



Original Article

Evaluation of human keratinocyte sheets transplanted onto porcine excised esophagus after submucosal dissection in an ex vivo model

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ABSTRACT

Background: The utility of endoscopic transplantation of epithelial cell sheets to ulcer sites after endoscopic submucosal dissection (ESD) has been shown to prevent scar stenosis after ESD of early esophageal cancer. Previously, our group reported use of an endoscopic transplantation device fabricated with a 3-dimensional printer. Cell sheets are transplanted to the esophageal wound site with the following procedure: first, a cell sheet harvested from temperature-responsive culture dishes is placed on the device's deflated balloon surface and transported to the wound site with endoscopic forceps; second, by applying pressure from inflating the balloon locally at the wound site, the cell sheet is successfully transferred and adhered to the wound tissue; third, the balloon is deflated, and the device is removed. By repeating the procedure, several cell sheets can be safely transplanted to a wider ESD area. Nonetheless, possible damage to cell sheets using this procedure has not yet been assessed.

Objective: Effects of endoscopic transplantation balloon inflation on cell viability and damage of normal human epidermal keratinocyte sheets resident on the device's balloon surface were evaluated by histology after sheet placement onto luminal surfaces in the ex vivo porcine submucosal dissection esophagus model. Endoscopic transplantation of these same cell sheets with conventional methods using a polyvinylidene fluoride (PVDF) cell sheet support membrane, balloon device transfer, and also using a novel modified balloon transfer procedure was also examined. Cell sheet transfer results obtained with these three procedures were compared.

Method: Normal human epidermal keratinocyte sheets were fabricated on temperature-responsive culture inserts. By temperature reduction to 20 °C, all cells were harvested as a single contiguous cell sheet. Freshly excised porcine esophagi purchased in a slaughter house were turned inside-out, and the exposed luminal mucosa and submucosal layers were removed by Cooper scissors. This luminal surface was then utilized as a transplantation bed in ex vivo cell sheet experiments. Cell sheets were adhered to the endoscopic transfer device balloon, expanded by balloon inflation and resulting cell viability was evaluated by trypan blue exclusion test after cell sheet trypsinization and dispersion. Cell sheets were transferred onto the esophagus lumen ex vivo using forceps and the balloon device, and also using a modified balloon transfer method. The obtained results were compared with those without balloon expansion, and evaluated for sheet thickness and luminal histology. Finally, TUNEL staining was performed to examine cell apoptosis.

Result: Cell sheets thinned after cell sheet balloon expansion, but no apoptosis was observed after these procedures.

Conclusion: Expanding keratinocyte cell sheets on a balloon endoscopic transfer device did not damage the cell sheets. This sheet transplantation method using the endoscopic balloon transfer device may be

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considered as a future standard cell sheet endoscopic transplantation procedure for repairing the esophagus.

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1. Introduction

In recent years, endoscopic submucosal dissection (ESD) is performed to locally remove lesions from early stage esophageal, gastro, and colon cancers. Although ESD is less invasive to the patient than conventional open surgery, esophageal ESD often causes esophageal scar stenosis at sites of large cancer resection. In order to treat esophageal stricture after ESD, balloon dilatation or esophageal stenting is used, but these treatments are very painful to patients. Repeat treatment is sometimes required since stenosis recurs; resulting patient QOL is reduced. Recently, local anti-inflammatory steroid injection to the ESD site and oral steroid administration are also shown to be useful for preventing esophageal stricture. But the complications of steroid administration such as susceptibility to infection, osteoporosis, and hyperglycemia remain concerns. Therefore, we previously developed a cell-based treatment to stricture using autologous oral mucosa epithelial cell sheets to prevent esophageal stenosis after ESD of early superficial cancer [1]. After a report of preclinical study using a canine model [1], we performed a human clinical study in three different hospitals [2–5]. Different endoscopists successfully transplanted cell sheets to patients, with good outcomes. These grafted epithelial cell sheets cover luminal ulcerations after ESD to protect wound surfaces; wound healing is promoted to reduce esophageal stricture as well. Originally, autologous oral mucosa epithelial cell sheets were transplanted using a PVDF support membrane grasped by endoscopic biopsy forceps. Subsequently, a novel procedure using a specialized endoscopic cell sheet transplantation balloon-based device was developed [6].

In this procedure, first, a cell sheet was placed on the endoscopy device deflated balloon surface and the balloon is inflated to examine and adjust the cell sheet positioning on the balloon surface. The balloon is then deflated with the adherent cell sheet attached, and endoscopy transfer device is inserted into the esophagus and maneuvered close to the ESD site. Then, the balloon is inflated and contacted with the ESD site to adhere and transfer the cell sheet onto the luminal surface of ESD site. Upon cell sheet transfer, the balloon is deflated and the device is removed.

This device and procedure facilitate endoscopic sutureless cell sheet transplantation, in particular, when the tumor is large, and when several cell sheets are required to prevent stenosis. While the previous procedure using manual transfer of a cell sheet on a PVDF support membrane is tedious and time-consuming, the endoscopic balloon transfer device procedure is brief, and operator-friendly [6]. We reported the utility of this novel transplantation device using inflatable balloon [7]. In the present study, we elucidate cell damage caused by balloon inflation of cell sheets and their tissue site transfer using a porcine ex vivo submucosal dissection esophagus model.

2. Materials and methods

2.1. A balloon device for endoscopic cell sheet transplantation

Full description and development of the endoscopic balloon-based cell sheet transfer device (provided by Cell Seed Inc., Tokyo,

Japan) was previously reported in detail [6]. The device comprises an air tube and a cell sheet container connected with the air tube, and surrounded by clinical-grade latex to produce an inflatable balloon. The air tube passes through an endoscope's biopsy channel (2.8 mm inner diameter) (Fig. 1A). The air tube also transmits linear rotational torque to the cell sheet container (Fig. 1B). Air is supplied through the air tube from an attached syringe to the balloon of the cell sheet container in order to inflate or deflate the balloon. A cell sheet is initially placed and stably adhered on the wet, completely deflated balloon surface with a minor amount of culture medium and water surface tension (Fig. 1C). In order to adjust the cell sheet position on the balloon surface, the balloon was inflated prior to device insertion into the esophagus. With final sheet positioning, the balloon is deflated again, and the cell sheet/balloon is protected in the attached storage container during manipulation, transportation and luminal insertion (Fig. 1D). The endoscopy device is manipulated into position in the esophagus and the attached container is transported close to an ESD site. Finally, the cell sheet is contacted, adhered and transferred onto the ESD site by inflating the balloon, requiring no suturing, and the endoscopy device and container withdrawn (Fig. 1E).

2.2. Fabrication of human keratinocyte sheets

Normal Human Epidermal Keratinocytes (NHEK, Lonza, Basel, Switzerland) were expanded approximately ten times in cell number in keratinocyte culture medium (KCM) [8], with mitomycin-C-treated 3T3 feeder layers as reported previously [9]. KCM was composed of the following components; Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, MO, USA) and F-12 medium (Sigma Aldrich) were mixed at a 3:1 ratio, and supplemented with 5 µg/mL insulin (Eli Lilly, Indianapolis, USA), 10 ng/mL epidermal growth factor (EGF, Higeta Shoyu, Chiba, Japan), 1 nM cholera toxin (List Biological Laboratories, Campbell, USA), 2 nM triiodothyronine (Wako Pure Chemicals, Osaka, Japan), 0.4 µg/mL hydrocortisone (Kowa Pharmaceutical, Tokyo, Japan), 100 unit/mL penicillin G (Fujifilm Wako Pure Chemicals, Osaka, Japan), 100 µg/mL streptomycin (Fujifilm Wako Pure Chemicals, Osaka, Japan), 0.25 µg/mL amphotericin B (Fujifilm Wako Pure Chemicals, Osaka, Japan), and 5% FBS (Moregate BioTech, Queensland, Australia). NHEK were frozen in a culture medium supplemented with anti-freeze reagents (TC protector, KAC, Kyoto, Japan), and stored in a gas-phase liquid nitrogen tank until use. Thawed NHEK were seeded on temperature-responsive cell culture inserts (Cell Seed Inc., Tokyo, Japan), at an initial cell density of 2×10^4 cells/cm², and cultured for 21 days at 37 °C in a humidified CO₂ incubator. Then, cells were harvested as a single contiguous cell sheet by reducing the temperature to 20 °C for 30 min on Day 21 (Fig. 2A).

2.3. Ex vivo porcine submucosal dissection model

Fresh pieces of porcine esophagus from a local slaughter house (Tokyo Shibaura Organs Co., Ltd, Shinagawa, Tokyo) (Fig. 2B–1) were used. Then, all layers of the esophagus were incised along the longitudinal axis (Fig. 2B–2) to create a flat tissue sheet. An ex vivo

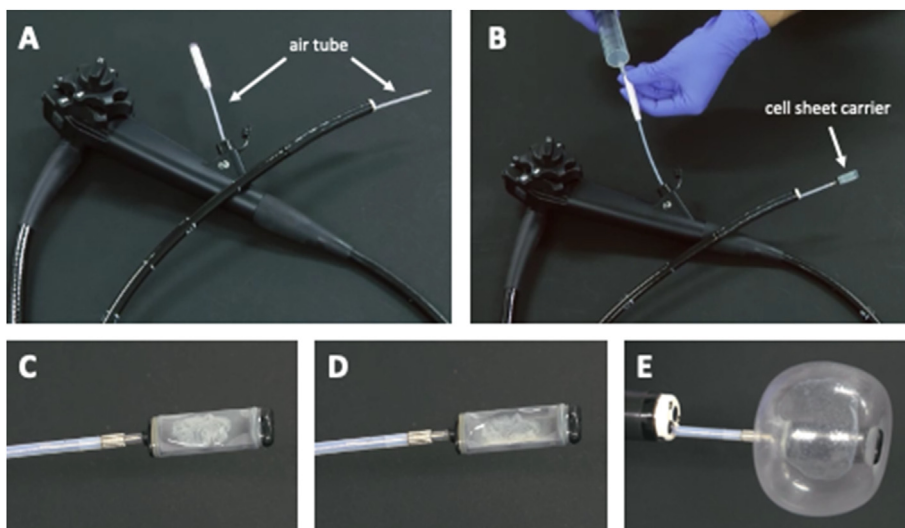


Fig. 1. Endoscopic balloon-based cell sheet transplantation device. (A) Pass the air tube through an endoscopic biopsy channel. (B) Connect cell sheet carrier and air tube. Inflate the balloon by injecting 20 cc of air with syringe. (C) The normal state of the balloon. (D) The contracted state of the balloon. (E) The expanded state of the balloon.

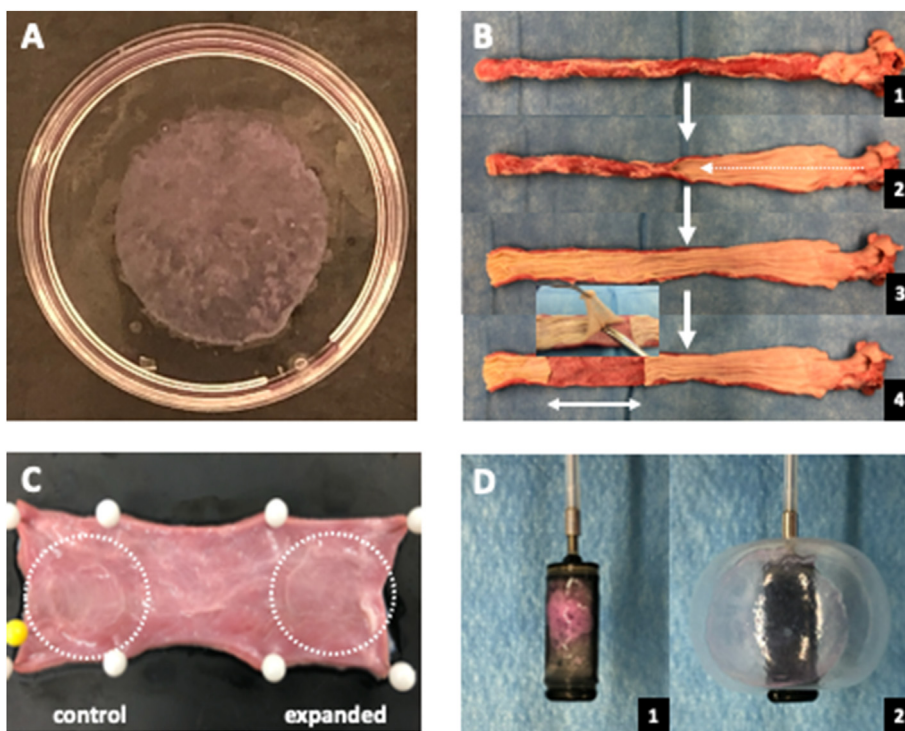


Fig. 2. (A) Normal Human Epidermal Keratinocytes (NHEK) cultured on temperature-responsive culture inserts were harvested as a cell sheet after incubation at 20 °C for 30 min. (B) Ex vivo porcine ESD model (1) Utilizing freshly excised slaughterhouse porcine tissue. (2) A longitudinal incision with Cooper scissors is made from the oral side of the esophagus to the gastric side (3,4) Dissection of about 10 cm of submucosa with Cooper scissors. (C) Transplantation of untreated and expanded cell sheets (control group and expanded group). (D) Expanded group (1) cell sheet is placed on the deflated balloon device. (2) Inflate the balloon for 30 s by injecting 20 cc of air to inflate then deflate repeatedly in sequence 3 times total.

porcine submucosal dissection model was fabricated by a full circumferential submucosal dissection of approximately 10 cm-long with Cooper scissors (Fig. 2B–3,4). On areas from which mucosal and submucosal tissues were dissected in this model, cultured NHEK sheets with (expanded) or without (control) cell sheet expansion using the endoscopic device balloon inflation (Fig. 2C). were grafted using forceps.

2.3.1. Balloon expansion and transfer of keratinocyte sheets to an ex vivo porcine submucosal dissection model

Cell sheets were histologically examined after transfer onto the luminal surface of the ex vivo porcine submucosal dissection model (Fig. 2C). Two groups of cell sheets – one with and one without expansion by inflating the balloon - were compared. NHEK sheets were transfer grafted aided by forceps to circumferential

submucosal dissected sites from the balloon surface without or with expansion by balloon inflation, designated as control and expanded groups, respectively (Fig. 2C). In the expanded group, cell sheets were subjected to the following procedure before grafting. First, NHEK sheets were placed on the device deflated balloon surfaces (Fig. 2D–1) using forceps, and 20 cc of air was injected into the deflated balloon and held for 30 s (Fig. 2D–2). The balloon was deflated by air removal, and the same inflation operation was repeated 3 times in total. The NHEK sheet did not detach, peel or tear during the repeated inflation/deflation sequence. Then, cell sheets were transferred from the inflated balloon onto luminal surfaces of the ex vivo porcine submucosal dissection model with forceps. In the control group, without the balloon expansion procedure, harvested cell sheets were directly grafted onto luminal surfaces of the ex vivo porcine submucosal dissection model from the deflated balloon surface with forceps. The grafted tissues were then subjected to histological analyses to assess cell sheet adhesion and thickness.

2.4. Cell viability from balloon-expanded cell sheets

To examine cell viability, NHEK sheets either with or without identical cell sheet balloon expansion (but not transferred to the ex vivo porcine tissues sites) were transferred from the balloon surface into culture dishes, and washed with PBS, and trypsinized for 15 min at 37 °C to disperse to individual cells. The enzymatic reaction was stopped with KCM and the cell suspension was filtered using a 40- μ m cell strainer (Biosciences, Franklin Lakes, USA), and transferred to a new 15 mL centrifuge tube, then centrifuged at 270 g for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended with KCM, and the positive (dead) and negative (live) cell numbers were counted after trypan blue staining.

2.4.1. Endoscopic transplantation of keratinocyte sheets to an ex vivo porcine submucosal dissection model

The ex vivo porcine submucosal dissection model (Fig. 3A 1–3) was inverted (Fig. 3A–3) and connected with an inserted endoscopic over-tube (Fig. 3A–4).

NHEK sheets were transferred to the luminal surface of the ex vivo porcine submucosal dissection model by three different endoscopically facilitated methods for comparisons: 1) Conventional PVDF cell sheet supporter method [1] (Fig. 3B and C), 2) endoscopy balloon device transfer method [6] (Fig. 4A and C), or 3) a modified balloon device transfer method (Fig. 4B and C) as follows.

First, fresh porcine esophagus (Tokyo Shibaura Organ Co., Ltd.) (Fig. 3A–1) was turned inside-out (Fig. 3A–2). The submucosal layer was full circumferentially dissected with Cooper scissors over the entire length of 20 cm and inverted again (Fig. 3A–3). This ex vivo porcine submucosal dissection model was inserted with an endoscopic over-tube (EMR tube; Create Medic, Tokyo, Japan) from the proximal oral to the distal gastric end, and the oral proximal esophageal end was fixed to the end of the over-tube by ligature with silk suture (Fig. 3A–4). Transfer of cell sheets to the luminal surface in this configuration was performed by 1) PVDF conventional support membrane method, 2) the balloon method or 3) a modified balloon transfer method ($n = 8$).

In the conventional support membrane transfer method, an NHEK sheet was placed on the PVDF support membrane (Merck Millipore, MA, USA) by forceps, the resulting sheet-membrane laminate was grasped with endoscopic biopsy forceps (Fig. 3B), and transported to the target site through an over-tube using an endoscope and forceps (GIF-XQ260; Olympus, Tokyo, Japan)

(Fig. 3C–1). The position and orientation of the cell sheet were adjusted so that the cell sheet faced the target tissue site by moving the forceps (Fig. 3C–2). By applying pressure onto the backside of the PVDF support membrane for 5 min, cell sheets successfully adhered onto luminal surfaces of the ex vivo porcine submucosal dissection model (Fig. 3C–3). The endoscope was removed after confirming that the cell sheet transferred and remained adherent to the tissue site (Fig. 3C–4). The cell sheet support membrane was left on the transferred cell sheet at the grafted site as in clinical settings.

The procedure for the balloon transfer method is shown in Fig. 4A. First, a piece of the cell sheet was adhered to the device's deflated balloon surface with a small amount of culture medium (Fig. 4A–1) using forceps, and the balloon was inflated with 20 cc to expand the adherent cell sheet (Fig. 4A–2). After adjusting the position of the cell sheet to the center of the balloon surface with forceps (Fig. 4A–3), the balloon was deflated again to store the cell sheet in the attached container (Fig. 4A–4). Immediately before insertion of the endoscopy device into the esophagus model, the balloon was re-inflated to ensure that the adherent sheet remains on the center of the balloon (Fig. 4A–5). The balloon is deflated to store the cell sheet in the device (Fig. 4A–6). Finally, the cell sheet is transported through the device over-tube, and transferred to the luminal surface of the target tissue site by inflating the balloon and applying pressure. All cell sheets were expanded by balloon inflation a total of three times in each single transfer procedure.

The modified balloon method is shown in Fig. 4B. In this procedure, the harvested cell sheet was placed at the center of the inflated balloon by forceps (Fig. 4B–1), and then the balloon was deflated, the cell sheet was stored in the container (Fig. 4B–2), and subjected to device-based transfer to the site using balloon inflation and tissue-sheet pressure. Therefore, in the modified balloon method, each cell sheet is expanded only once at the time of transfer/adhesion to luminal esophagus surface.

2.5. Histological analyses

The ex vivo porcine submucosal dissection model grafted with NHEK sheets were kept in a humid atmosphere at room temperature for 5 h, then cut into pieces. Each piece was subject to tissue fixation with 4% paraformaldehyde in PBS for a few days at room temperature. The fixed blocks were then routinely processed into 3- μ m thick paraffin-embedded sections. Hematoxylin and eosin staining was performed with conventional methods. The central part of each grafted cell sheet was observed under a microscope. Images were obtained using an Eclipse E800 Microscope virtual slide scanner (Nikon, Tokyo, Japan). The thickness of grafted NHEK sheets ($n = 8$) was measured with viewing software (NIS-Elements ver. 4.10, Nikon, Tokyo, Japan). Statistical analysis was performed by using a 2-tailed unpaired Student's *t* test (Microsoft Office, v.16, Microsoft; 2019).

2.6. Apoptosis assay

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) method using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, USA), according to the manufacturer's protocol. Images were obtained using an Eclipse E800 Microscope with viewing software (virtual slide scanner, Nikon, Tokyo, Japan). Positive controls were performed using the RNase-Free DNase (Promega, Madison, USA), according to the manufacturer's protocol.

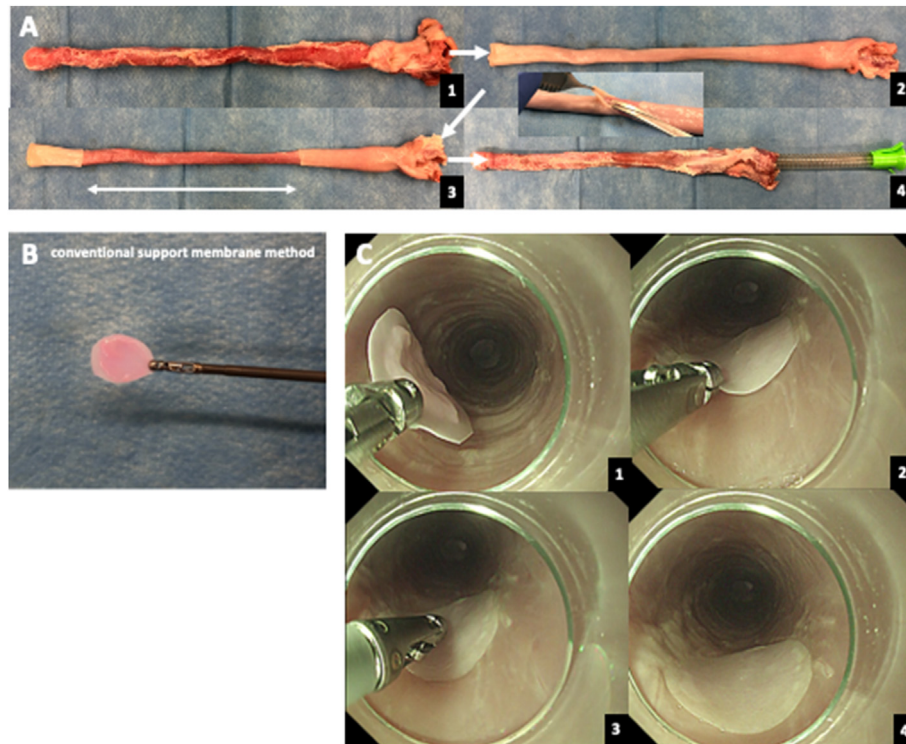


Fig. 3. Endoscopic NHEK sheet transplantation to luminal surface of ex vivo porcine ESD model. (A) Ex vivo porcine ESD model (1,2) Fresh porcine esophagus was turned inside-out. (3) The submucosal layer was full circumferentially dissected with Cooper scissors over the entire length of 20 cm. (4) Invert again and the oral proximal esophageal end was fixed to the end of the over-tube by suturing with silk suture. (B) The conventional PVDF support membrane method. Cell sheets placed on the support membrane are grasped with biopsy forceps and transplanted. (C) Endoscopic views of a cell sheet transplantation. (1) support membrane on which the cell sheet is placed is grasped with biopsy forceps and placed over the ulcer site using an endoscope. (2) Adjust the placement so that the sheet is transferred on the ulcer site. (3) Push the back of the support membrane in several places and press the sheet against the ulcer. (4) The endoscope was removed after confirming that the cell sheet transferred and remained adherent to the tissue site.

3. Results

3.1. Cell sheet expansion by balloon inflation

NHEK sheets were expanded by repeated balloon inflation and transferred to esophagus luminal surfaces. During cell sheet expansion using the balloon inflation, no cell detachment from balloon surfaces or sheet tearing was observed (Fig. 5A and B). Upon transfer, the basal layers of the cell sheets adhered spontaneously to host tissue surfaces without suturing. Comparison of cell sheet thickness between control and expanded groups is shown in Fig. 5C. The average thickness of cell sheets expanded three times via balloon was smaller than the control group. Cell sheets of both groups adhered to the ex vivo model, and integrated with the luminal surface within 5 h without suturing, no detachment was observed in fixation or sectioning. However, cell sheets each composed of several layers of stratified squamous epidermal cells significantly thinned (Fig. 5A and B). Balloon-expanded cell sheet thickness was reduced to one third of the control sheets' thickness (Fig. 5C), however, cell viability after cell dispersion by trypsinization, was unchanged (Fig. 5D). Cell viability for the control and expanded groups was $93.0 \pm 1.45\%$ and $93.1 \pm 2.02\%$, respectively. No significant difference was found between the two groups.

3.2. Endoscopic transplantation of cell sheets

Endoscopic views of cell sheet transplantation are shown in Fig. 4C. The essential procedure for endoscopic transplantation is

the same for both balloon methods. In each case, the cell sheet was delivered to the ulcer site through the over-tube, and the balloon-adherent cell sheet was faced to the ulcer surface by insertion/extraction and rotation of the device (Fig. 4C–1). After adjusting the cell sheet into the correct position and orientation, the balloon was expanded by slowly injecting 20 cc of air (Fig. 4C–2), and the cell sheet was pressed against the submucosa-dissected esophagus luminal surface for 30 s (Fig. 4C–3). After deflating the balloon (Fig. 4C–4), cell sheet was transferred, firmly spread and confirmed to be grafted onto the esophagus lumen (Fig. 4C–5,6). Then, the balloon device was removed.

Cell sheets were successfully grafted onto ex vivo luminal surfaces of esophagus using three methods. The conventional method using a PVDF support membrane requires the operator to apply pressure for a few minutes to transfer the cell sheet and PVDF membrane onto luminal tissue surfaces. The balloon device-placed cell sheets are more easily and stably adhered to tissue surfaces in shorter times. This method can be leveraged to cover larger surfaces with several cell sheets. However, during balloon inflation and pressing cell sheets against the tissue, the possibility of cell sheet damage cannot be excluded. In the single cell sheet transplantation procedure, cell sheets were expanded three times by balloon inflation. A possible risk of cell damage during each expansion cannot be excluded.

For this reason ex vivo transplantation of cell sheets onto excised porcine esophagus in the present study compared the previously reported conventional method [1,3,10], repeat 3-inflation balloon device transfer method, and a new, modified single-inflation balloon device transfer method. Cell sheets were

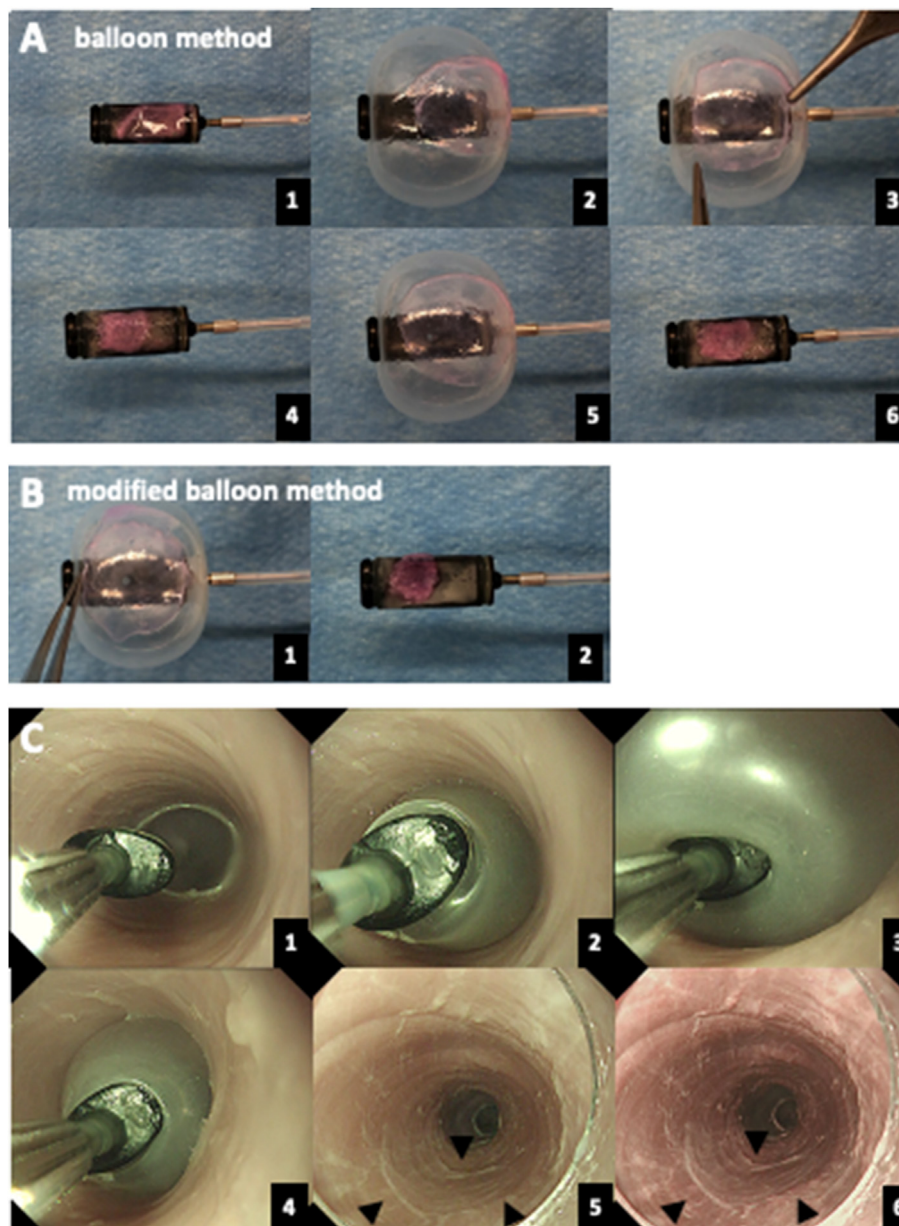


Fig. 4. Endoscopic transplantation of keratinocyte sheets to an ex vivo porcine submucosal dissection model. **A)** Balloon inflation method. (1) cell sheet placed on the deflated balloon device with a some culture medium. (2) Unfold the sheet by expanding the balloon. (3) Center the sheet on the balloon with forceps. (4) Shrink the sheet by deflating the balloon. (5) Ensure sheet remains unfolded in the center of the balloon by Inflating the balloon. (6) Store the cell sheet in the device by air suction. **(B)** Modified balloon method. (1) Unfold the sheet in the center of the expanded balloon with forceps. (2) Store the cell sheet in the device using balloon deflation. **(C)** Endoscopic views of a cell sheet transplantation. (1) Delivering the cell sheet to the ulcer site through the over-tube. Cell sheet faces the ulcer site by insertion/extraction and rotation of the device (2,3) Attach the cell sheet to the ulcer site for about 30 s by inflating the balloon. (4) Deflate the balloon. (5) Sheet transplant findings under normal observation. (6) Sheet transplant findings under NBI (narrow band imaging) observation.

examined histologically after balloon inflation and transplantation.

Comparative histology of the ex vivo esophagus post-transplantation with cell sheets with the conventional support membrane method, balloon method and modified balloon method is shown in Fig. 6A, B and C, respectively. Comparison of cell sheet thickness among these grafting methods is shown in Fig. 6D. Cell sheets grafted by the balloon method and the modified balloon method were both significantly thinner than the conventional cell sheet PVDF support membrane transfer method. No significant difference was observed in thickness between cell sheets grafted by the 3-inflation and modified single-inflation balloon methods.

3.3. Apoptosis assay

TUNEL-positive cells were not detected in all NHEK sheets grafted by the three methods (Fig. 7A–C), whereas in the positive control in which tissue specimens were treated with DNase, TUNEL-positive cells displaying brown-stained nuclei were detected in all three groups (Fig. 7D–F).

4. Discussion

Esophageal cancer is the sixth most common cause of cancer-related deaths globally [11], and an estimated 455,800 new

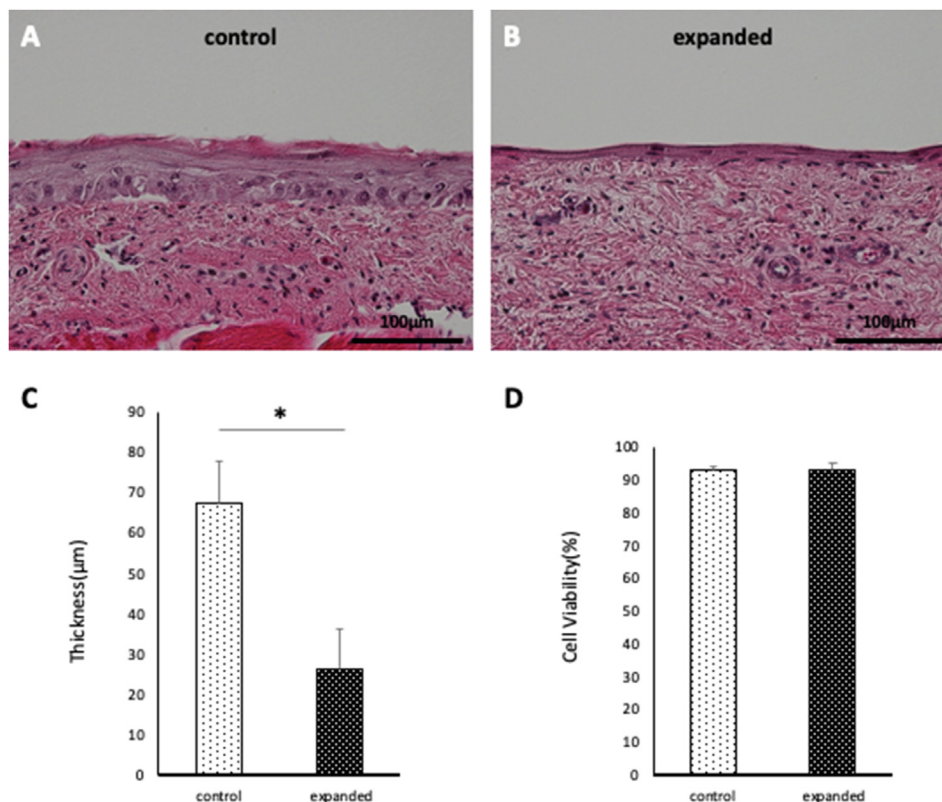


Fig. 5. (A) Histological analysis of cell sheets without treatment 5 h after transplantation (H&E) (control) (B) Histological analysis of balloon-expanded cell sheets 5 h after transplantation (H&E) (expanded). (C) Comparison of cell sheet thickness between control group and balloon-expanded group. Each data point represents the mean \pm SD, * $p < 0.05$. (D) Comparison of cell viability between control group and balloon-expanded group. Each data point represents the mean \pm SD.

esophageal cancer cases and 400,200 deaths occurred in 2012 worldwide [12]. The highest rates are found in Eastern Asia and in Eastern and Southern Africa and the lowest rates are found in Western Africa. The 2 main types of esophageal cancer are squamous cell carcinoma and adenocarcinoma. Differences between Japan and Europe and the United States for esophageal cancer include differences in histological type and site of occurrence. In Japan, squamous cell carcinoma accounts for more than 90% of the histological types of esophageal cancer, and the most common site of occurrence is the central thoracic esophagus. On the other hand, in Europe and the United States, most esophageal cancers are adenocarcinomas [13], and the site of occurrence is mainly the lower esophagus. Squamous cell carcinoma accounted for more than 90% of all esophageal tumors in the United States in the 1960s, but the prevalence of esophageal adenocarcinoma of Barrett's epithelial origin formed by persistent inflammation of the lower esophagus due to gastroesophageal reflux disease has increased significantly in the last 20 years [14].

Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), both endoscopic resections for early cancer, were developed in Japan [15,16] and are widespread in Asia, Europe and the United States. Japanese esophageal cancer clinical practice guidelines [17] examine endoscopic treatment in detail, and clinical T1a-epithelium (EP) or lamina propria (LPM) is an absolute indication for endoscopic treatment. In addition, patients with clinical T1a-muscularis mucosae (MM) or T1b-submucosa (SM1) without lymph node metastases are considered relative indications. Endoscopic resection of esophageal cancer is a minimally invasive but highly effective treatment. However, patients with extensive resection of more than 3/4 of the lumen circumference frequently develop intractable stenosis after surgery [18–21] and require

frequent balloon dilation [22], which significantly reduces the patient's quality of life. In recent years, various reports have indicated that local steroid injection therapy [23–28] and oral steroid administration [29–32] are useful for postoperative stenosis. Nonetheless, local steroid injections to prevent stenosis carry the risk of delayed perforation due to weakened walls [33], and oral steroids carry the risk of diabetes and serious infections [34].

We have described a distinct regenerative medicine strategy in which tissue pieces are collected from the autologous oral mucosa, prepared using a temperature-responsive culture dish, and endoscopically transplanted to the surface site of the esophageal ulcer after ESD [3,10]. Clinical trials have shown the usefulness and safety of this cell sheet therapy for prevention of esophageal strictures [2–5]. Cell sheet therapy has no serious complications or contraindications, and may be indicated even when steroid use is difficult. Since cell sheets are very thin and difficult to handle, conventional endoscopic transplantation has exploited a PVDF support membrane to deliver cell sheets to the ESD site [1,3,10]. However, this procedure is tedious, time-consuming and not simple for every operator. Therefore, we have innovated a novel balloon-type endoscopic transplantation device to facilitate cell sheet placement [6]. We reported that the device can improve cell sheet transplantation over a wide tissue area in a shorter time, but with possible risks that the cell sheet might be damaged by repeated balloon inflation/deflation requirements.

In the present study, we now elucidate how balloon expansion affects adherent cell sheets. While *in vivo* animal experiments were ideally desired, this study was conducted utilizing an *ex vivo* esophagus submucosal dissection model newly developed for this study. Before conducting endoscopic transplantation of cell sheets to the model, harvested cell sheets were expanded by balloon

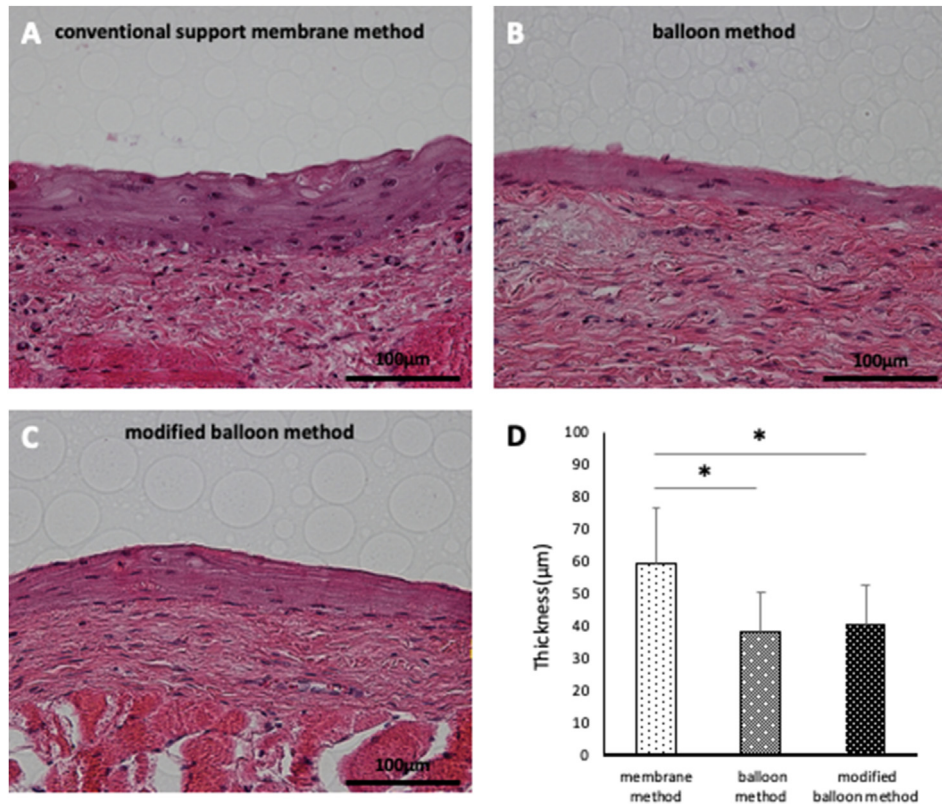


Fig. 6. (A) Histological analysis of cell sheets transplanted with the conventional PVDF support membrane method (H&E). (B) Histological analysis of cell sheets transplanted with 3-cycle balloon inflation method (H&E). (C) Histological analysis of cell sheets transplanted with single-inflation modified balloon method (H&E). (D) Comparison of cell sheet thickness between conventional support membrane method, 3-cycle balloon method and single-inflation modified balloon method. Each data point represents the mean ± SD, *p < 0.05.

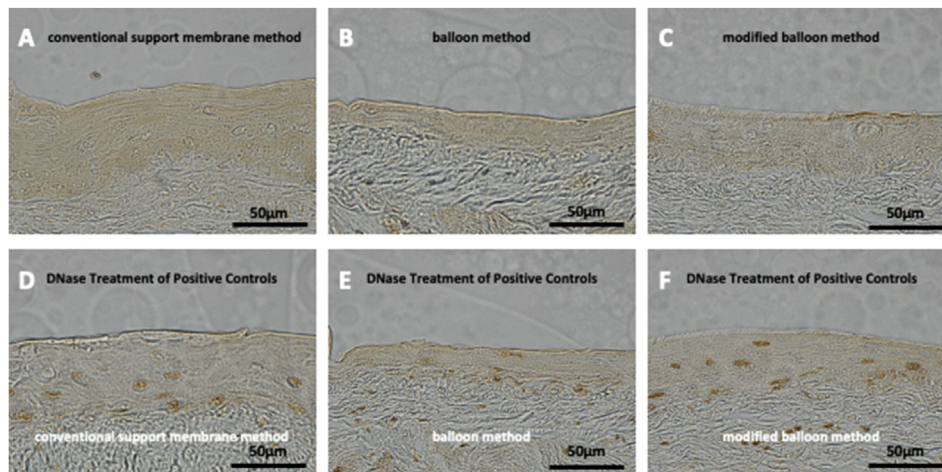


Fig. 7. (A) Apoptotic cells were not detected by TUNEL staining in cell sheets transplanted by the conventional support membrane method. (B) Apoptotic cells were not detected by TUNEL staining in cell sheets transplanted by balloon method. (C) Apoptotic cells were not detected by TUNEL staining in cell sheets transplanted by modified balloon method. (D) Apoptotic cells were detected in the positive control in cell sheets transplanted by the conventional support membrane method. (E) Apoptotic cells were detected in the positive control in cell sheets transplanted by balloon method. (F) Apoptotic cells were detected in the positive control in cell sheets transplanted by modified balloon method.

inflation three times and transferred onto the surface of the ex vivo esophagus submucosal dissection model in order to evaluate the effects of balloon expansion on cell sheets. Results suggested that the cell sheet was stretched and thinned by the expansion procedure. Nonetheless, no significant difference was observed in cell viability. No particular harmful effect on cells were observed.

Next, using an endoscope, we compared cell sheets transplanted by conventional PVDF membrane transfer methods with cell sheets

transplanted using the balloon transfer endoscopic device. The result here, similar to the previous result, was that cell sheets expanded and then transplanted using the balloon transfer device were thinner than sheets transplanted by the PVDF support membrane method lacking expansion. In addition, TUNEL staining showed no apoptotic cells observed in any of the three transplantation methods. Therefore, the balloon method was considered to produce no harmful cells effects.

The repeated 3-inflation balloon method and the modified single-inflation balloon method differ only in the number of cell sheet expansions before pressing cell sheets onto the luminal surface of the esophagus. No significant differences are observed in grafted cell sheet thickness or cell death due to apoptosis between these methods.

5. Conclusion

Overall, we conclude from this study that endoscopic cell sheet transplantation into the esophagus using an endoscopic inflatable balloon device cell sheets can address a wide tissue repair area in a short time without damaging the transferred epithelial cells, compared with the conventional membrane support transfer method.

Declaration of competing interest

The endoscopic balloon-based cell sheet transfer device was provided from CellSeed Inc. Tokyo Women's Medical University received research fundings from CellSeed Inc.

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