Construction of tissue-engineered laryngeal cartilage with a hollow, semi-flared shape using poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) as a scaffold

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Abstract. The aim of the present study was to construct tissue-engineered laryngeal cartilage with a hollow, semi-flared shape using a poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHH) scaffold. Porous PHBHH was prepared and formed into a hollow, semi-flared shape, and the cell-material composites were cultured for one week in vitro prior to implantation in vivo. Cells of the nine rabbits of the experimental group were filled and encapsulated in the myofascial flap-shaping material composite for in situ implantation. The three rabbits in the control group were treated with the shaping material without the chondrocytes. Cartilage regeneration was assessed at six, 12 and 18 weeks after surgery. In the experimental group, the shape and porosity of the material were ideal, the cells exhibited good adhesion with the material and the myofascial flap blood supply was rich. The engineered laryngeal cartilage with the hollow, semi-flared shape was ideally formed, and the cartilage formed at six weeks after the surgery. Further maturation of the cartilage was observed at 12 and 18 weeks after the surgery. PHBHH was a suitable material for the formation of a hollow, semi-flared shape with good cellular compatibility. Myofascial flap filling and wrapping can be used to build tissue-engineered laryngeal cartilage with a hollow, semi-flared shape.

Introduction

The study of tissue-engineered cartilage with the basic goals of predetermined shaping and regeneration has provided novel ideas and techniques for research into laryngeal cartilage erosion (1-3); however, due to the special nature of the morphology, location and function of laryngeal cartilage, tissue engineering research has not, to date, exhibited its full advantages in the reconstruction of laryngeal cartilage (4). This is not only due to the large quantity of seed cells and biomaterials required for the construction and shaping of the tissue-engineered laryngeal cartilage, but also due to the difficulty of constructing the ideal irregular, hollow, three-dimensional structure. Tissue-engineered laryngeal cartilage with a hollow, semi-flared shape should show good biocompatibility, biodegradability and structures of the pores inside the biological materials, as well as sufficient mechanical strength and flexibility (5). How to ensure that the cartilage maintains its shape and structure and how to develop the potential of cartilage reshaping applications are two problems currently faced in the process of tissue-engineered, hollow, semi-flared laryngeal cartilage construction. The exploration of hollow structural biomaterials that are more suitable for shaping and tissue-engineered cartilage regeneration, as well as the practical application of the cartilage tissue engineering regenerative technology, is the only way to improve laryngeal cartilage repair and reconstruction. In the present study, on the basis of previously published tissue engineering findings (6,7), the new biomaterial poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHH) was used in the shaping of laryngeal tissue-engineered cartilage tissue into a hollow, half-flared shape, and the compatibility of the PHBHH with chondrocytes was investigated. The aim of the study was to determine the feasibility of constructing laryngeal cartilage tissue with a hollow, half-flared shape using the muscle wrapping and filling method and using the fascia lining of a pedicled myofascial flap, in order to provide an experimental basis for the tissue engineering of laryngeal cartilage with a hollow, half-flared shape through the transplantation of myofascial tissue.

Materials and methods

Chondrocyte harvest. Costal and articular cartilages were obtained under sterile conditions from young New Zealand rabbits of both genders, aged between three and seven days,
The shaping of the small end of the hollow, semi-flared PHBHH, and the three longitudinal wire supports were located in the sides of the PHBHH shape and in the middle of the front. The steel support frame was placed tightly inside the PHBHH model with no special fixation. The hollow, semi-flared PHBHH objects was aided by the inner surface of a steel wire with a diameter of 0.4 mm, which was uninterruptedly woven into the form of two approximate rings and three longitudinal supports. Compared with the PHBHH with the hollow semi-flared shape, the larger ring fluctuated correspondingly up and down along the semiflared shape of the corners and edges of the stent, mainly helped the shaping of the edges and rear parts with less material of the PHBHH with hollow semi-flared shape. The smaller ring was nearly round, which assisted in the shaping of the small end of the hollow, semi-flared PHBHH, and the three longitudinal wire supports were located in the sides of the PHBHH shape and in the middle of the front. The steel support frame was placed tightly inside the PHBHH model with no special fixation. The hollow, semi-flared PHBHH constructions and the steel wire support frame were disinfected by immersion into 75% alcohol and then rinsed three times with PBS, prior to being dried under sterile conditions. Adhesion was promoted by immersing the PHBHH and the frame into sterile poly-L-lysine aid (relative molecular mass, 1.89 million; Sigma-Aldrich) for 1 h, prior to drying.

Compounding of chondrocytes with PHBHH porous materials and culturing in vitro. Following the adjustment of the cell suspension to a density of $5 \times 10^4$ /ml, the chondrocytes were inoculated in the pre-dried shaping materials by culture medium containing ~30% wet culture medium, a small amount of cell suspension each time when inoculated, was scattered and multicasted to make the cells distribute as evenly as possible, the inoculated cells of the inner surface of the shaping hollow material were pipetted using a bend head. The cells were placed in a CO₂-saturated humidity incubator (Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂ for 0.5 h, and then removed and agitated. The cell suspension around the bottom of the material was sucked out and inoculation was performed from top to bottom twice, prior to further incubation for 1 h. Fresh culture medium was then added slowly along the sidewall so that the composite was infiltrated by 2-3 cm. The medium was changed once every 48 h. Cell growth and adhesion in the
Design of the pedicled myofascial flap. Intravenous anesthesia was induced with 3% barbiturate. The side of the back skin of the rabbit was disinfected and shaved, and the middle portion of the sacral spine muscle and fascia was isolated from the distal side of the rabbit's back to form the myofascial tissue flap with the pedicle for the proximal and distal sides of the flap. The filled myofascial flap with a conical hollow portion was designed according to the size of the chondrocyte-PHBHH composite. The fascial layer of the myofascial flap, similar to a conical shape, was then stabbed into the form of a net with a scalpel blade for filling in the prepared hollow part of the composite. During flap preparation, the sacral spinal fascia in the sides of the depression was freed, simply sutured, connected and placed in the recessed slots, and the fascia in the depressed trench was subjected to poration for the in situ implantation of the PHBHH-chondrocyte complex. Following implantation, the remaining sacral spine muscle and fascia were separated to enclose the chondrocyte-PHBHH complex. Autologous fascia tissues were used for each implanted chondrocyte-PHBHH composite.

Implantation in vivo. The chondrocyte-PHBHH composites were co-cultured in vitro for 7-10 days, until abundant extracellular matrix was observed using inverted microscopy. The composites were removed subsequent to reaching a certain degree of support (hardness). The in situ implantation was designed, filled and wrapped in the body according to the design of myofascial tissue, and the distal portion of the filled myofascial flap was fixed. The subcutaneous skin was sutured. Each batch of surgical procedures was performed by the same group of technicians, and the surgical procedures and technology used were consistent. The rabbits were administered an intramuscular injection of 800,000 units penicillin, and the surgery was then repeated the next day for a total of four times.

Experimental observation and evaluation
Poration, shaping and cellular compatibility of PHBHH. The PHBHH composites were observed and assessed for shapes similar to the hollow, semi-flared laryngeal cartilage morphology. The composite pore continuity and chondrocyte adhesion, distribution and growth were observed using SEM.

Construction of tissue-engineered, hollow, semi-flared laryngeal cartilage. The animals were anesthetized and the implants were removed: Three rabbits from the experimental group and one from the control group were selected at each time-point, six, 12 and 18 weeks after surgery, respectively. The gross morphology of the tissue-engineered laryngeal cartilage was observed, and hematoxylin and eosin (HE), Masson's trichrome and Alcian blue/periodic acid Schiff reaction (AB/PAS) staining were performed, as well as collagen type II immunohistochemical detection for the evaluation of cartilage formation.

Results
Fabrication of PHBHH porous models. The porous PHBHH composite was prepared by solvent casting, compression molding and particulate filtering, and had a hollow, semi-flared shape, which was substantially similar to laryngeal cartilage morphology (Fig. 1). Following the filtering and demineralization, the whole structure was porous and spongy. The porosity was measured using the ethanol static volumetric measurement method (92±2%), and the pore size and thickness were found to be 100-150 µm and ~1.5 mm, respectively.

Experimental observation in vitro. At 24 h after the inoculation of chondrocytes into the hollow, semi-flared PHBHH biomaterials, the chondrocytes were observed to attach to the surface of the lower edge of the material under an inverted microscope. Following co-culture of the chondrocytes and PHBHH for one week, a jelly-like matrix, secreted by the chondrocytes, was visible at edge of the material (Fig. 2A).

SEM. The simple PHBHH material was porous and spongy, exhibiting a pore size of 100-150 µm (Fig. 2B). SEM showed
that the cells (single, string or cluster) were distributed at the surface and in the cavernous, spongy hollow of the chondrocyte-PHBHH complex. Mucus-like stromal substances around the cells exhibited interlinking adhesions (Fig. 2C), and numerous small projections were visible under high-magnification microscopy, which may have been fused matrix components secreted by the chondrocytes (Fig. 2D).

**General morphology.** The materials were harvested six weeks after implantation, and the implants and connective tissue myofascial adhesions were wrapped together. Dense, tiny blood vessels were distributed on the connective tissue. Subsequent to stripping away part of the wrapped organization it was observed that the filled myofascial tissue was closely in contact with the implants. The steel wire support frame, filled packages and fascia tissues were removed, and it was found that the general form of the implants in the two cases was consistent with the morphology prior to implantation (Fig. 3A). The specimens possessed a certain hardness with smooth inner and outer surfaces. In one case the implant had collapsed, showing angular release and a loss of the predetermined hollow, semi-flared shape (Fig. 3B). At 12 weeks, only two complete specimens of the three experimental group implants remained for harvesting; as the third collapsed; the general form showed a hollow, semi-flared, tissue-engineered laryngeal cartilage. When harvested at 18 weeks, the implant shape of the three cases was consistent, and the milky white, hollow, semi-flared, tissue-engineered laryngeal cartilage exhibited a realistic shape (Fig. 4). In the control group, one implant was removed at each corresponding time-point. The retained hollow, semi-flared, tissue-engineered laryngeal cartilage at six weeks (Fig. 3C) was significantly less than that of the experimental group at the same time-point (Fig. 3A). At 12 and 18 weeks the gross morphology of the implants in the control group had almost disappeared; only the built-in medical steel support frame was visible in the implant area.
Histological observation

**HE staining.** At the six-week time-point the cells of the experimental group exhibited a circular, oval or polygonal morphology, and single cells were scattered. Incomplete degradation ‘impurities’, such as material around the PHBHHH, were visible among the cells, and inflammatory cell infiltration was present around the cartilage (Fig. 5A). At 12 weeks, the majority of the cells exhibited an oval morphology; two to three cells were commonly clustered together and showed a uniform stromal staining. The morphology of the cells at 18 weeks was similar to that at 12 weeks; the ‘impurities’ had disappeared, but a small number of inflammatory cells remained in the surroundings. Cartilage-like cells in the control group were visible amongst the collapse of the pores and degradation of the material at six weeks, and few other tissue cells were scattered on the surface (Fig. 5B).

**Masson’s trichrome staining.** Pale green objects were observed in the chondrocytes and cartilage matrix following cytoplasmic staining of the experimental group at the sixth week, and the presence of the green pollutants increased significantly at the 12th week, with deeper staining (Fig. 6A). The cell morphology, distribution and coloring at 18 weeks were not different from the observations at 12 weeks.

**AB/PAS.** The matrix was purple at the six-, 12- and 18-week time-points (Fig. 6B).

**Immunohistochemistry for type II collagen.** At six weeks, immunohistochemistry for type II collagen in the experimental group (Boster Biotechnology Co., Ltd., Wuhan, China) revealed weakly positive staining, which was mainly distributed in the cytoplasm, with very small amounts in the matrix. At 12 weeks, the positive staining in the cytoplasm was significantly enhanced, and increased type II collagen-positive staining was detected in the matrix (Fig. 6C). Similar results were observed at 18 weeks.

Discussion

Different from the regeneration of sheet cartilage tissue by tissue engineering technology, the construction of hollow, semi-flared, tissue-engineered laryngeal cartilage has a higher demand for biocompatibility, biodegradability, pore structure, mechanical strength and the shaping of extracellular matrix materials (10). Biomaterials that are currently used in cartilage tissue engineering include natural biomaterials, such as collagen, fibrin and chitosan; synthetic biomaterials, such as polylactic acid; microbial biosynthetic material, such as types of biological polyhydroxyalkanoates and copolymers; and composite materials, such as polylactic acid-collagen and fibrin-polyurethane (11-14). The biomaterials each have individual advantages and disadvantages in terms...
of biocompatibility, degradation suitability, cell adhesion, metabolic microenvironment, mechanical strength, shaping and flexibility. Natural biological materials lack sufficient mechanical strength, while artificial and compound biomaterials are expensive and involve complex processing, but are easy to shape (15-19).

Polyhydroxyalkanoate is a natural substance produced by microorganisms and exhibits characteristics such as good biocompatibility, no induction of inflammation, no degradation and reduced rejection (20,21). As more types of polymers become available, and as the compatibility among the polymers is enhanced, tissue engineering scaffolds with improved strength, toughness, biocompatibility and controlled degradation are likely to be able to be obtained through the advantages resulting from the combination of different polymers (6).

In the present study, the selected material PHBHH, a copolymer of poly-3-hydroxybutyrate and poly-3-hydroxyhexanoate ester, not only had the advantages common to biological materials belonging to the polyhydroxyalkanoate class, but could also be found at low prices and from abundant sources. PHBHH was made into a hollow, semi-flared, tissue-engineered, larynx-shape model by solvent casting and compression molding methods; the material maintained a good shape subsequent to desalting by filtering and exhibited considerable mechanical strength. The material was porous and spongy under the electron microscope, the pore size was consistent with the screening of the salt, the porosity was high, which was in line with the basic requirements for a porous structure, and the model showed a certain degree of mechanical strength. Following the co-culture of the cartilage cells with the PHBHH composite material in vitro, the model showed good cell adhesion, distribution and vigorous growth; however, the mechanical strength was still insufficient to maintain the predetermined shape upon implantation, which highlighted the necessity of the auxiliary, in-built support frame. The implantation of simple PHBHH laryngeal cartilage scaffolds, without cells, was taken as the control group; after six weeks an increased loss of general shape was observed in the control group compared with the experimental group, in which a chondrocyte-PHBHH composite had been implanted. Under the microscope, no cartilage-like structure was observed in the control group, and more incomplete degradation impurities of PHBHH were apparent in the cartilage tissue. The shape of the control group implants was lost at the 12th and 18th weeks, suggesting that implantation of the material for six weeks resulted in significant, but not complete, degradation and absorption. Implantation of PHBHH alone, without seed cells, led to an ineffective generation of cartilage tissue. By contrast, after 12 weeks of in vivo implantation in the experimental group, mature, hollow, semi-flared, tissue-engineered laryngeal cartilage could be obtained.

Although basic research in cartilage tissue engineering has achieved encouraging results (22,23), the functional repair and reconstruction of tissue-engineered laryngeal cartilage has not yet been implemented in a practical sense (24,25). The factors impeding this implementation are the blood supply and mucosal and supportive defects of the regenerated cartilage. The limitations in the regeneration of cartilage could be overcome by combining the advantages of tissue engineering technology and the predetermination of cartilage morphology with the advantages of the use of a tissue flap to provide a blood supply. Through this strategy, and using the characteristics of epithelization (26), an affiliation could be achieved between the fibrous tissues and the tissue flap. The tissue flap could be prepared using the design principle of filling the lining of the cavity fascia and wrapping the outer cavity with muscle to adhere to the tissue-engineered cartilage. The complex could then be used to reconstruct the laryngeal cartilage with scaffold functions according to the common nearest transfer or graft method (27-31). This technique is likely to overcome the problems in vascularization, mucosalization and support in cartilage repair and reconstruction for laryngeal cartilage.

Based on the above idea, the present study attempted to take a fascia tissue lining and use myofascial tissue flap filling and wrapping technology to build a predetermined, hollow, semi-flared, tissue-engineered laryngeal cartilage. In the experimental procedure, the myofascial tissue of the fascial portion was placed on the surface of the proposed cartilage construction for contact. Fascia poration not only enhanced blood circulation, but was also conducive to adhesion between the structures. Experimentally designed muscle flap was filled with a rich blood supply, while the seperated flap, which remained in the recessed part, also formed an equally rich blood supply. The cultured chondrocyte-hollow, semi-flared PHBHH composite was placed off the depression of the tissue flap and cultured for a certain period of in vitro. The cavity was filled with fascia and muscle lining, and the periphery was supported by the tissue around depression or recessed slots. The upper part of the complex was covered and wrapped with myofascial tissues. The results of the study showed that the
application of myofascial tissue with a flap blood supply with functional filling and wrapping technology could successfully lead to the generation of hollow, semi-flared, tissue-engineered laryngeal cartilage. Difficulties may arise, however, in avoiding the collapse of the samples, considering the multiple steps in the construction of the hollow, semi-flared, tissue-engineered laryngeal cartilage.

This study has raised the potential for a common transfer transplantation with complex of the tissue flap and hollow, semi-flared, tissue-engineered cartilage, and has indicated the success of the myofascial flap filling and wrapping method in generating hollow, semi-flared, tissue-engineered laryngeal cartilage for functional repair and reconstruction. The study has also demonstrated the potential of the mucosal epithelial transformation of an ideal hollow cartilage surface; however, the results of this study are preliminary, and the design of laryngeal cartilage scaffolds based on this design concept, and the construction, transplantation and practical applications of the composite tissues, should be the direction of future studies.

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