QUANTIFICATION OF INTRAEPIDERMAL NERVE FIBER DENSITY AS A DIAGNOSTIC TOOL FOR NEUROPATHIES IN DOGS

A Thesis

by

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ABSTRACT

Skin biopsies have been extensively used for the diagnosis of small fiber neuropathies in humans. Although inherited and acquired neuropathies have been documented in dogs, they can be difficult to unequivocally diagnose in the living animal. The use of skin biopsies for the diagnosis of neuropathies in dogs has not been evaluated.

In this thesis, skin biopsies were collected and stained using immunohistochemistry for protein gene product 9.5 (PGP 9.5). Intraepidermal nerve fiber density (IENFD) was quantified using the published guidelines for the diagnosis of peripheral neuropathies in humans.

First, skin biopsies were collected *post mortem* from adult hound dogs using a 3mm biopsy punch from the skin overlying the larynx. The entire neck was harvested and stored at 4°C for 7 days. Punch biopsies were collected at day 0, day 1, day 2, day 5 and day 7. IENFD was determined for each sample collection day for each dog and compared. A gradual reduction in IENFD was observed over time. Higher mean IENFDs were observed at day 0, day 1 and day 2. There was no significant difference in IENFD between any of the subsequent collection days ($p=0.12$). These results suggest that refrigeration at 4°C may be acceptable for up to 48 hours after collection, before the samples need to be placed in Zamboni's fixative.

Then, dogs diagnosed with laryngeal paralysis were recruited to evaluate IENFD in a well-established example of canine neuropathy. Skin biopsies were collected using a 3mm biopsy punch from the skin overlying the larynx. IENFD was determined for these

dogs and compared to controls. There was no significant difference between the IENFD of the dogs diagnosed with laryngeal paralysis and the control dogs ($p=0.28$). These results suggest that it might be difficult to use skin biopsies for diagnosis of peripheral neuropathy in dogs but several modifications of our technique, such as taking samples from the distal hind limb, might be worth considering as means to improve precision of measure of fiber density.

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The data analyzed for Chapter III and IV was provided by Dr. Nick Jeffery. All other work conducted in the thesis was completed by the student independently.

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NOMENCLATURE

CLPP	Canine Laryngeal Paralysis Polyneuropathy
EMG	Electromyographic
IENFD	Intraepidermal Nerve Fiber Density
IENF	Intraepidermal Nerve Fiber
LP	Laryngeal Paralysis
NCV	Nerve Conduction Velocity
PGP 9.5	Protein Gene Product 9.5
SE	Standard Error

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Peripheral neuropathies

Peripheral neuropathies are disorders resulting in dysfunction of peripheral nerves[1,2]. Peripheral nerves can be categorized as motor, sensory or autonomic and each can be affected by neuropathies[1-6]. The symptoms of peripheral neuropathies are variable based on the types of nerves affected, and mixed disease processes are noted[2,4-6]. Numerous etiologies have been reported to result in a peripheral neuropathy, such as diabetes mellitus[2,4-7], vitamin deficiencies[4,5,7], leprosy[2,4-6], paraneoplastic syndromes[2,4-7], administration of chemotherapeutics[4,7], Guillain-Barré syndrome[2,4-7], Charcot-Marie-Tooth disease[2,4,5,7], amyloidosis[2,4-7], and many more.

Clinical signs reported in humans vary depending on the nerves affected, the underlying disease process, and the progression of the disease[2,4-7]. These can include pain, loss of sensation, numbness, tingling sensation, weakness, muscle wasting, and loss of reflexes[2,4-7]. Diagnosis of neuropathies can be complex and often involves a combination of electromyographic testing[1-3,7], nerve conduction studies[1-3,6-7,] muscle and nerve biopsies[1,2,6,7,9], and skin biopsies.[1,2,7-12]

Canine neuropathies

Sensory and motor neuropathies, both inherited and acquired, have been documented in dogs[13-18]. As noted in humans, clinical signs vary and can include ataxia[17,18], weakness[14,15,17, 18,] muscle atrophy[13,14,18,17], self-mutilation[15,18,19], and reduced

or absent spinal nerve reflexes[13,14]. Many of the subjective clinical signs that are prominent features of sensory neuropathies in humans will be difficult to appreciate in a non-verbal species. It is probable that peripheral neuropathies generally, and sensory neuropathies specifically, are greatly underdiagnosed or misdiagnosed in dogs.

Inherited neuropathies are typically noted in younger animals, often of a specific breed, such as Alaskan Malamutes with distal sensory polyneuropathy and a distal axonopathy resulting in laryngeal paralysis in Bouviers des Flandres[14,15,18]. These and other degenerative neuropathies are considered to be inherited due to their association with a particular breed or a familial link noted between affected individuals[14,18]. Certain of these conditions carry similarities to neuropathies documented in humans such as Charcot-Marie-Tooth disease and giant axonal neuropathy[15,17,18]. Young Border collies have been affected by progressive proprioceptive ataxia and self-mutilation between 2-7 months of age[15,20-23]. This condition was further classified as a sensory and autonomic neuropathy due to an inversion disruption of the FAM134B gene[20]. This gene has also been implicated in a human neuropathy[24]. Prognosis for these dogs is poor, with progression of neurologic signs expected[21-23]. Certain hunting breeds such as the French spaniel, German short-haired pointer, English pointer, English springer spaniel, and the English cocker spaniel, have been diagnosed with a sensory neuropathy attributed to an autosomal recessive inheritance to a mutation in the GDNF gene, resulting in insensitivity to pain, and progressing to self-mutilation[15,25-27]. Additional conditions have been documented, such as dancing Doberman disease and a sensimotor polyneuropathy in Rottweilers, although

their pattern of inheritance and specific genes affected have not yet been fully determined[14,17,18].

Acute canine polyradiculoneuritis (Coonhound paralysis) is one of the most common peripheral neuropathies recognized in dogs[13,16]. The exact pathogenesis of this condition is not known but is suggested to be immunologically-mediated[13,16]. Similarities have been noted between acute canine polyradiculoneuritis and Guillian-Barré syndrome in humans, as both result in acute generalized paralysis[13,16,29,30]. Clinical signs can be similar to those of botulism, tick paralysis or myasthenia gravis, causing acute lower motor neuron tetraparesis[16]. Other than acute canine polyradiculoneuritis, most acquired canine peripheral neuropathies are chronic and progressive[16]. For example, canine ganglioradiculitis can present with pelvic limb ataxia in the absence of paresis with self-mutilation and dysphagia[16,19]. Adult dogs of various breeds have been affected by this condition[16,19,31-33], which is characterized by sensory loss and mononuclear inflammation of sensory neurons[19,31-33]. Numerous other etiologies have also been associated or hypothesized to result in acquired peripheral neuropathies[16,19].

Diagnosis of the many etiologies of peripheral neuropathies can be challenging[16], but often relies on comprehensive laboratory testing to help determine an underlying cause, as well as a combination of electromyographic testing, nerve conduction testing, and muscle and nerve biopsies to try to confirm the neuropathy[14,16]. In veterinary patients, nerve and muscle biopsies are performed under general anesthesia[18,34], which also makes these procedures more invasive and costly. Electromyographic and nerve conduction testing require referral to a specialty center, such as a veterinary teaching hospital.

Canine laryngeal paralysis polyneuropathy

Laryngeal paralysis is a cause of upper respiratory stridor and dyspnea in older, large breed dogs[35-37]. Dyspnea occurs secondary to the lack of abduction of arytenoid cartilages during inspiration, due to dysfunction of the recurrent laryngeal nerves[38-40]. Mounting evidence has demonstrated that a generalized peripheral neuropathy in dogs could result in the acquired form of laryngeal paralysis[38,41-43]. Neurologic signs, consistent with a polyneuropathy, such as generalized weakness including difficulty rising, paresis, and dysphagia, may be present initially along with the more classical respiratory signs or may develop several months later[37,38,40-43]. The prevalence of a generalized neuropathy associated with laryngeal paralysis is unknown.

On electromyography, dogs with laryngeal paralysis were noted to have fibrillation potentials and positive sharp waves affecting muscles both proximally and distally in the pelvic limbs[38,41,43]. Fibrillation potentials and positive sharp waves have also been reported in the distal forelimbs and the intrinsic laryngeal muscles[38]. A distal distribution of abnormalities detected via electrodiagnostics suggest a dying-back neuropathy[38].

Nerve conduction velocities in dogs with laryngeal paralysis can also be slowed in the proximal and distal segments of the sciatic nerve[38,41,42] and the ulnar nerves[38,42,43]. Residual latency was increased in the tibial nerve and certain cranial nerves[42]. Abnormalities in electrodiagnostic procedures performed on the thoracic and pelvic limbs were consistent with a generalized polyneuropathy and functional impairment of the lower motor neurons[43]. The detection of electromyographic changes paired with nerve conduction abnormalities suggest a disease affecting axons rather than a demyelinating

process, and the more pronounced changes in the distal nerves is consistent with a length dependent process[42,43].

Under histologic examination of muscle biopsies of dogs with laryngeal paralysis, mild to moderate type I and II angular myofiber atrophy was noted[41]. Scattered angular atrophic fibers, evidence of fiber hypertrophy, and giant whorled cells have also been reported[38]. Large and small grouped atrophy was observed on muscle biopsy of the cricoarytenoideus dorsalis and cranial tibial muscles[43]. Nerve biopsies of dogs with laryngeal paralysis have demonstrated loss of large-caliber nerve fibers, axonal degeneration, and endoneurial fibrosis[43]. Demyelination and remyelination, thought to be secondary to degenerative changes in the nerves[38], and axonal loss [39] have also been noted on nerve biopsy, consistent with a neuropathy.

The abnormalities in electromyographic testing, nerve conduction studies, and biopsies described in previous studies are consistent with the hypothesis that laryngeal paralysis is a component of a more generalized peripheral polyneuropathy[38,41-43].

Protein gene product 9.5

Protein gene product (PGP 9.5) is an ubiquitin C-terminal hydrolase specific to neurons which is widely distributed in central and peripheral nerve cells[45]. It is used as a general neuronal marker, as it is found in the cytoplasm of all types of efferent and afferent nerve fibers[46] and is involved in controlling the degradation of proteins found in neurons[47]. It labels nerve fibers within the peripheral nervous system in humans[46,48,49]. Based on these characteristics, PGP 9.5 is useful in studying the innervation of skin and changes during reinnervation[46]. Early work in humans demonstrated that PGP 9.5 antibodies labelled a

larger number of sensory and autonomic fibers in human skin than other neuronal markers previously used[46,50].

Use of skin biopsies for diagnosis of neuropathies in people

Guidelines for the collection and use of skin biopsies for the diagnosis of small fiber neuropathy have been established by the European Federation of Neurologic Societies and the Peripheral Nerve Society[11,12]. A skin biopsy obtained with the use of a 3mm disposable punch using sterile technique and local anesthesia is recommended to obtain samples from patients. Sutures are not required and no major complications have been reported with biopsy collection. The biopsy is most often taken from the distal leg, 10cm above the lateral malleolus however, other locations such as the calf, paraspinal region and the upper lateral aspect of the thigh have also been used. Collection from multiple sites is required to demonstrate a length-dependent loss of nerve fibers. Bright-field immunohistochemistry and indirect immunofluorescence with or without confocal microscopy have been used to assess intraepidermal nerve fiber density (IENFD)[11,12].

Diagnosis of neuropathies using skin biopsies was suggested by Levy *et al*. in 1989, when diabetic patients were examined for reduction in cutaneous nerve density. Samples from the diabetic patients revealed reduced dermal innervation when compared to control individuals, sometimes in the absence of electrophysiological abnormalities[51]. Diabetes mellitus induces early degeneration of skin nerve fibers and this correlates with the duration of the disease, with fewer nerve fibers noted with a longer disease duration[52]. An inverse association has been found between IENFD and the severity of the neuropathy in diabetic patients[53,54]. The use of skin biopsies and the quantification of intraepidermal

nerve fibers can be used for the early diagnosis of diabetic neuropathies or for assessing its progression or response to therapy[55,56].

Further investigations into various diseases associated with neuropathies have been performed. Reduced epidermal fiber density can be noted without symptoms of polyneuropathy or changes in nerve conduction studies[57] and may be the first sign of a neuropathy[58,59]. Skin biopsies are a sensitive method to diagnose idiopathic small fiber neuropathy, even in the absence of abnormalities in nerve conduction studies or nerve biopsies[60-62]. Serial biopsies can also be used to monitor progression of disease, with a decrease in the IENFD over time[63]. Patients with idiopathic small fiber neuropathy often have reduced nerve fiber density at the calf with more normal nerve fiber density proximally, thought to indicate a length-dependent process[64 65]. Severe length-dependent neuropathies can progress to also affect proximal sites[66]. Other conditions such as Guillain-Barré syndrome, chronic inflammatory demyelinating neuropathy, HIV infection, Fabry disease, Friedreich ataxia and familial dysautonomia also affect IENFD[67-73]. Cutaneous nerve fibers can also be affected in leprosy and their quantification in skin lesions could serve as an additional diagnostic tool in detecting neuropathy[74]. Small fiber neuropathy has been reported in patients with hypothyroidism and demonstrated via skin biopsy, with improvement noted after thyroid hormone replacement therapy[75-77]. In Ross syndrome, erythromelagia, complex regional pain syndrome type I, and late onset restless leg syndrome, loss of intraepidermal nerve fibers have been reported[78-83]. In Parkinson's disease, loss of epidermal nerve fibers and Meissner corpuscles were noted with more significant changes on the more affected side[84]. Patients with amyotrophic lateral sclerosis

(ALS), which is typically considered a motor neuron disease, had reduced IENFD, suggestive of a sensory axonopathy component to this disease[85].

Skin biopsies are advantageous in the diagnosis and monitoring of neuropathies in humans because they are simple, safe, and quick[12]. As the technique has been further studied and optimized, it also may provide a more reliable diagnosis for small fiber neuropathies, even in the face of normal nerve conduction studies and nerve biopsies[60-62].

Evaluation of epidermal innervation in dogs

In dogs, antibodies to PGP 9.5 have been used to demonstrate the innervation of mammary gland tissue, dorsal root ganglia, cornea, laryngeal taste buds, and skin[86-90]. Epidermal innervation was evaluated at various locations using a technique adapted from the human guidelines for diagnosis of neuropathies in young, healthy beagles[87]. The pattern of innervation of the epidermis was noted to be similar to that found in humans and rats, with frequent projections through the epidermal barrier[48,87,91,92]. Bundles of nerve fibers were found in the dermis and a higher density of nerve fibers was found at the base of hair follicles[87]. These findings suggest that skin biopsies could be used as a simple, low morbidity procedure for diagnosing neuropathies in dogs[87].

Purpose

The long-term goal of this research is to develop skin biopsies as a reliable diagnostic test for dogs with neuropathies. As a first step toward that goal, the effect of delayed sample fixation and refrigerated storage on nerve fiber integrity for the evaluation of IENFD is described. Secondly, this thesis determined quantification of IENFD in dogs diagnosed with laryngeal paralysis, as a model for other neuropathies in the dog. This

work is of clinical significance because if reduction in IENFD can be determined in dogs with canine laryngeal paralysis polyneuropathy as a model, it is possible that this technique can be applied to diagnose other neuropathies. As this is a simple technique, it could be performed by a general veterinary practitioner, without the need for referral to a veterinary neurologist for the current, more invasive techniques used for diagnosis. The effect of refrigeration and delayed fixation of the skin biopsy could have clinical relevance as samples may not be able to be immediately processed for immunohistochemical evaluation if the samples need to be shipped to a diagnostic laboratory. The use of skin biopsies for the diagnosis of neuropathies represents a new

CHAPTER II

GENERAL MATERIALS AND METHODS

Sample collection

In all cases, samples of skin were collected using 3mm punch biopsies from the lateral neck, overlying the larynx. The hair on the lateral aspect of the neck overlying the larynx was clipped. Ulnar nerve was collected from canine cadavers to provide positive and antibody-negative controls.

Tissue processing

The skin samples were fixed in Zamboni's fixative (2% paraformaldehyde with 0.2% picric acid in 0.1M phosphate buffer, Newcomer's supply, Middleton, WI, USA) by immersion for at least 72 hours at 4°C and then immersed in 30% sucrose for 72 hours. Excess subcutaneous adipose tissue was removed with a blade, and the punch biopsy piece was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA). Samples were frozen and stored at -80°C if there was a delay in processing and staining. Using a cryostat, 20 μm sections were cut from the punch biopsy samples and collected on SuperFrost Plus slides (VWR, Radnor, PA, USA). Slides were frozen and stored at -80°C if there was a delay in staining.

The sections were pretreated with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity of the skin. The slides were then washed in phosphate-buffered saline (PBS) on an orbital shaker at gentle speed for three cycles of 10 minutes each. Then, the sections were treated with 1% sodium borohydride in PBS for

7 minutes to help reduce background staining and retrieve antigen from the fixed tissue. The slides were then washed in PBS on an orbital shaker at gentle speed for three cycles of 10 minutes each. The sections were incubated with a blocking solution of 4% normal goat serum (Jackson ImmunoResearch Laboratories, INC., West Grove, PA, USA) in PBS containing 0.2% Triton X-100 (Life Technologies, Frederick, MD, USA) to reduce non-specific binding of secondary antibodies for one hour at room temperature in a humidified black slide box. They were then incubated at room temperature overnight with the primary antibody, rabbit polyclonal anti-human protein gene product 9.5 (PGP9.5) (EMD Millipore Corporation, Temecula, CA, USA) diluted 1:6000 in the blocking solution in a humidified black slide box.

Following incubation overnight, the slides were washed in PBS on an orbital shaker at gentle speed for three cycles of 10 minutes each. The sections were incubated with the secondary antibody solution, biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vectastain Elite ABC Reagent, Vector Laboratories, Burlingame, CA, USA), for an hour in a humidified black slide box to amplify the detected stain. The solution was made according to the manufacturer's instructions and then diluted 1:4 using PBS. The slides were washed in PBS on an orbital shaker at gentle speed for three cycles of 10 minutes each. The slides were incubated with an avidin-biotin horseradish peroxidase solution (Vectastain Elite ABC Reagent, Vector Laboratories, Burlingame, CA, USA) for 30 minutes in a humidified black slide box to further amplify the detected stain and then washed in PBS on an orbital shaker at gentle speed for three cycles of 10 minutes each. Next, a horseradish peroxidase substrate (ImmPACT VIP Peroxidase substrate kit, Vector

Laboratories, Burlingame, CA, USA) was added and incubated for 4 minutes in a humidified black slide box. Three cycles of washes in deionized water for 10 minutes each on an orbital shaker at gentle speed were then performed. The sections were dehydrated in 95% ethanol for 5 minutes, two washes in 100% ethanol for 5 minutes each and two washes in d-limonene (Histoclear, National Diagnostic, Hatfield, PA, USA) for 5 minutes each and coverslipped with permanent mounting media.

Cadaveric ulnar nerve was processed in the same manner to provide positive controls. Primary and secondary antibody solutions were withheld to provide primary antibiotic negative and secondary antibody negative controls, respectively.

Quantitative analysis of epidermal nerve fibers

Sections were examined from each skin sample and were photographed with a digital camera at 20x magnification. Unbroken, unfolded epidermis suitable for quantification was identified and measured using the line measuring program in Image J 1.40g (National Institute of Health). The density of intraepidermal nerve fibers was expressed as the number of nerve fibers per millimeter of basement membrane. The nerve fibers were counted by a single observer in each skin sample at a magnification of 400x in the portion of skin previously been measured. The rules for counting the nerve fibers followed those described by the guidelines of assessing nerve fibers density in human skin (11,12) and previous work in dogs (87). Each nerve fiber that crosses the basement membrane is counted. Axons that branch within the epidermis are counted as single axons. Crossing of the basement membrane must be observed to be counted as a crossing.

CHAPTER III

IMPACT OF SAMPLE REFRIGERATION AND STORAGE ON INTRAEPIDERMAL NERVE FIBER INTEGRITY

Introduction

Skin biopsies for evaluation of intraepidermal nerve fiber density (IENFD) have been extensively used for the diagnosis and monitoring of small-fiber neuropathies and other neuropathies in humans. Over the past 30 years, the technique has gained widespread use. Skin biopsies can be performed at major medical centers or can be performed locally at physicians' offices. The European Federation of Neurological Societies (EFNS) and Peripheral Nerve Society (PNS) guidelines on the use of skin biopsies in the diagnosis of small fiber neuropathy recommend immediate fixation of the biopsy sample in fixative following collection for 24 hours, followed by immersion in a cryoprotective solution for one night[12].

The Johns Hopkins Cutaneous Nerve Lab will provide a cutaneous nerve biopsy test kit to physicians performing skin biopsies which advises refrigeration of the biopsy sample in fixative solution for no longer than 24 hours and followed by washing the sample in phosphate buffer. The sample should then be placed in the provided cryoprotective solution to be shipped using a refrigerated shipper for processing at the John Hopkins Cutaneous Nerve Lab or further stored at 4^{o}C [93]. Comparison of IENFD of skin samples stored in Zamboni's fixative at cooled and ambient temperatures determined

that fewer nerve fibers could be detected in the samples kept at room temperature, which could result in the misdiagnosis of a small-fiber neuropathy based on reduced IENFD[94].

There are currently no guidelines on the collection, storage and processing of samples for the diagnosis of peripheral neuropathies in dogs. In previous work by de Medeiros *et al.* (2009), the canine skin samples were immediately placed in Zamboni's fixative for at least 72 hours at 4°C and then immersed in 30% sucrose for 72 hours[87]. Nerve fibers were successfully stained and IENFD could be determined. The goal is to develop a reliable diagnostic test, allowing for the diagnosis of neuropathies using skin biopsies, aiding in timely and accurate recognition of these diseases in dog. In order for such a technique to be accessible to general veterinary practitioners, the impact of sample storage prior to processing on IENFD requires evaluation, because samples will need to be shipped to diagnostic laboratories for processing and quantification of IENFD. While most general veterinary practitioners may have access to formaldehyde for tissue fixation, human skin specimens fixed in formalin resulted in a more fragmented appearance of nerve fibers when compared to Zamboni's fixative[95,96]. The objective of this chapter was to evaluate the effects of delaying sample fixation with refrigeration at 4ºC on IENFD. We hypothesized that delaying sample fixation with refrigeration would result in a reduction in IENFD after 24 hours following euthanasia.

Materials and methods

Samples of skin were collected *post mortem* from adult hound dogs euthanized for reasons unrelated to this study. The entire neck was harvested and stored at 4°C for 7 days. Six punch biopsies were collected at day 0 (day of euthanasia), day 1, day 2, day 5 and

day 7 from the skin overlying the larynx. Tissue samples were processed and quantitative analysis of epidermal nerve fibers was performed as outlined above in Chapter II.

Statistical analysis

The density of nerve fibers in each dog at each day was compared using the Friedman test, which is a repeated measure test, thereby controlling for the effect of individual animal difference in nerve sample densities within each dog when comparing different time points (GraphPad Prism version 7.0). The level of significance was set at $p<0.05$.

Results

Six adult hound dogs, 2 intact females and 4 intact males, were sampled. Average body weight was 30.5kg (range 22.3-36.8kg) and average age was 8.7 years (5.07-13.5 years). Clinical history was unknown.

Positive and negative controls were examined. Axonal staining was noted in the positive control (Figure 1 A). There was no axonal staining noted in either of the negative controls (Figure 1 B and C).

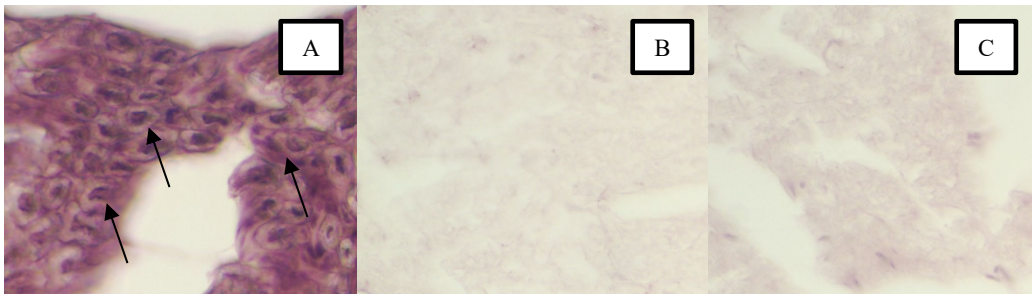

Figure 1: Control slides of stained cadaveric ulnar nerve. A: positive control with axons stained purple indicated by the black arrows. B: negative control with primary antibody (rabbit polyclonal anti-human PGP 9.5) omitted. C: negative control with secondary antibody (biotinylated goat anti-rabbit IgG) omitted. No stained axons are visible in B or C.

The total number of intraepidermal nerves fibers in ten 20 µm sections of stained skin sections for each sample collection day for each dog was recorded (Figure 2, Figure 3). IENFD was determined for each sample collection day for each dog and compared. The results of each sample collection day are summarized in Table 1.

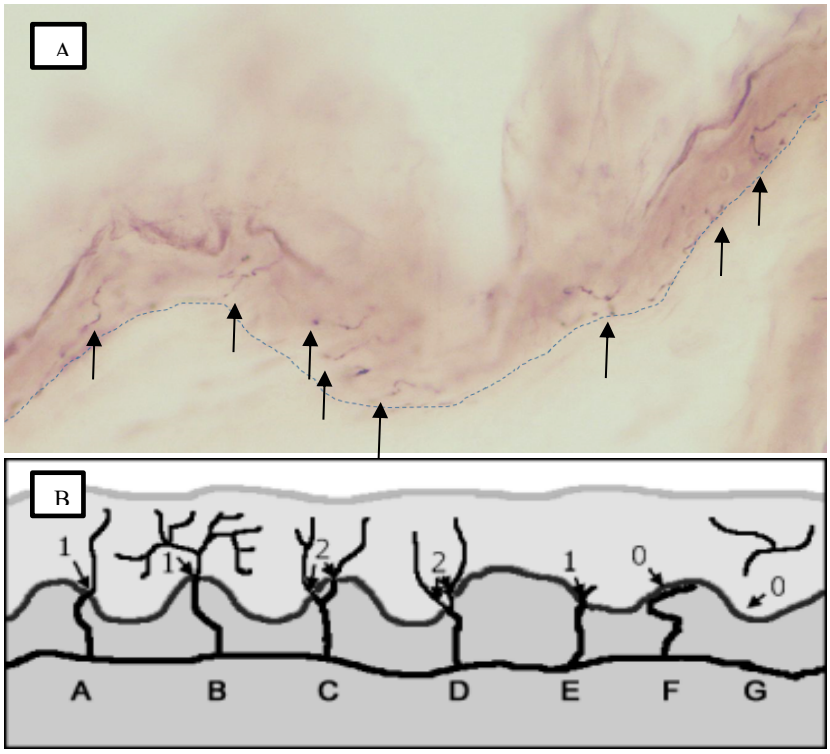

Figure 2: Epidermal innervation and intraepidermal nerve fiber counting rules. .A: Epidermal innervation example from F2 on day 0 (day of euthanasia). Arrows represent stained nerve fibers. The basement membrane is outlined using the grey hatched line. B: Intraepidermal nerve fiber counting rules (Reprinted from Lauria, G et al. European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society[11]. Used without permission according to Fair Use Analysis)

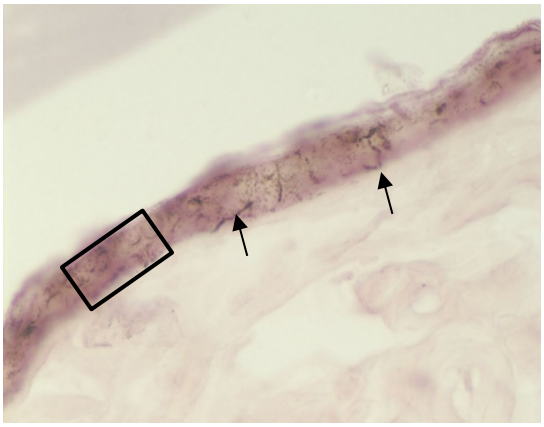

Figure 3: Skin section with melanin granules within the epidermis. Intraepidermal nerve fibers (arrows) with a defined linear structure that differentiate from the melanin granules (highlighted in the black rectangle).

Dog	Day 0	Day 1	Day 2	Day 5	Day 5
F1	2.46	0.43	1.48	0.52	0.64
F2	6.19	0.86	1.22	0.82	1.30
M3	0.55	0.23	1.42	0.04	0
M4	0.20	0.79	0.09	0.13	0
M5	0.06	1.71	0.03	0.08	0.03
M6	0.29	1.37	0.24	0.84	0
Mean (SE)	1.625 (0.982)	0.915 (0.221)	0.747 (0.284)	0.405 (0.151)	0.328 (0.220)

Table 1: Densities of intraepidermal nerve fibers (number/mm of basement membrane) in the skin of the neck overlying the larynx in dogs at each collection day. Mean (se) for each sample collection day are represented in the last row of the table.

A gradual reduction in the IENFD was observed over time (Figure 4). There was no significant difference in IENFD between any of the subsequent collection days when compared to day 0 (Friedman test p=0.12) (Figure 4, Figure 5). Three dogs had an increase in nerve fiber density counts observed between day 0 and day 1 (Table 1, Figure 5). Day 7 was the only day when a density of 0 nerves/mm was found.

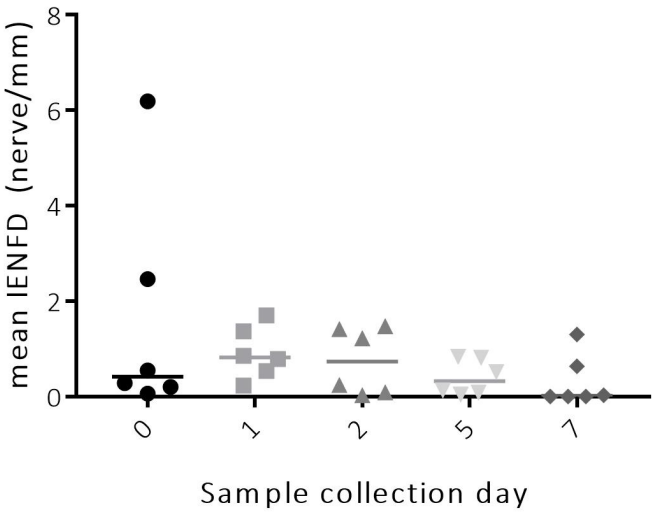

Figure 4: Densities of intraepidermal nerve fibers (number/mm of basement membrane) in the skin of the neck overlying the larynx in dogs at each collection day. Each dog is represented as a data point. Horizontal bars represent the median. Significant difference was not detected between any of the sample collection days (p=0.12).

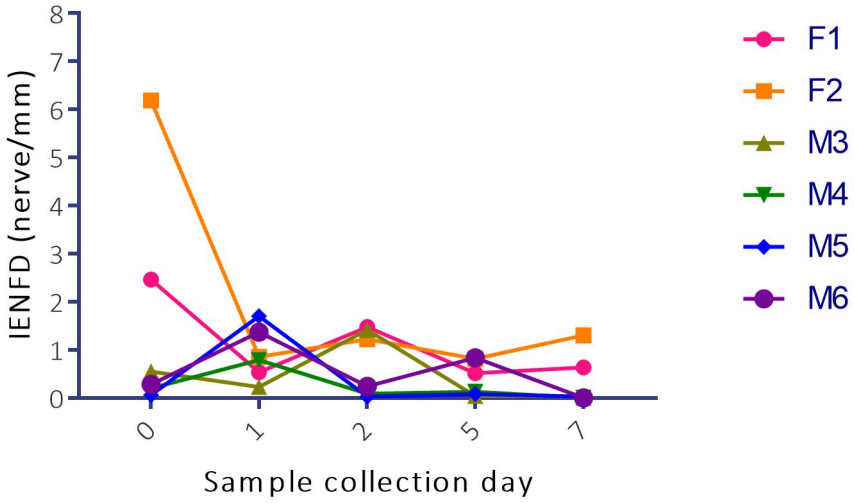

Figure 5: Individual densities of intraepidermal nerve fibers (number/mm of basement membrane) in the skin of the neck overlying the larynx in dogs at each collection day. Each dog is represented as a data point.

Discussion

The results of this study demonstrate that the immunohistochemistry protocol outlined by de Medeiros *et al.* (2009) could be successfully replicated to quantify IENFD in dogs. Nerve fibers could be observed crossing the basement membrane.

Although the difference was not statistically significant, there was a gradual reduction in the IENFD observed over day 0 to day 7. In three dogs, a density of 0 nerves/mm was noted at day 7. No other days had an IENFD of 0 nerves/mm. Although not statistically significant, higher mean nerve densities were observed at day 0, day 1 and day 2. These results suggest that refrigeration at 4°C may be acceptable for up to 48 hours after collection, before the samples need to be placed in Zamboni's fixative. This could permit transportation of a refrigerated biopsy sample to diagnostic laboratories within 48 hours if there is no access to Zamboni's fixative at the time of sample collection. Sample collection may also be delayed for up to 48 hours after death or euthanasia, allowing for investigation of peripheral neuropathy as part of a *post mortem* examination, if indicated.

When compared to the previous IENFD results obtained by de Medeiros *et al.* (2009), the IENFD of the skin of the neck was lower. In that study, the mean density of intraepidermal nerve fibers in the skin of the neck was 9.46 mm^{-1} [87]. In this study, the greatest IENFD found in one dog was 6.18 $mm^{-1}$and the mean density at day 0 was 1.63 mm^{-1}. This may have been related to the differences in collection techniques, with larger skin samples of 1cm by 0.5cm used by de Medeiros *et al.* (2009) whereas only 3mm punch biopsies were used in this study[87]. A larger piece of skin may increase the likelihood of obtaining an area with a high IENFD. In healthy human children and adolescents, intra-

biopsy epidermal nerve fiber density was higher between images from the standard 3mm biopsies when compared to variation between images obtained by the suction blister method[97]. The suction blister method allows for evaluation of a greater surface area in each image when compared to the biopsy images for quantification of epidermal nerve fibers. It is possible that the variability between dogs and between sample collection days in this study was related to the smaller individual surface area examined with the standard 3mm biopsy technique. This was attempted to be addressed by evaluating 10 sections for each dog for each sampling day. The IENFD varied widely between different areas of the same section and between different sampled body regions in young healthy beagles[87]. The pattern of innervation was described as patchy with certain areas with very high and very low densities of intraepidermal nerve fibers noted[87]. This was similar to the findings in the present study.

Healthy children and adolescents had more than 3 times higher IENFD when compared to the normal ranges in healthy adults[97]. In humans, the youngest subjects examined (16-20 years) had higher densities than other older groups, but overall there was no effect of increasing age on IENFD[95]. Further studies revealed a significant decrease in IENFD with increasing age[10,98-102]. Dogs of 15 months of age were used in the previous study by de Medeiros *et al.* (2009)[87]. The effect of increasing age on IENFD in dogs is not known but could follow a similar trend to that in humans. In addition, breed-specific differences are unknown. Race has been compared in certain studies: no difference has was found IENFD between Caucasian and non-Caucasian healthy controls, however this study compared 461 Caucasian subjects to 34 subjects of different races[101]. Chinese

individuals were found to have significantly higher IENFD at the distal leg when compared to Finnish individuals[103].

Three dogs had a higher nerve fiber density recorded between day 0 and day 1. Although sample collection was performed on the skin overlying the larynx at each day, individual variations in nerve fiber density at different locations could result in different densities being noted on different sample collection days. This finding raises the question of whether the overall trend of a decrease in IENFD observed could be due to variation based on the location of the skin and not due to sample refrigeration. In humans, no significant variation is observed in IENFD determined in adjacent biopsies from the same site[95]. This has not yet been evaluated in dogs. When skin biopsies are performed for regional conditions such as nostalgia paraesthetica, reduced IENFD is noted in pathological areas but normal innervated skin can be seen when a healthy dermatome is sampled[104]. Sensation of the neck in dogs is provided by the terminal segments of spinal cervical nerves III, IV and V[105]. It is possible that individual differences in dermatomes or the dermatome affected by the neuropathy could be responsible for the variability between samples collected.

Only one observer performed the IENFD counts in this study. Previous studies have evaluated both intra- and inter-observer reliability[98,99]. In healthy individuals, blinded inter-observer variability was higher than intra-observer variability, but these values were numerically low with values of 0.4±1.5 fibers/mm and 0.2 ± 1.2 fibers/mm, respectively[99]. Both intra-observer and inter-observer variabilities were determined to be acceptable based on Bland-Altman plots[99]. Bakkers *et al.* (2009) found good intra- and inter-observer

reliability when evaluating blinded researchers at one institution[98]. When three institutions were assessed for inter-observer reliability in healthy controls, inter-observer reliability between the researchers was rated as good[98]. As this is not an automatic technique, variability is expected. The rules outlined by the European Federation of Neurological Societies/Peripheral Nerve Society guidelines were followed when evaluating the biopsy samples in this study. Intra- and inter-observer reliability have not been assessed in the quantification of IENFD in dogs and should be considered in future studies.

Limitations of this study include the small sample size, as it is possible that a significant difference between days may have been detected with additional subjects (*i.e.* 'type II error'). The clinical history of these dogs was unknown and a clinical examination was not performed on these dogs prior to sample collection, thus the presence of laryngeal paralysis, any neuropathy or other condition which may affect IENFD is unknown. Dogs served as their own controls at day 0 to determine the impact of refrigeration and delayed tissue fixation on IENFD. However, IENFD was very variable between dogs and between individual sections amongst dogs. This could represent an inherent variability in IENFD between individuals and between even slightly different locations on the skin, raising the question of whether this method would be feasible to use as a diagnostic test for dogs with neuropathies. Healthy humans of the same age and gender have been noted to have a large variability in IENFD, which could be a result of other factors such as genetics, the environment and individual differences in metabolic parameters[101]. A similar trend could be found in dogs. Further studies would be required to evaluate for individual variation and the effect of age and breed on IENFD. Although inter-individual variability of IENFD

may be too great in dogs to allow for establishing an absolute reference interval for diagnosis of neuropathies, the technique could still be useful for monitoring changes in one individual over time. Despite these limitations, the results may prove useful as a foundation for the guidelines for diagnostic laboratories, once the feasibility of this technique is determined.

CHAPTER IV

INTRAEPIDERMAL NERVE FIBER DENSITY IN DOGS DIAGNOSED WITH CANINE LARYNGEAL PARALYSIS

Introduction

Canine peripheral neuropathies pose a challenge for veterinarians, both in the recognition of a neuropathy and the diagnosis of the etiology[16]. Sensory and motor neuropathies have been documented in dogs, with certain bearing similarities to Charcot-Marie-Tooth disease in humans[13-18]. Clinical signs are often vague or difficult to recognize and can include ataxia[17, 18], weakness[14,15,17,18], muscle atrophy,[13,14,17,18] self-mutilation[15,18,19] and reduced or absent spinal nerve reflexes[13, 14]. Diagnosis typically relies on electromyographic testing, nerve conduction testing, and muscle and nerve biopsies, requiring general anesthesia and referral to a specialty center[14,16,18,34].

In humans, peripheral neuropathy is frequently diagnosed by skin biopsy, by determining intraepidermal nerve fiber density (IENFD) with immunohistochemical staining for protein gene product 9.5 (PGP 9.5). Changes have been described in sensory, motor, mixed and autonomic neuropathies[66,71,81]. Reduction in IENFD suggests neuropathy, even in the absence of abnormalities in nerve conduction studies or nerve biopsies[60-62]. Serial biopsies can also be used to monitor for progression of disease[63] or response to treatment[75-77].

Epidermal innervation has been examined at various anatomical locations using a technique adapted from the human guidelines for diagnosis of neuropathies using young,

healthy beagles[87]. Epidermal nerve fiber quantification was successful, suggesting this technique could be the basis of a new diagnostic test for peripheral neuropathies or neuropathic pain in dogs. Abnormalities in electromyographic testing, nerve conduction studies and nerve and muscle biopsies have provided evidence that laryngeal paralysis is a component of a more generalized polyneuropathy[38,41-43]. As laryngeal paralysis is well-known and its clinical signs can be easily recognized, dogs with laryngeal paralysis could serve as an ideal patient population for the investigation of techniques for the diagnosis of neuropathies. There are currently no guidelines on the diagnosis of peripheral neuropathies in dogs using skin biopsies. The objective of this chapter was to quantify IENFD in dogs diagnosed with laryngeal paralysis as a model for diagnostic evaluation of other neuropathies. We hypothesized that dogs with laryngeal paralysis would have reduced IENFD when compared to controls.

Materials and methods

Dogs were recruited through the Texas A&M University Small Animal Hospital. Dogs with a diagnosis of laryngeal paralysis via observation of absent unilateral or bilateral arytenoid cartilage movement during direct laryngeal exam under a light plane of propofol anesthesia or with a history of a previous arytenoid lateralization were included in the study. If respirations were not adequate for diagnosis, doxapram HCL (Dopram-V, Fort Dodge Animal Health, Overland Park, KS, USA) was administered at the discretion of the person conducting the laryngeal exam. The study was approved by the Texas A&M University Institutional Animal Care and Use Committee (protocol AUP IACUC 2017-0251). Written informed consent was provided for all animals (Appendix A).

Skin samples were collected while the dogs were under general anesthesia for other procedures (diagnostic imaging, surgery, other diagnostic procedure) or under heavy sedation at the discretion of the attending clinician if no further procedures were required to be performed. The lateral aspect of the neck overlying the larynx was clipped and aseptically prepared using chlorhexidine scrub and isopropyl alcohol. Skin was collected from the lateral aspect of the neck overlying the larynx via three 3mm punch biopsies using sterile technique. The biopsy sites were each sutured using a single skin suture of 3-0 nylon (Monosof, Covidien, Manfield, MA, USA) or poliglecaprone 25 (Monocryl, Ethicon, Somerville, NJ, USA). Following sample collection and any other concurrent required procedures, dogs were recovered from anesthesia or sedation. Dogs were discharged following recovery at the discretion of the attending clinician.

Tissue samples were processed and quantitative analysis of epidermal nerve fibers was performed as outlined in Chapter II. Day 0 samples of the dogs used in Chapter III were used as controls. If the epidermis was absent on evaluation of the slides, this section was excluded from analysis.

Statistical analysis

The IENFD of control dogs and dogs diagnosed with laryngeal paralysis was compared using the Mann-Whitney U test, allowing to compare of two non-normally-distributed populations (GraphPad Prism version 7.0). The level of significance was set at $p<0.05$.

Results

Eight client-owned dogs were recruited. No dogs were excluded once enrolled and data from all animals was included in the analysis. Breeds included were Labrador retriever (4), mixed breed (2), one golden retriever and one American pit bull terrier. Ages ranged from 9 to 13 years, with a mean of 11.75 years (SE 0.5261). Body weight ranged from 27.8 to 50.2kg, with a mean of 38.3kg (SE 2.608). There were 7 castrated males and one spayed female. Demographic data is summarized in Table 2.

Clinical examination

Neurologic examination was performed in 5 of the dogs (Table 2). Neurologic examination was not performed due to the in the remaining three dogs as they were already under general anesthesia prior to sample collection. Neurologic abnormalities consistent with peripheral neuropathy were noted in all 5 dogs examined and are described in Table 2. Bilateral laryngeal paralysis was diagnosed in 6 dogs. Left unilateral laryngeal paralysis was diagnosed in one dog that did not undergo surgical intervention. One dog had previously undergone a left unilateral arytenoid lateralization and an upper airway examination was not performed at the time of sample collection.

Table 2: Demographic information and neurologic signs noted on examination of dogs diagnosed with laryngeal paralysis. CM = castrated male. SF = spayed female

	Breed	**Gender**	**Weight (kg)**	**Age**	**Neurologic signs**
1	Labrador retriever	CM	43.3	11	Did not assess, limb amputation performed for distal tibial osteosarcoma, diagnosed with bilateral laryngeal paralysis previously (2/2018)
2	Labrador retriever	CM	37.8	11	Decreased postural reactions in pelvic limbs, no patellar reflexes, reduced hock flexion bilaterally during withdrawal, bilateral laryngeal paralysis
3	Labrador retriever	CM	41.8	11	Proprioceptive deficits and reduced reflexes in all four limbs, worse in the pelvic limbs, absent patellar reflexes bilaterally and reduced withdrawals in pelvic limbs, temporalis muscular atrophy bilaterally, bilateral laryngeal paralysis
4	Golden retriever	CM	34.7	13	Absent patellar reflex bilaterally, delayed proprioception in the pelvic limbs bilaterally, incomplete withdrawals bilaterally in pelvic limbs, bilateral laryngeal paralysis
5	American pit bull terrier	SF	27.8	9	Did not assess, bilateral laryngeal paralysis. Later diagnosed with neuritis on *post mortem* examination.
6	Mixed breed	CM	40.8	13	Ambulatory tetraparesis, previous arytenoid lateralization, complete neurologic examination not performed
7	Labrador retriever	CM	50.2	13	Reduced proprioceptive responses in the pelvic limbs, reduced patellar reflex, normal withdrawal reflexes in all four limbs, forelimbs normal, cranial nerves normal, mild temporalis muscular atrophy bilaterally, left laryngeal paralysis , right side had appropriate function
8	Mixed breed	CM	29.8	13	Reduced proprioceptive responses in pelvic limbs, normal withdrawal reflexes with cross extensor in pelvic limbs, clonus on patellar reflex, forelimbs normal, bilateral laryngeal paralysis

Intraepidermal nerve fiber density

The total number of intraepidermal nerves fibers in a mean of 13 (range 9-15 sections) 20µm stained tissue sections for each dog diagnosed with laryngeal paralysis were counted (Figure 6).

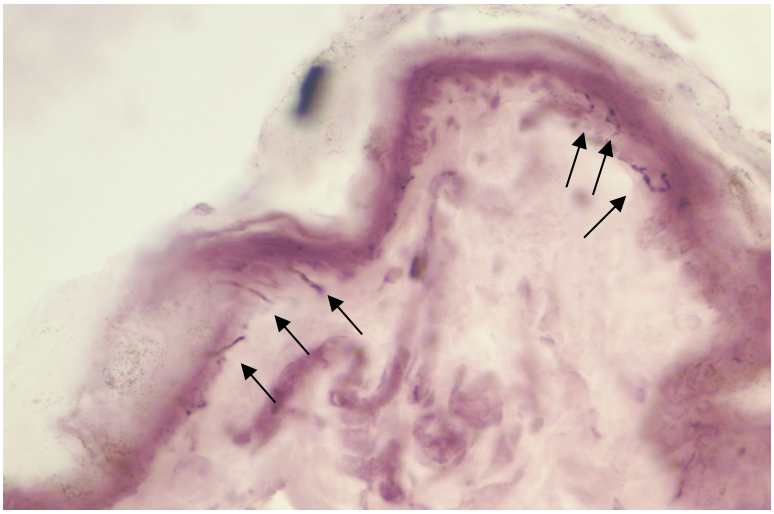

Figure 6: Epidermal innervation example from dog 1. Arrows represent stained nerve fibers.

The IENFD obtained was compared to the mean IENFD of control dogs (Figure 7). The mean of the control dogs was 1.625 nerves/mm (SE 0.982) and the mean of the CLPP dogs was 2.317 nerves/mm (SE 0.584). There was no significant difference between the IENFD of the dogs diagnosed with laryngeal paralysis and the control dogs (Mann Whitney test p=0.28) (Figure 7). No complications were reported from the skin biopsies.

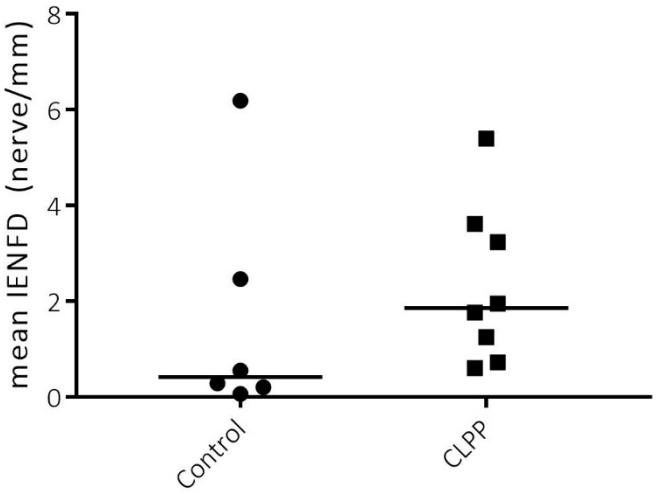

Figure 7: Densities of intraepidermal nerve fibers (number/mm of basement membrane) in the skin of dogs with CLPP and control dogs. Each dog is represented as a data point with horizontal bars representing the median. No significant difference was found (p=0.28).

Discussion

The objective of this study was to determine whether dogs diagnosed with canine laryngeal paralysis polyneuropathy had reduced IENFD compared to controls. All 8 dogs were diagnosed with laryngeal paralysis and the 5 dogs that underwent neurologic examination had abnormalities consistent with a peripheral neuropathy. No dogs were reported to have any complications with the sampling site of the skin biopsies, supporting that this is a simple, safe and low morbidity procedure.

Despite these clinical findings, there was no significant difference between the IENFD of the dogs diagnosed with laryngeal paralysis and the control dogs. This could indicate that there is no alteration in IENFD in dogs with CLPP. Alternatively, the ability to detect a difference between the affected and control groups could have been compromised by the high individual variability in epidermal innervation or by the choice

of the control samples. Dogs used in Chapter III were used as controls but they were not age- or breed- matched controls, although their mean age and weight was comparable to the dogs recruited with laryngeal paralysis. Samples were obtained shortly after these control dogs were euthanized and their clinical history was unknown. It cannot be ascertained whether these dogs had laryngeal paralysis, another neuropathy or underlying disease which could have impacted the IENFD of the control dogs.

Previous work by de Medeiros *et al.* (2009) determined that IENFD of the skin of the neck of 15 month old beagles was 9.46 nerves/mm (SE 0.66, confidence interval 8.01-10.9), which is higher than both our control dogs and the dogs diagnosed with laryngeal paralysis[87]. The impact of age and breed on IENFD has not been established in dogs. In humans, a decrease in IENFD can be expected with increasing age[10,98,99,100,102,106]. Chinese individuals were found to have significantly higher IENFD at the distal leg compared to Finnish individuals[103].

CLPP is hypothesized to affect longer nerves first, which could be consistent with a length-dependent process as noted with small fiber neuropathies in humans. The neck was chosen as the site of sample collection in dogs with CLPP because this is where the incision for a unilateral arytenoid lateralization is performed. This location was selected to decrease possible morbidity from collecting skin samples from multiple locations. In humans, the skin of the trunk appears to have a higher IENFD when compared to the calf[104]. It was hypothesized that a greater change in IENFD would be seen in the skin of the neck of dogs with CLPP if there was a greater initial density. However, this was not observed in this study. In human patients with idiopathic small-fiber neuropathy, reduced

IENFD can be observed at the calf whereas IENFD can still be normal proximally[64,65] with progression to affect the proximal sites in more severe length-dependent neuropathies[66]. It is possible that dogs with CLPP may have had more significant changes in IENFD at the level of the crus compared to the thigh, as observed in humans. In subsequent studies, it may prove useful to compare the IENFD of the crus, thigh, and neck in dogs affected by CLPP and compare these values to age- and breed- matched controls that do not have clinical signs consistent with a peripheral neuropathy.

The control dogs used were all intact dogs, whereas the dogs diagnosed with laryngeal paralysis were all sterilized. Guidelines for the evaluation of neuropathies in humans recommend using age- and gender-matched controls[100]. In humans, men had lower normal IENFD of skin biopsies from the distal leg compared to women[10,100,101]. Normal values were found to be equivalent by Lauria *et al.* (2010) in men and women in age groups of 70 years of age and greater[100], whereas persistent differences were noted by Collongues *et al.* (2018) and Provitera *et al.* (2016)[10,101]. Neither study commented on hormonal status in their patient population. The impact of gender on IENFD in dogs has not been determined and whether hormonal status may impact IENFD is not known. Experimental studies have demonstrated neuroprotective effects of progesterone and its derivatives[106] and testosterone derivatives[107] in rats with experimentally-induced diabetes as improvement of IENFD was noted with supplementation. In the present study, there was no statistical difference between the intact control dogs and the sterilized dogs diagnosed with laryngeal paralysis.

The majority of studies in humans have used IENFD in the context of sensory neuropathies whereas CLPP would be considered a motor neuropathy. Patients with amyotrophic lateral sclerosis (ALS), which is a motor neuropathy, have reduced IENFD but this was attributed to a distal sensory axonopathy in addition to the motor neuropathy[85]. Guillian-Barré syndrome is an acute motor neuropathy reported in humans in which a reduction of cutaneous innervation was noted, suggesting a small fiber neuropathy in these patients[71]. In this current study on dogs, an assumption was made that changes in IENFD would be detected for a motor neuropathy. However, this may not be possible unless there is a component of a sensory neuropathy in CLPP. Further work is needed to determine whether CLPP can be detected via skin biopsies or whether there is also a sensory component to this neuropathy.

Limitations of this study include the small sample size and the variability of the IENFD between dogs. IENFD was highly variable between dogs and variable between sections amongst individual dogs, as noted in Chapter III. This could represent an inherent variability in IENFD between individuals and between different areas of the skin of the neck, despite the presence of a peripheral neuropathy, raising the question of whether this method would be feasible to use as a diagnostic test for dogs with neuropathies. Further studies would be required to evaluate for individual variations and whether this technique can be applied for the diagnosis of neuropathies in dogs. Despite these limitations, the results serve as an initial, important advance in the determination of the use of IENFD for the diagnosis of peripheral neuropathies in dogs.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis outlines an evaluation of a new potential diagnostic technique to aid in the diagnosis of neuropathies in dogs. While evaluation of IENFD has been determined to be a useful diagnostic technique in humans with neuropathies, we have not yet established the plausibility of this technique for the diagnosis of neuropathies in dogs. Nevertheless, this work serves as an advance in the current knowledge regarding the use of skin biopsies for the diagnosis of neuropathies in dogs and may serve as a launching point for future studies to determine if IENFD could be used to aid in the diagnosis of CLPP and other neuropathies in dogs.

In both chapters, the previous immunohistochemistry protocol outlined by de Medeiros *et al.* (2009) was successfully performed in order to evaluate IENFD in dogs. Nerve fibers could be observed crossing the basement membrane.

In Chapter III, skin was stored for 7 days to determine the impact of refrigerated storage on nerve fiber integrity prior to sample fixation in Zamboni's fixative. Results suggested that skin samples could be stored at 4^{o}C for up to 48 hours before fixation. Subjectively, there was a trend in the reduction of epidermal nerve fiber density was observed beyond this time, with a decrease in IENFD observed with increased storage time, however, this difference was not significant, likely representing a type II error because of small sample size and large inter-individual variability. The reduction in IENFD is potentially an important finding because it suggests that it is feasible for sample

collection to be performed by a general practitioner with subsequent transportation of a refrigerated biopsy sample to a diagnostic laboratory within 48 hours of sample collection, without requirement for immediate access to Zamboni's fixative at the time of collection.

In Chapter IV, IENFD of dogs diagnosed with laryngeal paralysis was determined and compared to that of control dogs. Although there was no significant difference between the IENFD of the skin of the neck of dogs in the control and CLPP groups, again, possibly a consequence of type II error associated with small sample size, this work does serve to advance the determination of the feasibility of this technique in dogs. A large amount of IENFD variability was noted between dogs and between sections from individual dogs. These results do not rule out the use of skin biopsies in diagnosis of neuropathies in dogs but more work is needed to establish the plausibility and utility of this technique.

Future studies are necessary to further elucidate whether IENFD can be used to define a reference interval for IENFD in normal dogs or whether inherent IENFD variability precludes this from being a viable diagnostic technique. A reference interval could allow for this procedure to become a diagnostic test for neuropathies. If inter-individual variability of IENFD in dogs were too great to allow for establishing an absolute reference interval for diagnosis of neuropathies, further work could establish if the technique could still be useful for monitoring changes in one individual over time. Additional work could determine if skin biopsies in other locations, such as at the level of the gastrocnemius, may be more accurate for the determination of reduced IENFD in dogs

affected by CLPP or other neuropathies. Additional evaluation could also determine whether information regarding progression of disease or prognosis, can be attained from skin biopsies in dogs. If proven reliable, evaluation if IENFD in the skin of dogs could provide a low-morbidity, simple, inexpensive, and widely available diagnostic test, without the need for referral to a specialist.

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APPENDIX A

INFORMED OWNER CONSENT FORM

MEASUREMENT OF SKIN NERVE DENSITY IN DOGS

Nick Jeffery, Professor, Neurology & Neurosurgery
Michelle Hasiuk, Resident in Small Animal Surgery

1. Purpose of the project
To measure skin nerve density (the number of nerves in a defined area of skin) in dogs that have signs of nerve disease and compare it with normal dogs.

2. Eligibility for participation
Dogs will be eligible if they:

- Are any age, size, breed, or sex
- Are scheduled to undergo anesthesia for diagnostic or treatment purposes
- Are diagnosed with signs of nerve disease, such as swallowing or breathing difficulties or limb weakness

3. Expected duration of participation
Participation may require up to 5-10 minutes extra time for skin sample collection while the patient is under anesthesia.

4. Description of procedure
The skin on the side of the neck where the samples will be taken will be clipped of hair and prepared with antiseptic solution. Up to six, 3-5 mm (1/8 inch) diameter skin biopsy samples (punch biopsies) will be obtained from the side of the neck while the dog is under anesthesia. The holes in the skin made by this technique are small so that placing sutures is often not necessary, but if required, a single suture may be placed.

5. Possible discomforts and risk
Punch biopsies: Possible risks associated with punch biopsies include slight bleeding, focal bruising, minimal scarring, and secondary infection. The impact from the biopsies are minor and should entail minimal discomfort during healing. Appropriate medications will be prescribed if any of these complications should arise.

General anesthesia: Potential side effects of general anesthesia are: abnormal behavior, vomiting, aspiration pneumonia, decreased appetite, sore throat, coughing, nausea, drowsiness, disorientation, itching, allergic reactions (including anaphylaxis), malignant hyperthermia (high body temperature), decreased heart rate, decreased blood pressure, and possible death.

6. Possible benefits of study

Your animal will receive no direct benefits from participation in this study.

7. Alternative diagnostics, procedures, or treatments
None

8. Confidentiality
Owner and patient confidentiality will be maintained. No identification of individuals shall be made when reporting or publishing the data arising from this study.

9. Financial obligations
There are no financial obligations by the owner to Texas A&M University for participation in this study.

10. Compensation or therapy for accidental injury or complications
If the biopsy site becomes infected, the cost of bacteriological culture and a course of appropriate antibiotics of up to three weeks will be paid for by the investigator.

11. Primary contact person
To obtain further information regarding this study contact:

Nick Jeffery, BVSc, PhD, MSc, DipECVS, DipECVN, DipSAS (soft tissue), FRCVS
Professor
Department of Small Animal Clinical
Sciences 4474 TAMU
College Station, TX 77843
979-213-3366

12. Participation and right to withdraw
Enrolling your animal for participation in this study is voluntary, and refusal to participate involves neither penalty nor loss of care to which your animal is otherwise entitled. You have the right to withdraw your animal from the study without penalty at any time and for any reason.

13. Termination of participation by principal investigator
The investigator has the right to terminate the study for any or all participants at any time and for any reason.

14. Unforeseen risks
Unforeseen risks might arise at any time during the study. The investigator will promptly inform you of any new information that may affect your willingness to continue to have your animal participate in the study.

MEASUREMENT OF SKIN NERVE DENSITY IN DOGS

Nick Jeffery, Professor, Neurology & Neurosurgery
Michelle Hasiuk, Resident in Small Animal Surgery

I, _____(name), of

_____ (address)

_____ (City, Zip)

hereby consent to the participation of the following animal in the study cited above. I certify that I am the legal owner (or agent of the owner) of, and am responsible for this animal. I have read, received a copy of, and understand the Informed Owner Consent Form.

Animal Details

Name: _____

Breed: _____

Age: _____

Signature of Owner or Agent: _____ Date:_____

Signature of Investigator: _____ Date:_____

Witness: _____ Date:_____

I have received a copy of the consent form

_____

This consent form has been reviewed and approved by the Clinical Research Review Committee of the Texas A&M University College of Veterinary Medicine & Biomedical Sciences. If questions arise regarding your rights as a participant, please contact Dr. Robert Burghardt; Associate Dean for Research & Graduate Studies; College of Veterinary Medicine & Biomedical Sciences at Texas A&M University; phone: 979-845-5092; CRRC@cvm.tamu.edu.