Tissue-engineered artificial oesophagus patch using three-dimensionally printed polycaprolactone with mesenchymal stem cells: a preliminary report

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INTRODUCTION

Several congenital and acquired medical conditions necessitate oesophageal reconstruction [1]. The conventional method is reconstruction using visceral organs such as the stomach, jejunum and colon, but these methods are invasive and can result in post-operative complications and changes in the normal gastrointestinal physiology. Thus, tissue engineering of an artificial oesophagus is needed. Various materials have been applied for artificial oesophageal replacement including acellular scaffolds, decellularized matrices and synthetic materials [2]. Despite these endeavours, the ideal materials for artificial oesophagus that meet the anatomical, functional and physiological requirements have not been identified.

Among various biodegradable synthetic materials, polycaprolactone (PCL) has excellent mechanical strength and durability with biocompatibility [3]. PCL is water-soluble and compatible with current 3D printing (3DP) systems. The tailorable characteristics of PCL, which originate from its very low glass transition temperature and melting point, enable its use in 3DP devices [4, 5]. A previous study reported promising results of a PCL mesh as a scaffold for an artificial oesophagus, but a pseudo-diverticulum developed around the mesh due to rapid degradation of PCL [6]. In addition, a human-derived fibrin has favourable features including high biocompatibility and biodegradability, and therefore is useful as a matrix for cell delivery [7]. We formulated the hypothesis that

OBJECTIVES: There has been a recent focus on 3D printing with regard to tissue engineering. We evaluated the efficacy of a 3D-printed scaffold coated with mesenchymal stem cells (MSCs) seeded in fibrin for the repair of partial oesophageal defects.

METHODS: MSCs from rabbit bone marrow were cultured, and a 3DP polycaprolactone (PCL) scaffold was coated with the MSCs seeded in fibrin. The fibrin/MSC-coated 3DP PCL scaffold was implanted on a 5 × 10 mm artificial oesophageal defect in three rabbits (3DP/MSC group) and 3DP PCL-only scaffolds were implanted in three rabbits (3DP-only group). Three weeks post-procedure, the implanted sites were evaluated radiologically and histologically.

RESULTS: None of the rabbits showed any infection, stenosis or granulation on computed tomography. In the 3DP/MSC group, the replaced scaffolds were completely covered with regenerating mucosal epithelium and smooth muscle cells as determined by haematoxylin and eosin and Desmin staining. However, mucosal epithelium and smooth muscle cell regeneration was not evident in the 3DP-only group.

CONCLUSIONS: Use of the 3DP scaffold coated with MSCs seeded in fibrin resulted in successful restoration of the shape and histology of the cervical oesophagus without any graft rejection; thus, this is a promising material for use as an artificial oesophagus.

Keywords: 3D-printing • Oesophageal regeneration • Fibrin • Tissue engineering • Mesenchymal stem cell
PCL coated with mesenchymal stem cells (MSCs)/fibrin would facilitate the regeneration of oesophageal tissue (including smooth muscle cells) after implantation.

In this preliminary study, we investigated the outcomes of a 3DP PCL-only scaffold and fibrin/MSC-coated 3DP PCL scaffolds in the reconstruction of a wedge-shaped cervical oesophageal defect in a rabbit model. The clinical, radiologic and histological characteristics of a PCL-only scaffold and hybrid oesophageal implant composed of fibrin/MSCs with a 3DP PCL scaffold for oesophageal reconstruction were evaluated.

MATERIALS AND METHODS

Preparation of 3D-printed oesophageal scaffold

The 3DP scaffold was fabricated using PCL (Sigma-Aldrich, St Louis, MO, USA, Catalog No. 440744) as previously described [5, 8, 9]. The polymer pellets were melted at 130°C in a heating cylinder, and 3DP scaffolds were constructed using the Bioplotter® System (EnvisionTEC GmbH, Gladbeck, Germany). A 3DP scaffold (5 × 10 mm) was plotted in a layer-by-layer manner through the extrusion of melted PCL onto a cylinder [8]. The thickness of the PCL scaffold was 2 mm, and the grid created by deposition of PCL was 0.5 × 0.5 mm in size. The pores of the 3DP PCL scaffold were visualized by scanning electron microscopy (SEM) (Fig. 1).

Isolation and implantation of mesenchymal stem cells

The Committee for Ethics in Animal Experiments, Ajou University School of Medicine, approved this study. Bone marrow-derived MSCs were isolated from rabbit femurs and tibias (New Zealand white rabbits, male, 3 months of age) [8, 10, 11]. Under anaesthesia with zolazepam (4.0 mg/kg) (Virbac) and tiletamine (4.0 mg/kg) (Virbac Ltd, Carros, France), the bone marrow was aspirated at the femur and the tibia with an 18-gauge needle [8]. Aspirated materials were diluted in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal bovine serum, 100 µg/ml streptomycin (Gibco BRL) and 100 U/ml penicillin G (Gibco BRL, Grand Island, NY, USA). Non-adherent cells were removed by irrigating in phosphate-buffered saline and subsequent changes of medium. Culture medium was exchanged every 3 days. The cells were trypsinized and subcultured at ~50% confluence. The MSCs were passaged two times prior to their use in experiments.

MSCs were pelleted by centrifugation and reseeded in a human-derived fibrinogen (9–18 mg/ml) (Mokam Research Center, Suwon, South Korea) solution [8, 9]. An MSC suspension of 5 × 10^5 cells/ml was then mixed homogeneously with 110 KIU/ml aprotinin (Mokam Research Center), 60 U/ml thrombin (1000 U/mg protein) (Sigma, St Louis, MO, USA), 50 U fibrin stabilizing factor XIII and 50 mM CaCl₂ [9]. The 3DP scaffolds, both pores and PCL strands, were completely coated with the fibrin-containing MSCs as a thin layer. The scaffold was split into cubes (5 × 3 × 1 mm). After platinum coating using a model SC 500K plasma sputter (Emscope, West Sussex, UK), each scaffold was examined with a model S-4800 SEM (Hitachi, Tokyo, Japan) operating at 10 kV. A live/dead cell assay confirmed that over 90% of the implanted MSCs in the fibrin-coated 3DP scaffold were viable (Fig. 2).

Surgical procedures

Six rabbits were prepared and randomly assigned to the 3DP-only group (3DP PCL-only scaffold, n = 3) and 3DP/MSC group (fibrin/MSC-coated 3DP PCL scaffold, n = 3). Under anaesthesia, an 8-Fr Levin tube was inserted through the nose under laryngoscopic guidance. The rabbit was placed in a supine position with the neck extended. A vertical skin incision was made at the midline and the strap muscles were divided. Following appropriate exposure of the cervical oesophagus, a 10 × 5 mm, wedge-shaped oesophageal defect was made in the cervical oesophagus using a scalpel and electrocautery (Fig. 3A). In the 3DP/MSC group (n = 3), the defect was covered with the fibrin/MSC-coated 3DP PCL scaffold, and was sutured using Vicryl 5-0 (Ethicon, Inc., Somerville, NJ, USA) interrupted sutures (Fig. 3B and C). In the 3DP-only group (n = 3), the defect was covered with the 3DP PCL-only scaffold in an identical manner. Following the operation, oral ingestion was prohibited for 1 week and rabbits were fed cat milk through a Levin tube. Mouth feeding was started 7 days after the procedure.

Radiological and histological assessment

Computed tomography (CT) scan was performed using a CT scanner system (Brilliance 64; Philips, Eindhoven, Netherlands) at 3 weeks postoperatively. Axial images were taken at 1 mm intervals. The animals were sacrificed after the radiological examination. The cervical oesophagus (including the implanted site) was resected and subjected to visual inspection. The specimen was then processed for light microscopy. After fixation in 10% neutral-buffered formalin for 24 h, the dissected tissues were embedded in paraffin and cut into sections of 4 µm thickness; the sections were deparaffinized and rehydrated, and stained with haematoxylin and eosin (H&E). Next, immunohistochemistry using Desmin staining was performed to evaluate the generation of smooth muscle layers.

RESULTS

Clinical and radiological outcomes

All of the six rabbits survived the procedure and showed no signs of complications, such as infection. All of the rabbits started oral feeding at 7 days post-procedure and showed no complications. Axial CT scan images also showed no definite signs of leakage, granulation or infection around the cervical oesophageal implants in both groups (Fig. 4A and B).

Histological and immunohistochemical outcomes

Upon visual inspection, both groups showed a patent oesophageal lumen and no stenosis or obstruction. The implanted 3DP PCL scaffolds remained in place in both groups. Mucosal epithelium and smooth muscle were observed within the 3DP PCL scaffold in the 3DP/MSC group, but not the 3DP-only group, as determined by H&E staining (Fig. 5A and B). Immunohistochemical Desmin staining revealed the regeneration of smooth muscle cells in the 3DP/MSC group, but not in the 3DP-only group (Fig. 6A and B).
DISCUSSION

The ideal artificial oesophagus must meet several requirements. For example, it should not cause infection, stenosis or leakage; must be resistant to the low pH environment due to the presence of gastric acid; and ideally, would coordinate the peristaltic movement of the gastrointestinal tract. Several studies on tissue engineering for oesophageal replacement have been performed [1, 2]. Acellular scaffolds, such as polyethylene plastic and silicon, did not facilitate epithelial regeneration and resulted in poor outcomes, such as infection and severe oesophageal stenosis. Decellularized matrices, such as small-intestinal sub-mucosa or decellularized xenograft oesophagus, did not result in stenosis after replacement of the mucosal defect, but their mechanical durability is not appropriate for maintaining the oesophageal lumen. Furthermore, these previous studies mainly focused on regeneration of the mucosal epithelium and preventing stenosis. The native oesophagus has three layers: the mucosa, which consists of squamous epithelial cells; the sub-mucosal layers, which are composed of collagen types I and III; and the muscularis externa, which is composed of inner circular and outer longitudinal smooth muscle cells. To mimic the physiology of the native oesophagus, an artificial oesophagus must have such a multiple-layer structure. However, previous studies of the artificial oesophagus only focused on the regeneration of mucosal epithelium without consideration of the multiple-layer structure [1, 2].

The novelty of the present study lies in its focus on regeneration of both mucosal epithelial cells and smooth muscle cells to produce a multiple-layer structure, compared with previous studies, which only focused on mucosal regeneration [1]. The regeneration of smooth muscle cells is critical for the restoration of a functional oesophagus. Re-epithelialization is also important for preventing infection, formation of granulation tissue, fibrosis and eventual stenosis. We hypothesized that a 3DP scaffold with both naturally derived fibrin and synthetic PCL could be used to create a tissue-engineered artificial oesophagus comprising multiple layers of mucosal epithelium and smooth muscle. As expected, the implanted 3DP PCL-only scaffold and fibrin/MSC-coated 3DP scaffold would be beneficial for controlling infection, facilitating epithelialization and inhibiting stenosis.

Figure 1: SEM images of PCL 3D-printed scaffolds with a fibrin coating containing mesenchymal stem cells. PCL strands formed a 3D grid structure; SEM: scanning electron microscope; PCL: polycaprolactone.

Figure 2: Live/dead assay of implanted MSCs on the scaffold. Live cells are stained with green fluorescent calcein-AM, and dead cells are stained with red fluorescent ethidium homodimer-1. Cells were enumerated using the ImageJ software. The survival of implanted MSCs was determined by the green: red fluorescence ratio; over 90% of cells remained viable following implantation. Scale bar: 100 μm in the upper column, 400 μm in the lower column. MSCs: mesenchymal stem cells; AM: acetoxymethyl ester.
PCL scaffold successfully reconstructed a defect of the cervical oesophagus at 3 weeks post-procedure in this study. Although both scaffolds did not show leakage and infection, regenerated mucosal epithelium and smooth muscle cells were observed only with the fibrin/MSC-coated 3DP PCL scaffold. The reason for the lack of leakage or infection with the 3DP PCL-only scaffold lies in the maintenance by 3DP PCL of the tissue integrity of the cervical oesophagus without degradation. The fibrin/MSC-coated 3DP PCL scaffold provided a mechanically stable and biochemically favourable cellular environment for the regeneration of both smooth muscle and epithelial cells [8]. The technique of 3DP has recently been employed for the hybridization of scaffolds [12, 13]. It enables the fabrication of viable tissue architecture by depositing the biomaterials in a layer-by-layer fashion. In addition, 3DP enables the fabrication of a 3D solid scaffold of any shape from digital images, and produces accurate and reproducible scaffolds at minimal cost compared with conventional fabrication techniques [8, 14]. In spite of the difficulty in material selection, we assumed that application of the 3DP technique would facilitate oesophageal reconstruction using engineered tissue.

PCL is a synthetic, absorbable polymer that can be widely applied in the field of tissue engineering [8]. It has an average molecular weight of 80,000 Da with a melting point of 60°C, and as such, can be applied in a 3D printer with no requirement for toxic solvents [5, 12]. PCL also has good mechanical properties and biodegradability by hydrolysis [15]. In a recent paper focusing on tissue-engineered artificial oesophagus, Diemer et al. reported the application of a PCL mesh as a scaffold for an artificial oesophagus [6]. However, PCL has limitations in recruiting or delivering cells that can regenerate tissue. We envisaged a hybrid scaffold using PCL and a naturally derived material, fibrin. In previous reports, our group reported successfully applications of a fibrin-based scaffold containing chondrocytes to tracheal reconstruction and vocal-fold augmentation [15, 16]. Based on this work, MSCs seeded in human-derived fibrin were coated on the 3DP PCL scaffold. Fibrin also has several advantages as a biological material for tissue engineering, such as biodegradability, biocompatibility, abundant fibronectin and a high affinity for biological tissue. Fibronectin increases the expression of proteins related to smooth muscle cell differentiation [17]; therefore, fibrin glue can facilitate

Figure 3: Insertion of a tissue-engineered tracheal implant using a 3DP scaffold. (A) The 3DP scaffold composed of PCL coated with fibrin-containing MSCs. The 3DP scaffold was 5 × 10 mm, with a thickness of 2 mm. The grid comprising horizontal and vertical strands of PCL was 0.5 × 0.5 mm in size. (B) A partial cervical oesophageal defect, approximately 5 mm wide by 10 mm long, was made in the cervical oesophagus of a New Zealand white rabbit. (C) Insertion of the oesophageal implant into the oesophageal defect. The incision was sutured in an interrupted manner using 5-0 Vicryl sutures. 3DP: 3D-printed; PCL: polycaprolactone; MSCs: mesenchymal stem cells.

Figure 4: CT findings at three weeks post-procedure. (A) Axial CT of the 3DP-only group. (B) Axial CT of the 3DP/mesenchymal stem cell group. Implanted grafts did not exhibit radiological signs of infection or leakage in both groups (red circles). Scale bar: 6.0 cm. CT: computed tomography; 3DP: 3D-printed; PCL: polycaprolactone; MSCs: mesenchymal stem cells.
a favourable environment for the survival and differentiation of MSCs into smooth muscle cells. In the present study, the live/dead assay showed that over 90% of the implanted MSCs survived on the scaffold. In spite of these advantages in cell delivery, fibrin is absorbed after only 1–4 weeks under in vivo environments [18, 19]. The 3DP PCL scaffold can compensate for the early degradation and weak mechanical properties of fibrin [8].

In the present investigation, regeneration of smooth muscle cells was confirmed by immunohistochemical staining for Desmin in the area in which the fibrin/MSC-coated 3DP PCL scaffold was implanted. Desmin is the major muscle-specific intermediate filament protein in the dense bodies of smooth muscle cells [20]. Its expression is strictly regulated in the context of developmental programmes [21]. Desmin expression in fibrin/MSC-coated 3DP PCL scaffolds suggests active regeneration of smooth muscle cells. The regenerated smooth muscle cells may have derived from the implanted MSCs or migrated from the adjacent normal oesophageal tissue. MSCs can differentiate into cells of mesengenic lineage, such as smooth muscle cells, chondrocytes, adipocytes, osteoblasts and neural cells in the appropriate environments [8, 22, 23].
In addition, MSCs have considerable paracrine immunomodulatory and regenerative effects [24]. MSCs produce various cytokines, chemokines, growth factors and extracellular matrix molecules. The paracrine effect of MSCs might modulate the microenvironment of tissue, thereby facilitating the recovery of damaged tissue. The primary aim of cell therapy with MSCs is regeneration and replacement of damaged tissues by differentiated MSCs, and recent studies have focused on their paracrine effects [8, 25].

Despite the promising findings of the 3DP scaffold with PCL in the present study, this approach might have several limitations for oesophageal reconstruction. Firstly, because this was a preliminary study, the reconstructive outcome was only evaluated at 3 weeks post-procedure. Before clinical application, larger scale animal studies with long-term follow-up are necessary. Secondly, the analyses in this study were not quantitative, but rather, were only qualitative. Future studies should involve tracing of implanted cells and evaluation of smooth muscle regeneration. Finally, this study involved reconstruction of an only partial defect of the cervical oesophagus. Circumferential reconstruction is more important in clinical situations; therefore, future studies should assess circumferential reconstruction in longer segments of the oesophagus. In addition, we are planning to use the 3DP scaffold coated with MSCs in pathological conditions such as the animal model of Boerhaave’s syndrome and in other locations such as the mid-oesophagus and the esophago-gastric junction instead of the cervical oesophagus, with sufficient number of animals. The physical properties of the scaffold, such as acid resistance and motility, should be also investigated in vitro. Despite these limitations, the present study is the first report of an artificial oesophagus with a multiple-layer structure and use of the 3DP technique for production of an artificial oesophagus using an animal model.

In conclusion, bioimplants comprising a 3DP PCL scaffold and fibrin/MSCs facilitated successful reconstruction of the rabbit cervical oesophagus, with regeneration of mucosal epithelium and smooth muscle cells at 3 weeks after implantation. Thus, the 3DP PCL scaffold with allogenic MSCs represents a promising material for the anatomical and functional reconstruction of the oesophagus.

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REFERENCES


