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## Independent Study Projects

### Title

Histologic and morphometric evaluation of normal aged canine muscle : baseline data for future evaluation of aged dogs with suspected sporadic inclusion body myositis.

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### Author

Chang, Brian

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**HISTOLOGIC AND MORPHOMETRIC EVALUATION OF  
NORMAL AGED CANINE MUSCLE: BASELINE DATA FOR FUTURE EVALUATION OF AGED  
DOGS WITH SUSPECTED SPORADIC INCLUSION BODY MYOSITIS**

**ABSTRACT**

**Introduction:** Sporadic inclusion body myositis (sIBM) is the most common myopathy in people over 40 years of age. However, sIBM is a poorly understood disease with no effective treatments to date. Currently there are no reliable animal models for sIBM which is a hindrance to development of new therapies. While other inflammatory myopathies are well documented in dogs, spontaneously occurring sIBM was only recently reported (Shelton et al. 2009). The identification of sIBM in dogs raises the possibility that canine sIBM may be a useful animal homologue for the comparable human disease. As sIBM is associated with aging, and baseline information on normal aged canine muscle not available, in-depth studies of normal aging changes in canine muscle were performed.

**Methods:** Fresh frozen muscle biopsies from five aged dogs (>10 years) without clinical signs of neuromuscular disease were sectioned and examined by light microscopy using a standard panel of histologic stains and enzyme reactions. The following histologic features were observed and quantitated morphometrically using NIH Image™ software: (1) variability in myofiber diameter, (2) fiber type proportions, (3) ratio of capillaries to muscle fibers, and (4) percentage of muscle area composed of endomysial connective tissue.

**Discussion:** This study provided baseline data that will help characterize normal aged canine muscle. Future studies will compare normal aged canine muscle to normal young adult (< 5 years) canine muscle, and to the muscle of aged dogs with suspected sIBM. Together these studies will provide useful baseline data for interpretation of muscle changes in future studies of canine sIBM and other myopathies associated with aging. Ultimately, the goal is to establish the dog as a reliable homologue for sIBM, and facilitate research into the pathogenesis and potential treatments of sIBM.

## **Background**

Sporadic inclusion body myositis (sIBM) is an inflammatory myopathy in humans characterized by progressive weakness and wasting affecting distal and proximal muscles of the arms and legs. It is the most common inflammatory myopathy and the most debilitating muscle disease in aging humans, with a prevalence of 51.3 per million in people over 50 years of age.<sup>1</sup> While sIBM is usually not fatal, it increases the risk of injury due to falls, and often renders patients unable to perform activities of daily living. Dysphagia occurs in 40 to 85% of patients with sIBM and can lead to death from aspiration pneumonia.<sup>2</sup>

Some authors have implicated autoimmune processes in the pathogenesis of sIBM, while others blame the disease on degenerative processes and accumulation of misfolded proteins.<sup>3,4</sup> However, there are flaws with each hypothesis, and the precise cause of sIBM is still unknown. Lack of understanding of the mechanism of myofiber injury in sIBM is a major reason that a treatment to slow or stop the progression of sIBM is not yet available. Patients with sIBM do not respond to anti-inflammatory or immunosuppressant drugs. Thus, management is symptomatic and focuses on prevention of falls.

Research on the cause(s) and potential treatment of sIBM has been hindered by the lack of an animal model that accurately reproduces the inflammatory and degenerative processes of sIBM. The study of other neuromuscular diseases has been enhanced by the identification of large animal models with spontaneously occurring diseases, such as lipid storage myopathy in the cocker spaniel,<sup>5</sup> myasthenia gravis in the Newfoundland,<sup>6</sup> and dystrophin-deficient muscular dystrophy in the Labrador retriever and golden retrievers.<sup>7</sup> However, in the case of sIBM, the current rodent models are flawed and could lead to clinical trials of drugs that are harmful and lack proper scientific foundation.<sup>8</sup>

In 2009, Shelton et al. confirmed for the first time that a vacuolar myopathy with many

similarities to human sIBM affects aged dogs.<sup>9</sup> Clinical and pathological aspects of human sIBM were identified in two dogs in this study, providing hope that naturally occurring canine sIBM could be further studied to gain insight into the mechanism of myofiber injury in human sIBM. While young adult canine muscle has been studied extensively, little data exists on the characteristics of normal aged canine muscle. Such data is necessary for the future use of canine homologues in the study of sIBM, allowing the researcher to determine which pathological changes are due to normal muscle aging and which are due to the disease. The goal of this study is to gather baseline data on the histochemical and morphometric characteristics of aged canine muscle.

## **Methods**

A total of 15 muscle biopsy specimens, including the quadriceps, triceps and cranial tibial muscles from each of five dogs, were analyzed. The dogs represented both sexes and were all over ten years of age. The dogs were “normal” in the sense that they did not have clinically detectable neuromuscular disease; that is, any disease of the muscle or peripheral nerve. However, they may have had central nervous system and orthopedic diseases that are common with aging.

Muscle specimens were obtained from the necropsy floor of UC Davis School of Veterinary Medicine. Unfixed muscles were flash frozen in isopentane pre-cooled in liquid nitrogen, stored at -80 °C, and shipped to UCSD School of Medicine on dry ice. Upon receipt of the muscle specimens at UCSD, cryosections (8 µm) were cut, processed using a standard panel of histological and histochemical stains and reactions, then evaluated by light microscopy.

The following stains and histochemical reactions were applied to the 15 muscle specimens: (1) hematoxylin and eosin, (2) Gomori modified trichrome, (3) NADH dehydrogenase, (4) succinic dehydrogenase and cytochrome C oxidase, (5) myofibrillar ATPases at pH 9.8 and 4.3, (6) esterase, (7) acid phosphatase, (8) periodic acid-Schiff, (9) oil red O, and (10) protein A-peroxidase. These

stains comprise the complete muscle profile.<sup>10</sup>

Qualitative Studies: Using these stains and reactions, the following muscle features were subjectively observed and analyzed: (1) fiber size (atrophy, hypertrophy, hypotrophy) and profile (polygonal, round, angular); (2) fiber type proportions and distribution patterns; (3) number and position of nuclei (peripheral, central, random); (4) myonecrosis and regeneration; (5) cellular infiltration; (6) connective and vascular tissue morphology; and (7) intramuscular nerve morphology.<sup>10</sup>

Quantitative Studies: Myofibers were further characterized morphometrically by area and diameter. Light microscopic images in transverse planes were evaluated with NIH Image™ software (NIH Image, Bethesda, MA). Cross-sectional area of all fiber types was determined using at least 100 muscle fibers of each fiber type. Only areas without artifacts and myofibers with distinct borders were measured. To determine myofiber size variation in aged dogs, coefficients of variability of each muscle fiber type was calculated. In addition, the percentage of type 1 versus type 2 fibers was determined.

Cryosections were then stained with anti-Collagen VI antibody (FITC) to determine the proportion of each muscle section composed of endomysial connective tissue. This was done by using NIH Image™ software to measure the cross-sectional area of each muscle fiber in the field of view, then summing all the cross-sectional areas, then subtracting this value from the total area of the field of view, and finally dividing this value by the total area of the field of view. The number of nuclei was also counted for each field of view, and the ratio of nuclei to myofibers was obtained. Finally, cryosections were stained with anti-von Willebrand Factor antibody which labels canine muscle capillaries, and the capillary and myofiber numbers in each high-power field were counted.

## Results

Specimens were examined from three different muscles (cranial tibial, triceps, and quadriceps) from five aged dogs without clinically evident neuromuscular disease. These dogs will henceforth be identified as Dog 1, Dog 2, Dog 3, Dog 4, and Dog 5.

### *Fiber Typing and Morphometry*

Except for the cranial tibial muscle of Dog 2 and the triceps muscle of Dog 5, there was an overall preponderance of type 2 fibers compared to type 1 fibers in all three muscle types, and in all five dogs. The greatest disparity occurred in the quadriceps of Dog 2, in which 92% of the fibers were type 2. (Table 1, Figure 1.)

The average Type 1 fiber diameter for the cranial tibial muscle was 42.5  $\mu\text{m}$  when based on area, and 50.1  $\mu\text{m}$  when based on perimeter. The average Type 1 fiber diameter for the triceps muscle was 58.3  $\mu\text{m}$  when based on area, and 67.2  $\mu\text{m}$  when based on perimeter. The average Type 1 fiber diameter for the quadriceps muscle was 50.7  $\mu\text{m}$  when based on area, and 59.7  $\mu\text{m}$  when based on perimeter. (Table 1, Figure 2.)

The average Type 2 fiber diameter for the cranial tibial muscle was 48.2  $\mu\text{m}$  when based on area, and 57.5  $\mu\text{m}$  when based on perimeter. The average Type 2 fiber diameter for the triceps muscle was 53.4  $\mu\text{m}$  when based on area, and 65.5  $\mu\text{m}$  when based on perimeter. The average Type 2 fiber diameter for the quadriceps muscle was 49.7  $\mu\text{m}$  when based on area, and 60.1  $\mu\text{m}$  when based on perimeter. (Table 1, Figure 2.)

For both type 1 and type 2 fibers, the triceps muscles had the largest average diameter by both area and perimeter. The quadriceps had the second largest average diameter by both measures for both fiber types, and the cranial tibial muscles had the smallest average diameter by both measures for both fiber types. (Table 1.)

### *Ratio of Capillaries to Myofibers*

For 11 out of the 15 muscle specimens, there was a greater number of capillaries than myofibers in any given field of view. The four exceptions were the cranial tibial muscle of Dog 4, the triceps muscle of Dog 1 and Dog 3, and the quadriceps muscle of Dog 4. In these, there were more myofibers than capillaries counted in the field of view. The average ratio of capillaries to myofibers across all cranial tibial specimens was 1.28, the average ratio across all triceps specimens was 1.27, and the average ratio across all quadriceps specimens was 1.23. (Table 2, Figure 5.)

### *Percentage of Muscle Area Composed of Endomysium, and Ratio of Nuclei to Myofibers*

Across all five cranial tibial muscle specimens, an average of 21.3% of the visible field was composed of endomysium. This figure was 19.2% for the triceps muscle, and 18.4% for the quadriceps muscle. For the cranial tibial muscle specimens, the average ratio of nuclei to myofibers was 2.38, with a range from 1.61 to 3.42. For the triceps muscle specimens, the average ratio of nuclei to myofibers was 2.16, with a range from 0.99 to 3.50. For the quadriceps muscle specimens, the average ratio of nuclei to myofibers was 2.03, with a range from 1.10 to 2.73. (Tables 2, Figures 3 and 4.)

Table 1. Fiber type distribution summary

Muscle	Type I			Type II		
	%	Fiber diameter by area ( $\mu\text{m}$ )	Fiber diameter by perimeter ( $\mu\text{m}$ )	%	Fiber diameter by area ( $\mu\text{m}$ )	Fiber diameter by perimeter ( $\mu\text{m}$ )
<b>Cranial Tibial</b>	37.4	42.5	50.1	62.6	48.2	57.5
<b>Triceps</b>	31.4	58.3	67.2	68.6	53.4	65.5
<b>Quadriceps</b>	23.0	50.7	59.7	77.0	49.7	60.1
<b>Overall</b>		10.5 (28.8-67.8)	59.0 (35.0-78.6)		50.5 (32.0-63.3)	61.0 (38.4-76.4)

Table 2.

Dog	Muscle	Capillaries to Myofibers Ratio (Capillaries/ Myofibers)	Nuclei to Myofibers Ratio (Nuclei/ Myofibers)	Field of View Composed of Endomysium (%)	Type I			Type II		
					%	Diameter by area ( $\mu\text{m}$ ) / CV	Diameter by perimeter ( $\mu\text{m}$ ) / CV	%	Diameter by area ( $\mu\text{m}$ ) / CV	Diameter by perimeter ( $\mu\text{m}$ ) / CV
Dog 1	Cranial Tibial	1.69 (135/80)	3.42 (106/31)	18.1	39	39.6 $\pm$ 10.6 / 0.27	46.0 $\pm$ 12.2 / 0.27	61	63.3 $\pm$ 12.3 / 0.19	76.4 $\pm$ 15.6 / 0.20
	Triceps	0.92 (122/132)	2.23 (58/26)	15.4	32	67.8 $\pm$ 14.7 / 0.22	78.6 $\pm$ 17.0 / 0.22	68	61.1 $\pm$ 14.7 / 0.24	72.4 $\pm$ 17.4 / 0.24
	Quadriceps	1.42 (129/91)	2.57 (59/23)	12.8	24	55.1 $\pm$ 12.9 / 0.23	62.3 $\pm$ 13.9 / 0.22	76	54.5 $\pm$ 12.1 / 0.22	64.3 $\pm$ 14.5 / 0.23
Dog 2	Cranial Tibial	1.44 (323/225)	1.61 (145/90)	23.0	70	28.8 $\pm$ 7.5 / 0.26	35.0 $\pm$ 8.9 / 0.25	30	32.0 $\pm$ 10.4 / 0.32	38.4 $\pm$ 12.5 / 0.33
	Triceps	1.81 (205/113)	2.08 (328/158)	19.0	11	60.0 $\pm$ 10.0 / 0.17	68.8 $\pm$ 10.3 / 0.15	89	59.1 $\pm$ 9.8 / 0.17	73.6 $\pm$ 12.7 / 0.17
	Quadriceps	1.43 (197/138)	2.32 (308/133)	18.6	8	54.7 $\pm$ 9.8 / 0.18	69.0 $\pm$ 14.3 / 0.21	92	51.5 $\pm$ 10.3 / 0.20	65.9 $\pm$ 13.1 / 0.20
Dog 3	Cranial Tibial	1.03 (121/117)	2.85 (259/91)	21.6	18	53.1 $\pm$ 12.7 / 0.24	64.1 $\pm$ 14.4 / 0.22	82	49.3 $\pm$ 8.9 / 0.18	58.6 $\pm$ 11.3 / 0.19
	Triceps	1.03 (70/68)	3.50 (315/90)	21.2	10	65.1 $\pm$ 9.9 / 0.15	73.6 $\pm$ 11.0 / 0.15	90	54.3 $\pm$ 12.2 / 0.22	66.3 $\pm$ 14.9 / 0.22
	Quadriceps	1.55 (143/92)	2.73 (257/94)	18.9	26	58.3 $\pm$ 9.9 / 0.17	70.2 $\pm$ 12.2 / 0.17	74	48.9 $\pm$ 10.4 / 0.21	60.5 $\pm$ 13.0 / 0.22
Dog 4	Cranial Tibial	0.82 (139/170)	1.71 (299/175)	21.6	21	51.3 $\pm$ 7.2 / 0.14	59.3 $\pm$ 10.7 / 0.18	79	57.2 $\pm$ 7.9 / 0.14	68.3 $\pm$ 11.1 / 0.16
	Triceps	0.85 (172/203)	0.99 (97/98)	23.4	18	39.1 $\pm$ 4.4 / 0.11	45.3 $\pm$ 5.6 / 0.12	82	42.0 $\pm$ 6.5 / 0.15	50.1 $\pm$ 8.4 / 0.17
	Quadriceps	0.59 (138/235)	1.43 (70/49)	23.9	26	36.8 $\pm$ 6.5 / 0.18	43.4 $\pm$ 8.8 / 0.20	74	46.3 $\pm$ 8.8 / 0.19	54.5 $\pm$ 12.3 / 0.23
Dog 5	Cranial Tibial	1.42 (241/170)	2.33 (284/122)	22.1	39	39.6 $\pm$ 10.6 / 0.27	46.0 $\pm$ 12.2 / 0.27	61	39.2 $\pm$ 13.5 / 0.34	45.6 $\pm$ 15.1 / 0.33
	Triceps	1.74 (212/122)	2.01 (219/109)	17.1	86	59.4 $\pm$ 10.9 / 0.18	69.5 $\pm$ 12.2 / 0.17	14	50.7 $\pm$ 7.3 / 0.14	65.1 $\pm$ 12.3 / 0.19
	Quadriceps	1.15 (161/140)	1.10 (137/125)	17.9	31	48.5 $\pm$ 7.8 / 0.16	53.8 $\pm$ 7.9 / 0.15	69	46.4 $\pm$ 9.8 / 0.21	55.1 $\pm$ 11.3 / 0.21

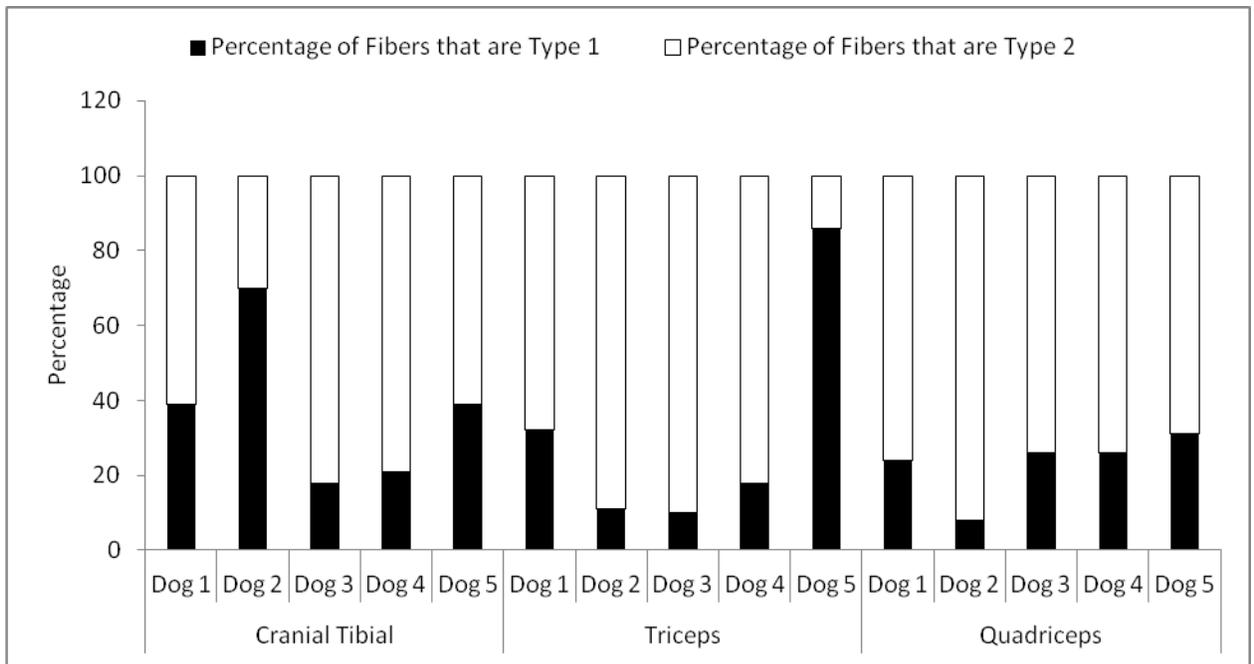


Figure 1. Proportions of Type 1 and Type 2 fibers in each specimen.

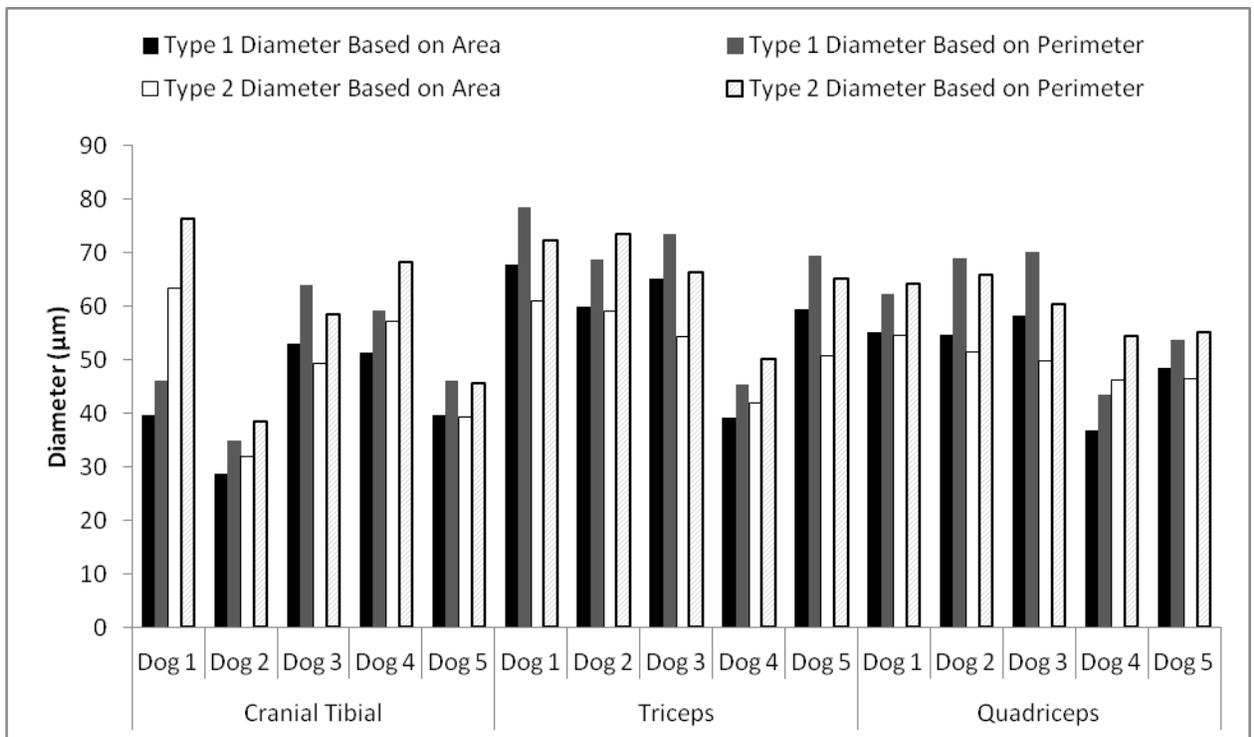


Figure 2. Fiber diameters by fiber type, based on area and perimeter, in each specimen.

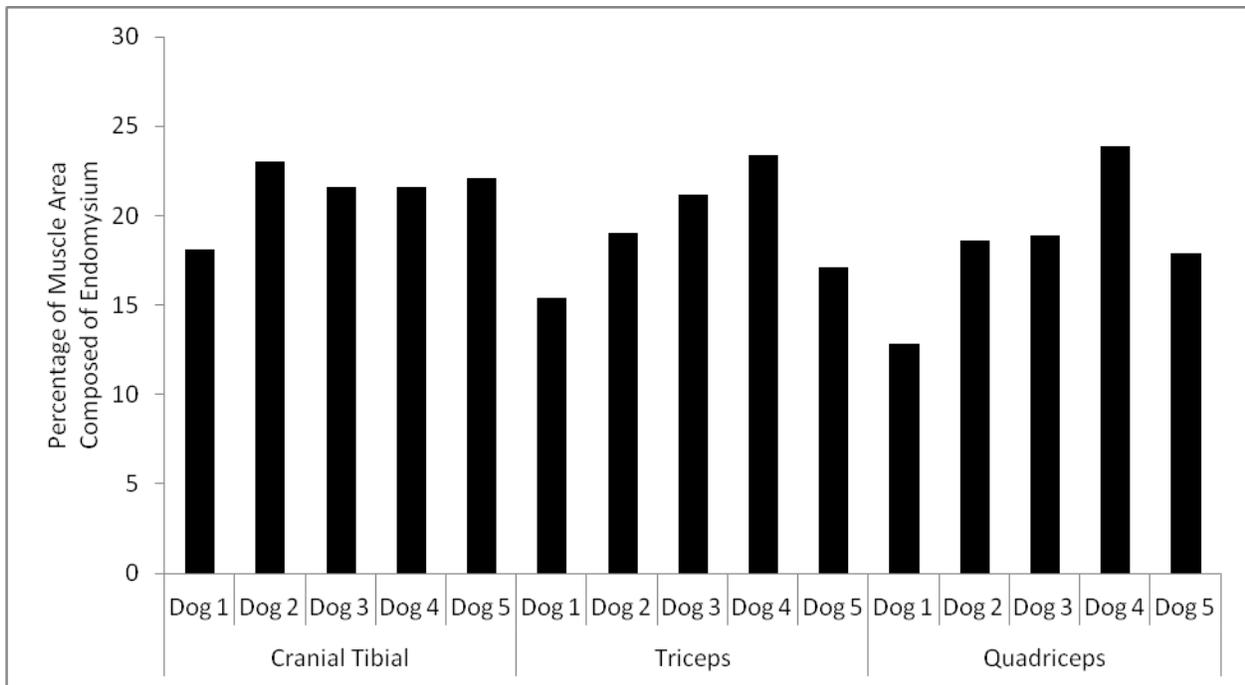


Figure 3. Percentage of muscle area composed of endomysium in each specimen.

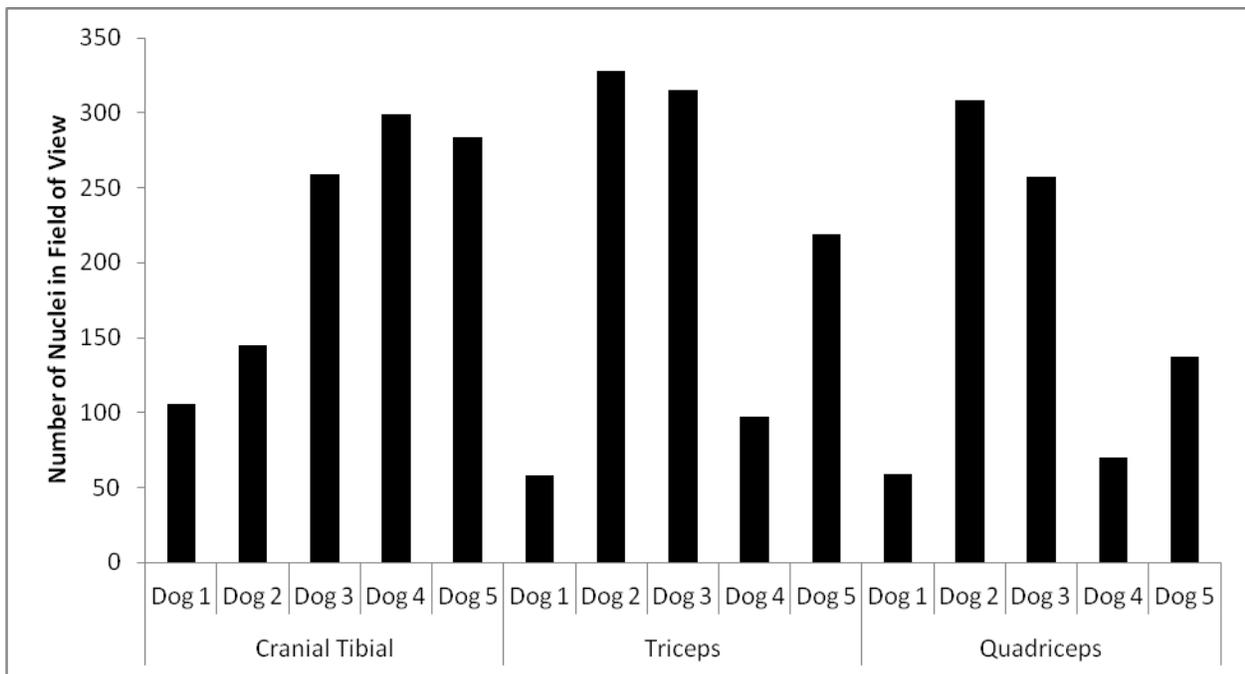


Figure 4. Number of nuclei in field of view in each specimen.

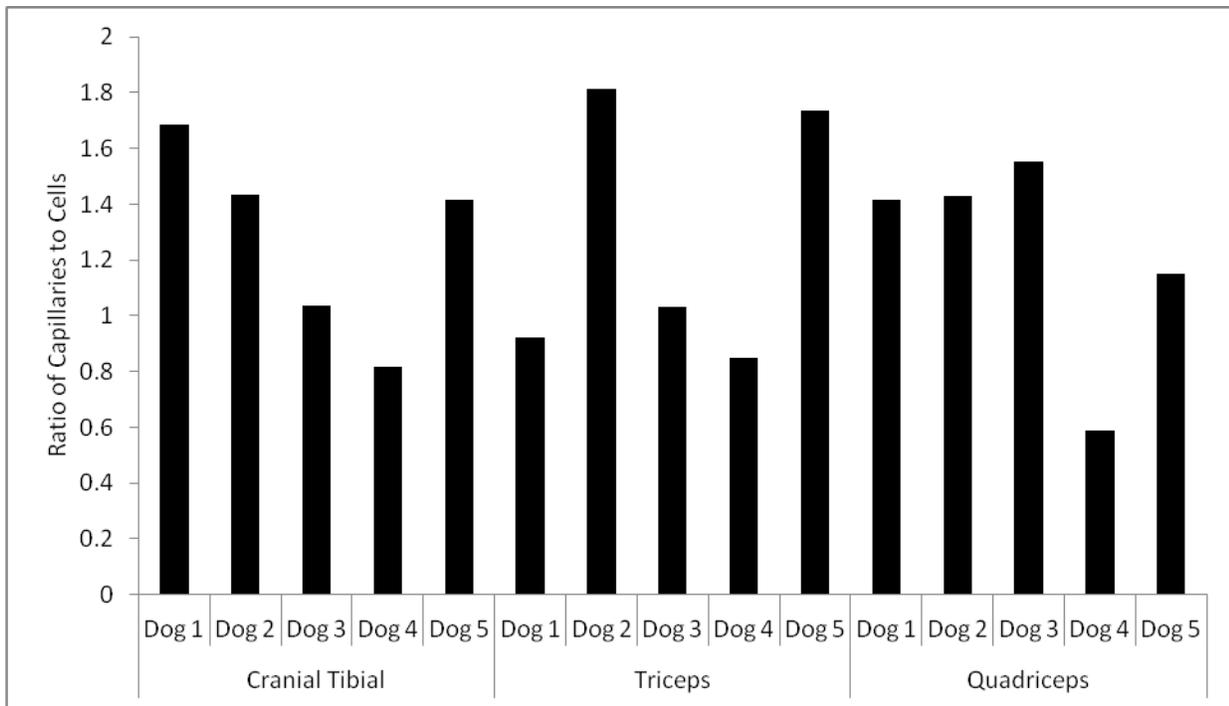
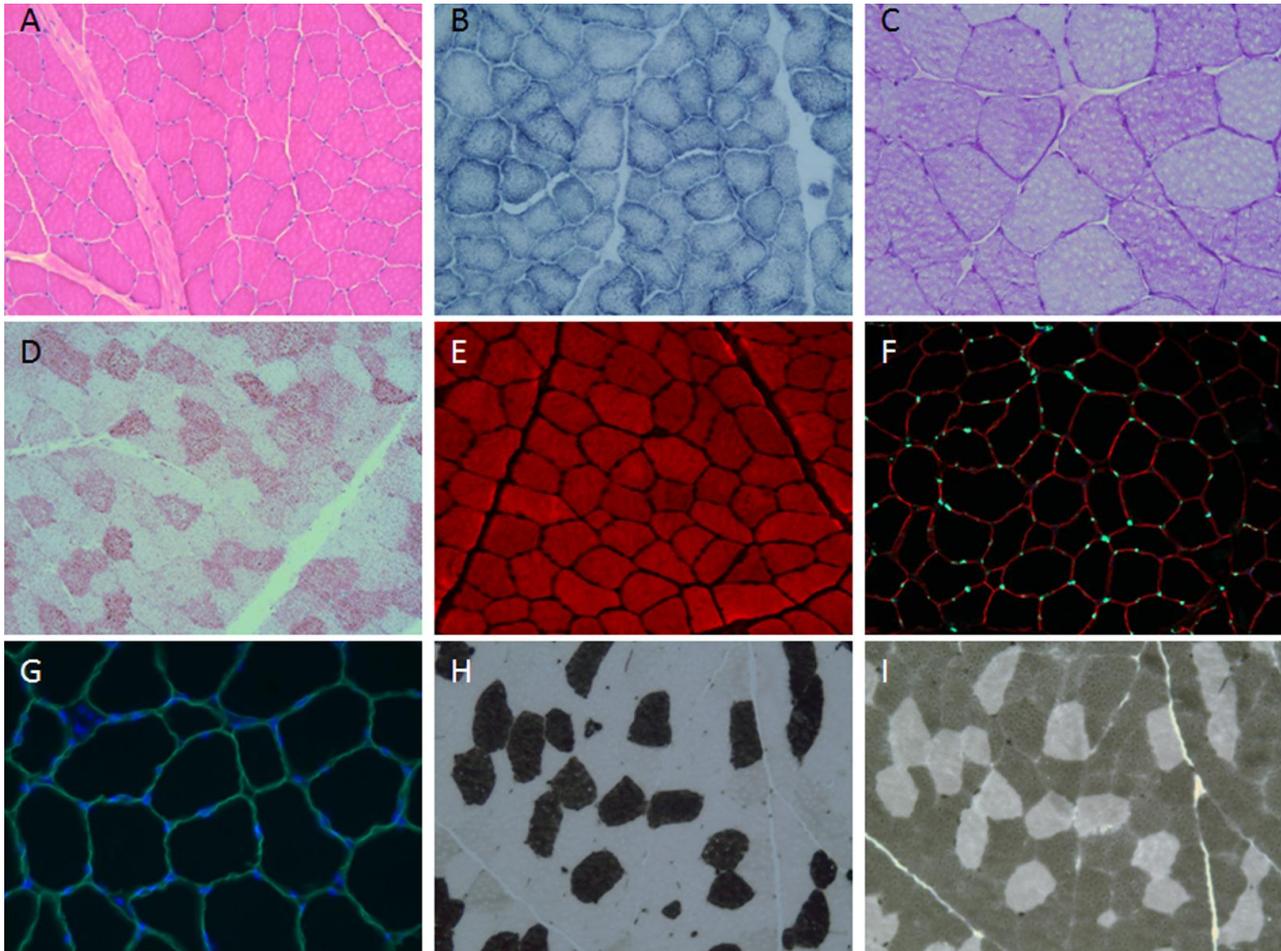


Figure 5. Ratio of capillaries to cells in field of view in each specimen.

## Representative Images



- A. Dog 3 Quadriceps stained with hematoxylin & eosin, magnified 20x.
- B. Dog 3 Triceps stained with NADH, magnified 20x.
- C. Dog 1 Cranial tibial muscle stained with Periodic Acid-Schiff, magnified 40x.
- D. Dog 2 Triceps stained with oil Red O for detection of neutral lipid droplets, magnified 20x.
- E. Dog 1 Cranial tibial muscle stained with Congo Red. There is an absence of congo red positive inclusions or deposits in normal aging canine muscle.
- F. Dog 1 Cranial tibial muscle stained with anti-von Willebrand Factor antibody and with Congo Red. The bright green spots correspond to capillaries.
- G. Dog 1 Cranial tibial muscle stained with anti-Collagen VI antibody (FITC). Areas of green correspond to endomysium, and bright blue spots correspond to nuclei.
- H. Dog 1 Triceps stained at pH 4.3. The dark fibers are Type 1 and the light fibers are Type 2.
- I. Dog 1 Triceps stained at pH 9.8. The light fibers are Type 1 and the dark fibers are Type 2.

## **Discussion**

This project marks the first time that the histochemical and morphometric characteristics of normal aged canine muscle were studied. This study represents the first step in creating a standard control to which future cases of suspected sporadic inclusion body myositis and other problems associated with aging can be compared. Once the characteristics of normal aged canine muscle are known, it will be possible to determine whether the changes seen in a case of suspected sIBM are attributable to the actual disease process, or just to the normal aging process. This will help investigators correctly identify sIBM in dogs and establish the dog as a model for sIBM, which is an important step in elucidating the pathogenesis of this disease and ultimately finding effective treatments.

This study also produced a substantial quantity of images of normal aged canine muscle that were sectioned and visualized using a panel of stains and reactions. Future investigators can compare their muscle images to the images obtained in this study, to see whether their muscle features are consistent with normal aging or with pathology. This study found that normal aged canine myofibers retain a polygonal shape without obvious atrophy of either muscle fiber type. Nuclei remain around the periphery of the muscle fibers. No glycogen deposits, cellular infiltrates, necrosis, or other morphological changes are present. Future studies could quantitate the changes in lipid and nerve features in the myofibers.

Limitations in this study include the small sample size (only three muscles from five dogs for a total of 15 specimens) and the lack of breed diversity, which calls into question the generalizability of these data. Also, these dogs were considered “normal” in that they did not have neuromuscular disease, but there is no guarantee that these dogs did not have some other disease that could result in muscle changes. Finally, despite meticulous efforts to be consistent, the quality of the specimens did vary somewhat, which may have led to inaccurate data. This is a general

problem using clinical material as the time from euthanasia to collection of muscle specimens varied.

Future studies include a comparison of these data from normal aged canine muscle to normal young canine muscle. If no significant difference is found between young and aged canine muscle, then future cases of suspected sIBM can be compared to any normal dog muscle, not necessarily the ones from this study. However, if a difference is found between young and aged canine muscle, then the utility of this study will be established, as future cases of suspected sIBM can be compared specifically to the data from this study.

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