Chitosan tubes can restore the function of resected phrenic nerves

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Abstract

OBJECTIVES: We previously reported that the phrenic nerve could be morphologically repaired by implantation of a chitosan nanofibre tube (C-tube). In the current study, we investigated whether implantation of C-tubes could improve the function of an injured phrenic nerve using a beagle dog model.

METHODS: Seven beagle dogs underwent right thoracotomy under general anaesthesia. An approximately 5 mm length of the right phrenic nerve was resected. Five dogs had a C-tube implantation (C-tube group) and other two dogs did not have the C-tube implantation (control group). Diaphragm movements were longitudinally measured by X-ray fluoroscopy before surgery, immediately after the surgery, and 3, 6 and 12 months after the surgery. The diaphragm movement was determined by diaphragm levels at inspiration and expiration phases, and the excursion difference between them was calculated. At 12 months after the surgery, rethoracotomy was performed to examine electrical phrenic nerve conduction. The C-tube and phrenic nerve were then excised for histological assessment of nerve regeneration.

RESULTS: Three of the five animals of the C-tube group showed improvement of diaphragm movement with time. In these three animals, slow phrenic nerve conduction was observed. Histological assessment showed that the injured nerve was connected by newly regenerating nerve fibres surrounded by granulation tissue within the C-tube. On the other hand, the animals in the control group and two animals of the C-tube group showed neither improved diaphragm movement, nor electrical conduction to the diaphragm. No nerve fibre regeneration was found by histology.

CONCLUSIONS: Our results suggest that, in addition to morphological improvement, C-tube implantation can functionally improve the injured phrenic nerve by promoting phrenic nerve regeneration.

Keywords: Chitosan tube · Phrenic nerve · Nerve regeneration

INTRODUCTION

Extracted from crustacean shells and crab tendons, chitosan has been used as both an aesthetic and a wound dressing material because of its high biocompatibility and moisture retention, and its ability to control bacterial growth [1, 2]. Approximately 95% or more of the phrenic nerve is composed of motor neurons, and it regulates the complex diaphragmatic movements. The phrenic nerve is resected concomitantly in chest malignancy surgery or damaged by trauma or the like, which leads to respiratory failure. The development of nanotechnology in recent years has made it possible to fabricate a tube structure from chitosan using the electrospinning method. We have previously reported that the phrenic nerve was repaired morphologically by chitosan tube implantation [3]. However, it was not proven whether the chitosan tube implantation functionally improved the injured phrenic nerve. Therefore, in the present study, we investigated whether chitosan nanofibre tube (C-tube) implantation could functionally repair the injured phrenic nerve using an animal model.

METHODS

C-tube fabrication

C-tubes were prepared by the method that we reported previously [3]. Briefly chitosan with 93% deacetylation was dissolved in trifluoroacetic acid, and methylene chloride was added. The resulting solution was filtered to obtain chitosan trifluoroacetic acid. By spraying chitosan trifluoroacetic acid, fibre was spun by electrospinning to form a chitosan nanofibre tube around a 2-mm stainless steel bar. During this process, a magnetic field was applied along the long axis of the stainless steel bar, which served as a core for fibre tubes, and chitosan was aligned in the direction...
of nerve elongation. By the above process, a C-tube with a length of 1.8 cm and an internal diameter of 2 mm was prepared.

**Animal experiment**

The experiment was conducted with seven adult beagle dogs. All dogs were treated humanely in accordance with the ‘Principles of Laboratory Animals Care’ defined by the US National Society for Medical Research and the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The implementation guidance was approved by the Institute for Experimental Animals of Kanazawa University and in full compliance with the regulations set forth by this institution.

Ketamine (10 mg/kg, Daiichi Sankyo Co, Ltd, Tokyo, Japan) was administered as a preanaesthetic medication, and thoracotomy was performed by a right posterolateral incision under general anaesthesia (1% isoflurane, AbbVie, Tokyo, Japan). Approximately 5 mm of the right phrenic nerve was resected at a site ≏ 5 cm from the diaphragm. The injured phrenic nerve was contracted and the nerve defect became ≏ 15 mm long. The dogs were divided into two groups: a group of those with no C-tube implant (control group, n = 2), and a group of those that had a C-tube implant (C-tube group, n = 5) (Fig. 1). The stumps of the phrenic nerve in the C-tube group were sutured with 7-0 prolene (Ethicon, Somerville, NJ, USA) at both ends of the C-tube. Antibiotics were administered before the thoracotomy and before chest closure.

**Assessment of phrenic nerve regeneration**

*Measurement of diaphragm movement + video.* Diaphragmatic movement was observed and recorded under X-ray fluoroscopy (Arcadis Avantic, Siemens AG, Munich, Germany), before surgery, immediately after the surgery, and 3, 6 and 12 months after the surgery. The diaphragm was projected in the frontal plane with the subjects in a supine position. Anterior-posterior radiographic images of the diaphragm were obtained. Radiographic measurements of diaphragm excursion included lengths between the inferior endplate of the 13th thoracic vertebra (T13) and the superior dome of the diaphragm at the end of expiration (L\(^e\)) and at the end of the inspiration phase (L\(^i\)) (Fig. 2). The excursion differences (EDs) were calculated by L\(^e\) \(-\) L\(^i\) as the diaphragm movement. The improvement in diaphragm movement was defined as continual shortening of L\(^e\) and L\(^i\), and continual extension of ED.

Student’s t-test was used to compare the difference between the two groups. Statistical significance was defined by a value of \(P < 0.05\). All statistical analyses were performed with SPSS (version 22.0 for Windows, SPSS, Inc., Chicago, IL, USA).

*Examination of electrical phrenic nerve conduction.* Rethoracotomy was performed at 12 months after surgery, and electrical phrenic nerve conduction was examined under macroscopic observation of diaphragmatic movement. In both groups, electrodes were placed on the phrenic nerve, at the cranial side and the caudal side of the C-tube implant, respectively, and an electrical stimulus with the voltage at 10 V, stimulus time at 5 ms and frequency at 1 Hz was sent from the cranial side using an...
electric stimulator (SEN-3401, Nihon Kohden, Co, Ltd, Tokyo) and isolator (SS-203J, Nihon Kohden, Co, Ltd, Tokyo) system (Fig. 3). Electrical nerve conduction was recorded at the cranial and caudal electrodes, and diaphragmatic movement was observed.

**Histological assessment.** After the examination of electrical nerve conduction, the C-tube and phrenic nerve were excised en bloc, and the excised nerve was fixed with formaldehyde and serial sections were made in the long-axis direction of the phrenic nerve. Haematoxilin–eosin (HE) staining and immunostaining with anti-neurofilament antibody were performed to examine nerve fibre regeneration.

**RESULTS**

Results of seven dogs are summarized in Table 1. All dogs survived without signs of pneumonia or infection of the pleural cavity during the follow-up. The implanted C-tube was covered by granulation tissue without abscess formation indicating tube infection.

**Measurement of diaphragm movement + video**

Of the five cases in the C-tube group, improvement in diaphragmatic movement after C-tube implantation was observed in 3 cases. Flattening of the diaphragm and diaphragmatic movement that was substantially coordinated at the left and the right were confirmed at 6 months after surgery in 2 cases and at 12 months after surgery in 1 case (Videos 1 and 2). In these 3 cases, the Le and Li, which were extended by an elevated hemidiaphragm immediately after nerve resection, were shortened to near preoperative values at 6 and 12 months after nerve resection. The ED, which was shortened by dysfunction of the diaphragm immediately after nerve resection, was also extended gradually to near preoperative values at 6 and 12 months after nerve resection.

**Table 1:** Experimental results of phrenic nerve regeneration with a C-tube

<table>
<thead>
<tr>
<th>Group</th>
<th>C-tube (n = 5)</th>
<th>Control (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>1  2  3  4  5  6  7</td>
<td></td>
</tr>
<tr>
<td>X-ray examination</td>
<td>×  ○  ○  ×  ○  ×  ×</td>
<td></td>
</tr>
<tr>
<td>Phrenic nerve velocity</td>
<td>×  ○  ○  ×  ○  ×  ×</td>
<td></td>
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<tr>
<td>Histological assessment</td>
<td>×  ○  ○  ×  ○  ×  ×</td>
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X-ray examination: ○, diaphragm movement improved; ×, diaphragm movement not improved; phrenic nerve velocity: ○, phrenic nerve velocity recorded; ×, phrenic nerve velocity not recorded; histological assessment: ○, nerve fibre regeneration observed; ×, nerve fibre regeneration not observed.

**Video 1:** X-ray fluoroscopy immediately after C-tube implantation. The left diaphragm is elevated and essentially does not move.

**Video 2:** X-ray fluoroscopy 12 months after C-tube implantation. The diaphragm has become lowered to almost the original position, and diaphragmatic movement that is substantially coordinated at the left and the right is confirmed.
The average Le,Li at 12 months after the surgery were significantly shorter than those immediately after the surgery (Le; 10.1 ± 0.6 cm vs 11.2 ± 0.8 cm, P = 0.04, Li; 9.0 ± 0.7 cm vs 10.7 ± 0.8 cm, P = 0.03). The average ED at 12 months after the surgery was significantly longer than that immediately after the surgery (ED; 1.1 ± 0.1 cm vs 0.4 ± 0.1 cm, P = 0.02). The remaining 2 cases in the C-tube group and the 2 control animals, however, showed that the diaphragm remained elevated and essentially did not move (Fig. 4B). There was no difference in the average Le,Li and ED between 12 months and immediately after the surgery (Le; 10.4 ± 1.7 cm vs 10.3 ± 1.7 cm, P = 0.73; Li; 9.9 ± 1.9 cm vs 9.9 ± 1.7 cm, P = 0.79; ED; 0.6 ± 0.2 cm vs 0.4 ± 0.2 cm, P = 0.18).

**Histological assessment**

Histological examination revealed that the regenerated tissue inside the C-tubes had elongated cells that converged to bunches, which were stained pale pink with HE staining, and the periphery was covered with granulation tissue. This tissue was present uniformly from the central side to the peripheral side where the nerve was severed. These converged nerve fibres were also positive on immunostaining with anti-neurofilament antibodies and the resected nerve was connected by the regenerated nerve fibres (Fig. 6).

However, 2 cases of the C-tube group without diaphragm movement did not produce electrical nerve conduction and also did not show nerve fibre regeneration. Of these, 1 case had the inside of the tube filled with thin tissue, which histologically was only granulation tissue without nerve fibres found (Fig. 7). Another case showed that the tube itself had collapsed, and there was no growth of tissue inside the tube.

In 2 control cases, the nerve remained macroscopically resected, and stimulus nerve conduction could not be confirmed. There was no diaphragmatic movement from electrical stimuli coming from the cranial side from the resected part; on the other hand, diaphragmatic movement was observed from electrical stimuli of the caudal side from the resected part. Histologically, no nerve fibre regeneration was observed at the nerve resection site.

**DISCUSSION**

The results of this study demonstrated X-ray confirmation of improvement of diaphragmatic movement, as well as electrical nerve conduction, in beagle dogs in which nerve tissue had been regenerated inside the C-tube. Neither improvement in diaphragmatic movement nor electrical nerve conduction was observed in control dogs in which nerve tissue regeneration was not found. Thus, though not true of all cases, C-tubes can morphologically regenerate nerves, and the resulting nerve regeneration also restores phrenic nerve function.

When the phrenic nerve has been damaged by trauma or surgery for lung cancer, thoracic aortic aneurysm, thyroid cancer or the like, then the result is phrenic nerve paralysis, which leads to severe respiratory failure and decreased quality of life [5].
the treatment of phrenic nerve paralysis, past attempts have included diaphragm pacing, diaphragm plication and phrenic nerve reconstruction [6–8], but these methods do not necessarily produce physiological motor function recovery, and surgery is comparatively more invasive.

Research on nerve regeneration by nerve guide tubes has been advancing since the 1970s. In previous reports, the tubes have been made from materials such as silicon, polyglycolic acid (PGA) and collagen [9]. Lundborg et al. [10] succeeded in using a tube made of silicon to regenerate a 5-mm defect in the median nerve and the ulnar nerve in humans. However, being non-absorbent, silicon provokes inflammation of the surrounding tissue and leads to ischaemia of the regeneration site. Yoshitani et al. [11] used guide tubes made with PGA and collagen for 10-mm defects in the phrenic nerve in beagle dogs and reported nerve regeneration and functional recovery. However, PGA is relatively expensive, and the placement of PGA is desired at sites where there is rich blood flow in the surrounding tissue; therefore, there are still many limitations to nerve regeneration by guide tubes.

Chitosan is taken from crustacean shells and tendons, and has been investigated as a regenerative medicine material for promoting living tissue regeneration with high biocompatibility and the ability to control bacterial growth as a natural polymer [12]. C-tubes, which have also been used in nerve regeneration experiments, can be fabricated relatively inexpensively, and because they are made from crustacean shells and tendons, they are helpful in reusing waste material and are also good for the environment.

Previously, we implanted C-tubes into defects of the thoracic sympathetic nerves and phrenic nerves in beagle dogs and reported that the nerves were morphologically regenerated [3]. The improvement of diaphragm elevation in X-ray images and histological nerve fibre regeneration have been confirmed; however, it was not proved that the repaired phrenic nerve were actually functioning. To prove the functional repair of the phrenic nerves, we have designed an experiment in which diaphragm movement is quantified and the phrenic nerve conduction velocity recorded.

In the 3 cases (60%) in the C-tube group, the continual improvement process of diaphragm movement was measured. There were no previous reports of nerve regeneration showing the continual improvement of nerve function. In this study, we showed that it could take at least 6 months to improve phrenic nerve function by C-tubes.

However, the peripheral nerve conduction velocity was slower by 1/5 to 1/6 of the conventional velocity. There was also attenuation in the stimulus potential. Even when morphological nerve regeneration is observed, it is not proved whether the saltatory conduction is also repaired. Thus, long-term observation is required to confirm the possibility of total function recovery.

The limitations of this study include the fact that the number of dogs is very small, and that the measurement of diaphragm movement could be affected by anaesthesia and thoracotomy. With regard to the C-tube, though its biocompatibility in the body is high, it is still a non-absorbable material, which remains in the body permanently. Furthermore, nerves are not regenerated when the C-tube is made to collapse by the surrounding tissue. To maintain the patency of the tube’s lumen, C-tubes with more rigid duplex coating may need to be developed. Yoshitani et al. [11] described that implanted tubes covered by recipient biological tissue such as pericardial fat can create a better environment for the phrenic nerve regeneration. It is also not clear whether the diaphragmatic side of the phrenic nerve, which has become dysfunctional for a long period after the nerve resection, can be functionally regenerated over time. However, this experiment did confirm diaphragmatic movement with electrical stimulus at the caudal side from the resected part 1 year after the nerve was resected, and so it is possible that the peripheral nerve from the resected part could be functionally regenerated.

In recent years, research in regenerative medicine using induced pluripotency stem (iPS) cells, which can differentiate into
various types of cells has been applied in nerve regeneration [13]. C-tubes are expected to become a conduit for inducing regenerated tissue, and in the future, combined with the usage of iPS cells and improvements to C-tubes, could potentially be applied to humans with improvement in the nerve regeneration rate.

CONCLUSION

Although this was not observed in all cases, the C-tube can functionally repair the injured phrenic nerve. The functional recovery of the regenerated phrenic nerve may not be complete. While there is still room for improvement in terms of device and technique, we believe that the C-tube can be implemented to regenerate phrenic nerves.

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Conflict of interest: none declared.

REFERENCES


