Int J Clin Exp Med 2017;10(3):4687-4695
www.ijcem.com /ISSN:1940-5901/IJCEM0045381

Original Article

Phrenic nerve reconstruction by intercostal nerve neurotization on pigs

Hakan Şimşek[1], Akın Yıldızhan[2], Hakan Çermik[3]

Departments of [1]Neurosurgery, [2]Thoracic Surgery, University of Health Sciences, Haydarpaşa Sultan Abdulhamid Teaching and Research Hospital, Istanbul, Turkey; [3]Department of Pathology, Gülhane Teaching and Research Hospital, Ankara, Turkey

Received January 31, 2016; Accepted February 4, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: Background: Our aim was to investigate the motor outcome, effects on the diaphragm muscle, and delimit the regained nerve conduction capacity of the transected phrenic nerve, following its reconstruction to prepare the subjects to electrophrenic respiration. Methods: All one-year-old 12 healthy pigs (German Landrace) weighing 45 to 50 kg were used in the study. Three groups, each of which consisted of four subjects were composed; control group, anastomosis group, and anastomosis plus pericardial flap group. Initial nerve conduction studies were applied and data were recorded. All the subjects encountered left phrenic nerve transection and except the control group, distal stumps were immediately repaired with intercostal nerve neurotization and coaptation. One subject from each group was sacrificed at 1 h, 2 h, 6 h and 4 weeks after surgery, following final nerve conduction assessments for comparison. Results and conclusions: Harvested nerve distal stumps and diaphragm muscles were examined for histopathological changes. The results yielded that reconstructed nerves responded to stimulation in the end of four weeks and diaphragm was excitable thereafter. Wrapping the repair site with pericardial fat tissue did not prove to be superior to nerve repair alone. More detailed experimental studies are needed to find out the best estimates of timing for repair and neurostimulation.

Keywords: Phrenic nerve reconstruction, neurotization, diaphragm stimulation, electrophrenic respiration, electric stimulation

Introduction

Unilateral phrenic nerve injuries usually result in decrease in pulmonary function, whereas bilateral injury unfortunately leads to mechanic ventilator dependence. Phrenic nerve might either be affected solely or the individual might have encountered an injury as severe as the total spinal cord injury at or above the level of third cervical spinal cord segment [1]. Chronic mechanical ventilation via a tracheostomy has been the standard way of managing quadriplegic patients after a complete cervical spinal cord injury. Mostly attributed other reasons, which cause the patients to be respiratory-debilitated, are injury to the nerve during cardiac surgery, gross neck surgery, mediastinal surgery, interscalene or epidural nerve block procedures, chiropractic manipulations, trauma and tumor in the course of the phrenic nerve [1-8]. Blunt or penetrating injuries to thorax are known to cause phrenic nerve paralysis. While performing intra-thoracic surgery, particularly for tumor resection, and for other intentions, phrenic nerve may be iatrogenically transected and require coaptation and primary repair during surgery. If that is not possible, end-to-end anastomosis with intercostal nerve, accessory nerve transpositioning, or free nerve graft repair can be utilized to reconstruct phrenic nerve [1, 8-14]. Successfully repaired cases that restored diaphragmatic function have been reported in the literature [15, 16]. Some other studies concerning targeted gene expression to promote functional recovery have also been reported [17]. However, experimental and clinical studies are scarce, and lack in identifying the parameters, which would likely carry the outcomes up to more favorable levels. Therefore, the method applied during the surgery totally depends on the experience of the surgeon, and it is most of the time likely to be supplanted by the primarily intended surgical procedure. In order to avoid iatrogenic sequel and

Table 1. Summary of the procedures planned for each group

Groups	Subject number	Surgical procedure	Sacrification time (postoperative)
Control group	1	PNT	1 hour (h)
	2	PNT	2 h
	3	PNT	6 h
	4	PNT	4 weeks (we)
Anastomosis group	1	PNT+INN	1 h
	2	PNT+INN	2 h
	3	FST+INN	6 h
	4	FST+INN	4 we
Anastomosis and pericardial flap	1	FST+INN+PF	1 h
	2	FST+INN+PF	2 h
	3	FST+INN+PF	6 h
	4	FST+INN+PF	4 we

PNT: Phrenic nerve transection, INN: Intercostal nerve neurotization, PF: Pericardial flap.

Figure 1. During the surgical procedure, all the subjects had the invasive Diaphragm EMG and phrenic nerve conduction velocity studies to evaluate the condition of the nerve and diaphragm as baseline values before transecting the nerve. Data were recorded for comparison with the later records of the same subjects.

restore the impaired respiratory function, more experimental and clinical studies are needed. Present study was designed to investigate the motor outcome, effects on the diaphragm muscle, and delimit the overall respiratory functional recovery, following the reconstruction of the transected phrenic nerve using intercostal nerve.

Materials and methods

All one-year-old 12 healthy pigs (German Landrace) weighing 45 to 50 kg used in the study were provided by the Laboratory Animal Application and Research Section in the Research and Development Center in GATA. This study was approved by the Animal Welfare and Commission for Experimental Animal Use of GATA Ankara School of Medicine. Animals were randomly put into three groups (n = 4 for each group): control group (CG), anastomosis group (AG), and anastomosis and pericardial flap group (APFG). Group specifications are summarized in **Table 1**. Same experimental conditions were provided for all subjects. Oral intake was restricted 12 hours before the surgery and all the subjects received 0.01 mg/kg atropine via intramuscular (im) route 10 minutes prior to surgery.

Control group

Left phrenic nerves of the subjects in the control group (CG) were paralyzed by transecting the nerve 1 cm above the *vena cava inferior (VCI).*

Operative design

After premedication by im atropine, induction of anesthesia was performed with administration of im*tiletamine HCL* and *zolazepam HCL* mixture. Subject was then carried onto the operating table and positioned carefully. A venous access was obtained to the ear veins and following the intravenous (iv) administration of 2 mg/kg of bolus *propofol,* endotracheal intubation was carried out. All the subjects received 0.1 mg/kg of iv*vecuronium bromide* as the muscle relaxant. Then, general anesthesia was maintained by *isoflurane* (1.5%) with an oxygen-nitrous oxide mixture (40%:60%) [18]. Mechanical ventilator was set so as to keep a respiration frequency of 14 per minute, tidal volume 6 ml/kg and PEEP 6 mmHg. After proper anesthetization, the subject was placed on the table its left side up and fixed to the table. Left chest skin was shaved, cleaned and disinfected with *povidone-iodine* solution, and thereafter treated in sterile manner. Subject was covered sterile in a fashion to leave only the operative field bare. A thoracotomy incision

Table 2. Operative design

• Induction of anesthesia (imtiletamine HCL and zolazepam HCL 10 mg/kg)
• Placing the subject on the operating table and obtaining venous access to the ear vein
• Further bolus anesthetic infusion (propofol 2 mg/kg iv) and endotracheal intubation
• Muscle relaxant (0.1 mg/kg vecuronium bromide iv)
• General anesthesia maintained by inhalant anesthetics (1.5% isoflurane with a 40%: 60% oxygen-nitrous oxide mixture)
• Mechanical ventilation (tidal volume: 6 ml/kg, PEEP: 5 mmHg, frequency: 14/sec)
• Positioning the subject in lateral decubitus; left side up
• Preparation of the operation field (shaving, sterilizing, wrapping)
• Surgical incision (left thoracotomy in the 5th intercostal space)
• Identification, monitoring and transection of the phrenic nerve
• Coaptation of the intercostal nerve end and distal stump of the phrenic nerve
• Repair with 6/0 monofilament microsurgical polypropylene suture
• Transposing vascular pedicled pericardial fat tissue over the reconstructed nerve site (Last three steps are affiliated with anastomosis and anastomosis plus pericardial flap groups.)

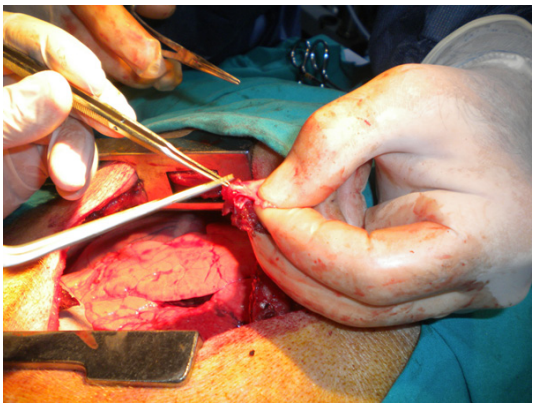

Figure 2. The fifth intercostal nerve was dissected from the neighboring arterial and venous tissue and prepared as one of the free stumps of the anastomosis. Neurons in the anterior horn remain viable at cord levels below the site of injury at high cervical spinal cord injuries. Although these cells are disconnected from the higher nervous system, they preserve their capacity to generate nerve stimuli enough to prevent axonal degeneration and promote nerve regeneration.

was made on the left side and thoracic cavity was accessed through the fifth intercostal space. Left phrenic nerve was identified on the surface of VCI and freed by dissecting gently. Phrenic nerve was then hung with a thick (number 0 silk) string and a nerve stimulator was used to confirm the integrity of the phrenic nerve that contracted the diaphragm on stimulation (**Figure 1**). Following diaphragm EMG and phrenic nerve conduction studies, the nerve was transected approximately 1 cm above the VCI. The steps of the procedure are outlined in **Table 2**. To prevent the unintentional retention of any surgical objects in the surgical wound, we performed a methodical wound exploration before closure of the thoracic cavity. During closure, full expansion of the lung was provided by positive respiratory pressure. Then, the subjects were followed up postoperatively until they were sacrificed at 1 h, 2 h and 6 h under general anesthesia. The last one in the group was extubated and we sacrificed it in the end of 4 weeks after surgery. Just before the sacrifice, diaphragm EMG and nerve conduction studies were performed again and data were recorded. Finally, diaphragms of the subjects were harvested for histopathologic evaluation.

Anastomosis group

Left phrenic nerves of the subjects in the anastomosis group were transected and paralyzed through the same steps that we have described for the control group. In this group, fifth intercostal nerve was identified and dissected from the surrounding tissue. It was cut from a distance that would allow to coaptation of the stumps of the phrenic and intercostal nerves (**Figure 2**). Left phrenic nerve was identified over the surface of the IVC and then transected with a sharp scalpel at the level of 6th or 7th thoracic vertebra level. The free ends of the distal phrenic nerve and proximal 5th intercostal nerve were drawn together and neurorrhaphy was performed in the epineurium using interrupted 6/0 microsurgical polypropylene suture under magnification (**Figure 3**). Operative site was checked for foreign body and after bleeding control; surgical wound was sutured in airtight fashion assuring full expansion of the lung. Then, the subjects were followed up postoperatively until they were sacrificed at the same time intervals of the control groups (1 h, 2 h, 6 h and 4 weeks, postoperatively). Just

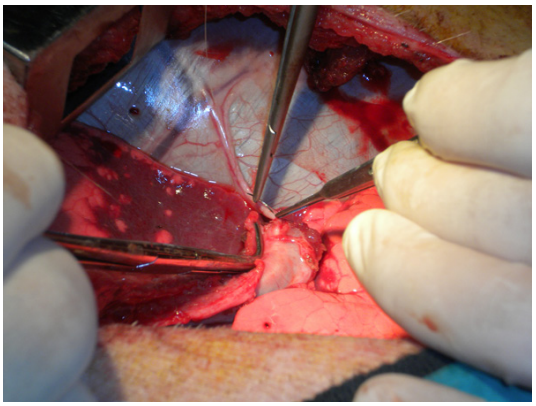

Figure 3. Distal stump of the phrenic nerve is also prepared for coaptation. Intercostal nerve end is approached into the anastomosis area and reconstruction was achieved using 6/0 polypropylene nonabsorbable, monofilament suture in the epineurium of both ends. After reconstructing a continuity of the intercostal nerve with the phrenic nerve, vascular pericardial fat tissue was reflected over the anastomosis site and wrapped by the tissue for supporting the healing process and neural regeneration.

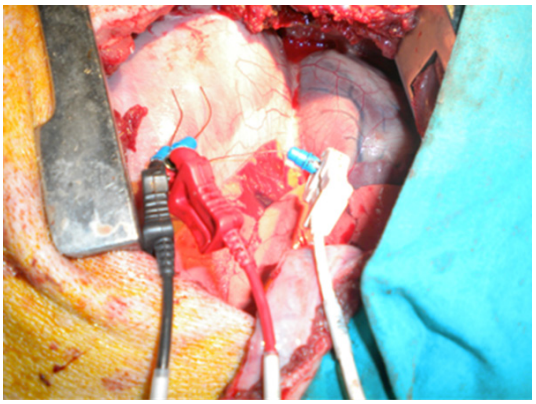

Figure 4. During the nerve conduction studies, three pins were placed in the diaphragm muscle and the phrenic nerve was stimulated 7 cm proximal to the diaphragm.

before transecting the phrenic nerve and the sacrifice, diaphragm EEG and nerve conduction studies were performed. Diaphragms of the subjects were resected totally for histopathologic evaluation during sacrifice.

Anastomosis and pericardial flap group (APFG)

All the steps in the anastomosis group were followed with a difference of additional transposition of a vascular pedicled pericardial fat tissue over the reconstructed nerve site. Electrophysiological studies, sacrificing subject, and harvesting diaphragm were all held in the same way as the anastomosis group (**Figure 4**).

EMG of the diaphragm and phrenic nerve conduction studies

All the subjects enrolled in the study had baseline phrenic nerve conduction velocity measurements. Initial nerve stimulations and recordings were carried out with an electromyograph before transecting the phrenic nerve to assure its integrity. Three pins were placed in the diaphragm muscle. Electric stimulations were given to the phrenic nerve 7 cm proximal to the diaphragm where it merges into the muscle and its motor nerve conduction velocity was calculated and recorded for each nerve (**Figure 4**).

Final nerve stimulations for NCS for the subjects, which underwent nerve reconstruction, were applied 5 cm proximal to the anastomosis site before sacrifice and data were again recorded individually for each nerve.

Histopathologic evaluation

In the end of the designed waiting time for the subjects, one from each group was sacrificed and diaphragms of the animals were totally resected for histopathological evaluation. All muscular tissues were placed in 10% buffered paraformaldehyde and they were left for 12 hours for fixation. Cross-sectional specimens were cut out and they were embedded in paraffin. Two slides for each nerve with specimens of 4 μm thickness were prepared and stained with hematoxylin and eosin (H&E). Single pathologist in blind fashion evaluated all slides with light microscopy. The number of atrophic muscle fibers were calculated for each case, as 0 = none, 1 = 1-5 (mild damage), 2 = 6-10 (moderate damage), 3 = more than 11 (severe damage) in 10 high power fields (HPF, ×400) (**Table 3**).

Statistical analysis

In this study, we analyzed the data using SPSS 11.5 for windows (SPSS Inc, Chicago, IL, USA). The study was tested by applying student-t and ANOVA tests as parametric, and Mann-Whitney U and Kruskal-Wallis techniques as non-parametric techniques. *P* value less than 0.05 ($P<0.05$) was accepted as significant.

Table 3. Overall outcome of nerve conduction studies and classification of histopathological findings in terms of atrophy

Group	Subject numbers	Time for sacrifice (hour) (week)	Nerve conduction+/-	Degree of atrophy
Control group	1	PO 1 h	-	3 (severe)
	2	PO 2 h	-	3
	3	PO 6 h	-	2 (moderate)
	4	PO 4 w	-	1 (mild)
Anastomosis group	5	PO 1 h	-	3
	6	PO 2 h	-	1
	7	PO 6 h	-	0 (none)
	8	PO 4 w	+ (2.3 m/s)	0
Anastomosis and flap group	9	PO 1 h	-	3
	10	PO 2 h	-	1
	11	PO 6 h	-	0
	12	PO 4 w	+ (1.3 m/s)	0

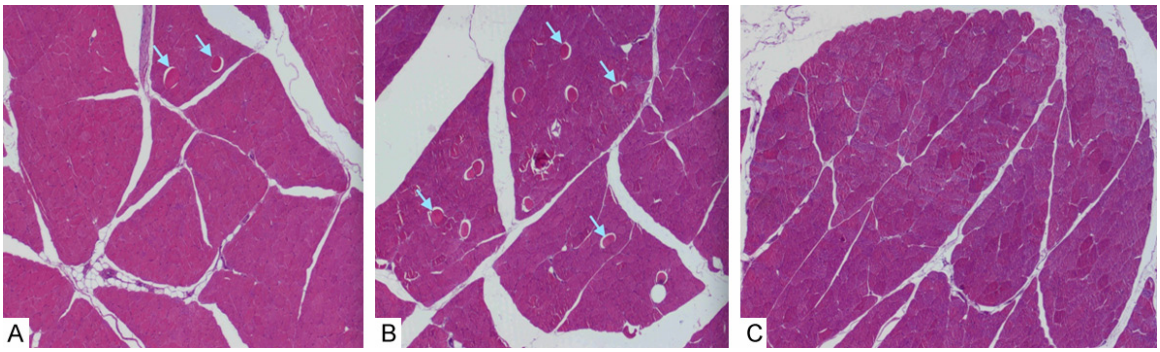

Figure 5. A. In mild damage, denervation atrophy of many fascicles was seen (arrow) (HE, ×40). B. Arrows point out randomly distributed severe denervation atrophy of several fasciculi (HE, ×40). Some of fibers are shrunk. C. Normal fasciculi of muscle in control group (HE, ×40).

Results

A total of 12 pigs were enrolled in the study. In the end we obtained 12 diaphragms for histopathological evaluation.

The phrenic nerve conduction velocity measurements prior to nerve transection yielded a mean PNCS of 8.7 meters/second (m/s). Before sacrifice, only the subjects undergoing nerve reconstruction received phrenic nerve conduction velocity measurements again. We could not get any sign of nerve conduction in the subjects, which were sacrificed at 1 h, 2 h, and 6 h after surgery. But NCS in two subjects that were followed up for four weeks yielded 2.3 and 1.3 m/s of nerve conduction velocity, respectively (**Table 3**).

We found denervation atrophy of many fascicles in some groups. Some of the fibers were shrunk (**Figure 5A-C**). Inflammation was not detected in any of the groups. The atrophy that took place in the myofibers of the muscle was more severe in the subjects that did not have nerve reconstruction. The two groups, which were subject to nerve reconstruction, did not reveal any significant difference in regard to atrophy or necrosis. Histopathological evaluation results were given in **Table 3**.

Discussion

Patients with high spinal cord injuries may totally be dependent to artificial ventilation and the use of the cumbersome mechanical ventilation devices restricts the mobility of the patients and moreover requires usually intensive care, though some of the families might be educated for home care. To substitute mechanic ventilation devices, electrophrenic stimulators have been used successfully for decades [19-24].

That the phrenic nerve encounters a gradual degeneration and fibrosis takes place at the motor-neuron end plate at patients with C3-5 spinal cord injury is a matter of utmost importance, because an excitable phrenic nerve viable for electro-stimulation is essential. People who have sustained spinal cord injury at the C3-5 level will not be able to propagate a nerve stimulus, nor respond to diaphragmatic pacing devices to start electrophrenic respiration due to the axonal loss in their phrenic nerves [3]. Another prerequisite for a working electrophrenic respiration is a responsive diaphragmatic muscle that did not undergo fibrosis. Absence of innervation of the diaphragm by a viable phrenic nerve precludes the success of electrophrenic stimulation [25-27]. Restoration of the phrenic nerve is the procedure of choice to keep the diaphragm in working condition if electrophrenic respiration is intended. Results in the literature so far have been promising, however, most of the data are released from individual cases. Literature search revealed that a time span of 3 months to one year was needed to condition thedenervated diaphragm in order to appropriately respond to stimulation and yield an effective tidal volume [3, 28, 29]. Fibrosis of the end organ occurs in one year, therefore, reconstruction of the injured phrenic nervehas to be achieved no later than a year after injury. Otherwise, functional recovery will not be recorded because of the irreversible fibrotic changes in the muscle [30].

This issue is a result of accommodating changes according to the demand. Metabolic and functional requirements determine which biological or chemical characteristics will be acquired or lost in order to better suit to the new conditions [31]. A decrease in metabolic demand as a consequence of exceedingly immobilization, bed rest, lesions of the spinal cord or dorsal roots, neuromuscular block, and peripheral nerve injury or blockade lead to the atrophy of the muscles. However, as in exercise and using the muscles contrary to gravity (anti-gravity muscles), or electrical stimulation of the peripheral nerve, some conditions generate an increase in functional and metabolic demand. Chronic stimulation of the fast twitch skeletal muscles with low-frequency electric current is achieved by implanting appropriate devices that causes the fast-twitch muscles to undergo a sequence of changes which in the end bring about complete transformation to a slow-twitch muscle [32]. In the first week, capillary density of the muscle increases, and after the first week of stimulation, some metabolic changes begin to occur that consist of shifting from anaerobic metabolism towards aerobic metabolism [31, 32]. Parallel to the increase in the capillary density and blood flow, resistance to fatigue is also observed. When metabolic conversion is completed, the muscle becomes fully resistant to fatigue as long as it is stimulated with the appropriate frequency and nerve integrity is preserved [31-34]. Muscles, which have acquired slow-twitch characteristics as a result of chronic low frequency stimulation, gradually regain their original fast contracting properties when stimulation is discontinued. This observation suggested that a slow muscle should be eligible to be transformed into fast twitch muscle when its motor nerve activity is eliminated [33, 35]. Several animal studies were designed to evaluate impact of elimination of the descending influence of lower motor neuron by cord section [26, 31, 36, 37]. It was strongly suggested that following spinal cord and dorsal root section, denervated muscles developed fast-twitch physiological properties; therefore, in order to maintain excitability of the diaphragm without fatigue, it is essential to establish the integrity of the phrenic nerve by neurotization if transected. A quadriplegic patient is a candidate for chronic electro-stimulation, so conditioning the muscle via phrenic nerve stimulation will be necessary to gradually prepare it for metabolic transformation that gives the muscle resistance to fatigue during the upcoming chronic electrophrenic respiration. The fourth or fifth intercostal nerves are used for intercostal nerve neurotization because; they reach to the phrenic nerve most easily, thus providing an anastomosis without tension. In cases of high cervical spinal cord injury, neurons in the anterior horn remain viable at cord levels below the site of injury. Although these cells are disconnected from the higher nervous system, they can propagate nerve stimuli enough to prevent axonal degeneration and promote nerve regeneration. But the stimuli these neurons generate are in not an orderly fashion, and that necessitates pacing after transferring one of these nerves to phrenic nerve [3, 19, 38]. Studies evaluating the condition of the phrenic nerve upon chronic

electrical stimulation had apparently shown that electro-stimulation of the phrenic nerve did not injure the nerve when applied properly [33]. No morphological changes that could be correlated with the duration of the application was discerned [33]. Therefore, a prompt restoration of integrity as mentioned is the most important step of desired functional recovery of respiration.

Successful nerve grafting depends on preservation of the potential for nerve conduction and the viability of motor end plates. Conventional teaching states that after a year or not more than 1.5 year of denervation, the motor end plates degenerate, thereby causing to fail regaining function despite a reconstructive treatment [8, 9, 11-14]. However, there are reports of nerve transplants well beyond this time period after initial injury that show functional recovery of the diaphragm. We think the issue here is that in cases where there is partial denervation, the motor end plates may receive a subthreshold level of signal enough to maintain viability of the motor end plates despite an absence of diaphragm contractility. Therefore, the exact time for reinnervation surgery is disputable.

In our study, we evaluated the acute histopathologic and electrophysiological effects of nerve injury. In addition, functional behavior of the diaphragm muscle and the nerve conduction velocity were studied in the end of four weeks to assess the extent of functional recovery achieved by nerve reconstruction. Our findings were consistent with that of the literature. Since we advocate reconstructing the integrity of the nerve and preserving contractility of the diaphragm as early as possible, we evaluated the very early changes in the diaphragm following transection and reconstruction of the nerve. Also in the end of four weeks-the generally estimated time of axonal regeneration for the studied length of phrenic nerve- histopathologic changes were researched. As we did not expect earlier recovery in nerve conduction studies, 1st, 2nd, and 3rd subjects in the three groups, which were sacrificed in the end of 1, 2, and 6 hours yielded negative EMG studies. However, EMG studies in the end of four weeks showed functional nerve conduction velocities. As the shortage of the study, there were a limited number of animals in each group and the outcome data needs to be verified with long term follow up results with larger groups to obtain statistical significance.

In our surgical technique we repaired the nerve by end-to-end coaptation either supported by wrapping the site with pericardial fat or leaving unwrapped. *Yoshitani* described a series of phrenic nerve repairs using nerve tubes in dogs, demonstrating that the diaphragm can regain function using neural tubes as a conduit for nerve regeneration, especially in the setting of a pericardial fat pad. In their series, the phrenic nerve was acutely transected and replaced with a 30-mm graft [10]. By 4 months, three of the four dogs receiving the nerve tube placed in a pericardial fat pad had regained function. In the group without use of a pericardial fat pad, only one of five dogs regained function [10]. However, we did not observe any significant superior nerve healing effect of using pericardial fat in terms of nerve conductivity and muscle contractility that shows successful nerve healing. Probably longer follow-up should have worked. According to our findings, damage to the lower motor neuron may cause myofiber atrophy.

We also detected that when myofibers lose their innervation, they become angular and shrink. In the early stage, denervation may cause atrophy of isolated myofibers, which are dispersed in a haphazard fashion. At an extreme stage of atrophy, almost all sarcoplasm is lost and the myofiber is reduced to a bunch of nuclei.

In conclusion, phrenic nerve neurotization using intercostal nerve in the reconstruction of the transected phrenic nerve yields early regeneration potential and is a reliable means of preparing the patient to electrophrenic stimulation if needed. Phrenic nerve should be repaired as early as possible. In order to avoid iatrogenic sequel and restore the impaired respiratory function, more experimental and clinical studies with larger groups and longer follow-ups are needed.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hakan Şimşek, Haydarpaşa Sultan Abdulhamid Egitimve Araştırma Hastanesi, Beyin Cerrahisi Bölümü, Tıbbiye Caddesi,

34668, Üsküdar, Istanbul, Turkey. Tel: +90 533 3884087; Fax: + 90 216 348 7880; E-mail: drhakansimsek@hotmail.com

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