Quantitative analysis of the anatomy of the epineurium of the canine recurrent laryngeal nerve

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(Accepted 24 August 1999)

ABSTRACT

The purpose of this investigation was to determine the amount of epineurium surrounding the recurrent laryngeal nerve (RLN) compared with a limb nerve, that to flexor hallicus longus (NFHL). Nerve samples were obtained from 10 adult dogs and studied using scanning electron microscopy and light microscopy to measure the relative proportion of epineurium and the relative proportions of adipose and collagenous tissue comprising the epineurium in both nerves. Significantly greater relative epineurial cross-sectional areas and adipose content were found in the RLN than in the NFHL. Based on observations on noncranial peripheral nerves, the findings indicate that the RLN is better protected against deformational forces associated with compression than stretching forces. The RLN may not be structured well for successful reinnervation after injury. The patterns observed for adipose tissue in RLN epineurial tissue appeared unique compared with those previously reported in peripheral nerves. The primary role associated with adipose tissue is to ‘package’ the nerve for protection. The RLN is considered to be a vital nerve in the body, as are other cranial nerves. The large proportions of adipose tissue in the epineurium may relate to the importance of protecting this nerve from injury.

Key words: Peripheral nerve; connective tissues.

INTRODUCTION

The motor and sensory functioning of the larynx is vital for such functions as breathing, swallowing and voice production. The nerves innervating the larynx are, therefore, of great clinical importance. These nerves, which follow a long course through the thoracic cavity and neck, may be damaged by trauma, surgical procedures to the neck, trachea, oesophagus, or heart. They may also develop an unexplained ‘idiopathic’ neuropathy. It is therefore important to understand their anatomy in terms of their ability to resist damage. Few studies, however, have described the connective tissue surrounding the laryngeal nerves. These tissue coverings (see Fig. 1) have been demonstrated to provide the primary structural framework and biomechanical protection in peripheral nerves. Information regarding the quantity and composition of these tissues in laryngeal nerves is important for determining their protective characteristics. This information could also contribute to surgical approaches such that nerve damage may be prevented. This study will fill in some of these gaps by studying the size and content of the outermost connective tissue layer, the epineurium, in the canine left recurrent laryngeal nerve.

Innervation of the larynx

The motor and sensory nerve fibres to the larynx travel with the vagus nerve before branching into the superior and recurrent laryngeal nerves (Dilworth, 1922; Lemere, 1932a, b; Armstrong & Hinton, 1951; Williams, 1951; Rustad & Morrison, 1952; Sunderland & Swaney, 1952; Vogel, 1952; Rustad,
1922; Lemere, 1932; during swallowing, and respiratory valving (Dilworth, 1922; Lemere, 1932; Armstrong & Hinton, 1951; Williams, 1951; Vogel, 1952; Rustad, 1954; Bowden, 1955; Wyke & Kirchner, 1976; McClure, 1979; Tucker, 1993). The right and left RLNs display asymmetric routes to the larynx. The left RLN travels with the vagus nerve until its branching point near the aortic arch in the thoracic cavity. After looping under the aortic arch, it ascends along the tracheo-oesophageal crease towards the larynx (Dilworth, 1922; Lemere, 1932; Armstrong & Hinton, 1951; Williams, 1951; Vogel, 1952; Rustad, 1954; Bowden, 1955; Wyke & Kirchner, 1976; McClure, 1979; Tucker, 1993). The right RLN does not descend as far inferiorly as that on the left. Its pathway branches from the vagus in the thoracic cavity near the subclavian artery. After looping around the subclavian artery, the left RLN travels superiorly towards the larynx along the tracheo-oesophageal groove.

Injuries to the recurrent and superior laryngeal nerves

Up to 90% of injuries to the recurrent (RLN) and superior (SLN) laryngeal nerves are related to peripheral nerve damage with the majority of these appearing postoperatively (New & Childrey, 1932; Glas, 1949; Meurman, 1950; Cunning, 1955; Huppler et al. 1955; Teixido & Leonetti, 1990; Mu & Yang, 1991). In general, the left RLN is affected 7–10 times more often than the right in man (Cunning, 1955; Huppler et al. 1955). The frequency of left RLN impairment is attributed to its longer path through the intrathoracic cavity.

While surgical or tumour mass aetiologies make up the majority of laryngeal paralyses, an estimated 16–31% of laryngeal paralyses have been labelled as idiopathic in man (Cunning, 1955; Holt et al. 1977; Ward & Berci, 1982; Agha, 1983). ‘Idiopathic laryngeal hemiplegia’ has also been identified in 5% of thoroughbreds and 9% of draught horses (Duncan et al. 1974, 1991a, b). Ward & Berci (1982) reported that less than 50% of human subjects with idiopathic laryngeal paralysis attain partial or full recovery of laryngeal function. For those with partial recovery, symptoms continued to be severe enough to warrant surgical intervention to improve voice production and/or swallowing. In horses, idiopathic laryngeal hemiplegia also has a poor prognosis (Duncan et al. 1974; Duncan & Hammang, 1987).

Anatomical studies related to RLN damage

Relatively little attention has been directed towards the connective tissues surrounding the nerve fibres. Three layers of connective tissue have been described in nerves; the endoneurium, perineurium and epineurium (see Fig. 1). The primary purpose served by these connective tissue layers is to provide structural support and protection to peripheral nerve (Sunderland & Bedbrook, 1949a; Sunderland & Bradley, 1961a–c; Sunderland, 1965, 1970, 1978, 1980, 1990; Lundborg, 1988; Peters et al. 1991).

The endoneurium is formed from collagen fibrils that form inner and outer layers around individual nerve fibres (Sunderland, 1980; Thomas & Olsson, 1985; Ushiki & Ide, 1990; Peters et al. 1991). The perineurium is generally considered to be a major component of the blood-nerve barrier because the structures present within this layer of the nerve prevent transport of selected substances into the endoneurium (Shanthatveerappa & Bourne, 1962; Sunderland, 1980; Bell & Weddell, 1984; Thomas & Olsson, 1985; Peters et al. 1991). The epineurium is the outermost layer of the nerve and is primarily composed of collagen fibrils and adipose tissue (Sunderland, 1945; Junqueira et al. 1979; Thomas & Olsson, 1985; Peters et al. 1991). Elastin fibres (250–500 nm in width) and elaunin filaments 10 nm width have also been located among epineurial collagen fibrils (Thomas, 1963; Thomas & Olsson, 1985; Peters et al. 1991). The epineurium has been described as having 2 anatomical portions (see Fig. 1), inner and outer (Thomas, 1963; Thomas & Olsson, 1985; Peters et al. 1991). In nerves containing multiple nerve fascicles, each fascicle is contained within an inner epineurium. An outer epineurium then surrounds the multiple inner epineuria to form a single nerve trunk. In this study, the nerves did not generally exhibit an outer epineurium. Therefore, the use of the term ‘epineurium’ in the remainder of this study will refer to the inner epineurium.

The extent of epineurium in nerve cross sections has been observed to increase where nerves cross joints (Thomas & Olsson, 1985). Sunderland reported a proportional relation between the amount of epineurium and the resistance of nerve fascicles to damage from compression (Sunderland & Bradley, 1961a–c; Sunderland, 1965, 1970, 1978, 1990). In
addition, he associated the amount of adipose tissue present in the epineurium with the degree of compressional forces to which the nerve may be exposed (Sunderland, 1945). Thus the amount and content of epineurium appears related to the potential vulnerability of the nerve.

**Connective tissue in the laryngeal nerves**

Only 4 studies have described the characteristics of connective tissues in the RLN (Sunderland & Swaney, 1952; Malmgren & Gacek, 1981; Quiney & Michaels, 1990; De et al. 1991). Quiney & Michaels (1990) reported the morphological changes in both vagus nerves subsequent to penetration of a tumour through the perineurium. Their assessment of the branches arising from the damaged vagus nerve described intact RLN structure.

Sunderland & Swaney (1952) described the variation in loose epineurial supporting tissue surrounding RLN nerve fascicles along the length of the right RLN taken from 4 human cadavers. Measurements revealed that approximately 23–33% of the nerve was occupied by nerve fascicles below the level of the inferior constrictor muscle. Above the inferior constrictor, the RLN cross-sectional area contained only 10–15% nerve fascicles. The lower branches of the RLN supplying the oesophagus, trachea and inferior constrictor muscle contained 67% nerve fascicles in the cross-sectional area.

Malmgren & Gacek (1981) studied the path of nerve fibres within the human and feline RLN as well as the connective tissue coverings of the nerve. They obtained 28 samples from individuals undergoing laryngectomy. The number of nerve samples derived from the right and left sides was not identified and the samples were restricted to the most distal portions of the nerve from the level of the cricoid cartilage to approximately 3 cm distally. They measured the amount of fascicle tissue and surrounding epineurial tissue in the human RLN samples. Epineurial tissue amounted to 70–90% in cross sections obtained just.
below the larynx. The number of nerve fascicles present within these cross sections ranged from 1 to 11. They did not describe the components of the epineurial tissue, but the large proportion of connective tissue was considered to serve a protective function. Unfortunately, their descriptions were restricted to a small segment of the RLN pathway.

De Pasquale et al. (1991) compared the connective tissue coverings of the bovine left RLN with that of the optic nerve. The left RLN was found to contain several nerve fascicles within a shared epineurium. The perineurium surrounding each nerve fascicle of the left RLN was described as ‘the typical architecture of alternating cell and collagen layers’. De Pasquale et al. (1991) did not specify the location of RLN sampling; thus, it was unclear if they sampled along the entire nerve or just from one section. It is therefore difficult to relate their findings to other descriptions of the RLN as presented by Sunderland & Swaney (1952) and Malmgren & Gacek (1981).

The connective tissue coverings play an important role in the protection, pathology and reinnervation after injury of the laryngeal nerves. Structural changes to the connective tissue coverings such as occur with scar tissue formation, may impair normal mobility (i.e. ‘gliding’) of nerve fibres through the surrounding connective tissue (Millesi et al. 1990). Inflammatory responses to surgery that generate long term compression on the RLN may reduce intraneural blood flow resulting in ischaemia (Lundborg, 1975). Rydevik et al. (1981) reported that compressive loads of as little as 20–30 mmHg can impair epineurial blood flow and 60–80 mmHg affects blood flow throughout the endoneurium.

Detailed information pertaining to the connective tissue components of the laryngeal nerves is lacking. Such information is important for a number of reasons. First, the laryngeal nerves serve vital roles by supplying muscles that regulate respiration, deglutition and voice production. Damage to these nerves does not often reverse itself and can compromise the ability of the individual to survive without some form of intervention.

Secondly, the fairly large proportion of idiopathic laryngeal paralyses in man as well as horses may suggest a predisposition of the RLN, particularly the left, towards impaired function. Information regarding the connective tissues in the nerve trunk of the RLN may enable us to improve understanding of conditions that lead to impaired function.

Thirdly, there is evidence to suggest the RLN requires more epineural protection in its distal regions than has been reported for other peripheral nerves (Sunderland & Swaney, 1952). This information suggests that the RLN has an increased amount of epineurium where the nerve occupies a more vulnerable position in the neck. What is not known is the proportion of epineurial tissue in the left RLN, which is more frequently impaired, along its length from the vagus to the larynx.

The purpose of this study is to determine whether the left RLN has a significantly greater proportion of epineurial tissue than other noncranial peripheral nerves. This question will be addressed by assessing whether epineurial tissue proportions and composition differ along the length of the left RLN as compared with a noncranial nerve selected for its similarities to the RLN, the nerve to flexor hallucis longus (NFHL). The NFHL was chosen for comparison with the RLN because of the following similarities. (1) It passes between tissue structures such as muscle and/or fascia as does the RLN; (2) it lies in an area of the body subject to mobility as occurs in the neck, and (3) it is primarily a motor nerve of similar nerve fibre composition and trunk size as reported for the left RLN.

The NFHL separates from the tibial nerve in the lower limb and pursues a deep course within the lower thigh. Large muscle groups surround it (Bennett, 1976; Evans & Christensen, 1979). Before its insertion into flexor hallucis longus, the NFHL travels through extensive amounts of adipose tissue (Bennett, 1976). The nerve thus travels through an area protected by surrounding structures but is inserted into a muscle located in a mobile area of the limb behind the knee and is likely to be subjected to stretching and compressive forces (Bennett, 1976).

Previous research has reported myelinated fibre diameters in the left RLN to range from 4 to 12 µm with an average nerve trunk cross-sectional area of 2.39 mm² just below the larynx in human cadavers (Malmgren & Gacek, 1981).

Myelinated nerve fibre diameters in the NFHL of the rat range from 1 to 14 µm with the highest proportion lying between 4 and 8 µm (Fraher et al. 1990). Nerve trunk cross-sectional area of the NFHL in the dog and cat has been measured as 1.71–3.2 mm² (Bennett, 1976).

Materials and Methods

Dog cadavers

Ten adult dogs of mixed breed were used in this investigation. There were 6 males and 4 females.
ranging in weight from 19 to 29 kg. Nine dogs were obtained on termination of a cardiological study and 1 following a neurophysiological study. All dogs were perfused within 1 h of death.

Tissue preparation

One dog underwent general intracardiac perfusion to prepare nerve tissue prior to sampling. The absence of heart tissue in the other 9 dog cadavers required a modified perfusion protocol. All these dogs had received 10000 units of heparin to reduce blood clotting. The supraaortic region was perfused first with physiological saline followed by fixative consisting of 2.5% glutaraldehyde with 0.1 M phosphate buffer. After perfusion of the head and neck areas, the same procedures were repeated through the femoral artery for preparation of NFHL tissue.

Nerve samples

Multiple samples each ~ 2 cm in length were obtained along the length of the NFHL and RLN to permit examination of variations in the amount of connective tissue at different sites. Samples were taken from 4 different sites in the left RLN. The defined locations were as follows: (1) immediately after the point of RLN branching from the left vagus nerve; (2) the area where the left RLN loops around the aortic arch and begins its ascent towards the larynx; (3) just below the superior aperture of the thoracic inlet prior to leaving the thoracic cavity; and (4) immediately prior to insertion into the larynx and division of the RLN into anterior and posterior branches.

NFHL nerve samples were obtained from 3 different locations. Fewer sites were sampled in the NFHL because of its shorter length compared with the RLN. The defined locations were (1) immediately after the point of branching from the main trunk of the tibial nerve; (2) the middle portion of the nerve between branching and insertion into the muscle; and (3) immediately prior to insertion into the muscle.

All nerve samples were stored in vials containing 2.5% glutaraldehyde and 0.1 M phosphate buffer. The nerves were processed within 30 d for viewing in a Hitachi S4000 scanning electron microscope (SEM).

Preparation for scanning electron microscopy

All nerve samples were prepared for SEM viewing beginning with 3 buffer rinses (pH 7.2–7.4) within 1 h. Following the rinses, nerves were immersed in 1% osmium tetroxide-buffer solution for 1 h followed by a rinse in distilled water. Graded dehydration using alcohols (25%, 50%, 75%, 95%, and 100%) was then carried out with subsequent placement in hexamethyldisilazane to complete dehydration of the tissue. After dehydration, steel razor blades were used to make transverse cuts on each nerve sample using a dissecting microscope. Once transected, samples were mounted on aluminium stubs and coated with gold and palladium using a SC500 Enscope sputter coater.

Photography and analysis

Images of the nerve sample cross sections were photographed with high resolution Kodak Polaroid Type 55 film. Film negatives were used to analyse the nerve samples. The positives were used for initial interpretation and for referencing negatives.

Analysis of the nerve cross section photographs was performed at the University of Iowa Image Analysis Facility. The nerve sample negatives were first digitised at 300 dpi using an Imapro QSC 1240 Flatbed Scanner and Adobe Photoshop 2.0 software program on a MacIntosh IICX computer. All images were acquired using a 250 range grey scale. Next, the nerve images were analysed using an Indigo Silicon Graphics computer with entry level graphics and MTrace software. Once digitised, the nerve image was cropped so that views of the nerve cross section and scale legend were contained in the image file. The image was then light intensity level enhanced and filter sharpened.

Measurement of epineurium

Epineurial measurements were obtained using MTrace software on the Silicon Graphics Indigo computer. All tracings of nerve cross sections were performed by hand. Tracings were made around the periphery of all epineurial nerve regions as well as around the nerve fascicles contained within the epineurial boundaries. These 2 types of tracing areas were then used to calculate the cross sectional area of connective tissue comprising the epineurium for each nerve sample.

Nerve cross section tracings

Cross section region of interest (ROI) lines were traced along the outer edge of each epineurial connective tissue border. Excess surrounding tissue was sometimes taken along with nerve tissue to
preserve the epineurium of nerve samples. In some cases, this made it difficult to differentiate the epineurium from surrounding tissues. Thus the following criteria were developed to improve investigator consistency in placing cross section tracing lines when tissue demarcation was not clearly present. (1) Excess surrounding tissue interfering with the view of the epineurium border was traced such that the excess tissue was not included in the cross sectional area. (2) Interruption of the integrity of the peripheral epineurial boundary due to artifactual space was not included as part of the epineurium area.

Nerve fascicle area tracings

Individual nerve fascicles were traced after tracing the epineurial periphery of the particular nerve sample was completed. RLN nerves generally contained 1–2 fascicles in sections 3 and 4 and occasionally presented more than 2 fascicles in sections 1 and 2. The NFHL samples presented a range of 2–20 nerve fascicles from proximal to distal.

Epineurial calculations

Epineurial area for each nerve sample was obtained from the ‘measurement’ function of the MTrace program. All cross section and fascicle ROI areas were obtained from calibrated pixel counts. Calibration of each photograph was performed by the MTrace program using the calibration scale provided in the bottom right corner of each SEM photograph.

Relative epineurial areas were then calculated for all nerve samples. Since nerve section diameters varied between and within dogs and nerve types, comparisons were made between normalised epineurial areas, i.e. epineurial areas were recorded as the percentage of the total cross-sectional area. An estimate of investigator consistency in tracing the epineurium and fascicle ROIs was obtained by repeating measurement of 2 nerve samples from each dog.

Preparation of tissue for histology

Histological examination of the nerve samples was undertaken to study the proportion of collagen and adipose tissue along the length of each nerve. Pilot data comparing the RLN with other noncranial peripheral nerves in the dog indicated a difference in the amount and distribution of these 2 tissue types. The relative quantity of each tissue type was measured in this study to determine whether the apparent difference viewed in preliminary examinations of the RLN and other noncranial nerves could be substantiated.

Prior to paraffin processing, all nerve samples were submerged in osmium tetroxide for 24 h. This osmication process was performed to stain fat tissue in the epineurium. Fat tissue appeared black. Nerve samples measuring 1 mm or less in diameter generally resulted in successful staining of adipose tissue in the sample but samples larger than this were not completely penetrated by osmium tetroxide (Bozzola & Russell, 1993). This resulted in the absence of stained adipocytes in the tissue below the sample surface. The sites where adipocytes had existed before paraffin processing were indicated by their tissue matrix surrounding an empty space.

On completion of osmication, nerve samples were embedded in paraffin for sectioning; 10–15 µm cross-sectional slices were cut using a Spencer AO (model 820) microtome. Thinner segments were not successfully obtained during the sectioning process. Sections were then stained with Mallory’s analine blue collagen stain.

Adipose and collagen tissue stains

As previously indicated, fat was stained black with osmium tetroxide. Differentiation of collagenous structures from other components within the nerve connective tissue was possible using Mallory’s analine blue collagen stain. With this trichrome stain, collagen was stained blue, elastin fibrils appeared pale pink, nuclei were red and myelin and erythrocytes were yellow (Sheehan & Harpchak, 1980).

Analysis of histological sections

The amount of collagen and fat present in the epineurium was measured from the cross sections. Elastin was not measured as it was not identified in the epineurium of these samples, although, successful staining of elastin was obtained in blood vessel walls. Since elastin fibres have a diameter between 250 and 500 nm, elastin was probably difficult to identify by light microscopy unless it occurred in large quantities in cross sectional view such as in vascular tissue.

Histological measures were obtained using a single chip Cohu RGB CCD colour camera (Picon 1:3.5 f = 100 mm macro lens) mounted onto a Balplan microscope (Bausch and Lomb). The camera transmitted the slide image to an Electrohome 38-D051MA-YU.
Canine recurrent laryngeal nerve

graphics screen via a PC-based (IBM AT) true-colour image analysis system (American Innovision, Videometric 150). The Videometric 150 (V150) image analysis system allowed measurement of stained areas on images captured on the Electrohome 38-D051 MA-YU graphics screen. All digitised images were calibrated for their magnification level.

The area of each nerve section occupied by adipose and collagenous tissue was measured on completion of calibration. Each pixel comprising the image was associated with a colour that was described numerically in the threshold function according to its brightness, hue and saturation. The pixels were highlighted for measurement by using the cursor to select a tissue colour. The pixel under the cursor was then highlighted along with all other image pixels with matching threshold values. It was necessary to repeat this highlighting procedure for the same general colour spectrum with different threshold values. For example, blue-stained collagen was indicated by a light blue-grey to royal blue tissue colour. Thus the highlighting process was repeated until the spectrum of ‘blue’ collagen tissue pixels was contrasted from the other stained tissues. The highlighting process was performed separately for adipose tissue and collagen.

Tissue areas were measured for collagen (blue stain) and adipose tissue (black stain and ‘white’ spaces). The adipose tissue was calculated in 2 steps. First, the area occupied by osmicated (black) adipose tissue was highlighted as described above. Next, the spaces left by dissolved adipose tissue were highlighted and the area occupied was calculated. The areas calculated for the black-stained adipose and ‘white’ adipose spaces were summed to obtain the total area occupied by adipose tissue for each sample. All measures were normalised so that comparisons could be made across nerve samples. Normalisation was attained by calculating the proportion of total highlighted epineurium occupied by each type of tissue. Since the total highlighted epineurium was composed either of collagen or adipose tissue, either type of tissue could be calculated from the other. Reliability estimates of histology measures were obtained to determine the consistency of the investigator in highlighting the stained adipose and collagenous tissue for area calculations. The nerve specimens used for SEM reliability measures were also used for obtaining histology reliability estimates.

**Statistical analysis**

Differences between relative epineurial areas from each nerve type (i.e. RLN and NFHL) were tested by analysis of variance (ANOVA) using a regression approach. This approach was used to analyse the relation between relative epineurial area, percentage collagen and fat measures and such variables as subject, nerve type, sample section of each nerve type, sex, and weight.

The ‘best fit’ equation was determined in a building block progression. The model equation was built in this fashion using all possible model combinations. Model combinations were tested until no further significant main and interaction effects were found. The final model was then used to report the results of the regression ANOVA.

Post hoc testing of pairwise comparisons were performed using the Bonferroni Test for Comparison. Significant interactions were tested using the Scheffé method to evaluate all possible combinations of data.

**RESULTS**

Reliability of repeated measures

Reliability estimates demonstrated that the overall distribution of measure variance related to tracing error and to histological tissue highlighting error did not significantly contribute to the variance obtained from epineurial or histological tissue area measures.

**Relative epineurial area results**

The comparison of relative epineurial area between nerve types demonstrated a significant difference with an $F_{(1, 64)}$ ratio $= 69.74$ ($P < 0.0001$). As shown in Table 1, RLN samples exhibited a larger average relative epineurial area than the NFHL samples.

There was also a significant difference between the sample sections taken within each nerve type ($F_{(3, 64)} = 19.42$, $P < 0.0001$). The Bonferroni (Dunn) $T$ test was performed to determine which sections were significantly different at $P < 0.05$ and a confidence interval (CI) of 95%. Because each nerve type had different numbers of sections, testing was performed within each nerve type. Results demonstrated significant differences between sections 1 and 4, 1 and 3, and 2 and 3 in the RLN. The NFHL exhibited significant differences between sections 1 and 3 and 2 and 3.

<table>
<thead>
<tr>
<th>Nerve type</th>
<th>N</th>
<th>Mean</th>
<th>s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLN</td>
<td>39</td>
<td>0.84</td>
<td>0.13</td>
</tr>
<tr>
<td>NFHL</td>
<td>30</td>
<td>0.64</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 1. Average relative epineurial area by nerve type
shown in Table 2, distal sections (i.e. sections 3 and 4) had larger average relative epineurial areas than the proximal nerve sections (i.e. sections 1 and 2) for both nerve types. The only consecutive sections demonstrating significant differences were sections 2 and 3 for both nerve types. In general, a gradual increase in relative epineurial area occurred from proximal to distal sections for both nerve types, although relative epineurial area measures declined slightly at section 4 in the RLN.

Additional factors such as sex, weight and subject (i.e. dog) were also tested but were not significant contributors to the overall variance of epineurial measures. Therefore, the final model for relative epineurial area was:

Relative epineurial area = nerve type + nerve section.

Variations in epineurial measures were thus related to the 2 independent variables of nerve type and the particular section samples within each type of nerve. This model accounted for 67% of the variance in the relative epineurium area measures.

Histological data analysis

As described previously, elastin was not found in measurable quantities in transverse sections of the epineurium of these samples. Thus histological results will only report on measures obtained for adipose and collagenous tissue.

Adipose and collagenous tissue measures were normalised such that they reflected a proportion of the total amount of analysed tissue. For example, a measure of 50% adipose tissue for one nerve sample would also have a measure of 100% minus 50%, or 50% collagen tissue. This being the case, statistical main effect and interaction results for adipose tissue were the same as those for collagenous tissue, with the exception of means and standard deviations. Therefore, the General Linear Models Procedure ANOVA results are presented for adipose tissue, but also apply for collagen.

Histological measures demonstrated a significantly different amount of adipose tissue and collagen for each nerve type with an $F_{(1, 53)}$ ratio = 25.86 ($P < 0.0001$). In addition, the measures for adipose and collagenous tissue were significantly different between sexes ($F_{(1, 53)}$ = 17.58; $P < 0.0001$) and across nerve sections ($F_{(3, 53)}$ = 5.81; $P < 0.0016$). As can be seen in Table 3, RLN samples exhibited a higher average percentage adipose tissue (and, therefore, less collagen) than for the NFHL samples. In addition, the samples obtained from female dogs had higher average adipose (and lower collagen) proportions than the male dogs (see Table 4).

An interaction was also indicated between nerve type and nerve section that was significant at $P < 0.07$ ($F_{(3, 53)}$ = 2.75). Due to the small number of animals in the subject population, this ‘borderline significant’ interaction was included in the model equation. The Scheffé post hoc test was performed to determine which combinations of sample differences were significant for this interaction. The following differences in adipose and collagen measures were found significant: (1) as a group, RLN samples exhibited significantly higher adipose and lower collagen proportions than samples from the NFHL nerve; (2) pairwise comparisons revealed significantly greater adipose tissue and less collagen in RLN sections 1, 3, and 4 compared with section 1 in the NFHL; and (3) RLN section 3 exhibited significantly greater adipose tissue and less collagen than measured for section 2 in the NFHL.

The final equation of the model describing the relation between adipose tissue proportions in the RLN and NFHL was (the same components in this equation apply to collagen):

$\% \text{ Adipose tissue} = \text{ nerve type } + \text{ section } + \text{ sex}$

$\quad \quad + (\text{nerve type}) \cdot (\text{section})$.

<table>
<thead>
<tr>
<th>Section</th>
<th>Mean RLN N s.d.</th>
<th>Mean NFHL N s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.73 10 0.13</td>
<td>0.55 10 0.12</td>
</tr>
<tr>
<td>2</td>
<td>0.78 9 0.13</td>
<td>0.62 10 0.10</td>
</tr>
<tr>
<td>3</td>
<td>0.94 10 0.02</td>
<td>0.76 10 0.07</td>
</tr>
<tr>
<td>4</td>
<td>0.89 10 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Relative epineurial area (mm²) by nerve type and section

<table>
<thead>
<tr>
<th>Section</th>
<th>Mean RLN N s.d.</th>
<th>Mean NFHL N s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51 9 16</td>
<td>25 8 9</td>
</tr>
<tr>
<td>2</td>
<td>40 9 19</td>
<td>34 9 15</td>
</tr>
<tr>
<td>3</td>
<td>58 8 9</td>
<td>46 9 12</td>
</tr>
<tr>
<td>4</td>
<td>54 9 11</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Average percentage adipose tissue for sections at different levels within each nerve type

<table>
<thead>
<tr>
<th>Section</th>
<th>Mean RLN N s.d.</th>
<th>Mean NFHL N s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>47 21 16</td>
<td>31 17 15</td>
</tr>
<tr>
<td>Female</td>
<td>57 14 12</td>
<td>44 10 14</td>
</tr>
</tbody>
</table>

Table 4. Percentage adipose tissue for sex and nerve type
Fig. 2. Comparison of percentage adipose tissue between the RLN (open circles) and NFHL (filled circles).

This equation accounted for 56% of the variance in the measures of percentage adipose tissue and collagen.

Figure 2 presents the average proportion of adipose tissue across nerve types and sections, respectively. The section-to-section trend in the RLN appeared nonlinear in contrast to the linear trend for the NFHL. In addition, RLN section 2 exhibited lower adipose tissue percentages than the other 3 RLN sections. The measures obtained for adipose tissue and collagen at the level of section 2 in the RLN was not significantly different from NFHL sections 1, 2, or 3. Similar to RLN section 2, RLN section 4 showed a slightly lower adipose tissue value than the preceding sample section. These ‘up and down’ vacillations in RLN tissue measures did not occur across the NFHL sections. Instead, in NFHL the proportion of adipose tissue generally increased distally. Only 1 dog exhibited the inverse of this general pattern across NFHL sections, i.e. a linear decrease in adipose tissue occurred from proximal to distal sections.

In general, the RLN demonstrated a relatively consistent proportion of adipose tissue from its origin to its most distal section. In contrast, the NFHL increased the proportion of adipose tissue from its origin to distal sections.

**Qualitative SEM observations**

Figure 3 presents SEM images across all sections within each nerve type from dog 3. These RLN and NFHL images present typical morphological patterns seen within and between each nerve type. These images display cross sectional views; portions of the nerve trunk sheath extending longitudinally along the sides of the nerve trunks can also be seen in Figure 3A, and C–G. Figure 4 displays the placement of epineurial boundaries for each of the images in Figure 3.

The most obvious difference observed between these 2 nerves was the larger number of fascicles generally seen in the NFHL compared with the RLN. The distribution of epineurial tissue around the fascicles also appeared to differ between nerves. The NFHL possessed less epineurial tissue around nerve fascicles than the RLN. With the exception of RLN section 1, the RLN fascicles were generally found in the centre of the nerve trunk surrounded by epineurial tissue. In addition, RLN cross sections appeared to be oval in shape. The NFHL had fascicles distributed across the entire cross section with an overall flattened oval appearance.

The 1st section of the RLN series shown in Figure 3A has 1 large and 1 small nerve fascicle. There were many variations at this level in the RLN between dogs. Generally, the nerve fascicles appeared large, or there were multiple fascicles. Most notable at section 1 was the smaller relative proportion of epineurium compared with the other RLN sections.

RLN section 2 in Figure 3B was not representative of its comparison RLN section 2 samples as seen in other dogs. This example of RLN section 2 displays a larger quantity of epineurium than usually occurred in the same section from other dogs. In addition, there was typically only 1 nerve fascicle at this level. What was most striking with RLN section 2 was the large quantity of adipose tissue surrounding the aortic arch and RLN. This large quantity of adipose tissue frequently made it difficult to dissect the nerve from surrounding tissue.

The relative proportions of epineurial tissue in RLN sections 3 and 4 were comparable (cf. Figs 3C, D and 4C, D). These RLN sections exhibited significantly larger relative proportions of epineurial tissue than RLN sections 1 and 2.

The NFHL sections in Figure 3 had a flattened oval shape and a larger numbers of fascicles than the RLN. NFHL section 1 (see Fig. 3E) exhibited larger and fewer nerve fascicles than seen in sections 2 and 3 (panels F and G in Fig. 3, respectively). Within the NFHL epineurium, the nerve fascicles appeared to be distributed across the nerve trunk in a linear fashion. This contrasted with the RLN, which typically contained fewer nerve fascicles embedded in large relative proportions of epineurium.

In general, the SEM morphological features of each nerve were qualitatively distinct. The RLN had a rounder shape with fewer nerve fascicles than the NFH nerve. In contrast, the NFHL had a flattened oval shape with larger numbers of nerve fascicles.
Fig. 3. SEM images of the RLN and NFHL for sections at different levels. Images A, B, C and D are examples of RLN sections 1, 2, 3 and 4, respectively. Images E, F and G are examples of NFHL sections 1, 2 and 3, respectively. All images were obtained from the same animal. Bars, 500 µm.
Qualitative histological observations

By comparing the RLN and NFHL sections, many patterns were seen in adipose and collagen tissue distributions. For example, adipose tissue in distal sections of the RLN (e.g. RLN section 4 in Fig. 5C) was evenly distributed around the nerve fascicles. In contrast, the adipose tissue appeared to accumulate between, but not entirely around distal NFHL sections (e.g. NFHL section 3 in Fig. 5G).
Fig. 5. Light microscopy sections of the RLN and NFHL at different levels. Images A and C are examples of RLN sections 1 and 4, respectively. Images B and D are magnified views of the boxed areas of A and C. Images E and G are examples of NFHL sections 1 and 3, respectively. Images F and H are magnified views of the boxed areas of E and G. Bars, 250 µm. P, perineurium; C, collagen; A_w, adipose tissue, black staining; A_W, adipose tissue, white spaces; BV, blood vessel.
A common pattern for NFHL nerves was an increase in the proportion of adipose tissue from proximal to distal nerve sections. The 1st and 2nd sections of the NFHL examples were primarily composed of collagenous tissue. However, the 3rd section showed nerve fascicles surrounded by more adipose tissue than seen in the initial 2 sections (cf. Fig. 5E–H). In contrast to adipose tissue, the amount of collagen became reduced distally as can be seen by comparing Figure 5, panels F and H. Although not as dramatic, the relative proportion of collagen in the RLN epineurium also diminished from section 1 to section 4 as seen in Figure 5, panels B and C. In contrast to the NFHL, the relative adipose tissue proportions did not change significantly in the RLN from proximal to distal sections as seen in Figure 5, panels b and c. Although the RLN did not exhibit changes in the relative proportion of adipose tissue along its length (see Fig. 5B, D), it exhibited significantly more adipose tissue on average than the NFHL nerve (cf. Fig. 5B, D and Fig. 5F, G). The NFHL exhibited significantly larger relative proportions of collagenous tissue primarily in section 1 than the RLN (cf. panels B and F, Fig. 5).

Another consistent observation between RLN nerve samples related to adipose tissue padding immediately surrounding the nerve trunk. RLN section 2 was consistently embedded in large quantities of fat. NFHL sections 1 and 2 were also typically contained within surrounding adipose tissue. In addition, these 2 sections in the NFHL were surrounded by fascial tissue that appeared to secure the nerve between large muscle groups in the leg.

In general, distinctive histological patterns of tissue distribution were seen between the various sections between nerve types. Thus, collagenous tissue was not present in large proportions between RLN sections compared with NFHL sections. In addition, the NFHL exhibited increasing proportions of adipose tissue distally while the RLN nerve samples had relatively constant proportions.

**DISCUSSION**

*Relative epineurial cross-sectional areas*

The results of this study demonstrate that the left RLN has a significantly greater relative epineurial area than the left NFHL. Of particular interest is the significant increase in relative epineurial area from the 1st to the last nerve section in both nerves. Sunderland & Swaney (1952) described this pattern for samples of the right RLN taken from its location near the inferior constrictor muscle in 4 humans. Increased proportions of epineurium in the distal left RLN may be a result of the need for additional protection as the nerve enters the laryngeal area. For the particular nerves studied, this may be related to location in areas of increased environmental ‘stress.’ For example, the left RLN leaves the protection of the thoracic cavity at the superior aperture of the thoracic inlet. Before this, the RLN is primarily exposed to compressive forces from the aortic arch and possibly from peristaltic contractions of the oesophagus. However, the rib cage and surrounding thoracic structures offer protection. Above the thoracic cavity, the nerve may be exposed to external damage such as from blows to the neck. Extra padding surrounding the RLN could help to prevent such damage. Other environmental ‘stresses’ to the RLN may relate to compression and elongation related to movements of the head such as flexion, extension and rotational movements. In addition, the larynx may impose these types of forces on the RLN during elevation and depression as occurs during swallowing and voice production.

The role of the epineurium as protection to nerves has been suggested in previous studies of noncranial peripheral nerves (Sunderland, 1945, 1965, 1970, 1978, 1990; Sunderland & Bedbrook, 1949a). In particular, Sunderland (1990) discussed the role of the epi-neurium in directing deformational forces away from nerve fascicles. In his view, the epineurium could effectively prevent damage from deforming forces associated with compression when there is a large amount of supportive tissue between nerve fascicles and when there are few and/or smaller fascicles within the nerve trunk. In contrast, nerves with large closely approximated nerve fascicles and small amounts of epineurial tissue are predicted to be at risk for injury. An example of this pattern was reported for the peroneal and tibial divisions of the sciatic nerve (Sunderland, 1953). The peroneal division was found more frequently to be impaired, or to exhibit more severe damage, than the tibial branch (Sunderland, 1953). The primary differences between the nerves were the morphological distribution and size of nerve fascicles within the epineurial tissue as well as their environment. The tibial branch of the sciatic has large amounts of epineurial tissue surrounding many small nerve fascicles. In contrast, the peroneal division of the sciatic nerve contains small amounts of epineurium surrounding small numbers of large nerve fascicles. In addition, the tibial branch travels in a less rigid environment in which stretch forces may be distributed along the length of the nerve. Stretching forces imposed on the peroneal nerve may result in...
structural damage associated with the fixed position of the nerve relative to the sciatic notch and the neck of the fibula. Such injuries are usually related to fractures or fracture-dislocations.

In the present study, the left RLN generally contained relatively less epineurium at its origin from the vagus nerve. As the left RLN ascended towards the larynx, the relative amount of epineurial tissue increased until the nerve began to branch near the larynx. One or 2 nerve fascicles surrounded by large amounts of epineurium were found at the most distal sampling segment (see Fig. 3D). This pattern of nerve morphology indicates that the left RLN may not be as well protected from damage to deforming forces associated with compression near its origin. As the nerve travels along the tracheo-oesophageal groove, it acquires increasing amounts of protective supportive tissue until entering the larynx.

The NFHL exhibited a different morphological pattern. This nerve travels through a significant amount of adipose tissue near its origin from the tibial nerve. At this point, the nerve generally exhibited small numbers of large nerve fascicles with small amounts of supportive tissue. As the nerve approached its termination, the number of nerve fascicles increased as did the amount of epineurium.

The other distinguishing feature for the 2 nerves was the general shape of the nerve trunk. The NFHL had more of a flattened oval shape while the left RLN had a rounder oval shape. The differences in shape may relate to the mechanical forces imposed on each nerve. The NFHL lay between large leg muscles and appeared relatively fixed within surrounding connective tissue. The RLN was not fixed by surrounding connective tissue and travelled ‘loosely’ along a groove-shaped space until reaching the laryngeal region.

The large numbers of nerve fascicles with smaller amounts of epineurium in the NFHL may not protect the nerve from injuries related to compression as well as the fascicular-epineurial morphology of the RLN. However, the NFHL may withstand stretching forces better. According to a series of stress-strain studies (Sunderland & Bedbrook, 1949b; Sunderland & Bradley, 1961a–c; Sunderland, 1965, 1970, 1978, 1990; Rydevik et al. 1990), larger numbers of nerve fascicles in the transverse area of a nerve trunk provide stretch resistance. Protection from stretching forces has been associated with increased amounts of perineurium in the cross-sectional area (Sunderland & Bradley, 1961a–c; Sunderland, 1978, 1990). With this in mind, the left RLN may be at risk for stretch injuries. Typically, 1 nerve fascicle was found in the midst of large amounts of epineurium in the RLN. Nerves with 1 large nerve fascicle have been shown to resist stretch poorly (Sunderland & Bradley, 1961a–c; Sunderland, 1978, 1990). Further, they may be predisposed to ischaemia on stretching. Sunderland (1951) observed that nerves with small numbers of fascicles tend to carry the blood supply superficially. With multiple nerve fascicles, blood vessels travel between fascicles and are afforded greater protection than the former. In the tibial nerve, a multifasciculated nerve, graded stretch of as little as 8% beyond resting length was shown to result in impaired venular flow (Lundborg & Rydevik, 1973). Complete cessation of intraneural blood flow occurred at 15% stretch beyond resting length (Lundborg & Rydevik, 1973). The gradual reduction in blood flow under stretch conditions was linked to constriction of blood vessels during elongation.

The RLN may be predisposed to less stretch resistance and, therefore, possible ischaemia resulting from elongated and constricted blood vessels (Sunderland & Bradley, 1961b; Lundborg & Rydevik, 1973). Impaired blood flow for short periods of time (e.g. 1–4 h) may temporarily impair the RLN while long periods of ischaemia (e.g. 8 h) could result in permanent structural damage (Lundborg, 1975).

The morphological patterns of the NFHL and RLN may reflect the particular forces to which they are most frequently exposed. For example, the NFHL is probably stretched more often than it is compressed. Its location is protected by the femur and fibula as well as surrounding muscle and connective tissue structures. In the event that deforming forces associated with compression are imposed on the NFHL, however, it is at greater risk than the RLN for damage to the nerve fibres within its fascicles.

In contrast, the left RLN is probably exposed to deforming forces associated with compression imposed by surrounding structures such as the aortic arch, the oesophagus, and surrounding structures in the neck region. Its rounded thick epineurium surrounding a single nerve fascicle may offer protection from damage related to compression. In the event that the RLN is stretched beyond its limits, however, it may be more likely to suffer damage to the nerve fibres within the fascicles. Further, it could undergo ischaemic changes related to constricted blood vessels.

Epineurium and nerve recovery after injury

The general morphology of the RLN appears advantageous for protection purposes. However, based
on information available from noncranial peripheral nerves, RLN nerve structure may present disadvantages for regeneration of damaged nerve fibres. For example, the amount of adipose tissue in the epineurium can influence the regenerative capacity of damaged nerves (Sunderland, 1945, 1951, 1965, 1970, 1978, 1980, 1990). Ideal conditions for successful regeneration of damaged nerve fibres include: (1) intact endoneurial tubes; (2) intact nerve fascicles; (3) localisation of nerve fibres within nerve fascicles; (4) widely separated nerve fascicles; and (5) small amounts of epineurial tissue (Sunderland, 1951).

Damage compromising the endoneurium of nerve fibres leads to nonhomologous nerve fibres finding their way into the wrong endoneurial tubes (Sunderland, 1945, 1970, 1978, 1980, 1981, 1990). The RLN, therefore, is at risk of this happening on injury to the endoneurium due to the intermingling of different nerve fibres between fascicles.

When the perineurium of damaged nerve fascicles is breached, regenerating nerve fibres may wander into the epineurial tissue and ‘terminate blindly’ amidst the supportive connective tissue (Sunderland, 1945, 1970, 1978, 1980, 1981, 1990). The probability for this is high in the RLN because of intermingled nerve fibres existing in 1 or 2 nerve fascicles surrounded by large amounts of supportive tissue.

Studies on nerve regeneration of the RLN in horses (Duncan & Baker, 1987), dogs (Green et al. 1992), and man (Crumley, 1991) have generally reported poor reinnervation success by RLN nerve fibres. Successful reinnervation has been reported in horses when the RLN was crushed near its termination (Duncan & Baker, 1987). In those cases, it was likely that nerve fascicles contained nerve fibres with similar destinations. Typically, however, ‘successful’ reinnervation in the larynx is characterised by synkinesis, that is, regenerating nerve fibres find their way into the wrong endoneurial tubes and eventually terminate in the wrong muscle. In the larynx, this is manifested by simultaneous contraction of adductor and abductor vocal fold muscles.

Sex differences in epineurial adipose composition

In addition to differences in epineurial composition between nerve types, a significant sex difference was found for adipose tissue proportions. Within each nerve type, females exhibited approximately 10% more adipose tissue than males. This finding has not been reported elsewhere. A factor that may relate to the adipose tissue sex difference is percentage body fat. Females are known to generally have a greater percentage body fat than males (Ross & Romrell, 1989). Sunderland (1945) reported a relation between the amount of adipose tissue in the epineurium and percentage body fat. He observed that a ‘general wasting’ occurred in epineurial adipose tissue in a fashion similar to that found elsewhere in the body. It should be noted, however, that an independent measure of the percentage of body fat was not obtained for the dogs used in the present study.

CONCLUSION

In conclusion, significant differences were found between the RLN and NFHL in the relative cross-sectional proportions of epineurial connective tissue as well as collagen and adipose tissue. Implications for the ability of each nerve type to withstand compression and elongation forces have been discussed in relation to their morphology and composition. Future studies are needed to clarify further the relation of the quantity and composition of the RLN epineurium to effects of biomechanical stresses on nerve integrity and function.

ACKNOWLEDGEMENTS

We thank Kathy Walters and Steve Speakman for assistance with preparation and analysis of the data and David Kuehn for the use of his imaging equipment. Funding for this project was provided by the National Center for Voice (P60 DC00976).

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