations using various animal models will be necessary.

In the present study, we used allogenic biosheets. By immersing the biosheets in 70% ethanol, most (but not all) of the cellular components were able to be detached from the biosheet. A previous study reported that bovine biosheets immersed in 70% ethanol exhibited self-organization without inflammation in a beagle abdominal wall xenograft model [17]. Consistent with this report, there was little rejection, which would otherwise be indicated by, for example, the accumulation of lymphoid cells with a spherical nuclear structure (Fig. 3a). In addition, we did not observe progressive scar tissue formation due to excessive immunological and inflammatory responses leading to stenosis and scaffold obstruction. These findings suggest that biosheets may have advantages over other extracellular matrix materials in the tissue remodeling process by reducing the immunological and inflammatory reaction. Although there are still many unresolved issues, including elucidation of the specific mechanisms underlying tissue reconstruction and the long-term outcomes of these transplanted biosheets in human beings, the results of this preliminary study are promising.

In conclusion, tracheoplasty using biosheets enabled the long-term maintenance of the patency of the trachea. Furthermore, this biomaterial showed the ability to self-organize without progressive scar tissue formation. These findings suggest that biosheets may have potential application as scaffolds for patch tracheoplasty.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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