Formation and evaluation of tissue-engineered tracheal cartilage using chondrocytes and biocompatible composite scaffolds in rabbits

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Formation and evaluation of tissue-engineered tracheal cartilage using chondrocytes and biocompatible composite scaffolds in rabbits

Directed by Professor Eun Chang Choi

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Formation and evaluation of tissue-engineered tracheal cartilage using chondrocytes and biocompatible composite scaffolds in rabbit

Directed by Professor Eun Chang Choi

This thesis consists of two parts as followings.

Part I:

Transplantation of Autologous Chondrocytes Seeded on a Fibrin/Hyaluronate Composite Gel into Tracheal Cartilage Defects in Rabbits: Preliminary Results

Part II:

Formation and evaluation of tissue-engineered tracheal cartilage using autologous chondrocytes and Poly-lactic-co-glycolic acid composite in rabbits

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ABSTRACT

Transplantation of Autologous Chondrocytes Seeded on a Fibrin/Hyaluronate Composite Gel into Tracheal Cartilage Defects in Rabbits: Preliminary Results

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(Directed by Professor Eun Chang Choi)

Reconstruction of tracheal defects is one of the most difficult procedures in head and neck surgery. To date, various reconstructing techniques have been used with no consensus on the best approach. This study investigated the feasibility of using a fibrin/hyaluronic acid (HA) composite gel with autologous chondrocytes for tracheal reconstruction. Chondrocytes from autologous rabbit auricular cartilages were expanded and seeded into a culture dish at high density to form stable tracheal cartilages mechanically using a fibrin/HA composite gel. A 1.0cm long by 0.5cm wide defect was created by a scalpel on the cervical tracheae of six rabbits. Tissue-engineered cartilages using fibrin/HA composite were trimmed and fixed to the defect boundaries with tissuecol. Postoperatively, the site was evaluated endoscopically, histologically, radiologically, and functionally. None of the six rabbits showed signs of respiratory distress. Postoperatively, in all cases, rigid telescopic examination showed that the implanted scaffolds were completely covered with regenerated mucosa without granulation or stenosis.

Histologically, the grafts showed no signs of inflammatory reaction and were covered with ciliated epithelium. Even when grafts were broken and migrated from their original insertion site, the implanted cartilages were well preserved. However, the grafts did show signs of mechanical failure at the implantation site. The beat frequency of ciliated epithelium on implants was very similar to that of normal respiratory mucosa. In conclusion, implants with autologous chondrocytes cultured with fibrin/HA showed good tracheal luminal contour, functional epithelial regeneration, and preservation of neo-cartilage without inflammation but lacked adequate mechanical stability.

Key words: tracheal reconstruction, tissue engineering, autologous chondrocyte, tracheal epithelialization, ciliary function

Part I:

Transplantation of Autologous Chondrocytes Seeded on a Fibrin/Hyaluronate Composite Gel into Tracheal Cartilage Defects in Rabbits: Preliminary Results

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I. INTRODUCTION

Tracheal defects, such as tracheal stricture and tracheomalacia, are related to human morbidity. Every year in the USA, approximately 150,000 patients experience complications associated with endotracheal intubation and mechanical ventilation, particularly patients with brain or spinal cord injuries requiring long-term intubation and ventilator support.[1] Chronic airway strictures show high mortality rates of 11 to 24% and pose significant clinical challenges. Other tracheal lesions such as tumors or trauma may also require tracheal resection with primary reconstruction.[2] Although the total resectable length is restricted to 30% of the trachea in children or to 6 cm in adults, replacement of longer sections will be practical with the development of reliable, functional tracheal replacements.

Various approaches for tracheal replacement have been described, including the use of autologous tissues,[3] auto-grafts,[4] allografts,[5] prosthetic materials,[6] or a combination of these approaches.[7] However, all of these approaches and

techniques carry risks and complications, such as immune rejection, poor graft integration, graft ischemia, or rejection, resulting in limited clinical use.[2]

Recently, regenerative medicine has been considered as a useful clinical discipline that ensures and enhances the quality of life in patients under- going organ reconstruction. Tissue bioengineering has already provided functional human organ replacements in various other fields.[8] However, possible impediments should be resolved to make tracheal tissue engineering successful. Factors contributing to unsatisfactory outcomes include the following: lack of ciliated epithelium, which contribute to auto-purification; infection or foreign body reaction; immunological complications; material failure; and repeated surgical interventions, including tracheostomy.

Along with the synthetic scaffolds, many naturally derived scaffolds have been developed and examined, both in vitro and/or in vivo, including hyaluronate (HA),[9,10] fibrin,[11] collagen,[12] alginate,[13] and chitosan scaffolds.[14] Recently, a combination of two or more biomaterials is commonly used to increase the benefits of each biomaterial. In a previous study, we examined the potential of fibrin/HA gel as a promising composite biomaterial to build a hyaline-like cartilage construction[15] and we demonstrated the replacement of the resected rabbit trachea with an allograft implant using a fibrin/hyaluronate composite gel.[16] Fibrin glue possesses several important features including biocompatibility and biodegradability it is fibronectin-rich and has high affinity for biological tissues. Fibronectin is an essential protein in the cartilage matrix for chondrocytes–extracellular matrix (ECM) interaction; however, the weak mechanical properties and early degradation of fibrin have been problematic [17]. HA is considered as a key component in the cartilage ECM with high hydrophilicity and molecular weight. HA binds to aggrecan, an assembly of large aggregating proteoglycans, and to the chondrocyte surface receptor cluster of differentiation 44.[18] It is believed that mutual interactions between chondrocytes, ECM molecules, and HA are critical in maintaining cartilage homeostasis.[19]

Fibrin/HA composite provide a favorable environment for chondrocytes to maintain their characteristic phenotype and synthesize cartilage ECMs as compared with fibrin only.[15]

However, in our previous study[15,16] one of the main limitations of this approach was absorption of implanted cartilages.[16]

We hypothesized that autologous tissue engineered implants have better biocompatibility and structure, supporting in vivo cartilage formation with specialized shapes. In this preliminary study to develop autologous tissue-engineered circumferential tracheal segments, we performed endoscopic, radiological, and histological characterizations of autologous tissue-engineered implants for tracheal defect repair in animal experiments using auricular chondrocytes cultured with fibrin/HA. We also studied the ciliary beat frequency (CBF) of the ciliated epithelium regenerated on the implants as functional evaluation of the reconstructed airway.

II. MATERIALS AND METHODS

1. Isolation of chondrocytes

Chondrocytes were isolated from the rabbit auricular cartilages (Figure 1A). All isolation and culture of chondrocytes were performed as described previously.[16] In brief, female rabbits, weighing about 250 g, were euthanized by overdose injection of Nembutal, and the knee cartilages were removed by sterile dissection. Pieces of cartilage were finely minced and washed with phosphate buffered saline (PBS). They were then digested in 0.2% (w/v) collagenase (Worthington Biochemical, Lakewood, NY) in PBS for 5 hours at 37℃. Using a 70 lm nylon cell strainer (Falcon, Franklin Lake, NJ), the cells were filtered, pooled, and centrifuged at 1200 rpm for 10 minutes. After washing twice with PBS, the cell pellet was re-suspended in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS)

(Gibco BRL), 100 U/mL penicillin G (Gibco BRL) and 100 μg/mL streptomycin (Gibco BRL). The cells were then plated at a density of 1.5 x 10^5 cells/cm^2 and placed at 37℃ in a 5% CO_2 incubator. The culture medium was changed every other day. The primary chondrocytes were passaged twice before the experiments (Figure 1B). This study was approved by the Committee for Ethics in Animal Experiments of Ajou University School of Medicine.

2. Preparation of fibrin/HA composite gel

To prepare fibrin/HA composite gel, chondrocytes were pelleted by centrifugation and then re-suspended in a solution containing both fibrinogen (9 mg/mL, Mokam Research Center, Suwon, Korea) and HA (molecular size of 3000 kDa; 10 mg/mL, LG Chemical Research Institute, Daejeon, Korea). The mixing ratio of fibrinogen to HA was 10:1 by volume. Chondrocyte suspension of 5 x 10^6 cells/mL was then mixed homogeneously with aprotinin (Mokam Research Center), 60 U/mL thrombin (1000U/mg protein: Sigma, St. Louis, MO, USA), the fibrin stabilizing Factor XIII (Green Cross, Young In, Korea), and 50mM $CaCl_2$ (Green Cross). Then, 250μL of the fibrin/HA mixture was dropped into an empty Petri dish to form a gel. Viable cells in the fibrin/HA composite were screened using a live/dead kit (Molecular Probes, Eugene, OR, USA). Specimens were stored for up to 4 weeks in the medium prior to in vitro examination and implantation (Figure 1C and 2).

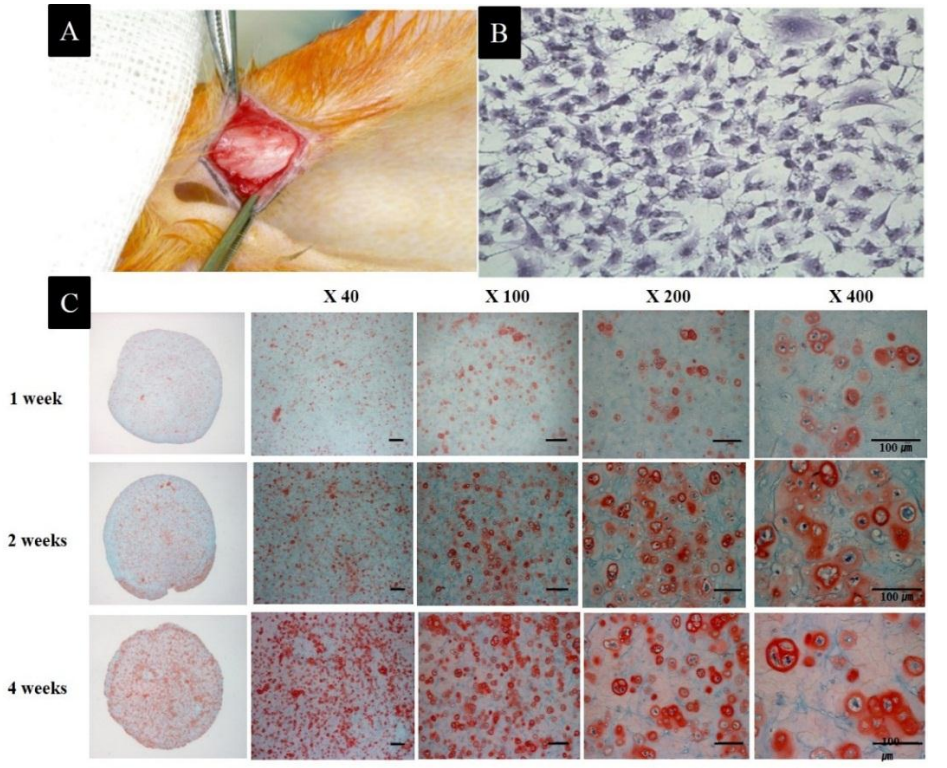

Figure 1. Isolation of chondrocytes and morphological findings of tissue-engineered cartilage using a fibrin/hyaluronate composite gel as observed at different time points. (A) Autologous chondrocytes were isolated from rabbit auricular cartilages. Pieces of cartilages were finely minced and the cells were then plated at a density of 1.5 x 10^5 cells/cm^2 as a monolayer culture and placed in a 5% CO_2 incubator at 37℃. (B) The primary chondrocytes were passaged twice before experiments. In Safranin O, the proteoglycan-rich matrix was stained in red. (C) Photographs of the stained slides taken at the first, second, and fourth week showed increased staining with time, indicating the relationship between proteoglycans in the extracellular matrix and the time period.

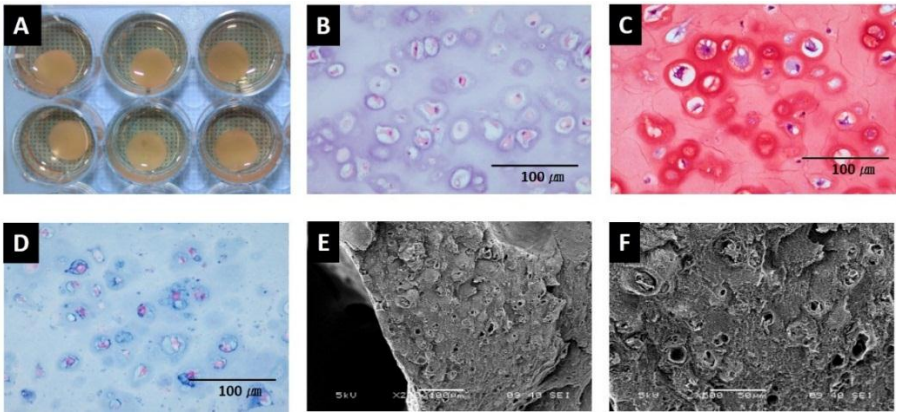

Figure 2. Histology and SEM images of tissue-engineered cartilages using a fibrin/hyaluronate composite gel as seen at 4 weeks. (A) A three-dimensional culture of chondrocytes embedded in the fibrin/hyaluronate composite matrix. (B) H&E staining showed that engineered tissues were composed of evenly spaced lacunae cells embedded in a basophilic matrix (bar scale: 100 mm). (C) The extracellular region was strongly stained by the characteristic red of safranin O. (D) Alcian blue staining confirmed the observations of H&E staining. (E, F) SEM images of implants reflected the surface characteristics of the fibrin/hyaluronate composite with chondrocytes.

3. Animals and surgical procedures

Under anesthesia by tiletamine (4.0 mg/kg; Virbac, Ltd., Carros, France) and zolazepam (4.0 mg/kg; Virbac, Ltd.), tracheal defects, approximately 5 mm wide by 10 mm long, were made using a scalpel (Figure 3A). The autologous cartilage with a fibrin/HA scaffold was trimmed and inserted into the resected site (Figure 3B and C). Finally, it was fixed and sealed to the defected boundaries with tissuecol (fibrinogen 110 mg+ thrombin 500 international unit (IU) + aprotinin 3000 KIU, DaLim Co., Seoul, Korea). Both sides of the split sternohyoid and sternothyroid muscles were then replaced over the graft and sutured. Finally, the incised skin was sutured. Detail surgical procedures were performed as described previously.[16]

Animal care, housing, and experimental procedures were conducted according to the Guidelines for Animal Experiments of Ajou University. Tardomyocel comp (benzathine penicillin G [100,000 IU], procaine penicillin G [25,000 IU], streptomycin [125,000 IU]); (Bayer Korea Ltd., Seoul, South Korea) were administered (0.1 ml/kg; subcutaneous injection) for 5 days to prevent postoperative infections.[16]

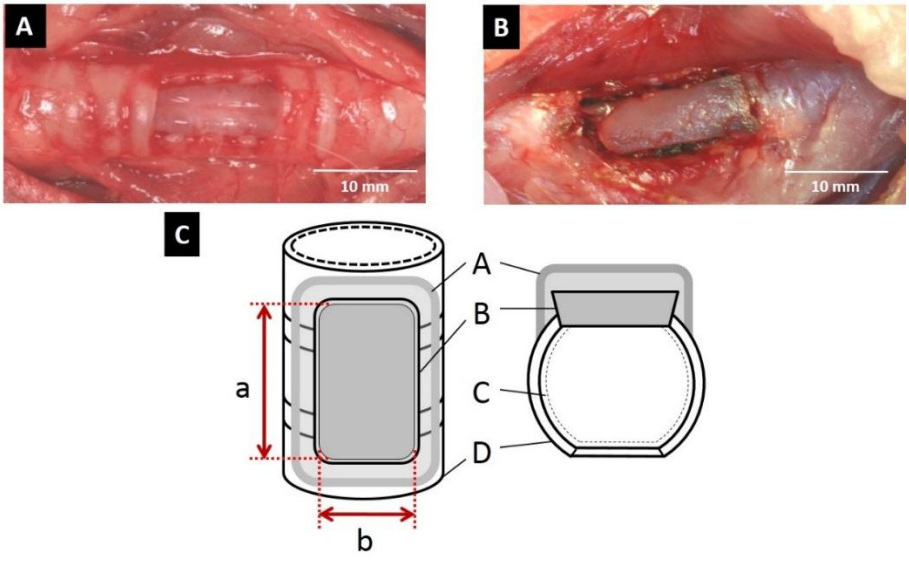

Figure 3. Implantation of tissue-engineered cartilages using a fibrin/hyaluronate composite gel. Under anesthesia, the cervical tracheae were exposed through a vertical skin incision and splitting of the sternohyoid and sternothyroid muscles along the median line. (A) Tracheal defects, approximately 5 mm wide by 10 mm long, were formed by a scalpel. An allograft cartilage with a fibrin/HA scaffold was trimmed and inserted edge to edge to the resected site instead of overlaying. (B) It was fixed and sealed to the defected boundaries with tissuecol. (C) A schematic of the technique described in this study. A: tissuecol, B: implant, C: mucosa, D: cartilage, a: length of implant, b: width of implant.

4. Endoscopic and radiological assessment

To monitor the progress of tracheal regeneration, endoscopic examinations were carried out periodically with an endoscopy system composed of a rigid endoscope (0 °, diameter 4 mm, Karl Storz, Tuttlingen, Germany) and a camera (Coolpix 3500, Nikon Co., Tokyo, Japan) with a light source (Head-light unit, Karl Storz). Anesthesia was inducted with tiletamine (4.0 mg/kg) and zolazepam (4.0 mg/kg) to facilitate these examinations.

A three-dimensional image of the rabbit's trachea was obtained using computed tomography (CT) performed on the operation site using a CT scanner system (Brilliance 64, Philips, Eindhoven, The Netherlands) 1, 3, and 6 months postoperatively.

5. Histology and scanning electron microscope (SEM)

Histological assessments including light microscopy and scanning electron microscopy were also performed to evaluate the regenerative status of the operated site at 3 and 6 months postoperatively.

After fixing with 10% formalin for 24 hours, the implanted samples were embedded in paraffin and sectioned in 5 lm thickness. The sections were stained with hematoxylin and eosin (H&E) for cell morphology. Both safranin O/fast green and alcian blue were used to locate the glycosaminoglycan (GAG) deposits in the specimens. The surface morphology of implant was examined using SEM (Hitachi S-800; Hitachi, Tokyo, Japan). The samples were fixed with 0.4% glutaraldehyde for 24 hours and then coated with gold/palladium for 20 minutes before SEM observation. Detail histology and SEM procedures were performed as described previously.[16]

6. Measurement of tracheal ciliary beating frequency (CBF)

Tracheal ring explants (1–2 mm thick) were prepared from the trachea, and measurement of tracheal CBF was performed as described previously.[16] Tracheal

ring explants (1–2-mm thick) were prepared from the trachea, and the trachea was washed with DMEM/F12 medium (DMEM/Ham's F-12; Gibco BRL), containing 10,000 Units/mL penicillin, 10,000μg/mL streptomycin (Gibco BRL) and kept in the medium. Briefly, actively beating ciliated cells were maintained at 24 ±0.5℃ by a thermostatically controlled heated stage and digitally analyzed using inverted microscope (Axiovert 40CLF; Carl Zeiss, Inc., Thornwood, NY) and high-speed digital camera (Moticam 2000; Motic Ltd., Causeway Bay, Hong Kong). Whole-field analysis was performed using software (Ammons Engineering, Mt. Morris, MI) that automatically analyzed the entire captured image of all ciliated cells in a given field. Amplitudes and frequencies of all cilia were collated, mapped to the screen image, and statistically analyzed to determine the frequency average and standard deviation (SD) of the entire image. Predominant frequencies of all motile cilia were sampled in at least six separate fields. Thus, all frequencies were expressed as mean ±SD from six separate fields.

7. Statistical analysis

Parameters of the data from at least six independent experiments were expressed as mean ± SD. Comparisons of the means of different groups were made using one-way analysis of variance (ANOVA). Statistical significance was set at $P<0.05$.

III. RESULTS

1. Assessment of in vitro culture of autologous chondrocytes with fibrin/HA composite

As shown in Figure 1C, chondrocytes kept their round shape and preserved their attachment on the fibrin/HA matrix. Among the whole cell population in vitro, more than 90% were viable cells in the fibrin/ HA composite at week 1. The cells

were homogeneously distributed in the matrix and exhibited round morphology along lacunae. The extracellular region was strongly stained in safranin O (red), indicating the presence of the proteoglycan-rich matrix (Figure 1C). At week 4, the accumulated glycosaminoglycan at the core region of the fibrin/HA composite in vitro was more significant than that at week 1. At 4 weeks, histological features of the tracheal implant hematoxylin and eosin (H&E) staining revealed a nearly normal hyaline cartilage (Figure 2A and B). Similar to that of a native trachea, the staining showed the presence of a mature cartilage surrounded by connective tissues. With safranin O staining, the tissue-engineered cartilage was organized into lobules with round, angular lacunae, each containing a single chondrocyte (Figure 2C). In addition, alcian blue showed intense staining of the entire matrix at 4 weeks, confirming the aforementioned observations (Figure 2D). In SEM, a large amount of the ECM produced by the engineered cartilage was observed. The HA fibers were found embedded in and distributed through the entire matrix (Figure 2E, F). In most areas, morphology and structure of the engineered cartilage were similar to those of the native cartilage.

2. Assessment of in vivo implants using autologous chondrocytes cultured with fibrin/HA

In total, six rabbits underwent the process of tracheal reconstruction with tissue-engineered cartilages. During recovery or postoperative period, none of the rabbits showed any signs of stridor or difficulty in breathing. All of the animals survived through the operation and no wound infection or signs of inflammation at the surgical sites were noted.

Telescopic findings under anesthesia showed that all six rabbits showed a complete recovery with no stenosis or granulation in the regenerated trachea (Figure 4A, B). Figure 4 shows the telescopic images of the rabbits with typical luminal portrayal of their successfully reconstructed trachea 1 month and 6 months after the operation. No obvious tracheal stenosis was observed on the CT

images at two different observational periods. Six months after surgery, axial CT images at the operated sites revealed a fine luminal portrayal of the reconstructed trachea (Figure 4C) and definite cartilage signal was detected in the three-dimensional images reconstructed from fine axial images (Figure 4D).

Gross assessments showed that all implants appeared to be healthy and integrated, and the luminal shape was comparable in size with that of a native rabbit trachea (Figure 5A and B). However, all grafts underwent partial breaking and migration from their original insertion site into the tracheal defect (Figure 5B and C). Histological assessments indicated that the cartilages closely resembled native tracheal cartilages with similar overall morphology (Figure 5C and D). H&E staining revealed the presence of mature chondrocytes and matrix surrounded by connective tissues as expected in native trachea. Three months after surgery, the epithelial lining was already complete at the operated site, and the concentration of cilia was nearly normal (Figure 5C and D). SEM images showed completely regenerated cilia on the external surface and lacunae cells, matrix, and fibrous tissues on the cross- sectional view (Figure 5E–H).

When normal tracheal mucosa was compared with the regenerated mucosa, the CBF of the regenerated ciliated epithelium was similar to the normal control data with no statistically significant difference of CBF between a normal trachea and implanted trachea (Figure 6A and B) ($P = 0.239$ by one-way ANOVA: control vs. implanted trachea at postoperative 3 months: $P = 0.169$; control vs. implanted trachea at postoperative 6 months: $P = 0.868$; postoperative 3 months [of implanted trachea] vs. postoperative 6 months: $P = 0.127$).

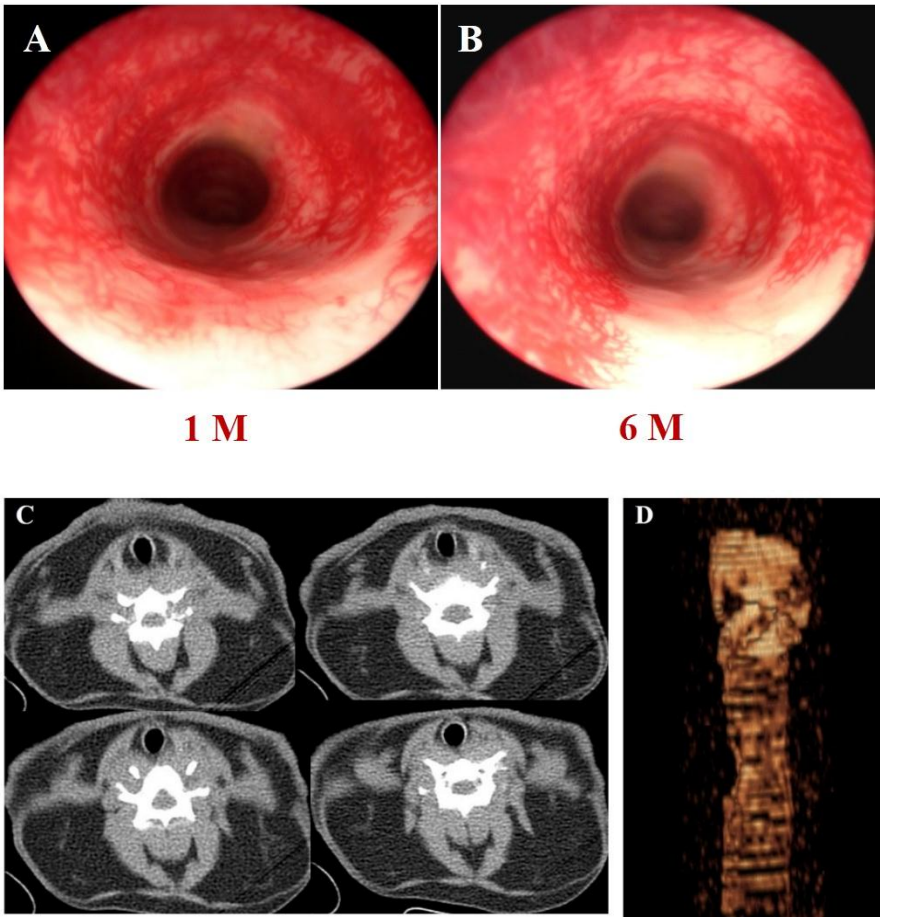

Figure 4. Findings of endoscopy and computed tomography of the rabbit trachea. The implant was a good fit on the tracheal defect in the rabbit. In images taken (A) 1 month and (B) 6 months after the operation, the implant was completely covered with regenerated mucosa without any granulation or displacement. Six months after surgery, axial CT images at the operated sites revealed a fine luminal portrayal of the (C) reconstructed trachea and (D) cartilage signal was clearly detected in the three-dimensional images reconstructed from fine axial images**.**

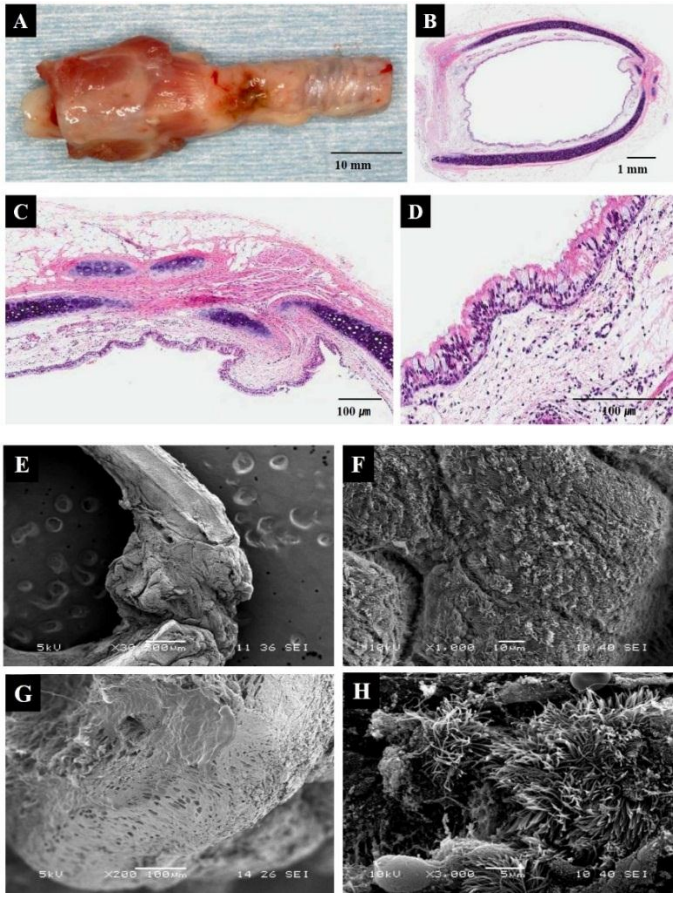

Figure 5. Histological evaluation and scanning electron microscopic images of tissue-engineered tracheal construction 6 months after surgery. Laryngotracheal complex, all grafts underwent partial buckling, breaking, and migration from their original insertion site into the laryngofissure. (C, D) The luminal surface layered with connective tissues is completely covered with epithelial cells and pseudostratified ciliated columnar epithelium (i.e., normal respiratory mucosa) is clearly observed. (E) Gross images of the implanted trachea and the adjacent normal trachea in scanning electron microscopic images. (F) The cross- section image of lacunae cells and fibrous tissues and (G, H) the scanning electron microscopic images show completely regenerated cilia on the external surface.

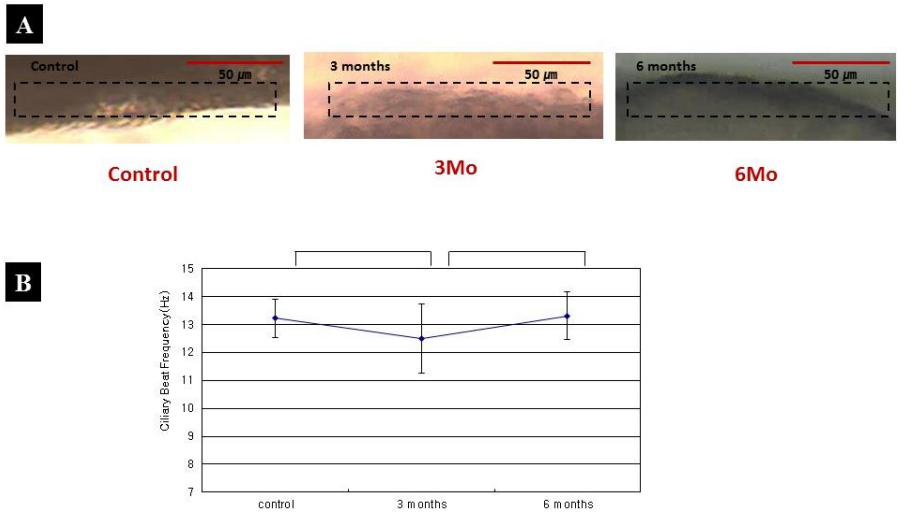

Figure 6. Measurement of trachea ciliary beat frequency (CBF). (A) Computerized spectrum analysis of CBF performed on the normal tracheal and the implanted trachea. (B) CBF on the implants was very similar to that of normal respiratory mucosa and there was no statistically significant difference in CBF between the normal trachea and the implanted trachea (P <0.05).

IV. DISCUSSION

A variety of materials have been used in tracheal prostheses, such as various inert materials alone[6,20] or in combination with autologous tissues, [7,21] but these methods did not show satisfactory results. There is, however, a general agreement that autologous tissues are the first choice in reconstructive surgery, particularly in tracheal surgery. Several reports have described experimental tracheal reconstruction with auto-transplants, but none of them has proven to be satisfactory for human use. However, this procedure is complicated by the need for a long term immunosuppressant therapy and a lack of sufficient donor tissue. Therefore, reconstruction of cartilage tissues used by tissue engineering these

days has become one of the most popular and prospective research themes due to the fact that chondrocytes rely on the permeation of tissue fluid for nutrition supply, thus exempting cartilage tissues from revascularization. Due to their low antigenic characteristics, allogeneic chondrocytes can be used to repair cartilage tissue defects.[22,23]

In our previous allogeneic study, we were unable to find any evidence of graft rejection or severe inflammation.[15,16] However, the limitation of our previous study was graft resorption.[16] Therefore, methods to decrease the absorption of implanted cartilages should be developed. In this study, we hypothesized that autologous tissue-engineered implants have better biocompatibility and structure supporting in vivo cartilage formation. Compared with our previous study, the use of autologous tissue-engineered cartilage implants for reconstruction of the trachea can be advantageous over the allogeneic approach in terms of preservation of implanted cartilages.

However, our present study has some limitations due to the fact that reconstruction was not necessary for partial tracheal resection. Small defects where at least 50% of the native cartilaginous support for the trachea was preserved could be allowed to heal by secondary intention with minimal eventual loss of lumen followed by successful re-epithelization. There- fore, this study was designed as a preliminary animal experiment for evaluation of tissue-engineered implants using a fibrin/HA composite gel and an intermediate step toward the ability to produce lengths of autologous tissue-engineered complete tracheal segments.

In the present study, a rabbit model was designed to determine the suitability of using autologous engineered cartilages as grafts in tracheal reconstruction. The meaningful results from this study were that tissue-engineered cartilages in tracheal defects of all rabbits were preserved, although the tracheal regeneration was incomplete, and that functional epithelial regeneration was observed. It was also noted that in each rabbit, the airway was maintained even in cases where the

grafts had shifted position or collapsed. The fact that the grafts were broken and have occasionally become dislodged from their original insertion point demonstrates that the compressive biomechanical forces exerted on the grafts were not always counteracted by the mechanical properties of the grafts themselves and that surgical placement is extremely important for graft success. What is unclear is the cause of the displacement of some of the grafts. In some cases, the graft material itself appears to have collapsed, but in other instances, the graft material may have simply shifted its position during or soon after surgical implantation.

In tracheal regeneration, functional epithelial regeneration is very important. Belsey summarized the requirements for tracheal replacement: a laterally rigid but longitudinally flexible tube and a surface covered with ciliated respiratory epithelium.[24] At the very beginning, many scientists were led to believe in the illusionary simplicity of tissue- engineered tracheal reconstruction and took it as no more than developing a tubular cartilage tissue. Later, animal examinations demonstrated the importance of an intact epithelial line to prevent the in-growth of granulomatous tissues and fatal airway obstruction. The cilia also help to expel the mucosal fluid and the adhering micro-particles. Therefore, the focus in tissue-engineered trachea studies has shifted currently toward substitution of re-epithelialization.[25,26]

From this perspective, we ascertained the importance of functional assessment of cilia and measured the CBF of implanted mucosa. Comparable with our previous study, the CBF of the regenerated ciliated epithelium was similar to the control data with no statistically significant difference in CBF between a normal trachea and an implanted trachea ($P<0.05$). However, to mention superiority of our approach (autologous chondrocytes cultured with fibrin/HA) in terms of functional re-epithelialization, it needs to be evaluated in long circumferential tracheal defects because most cases of tracheal reconstruction or secondary healing after partial resection demonstrated successful re-epithelialization.

V. CONCLUSION

This preliminary study shows that tracheal reconstruction after partial resection using implants with autologous chondrocytes cultured with fibrin/HA resulted in favorable epithelialization on the implants and preservation of neo-cartilage without graft rejection or inflammation but the graft lacked adequate mechanical stability. Further studies on the mechanical stability of cultured autologous cartilage implantation and the ability to produce autologous tissue-engineered circumferential tracheal segments are essential to advance our technique as a good substitute for conventional approaches.

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Part II:

ABSTRACT

Formation and evaluation of tissue-engineered tracheal cartilage using autologous chondrocytes and Poly-lactic-co-glycolic acid (PLGA) composite in rabbits

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(Directed by Professor Eun Chang Choi)

Reconstruction of trachea is still a clinical dilemma. Tissue engineering is a recent and promising concept to resolve this problem. This study evaluated the feasibility of chondrocytes cultured with fibrin/hyaluronic acid (HA) hydrogel and degradable porous poly (L-lactic-co-glycolic acid) (PLGA) scaffold for partial tracheal reconstruction. Chondrocytes from rabbit articular cartilage were expanded and cultured with fibrin/HA hydrogel and injected into a 5 x 10 mm-sized, curved patch-shape PLGA scaffold. After 4 weeks in vitro culture, the scaffold was implanted on a tracheal defect in eight rabbits. Six and 10 weeks postoperatively, the implanted sites were evaluated by bronchoscope and radiologic and histologic analyses. Ciliary beat frequency (CBF) of regenerated epithelium was also evaluated. None of the eight rabbits showed any sign of respiratory distress. Bronchoscopic examination did not reveal stenosis of the reconstructed trachea and the defects were completely recovered with respiratory

epithelium. Computed tomography scan showed good luminal contour of trachea. Histologic data showed that the implanted chondrocytes successfully formed neo-cartilage with minimal granulation tissue. CBF of regenerated epithelium was similar to that of normal epithelium. Partial tracheal defect was successfully reconstructed anatomically and functionally using allogeneic chondrocytes cultured with PLGA-fibrin/HA composite scaffold.

Key words: tracheal regeneration, poly (L-lactic-co-glycolic acid), chondrocyte, hyaluronate, fibrin

Part II:

Formation and evaluation of tissue-engineered tracheal cartilage using autologous chondrocytes and Poly-lactic-co-glycolic acid (PLGA) composite in rabbits

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I. INTRODUCTION

Tracheal reconstruction is required in cases of tracheal stenosis, trauma, or thyroid/laryngeal malignancies.[1,2] Replacement or reconstruction of the trachea is one of the most difficult procedures in head and neck surgery.[3] An optimal treatment has not yet been found, but current treatments for tracheal disease mainly involve surgical repair.[4,5] Total resection of pathologic trachea followed by end-to-end anastomosis is the favored treatment; however, the total resectable length cannot exceed 30% of the trachea in children or 6 cm in adults.[6] In addition, if indicated, reconstruction with various cartilage substitutions, such as costal cartilage, buccal mucosa, or artificial materials, has been performed after partial tracheal resection with limited success.[7]

There is no ideal substitute of tracheal cartilage because of the anatomical and functional characteristics of the trachea.[1] The trachea is composed of C-shaped cartilage, respiratory epithelium, and connective tissue including smooth muscle,

and supporting blood supply.[1] Tracheal cartilage is responsible for the mechanical airway framework, while the respiratory epithelium is essential for muociliary clearance.[6] Thus a reconstructed trachea should have mechanical strength and functional epithelium.[8] For ideal tracheal replacements, materials and cells for the tracheal construct should be airtight, accepted by the host, induce minimal inflammatory reaction, be capable of molding into various sizes and configurations, be capable of incorporation into surrounding tissues, and should be impervious to fibroblastic and bacterial invasion.[9] Since the first successful results of tissue-engineered cartilage implantation in small tracheal defects of mice,[10] increasing evidence has indicated the potential of tissue-engineering techniques.[11] Bioengineered tracheas have been made using a wide variety of materials and methods.[11] However, there is still no "gold standard" material to replace trachea because it does not meet the aforementioned requirements.[12] A reliable, functional tracheal replacement would overcome the limitation of tracheal resection length in performing end-to-end anastomosis.

In the past decade, poly- L-lactic-co-glycolic acid (PLGA) copolymer has been brought to the fore in various medical applications, especially regenerative medicine. Owing to its virtues of adjustability of degradation rates, mechanical strength, fair processability, and biocompatibility, PLGA has become the most popular biodegradable polymer. In addition, our group previously reported the feasibility of using allogeneic chondrocytes cultured with fibrin/hyaluronic acid (HA) gel for tracheal reconstruction. However, these approach had limited mechanical strength because of rapid degradation of fibrin/HA in in vivo condition.[8,13] We hypothesized that combining fibrin/HA gel with PLGA scaffold would generate a system having the requisite mechanical strength and durability, which could overcome the current limitation. In this study, we structurally and functionally characterized regenerated trachea by allogeneic chondrocytes cultured with the fibrin/HA hydrogel and injected into PLGA scaffold.

II. MATERIALS AND METHODS

1. Isolation of chondrocytes

Chondrocytes were isolated from rabbit knee cartilage as previously described.[14] Under anesthesia by tiletamine (4.0mg/kg; Virbac, Carros, France) and zolazepam (4.0mg/kg; Virbac), the knee cartilage was removed by sterile dissection. Pieces of cartilage were processed as described.14 After washing twice with phosphate-buffered saline (PBS), the cell pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100U/mL penicillin G (Gibco BRL), and 100 mg/mL streptomycin (Gibco BRL). The cells were plated at a density of 1.5 x 10^5 cells/cm^2 and placed at 37℃ in a 5% CO_2 incubator. The cell medium was changed every other day. The primary chondrocytes were passaged twice before the experiments.

2. Preparation of fibrin/HA composite gel

The cultured chondrocytes were pelleted by centrifugation and suspended in a solution containing both fibrinogen (9–18mg/mL; Mokam Research Center, Suwon, South Korea) and HA (molecular size 3000kDa; 10mg/mL; LGCI, Daejeon, South Korea). The mixing ratio of fibrinogen to HA was 10:1 by volume. The suspension of chondrocytes (1.0 x 10^6 cells/mL) was processed as previously described.[13] Then 250 mL of the fibrin/HA mixture was dropped into an empty Petri dish to form a gel. Each drop was transferred to a six-well culture plate and cultured in DMEM supplemented with 10% FBS and antibiotics.

3. Preparation of PLGA scaffold

Dry cylinder-shaped PLGA scaffold (Regen Biotech, Sungnam, Korea) 50mm in length, 30mm in inner diameter, and 3mm in thickness, with a pore size of 250–400mm was designed as a slightly sweeping, curved patch-like graft 10mm in width and 5mm in height (Figures. 1 and 2A). The graft was hydrated by complete immersion into 75% ethanol solution using aseptic conditions and kept in a refrigerator (4°C) overnight. Ethanol was removed completely by repeated washing with sterile, pyrogen-free cold water, PBS, and serum-free culture medium, and sterilized with ethylene oxide gas using a model EOG-300 apparatus (Delta Medical, Ansan, Korea). The mixtures of cultured chondrocytes (1.0×10^6 cells/mL) and fibrin–HA gel were implanted in the sterilized PLGA scaffold. The implanted chondrocytes in the scaffold were cultured in chondrogenesis-defined media [DMEM with 1.0mg/mL insulin from bovine pancreas, 0.55mg/mL human transferrin, 0.5mg/mL sodium selente (ITS), 50mg/mL ascorbic acid, 100mM dexamethasone, 40mg/mL L-proline, 1.25mg/mL bovine serum albumin (BSA), and 100mg/mL sodium pyruvate (Sigma-Aldrich, St. Louis, MO) for 4 weeks before tracheal implantation (Figure 2B).

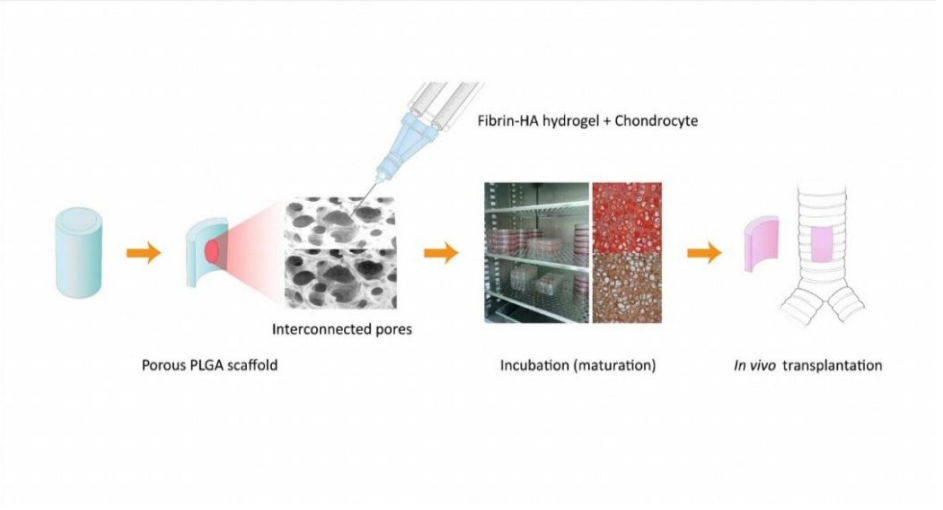

Figure 1. Schematic of preparation of PLGA-Fibrin/HA tracheal implant and procedure. Cylindrical commercially pre-made PLGA scaffold (Regen Biotech Inc., 50mm in length, 30mm in inner diameter and 3 mm in thickness, pore size 250 to 400 mm) was designed into curved patch like implant. Then, cultured chondrocyte and Fibrin-HA gel mixture implanted in the interconnected pores of designed PLGA scaffold. After 4 weeks maturation, the scaffold implanted in the tracheal defect of rabbits.

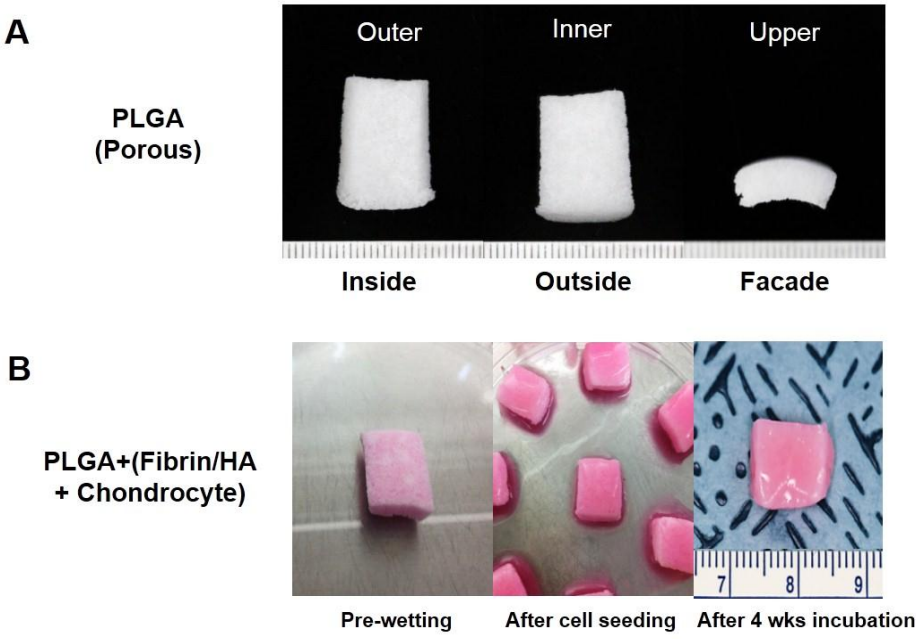

Figure 2. Preparation of PLGA-fibrin/HA scaffold with allogeneic chondrocytes. (A) The designed PLGA scaffold. The width and height was 10mm X 5 mm, the radius of curvature was 30 mm in the inner surface, and 3 mm in thickness. (B) Fibrin/HA hydrogel and chondrocyte mixture was implanted in the designed scaffold and then incubated to maturate in vitro up to 4 weeks before in vivo implantation.

4. Scanning electron microscopy (SEM)

Chondrocyte-seeded PLGA scaffold was assessed by scanning electron microscopy (SEM) to determine the pore connectivity and presence of seeded cells in the pore spaces (Figure 3). The scaffold was cut in cubes (5 x 3 x 1 mm^3) and fixed to the sample holder. After platinum coating using a model SC 500K plasma sputter (Emscope, West Sussex, UK), each sample was examined using a model S-4800 SEM (Hitachi, Tokyo, Japan) operating at 10 or 15 kV.

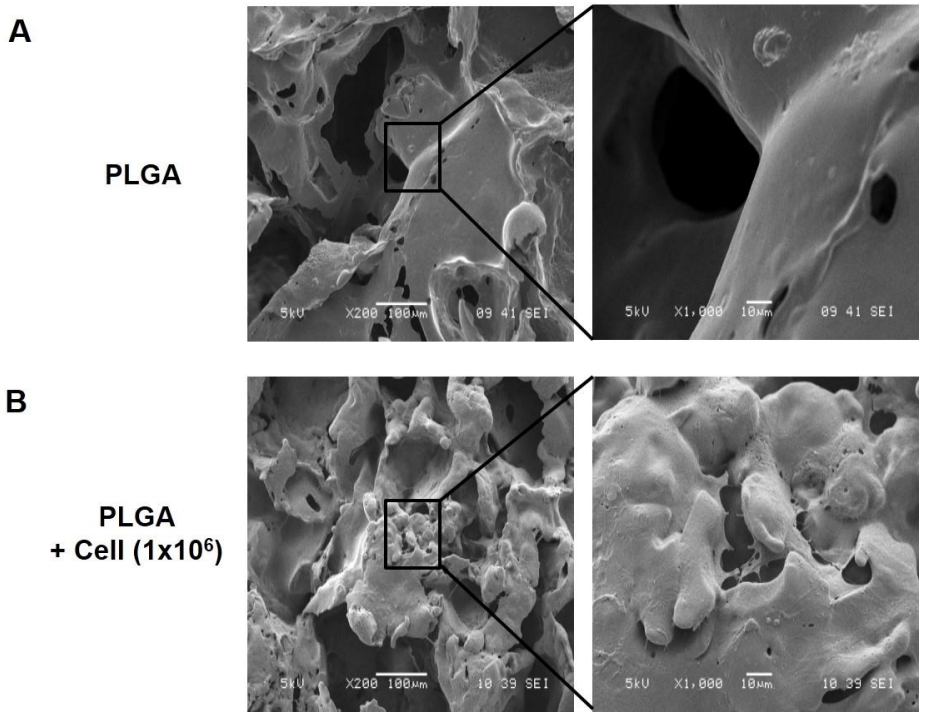

Figure 3. Scanning electron microscopy image of cell seeded PLGA scaffold. (A) Before cell seeding. Prepared PLGA scaffold showed good porosity and interconnection between pores. (B) After seeding of chondrocyte and fibrin/HA hydrogel mixture. Implanted chondrocytes were well settled down and an amount of ECM were produced in the pores of scaffold.

5. Animals and surgical procedures

Eight male, 12-week-old, 2.5-3.0 kg New Zealand white rabbits (Samtaco, Osan, Korea) were maintained in the central animal laboratory for 1 week. The animals were acclimatized for 1 week at 21 ±1 °C, 50 ±5 % humidity, and an automatic 12-h light-dark cycle. The rabbits were provided access to food and water ad libitum. Animal care and procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all experiments were approved by the Committee for Ethics in Animal Experiments of the Ajou University School of Medicine. Each rabbit was set in a supine position under anesthesia by tiletamine (4.0mg/kg) (Virbac) and zolazepam (4.0mg/kg) (Virbac) with the neck slightly extended. A vertical skin incision was applied and the sternohyoid and sternothyroid muscles were divided at the midline. After the cervical trachea was appropriately exposed, a 5 x 10 mm-sized tracheal defect was made with a scalpel and an electrocoagulator (Figure 4A). The defect was covered with the designed PLGA scaffold implanted with allograft chondrocyte suspended in fibrin/HA hydrogel, and it was fixed and sealed with GreenplastVR (Greencross, Suwon, Korea) (Figure 4B). The divided strap muscles were replaced over the graft and sutured. Finally, the skin was sutured meticulously.

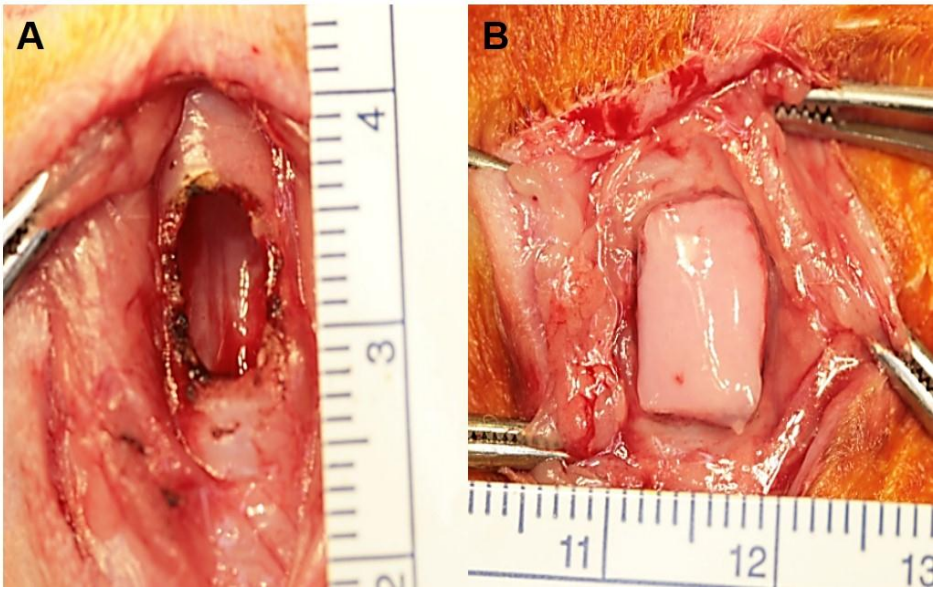

Figure 4. Insertion of tissue-engineered tracheal implant using PLGA-Fibrin/HA scaffold. (A) A tracheal defect, approximately 5 × 10 mm, was made. (B) The tracheal implant was trimmed and inserted to the tracheal defect. It was fixed and sealed to the defected boundaries with Greenplast®.

6. Bronchoscopic and radiologic evaluations

Bronchoscopic examination was done at 6 and 10 weeks after transplantation with a 4-mm-diameter rigid endoscope (Karl Storz, Tuttlingen, Germany) and a Coolpix 3500 camera (Nikon, Tokyo, Japan) with a light source (Head Light; Karl Storz). Computed tomography (CT) scan was performed using a Brilliance 64 CT scanner system (Philips, Eindhoven, The Netherlands) under anesthesia with the same method as described above at 6 and 10 weeks postoperatively. The axial images were obtained using 1-mm-thick sections and were used to reconstruct three-dimensional (3D) images of the trachea.

7. Histological examination

The animals were administered euthanasia after the bronchoscopy and CT scan. The trachea including implantation sites were resected and prepared for light microscopic examinations. After fixing with 10% neutral buffered formalin for 24 h, the treated samples were embedded in paraffin and sectioned at 4-mm thickness. The sections were stained with hematoxylin–eosin (H&E) and safranin-O (S-O) for light microscopic examination.

8. Measurement of tracheal ciliary beat frequency

Functional assessment of regenerated respiratory epithelium was measured with tracheal ciliary beat frequency (CBF), as previously described.14 Briefly, a piece of tracheal epithelium (1.2 mm thick) was harvested and washed with DMEM containing 10,000 U/mL penicillin and 10,000 mg/mL streptomycin, and kept in the medium. The ciliary beating video was captured with an Axiovert 40CLF inverted microscope (Carl Zeiss, Thornwood, NY) and a Moticam 2000 high-speed digital camera (Motic, Causeway Bay, Hong Kong). The captured images were analyzed using commercial software (Ammons Engineering, Mt. Morris, MI). Amplitudes and frequencies of cilia were sampled in at least six separate fields and statistically analyzed. One-way analysis of variance was used for statistical analyses of the data with the SPSS version 20.0 statistical software (SPSS, Chicago, IL). Data are expressed as mean6SD. A value of $p<0.05$ was taken to indicate statistical significance ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

III. RESULTS

1. Seeded chondrocytes survive in PLGA scaffold pores

SEM image of PLGA scaffolds revealed good porosity with sufficient space for implanted chondrocytes to adhere, proliferate, and interconnect between pores (Figure 3A). Injected allogeneic chondrocytes with fibrin/HA hydrogel were settled down in the entire pore of PLGA scaffold and the cells and extracellular matrix (ECM) produced by the chondrocyte occupied the pore spaces (Figure 3B).

2. Implant produces anatomically and functionally reconstructed trachea

All rabbits in each group survived until being euthanized at each time point of the histologic examinations. They were well nourished and in good general condition without body weight loss after the operation. None of the rabbits developed any signs of respiratory distress or wound infection. As shown in Figure 5A and B, gross assessments showed that all PLGA implants appeared to be healthy and well integrated with tracheal cartilage of recipient, and the shape near the implantation was comparable with that of a native rabbit trachea. However, in some cases a graft underwent slight migration from its original implant site (Figure 5B).

Bronchoscopy under anesthesia revealed that all eight rabbits had successful recovery of tracheal luminal contour with no sign of obstruction, stenosis, or granulation, and that the PLGA scaffolds were well covered with respiratory epithelium (Figure 5C and D), even though the color of intraluminal area of reconstructed was paler at 6 weeks postoperatively than at 10 weeks, indicating incomplete neovascularization at 6 weeks post-implantation. On both axial and 3D reconstructed view of CT scans, there was no sign of stenosis in any of the rabbits and tracheal contour was well maintained at 6 and 10 weeks (Figure 5E and F).

Histologic assessment 6 weeks after implantation revealed formation of newly developed cartilaginous tissue by H&E staining and the extracellular region of cartilage was strongly stained by characteristic red of S-O staining indicating the presence of proteoglycan-rich matrix. As shown in Figures 6 and 7, newly developed cartilages were evident in the periphery of implanted scaffold. Although not perfect, the neo-cartilages closely resembled native tracheal cartilage with an overall similar contour. In addition, under higher magnification, H&E-stained neo-cartilage revealed the presence of round- or angular-shaped lacunar structure containing a chondrocytes with the typical histologic character of cartilage.

Moreover, the luminal side of PLGA implant was already covered with epithelial lining even at 6 weeks post-implantation, but the lamina propria of epithelium in the vicinity of implanted scaffold was significantly thicker than that of native mucosa, with numerous cells noted under higher magnification indicating inflammatory process (Figure 6). In contrast, little inflammation and granulation was observed on the implant 10 weeks postoperatively using H&E staining (Figure 7). Furthermore, although the quantity of neo-cartilage formation of both examine times was not showed significant difference, the neo-cartilage at postoperative 10 weeks displayed more evenly spaced lacunae reflecting the presence of more mature cartilage than that of 6 weeks, and were surrounded by eosinophilic connective tissues (asterisk), seemingly perichondrium as expected in native trachea.

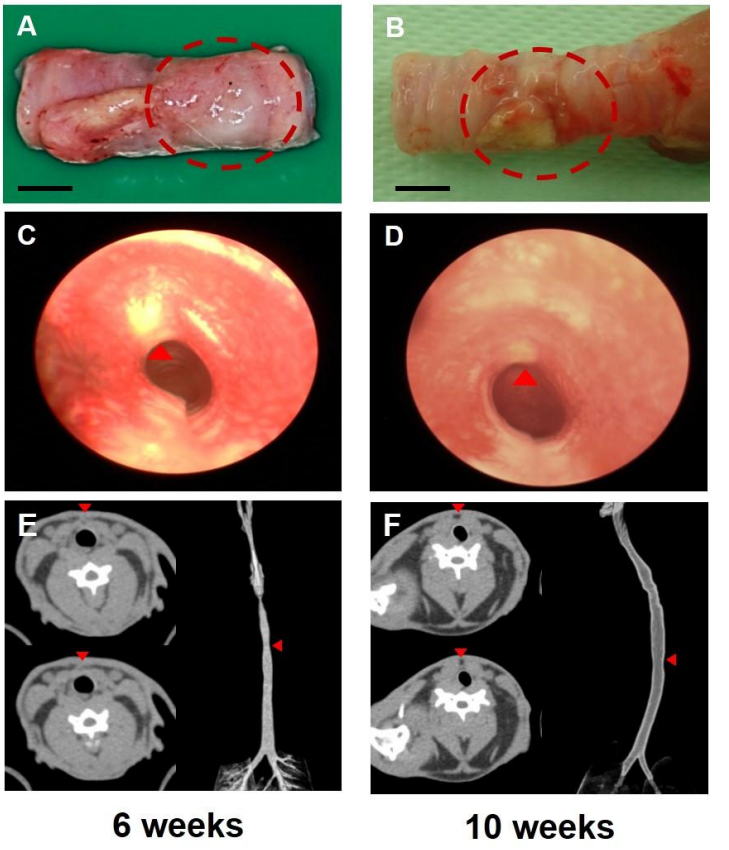

Figure 5. Structural evaluation of implant. (A, B) Gross morphology of representative reconstructed area (circle) of trachea at 6 and 10 weeks after PLGA-fibrin/HA scaffold implantation on the tracheal defect. The implant well maintained without definite stenosis or obstruction near the reconstruction. Scale bar denotes 5 mm. (C, D) Bronchoscopic findings of a representative reconstructed trachea 6 and 10 weeks after the operation. Endoscopic images revealed that the implant was completely covered with regenerated epithelium without any granulation or displacement even 6 weeks after implantation (arrow head). (E, F) Findings of computed tomography (CT) at 6 and 10 weeks after tracheal reconstruction. Axial CT and 3D reconstructed images of the implanted sites revealed a fine luminal patency of the repaired trachea, both 6 and 10 weeks after the operation.

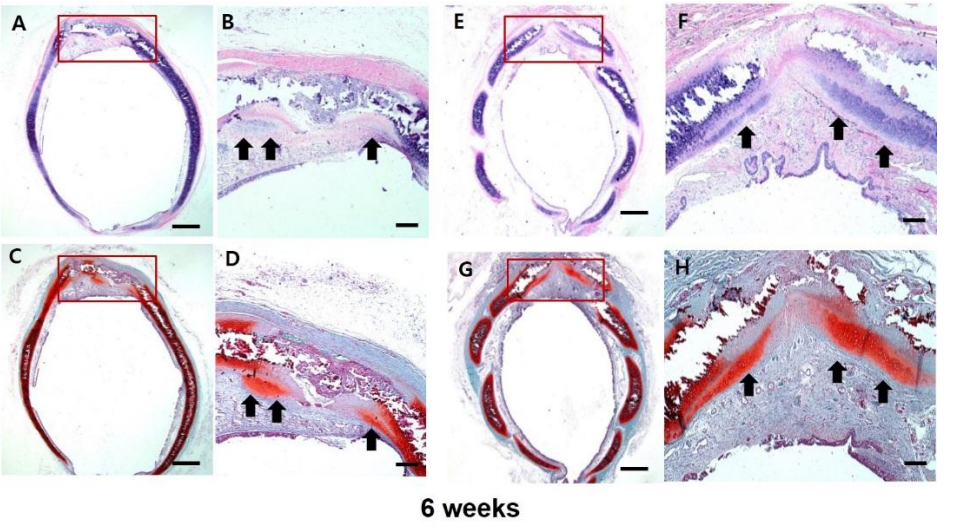

Figure 6. Histologic examination of implant six weeks after the operation.
(A), (B), (E), (F) The Hematoxylin and eosin staining of implanted material revealed neo-cartilage fragment on the periphery of the implanted area (arrow). (C), (D), (G), (H) The S-O staining at postoperative six weeks showed neo-cartilage fragment on periphery of the implant in consistent with H&E staining. Scale bar=2 mm in (A), (C), (E), (F), 500 μm in (B), (D), (F), (H).

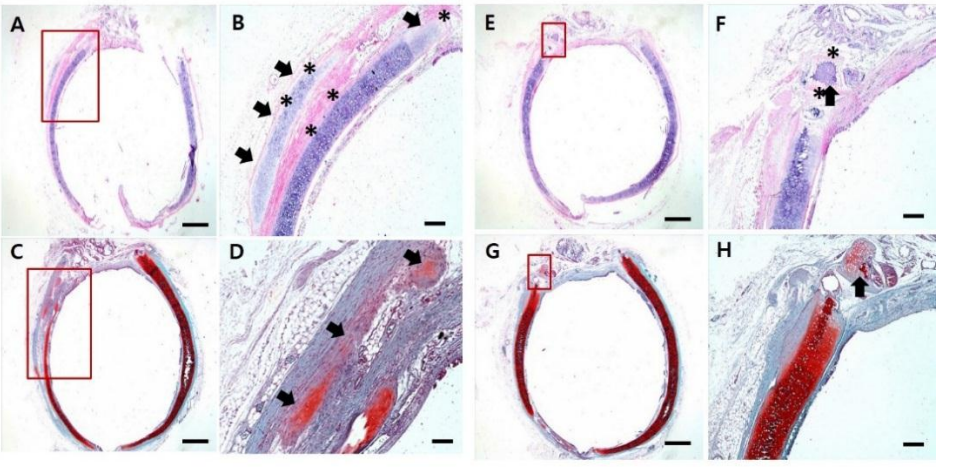

Figure 7. Histologic examination of implant ten weeks after the operation.
(A), (B), (E), (F) The Hematoxylin and eosin staining of implanted material revealed neo-cartilage fragment on the periphery of the implanted area (arrow). The neo-cartilage showed more evenly spaced lacunae cells embedded in a basophilic matrix than that of six week and encapsulated by eosinophilic connective tissue reflecting perichondrium regeneration (asterisk). (C), (D), (G), (H) The S-O staining at postoperative ten weeks showed neo-cartilage fragment on periphery of the implant in consistent with H&E staining. Scale bar=2 mm in (A), (C), (E), (F), 500 μm in (B), (D), (F), (H).

The CBF reflecting the function of cilia in the regenerated respiratory epithelium was analyzed and compared with that of normal epithelium near the reconstructed region. The CBF of regenerated epithelium decreased significantly about 15% compared with that of normal epithelium at 6 weeks after the implantation (p=0.0006), but restored gradually up to 94% of native CBF after 10 weeks (p=0.0013). There was no statistically significant difference in CBF between control and regenerated epithelium at postoperative 10 weeks (Fig. 8).

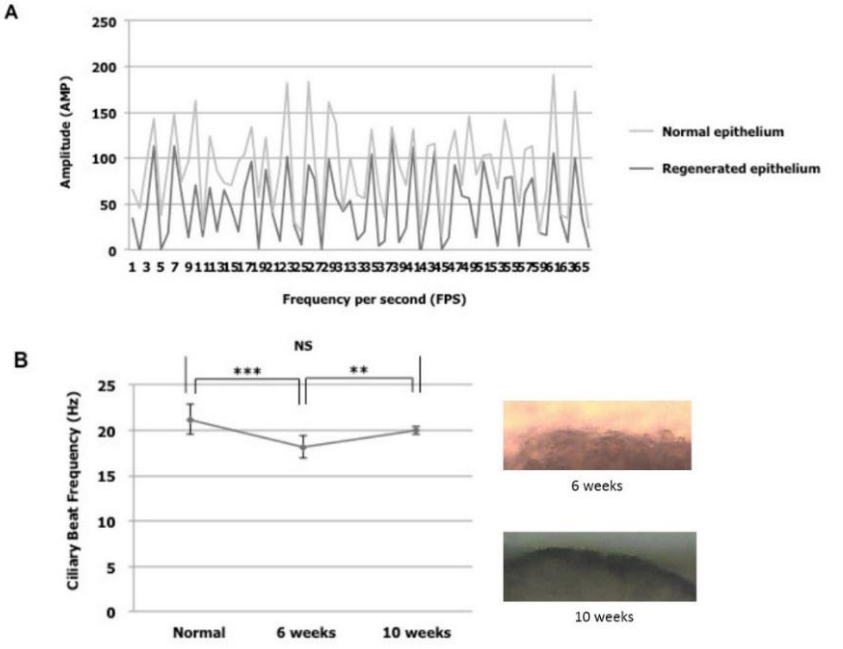

Figure 8. Measurement of trachea ciliary beat frequency (CBF).

(A) Computerized analysis of amplitude of ciliary beating of normal and regenerated tracheal epithelium, at ten weeks after the surgery. (B) Although, the CBF of regenerated epithelium was significantly decreased than that of normal epithelium at 6 weeks after implantation, the CBF of regenerated epithelium was almost recovered. Consequently, there was no statistically meaningful difference in CBF between normal and regenerated mucosa at ten weeks of the operation.

IV. DISCUSSION

The trachea is known as the "windpipe," which means essential in respiratory system[11] and also functions to trap inhaled foreign particles like dust and bacteria and expel them as phlegm.[15] The necessary conditions for an artificial tracheal graft to succeed are air tightness, proper mechanical strength comparable to normal cartilage frameworks, coverage with functional ciliated epithelium, and adequate blood supply to nourish these bioengineered frameworks and mucosa.[16] A variety of materials have been explored in tracheal implantation including biocompatible materials alone or in combination with autologous tissues or various growth factors.[17] However, no previously reported graft has proven satisfactory.[1,17]

In developing a biocompatible and functional artificial graft for tracheal defects, we focused on a chondrocyte-based technique. Chondrocytes are the only cells found in healthy cartilage and can remain viable in sterile conditions because they receive oxygen and nutrition by diffusion in the vicinity of blood vessels.[18],[19] Furthermore, owing to its limited need for vascular and lymphatic supply, implanted cartilage might have low antigenicity.[19] Allogeneic chondrocytes can be used to repair cartilage defects and it is already used in several orthopedic diseases such as osteochondral defects.[20] Chondrocytes readily express their original phenotype and synthesize ECM components, which contribute to mechanical properties necessary for weight bearing.[20] The authors previously applied chondrocytes to the reconstruction of tracheal defects[8,13] and vocal fold augmentation[21] using a fibrin/HA composite gel and, more recently, porcine cartilage-derived substance (PCS)[14] for partial tracheal defect. However, a fibrin/HA composite gel showed limited ability in achieving long-term chondrocyte survival and proper neo-cartilage formation in vivo and PCS revealed limited mechanical strength as a wind pipe.

In this study, we used PLGA copolymer as a framework of biodegradable tracheal implant and fibrin/HA hydrogel as an ECM support. Fibrin glue possesses abundant fibronectin, which is essential in the cartilage matrix, and HA is a key component in cartilage ECM.[8] Both are biocompatible and biodegradable materials, which already have been used in various medical fields.[22-25] Based on our previous study, fibrin/HA hydrogel provided a favorable environment for chondrocytes to maintain their characteristic phenotype and synthesize cartilage ECM, but had limited ability in achieving long-term chondrocyte survival and mechanically proper neo-cartilage formation.[8] Therefore, we tried to investigate a new proper, hybrid scaffold for chondrocyte implantation.

PLGA has been used for various medical applications,[26] and has been widely investigated, especially in orthopedic field, to repair the whole thickness cartilage defect with mesenchymal stem cells and satisfactory results were reported in vivo.[27,28] Chondrocytes also proliferate and synthesize ECM on PLGA scaffolds in vitro culture.[29] Advantages of PLGA include adjustability of degradation rates, good mechanical properties (especially toughness), and excellent processibility, which are necessary characteristics of porous scaffold in tissue engineering.[26,30] In addition, PLGA potentially retains many cytokines, growth factors, or other functional proteins that support chondrocyte viability, proliferation, and the production and maintenance of cartilaginous matrix.[14] Therefore, it can serve as not only structural base for cells but also biologic activator encouraging synthesis of ECM components and tissue regeneration. Moreover, PLGA is one of a few synthetic materials that have been used in products approved by the United States Food and Drug Administration (FDA) for various clinical applications.[11,26] Therefore, PLGA is a very promising biocompatible source of scaffold that can be applied more easily than other synthetic sources in clinical trials of tracheal reconstruction.

This study shows that the limitation of scaffolds made solely from fibrin/HA hydrogel can be ameliorated by combining the hydrogel with PLGA framework.

Tissue engineered grafts in tracheal defects of all rabbits were preserved without collapse or breakage. Although the tracheal regeneration was incomplete and we could not confirm the origin of neo-cartilage, the cartilage fragments found in the periphery of implanted area at both time of evaluation demonstrated more matured characteristics in a time-dependent manner. These results suggest that PLGA-fibrin/HA composite scaffold provides a favorable environment for chondrocytes to regenerate tracheal cartilage.

Functional epithelial regeneration was also observed. We believe that most of these epithelial cells migrated from the adjacent mucosa on to the implanted scaffold to form functional respiratory epithelium. Therefore, we suggest that PLGA-fibrin/HA composite scaffold provides a favorable circumstance to chondrocytes and ciliated epithelial cells.

In this study, a 4-week in vitro culture for maturation was needed to obtain proper mechanical strength for tracheal defect coverage. The optimal scaffold should provide an initial mechanical support and a 3D niche for transplanted cells until the regenerated tissue can stabilize the initial structure. This in vitro process, which integrates cells with the scaffold and generation of ECM, is a very important step and PLGA took much less time compared with 7 weeks in our previous study of PCS.

The main problem was fixation of the scaffold into the defect. In this study, commercially premade cylinder-shaped PLGA scaffold was used because the radius of curvature was similar to that of rabbit trachea, and we thought the implant might dovetail nicely with the tracheal defect. In addition, we applied fibrin glue to achieve complete air tightness of implant and not to dislodge from its original insertion area. However, in some cases, the implant became displaced laterally. It is unclear that what cause the displacement of graft, but the graft material may have shifted its position during or soon after surgical implantation. Performing interrupted suture the scaffold with recipient trachea could resolve this problem. Despite the PLGA-fibrin/HA composite scaffold showing

promising outcome in this investigation, circumferential tracheal reconstruction is still challenging. Future studies are warranted to extend our study into major tracheal reconstruction and clinical application. We are planning to perform further studies on the mechanical strength and durability of this composite scaffold to eventually apply this scaffold to circumferential defects in a clinical setting.

V. CONCLUSION

This study shows that tracheal reconstruction after partial resection using implants with allogeneic chondrocytes cultured with PLGA-fibrin/HA composite scaffold resulted in favorable mechanical and functional recovery. Chondrocytes cultured with PLGA-fibrin/HA composite scaffold could be a plausible implantable material for tracheal reconstruction.

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ABSTRACT (IN KOREAN)

가토에서 연골세포와 생체적합성 복합지지체를 이용한 조직공학적 인공 기관연골의 제작과 평가

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연구목적: 종양, 염증 및 선천성 장애 등의 다양한 원인에 의한 기관협착의 치료법은 여러 가지 수술법이 개발되어 있으나 효과의 한계가 있다고 알려져 있다. 이를 극복하고자 최근 발전하고 있는 조직공학 (tissue engineering) 의 기법을 이용한 치료법이 개발되어 시도되고 있고 이전의 자가 혹은 동종 이식기술과 융합된 조직공학적 방법을 이용한 기관재건술의 중요성이 다시 주목 받고 있다. 이에 본 연구에서는 보다 효과적인 생체적합성 인공이식물을 제작하고 평가하여 이를 기관협착증의 치료를 위해 이용할 수 있는 계기를 마련하고자 한다. 선행연구 (Part I) 에서 가토의 이개연골에서 채취하여 체외에서 배양한 자가연골세포와 섬유소 (fibrin)/ 히알루론산 (hyaluronate, HA) 의 복합지지체를 이용한 연구를 시행하고 이후 후행연구 (Part II) 에서 가토의 연골에서 채취하여 체외에서 배양한 연골세포와 PLGA (poly-lactic-co-glycolic acid)를 이용하여 복합지지체 형태의 인공연골 이식편을 제작한 후 이를 실험동물에서 만들어진

기관결손부위에 이식한 후 장기간의 추적관찰을 통해 이식 가능 여부를 알아보고자 한다.

연구방법: 뉴질랜드 화이트종 토끼를 대상으로 실험동물 모델을 제작하였다. Part I 에서 토끼 모델에서 이개연골을 채취하고 체외에서 배양하여 섬유소 (fibrin)와 히알루론산 (hyaluronate, HA)을 결합시킨 지지체와 혼합한 뒤 이식편을 제작하였다. Part II 에서는 무릎 초자연골에서 연골세포를 채취하고 체외에서 배양하여 섬유소 (fibrin)와 히알루론산 (hyaluronate)을 결합시킨 지지체와 PLGA 를 혼합한 뒤 이식편을 만들었다. 두 가지 실험에서 모두 이식을 위해 인위적으로 토끼의 기관연골을 1.0 x 0.5cm 크기로 제거한 뒤 이식편을 삽입 고정 하였다. 각각의 실험에서 사용된 이식편을 재건에 사용한 후 기능성과 안전성을 확인하였다. 수술 후 기관재건부위는 내시경 평가, 영상의학적 평가, 조직학적 평가 및 기관섬모세포 운동성 평가 등을 실시하였다. 제작된 이식편은 H&E 염색, Safrani O 염색과 주사전자현미경을 시행하여 평가하였다. 결손부위의 형태 변화를 보기 위해 수술 후 기관 내시경 검사, 3D 전산화 단층촬영을 시행하여 기관결손부위를 평가하였고 기관을 절제하여 조직학적 평가, 주사전자현미경 관찰 및 기관 상피세포의 미세섬모운동을 평가하였다.

결과: 자가연골세포와 fibrin/HA 의 복합지지체를 이용한 연구 (Part I)에서 수술 후 모든 경우 단단한 망원경 검사는 이식 발판이 완전히 과립 또는 협착 없이 재생 된 점막으로 덮여 것으로 나타났다. 조직학적으로 염증반응이 보이지 않고 전체적으로 섬모상피로 덮여 있었다. 이식편이 이동된 경우에도 이식 된 연골은 잘 보존되었다. 이식편의 섬모상피의 운동성은 보통 호흡기 점막의 그것과 매우 유사했다. 하지만 기계적인 힘을 부족한 것으로 보여졌다. 동종연골세포와 PLGA-Fibrin/HA 복합지지체를 이용한 실험 (Part II) 에

사용된 8 마리 모두 호흡곤란이나 이상 징후를 보이지 않았다. 이식 후 시행한 내시경검사에서 이식한 부위에 육아종, 가피, 분비물 등의 염증 소견은 없었으며 접합부의 분리나 결손 없이 점막이 완벽하게 재생되어 있었다. 3D 전산화 단층촬영에서도 기관 내경이 잘 유지됨을 확인하였다. 조직병리소견에서 이식편 주위의 염증은 보이지 않았고 기관 내 상피세포는 정상부위와 유사한 가중층섬모원주상피세포 (pseudostratified columnar ciliated epithelium) 가 관찰되었다. 기관 상피세포의 미세섬모운동평가는 정상과 유사한 정상 섬모운동을 보였다. 기관 일부를 재건하고자 시행한 실험에서 PLGA-fibrin/HA 복합지지체를 이용하여 해부학적 및 기능적으로 우수한 결과를 얻었다.

결론: 자가연골세포와 Fibrin / HA 복합지지체를 이용한 이식편은 염증없이 기관 내강의 윤곽, 기도점막상피 재생, 신생 연골의 보존을 보여 주었다 그러나 기계적 안정성이 부족함을 보여주었다. 동종연골세포와 PLGA-fibrin/HA 복합지지체를 이용한 기관의 재건은 구조적, 기능적으로 효과적으로 이식되었다. 배양된 연골세포와 PLGA-fibrin/HA 복합지지체를 이용한 인공이식편이 기관협착의 치료에 이용할 수 있는 유용한 재료로서의 가능성을 확인하였다.

핵심되는 말: 기관협착, 기관재건, 조직공학, 자가연골, 기관섬모세포, fibrin, hyaluronate, poly-L-lactic-co-glycolic acid

PUBLICATION LISTS

1. **Hong HJ**, Lee JS, Choi JW, Min BH, Lee HB, Kim CH. Transplantation of autologous chondrocytes seeded on a fibrin/hyaluronan composite gel into tracheal cartilage defects in rabbits: preliminary results. Artif Organs 2012;36:998-1006.

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