

# Histopathologic and immunohistochemical features of soft palate muscles and nerves in dogs with an elongated soft palate

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## OBJECTIVE

To histologically evaluate and compare features of myofibers within the elongated soft palate (ESP) of brachycephalic and mesocephalic dogs with those in the soft palate of healthy dogs and to assess whether denervation or muscular dystrophy is associated with soft palate elongation.

## SAMPLE

Soft palate specimens from 24 dogs with ESPs (obtained during surgical intervention) and from 14 healthy Beagles (control group).

## PROCEDURES

All the soft palate specimens underwent histologic examination to assess myofiber atrophy, hypertrophy, hyalinization, and regeneration. The degrees of atrophy and hypertrophy were quantified on the basis of the coefficient of variation and the number of myofibers with hyalinization and regeneration. The specimens also underwent immunohistochemical analysis with anti-neurofilament or anti-dystrophin antibody to confirm the distribution of peripheral nerve branches innervating the palatine myofibers and myofiber dystrophin expression, respectively.

## RESULTS

Myofiber atrophy, hypertrophy, hyalinization, and regeneration were identified in almost all the ESP specimens. Degrees of atrophy and hypertrophy were significantly greater in the ESP specimens, compared with the control specimens. There were fewer palatine peripheral nerve branches in the ESP specimens than in the control specimens. Almost all the myofibers in the ESP and control specimens were dystrophin positive.

## CONCLUSIONS AND CLINICAL RELEVANCE

These results suggested that palatine myopathy in dogs may be caused, at least in part, by denervation of the palatine muscles and not by Duchenne- or Becker-type muscular dystrophy. These soft palate changes may contribute to upper airway collapse and the progression of brachycephalic airway obstructive syndrome. (*Am J Vet Res* 2016;77:77–83)

An elongated soft palate is one of several morphological abnormalities of the upper airway that occur in dogs with BAOS. In addition to an elongated soft palate, other morphological abnormalities associated with BAOS include stenotic nares, everted laryngeal saccules, and tracheal hypoplasia. However, an elongated soft palate is the most common problem in dogs with BAOS.<sup>1–4</sup>

The negative pressure created during inspiration is increased in dogs with BAOS.<sup>4</sup> The increased negative pressure within the airway can cause inflammation and stretching of the pharyngeal tissue, which can lead to airway obstruction.<sup>4</sup> In healthy dogs, in-

creased negative pressure in the upper airway during inspiration activates soft palate muscles to prevent the airway from collapsing.<sup>5,6</sup> Because the activation of upper airway dilator muscles is important to prevent upper airway collapse, impairment of the function of the upper airway dilator muscle may lead to the progression of BAOS. Pichetto et al<sup>7</sup> observed atrophy, hypertrophy, Zenker necrosis, and regeneration of myofibers in resected soft palate muscle specimens from 11 dogs with mild BAOS and suggested that the pathological changes might be related to functional impairment during respiration. However, the pathologic effect of an elongated soft palate on the palatine musculature has not been thoroughly investigated.

The purpose of the study reported here was to assess the degree of atrophy, hypertrophy, Zenker necrosis, and regenerative changes observed in the palatine musculature (palatinus, levator veli, and palatopharyngeal muscles) of brachycephalic and mesocephalic dogs with an elongated soft palate for comparison

## ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BAOS	Brachycephalic airway obstruction syndrome
CV	Coefficient of variation
IQR	Interquartile range (25th to 75th percentile)
OSAS	Obstructive sleep apnea syndrome

with findings in healthy dogs. Because preliminary results indicated that, in dogs with an elongated soft palate, there was atrophy of myofibers following denervation or necrosis, regeneration, ring fiber formation, and sarcoplasmic mass characteristic of Duchenne- or Becker-type muscular dystrophy,<sup>8</sup> we also sought to examine whether denervation or dystrophin deficiency was associated with the histopathologic findings of palatine muscle lesions in those affected dogs by immunohistochemical analysis of palatine specimens with anti-neurofilament and anti-dystrophin antibodies.

## Materials and Methods

### Samples

One tissue specimen was surgically resected from each of the palates of 24 dogs with soft palate elongation. The specimens were collected during surgical intervention to relieve airway obstruction. Clinical records were reviewed, and breed, sex, age, clinical signs, blood test results, and drug history were recorded. One palatine muscle sample was also obtained after euthanasia by IV injection of pentobarbital and potassium chloride from each of 7 male and 7 female anesthetized Beagles (age range, 1 to 4 years) that had been used in undergraduate surgical practice. This surgical practice was approved by the Animal Care and Use Committee of Nippon Veterinary and Life Science University (approval code 11-80, 13-58).

### Histologic evaluation

All the specimens were fixed in neutral-buffered 10% formalin for 24 hours. The specimens were embedded in paraffin blocks, and 5- $\mu$ m sections were prepared. The sections were deparaffinized and stained with H&E or Masson trichrome stain.

### Immunofluorescence and immunohistochemical staining

Immunofluorescence staining was performed with an anti-dystrophin antibody.<sup>a</sup> After deparaffinization, serial sections from each specimen were placed in modified citrate buffer<sup>b</sup> and incubated in a solution containing 4% blocking reagent<sup>c</sup> at room temperature (approx 24°C) for 30 minutes. Sections were incubated overnight (approx 18 hours) at 4°C in a solution containing the anti-dystrophin antibody (1:50), followed by incubation for 30 minutes in a solution containing a fluorescent dye-labeled secondary antibody<sup>d</sup> (1:500). The sections were observed with a fluorescence microscope.<sup>e</sup> Additional sections of each specimen were examined to identify the distribution of peripheral nerve axons on the basis of binding of an anti-neurofilament antibody.<sup>f</sup> The sections were treated with H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase activity. The sections were placed in citrate buffer solution (pH, 6) and heated at 121°C for 10 minutes. After blocking the sections in a solution containing 4% blocking

reagent,<sup>c</sup> the sections were incubated at 4°C overnight in a solution containing the anti-neurofilament antibody (1:200). After the sections were washed with PBS solution, they were incubated in a solution containing a biotinylated secondary antibody<sup>g</sup> (1:500) for 30 minutes, followed by incubation for 30 minutes in a solution containing a horseradish peroxidase-streptavidin conjugate.<sup>h</sup> Diaminobenzidine was used as the horseradish peroxidase substrate, and hematoxylin was used as a nuclear counterstain. Specimens of the cerebrum and quadriceps femoris muscle harvested from one of the healthy study dogs were used as positive controls for neurofilament and dystrophin detection, respectively. For the negative control, sections were incubated with PBS solution instead of the primary antibodies.

### Evaluation of histopathologic abnormalities

One section each from 24 elongated soft palates and 14 control palates (1 section/palate/dog) was examined. The numbers of abnormal myofibers and peripheral nerve branches were evaluated in 10 randomly selected hpfs at 400X magnification. The number of myofibers with evidence of hyaline degeneration and regenerative changes was assessed, as was the number of neurofilament-positive axons, and the mean or median for each was calculated. We used software<sup>i</sup> to measure the diameter of 50 myofibers from 10 randomly selected hpfs,<sup>i</sup> and the mean and SD were calculated. The frequency distributions of the mean myofiber diameters were measured, and the curves of the frequency distributions for the dogs with and without an elongated soft palate were graphed. This method allowed assessment of the minimum myofiber diameter, defined as the maximum diameter across the lesser aspect of a cross-sectioned myofiber. Measurements of obliquely cut myofibers will provide erroneously large diameter values.<sup>9</sup> Myofibers with hyaline degeneration and necrosis were eliminated from measurement. The frequencies of atrophic and hypertrophic myofibers were quantified on the basis of the CV, which was calculated as previously described<sup>9</sup>:

$$CV = SD \times (1,000/\text{mean fiber diameter})$$

### Statistical analysis

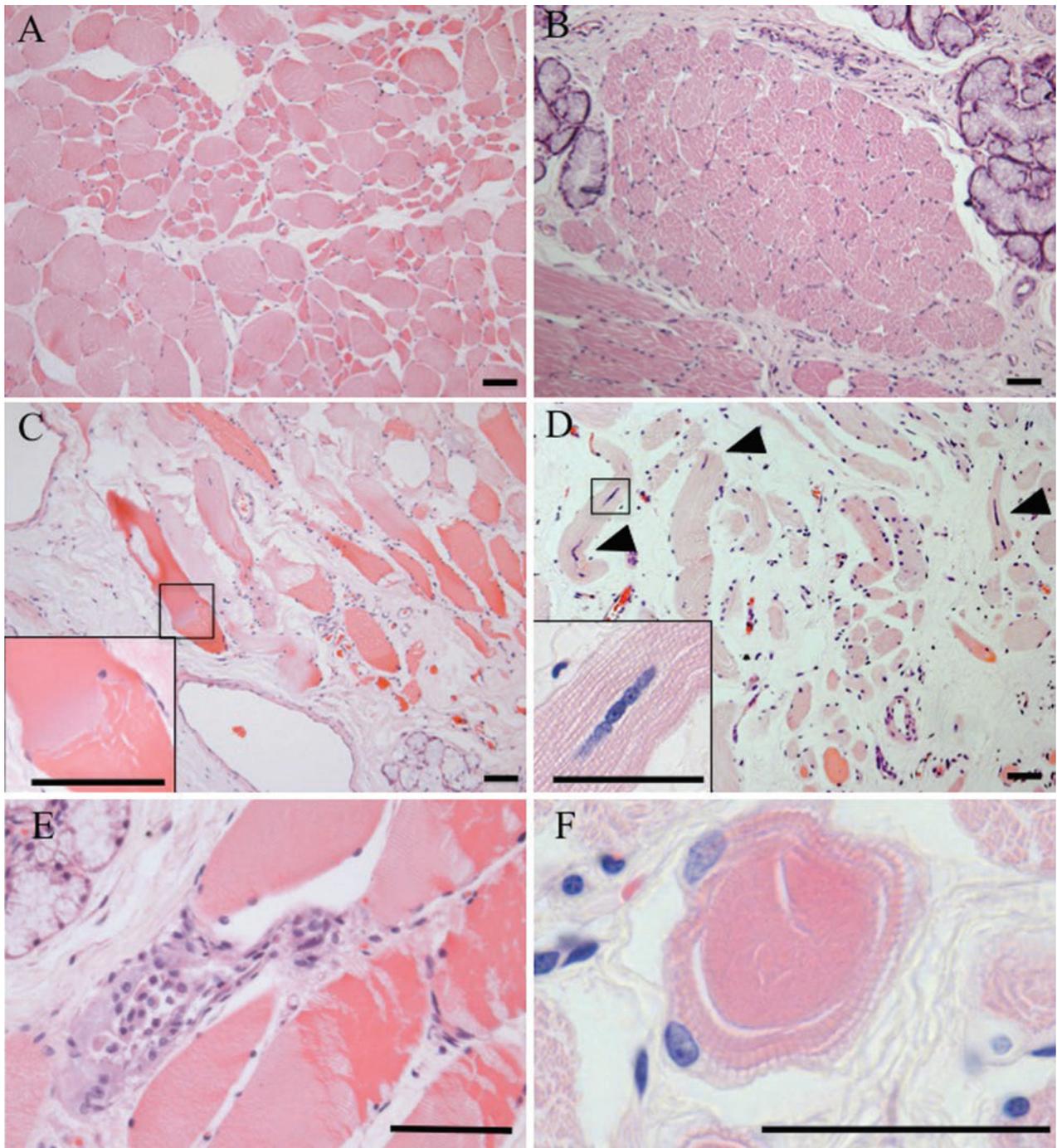
The CV data are reported as the mean  $\pm$  SD, and the intergroup differences in CV were compared by means of Student *t* tests. The numbers of regenerated myofibers/10 hpfs, hyaline-degenerated myofibers/10 hpfs, and peripheral nerve branches/10 hpfs are reported as the median (IQR), and the intergroup differences were compared by means of the Mann-Whitney *U* test. Results of comparisons with values of *P* < 0.05 were considered to represent significant differences. All statistical analyses were performed with spreadsheet<sup>j</sup> and statistical<sup>k</sup> software programs.

## Results

### Dogs

Seventeen of the 24 (70.8%) dogs with an elongat-

ed soft palate were male, and 7 (29.2%) were female. The brachycephalic breeds included French Bulldog (n = 6), Pug (5), Pomeranian (3), Shih Tzu (2), Cavalier King Charles Spaniel (2), Pekinese (1), and Bulldog (1).



**Figure 1**—Representative photomicrographs of sections of palatine muscle specimens from dogs with an elongated soft palate and healthy dogs (controls) illustrating histologic alterations of myofibers in soft palate muscle in affected dogs. **A**—In a dog with an elongated soft palate, both atrophic and hypertrophic fibers are present within fasciculi. H&E stain; bar = 50  $\mu$ m. **B**—In a soft palate muscle specimen from a control dog, myofibers are almost uniform. H&E stain; bar = 50  $\mu$ m. **C**—In a dog with an elongated soft palate, hyaline degeneration of soft palate muscle tissue is observed. H&E stain; bar = 50  $\mu$ m. Bottom left inset—Higher magnification of the outlined square. Notice the homogeneous acidophilic cytoplasm and loss of cross-striations of the myofibers. H&E stain; bar = 50  $\mu$ m. **D**—In an elongated soft palate specimen, there is central rowing of nuclei (arrow heads) indicative of myofiber regeneration. H&E stain; bar = 50  $\mu$ m. Bottom left inset—Higher magnification of the outlined square. Notice the central chained nuclei of myofibers. H&E stain; bar = 50  $\mu$ m. **E**—In another elongated soft palate specimen, phagocytosis of degenerated fibers by macrophages is evident. H&E stain; bar = 50  $\mu$ m. **F**—Ring fiber formation is detectable in a section of elongated soft palate tissue. H&E stain; bar = 50  $\mu$ m.

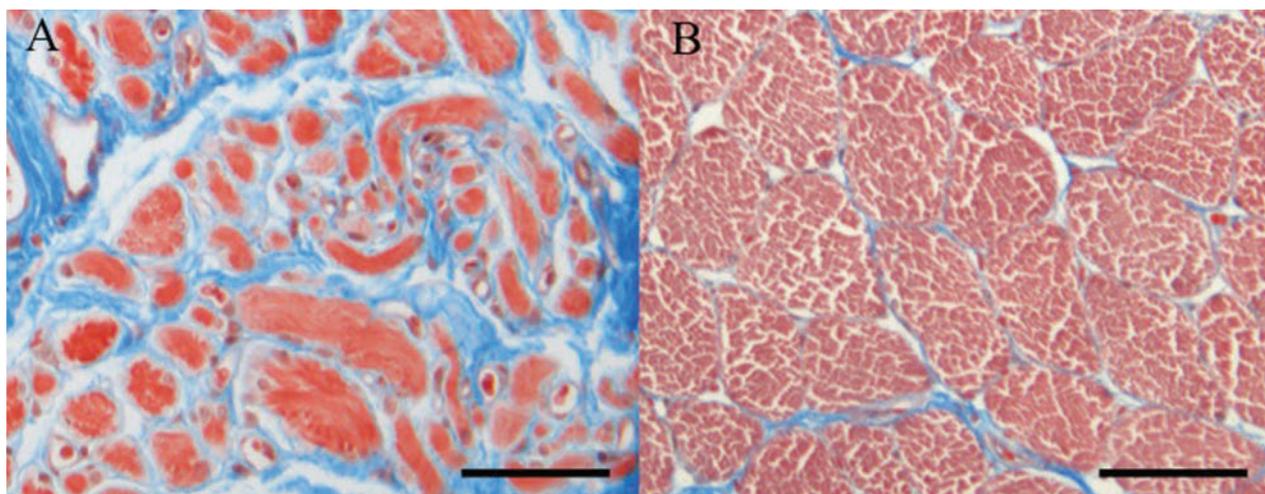
Mesocephalic breeds with an elongated soft palate included Beagle (n = 2), Labrador Retriever (1), and Yorkshire Terrier (1). Ages of the dogs with an elongated soft palate ranged from 1.1 to 13 years, with a mean age of 6.9 years. Seven dogs had a history of corticosteroid treatment for an elongated soft palate or other diseases. Corticosteroid treatment in the dogs with an elongated soft palate could not be determined from the clinical records of 16 dogs. For the dogs with an elongated soft palate, serum biochemical analysis revealed slightly to moderately high AST activity in 4 dogs and high ALT activity in 1 dog. However, data regarding circulating AST and ALT activities were not available for 14 and 11 dogs with an elongated soft palate group, respectively.

### Histologic evaluation and immunohistochemical analysis

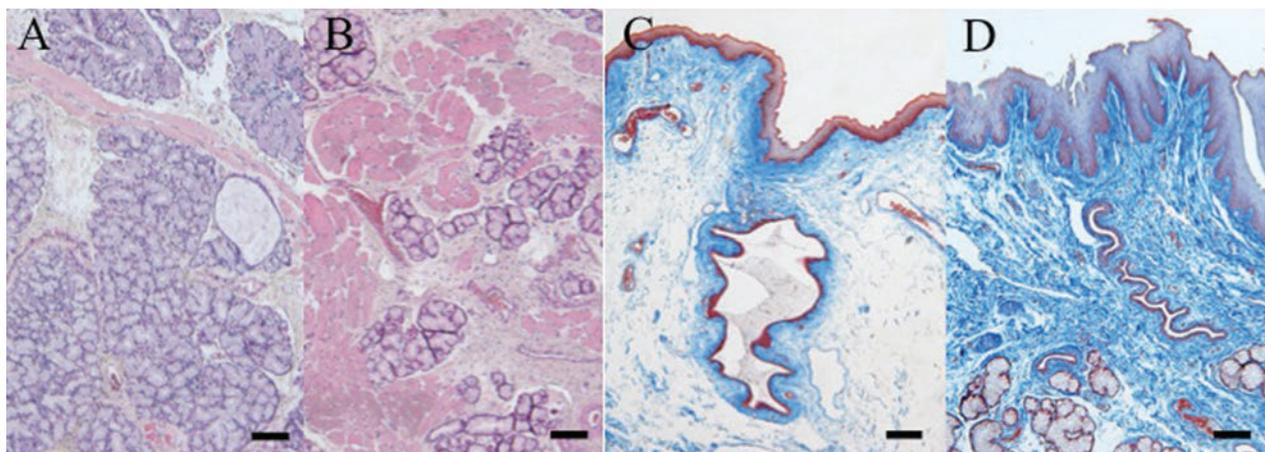
Both atrophic and hypertrophic myofibers were identified within individual fasciculi of the palatine

muscles of dogs with an elongated soft palate, whereas such myofibers were rare in the palatine muscles of the control dogs (**Figure 1**). Atrophic myofibers were visible in small groups within the fasciculi. Hyaline degeneration and regenerative changes, including central nuclear rowing and multinucleated cells, were also observed in the palatine muscle specimens from dogs with an elongated soft palate, whereas these types of abnormalities were rare in specimens from the control dogs. Phagocytosis of degenerated fibers by macrophages and ring fiber formation were observed in the palatine muscle specimens from dogs with an elongated soft palate but were absent in those from the control dogs.

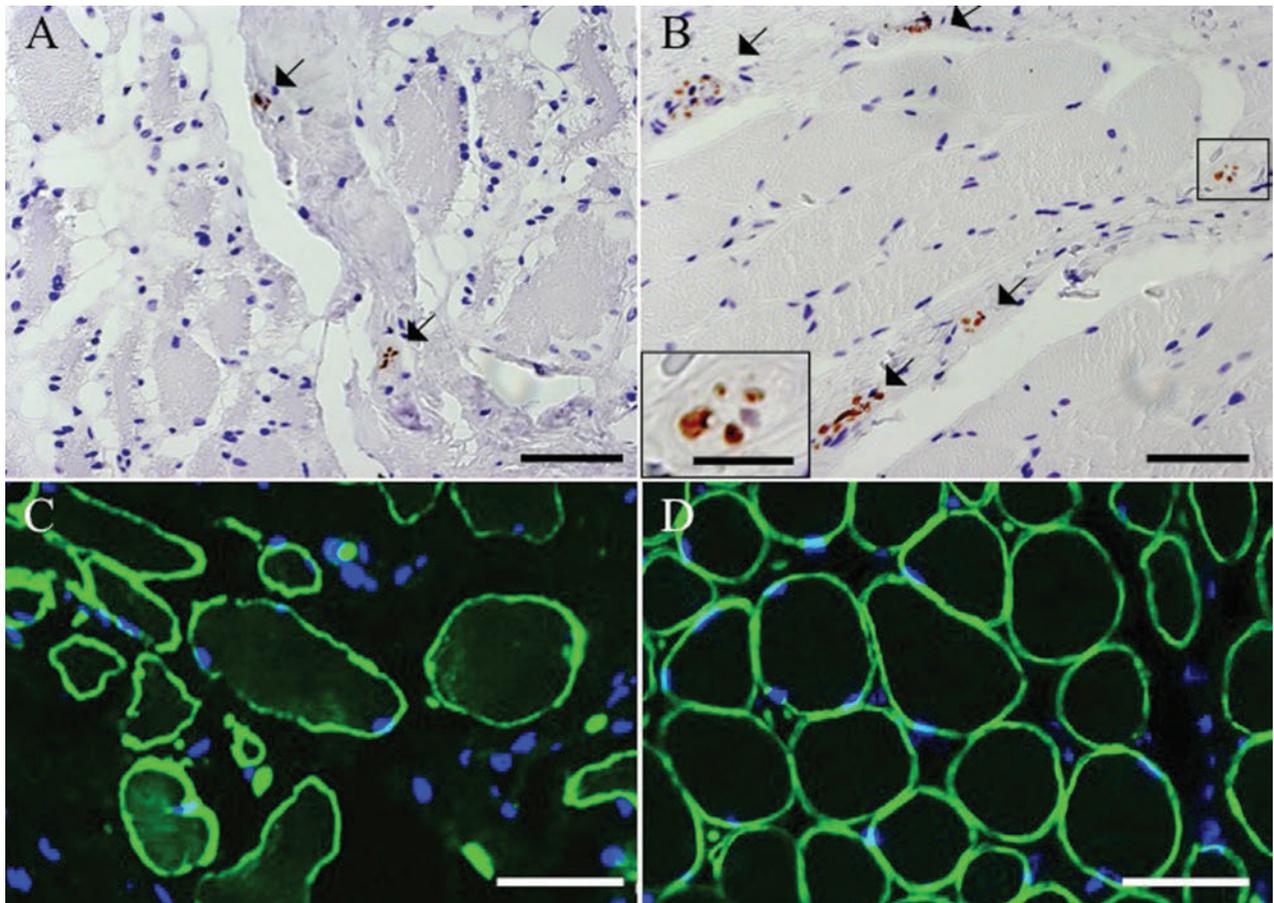
Mild fibrosis sometimes occurred around the affected fasciculi (**Figure 2**). Lobular hyperplasia of the mucous glands was observed in the specimens from dogs with an elongated soft palate, which was accompanied by edema at the lamina propria mu-



**Figure 2**—Representative photomicrographs of sections of palatine muscle specimens from a dog with an elongated soft palate (A) and a healthy control dog (B). Mild fibrosis is present around some atrophic myofibers in the elongated soft palate specimen, but not in the control specimen. Masson trichrome stain; bar = 50  $\mu$ m.



**Figure 3**—Representative photomicrographs of sections of palatine muscle specimens from a dog with an elongated soft palate (A and C) and a healthy control dog (B and D). Notice the severe lobular hyperplasia of the mucous glands in the elongated soft palate (A), compared with findings in the control specimen (B). H&E stain; bar = 100  $\mu$ m. Severe edema is present at the lamina propria mucosa, and a dilated duct of a mucous gland is visible in the elongated soft palate specimen (C); the control specimen is unaffected (D). Masson trichrome stain; bar = 100  $\mu$ m.



**Figure 4**—Representative photomicrographs of sections of palatine muscle specimens from 2 dogs with an elongated soft palate (A and C) and 2 healthy control dogs (B and D) following immunostaining for axonal neurofilament (A and B) and dystrophin (C and D). The elongated soft palate muscle specimen and control specimen are neurofilament positive. However, in the control specimen, the peripheral nerve branches (arrows) are evenly distributed among the fasciculi of myofibers (B); decreased peripheral nerve branches (arrows) are visible in the elongated soft palate specimen (A). Anti-neurofilament antibody staining; bar = 50  $\mu$ m. Inset = Axon staining reveals neurofilament positivity in a nerve branch. Anti-neurofilament antibody staining; bar = 25  $\mu$ m. Cell membranes of myofibers from both the elongated soft palate (C) and control (D) specimen were consistently dystrophin positive (green), although those of the former are irregular. Anti-dystrophin antibody staining; bar = 50  $\mu$ m.

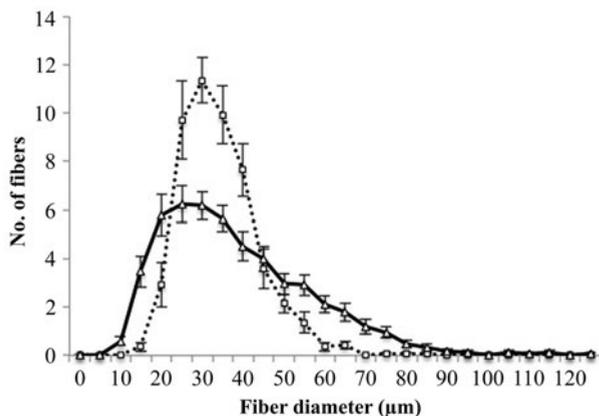
cosa and by ducts that were severely dilated with accumulated mucus (**Figure 3**).

The localization of nerve fibers was accentuated by immunostaining for axonal neurofilament. As a result, counting the number of nerve fibers was not difficult. The immunohistochemical analysis revealed a uniform distribution of peripheral nerve fibers among the myofibers and along the length of the muscle fasciculi in the control dogs (**Figure 4**). The number of peripheral nerve fibers in the palatine muscle specimens from dogs with an elongated soft palate was lower than that in the specimens from the control dogs. Despite the muscular abnormalities observed in the specimens from dogs with an elongated soft palate, dystrophin staining of the myofiber cell membranes was observed in the specimens from the dogs with an elongated soft palate and the control dogs, with the exception of only a few degenerated myofibers with their disrupted cell membrane seen in almost all elongated soft palate specimens.

### Histologic assessment

The CV for palatine myofibers from dogs with an elongated soft palate was significantly ( $P < 0.001$ ) higher (mean  $\pm$  SD, 452  $\pm$  95.6; median, 465 [IQR, 349 to 581]) than that of the palatine myofibers from the control dogs (mean  $\pm$  SD, 275  $\pm$  74.4; median, 277 [IQR, 169 to 386]). The number of palatine myofibers with hyaline degeneration (per 10 hpfs) in the dogs with an elongated soft palate (mean  $\pm$  SD, 4.91  $\pm$  5.78; median, 3.00 [IQR, 0.00 to 11.0]) was significantly ( $P = 0.001$ ) higher than that in the control dogs (mean  $\pm$  SD, 0.500  $\pm$  1.00; median, 0.00 [IQR, 0.00 to 0.00]). The number of myofibers with regenerative features in the dogs with an elongated soft palate was higher than that in the control dogs, but the intergroup difference was not significant.

No significant difference in myofiber diameter was observed between the dogs with an elongated soft palate (mean  $\pm$  SD, 36.1  $\pm$  18.7  $\mu$ m; median, 31.9  $\mu$ m [IQR, 7.1 to 56.7  $\mu$ m]) and the control dogs (mean  $\pm$  SD, 31.6  $\pm$  9.54  $\mu$ m; median, 30.3  $\mu$ m [IQR, 18.6 to



**Figure 5**—Frequency distribution curves of myofiber diameters in soft palate specimens from 24 dogs with an elongated soft palate (triangles; solid line) and 14 healthy control dogs (squares; dotted line). The diameters of 50 myofibers in each dog were measured; data represent mean  $\pm$  SE. To indicate the interval estimation of the mean distribution curve of the 2 groups, the error bar is shown as SE. Both atrophic and hypertrophic myofibers were detected more frequently in the elongated soft palate tissues, compared with the control tissues. The diameters of myofibers most commonly observed were 20 to 25  $\mu$ m and 25 to 30  $\mu$ m in the dogs with an elongated soft palate and control dogs, respectively.

42.0  $\mu$ m]). The frequency distribution curves of the mean myofiber diameters (**Figure 5**) revealed that atrophic and hypertrophic fibers were more numerous in the specimens from the dogs with an elongated soft palate than in the specimens from the control dogs. The diameter of myofibers most commonly observed in each specimen ranged from 20 to 25  $\mu$ m and 25 to 30  $\mu$ m in the dogs with and without an elongated soft palate, respectively. The mean number of nerve branches (per 10 hpfs) in the specimens from the dogs with an elongated soft palate was significantly ( $P = 0.012$ ) lower (mean  $\pm$  SD,  $5.90 \pm 5.34$ ; median, 4.00 [IQR, 0.00 to 10.0]) than that in the specimens from the control dogs (mean  $\pm$  SD,  $13.6 \pm 9.84$ ; median, 11.5 [IQR, 0.00 to 28.0]).

## Discussion

The results of the present study indicated that the degree of hyaline degeneration, atrophy, and hypertrophy of myofibers in palatine muscle specimens was more severe in dogs with an elongated soft palate, compared with findings in healthy dogs. A previous study<sup>10</sup> found that the CV calculated as an index of atrophy and hypertrophy of palatine myofibers in healthy humans was 185 to 325. The results of the present study indicated that the mean CV for palatine myofibers (452) in dogs with an elongated soft palate was higher than that in healthy human palatine muscles. It is unlikely that these abnormalities represent artifacts of surgical resection or the fixative procedure because the hypertrophy, atrophy, and regeneration of myofibers were strongly indicative of a long time course.

One likely cause of lesions in palatine muscles is corticosteroid use, which can induce myopathy simi-

lar to that observed with atrophy of type 2 fibers and can result in proximal muscle weakness.<sup>11</sup> In the present study, 7 of 8 dogs with an elongated soft palate had documented histories of corticosteroid treatments. Although it is unclear whether the palatine muscle lesions in these dogs resulted from corticosteroid use, this seems unlikely because none of the dogs with an elongated soft palate developed proximal muscle weakness (a common sign of glucocorticoid excess) and the serum activities of AST and ALT were high in only a few of the affected dogs. Further study will be needed to clarify the association between the palatine muscle lesions and corticosteroid use, given that serum biochemical data were not obtained from all dogs with an elongated soft palate.

The immunohistochemical analysis of tissue sections revealed that the palatine myofibers of dogs with an elongated soft palate were not dystrophin deficient, which is a characteristic feature of Duchenne- or Becker-type muscular dystrophy. In addition, there were significantly fewer peripheral nerve branches innervating the palatine muscles of the dogs with an elongated soft palate, compared with findings in the control dogs. The reduced number of peripheral nerve branches indicated that denervation of the palatine muscle had occurred in the dogs with an elongated soft palate, which likely contributed to neurogenic myopathy. In humans, the muscles of the soft palate, including the uvulae, levator veli, and palatopharyngeus muscles, are innervated by the lesser palatine nerve and pharyngeal plexus, which originate in the trigeminal and glossopharyngeal nerves, respectively.<sup>12</sup> The activation of these nerves is required to prevent upper airway collapse. De Bellis et al<sup>13</sup> found that uvular neurostimulation was reduced in humans with OSAS and hypothesized that OSAS had a neurogenic origin. Friberg et al<sup>14</sup> reported the presence of neurogenic lesions of soft palate muscles in humans with OSAS and hypothesized that nerve lesions caused by snoring trauma were a contributing factor in upper airway collapse. These hypotheses regarding the etiopathogenesis of OSAS can also be applied to the myopathogenesis observed in dogs with an elongated soft palate because the histopathologic changes that occur in palatine muscles and nerve fibers in these 2 diseases are similar.<sup>15,16</sup>

In the present study, 4 of 24 dogs with an elongated soft palate were mesocephalic breeds. Besides, some histopathologic changes were detected in control Beagles—dogs that are classified as a mesocephalic breed—although the severity of the changes was very low. The results indicated that palatine muscle in mesocephalic dogs may be also affected by chronic mild damage, which leads to the subsequent degeneration of myofibers. Therefore, palatine muscle damage might be one of the causes of elongated soft palate in mesocephalic breeds.

Results of the present study indicated that myofibers of dogs with an elongated soft palate undergo hyaline degeneration, atrophy, and hypertrophy and

that these changes may be caused by physical stress such as snoring or negative pressure and, at least in part, by palatine muscle denervation. These pathological changes may contribute to upper airway collapse and the progression of BAOS in affected brachycephalic dogs.

## Footnotes

- a. Mouse Monoclonal Antibody Dystrophin (Rod Domain), Novocastria, Leica Biosystems, Peterborough, Cambridgeshire, England.
- b. Target Retrieval Solution, pH 9, Dako, Carpinteria, Calif.
- c. Block Ace, Dainippon Pharma, Cyuuou, Osaka, Japan.
- d. Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Antibody, Molecular Probes, Eugene, Ore.
- e. Axiovert 200 M, Carl Zeiss, Oberkochen, Germany.
- f. Monoclonal Mouse Anti-Human Neurofilament Protein, Dako, Carpinteria, Calif.
- g. Polyclonal Goat Anti Mouse Immunoglobulin/Biotinylated, Dako, Carpinteria, Calif.
- h. Peroxidase-Conjugated Streptavidin, Dako, Carpinteria, Calif.
- i. Q Capture Pro, Nippon Roper, Fukagawa, Koutou, Japan.
- j. Microsoft Excel for Mac 2011, Microsoft Corp, Redmond, Wash.
- k. Statcel2, OMS, Tokorozawa, Saitama, Japan.

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