Esophageal replacement by hydroxylated bacterial cellulose patch in a rabbit model

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Background/aim: To repair esophageal defects by hydroxylated and kombucha-synthesized bacterial cellulose (HKBC) patch in a rabbit model.

Materials and methods: Semicircular esophageal defects 1 cm in length of the cervical esophagus were initially created in 18 Japanese big-ear rabbits and then repaired with HKBC patch grafts. The clinical outcomes including survival rate, weight change, food intake, and hematological and radiologic evaluation were observed. After X-ray evaluation, the rabbits were sacrificed sequentially at 1, 3, and 6 months for histopathologic analysis with light microscopy and scanning electron microscopy.

Results: Survival rate during the first month was 88.9% (n = 16). Two rabbits died from anastomotic leakage during the entire follow-up. Postoperatively, feeding function and body weight were gradually restored in the surviving animals. No hematological abnormalities were found, and no obvious anastomotic leakage, stenosis, or obstruction was observed under X-ray examination. The histopathologic results showed a progressive regeneration of the esophagus in the graft area, where the neo-esophagus tissue had characteristics similar to native esophageal tissue after 3 months of surgery.

Conclusion: HKBC is beneficial for esophageal tissue regeneration and may be a promising material for esophageal reconstruction.

Key words: Artificial esophagus, kombucha, bacterial cellulose, biological material

1. Introduction
Surgical resection of the esophagus due to tumors or other disease requires esophageal reconstruction using a variety of materials. Although autologous stomach tissue and the small or large intestine have been widely regarded as the first choice for such clinical interventions, their use is limited due to high morbidity or various potential postsurgical complications that greatly impair the patient’s quality of life (1). Therefore, seeking alternatively promising supplements or replacements has attracted much research interest. In recent years, great efforts have been made to develop new artificial esophageal substitutes by using various synthetic and natural biomaterials, such as silicone/collagen hybrid, polyglycolic acid, allogenic aorta, or other tissue engineering materials (2–8).

Bacterial cellulose (BC), as well as its chemical modifications, is a widely used biomaterial due to its unique characteristics such as biocompatibility, high purity, high crystallinity, and remarkable mechanical properties (9–11). It has found diverse applications in the field of tissue engineering, for example, it can be used to prepare artificial scaffolds for vascular grafts, wound dressings, artificial cartilage, and dental implants (12–15). However, there seem to be no reports on BC-based material utilization for esophageal replacement. This is probably because BC does not have the characteristics of degradability and absorbability in the bodies of mammals. A previous study demonstrated that the center areas of nonabsorbable materials do not allow proper tissue growth during the regeneration of esophagus tissue (16). A recent study, however, suggested that the in vivo degradability of BC could be greatly improved by incorporating cellulases enzymes and buffer ingredients (17), and thus BC can achieve good absorbability. This interesting finding inspired new endeavors for applying BC in esophageal defect repair in the current study.
Kombucha is obtained from a symbiotic culture of acetic bacteria and yeast strains during fermentation of sugared black tea (18). During fermentation, a jelly-like membrane forms, which is essentially composed of cellulose fibers, can be secreted by the kombucha strains. Among a diverse array of BC forms that are synthesized from different species of bacteria, a special BC form can be easily and effectively synthesized from kombucha (19). In the present study, therefore, we attempted to prepare kombucha synthesized BC (KBC) patches, which were further modified with glycerol, to improve the mechanical properties for artificial esophagus substitutes, and to repair the partial cervical esophageal defect in a rabbit model.

2. Materials and methods

2.1. Preparation of KBC patches for artificial esophagus substitutes

Kombucha strains were purchased from Taiyuan Kampuchea Biological Technology Co., Ltd. According to the instructions, a typical ferment mainly consisted of five kinds of bacteria (Acetobacter xilinum, Acetobacter xilinoides, Acetobacter ketogenum, Bacterium gluconicum, and Bacterium xilinum) and yeasts (Saccharomyces ludwigii, Schizosaccharomyces pombe, etc.). Black tea was purchased from the local market. Black tea (10 g) and sucrose (50 g) were added to 600 mL of water and boiled for 5~10 min. The sugared tea solution was cooled to room temperature and tea leaves were removed by filtration. Then 5 g of yeast extract and 0.5 g of peptone (both from Sigma, St. Louis, MO, USA) were added. The total volume and pH value of the solution were adjusted to 1000 mL and 4.5~5.5 with water and acetic acid, respectively. Kombucha strains were then poured into the solution and allowed to incubate at 30 °C for 7 days in static culture conditions. The resultant membranes were washed with water and then boiled in 0.05 M NaOH for 30 min to eliminate the attached bacteria and other impurities. After complete drying at 37 °C, the KBC membranes were then immersed in glycerol for hydroxylation of 24 h, rinsed with water, and dried. The so-obtained hydroxylated KBC (HKBC) membranes were cut into patches 1 × 1.5 cm in size. Gelatinous KBC membrane and HKBC patches were observed under a scanning electron microscope (SEM) (Hitachi, S-3400N, Japan). In addition, tensile tests of the KBC and glycerol modified KBC membranes were performed on a universal tensile testing machine (M350-20KN CX) at a cross-head speed of 0.5 mm/min.

2.2. Animals

All experimental procedures involving animals were conducted as per institutional animal care guidelines and approved ethically by the Administration Committee of Experimental Animals.

Thirty-six male Japanese big-ear rabbits, weighing between 2.5 and 2.9 kg (average, 2.72 kg) were randomly divided into 2 groups: I, HKBC group (18 animals); II, autologous cervical fascia (control group, 18 animals). There were no significant differences in the preoperative weights between the two groups. All animals underwent a conventional examination and quarantine before the operation.

2.3. Surgical procedure

The animals were fasted for 8 h before the operation. After routine surgical preparation, 3% sodium pentobarbital was given intravenously (1 mL/kg) and atracurium (1 mg/kg) was used to maintain muscle relaxation. Endotracheal intubation was used to provide respiratory support. The cervical esophagus 3 cm in length was exposed and completely mobilized via a midline incision after sequential incision of the skin, subcutaneous tissue, and muscle layer. Then a semicircular and transmural resection of the anterior wall of 0.5 × 1 cm was performed. In group I, the defects were patched by HKBC membranes and 5-0 polypropylene sutures, followed by a routine surgical procedure of closure. A HKBC patch 1 × 1.5 cm in size was autoclaved (120 °C, 20 min) for sterilization prior to use. In group II, the defect was patched by autologous cervical fascia 1 × 1.5 cm in size (Figure 1). A nasogastric tube was inserted in each animal but no external drainage was used.

Figure 1. Macroscopic view of esophageal replacement by a HKBC patch (a) or autologous fascia (b).
After the operation, intravenous nutrition, which was 5% glucose in a total volume of 60–80 mL every day, was initiated on postoperative day 2. Milk feeding and water supplement through a nasogastric tube were adopted for the next 3 days after surgery. Then the animals received semifluid food for 3 days after removal of the nasogastric tube and standard food thereafter. All animals were treated with analgesics and antibiotic drugs after the surgical intervention to minimize discomfort and prevent infection.

2.4. Postoperative observations
Postoperative monitoring in the two groups was performed daily for 3 weeks, followed by monthly examinations. Weight changes, as well as hematological indicators, including the counts of red blood cell (RBC), white blood cell (WBC) and blood platelet (PLT), alanine amino transferase (ALT), aspartate amino transferase (AST), total protein (TP), blood urea nitrogen (BUN), and electrolyte (K⁺, Na⁺ and Cl⁻) concentration were detected at 1, 2, and 3 weeks and 1, 3, and 6 months after surgery, respectively. In addition, esophageal radiography was performed through iodine solution intake to evaluate the continuity of the esophagus at 3 and 6 months postoperation.

2.5. Histopathological analysis
In the two groups, the rabbits were sacrificed sequentially for histopathological analysis by light microscope (LM) and SEM (S-3400N, Hitachi) at postoperative 1, 3, and 6 months. The cervical esophagus grafts and the surrounding tissues were dissected en bloc under anesthesia for macroscopic analysis, which consisted of the evaluation of graft appearance and consistency. Specimens were fixed in a 4% paraformaldehyde solution, dehydrated in a graded series of ethanol, and then were embedded in paraffin and sliced, followed by hematoxylin and eosin staining for LM examination. For SEM observation, samples were fixed in 4% glutaraldehyde and further postfixed with 1% OsO₄, dehydrated in a graded series of ethanol, which was replaced by tert-butyl alcohol, and then dried in a freeze drier (Hitachi, ES-2030). Afterwards, the samples were mounted for SEM observation after metal coating.

2.6. Statistical analysis
The data were expressed as means ± SD. Statistical analyses were performed using the Mann–Whitney U-test and chi-squared test. A P-value of <0.05 was considered statistically significant.

3. Results

3.1. Morphological observation and mechanical analysis for KBC patches
Freshly prepared KBC matrix is a translucent and gel-like membrane with a diameter of 15.0 cm and thickness of 0.5 cm. SEM results showed that KBC fibers with a diameter of less than 100 nm formed a three-dimensional interwoven network after freeze-drying. However, the ultrastructural characteristics of HKBC patches, which were essentially made of polyhydroxy-modified KBC film in a natural drying method, constituted a very dense structure by crisscrossing fibers (Figure 2A).

Figure 2B shows typical stress–strain curves of pristine KBC dry films and HKBC films, respectively. The tensile strength values of the KBC and HKBC membrane were approximate 179 Mpa and 236 Mpa, and the values of elongation at break were about 5% and 5.4%, respectively. Both the values of tensile strength and elongation at break of HKBC films were higher than those of pristine KBC films.

![Figure 2](image-url)
3.2. Postoperative observations

Mortality: All animals survived the surgical procedure. However, in the KBC group, two rabbits died during the follow-up because of anastomotic leakage at 11 and 13 days postoperation. In the control group, 4 animals died of anastomotic leakage on days 10 (2 animals), 13, and 16 after the operation. All surviving animals had mild dysphagia or vomiting for approximately 3–5 days after resuming oral feeding, which gradually disappeared without treatment, and appeared healthy with good appetite thereafter. The classification for dysphagia was adopted according to previous research (a scale of 0–4) (20).

Body weight in the 2 groups began to fall after the operation and reached the minimum of about 2.4 kg, which was approximately 85% of preoperative weight, 2 weeks after implantation. However, there were significant differences in weights at 2 weeks (P = 0.012) and 3 weeks (P = 0.03) between the two groups. Thereafter, they regained an average weight of 2.79 kg (mean value) 3 months after the operation and then maintained a stable weight (Figure 3).

3.3. Hematological and serum biochemistry

In both groups, there were no significant differences in blood counts at the same time point. However, the number of leukocytes in the same group was significantly higher at 1 week than at the other time points (P < 0.01). Similarly, there were no significant differences in the serum biochemical parameters linked to liver function, renal function, or electrolyte level between the two groups at different postsurgery times.

3.4. Macroscopic appearance of the reconstructed esophagus

Macroscopic examination of animals that died of anastomotic leakage in the two groups showed that the grafts were partially detached from the native esophagus. The defects in the two groups were greatly reduced to about 70% of the sizes of the original resections (Figures 4a and 4b). Six months postoperation the HKBC patches in the reconstructed artificial esophagus had exfoliated completely, and the inner surface of grafts areas showed mucosal tissue normal in appearance. In addition, there were no obvious signs of scar formations, and the reconstructive esophagus had become integrated into the
host tissue (Figure 4c). In the control group, the surviving animals had similar results to the HKBC group, but the regenerative areas showed a mild stricture (Figure 4d).

3.5. X-ray examination

Iodine solution for esophageal radiography was used to evaluate the continuity of the esophagus in the remaining rabbits in both groups at postoperative 3 and 6 months. The results indicated that there was no obvious anastomotic leakage, stenosis, or obstruction in surviving animals in the two groups. The reconstructed esophageal tissue maintained good continuity.

3.6. Histopathological evaluation

Histopathologic analysis of the tissues removed from the animals in the HKBC group after 1 month showed a lack of intact epithelium, chronic inflammation of the subjacent tissue, characterized by a few of both neutrophilic granulocyte and mononuclear inflammatory cells, and a variable amount of dense, disorganized, fibrous connective tissue. Muscular tissue, which normally constitutes the muscularis externa, could not be seen in the area of the artificial esophagus (Figure 5a). In the control group, there was also a chronic inflammatory reaction adjacent to the superficial fibrous connective tissue, but the regenerative esophagus tissues (Figure 5d) were thinner than those of the HKBC group. Three months postoperation the regenerated esophagus in the HKBC group was covered with 3–8 layers of epithelial cells, which there were only 2–6 layers in the control group, and no inflammatory cells infiltration could be seen (Figure 5b). The muscle layer could also be observed, but a functional arrangement of circular and longitudinal muscles was not formed in either group. Histopathologic results 6 months postoperation in both groups showed that the mucous layers were intact and well-organized, the submucosal muscle layers were complete, and the mucous glands had proliferated well. In addition, the outer longitudinal and inner circular muscle layers were clearly arranged (Figure 5c).

SEM results (Figure 6a–6l) provided further evidence of progressing tissue regeneration. Many longitudinal arrangements of mucosal folds were gradually formed in HKBC grafts after 3 months postoperation. In addition,

![Figure 5. Histologic characteristics of the regenerated esophagus tissues after surgical implantation at different time points. A) HKBC implantation at postoperative 1 month. The epithelium layers were replaced by fibrous connective tissues (black arrow) and inflammatory cells can be seen (white arrow). B) HKBC implantation at postoperative 3 months. Several layers of epithelium cells (thick arrow) and muscle layer (double arrows) were observed. C) HKBC implantation at postoperative 6 months. The mucosa layer (thick arrow), submucosa, and the outer longitudinal and inner circular muscle layers were clearly arranged. D), E), F) The three images represent the control group at postoperative 1, 3, and 6 months, and show a similar structure feature to the HKBC group. H&E stain ×20 original magnification.](image)
the inner surface of HKBC grafts was very similar to that of normal esophageal tissue at 6 months postoperation. The reconstructed esophagus showed good morphological characteristics.

4. Discussion
Esophageal cancer is a common cause of digestive tract tumors. After resection of tumors, the esophagus needs to be reconstructed to restore digestive tract continuity and feeding function. Despite the many esophagus reconstruction techniques available (21–23), an ideal esophageal substitute has yet to be found. Esophageal replacement remains associated with various complications, including anastomotic leakage, stenosis, and poor quality of life.

The ideal substitute should be nontoxic and absorbable, without immunological rejection or carcinogenicity, and easily obtainable (24). Our choice to use KBC as a substitute for conventional BC to replace esophageal defects was inspired by its unique properties (25,26), rich resource, and lower cost. In this investigation, KBC dry membrane was hydroxylated with glycerin to improve its mechanical properties. The modification was achieved by introducing multiple hydroxy groups into the cellulose polymers. Our previous study demonstrated that KBC showed a typical structural characteristic of cellulose Iα and possessed good biocompatibility in vitro and in vivo (19). The superior biocompatibility of HKBC was also verified by a gradually reduced inflammatory response in the graft region or by monitoring of hematological parameters. The mild inflammatory reaction observed by histological analysis 1 month after the operation was due to the stress response after surgical trauma and material implantation. Hydroxylation of KBC brought about a relative improvement in hydrophilicity, which was beneficial to cell growth (27), and may be more suitable for esophageal repair. Autologous fascia has been investigated as an autologous transplantation for many decades (28–30). In the present study, autologous fascia was used as a control for esophageal substitution to compare with HKBC.
Although the experimental animals in the two groups in this investigation showed a certain degree of dysphagia or vomiting after oral feeding resumed, this symptom seemed to be caused by stimulation of the nasogastric tube, as the indwelling tube in vivo for 1 week could cause a mild injury to the digestive tract. Changes in body weight showed that surgical implantations resulted in short-term weight loss, which may be due to the surgical trauma and change in dietary pattern, but the degree of weight loss in the HKBC group was significantly lower than that in the control group 2–3 weeks after the operation. Differences in weight changes between the two groups had gradually disappeared 3 months of surgery. Postoperative examinations of mortality, weight changes, and other postoperative complications after implantation of this study, however, suggested that HKBC is a promising material for esophageal replacement after undergoing further evaluation.

Our research found that either HKBC patches or autologous fascia grafts were reliable esophageal substitutions in the surviving rabbits. However, the 8% anastomotic dehiscence in the HKBC treated group was significant lower than that in the control group. Anastomotic fistula, as is well known, is one of the main reasons for failure of esophageal replacement, and its occurrence has a strong correlation with surgical technique (31) and the biomechanical characteristics of materials (32). Although nondegradable materials were considered unsuitable for esophageal replacement, Lynen Jansen et al. (32) demonstrated that the anastomotic leakage rate was higher when using absorbable mesh rather than nonabsorbable material of polyvinylidene fluoride mesh in a rabbit model. A low incidence of anastomotic leakage was also obtained in this study and anastomotic leakage occurred in 2 rabbits after surgery in the experimental group. To reduce the rate of leakage, grafted animals after the operation received the special nutritional support of intravenous nutrition for 2 days, nasogastric nutritional supplements in the subsequent 3 days, and gradually a normal diet. These measures effectively avoided the leakage generally caused by early oral feeding or gastroesophageal reflux, thus promoting the tissue growth of grafts. Urschel et al. (33) reported that cervical anastomoses have a higher leak rate than thoracic anastomoses and suggested that anastomotic location may be an important factor affecting the occurrence of leaks. In contrast, a retrospective review to assess the effect of anastomotic location conducted by Blewett et al. (31) manifested that cervical anastomoses do not have a higher incidence of leaks than thoracic anastomoses.

Histological examination of the HKBC repaired esophagus at the defect site 6 months postoperation showed total regeneration of mucosa, including luminal epithelium, lamina propria, muscularis interna, and submucosa, and nearly complete regeneration of the muscularis externa, which was structurally similar to normal esophagus tissue. The neo-esophagus was mainly constituted by fibrous connective tissue at an early stage in both groups and then was progressively replaced by adjacent esophageal tissue. The remodeling events that occurred in both groups were similar in equivalent time range periods during this study. The gradual in-growth of host cells from native esophageal tissue may be local adult stem cells (32). Complete epithelialization of the graft seems to require at least 3 months; this time was longer than the results obtained by Lopes et al. for cervical esophagoplasty in a rat model (34). In fact, the time required for good re-epithelialization of grafts differed in previous reports (5,32,35). This may be related to the animal model, defect area, or the degree of inflammatory reaction after implantation. Muscularis and submucosa were observed 3 months postsurgery and were well formed at 6 months. These results suggested that the structural remodeling and functional reconstruction of the neo-esophagus are at the rapid development stage during the 2 to 3 months following the operation, and also proved that the early stages of fibrous tissues are important to limit the inflammatory reaction, which will greatly inhibit tissue regeneration. However, excessive fibrous tissues are harmful and will result in a stricture by concentric retraction. The SEM results were consistent with those of LM analysis. Neogenetic mucosal folds of regenerated esophagus were gradually formed in a longitudinal arrangement pattern, very similar to the normal structure of esophageal mucosa, and similar results were also shown in the muscular layer of the HKBC graft, suggesting the structural and functional remodeling was completed about 3 months after the implantation of HKBC grafts. Compared with big animals such as dogs and pigs, rabbits are docile, convenient, low cost, and easy to obtain, but those big animals should be used in follow-up studies to verify this new method and explore experimental conditions. Although the histological results indicated that both HKBC and fascia can promote the regeneration of the esophagus, reconstruction by autologous fascia seemed more likely to result in a postoperative anastomotic leak.

In conclusion, our results demonstrated that KBC is a promising material for esophageal replacement. For a comprehensive evaluation of KBC as a substitute for clinical application, a full-circumferential resection repair would be required.

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References


