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Experimental study on neurorrhaphy of the recurrent laryngeal nerve in dogs

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Abstract

The effectiveness of anastomosis of a divided recurrent laryngeal nerve was evaluated in six adult mongrel dogs. Videolaryngoscopy and evoked compound muscle action potentials in the intrinsic laryngeal muscles were performed at six months and the posterior cricoarytenoid muscles and recurrent laryngeal nerves were processed for histomorphometric studies. Recovery of compound muscle action potentials in all re-innervated muscles and histomorphometric findings confirmed a good grade of axonal regeneration. The most significant histomorphometric changes observed were: a reactive hypertrophy of type I fibres in the posterior cricoarytenoid muscles of the re-innervated side, and a high nerve fibre density in the distal stump to the anastomosis. However, incomplete recovery of motion and fasciculated movements of the re-innervated vocal folds were observed. Reduction of effective motor units in the re-innervated muscles might be a factor that cause incomplete restoration of vocal fold movements.

Key words: Recurrent laryngeal nerve; Anastomosis, surgical; Dogs

Introduction

Several surgical procedures have been proposed for rehabilitation of vocal fold paralysis, and many of these procedures are specifically directed toward restoration of functional neural control to the paralyzed vocal fold. Horsley (1909) reported the first successful vocal fold re-innervation, describing nearly complete function return following neurorrhaphy of the recurrent laryngeal nerve (RLN). Since then, attempts have been made to repair the injured RLN, with conflicting results. It may be concluded from many reports that misdirection of regenerated nerve fibres, demonstrated by electromyographical investigations, is a definite problem to functional restoration after neurorrhaphy for recurrent laryngeal nerve paralysis and results in bizarre behaviour, paradoxical movement or spasmodic action of the vocal fold (Siribodhi, 1963; Dedo, 1971; Sato and Ogura, 1978). However, results from some investigations do not provide electromyographic evidence indicating misdirection of the regenerated nerve fibres, and suggest that reduction in the number of re-innervated motor units or failure of the sprouting axons to reach muscle fibres are the main causes of incomplete recovery of vocal fold movements (Gordon and McCabe, 1968; Mu and Yang, 1990).

There is essentially no data in the literature on the histochemical evaluation to support the effectiveness on the process of laryngeal muscle re-innervation. The best sign of re-innervation was considered to be loss of the expected mosaic pattern of muscle fibre arrangement, i.e., re-innervation would be noted by muscle fibre type grouping. However, muscle fibre type grouping is also observed in the laryngeal muscles when the recurrent nerve is excised, because spontaneous re-innervation can be provided from axon sprouts of the adjacent normally innervated muscles (Hatsuyama, 1966), or from regenerated axons of the transected recurrent laryngeal nerve (Crumley and McCabe, 1982; Zealear *et al.*, 1994).

Because several issues regarding the effectiveness of recurrent nerve anastomosis have not as yet been resolved, an experimental evaluation using dogs was made. The purpose of this study was firstly, to observe vocal cord function after RLN neurorrhaphy; secondly to evaluate electrophysiologically RLN regeneration; and thirdly to determine histochemical and histomorphometric changes of the posterior cricoarytenoid muscles (PCA) and recurrent laryngeal nerves following RLN anastomosis.

Materials and methods

Six healthy adult mongrel dogs (14 to 20 Kg) were

This study was performed in accordance with the Policy on Humane Care and Use of Laboratory Animals; the animal use protocol was approved by the Department of Surgery and Medicine of 'Universidad de Cantabria'.

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used in this study. Under halothane inhalation anaesthesia, a vertical midline neck incision, 10 cm long, was made to expose the left RLN which was sectioned and then sutured immediately 2 cm below the cricothyroid joint. Nerve suturing was performed under the operating microscope and four to five sutures of 10-0 black monofilament nylon swagged to a fine needle were placed through the perineurium. Care was taken to maintain as closely as possible the correct orientation of the segments. Before and after surgery the larynx was examined by direct laryngoscopy, and the resulting left vocal fold paralysis as verified in all cases.

All animals were examined at six months after the above described surgical procedures. Videolaryngoscopy was performed under intravenous pentobarbital anaesthesia, and vocal fold motion was recorded on videotape during spontaneous respiration. After this examination, both recurrent nerves of each dog were exposed and sectioned 3 cm below the cricothyroid joint. Utilizing two silver electrodes the distal stumps were stimulated in the form of 2-millisecond (ms) square-wave pulses provided by a digital nerve stimulator. In the left (operated) side stimuli were always given proximal to the neurorrhaphy site. Evoked compound muscle action potentials were recorded with unipolar concentric needle electrodes DISA 15K53 inserted into the ipsilateral PCA and thyroarytenoid (TA) muscles. These signals were routed to a Tektronix digital oscilloscope and amplitude, duration and latency of the evoked compound muscle action potentials on the left and right sides were analysed and compared by paired Student's *t* tests. Following on from this, both laryngeal superior nerves were sectioned, and evoked compound muscle action potentials were recorded again.

Finally, each animal was killed by use of barbiturate overdose (Buthenasia). Through a vertical midline neck incision the larynx was excised in continuity with a 5 cm segment of both recurrent laryngeal nerves. Gross appearance of the PCA muscles was assessed.

Immediately after euthanasia, mid-belly muscle specimens of left and right posterior cricoarytenoid muscles were obtained from all dogs. Muscle specimens were frozen in isopentane, cooled in liquid nitrogen, and serial cross sections, 5 μm thick, were cut throughout the length of the muscle. In addition to routine haematoxylin and eosin staining, the muscles were processed for histochemical reactions: (ATPase) adenosine triphosphatase (Guth and Samaha, 1970), and (NADH) nicotinamide-adenine dinucleotide dehydrogenase (Novikoff *et al.*, 1961).

Nerve specimens of both RLN were obtained from all dogs. Two cm segments of the unoperated nerve, and from above and below the neurorrhaphy site were removed, and then fixed in three per cent glutaraldehyde, postfixed in one per cent osmium tetroxide, washed in phosphate buffer, transferred through graded acetone solutions, and then processed for embedding in Spurr plastic medium. Semithin sections of 1 μm were cut transversely and stained with paraphenylenediamine (Estable-Puig *et al.*, 1965; Braund *et al.*, 1988).

For quantative studies, transverse muscle sections were projected onto a monitor, using a computerized video-display image analysis system (Kontron Mop-Videoplan analyzer). Serial 1-cm sections were examined throughout the length of the muscle; 100 fibres were counted per section. The smallest fibre diameter of type-1 and type-2 fibres was determined, and the muscle fibre type composition of each muscle was determined by counting type-1 and type-2 fibres.

The fibre diameters and their relative compositions were plotted as histograms for each fibre type and for each muscle on both operated and non-operated sides. Percentages of type-1 and type-2 fibres were determined; and the mean fibre diameter and standard deviation of each fibre type were calculated.

Transverse sections of nerve were projected onto a monitor, using the same computerized video-display image analysis system for quantitative studies. Axonal counts, nerve area and diameters of myelinated nerve fibres were determined. In all samples all nerve fibres were counted. The nerve fibre diameters and their density (number of fibres/mm^2) were plotted as histograms for each nerve: non-operated side, and proximal to the anastomosis and distal to the anastomosis in the operated side.

Results

All of the animals survived the planned duration of the study and none of them developed complications. Pre-operatively, all six dogs had normal spontaneous cord mobility.

Endoscopic studies

Videolaryngoscopy at six months showed recovery of spontaneous adduction and abduction of the vocal fold on the side of the recurrent nerve section and anastomosis in four of the six dogs. Both adduction and abduction were synchronous with the normal vocal fold, although re-innervated vocal fold showed lesser motion amplitude. Only slightly weak motion of the re-innervated vocal fold was observed in the remaining two dogs. In all cases frequently fasciculated movements were observed in the vocal folds of the re-innervated side.

Electrophysiological data

In all instances, a biphasic compound muscle action potential was obtained, with an initial steep negative deflection followed by another steep positive peak before returning to base line.

Results of amplitudes, latencies and durations of the potentials are given in Table I. Amplitudes were greater in the muscles on the control side, whereas latencies and durations were greater in the muscles of the re-innervated side. In all instances, differences between re-innervated and control sides were

TABLE I
MEAN RESULTS OF EVOKED MUSCLE ACTION POTENTIALS

		Unoperated side	Operated side	Difference
Amplitude (mV)	PCA:	194 ± 73	104 ± 35	(p<0.05)
	TA:	267 ± 51	79 ± 33	(p<0.05)
Latency (ms)	PCA:	2.74 ± 0.36	3.56 ± 0.68	(p<0.01)
	TA:	3.72 ± 0.20	4.49 ± 0.30	(p<0.01)
Duration (ms)	PCA:	11.7 ± 1.64	13.9 ± 2.69	(p<0.05)
	TA:	14 ± 3.16	16 ± 3.06	(p<0.05)

Potentials recorded in the posterior cricoarytenoid muscle (PCA) and thyroarytenoid muscle (TA)

significant. No differences were observed in compound muscle action potentials obtained after section of both laryngeal superior nerves.

Histomorphometric evaluation

PCA muscles

There was no difference in the gross morphology appearance between the right PCA muscles and left re-innervated muscles. Using the oxidative stain NADH-TR, PCA muscles of the unoperated side showed a mosaic pattern, with the darker staining type-1 fibres appearing smaller than the lighter type-2 fibres (Figure 1A). Use of ATPase revealed light-staining type-1 and dark staining type-2 fibres. PCA muscles of the re-innervated side preserved a random mosaic pattern, with minimal isolated areas of atrophic fibres, although muscle fibre type grouping was clearly present in areas of all re-innervated muscles (Figure 1B).

The averaged mean fibre diameters and standard deviations of type-1 and type-2 fibres from the non-denervated and re-innervated PCA muscles are given in Table II. Comparison of the mean fibre diameters between normal muscles and re-innervated muscles showed that mean type-1 fibre diameter was greater in each of the re-innervated muscles than in the control muscles, and this difference was significant (p<0.001). However, no significant differences were found in any cases with respect to type-2 fibre diameters. The histographic distribution of fibre type diameters was unimodal in all muscles. Type-1 fibres ranged from 20 μm to 60 μm with a peak at 30-40 μm, and type-2 fibres ranged between 20 μm and 70 μm with a peak at 40-60 μm. PCA muscles of the re-innervated side showed slight displacements on the right of the type-1 fibres (Figure 2).

TABLE II
MEAN FIBRE DIAMETERS AND FIBRE TYPE PERCENTAGES FROM POSTERIOR CRICOARYTENOID

	Mean diameter (μm)	Number counted	Fibre (%)
Control side			
Type-1	29.05 ± 3.55	600	51.09%
Type-2	39.04 ± 5.87	600	48.91%
Operated side			
Type-1	33.47 ± 5.58	600	50.91%
Type-2	40.56 ± 7.22	600	49.09%

Recurrent nerves

In cross-sectional nerve preparations, recurrent nerves consisted of one or two funiculi, and a variety of patterns was observed; some fascicles contained mixtures of small and large-diameter fibres (Figure 3A), whereas others contained either predominantly small- or large-diameter fibres. In the operated side (Figure 3B), axonal sprouting images were frequently found in all samples of both stumps of the anastomosis. There was no evidence of focal or multifocal fibre loss, and isolated areas of axonal degeneration were observed in only two cases.

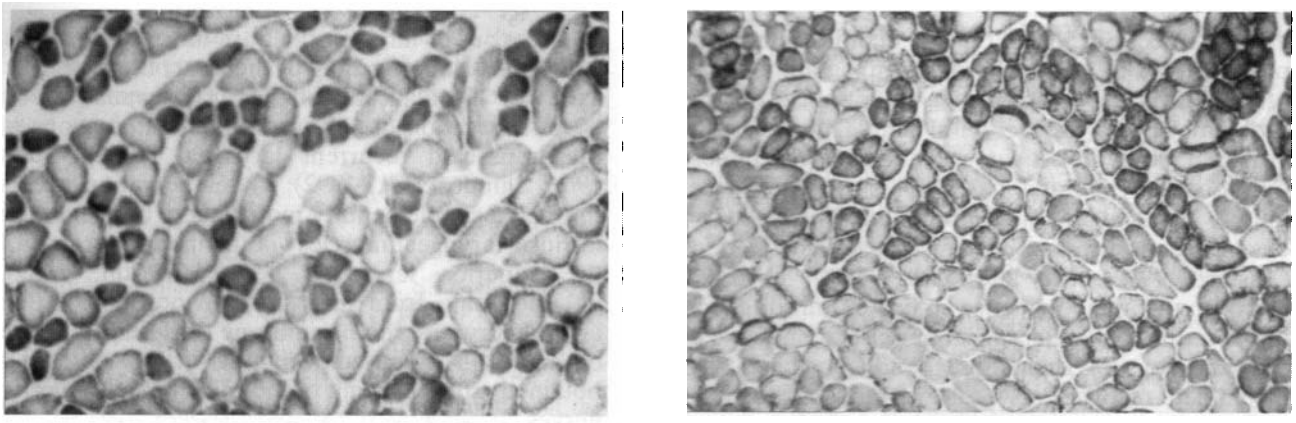
Nerve fibre density (expressed as nerve fibres/mm^2) of cross-sectional nerve preparations are given in Table III. Mean nerve fibre density of the right recurrent laryngeal nerves was 8183 ± 1258, whereas mean densities of distal and proximal stumps to the anastomosis of the left recurrent laryngeal nerves were not possible due to wide dispersion of values. It was significant that nerve fibre density was greater in the distal stump to the anastomosis than in the proximal stump in five out of six dogs.

Discussion

It may be concluded from many reports that RLN neurorrhaphy achieves nerve fibre regeneration and laryngeal muscles re-innervation, but does not achieve a return to normal vocal fold motion. It has generally been accepted that incomplete recovery of motion of the re-innervated vocal fold is caused by misdirected nerve regeneration (Ruedi,

TABLE III
NERVE FIBRE DENSITY OF RECURRENT LARYNGEAL NERVES

	Anastomosed left RLN		
Dog	Proximal stump	Distal stump	Right RLN (Control)
S-1 Density	6698/mm^2	9601/mm^2	9471/mm^2
S-2 Density	5552/mm^2	8048/mm^2	8326/mm^2
S-3 Density	5494/mm^2	6007/mm^2	8519/mm^2
S-4 Density	11524/mm^2	14585/mm^2	6313/mm^2
S-5 Density	6791/mm^2	8737/mm^2	9429/mm^2
S-6 Density	10197/mm^2	2437/mm^2	7135/mm^2

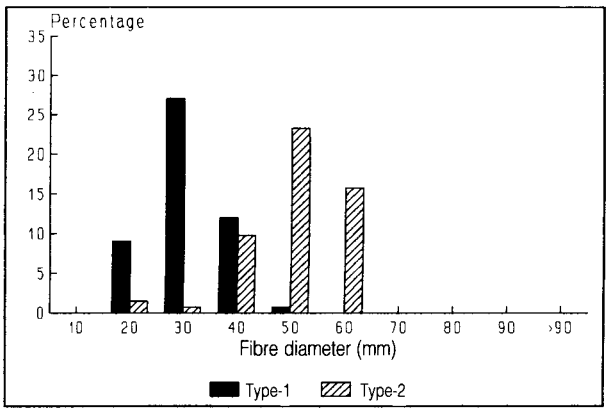


(A) (B)

FIG. 1

Posterior cricoarytenoid muscle (PCA) histochemistry. A) Mosaic pattern of small dark-staining type-1 fibres and larger light-staining type-2 fibres in the right normal PCA muscle (NADH staining; ×125). B) Re-innervated left PCA muscle showing typical 'type grouping' (NADH staining; × 80).

Right PCA muscle (control)



Left PCA muscle (Re-innervated)

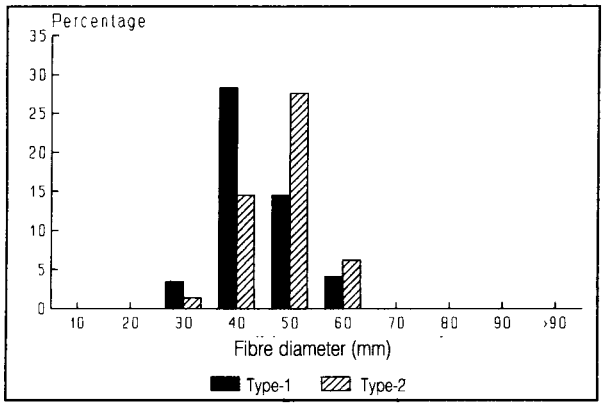
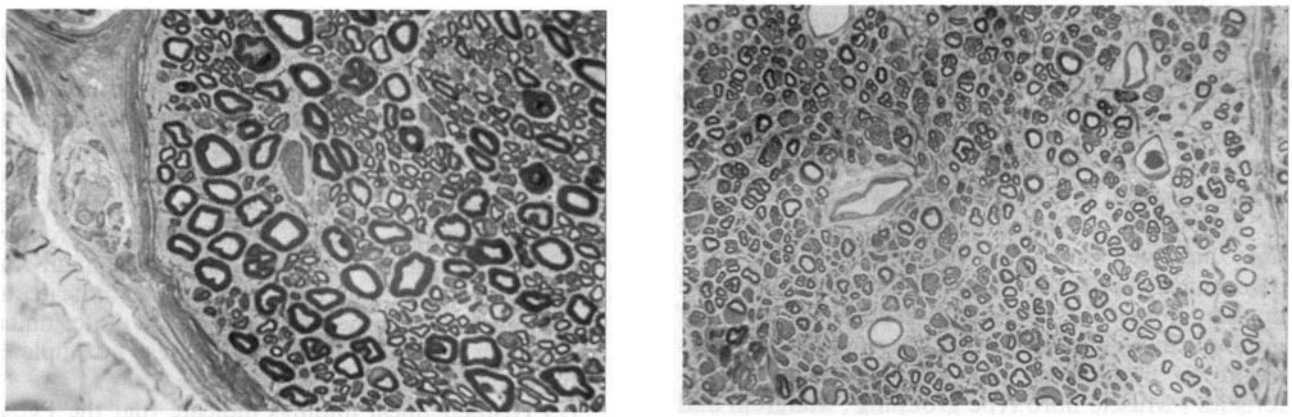


FIG. 2

Fibre type distribution histogram of PCA muscles from dog S-2. Note hypertrophy of type-1 fibres in the reinnervated PCA muscle.



(A) (B)

FIG. 3

Recurrent laryngeal nerves (RLN). Paraphenylenediamine stain; original magnification ×500). A) Section of normal RLN containing a mixture of small- and large-diameter fibres. B) Section of anastomosed RLN (distal stump to the anastomosis). Note many myelinated axons and 'sprouting' images.

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1959). Since Siribodhi (1963) demonstrated this phenomenon by means of electromyography, many others have also shown the importance of this problem (Dedo, 1971; Sato and Ogura, 1978). However, some investigators noted that the connections of the regenerated nerve fibres with the correct muscle fibres were more frequently indicated on electromyography than the misdirected connections (Tashiro, 1972). Mu and Yang (1990) observed electromyographic evidence indicating misdirection in the re-innervated laryngeal muscles in only one of the five dogs of their study; and concluded that incomplete recovery of vocal fold movements might be due to a reduction in the number of re-innervated motor units or failure of the sprouting axons to reach muscle fibres. Compound muscle action potentials obtained in our study showed lesser amplitudes and greater latencies in the re-innervated TA and PCA muscles than in the TA and PCA muscles of the unoperated side. These results might, therefore, indicate a reduction of motor units and a delay of nervous conduction as causes of incomplete recovery of vocal fold movements.

Reliable evaluation of the degree of recovery of fold mobility requires understanding of the remaining action of the superior laryngeal nerve and its cricothyroid muscle. Since the time of Wagner (1890) and Grossmann (1897) it has been recognized that contraction of cricothyroid muscle not only gives tension but also some adductive effects to the vocal fold. Although a cricothyroid muscle's ability to medialize the affected vocal fold once the denervated muscles have atrophied seems proven (Regenbogen, 1989), it has been observed that changes in vocal fold position following superior laryngeal nerves transection were minor (Woodson, 1989). It is also important to correctly evaluate the active fold motion, because passive movement of the vocal folds from the midline to the intermediate position should not be confused with normal abduction (Marie *et al.*, 1988). However, endoscopic results of this study showed recovery of spontaneous adduction and abduction of the re-innervated vocal fold in four of the six dogs, and both adduction and abduction were synchronous with the normal vocal fold, although re-innervated vocal folds showed lesser amplitude of motion and frequently fasciculated movements. On the other hand, no paradoxical glottic movements of expiratory abduction and inspiratory adduction were observed in our study.

The best sign of re-innervation was considered to be loss of the expected mosaic pattern of muscle fibre arrangement. Since the re-innervating axon determines the characteristics of the re-innervated muscle fibre, the normally random mosaic of different muscle fibre types is interrupted by groups of muscle fibres with similar histochemical characteristics ('Muscle fibre type grouping', Malgrem and Gacek, 1981). However, the fact that laryngeal denervated muscles undergo spontaneous re-innervation and muscle fibre type grouping is also observed in denervated muscles. Different possible explanations have been proposed to account for this spontaneous re-innervation: a) re-innervation occurs from collateral branching of the intact intramuscular axons of the adjacent muscles (Hatsuyama, 1966); b) re-innervation occurs from regenerated axons of the transected recurrent laryngeal nerve (Guth, 1962; Shindo *et al.*, 1992); c) reinnervation occurs through a supplementary motor nerve branch to the intrinsic laryngeal muscles via the internal branch of the superior laryngeal nerve (Martin, 1989), although data from this study do not support the last theory as no significant differences were observed in compound muscle action potentials obtained after section of both laryngeal superior nerves.

Comparison of the mean fibre diameters between re-innervated PCA muscles and normal PCA muscles showed that mean type-1 fibres diameters were greater in each of the re-innervated muscles than in the control muscles, and this difference was significant ($p<0.001$), while no significant differences were found in mean type-2 fibre diameters. Data of reactive hypertrophy of type 1 fibres are consistent with a previous study (Martin, 1989).

Morphometric findings of the right unoperated RLN nerves were similar to those reported by Braund *et al.* (1988). However, it was impossible to compare results from the proximal and distal stumps to the anastomosis of the left recurrent because no data were found in the literature. In previous reports axonal counts indicated, with some exceptions, that the number of neurilemma sheaths decreased when progressing from the proximal to the distal side of the neurorrhaphy, although nerve preparations were not processed for semi-thin sections, and nerve fibre density (expressed as nerve fibres/mm^2) was not determined (Gordon and McCabe, 1968; Tashiro, 1972). In the present study nerve fibre density was greater in the distal stump to the anastomosis than in the proximal stump in five out of six dogs; thus, a good grade of axonal regeneration in the anastomosed nerves is achieved.

Conclusions

Results obtained through the present investigation permitted the following conclusions:

(1) Recovery of compound muscle action potentials in all re-innervated TA and PCA muscles when stimuli were given proximal to the neurorrhaphy site means that the regenerated nerve fibres can pass through the anastomosis site and into the intrinsic laryngeal muscles. Lesser amplitudes and greater latencies in the re-innervated muscles than in muscles of the unoperated side might indicate a reduction in the number of re-innervated motor units and might be a factor that causes incomplete restoration of vocal fold movements.

(2) Histochemical findings indicate that the PCA muscles of the re-innervated side showed evident signs of re-innervation. The most significant histomorphometric change observed in the re-innervated PCA muscles was a reactive hypertrophy of type I fibres.

(3) Histomorphometric examinations of the recurrent nerves showed a high nerve fibre density in the distal stump to the anastomosis, which indicates a good grade of axonal regeneration in the sutured nerves.

References

Braund, K. G., Steiss, J. E., Marshall, A. E., Jagjivan, R. M., Toivio-Kinnucan, M., Amling, K. A. (1988) Morphologic and morphometric studies of the vague and recurrent laryngeal nerves in clinically normal adult dogs. *American Journal of Veterinary Research* **49:** 2111–2116.

Crumley, R. L., McCabe, B. F. (1982) Regeneration of the recurrent larygneal nerve. *Otolaryngology - Head and Neck Surgery* **90:** 442–447.

Dedo, H. H. (1971) Electromiographic and visual evaluation of recurrent laryngeal nerve anastomosis in dogs. *Annals of Otolaryngology* **80:** 664–668.

Estable-Puig, J. F., Bauer, W. C., Blumberg, J. M. (1965) Paraphenylene diamine staining of osmium-fixed plastic-embedded tissue for light and phase microscopy. *Journal of Neuropathology and Experimental Neurology* **24:** 531–535.

Gordon, J. H., McCabe, B. F. (1968) The effect of accurate neurorrhaphy on reinnervation and return of laryngeal function. *Laryngoscope* **78:** 236–250.

Grossman, M. (1897) Experimentelle beitrage zur tehre von der 'Posticuslahmung'. *Archiv fur Laryngologie und Rhinologie* **6:** 282.

Guth, L. (1962) Neuromuscular function after regeneration of interrupted nerve fibres into partially denervated muscle. *Experimental Neurology* **6:** 129–141.

Guth, L., Samaha, F. J. (1970) Procedure for the histochemical demonstration of actomyosin ATPase. *Experimental Neurology* **28:** 365–367.

Hatsuyama, Y. (1966) Histological studies on reinnervation of denervated muscle, with special reference to collateral branching. *Electromyography* **6:** 71–72.

Horsley, J. A. (1909) Suture of the recurrent laryngeal nerve with report of a case. *Transactions of the Southern Association of Surgery and Gynecology* **22:** 161–170.

Malgrem, L. T., Gacek, R. R. (1981) Histochemical characteristics of muscle fibre types in the posterior cricoarytenoid muscle. *Annals of Otology, Rhinology and Laryngology* **90:** 423–429.

Marie, J. P., Dehesdin, D., Andrew-Guitrancourt, J., Ducastelle, T. (1988) Réhabilitations des Paralyses Laryngées. Etude expérimentale chez le chien. *Revue de Laryngologie Otologie Rhinologie (Bordeaux)* **109:** 39–42.

Martin, F. (1989) Collateral reinnervation of supposedly denervated laryngeal muscles in dogs. *Laryngolrhinootologie (Stuttgart)* **68:** 57–61.

Mu, L. C., Yang, S. L. (1990) Electromyographic study on end-to-end anastomosis of the recurrent laryngeal nerve in dogs. *Laryngoscope* **100:** 1009–1017.

Novikoff, A. B., Shin, W. Y., Drucker, J. (1961) Mitochondrial localization of oxidative enzymes: staining results with two tetrazolium salts. *Journal of Biophysics, Biochemistry and Cytology* **9:** 46–61.

Regenbogen, E. (1989) Glottal closure in the hemiparalyzed canine larynx. *Laryngoscope* **99:** 711–714.

Ruedi, L. (1959) Histology and function of the larynx. *Journal of Laryngology and Otology* **73:** 1–20.

Sato, F., Ogura, J. H. (1978) Neurorraphy of the recurrent larygneal nerve. *Laryngoscope* **88:** 1034–1041.

Shindo, M. L., Herzon, G. D., Hanson, D. G., Cain, D. J., Sahgal, V. (1992) Effects of denervation on laryngeal muscles: a canine model. *Laryngoscope* **102:** 663–669.

Siribodhi, C. (1963) Electomyographic studies of laryngeal paralysis and regeneration of laryngeal motor nerve in dogs. *Laryngoscope* **73:** 148–164.

Tashiro, T. L. (1972) Experimental studies on the reinnervation of larynx after accurate neurorrhaphy. *Laryngoscope* **82:** 225–238.

Wagner, R. (1980) Die mediansiellung der stimmubun derbei der rekusreuslahmung. *Archiv fur Pathologie, Anatomie und Physiologie* **120:** 437–459.

Woodson, G. E. (1989) Effects of recurrent laryngeal nerve transection and vagotomy on respiratory contraction of the cricothyroid muscle. *Annals of Otology, Rhinology and Laryngology* **98:** 373–378.

Zealear, D. L., Hamdan, A. L., Rainey, C. L. (1994) Effects of denervation on posterior cricoarytenoid muscle physiology and histochemistry. *Annals of Otology, Rhinology and Laryngology* **103:** 780–788.

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