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Original Article

Assessment of Esophageal Reconstruction via Bioreactor Cultivation of a Synthetic Scaffold in a Canine Model

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Objectives. Using tissue-engineered materials for esophageal reconstruction is a technically challenging task in animals that requires bioreactor training to enhance cellular reactivity. There have been many attempts at esophageal tissue engineering, but the success rate has been limited due to difficulty in initial epithelialization in the special environment of peristalsis. The purpose of this study was to evaluate the potential of an artificial esophagus that can enhance the regeneration of esophageal mucosa and muscle through the optimal combination of a double-layered polymeric scaffold and a custom-designed mesenchymal stem cell-based bioreactor system in a canine model.

Methods. We fabricated a novel double-layered scaffold as a tissue-engineered esophagus using an electrospinning technique. Prior to transplantation, human-derived mesenchymal stem cells were seeded into the lumen of the scaffold, and bioreactor cultivation was performed to enhance cellular reactivity. After 3 days of cultivation using the bioreactor system, tissue-engineered artificial esophagus was transplanted into a partial esophageal defect (5×3 cm-long resection) in a canine model.

Results. Scanning electron microscopy (SEM) showed that the electrospun fibers in a tubular scaffold were randomly and circumferentially located toward the inner and outer surfaces. Complete recovery of the esophageal mucosa was confirmed by endoscopic analysis and SEM. Esophagogastroduodenoscopy and computed tomography also showed that there were no signs of leakage or stricture and that there was a normal lumen with complete epithelialization. Significant regeneration of the mucosal layer was observed by keratin-5 immunostaining. Alpha-smooth muscle actin immunostaining showed significantly greater esophageal muscle regeneration at 12 months than at 6 months.

Conclusion. Custom-designed bioreactor cultured electrospun polyurethane scaffolds can be a promising approach for esophageal tissue engineering.

Keywords. *Esophagus; Nanofiber; Bioreactor; Tissue Engineering; Mesenchymal Stem Cell*

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IINTRODUCTION

Despite recent advances in surgical resection of the esophagus for conditions such as congenital anomalies and esophageal cancer, the morbidity of esophagectomy remains high, with an estimated postoperative complication rate ranging from 30% to 60% [1,2]. The esophagus has little redundancy, and reconstruction therefore typically utilizes an alternative autologous tissue. Currently, the standard reconstruction of the native esophagus involves gastric pull-up conduits or colon interpositions to re-establish luminal continuity. However, the use of these esophageal substitutes can cause surgical morbidity and mortality [2,3].

Although tissue engineering promises to be an effective regenerative strategy, the use of biomaterials for the regeneration of multi-layered functional esophagus remains a challenge [4]. Alternative conduits must allow foods and liquids to pass to the stomach while withstanding leakage and rupture, necessitating mechanical characteristics capable of supporting this function. To accommodate an oral bolus, the esophagus must expand from the resting collapsed state to a dilated state and then revert back to a collapsed state after the bolus has passed—and this action must be performed repetitively. Therefore, an engineered scaffold should have characteristics similar to the native esophageal structure to achieve functional properties.

In previous studies, we tested polymeric scaffolds with various structures (e.g., three-dimensional [3D] printing and electrospinning) for 3D esophageal reconstruction [5-8]. Of the various structures that were investigated, the strong elasticity of polyurethane (PU) electrospun nanofibers was found to provide the most appropriate physical properties for withstanding esophageal peristalsis [5,9]. In addition, the differentiation potential into esophageal epithelium and muscle was suggested for a double-structured fibrous layer having a different arrangement [10]. At the same time, the efficacy of mechanical stimuli for esophageal tissue engineering through bioreactor culture was verified. The ultimate goal of bioreactor cultivation is to cultivate tissue *in vitro* that is completely identical to the tissue of interest biologically and structurally. Many studies of specific bioreactors have been designed to imitate a tissue/organ prior to implantation and investigate whether cell differentiation/maturation is enhanced after receiving a physiological mechanical stimulus. However, few tissue engineering studies for esophageal reconstruction have conducted efficacy testing in large animal models under biomechanical stimulation for esophageal regeneration.

Therefore, the purpose of this study was to evaluate the potential of a novel custom-designed bioreactor system optimally preconditioned for the esophageal environment and a two-layered PU electrospun fiber scaffold fabricated to mimic the native structure of the esophageal layered structure in a beagle model for esophageal reconstruction (Fig. 1).

HIGHLIGHTS

- A two-layered polyurethane nanofiber seeded with stem cells in a bioreactor improved esophageal reconstruction.
- Regeneration of the esophageal muscle and mucosa was confirmed at 12 months after transplantation into a canine model.
- A tissue-engineered scaffold may be clinically useful to create a bioartificial esophagus.

MATERIALS AND METHODS

Fabrication of polymer scaffolds

Esophageal polymeric scaffolds were fabricated by electrospinning as described previously (Fig. 2A) [10]. Briefly, 30 wt% polyethylene oxide (Mw=100,000, Sigma) solution in distilled water was electrospun onto rotating stainless steel mandrels (diameter=2 cm) to 2 mm thickness. Then, 20 wt% PU (Pellethane, Lubrizol LifeSciences) solution in N,N-dimethylformamide (Junsei Chemical Co.) was also electrospun onto the collector at the same thickness. The inner layer was made at a linear speed of 0.31 m/s (1,200 rpm) so that random fiber arrangement could occur. On the other hand, the outer layer rotated the mandrel at a linear speed of 3.14 m/sec (12,000 rpm), which led to a circumferential orientation of the fibers. In both cases, the feeding rate of the solution was fixed to 0.5 mL/hr. The distance between the nozzle and flat sheet collector was set precisely at 25 cm, with 15 kV generated by a power supply. The prepared double-layered esophageal scaffold was dried overnight in a vacuum oven (38 °C) to remove residual organic solvent. The mechanical strengths of two-layered PU scaffolds were measured using a tensile test machine (AG-5000G; Shimadzu) which was equipped with a 50-kgf load cell at a crosshead speed of 10 mm/min. Specimens were prepared in the form of a standard dumbbell-shape (n=4).

Bioreactor cultivation and cell viability

For cell seeding, scaffolds were sterilized by soaking in 70% ethanol under ultraviolet (UV) irradiation. After 2 days of prewetting with basal medium, scaffold were coated with 10 μg/mL fibronectin (Sigma-Aldrich) for 1 hour to promote cell adhesion. Then, human adipose-derived mesenchymal stem cells (hMSCs, R7788-110; STEMPRO) at passage 4 were suspended at a density of 1×10^6 cells/mL in Matrigel (354234; Corning) containing growth media (MesenPRO RS, Basal medium with growth supplement), and the suspension was evenly coated on the outer and inner layers of the polymer scaffolds. Cell seeded scaffolds were then incubated under a horizontal rotation at 1 rpm overnight for stable cell attachment. Subsequently, the esophageal scaffold was removed from the rotating system, and transferred into the chamber in a bioreactor device (Fig. 2B). Mechanical stimuli were applied with a hollow organ bioreactor along with

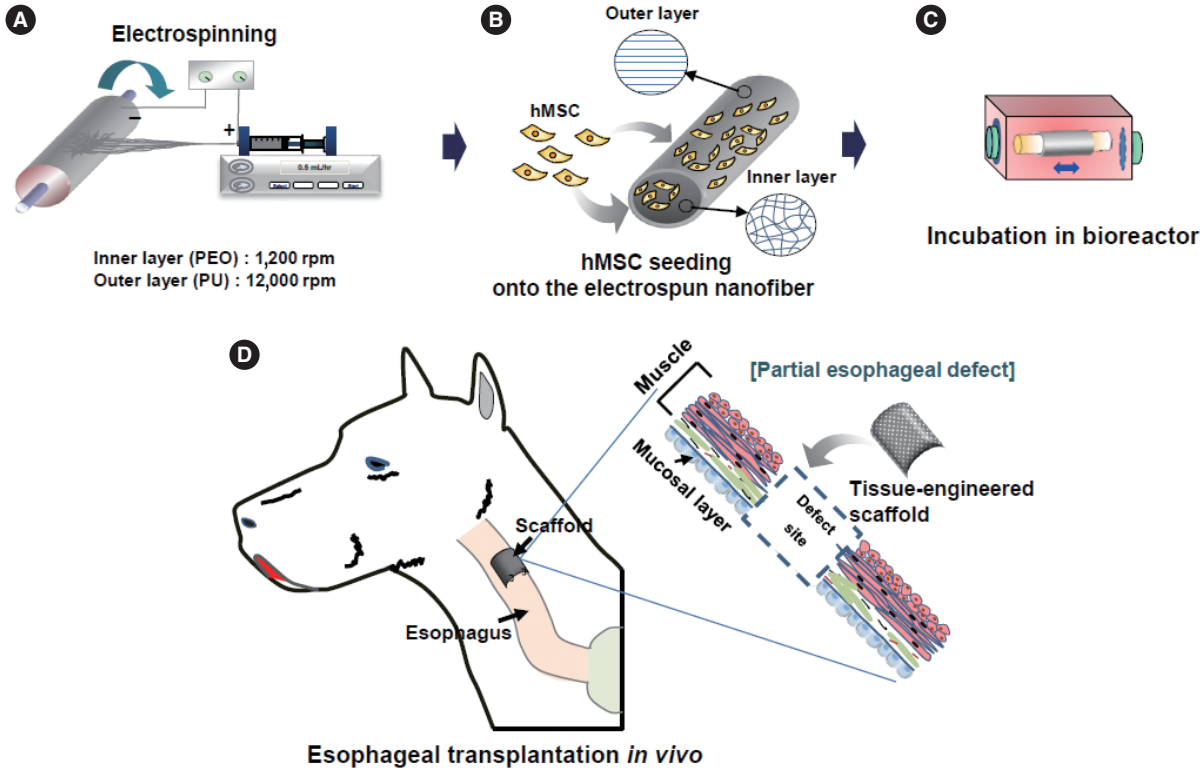

Fig. 1. Schematic illustration of the process used to fabricate two-layered tubular scaffolds and esophageal transplantation involving the tissue-engineered technique. (A) Two-layered tubular scaffolds were prepared by electrospinning at different rotation rates of the mandrel. (B) Human adipose-derived mesenchymal stem cells (hMSCs) were inoculated on the inner wall of tubular scaffolds with different fibrous structures for regeneration of esophageal mucosa. (C) These scaffolds were incubated in a bioreactor system. (D) The tissue-engineered esophageal scaffolds were then implanted into partial esophageal defects in a beagle model. PEO, polyethylene oxide; PU, polyurethane.

same growth medium inside and outside of the scaffold as described in our previous study (Fig. 2C) [10]. A custom-designed perfusion-type bioreactor was designed to maintain steady pulsatile flow (Fig. 2D) [5]. Pulsatile flow can cause shear stresses in the esophageal grafts as well as intraluminal pressure and circumferential stretching for simultaneous differentiation into epithelial and muscle lineages. The differentiation behavior of these hMSCs into epithelial cell and smooth muscle cell lineages following the administration of chemical agents under bioreactor cultivation has been demonstrated in previous studies [10]. Prior to esophageal transplantation, the hMSC-seeded tubular scaffolds were incubated under a bioreactor system for 3 days. Cell cytotoxicity on the two-layered PU nanofiber scaffold (inner & outer surface) without bioreactor cultivation was analyzed after 5 days using a live and dead viability/cytotoxicity assay (Molecular Probes) according to the manufacturer's instructions. hMSCs were grown on tissue culture plate to be used as controls. Images were captured via confocal microscopy (LSM510 META, Carl Zeiss). A CCK-8 assay kit (Dojindo) was used to determine cell proliferation on days 2 and 5. Each specimen was transferred to a new tissue culture plate (12-well) and 1ml of fresh medium was added to each well. Then, a 10% CCK-8 solution was filled and incubated at 37 °C for 2 hours. The reaction solution (100 μL) was transferred to a 96-well plate and by using a microplate reader (Thermo Scientific), the absorbance (OD value) was measured at the wavelength of 450 nm.

Orthotopic esophageal scaffold implantation

This study was carried out in strict accordance with the guidelines of the Animal Research Committee, Seoul National University Hospital. All protocols and experimental design parameters were reviewed and approved by the Institutional Animal Care and Use Committee of the Seoul National University Hospital (No. 17-0204-S1A0).

Four healthy male beagles aged 2 years and weighing 17.50–25.50 kg were used (OrientBio). The animals were divided into two groups as follows: (1) implantation of PU nanofiber scaffold (Graft group) (n=4), (2) for histological analysis, normal esophagus was collected at 5 cm away from the transplant site to replace the normal group (normal group). In addition, the animals were euthanized at 6 and 12 months after transplantation. All operative procedures were performed under general anesthesia following 24 hours of fasting. Next, a 10-mg subcutaneous injection of nalbuphine (Nalbuphine, Merck), an intramuscular injection of 0.3 mg/kg of midazolam (Hypnovel, Roche), and 5 mg/kg of ketamine (Ketamine Virbac) were administered. Endotracheal

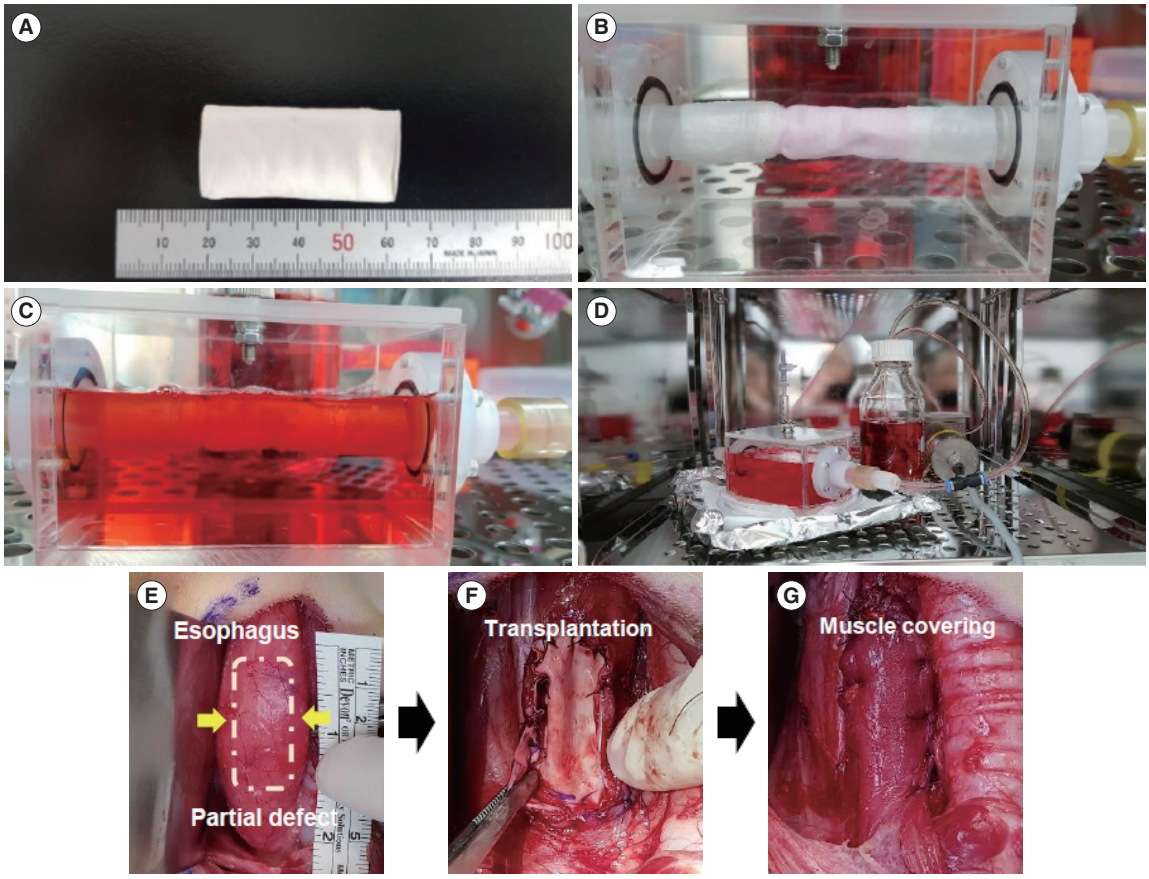

Fig. 2. Bioreactor cultivation of mesenchymal stem cell-inoculated tubular scaffolds and transplantation into esophageal defects in a beagle model. (A) The length and diameter of the prepared two-layered tubular scaffold are 4.5 cm and 2 cm, respectively. After human adipose-derived mesenchymal stem cells were seeded on the two-layered tubular scaffold, it was incubated in a horizontal rotation system for 1 day and then transferred to the bioreactor chamber (B). After filling the chamber with the culture medium (C), the mechanical stimuli were applied at a predetermined time (D). Scaffolds cultured with a bioreactor system were cut to fit the partial defect site of the beagle esophagus and implanted into the esophageal defect (E-G). The graft was covered with a sternocleidomastoid muscle flap for stable regeneration (G).

intubation was then achieved with 0.2 mL/kg/hr of propofol (Diprivan 1%, AstraZeneca). During surgery, all animals were perfused with a balanced crystalloid solution (10 mg/kg/hr), which was suppressed at the end of the operative procedure. Anesthesia was maintained by inhalation of 1% isoflurane and additional propofol. Mechanical ventilation were set to a total volume of 10 mL/kg, with a respiratory rate of 10–20 breaths/min and 60% of the fractional inspired oxygen. One intravenous ampoule of ampicillin (1 g)-sulbactam (0.5 g) was administrated before surgery as antibiotic therapy. Analgesia was provided by an intramuscular injection of 13 mg of nalbuphine (Nalbuphine1, Merck) before surgery. The detailed surgical procedure is as follows. The neck was shaved, and standard draping of the surgical site was performed for aseptic surgery. A ventral midline incision was made, and the strap muscle was divided to isolate the tracheoesophageal structure (Fig. 2E). A circumferential 5×3 cm-long resection of one-third of a partial full-thickness esophageal defect including esophageal muscle and mucosa was created (120 degrees of circumference). Reconstruction was supported by interrupted suture for the anastomoses of the 5×3 cm long PU graft (Fig. 2F). Esophageal graft was then covered with a sternocleidomastoid muscle flap for stable fixation and vascular supply of the implanted site (Fig. 2G). The muscle and skin tissues were closed with a 4-0 Vicryl suture.

The dogs recovered from anesthesia, were extubated and were monitored in the recovery room until they were resting comfortably in the sternal position. The dogs were kept in a cage overnight and returned to their larger run housing on postoperative day one. The dogs were given prophylactic antibiotics consisting of cefazolin (1 g/day intramuscularly) for 10 days. Analgesia was provided by intramuscular injection of 13 mg of nalbuphine 2/day (Nalbuphine) until postoperative day 5 and thereafter for analgesia as needed. NPO (nothing per oral) was continued for 6 d after surgery with intravenous total parenteral nutrition support (3,000 kcal/day, Combiflex Lipid Peri Inj) (Supplementary Fig. 1A). The oral liquid diet was started 7 d after surgery (Supplementary Fig. 1B, Supplementary Video 1), a half solid diet was started on the 10th day, and a normal diet (Oriental Yeast) was

resumed 3 weeks after surgery.

Postoperative monitoring was performed daily of the animal's survival and weight, assessing respiratory function and dietary conditions, behavior, and surgical complications such as signs of leakage. Animals were killed if the serious complication was not cured within 1 week. Intravenous administration of potassium chloride (KCL; 1–2 mmol/kg) was used to the euthanize the dogs under anesthesia at 6 and 12 months following surgery.

Endoscopic assessments and computed tomographic analysis

Esophagogastroduodenoscopy (EGD) was performed at 1, 6, and 12 months after implantation to evaluate esophageal structure and function. The animals were anesthetized (by 20 mg/kg ketamine alone) for EGD. EGD was systematically performed to evaluate esophageal patency and assess the inner aspect of the graft sites.

In order to evaluate the functional regeneration of the neoesophagus, a computed tomography (CT; GE Medical Systems) scan after the gastrografin swallow was performed at 1 month, 3 months and 6 months after implantation. The contrast medium, an amidotrizoic acid (Gastrografin; Schering) mixed with milk, was given 15 minutes before image scanning. The patency of the neoesophagus and food passage were evaluated.

Morphological examination of esophageal mucosa

The dogs were euthanized at 6 and 12 months after implantation. Immediately after euthanasia, the cervical esophagus, the grafts and the surrounding tissues were harvested. Scanning electron microscopy (SEM) analysis was performed to observe the morphological characteristics of the regenerated esophageal mucosa. Specimens were prefixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and were immediately postfixed for 2 hours in 1% osmium tetroxide (OsO_4; Sigma Aldrich) dissolved in phosphate buffered saline. Samples were treated in t-butyl alcohol after dehydration through a graded series of ethanol and dried in a freeze dryer. The fixed esophagus specimens were coated with platinum using a sputter coater (IB-5, Eiko) under argon atmosphere conditions. Samples were observed by an SEM (Model S-3000N, Hitachi) operated at an accelerating voltage of 25 kV.

Histological examination

The autopsy was carried out after the scheduled sacrifice. The gross finding of the grafts were accessed, and the graft sites and normal cervical esophagus were immediately fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned serially into 5 μm-thick sections in the transverse direction. The tissue slides were deparaffinized and dehydrated in a graded series of alcohol (in phosphate-buffered saline [PBS]) for 5 minutes each. The tissue slides were then stained with hematoxylin and eosin (H&E) and Masson's trichrome, following standard histological procedure. Elastin staining was also performed using the Elastin (Modified Verhoff's) stain kit (ES4807) according to the manufacturer's instructions. Histological images were captured in triplicate for each group using a light microscope (Olympus).

Immunohistological analysis

For immunohistochemistry, the specimens were immersed in 3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase. The slides were washed with PBS and then incubated with 3% bovine serum albumin to block nonspecific responses. The tissue sections were subsequently reacted with anti-keratin 5 (1:200 dilution, rabbit polyclonal antibody; Abcam) along with the anti-mouse Alexa Fluor 594 secondary antibodies (ab150116, Abcam). The thickness of the regenerative esophageal epithelium was calculated through the area indicated in red using the ImageJ tool bar (n=5). Tissue sections for alpha-smooth muscle actin (α-SMA; 1:50 dilution; mouse monoclonal antibody; ABIN126702) and von Willebrand factor (vWF; 1:200 dilution; rabbit monoclonal antibody; Abcam) were subsequently incubated using the horseradish peroxidase-conjugated kit (Vectastain), and they were visualized using the chromogenic substrate 3,3´ diaminobenzidine (Vector, pk-7800). Hematoxylin was used to counterstained cell nuclei. Images were captured by a fluorescence microscope (BX43-32FA1-S09; Olympus Optical, Japan). α-SMA+esophageal muscle was calculated from the brown-color area around the graft site using ImageJ (n=6). The number of vWF-positive vessels was calculated using ImageJ software. Five areas (high-power field; at ×400 magnification) were measured per slide (n=6 per group) by a blinded observer.

Statistical analysis

The data are expressed as median [interquartile range]. Statistical significance was determined via one-way analysis of variance with Kruskal-Wallis nonparametric test (GraphPad Prism 5, GraphPad Software).

RESULTS

Mechanical properties and cytotoxicity of the two-layered PU scaffold

The mechanical properties of two-layered PU nanofiber (dumbbell-shape, ASTM D412) were examined by the tensile test (Supplementary Table 1). The tensile strength was measured at 12.25±1.86 MPa, the Young modulus at 1.57±0.25 MPa, and elongation at 48.4%±7.8%. The low Young modulus and high elongation value represented a suitable degree of flexibility for esophageal implants. The cytotoxicity of the two-layered PU nanofiber itself was also evaluated by a CCK-8 assay without bioreactor culture. After uniform cell adhesion onto the PU nanofiber, hMSCs were monitored for 5 days. To investigate cell cytotoxicity on the PU scaffold, live and dead staining was done on day 5

and showed that most hMSCs were viable and spread well on the nanofiber structure, indicating that the extracellular matrix (ECM)-like structure of the nanofibers provided a favorable environment (Supplementary Fig. 2A). In particular, hMSCs on aligned fibers (outer layer) showed greater elongation. The CCK-8 assay confirmed that hMSC proliferation increased over time on the inner and outer layers of the cylindrical PU scaffold (Supplementary Fig. 2B).

Esophageal transplantation and the postoperative clinical course

The surgical procedure was successfully completed in all dogs, and there was an uneventful immediate postoperative course in three of the four dogs. A cervical abscess from anastomosis leakage was identified after postoperative day 5 in one of the four dogs. This animal then had to be euthanized as it showed signs of uncontrollable neck swelling due to the persistence of the infection. It was assumed that the saliva leakage was caused by rupture of the implanted PU scaffold. The rest of the beagles showed no signs of esophageal stricture or infection at the final examination. They also continued to eat and drink normally until the scheduled period (12 months after surgery). The mean weight loss was 17.4%±7.7%, and all dogs regained their preoperative weight after 1 month (Supplementary Fig. 1C). The appearance and attitudes of the beagles were also very similar to the preoperative status, and they remained active (Supplementary Video 2). As the study progressed through its first 6 months, the weight of all three animals slowly increased from approximately 10–11 kg to 12–15 kg from their initial weight. There was a much greater increase in weight from the sixth month to the 12th month, with their weights at the time of sacrifice being nearly 1.5 times the initial weights.

Endoscopic examination

The endoscopic examinations of the dogs showed the rapid formation of a normal-appearing mucosal surface by approximately 1 month postoperatively (Fig. 3A). No evidence of active inflammation, erosion, scarring, or necrosis was observed in the endoscopic examinations of the animals' esophagus. Throughout the 12-month study, endoscopy was performed, and the images exhibited complete re-epithelization of the regenerated tissue with what appeared to be a complete epithelium at the 12-month point (Fig. 3A). At 1, 6, and 12 months after implantation, CT demonstrated a normal lumen with no signs of leakage or stricture (Fig. 3B).

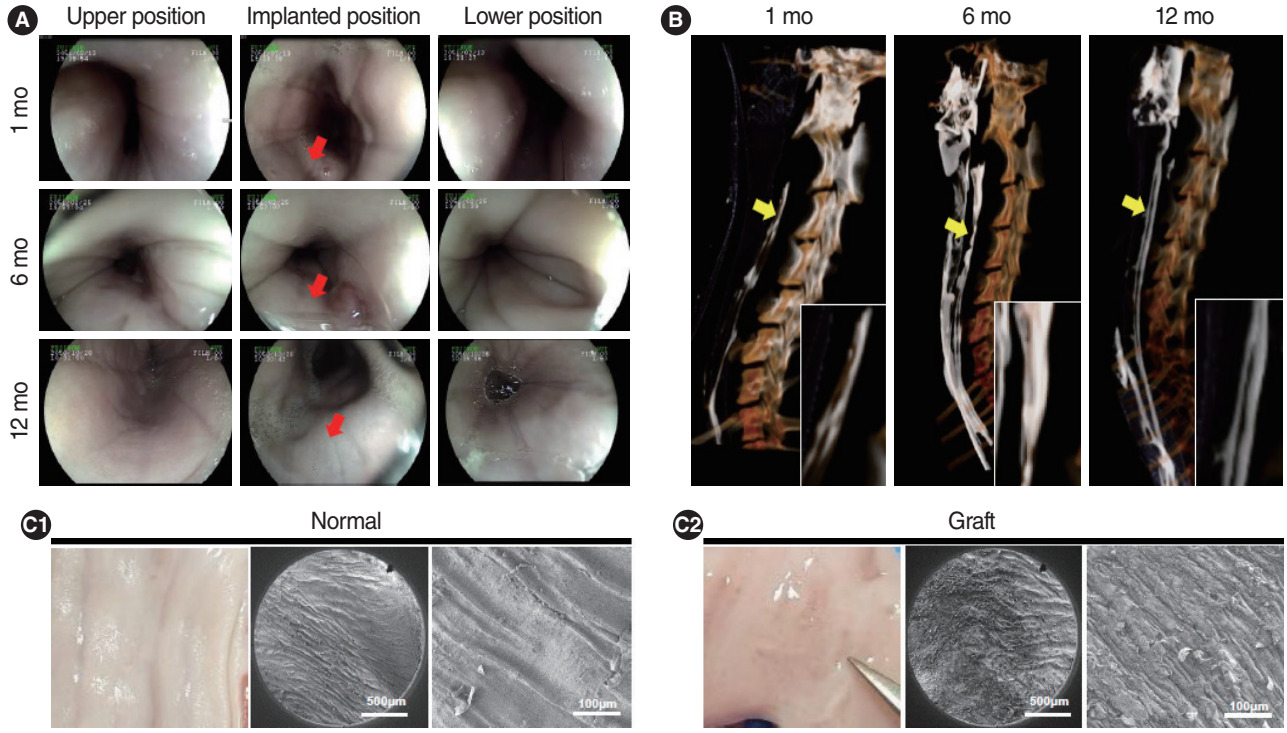

Fig. 3. After transplantation, esophagogastroduodenoscopy was performed at 1, 6, and 12 months to observe the surface conditions inside the esophagus (A). The implanted sites (red arrows) were completely covered with newly formed mucosal layers, showing no stenosis or inflammation. Three-dimensional computed tomography scans were performed at 1, 6, and 12 months after implantation to observe microleakage at the transplanted sites (yellow arrows) (B). No leakage of contrast medium was observed at the graft sites. The morphology of the regenerated mucosal surface at 12 months after transplantation was examined by gross images and scanning electron microscopy analysis of the collected graft sites (C).

Morphological analysis

The gross findings of the esophageal mucosa from the collected esophagus samples showed clear normal mucosal folds, whereas the graft group intermittently exhibited rough surface scars (left images in Fig. 3C). We conducted SEM analysis to interpret the morphology of these regenerated mucosal layers from a more microscopic perspective. Typical morphology of the wavy lumen was confirmed in the normal mucosa. The morphology of the graft group showed non-directional rough wrinkles.

Histological evaluation

Whole histology of the regenerated esophagus was examined by H&E staining (Fig. 4A). The regenerated esophageal epithelium was revealed to be highly complex in wrinkles and arranged atypically. The regenerated submucosa was considerably thick. However, the transplanted scaffold around the regenerated submucosal layer could not be found. It is presumed that the grafted scaffold was detached from the regenerated submucosal layer by continuous peristalsis of the esophagus. In particular, esophageal gland remodeling was observed sporadically at 6 months after transplantation (yellow arrows) (Fig. 4B). The morphology and thickness of the regenerative esophageal epithelium showed similar morphology to that of the normal epithelium through Masson's trichrome staining (Fig. 4C).

Re-epithelialization and elastin contents

Regeneration of the esophageal epithelium at 6 and 12 months after transplantation was identified by keratin-5 immunostaining. Fig. 5A represents the histological morphology of the regenerated esophageal epithelium, indicated in red. The newly regenerated epithelial layer was significantly hyperplastic compared to the normal epithelium at the implanted sites. In the next 12 months, the histological morphology of the stratified squamous epithelium became more similar to that of the normal epithelium (inset box). Quantitative analysis showed that the epithelial thickness tended to decrease slightly at 12 months compared with the thickness at 6 months (Fig. 5B). It was clear that the ECM-like properties of the PU nanofiber scaffolds had a structural effect on

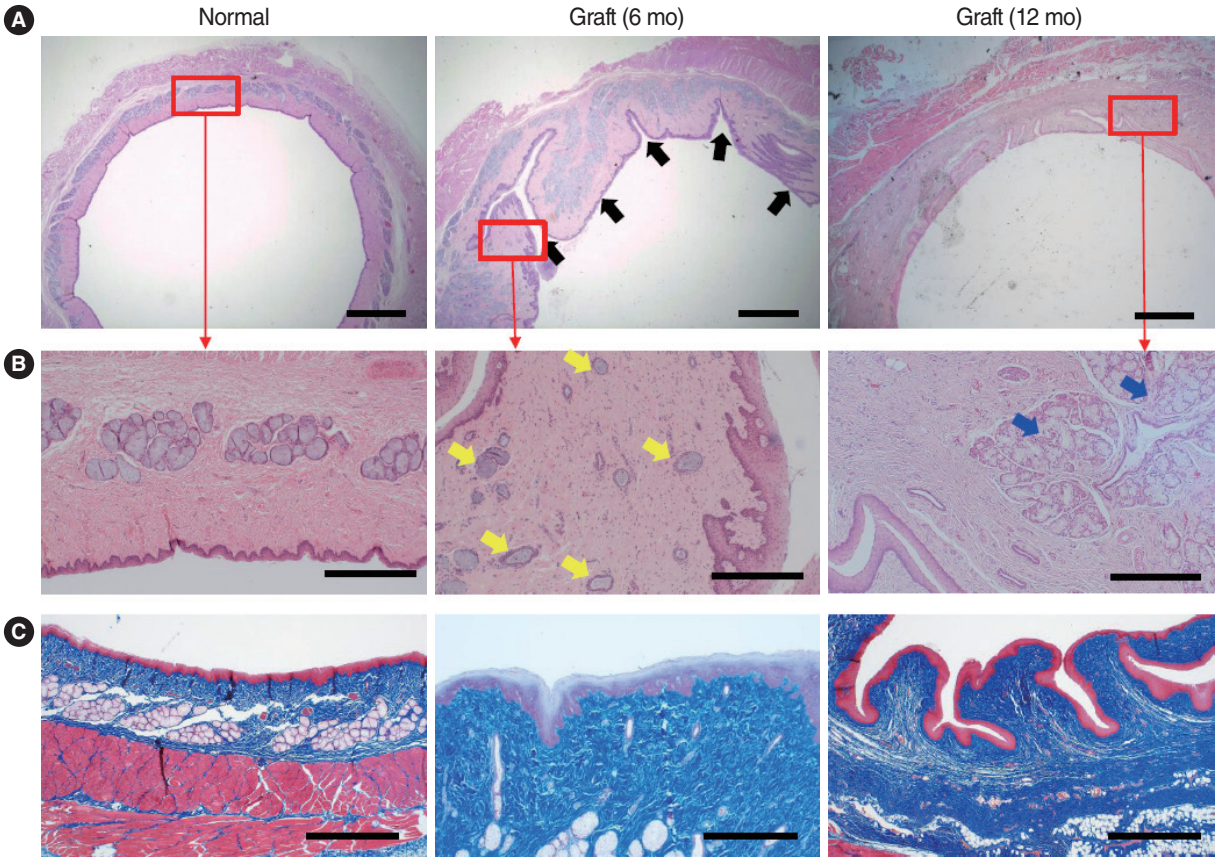

Fig. 4. Whole histology of the regenerated esophagus 6 and 12 months after transplantation (A) (H&E; scale bar, 2 mm). At 6 months after transplantation, regeneration of the mucosal layer was clearly observed (black arrows), but regeneration of the muscle layer was incomplete. It was confirmed that the muscle layer was also regenerated at 12 months. In addition, a newly regenerated esophageal gland (indicated by yellow arrows) was observed in the thickly formed submucosa (B) (H&E; scale bar, 200 μm). At 12 months, the esophageal gland was similar to normal (blue arrows). The histological morphology of the esophageal epithelium and submucosa was clearly characterized by Masson's trichrome staining (C; scale bar, 500 μm).

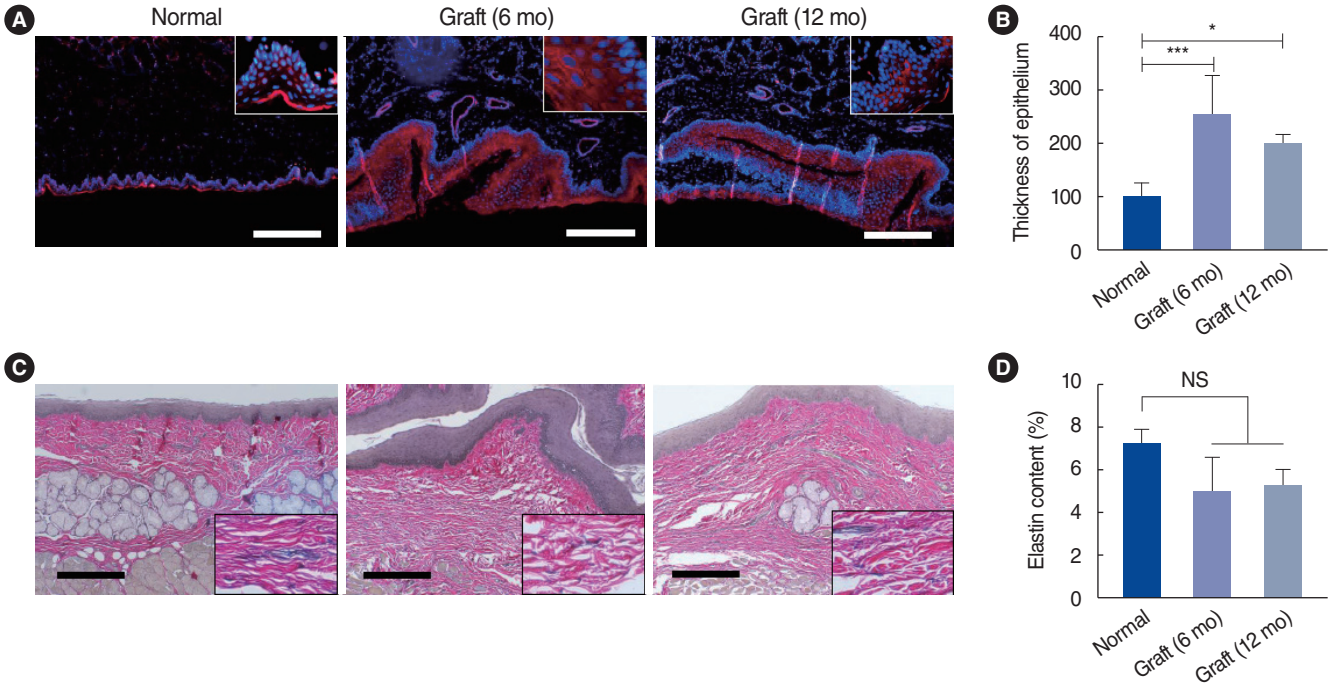

Fig. 5. Re-epithelialization and elastin distribution at 6 and 12 months after scaffold implantation into a partial full-thickness esophageal defect. (A) Keratin-5 immunostaining demonstrated the regenerated esophageal epithelium at 6 and 12 months after implantation (scale bar, 200 μm). (B) The regenerated epithelium was significantly thicker than the normal epithelium (*P<0.05 and ***P<0.001). (C) The regeneration of elastin fibers, indicating the mechanical elasticity of esophageal tissue, was confirmed by elastin immunostaining (scale bar, 200 μm). At 12 months after implantation, elastin fibers with morphologies similar to normal were abundantly observed. (D) No significant differences were found in elastin fibers between the transplanted group (6 and 12 months) and the normal group. NS, not significant.

improving re-epithelialization.

Elastin staining confirmed that remodeling of elastin fibers had occurred in the newly formed submucosal layer (Fig. 5C). There was an abundant distribution of elastin fibers at 6 months, and morphology similar to that of normal tissue was confirmed at 12 months. Additionally, at 12 months after transplantation, the elastin distribution was not significantly different from that observed in normal tissue (Fig. 5D). The abundant presence of elastin components means that the elastic properties of the regenerated esophageal tissue were also similar to those of normal tissue.

Muscle regeneration and neovascularization

α-SMA immunostaining was performed to investigate whether the tissue-engineered esophagus could affect esophageal muscle regeneration. Strong brown colors were observed around the implanted sites (Fig. 6A). α-SMA$^+$ muscle was observed intermittently 6 months after transplantation. In particular, the alignment of the regenerated muscles was irregular and abnormal. In comparison, with Masson's trichrome staining, the 6-month sample showed a dense penetration of collagenous tissues (blue color) between muscle layers (red color) (Fig. 6B). It was also difficult to distinguish between the circular and longitudinal muscles found in normal muscles. However, the amount of regenerated muscle increased over time (Fig. 6C). It is assumed that the hMSC-inoculated PU scaffold may have been effective for quantitatively increasing the amount of muscle regenerated to replace the lost esophageal muscle. However, it was not possible to accurately characterize the structural advantages of the inner and outer layers of the scaffold for muscle regeneration.

Immunostaining for vWF, an endothelial marker, was performed to confirm neovascularization in the esophageal submucosa, including the lamina propria, around the implanted site (Fig. 6D). New blood vessels were abundantly observed at both 6 and 12 months around the regenerated esophageal submucosa (black arrows). Quantitatively, both time points showed significantly higher neovascularization than in the normal esophageal layer (Fig. 6E).

DISCUSSION

Several strategies have been proposed for tissue-engineered esophagus development. With regard to cellular orientation, precisely controlled nano- or micro-patterned scaffold surfaces have been employed to promote muscle cell differentiation [11,12]. Diverse bioreactor systems enabling the mechanical stimulation of cell-seeded scaffolds have been developed, serving as platforms that promote myotube alignment and muscle maturation [13]. Therefore, scaffolds, cells, and optimal culture conditions must be combined appropriately for successful tissue engineering. Ideally, the

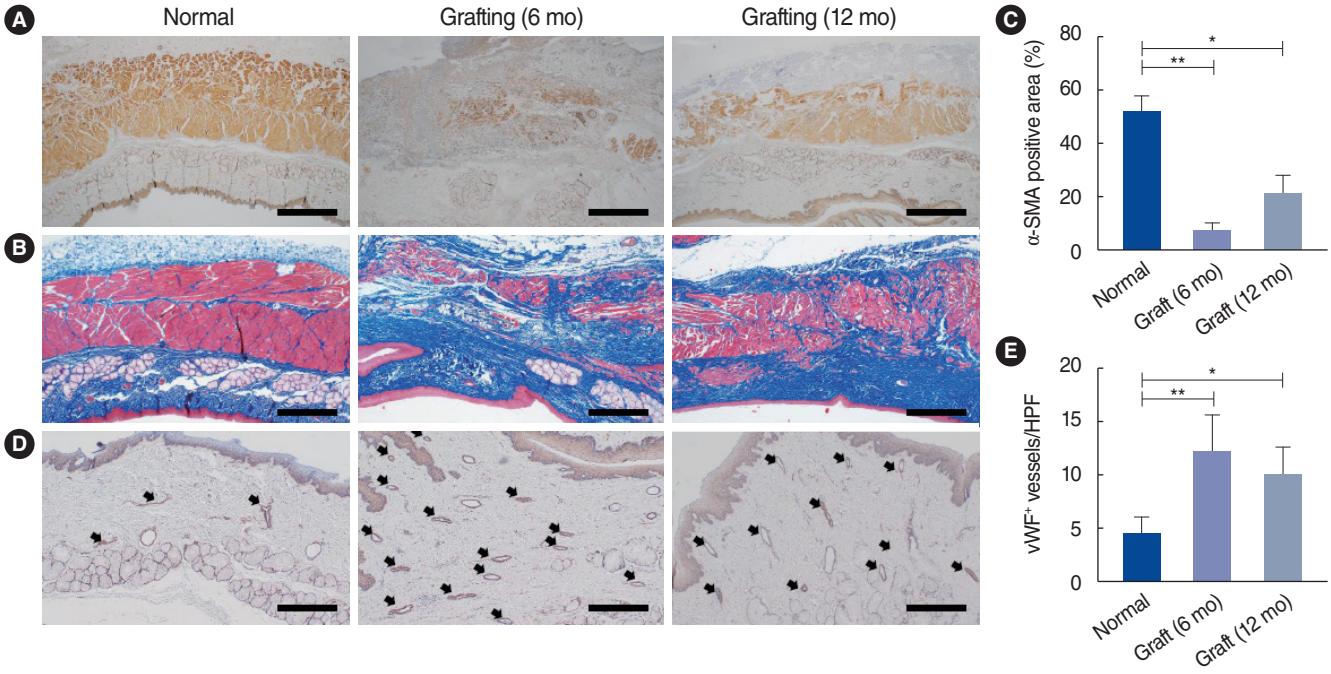

Fig. 6. Immunohistochemical staining of regenerative esophageal muscle and neovascularization at 6 and 12 months after implantation. (A) Representative images of alpha-smooth muscle actin (α-SMA) immunostaining in the reconstructed esophagus after surgery (scale bar, 500 μm). α-SMA-positive signals exhibited newly regenerated muscle adjacent to the implanted sites. (B) Masson's trichrome shows the morphology of the regenerated esophageal muscle and the distribution of collagen remodeling (scale bar, 500 μm). (C) Quantitative analysis of the α-SMA-positive area. (D) Representative image showing the regenerated blood vessels by von Willebrand factor (vWF) expression. The arrows represent vWF-positive vessels (scale bar, 200 μm). (E) Statistical analysis of the number of vWF-positive blood vessels per high power field. *P<0.05, **P<0.01.

scaffold should be similar to the native tissue in terms of structure and mechanical behavior. The esophagus is a hollow organ with a luminal non-directional epithelial layer, submucosal connective tissue, and outer aligned muscular layer [14]. In addition, nanofiber scaffolds have been used frequently in esophageal tissue engineering, and they have been suggested to exhibit good mechanical properties and to promote esophageal regeneration [8].

The esophagus has a number of tissue-engineering options to consider for reconstruction due to its relatively complex structural and physical characteristics compared to other organs [15]. An ideal tissue-engineered esophagus should be extremely protective of the lumen and take into account the cell lines necessary for regeneration and the physical properties necessary for peristalsis and structural stability [16]. In addition, after oral intake, the esophagus should be enlarged to accommodate the oral bolus without rupture or leakage. At present, despite the remarkable development of various biomaterials, no artificial esophageal products have been approved by regulatory agencies. From these trends, it is clear that large animal models capable of demonstrating the safety and efficacy of tissue-engineered constructs need to be developed. Therefore, the aim of this study was to determine whether histological and functional replacement of the esophagus is possible using synthetic scaffolds cultured with a custom-designed bioreactor system optimally preconditioned for the esophageal environment after partial esophageal resection in a large animal model. Beagles are suitable for these tests, because they have structural features and diameters similar to those of humans. Moreover, unlike other large animals, it is relatively easy to manage their nutrition supply after esophageal transplantation.

The initial esophageal scaffold that was attempted was an acellular collagen sponge [17]. Despite modifications and implantation by various routes, those scaffolds faced developmental barriers due to the lack of elasticity and microleakage of saliva. Subsequent attempts at esophageal constructs were tissue-derived implants that had undergone decellularized processes [1,18]. This approach clearly had the advantage of excellent tissue elasticity and prevented microleakage of saliva, but it had the disadvantage of causing severe stenosis. These scaffolds were also potentially vulnerable to a secondary immune response at any time. Some researchers tried to overcome stenosis using esophageal stent implantation [17,19]. However, this inevitably required a reoperation to remove the stent, which could cause additional complications.

Therefore, for an alternative artificial esophagus, several important factors must be considered in animal research. The ideal esophageal substitute should be biocompatible, the scaffold should be accessible to adhesion and migration of surrounding epitheli-

al cells and muscle cells, and it should not cause inflammation and infection. The mechanical properties of the esophagus are essential because esophageal tissue is constantly exposed to maximum stretching from esophageal peristalsis [5]. Therefore, it is important for implanted scaffolds to have characteristics similar to the native esophagus. The burst and dilation strength of these scaffolds should be excellent. Otherwise, the transplantation site may collapse, causing saliva to leak and thereby resulting in severe conditions such as mediastinitis. In contrast, scaffolds that are too hard could bulge into the esophageal lumen, blocking the passage of food.

Medical PU is suitable as a base material or esophageal reconstruction for several reasons. First, PU is a highly biocompatible and non-degradable polymer [10]. It also offers excellent mechanical and physical properties. We verified its high elasticity by manufacturing PU nanofiber membranes in previous studies [9]. This mechanical stability allows the scaffold to withstand swallowing stress during tissue regrowth when surgically implanted. PU nanofibers exhibit excellent cell adhesion and proliferation and can provide an environment favorable for initial mucosal formation at the implanted esophageal anastomosis [5]. In addition, the toughness and flexibility of PU can significantly reduce the possibility of anastomosis collapse caused by esophageal peristalsis. However, structural modification with another high-strength biodegradable polymer is required for transplantation of esophageal circumferential defects. Therefore, this study implanted double-layered PU nanofibers into a partial esophageal defect model.

It is obvious that scaffolds are better when they are closely similar in structure and composition to the relevant tissues. For tubular scaffolds (e.g., blood vessels), many studies have used electrospun scaffolds, although the materials used have varied among studies [20]. In this study, a double-layered scaffold was fabricated by electrospinning, while the structure of the esophagus was taken into consideration, with the inner surface being randomly oriented and the outer surface being circumferentially oriented. That is, the outer region was designed based on the layer in which the muscle cells are located. This layer, where muscle cells are circumferentially oriented, is expected to have sufficient mechanical strength to provide support against the expansion of the esophagus when food is being passed. The fibers mainly ranged in size from 200 to 500 nm [10]. Although the effects of micro-/nano-topography were not fully explored in the present study, our previous study demonstrated the effect of a nanofibrous substrate on esophageal epithelial differentiation [9], while another study showed that an aligned micro-fibrous substrate improved muscle differentiation and function. Therefore, we hypothesized that the fabricated double-layered scaffold might play a positive role in esophageal tissue engineering by inducing the differentiation of hMSCs to esophageal epithelial and muscle lineages simultaneously.

In the present study, we used MSCs, which are a promising therapeutic tool for tissue engineering due to their differentiation potential and proangiogenic, immunomodulating, and anti-inflammatory properties, as has been shown for cartilage, bone, and myocardial regeneration [21,22]. We expected to accelerate the formation of early esophageal mucosa from the inner surface of MSC inoculation. The esophageal epithelium, which is covered with stratified squamous epithelial cells, protects the underlying tissue from physical or biochemical damage [23]. *in vitro* studies of esophageal epithelial differentiation have enhanced our understanding of epithelial homeostasis and provided a theoretical foundation for esophageal tissue engineering. Specifically, MSCs have been reported to maintain epithelial homeostasis by continuously repairing necrosis or damaged esophageal epithelium [24]. Thus, epithelial differentiation and reconstruction are important processes that ensure esophageal function and are associated with life preservation in the recipient. The nanoscale surface properties and mechanical forces that occur during differentiation induction are known to play an important role in the epithelial differentiation of MSCs. Mechanical forces have been reported to affect the development and function of epithelial tissue [25]. The main mechanical force on the esophagus is believed to be the intraluminal pressure applied during swallowing. This intraluminal pressure is caused by peristalsis of the food mass. In a previous study, we converted this pressure into shear stress and applied it to a bioreactor system [5,9]. Based on this technical theory, the MSC-seeded PU scaffolds were transplanted into the esophagus after 3 days of dynamic culture using a bioreactor.

After orthotopic esophageal scaffold implantation, wound healing requires coordinated signal transduction between cells and the ECM and signaling molecules [26]. It is also suspected that MSCs are involved in the regulation of the inflammatory response and promotion of healing. This phenomenon can be explained by MSCs secreting growth factors involved in wound healing, such as transforming growth factor, basic fibroblast growth factor, and platelet-derived growth factor [27]. Although this issue was beyond the scope of our study, re-epithelialization via MSCs has been reported to have anti-inflammatory and anti-fibrotic effects [28]. Finally, MSCs can help muscle cell regeneration through a paracrine effect [5,10]. This paracrine effect will have significant advantages in the remodeling of the esophageal muscle through signaling processes that stimulate angiogenesis and inhibit fibrosis. These results were verified by numerous blood vessels under vWF immunostaining beneath regenerated epithelial tissue (Fig. 6D and E). Additionally, MSCs have heterogeneous immunosafety because they have immune privileges and, more importantly, immunomodulatory capacities.

This study demonstrated multi-layered esophageal regeneration of esophageal defects; however, there are some limitations to this method. In fact, the greatest obstacle to the clinical application of an artificial esophagus is the choice of an appropriate grafting material and early tissue regeneration to prevent post-

operative complications such as anastomotic leakage and stenosis. Although the scaffold itself is very difficult to tear, extensive tissue infection and complications due to anastomotic leakage resulted in one death. Furthermore, we explored this possibility by applying a minimal animal model, with consequent limitations in confirming the absolute therapeutic efficacy of MSCs due to the absence of a control group.

Despite the aforementioned limitations, the study clearly demonstrates the potential for human therapeutic use, as bioreactor-cultured electrospun PU scaffolds were capable of esophageal regeneration in a dog treatment model.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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SUPPLEMENTARY MATERIALS

Supplementary materials can be found online at https://doi.org/10.21053/ceo.2022.01522.

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