Optimal bovine collagen concentration to achieve tracheal epithelial coverage of collagen sponges

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気管上皮再生誘導性を有するコラーゲンスポンジスキャフォールドを
作製するための最適なウシコラーゲン濃度

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ABSTRACT

Objectives: Artificial tracheas prepared using collagen sponge and polypropylene mesh have been implanted in patients who received tracheal resections, but epithelialization in the reconstructed area was slow. We determined the optimal bovine atelocollagen concentration necessary for rapid and complete tracheal epithelial coverage of collagen sponge implants.

Study Design: Preliminary animal experiment.

Methods: Collagen sponges were made using lyophylizing 0.5%, 0.7%, and 1.0% atelocollagen solutions and analyzed using scanning electron microscopy. Partial tracheal defects were prepared in rabbits and reconstructed using sponges. Epithelial regeneration in the reconstructed area was evaluated by endoscopic, histological, and scanning electron microscope analyses.

Results: All sponges had a membranous structural framework, and numerous fibrous structures filled the spaces within the framework in the 0.5% sponges. The membranous structure in the 0.7% sponges branched at many points, and inter-membrane spaces were frequently observed. Conversely, the membranous structure in the 1.0% sponges was relatively continuous, thick, and closely arranged. Two weeks after implantation, the tracheal defects were entirely covered with

epithelium in 2/4 and 3/4 of the 0.5% and 0.7% sponge-implanted rabbits, respectively. The collagen sponges remained exposed to the tracheal lumen in 4/4 rabbits in the 1.0% sponge group. Ciliogenesis in the center of the epithelialized region was detected only in the 0.7% sponge group.

Conclusion: Collagen sponges prepared from various concentrations of bovine atelocollagen had different structures. Complete epithelial coverage was achieved in more rabbits implanted with sponges prepared using a 0.7% bovine atelocollagen solution than with those from 0.5% and 1.0% solution.

Key Words: structure, reconstruction, artificial trachea, prosthesis, cilia

Level of Evidence: N/A

INTRODUCTION

Tracheal resection is required for patients suffering from severe tracheal stenosis caused by traumatic injury, inflammation, and malignant tumors in trachea and thyroid. Resection in a large part of the trachea (>6 cm in adults, one-third of the length of the trachea in juveniles) is difficult to be closed by end-to-end anastomosis; therefore, large tracheal defects are reconstructed using pieces of other organs or prostheses.[1,2] However, harvesting autologous tissues, such as mucosa, skin, and auricular and costal cartilages, for replacement of tissue defects increases surgical stress and sometimes causes functional or cosmetic problems in residual tissues. An additional problem is that only relatively small areas can be reconstructed from autologous tissue implantation. Prostheses have the advantage that they can be prepared without damaging normal tissues. Stainless steel, silicone, and polytetrafluoroethylene have been used for tracheal reconstruction. However, these materials often fail to be incorporated into the trachea and remain uncovered by tracheal epithelium for a long period. The uncovered state causes infection because of the loss of epithelial barrier function, and in the worst case, tracheal stenosis by hypertrophy of granulation tissue can occur. Allografts may be another option. Allogenic aortae and tracheae have been implanted to reconstruct

tracheal defects, but patients implanted with allografts must take immunosuppressants for a long period. Limited availability is also a problem when using allogenic tissues.[3–5]

Based on the concept of "tissue engineering" proposed by Langer and Vacanti,[6] Nakamura et al. developed an artificial trachea that is capable of inducing *in situ* tissue regeneration. The artificial trachea is composed of polypropylene mesh and collagen sponge, which is made by lyophilizing collagen solution; the former is used for reconstruction or physical support of various tissues, and the latter allows cell adhesion and invasion.[7] Our group has clinically used the artificial trachea for tracheal/laryngeal tissue regeneration since 2002. The artificial trachea has been successfully applied to patients with partial defects in cervical tracheae and cricoid cartilages, which were generated by resecting areas suffering from stenosis and thyroid cancer invasion. However, a problem is that epithelial tissue formation in the luminal surface of the graft has taken approximately 2 months.[8,9] Further promotion of epithelialization is required to prevent postoperative infection and restenosis.

Scaffold, cell type, and biologically active molecules are 3 factors considered to be important for tissue engineering.[6] To hasten epithelialization in

the artificial trachea, the artificial trachea has been previously modified with respect to each of these three factors.[10–13] Collagen derived from various animals and fishes is commercially available, and the most common resource of clinically used collagen is cattle. In our country, only cattle-derived atelocollagen is approved for pharmaceutical use. Thus, bovine tissue is the most reliable resource of atelocollagen. Although our previous reports indicate that collagen sponge prepared from porcine atelocollagen is capable to induce tracheal epithelium regeneration[14], it has been reported that collagen sponge scaffolds prepared from porcine and bovine collagen have different structures.[15] Because the structure of the collagen sponge scaffold is known to affect cell behavior, collagen sponges with various structures have been prepared by modifying methods of crosslinking, collagen concentration, and temperature for freezing the collagen solution.[16] However, concentrations of bovine collagen that are optimal for tracheal epithelial regeneration have never been reported.

In this study, we prepared collagen sponges from various concentrations of collagen solutions. The structural differences in the sponges were analyzed using scanning electron microscopy (SEM), and the effects on tracheal epithelial regeneration were examined in an implantation study using a rabbit partial

tracheal defect model.

MATERIALS AND METHODS

Animal Care

All animal studies were performed under the control of the Animal Care and Use Committee in accordance with the Guidelines for Animal Experiments of Fukushima Medical University.

Preparation of bovine collagen sponges

Collagen sponges were prepared from bovine type I atelocollagen (KOKEN, Tokyo, Japan) using a method for porcine collagen sponge preparation described previously. Because the porcine collagen sponge had been conventionally prepared on a sheet of polypropylene mesh to provide adequate mechanical properties for tracheal reconstruction, the bovine collagen sponge was also prepared on a polypropylene mesh sheet. Briefly, atelocollagen was dissolved in a hydrochloric acid solution (pH 3.0), frozen with a polypropylene mesh sheet at −80° C for 16 h, and lyophilized in a laboratory freeze-dryer (FDU-2200; TOKYO RIKAKIKAI, Tokyo, Japan) to form a sponge-like structure. Collagen sponges were prepared

from 0.5, 0.7, and 1.0% bovine atellocollagen solutions (0.5%, 0.7%, and 1.0% sponges).

Analysis of collagen sponge structure by SEM

Collagen sponges were immersed in a 1:9 mixture of 0.5 M Tris–HCl (pH 7.4) and methanol, fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and further fixed with 1% osmium tetroxide (Wako Pure Chemical Industries, Osaka, Japan). After transfer into methanol, the sponges were vertically cut by using a razor blade and a hammer in liquid nitrogen. The samples were transferred to tert-butyl alcohol (Nacalai Tesque, Kyoto, Japan) and freeze-dried. Osmium-coated samples were observed under a SEM (Model SU8220; Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 5 kV. Images of the upper surface and vertical section images were taken.

For quantitative analysis of the thickness of the membranous collagen structure that formed the framework of the lyophilized collagen sponges, 100 vertical section images were randomly taken from each collagen sponge. The thicknesses of >100 membranous structures, which section was clear enough to measure thickness, in each collagen sponge were measured using ImageJ image

analysis software (downloaded from the National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij). Data are shown as the distribution of the thickness measured from the thickest 100 membranous structural areas in each collagen sponge.

Implantation of collagen sponges

The collagen sponges were implanted into Japanese white rabbits (male, 12 weeks of age; Japan SLC, Shizuoka, Japan). The rabbits were anesthetized by intramuscular injection of a cocktail of medetomidine hydrochloride (0.2 mg/kg; Nippon Zenyaku Kogyo, Fukushima, Japan), midazolam (1.0 mg/kg; Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (0.2 mg/kg; Meiji Seika Pharma, Tokyo, Japan). Following preparation of tracheal defects (5 × 10 mm) in the anterior portion of the trachea, the defects were covered with the collagen sponges. Collagen sponges prepared from 0.5%, 0.7%, and 1.0% were implanted into 4 rabbits for each concentration.

Endoscopic observation

At 1 and 2 weeks after implantation, the rabbits were anesthetized as

described above, and the luminal surface of the implanted area was observed by using a bronchoscope (VISERA ELITE; Olympus, Tokyo, Japan).

Histological analysis

At 2 weeks after implantation, the rabbits were euthanized by intramuscular administration of pentobarbital sodium (Kyoritsu Seiyaku Corporation, Tokyo, Japan) under general anesthesia induced as described above, and the trachea was excised en bloc with the sternohyoid and sternothyroid muscles.

The samples were fixed with paraformaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin. They were sliced into 4-μm sections, deparaffinized in xylene, rehydrated through a graded ethanol series, and subjected to hematoxylin and eosin (H&E) staining. H&E-stained sections were observed under a light microscope (BX-51; Olympus). The epithelialized and ciliated areas were evaluated by measuring lengths of newly formed epithelial (*Le*) and non-epithelial tissues (*Ln*), and ciliated region of newly formed epithelial tissue (*Lc*) along the luminal-surface line in the reconstructed area. *Le, Ln* and *Lc* were measured from 4 sections of an individual rabbit ($Le_{1\text{-}4}$, $Ln_{1\text{-}4}$ and $Lc_{1\text{-}4}$,

respectively). The ratio of epithelialized (*Re*) and ciliated (*Rc*) areas in an individual rabbit were calculated according to the following equation:

$$Re = \frac{1}{4}\sum_{n=1}^{4}\frac{Le_n}{Le_n + Ln_n}$$

$$Rc = \frac{1}{4}\sum_{n=1}^{4}\frac{Lc_n}{Le_n + Ln_n}$$

Data are shown as a plot of *Re* and *Rc* values.

Homogeneity of variance and normality of the data was assessed by Levene's and Shapiro-Wilk tests. If the *p*-values of both tests were more than 0.05, Tukey's HSD test was performed. If not, Kruskal-Wallis test was performed. $P < 0.05$ evaluated by Tukey's HSD or Kruskal-Wallis tests was considered significant.

Immunohistological analysis

The following primary antibodies were used for immunohistological analysis: AE1/AE3 mouse monoclonal antibodies against cytokeratin (Dako Japan, Tokyo, Japan), ßIV-tubulin (Sigma-Aldrich, St Louis, MO), and type I collagen (Southern Biotech, Birmingham, AL) for detection of epithelial cells, cilia, and collagen sponges, respectively. ImmPRESS horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G and alkaline phosphatase (AP)-conjugated anti-mouse immunoglobulin G (both from Vector Laboratories, Burlingame, CA)

were used as secondary antibodies.

The deparaffinized sections were incubated with a blocking solution (Dainippon Pharmaceutical Corporation, Osaka, Japan). They were then reacted sequentially with the primary and secondary antibodies for 60 minutes at 37°C. HRP- and AP-conjugated secondary antibodies were detected with 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) and Vector Red (Vector Laboratories), respectively.

SEM analysis for the luminal surface of reconstructed trachea

The paraformaldehyde-fixed tracheae were further fixed with 2% glutaraldehyde. Following post-fixation with 1% osmium tetroxide, the samples were freeze-dried as described above and observed under an SU8220 SEM at an accelerating voltage of 2 kV.

RESULTS

Structure of collagen sponges

The macroscopic appearance of the 0.5%, 0.7%, and 1.0% sponges was same (Fig. 2A). To analyze fine structure, the sponges were vertically cut, and the internal structures of the sponges were observed by SEM (Fig. 2B). All of the sponges had membranous structural frameworks, but many fibrous structures were observed to fill the spaces between the membranous framework in the 0.5% sponge. Increasing collagen concentration tended to make the membranous structures thicker (Fig. 2C). Median thickness of the membranous structure in 0.5, 0.7 and 1.0% sponges were 0.625, 1.795 and 2.134 μm. The membranous structures in the 0.7% sponge branched at many points, which produced many inter-membrane spaces. In contrast, the membranous structures in the 1.0% sponge were relatively continuous and closely arranged. Fibrous structures were observed on the upper surfaces of all sponges, but the upper surfaces of the membranous frameworks tended to show structural properties similar to those of the internal areas.

Endoscopic observation

All of the rabbits implanted with the 0.5%, 0.7%, and 1.0% sponges survived at least 2 weeks. Either severe stenosis or deformity was not observed in the trachea of any groups until the end point of the implantation study.

At 1 week after implantation, the implanted sponges were exposed to the tracheal lumen in all groups (Fig. 3). At 2 weeks after implantation, the tracheal defects were covered with newly organized tissues in 2/4 rabbits in the 0.5% sponge-implanted group and in 3/4 rabbits in the 0.7% group. However, the collagen sponges remained exposed to the tracheal lumen in 4/4 rabbits implanted with the 1.0% sponge. Moreover, 2/4 rabbits in the 1.0% sponge-implanted group exhibited granulation tissues in the luminal surfaces of the tracheae.

Histological and immunohistological analyses

Paraffin-embedded sections were prepared from the tracheal samples at 2 weeks post-implantation. Epithelialization in the center of the reconstructed area was evaluated by observing H&E-stained sections (Fig. 4). The reconstructed area was entirely covered by newly formed epithelia in 3/ 4 rabbits in the 0.7% sponge group. The epithelia were morphologically similar to the pseudo-stratified epithelium, and some cells in the newly formed epithelia had cilia on their apical

region (Table 1). Although the reconstructed area was also epithelialized in 2/4 rabbits in the 0.5% sponge group, the area was covered with the stratified squamous epithelium and cilia were absent. In contrast to the results of the 0.5% and 0.7% sponge-implanted groups, 4/4 rabbits implanted with the 1.0% sponge did not form epithelial tissue in the center of the reconstructed area. The frameworks of the sponges were still exposed to the tracheal lumen in all 4 rabbits in the 1.0% sponge group.

The findings from H&E-stained sections were confirmed by immunostaining for cytokeratin, βIV-tubulin, and type I collagen (Fig. 4). Cytokeratin-positive epithelial cells covered the reconstructed area in 2/4, and 3/4 rabbits in the 0.5% and 0.7% sponge groups but not in any rabbits in the 1.0% sponge group. βIV-tubulin-positive ciliated cells were detected only in the epithelialized area of the 0.7% sponge-implanted rabbits. The frameworks of the collagen sponges, which were intensely stained with anti-type I collagen antibody, were exposed to the tracheal lumen in the 1.0% sponge group. Faint staining for type I collagen was detected in sub-epithelial tissues formed in the 0.5% and 0.7% sponge-implanted groups, probably because of the synthesis of type I collagen by mesenchymal cells.

Analysis of epithelialized and ciliated areas

To quantify the effect of the collagen sponges on epithelial regeneration, the ratio of epithelialized and ciliated areas in the H&E-stained sections was measured. The reconstructed area in 2/4 rabbits in the 0.5% sponge group and 3/4 rabbits in the 0.7% sponge group were fully covered by epithelial cells (Fig. 5A). However, not even half of the reconstructed area was epithelialized in any of the 4 rabbits in the 1.0% group. When multiple comparison by Kruskal-Wallis test was performed differences among the groups were statistically significant. Over one-half of the reconstructed area were covered with ciliated cells in 3/4 rabbits in the 0.7% sponge group, but only in 1/4 and 0/4 rabbits in the 0.5 and 1.0% sponge groups (Fig. 5B). Mean ciliated area in 0.7% sponge group was significantly large compared to that in 1.0% sponge group（Tukey's HSD test）.

SEM analysis of the luminal surface of the reconstructed trachea

The tracheal samples obtained from each rabbit in the 0.5%, 0.7%, and 1.0% sponge-implanted groups were analyzed by SEM (Fig. 6). Cells covering the luminal surface of the reconstructed area in the 0.5% and 0.7% sponge groups were

tightly arranged, and some of the cells in the 0.7% sponge group had cilia. However, the cells were sparsely distributed in the 1.0% sponge group, and the membranous structures of the collagen sponge were observed in the intercellular spaces.

DISCUSSION

A graft ideal for tracheal tissue engineering is believed to require diverse characteristics, such as biocompatibility, liquid- and air-tightness, non-toxicity, non-carcinogenicity, supportive capacities for cell engraftment and neovascularization, reasonable mechanical strength to avoid collapse, non-immunogenicity, and minimal inflammatory response.[2,4]

Atelocollagen-based materials are widely used for tissue engineering because of their biological safety. Atelocollagen barely stimulates host immunogenic and inflammatory responses, because telopeptide, a major site that stimulates immune and inflammatory responses in native collagen, are enzymatically removed in atelocollagen.[17] Bovine tissues are the most reliable atelocollagen resource for clinical use among candidate resources of atelocollagen. Scaffolds prepared from bovine atelocollagen have been clinically used to repair dermal, mucosal, and nerve defects. Moreover, the bovine atelocollagen scaffolds are biocompatibile and supportive for cell engraftment and neovascularization. These scaffolds induce tissue reorganization and re-innervation by allowing cell invasion and growth.[18] Thus, clinical applications of bovine atelocollagen for tracheal tissue engineering may soon be available. The safety of polypropylene mesh has also been

confirmed by various clinical studies. Therefore, an artificial trachea made using bovine atelocollagen and polypropylene mesh is considered compatible with many of the requirements for an ideal tracheal graft.

Modification of the scaffold structure is one of the strategies used to improve the regenerative effects of grafts.[4,18,19] However, bovine collagen-based materials have hardly been used in tracheal reconstruction studies. There are no published reports describing the proper structure of a bovine collagen-based scaffold for tracheal epithelium regeneration. Our previous studies using porcine collagen sponges have shown that epithelialization in the reconstructed trachea initially required sub-epithelial tissue formation, which is achieved by mesenchymal cell invasion into the collagen sponge.[10,20,21] It is well accepted that degrees of cell migration and sprouting of blood vessels into 3D porous scaffolds depend on the pore size of the scaffolds. [22-24] Although the pore size most suitable for sub-epithelial tissue formation and angiogenesis in tracheal mucosa is not known, many reports have suggested that 3D scaffolds enriched with >100-μm pore diameters are beneficial for cell invasion.[25-30] In our study, it was difficult to measure the pore sizes of the bovine collagen sponges, but the inter-membrane spaces in the 0.7% sponge appeared to be larger than those in the 0.5% and 1.0%

sponges. Therefore, it may be possible that cells migrate more easily into the 0.7% sponges than in the 0.5% and 1.0% sponges. Reorganization of the provisional matrix provided by 3D scaffolds is also important for tissue regeneration.[31,32] Membranous structures in the 1.0% sponges tended to be thicker than those in the 0.5% and 0.7% sponges. Moreover, histological and SEM analyses revealed that the collagen frameworks of the 1.0% sponges remained undigested >2 weeks post-implantation. Taken together, these results suggest that cell migration and tissue reorganization in 1.0% sponge are restricted by closely arranged and poorly degradable thick collagen membranes.

Generation of ciliated cells in the center of the epithelialized region was detected only in the 0.7% sponge group, which indicated that the 0.7% sponges provided the best provisional matrix for tracheal epithelium regeneration among the three types of sponge. Nakaegawa *et al.* reported that implantation of collagen sponge prepared from 1.0% porcine atelocollagen solution made a similar effect on epithelialization and ciliogenesis.[32] The porcine atelocollagen constituted with approximately 70% of type I collagen and 30% of type III collagen, while bovine atelocollagen solution used in the present study contained approximately 90% type I collagen and 10% type III collagen. The porcine atelocollagen-derived sponge was

abundant in porous structure, which was not observed in the sponge prepared from 0.7% bovine atelocollagen, but both of the collagen sponges shared a similarity in abundance of inter-membrane spaces. However, in this study, the physical properties of the sponges could not be precisely analyzed. Further research is required to understand the properties suitable for inducing biological mechanisms during tracheal epithelium regeneration.

CONCLUSION

This study demonstrated that a tracheal graft could be prepared using bovine collagen sponge and polypropylene mesh. Collagen sponges prepared from various concentrations of bovine atelocollagen yielded different structures and effects on tracheal epithelium regeneration. Our results showed that coverage from tracheal epithelium regeneration was complete in a greater number of sponges prepared using a 0.7% bovine collagen concentration than in sponges prepared using 0.5% and 1% concentrations. Ciliogenesis in the center of the epithelialized region was detected only in the 0.7% sponge group.

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Figure Legends

Fig. 1. Procedures for implantation of collagen sponges. Collagen sponges were prepared from 0.5%, 0.7%, and 1.0% collagen solutions on polypropylene mesh sheets (A). Shown are tracheal defects, 5-mm wide and 10-mm long, that were prepared in the rabbit tracheae and covered with the collagen sponges (B).

Fig. 2. Structures of the 0.5%, 0.7%, and 1.0% sponges. Macroscopic images of the sponges taken from above (A). SEM images of the vertical sections and upper surfaces of the sponges (B). Bar: 200 μm. Arrows indicate membranous structure (yellow), fibrous structure (blue), inter-membrane space (red). Shown are examples of the thicknesses of >100 membranous structural areas in each collagen sponge that were measured (C). Data are shown as the distribution of the thickness measured from the thickest 100 membranous structures in each collagen sponge.

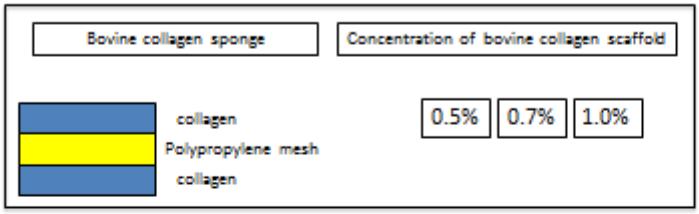
Fig. 3. Endoscopic images of the reconstructed trachea at 1 and 2 weeks post-implantation. Representative images obtained from the 4 rabbits in each of the 0.5%, 0.7%, and 1.0% sponge groups are shown.

Fig. 4. Histological examination for epithelial regeneration at 2 weeks after implantation. Shown are paraffin-embedded sections prepared using 2-week post-implantation tracheal samples that were subjected to H&E-staining (1st and 2nd lining) or immunostaining for cytokeratin (red, 3rd lining), βIV-tubulin (red, 4th lining), and type I collagen (brown, 5th lining). The nuclei were counterstained with hematoxylin in the immunostained sections. Bar: 1 mm (1st lining), 20 μm (2–5th lining). Dotted line indicates implanted region. Representative images obtained from the 4 rabbits in each of the 0.5%, 0.7%, and 1.0% sponge groups are shown.

Fig. 5. The ratio of epithelialized and ciliated area in the reconstructed trachea at 2 weeks after implantation. Data plots of the ratio of regenerated epithelia (A) and cilia (B) in the 0.5%, 0.7%, and 1.0% sponge groups are shown ($n = 4$). Asterisk indicates significant difference between groups ($p < 0.05$).

Fig. 6. SEM images of the reconstructed trachea at 2 weeks after implantation. Luminal surfaces of the reconstructed tracheae in the 0.5%, 0.7%, and 1.0 % sponge groups are shown. Bar: 10 μm (top),2 μm (bottom).Figure 1

A


Bovine collagen sponge
Concentration of bovine collagen scaffold
collagen
Polypropylene mesh
collagen
0.5%
0.7%
1.0%

B

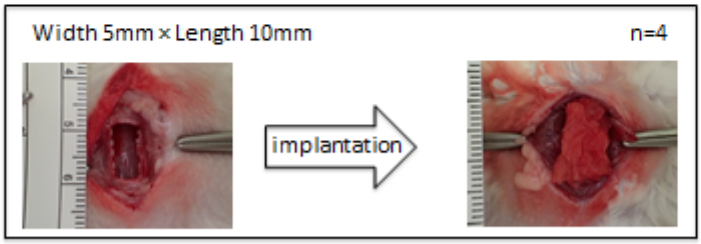

Width 5mm × Length 10mm
n=4
implantation

Figure 2

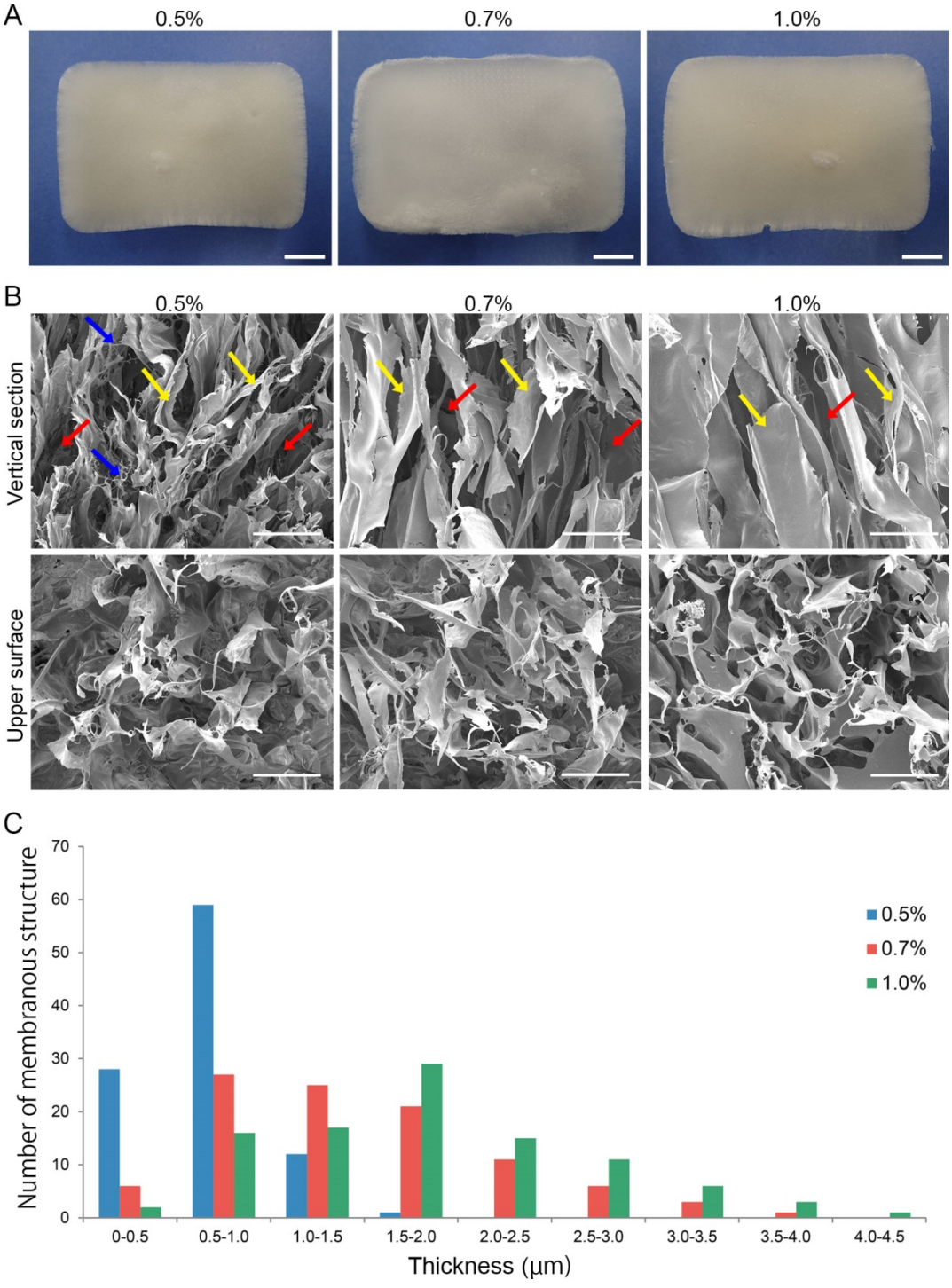

A
0.5%
0.7%
1.0%
B
0.5%
0.7%
1.0%
Vertical section
Upper surface
C
Number of membranous structure
70
60
50
40
30
20
10
0
0.5%
0.7%
1.0%
0-0.5
0.5-1.0
1.0-1.5
1.5-2.0
2.0-2.5
2.5-3.0
3.0-3.5
3.5-4.0
4.0-4.5
Thickness (μm)

Figure 3

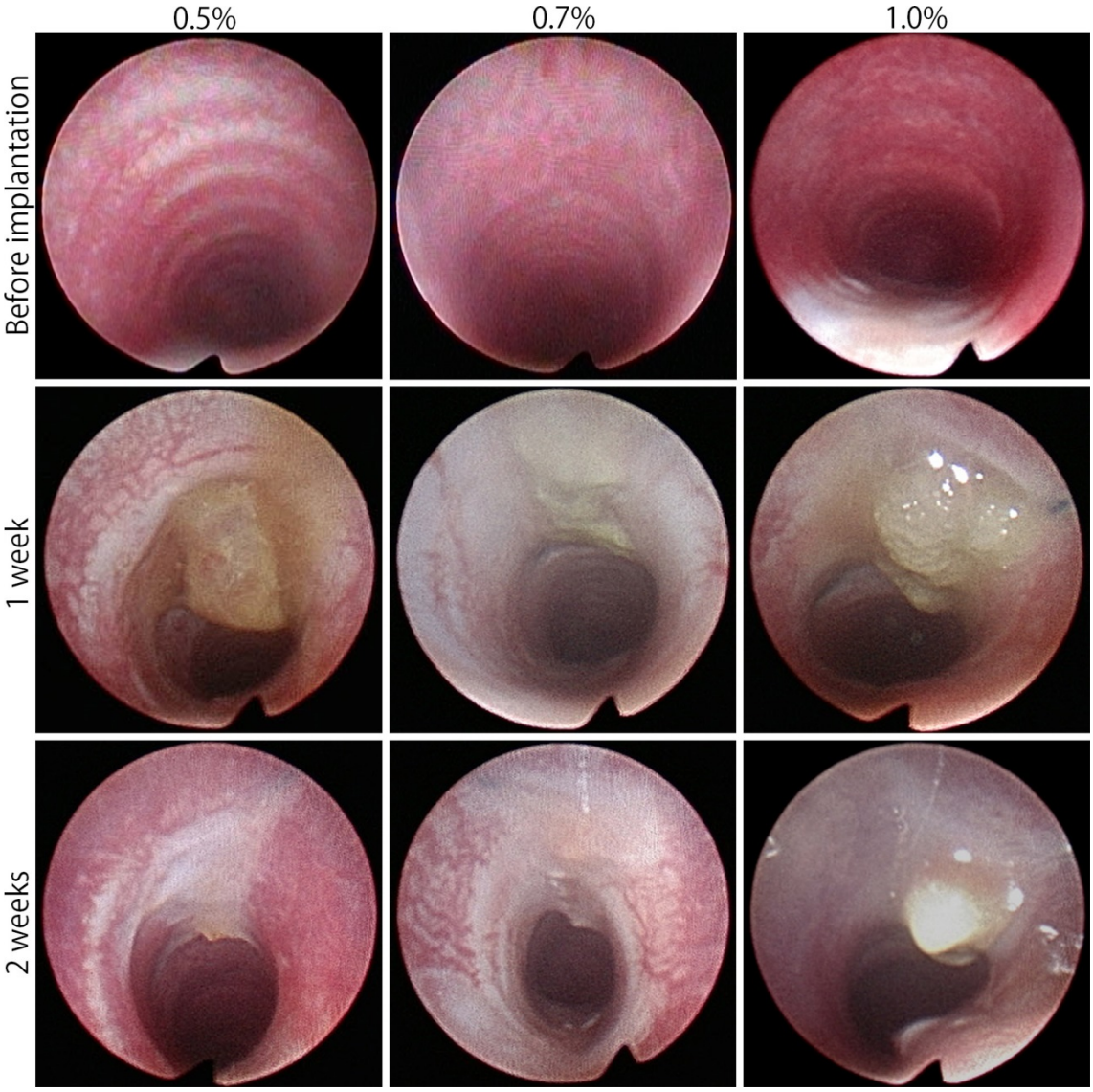


Figure 4

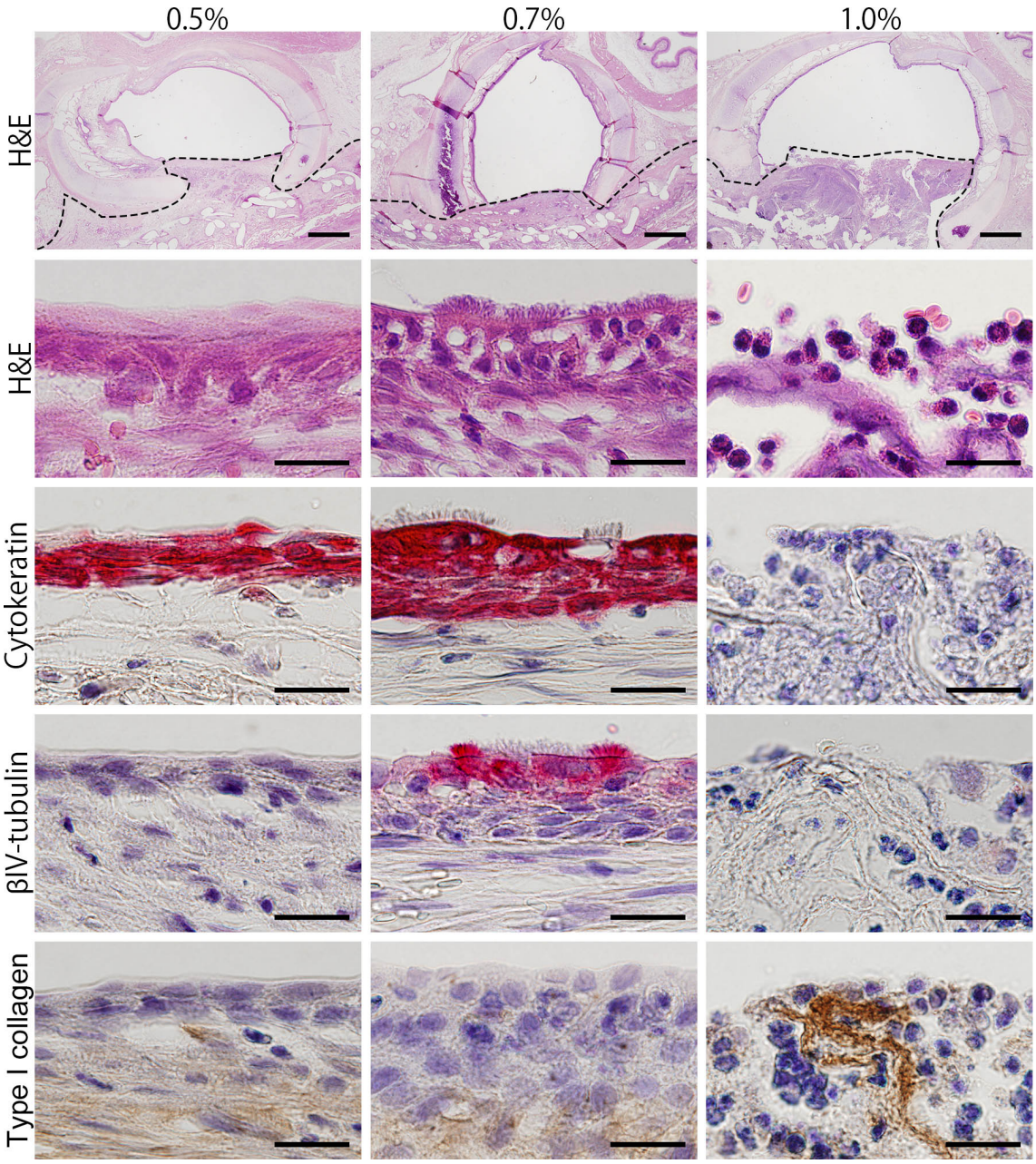


Figure 5

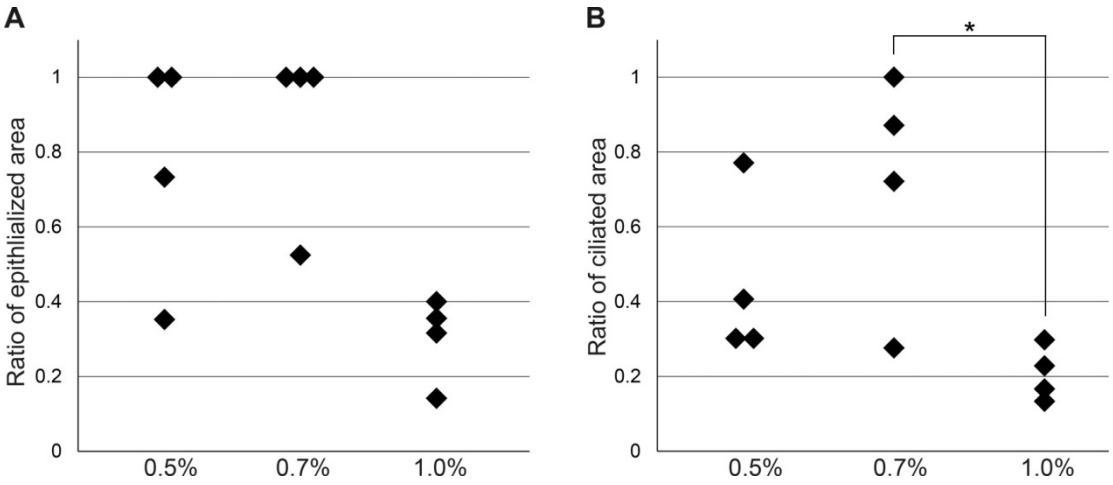

A
Ratio of epithlialized area
1
0.8
0.6
0.4
0.2
0
0.5%
0.7%
1.0%
B
Ratio of ciliated area
*
1
0.8
0.6
0.4
0.2
0
0.5%
0.7%
1.0%

Figure 6

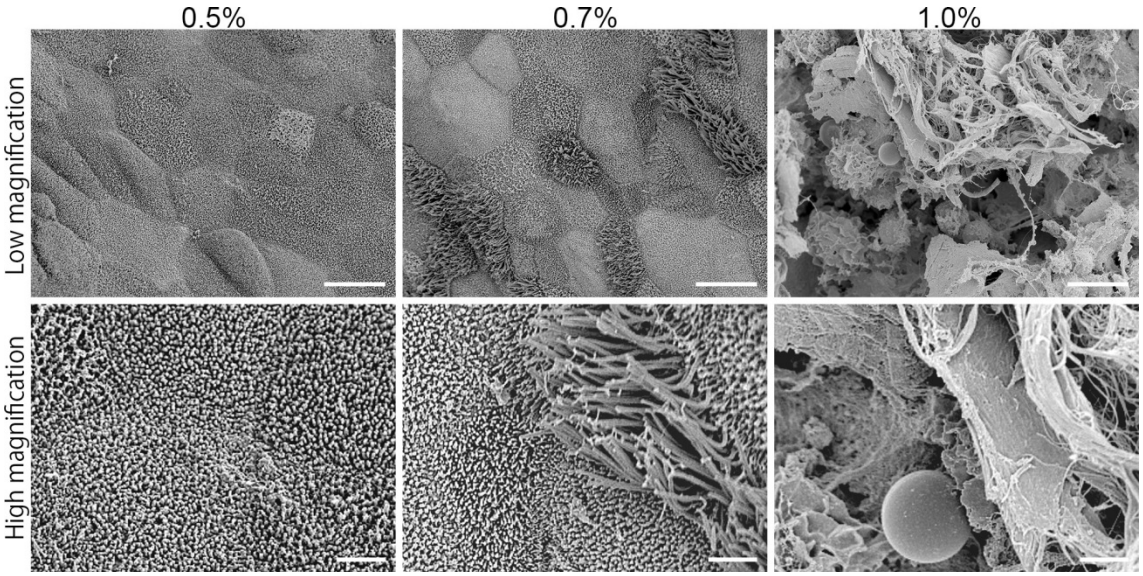


Table 1: Ciliogenesis in the center of reconstructed trachea at 2 weeks after collagen sponge implantation.

	0.5%	0.7%	1.0%
#1	−	+	−
#2	−	+	−
#3	−	−	−
#4	−	+	−

+: With cilia, −: Without cilia.