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Dilated Cardiomyopathy in Juvenile Portuguese Water Dogs

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Dilated cardiomyopathy recently has been recognized in juvenile Portuguese Water Dogs. The purpose of this study was to evaluate unaffected and affected puppies by physical examination, electrocardiogram (ECG), echocardiogram, specific biochemical assays, and ultrastructure to document disease progression and to develop a method of early detection. Results of segregation analysis were consistent with autosomal recessive inheritance. Of 124 puppies evaluated clinically and echocardiographically, 10 were affected. No significant differences were found between unaffected and affected puppies for blood and myocardial carnitine or taurine concentrations, serum chemical variables, results of ophthalmological examinations, ECGs, or measurement of urine metabolites. Ultrastructural examination of myocardium from affected dogs revealed myofibrillar atrophy and small regions of myofibrillar degeneration, most prominently at the region of the intercalated discs. Only echocardiography allowed detection of affected puppies before clinical signs became evident. Echocardiography revealed a significant difference in the shortening fraction, E point to septal separation, and the end systolic and diastolic left ventricular internal diameters. Affected puppies were detected 1–4 weeks before the development of acute congestive heart failure.

Key words: Dog; Idiopathic dilated cardiomyopathy; Inherited cardiomyopathy.

Idiopathic dilated cardiomyopathy (DCM) is characterized by cardiac chamber enlargement and markedly decreased contractile function.[1] Diagnosis is typically made by thoracic radiography, electrocardiography, and echocardiography. In a recent retrospective study examining 189 cases of canine DCM with secondary congestive heart failure, age of onset ranged from 3.5 to 13 years with a mean and median of 6.6 years.[2] In contrast, Portuguese Water Dogs (PWDs) develop a form of cardiomyopathy that occurs much earlier in life. Affected dogs die within the 1st 7 months (13 ± 7.3 weeks),[3] hence the term juvenile DCM (JDCM). JDCM of PWDs is not responsive to medical management and fulminant congestive heart failure develops rapidly,[3] with death often resulting before the recognition of clinical signs of cardiovascular disease. Although rare cases of juvenile onset have also been recognized in humans, human DCM is much more common between the ages of 20 and 50 years.[4] The mode of inheritance in human DCM is typically autosomal dominant, but reports have been made of autosomal recessive, X-linked, and mitochondrial inheritance.[4] JDCM in the PWD follows an autosomal recessive pattern.[3] The purpose of this study was to further characterize the clinical presentation and progression of JDCM in PWD, and to determine the usefulness of candidate metabolic and protein markers of cardiomyopathy that have been identified in other breeds and species for the detection and characterization of DCM in the PWD.

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Materials and Methods

All experiments were performed in accordance with protocols approved by the University of Pennsylvania Animal Care and Use Committee. During the course of this study, 124 client-owned PWD puppies, including 10 that died of DCM, were evaluated. For comparisons of affected to unaffected dogs, puppies surviving to greater than 7 months of age without evidence of DCM were considered to be unaffected, and were used retrospectively for comparison with affected puppies. Serial clinical evaluations were performed in 4 litters that contained affected puppies. In 3 of these litters (27 puppies), physical examinations and electrocardiograms (ECGs) were performed weekly with echocardiograms performed 2 times per week in abnormal pups. In 1 of these litters (8 puppies), ophthalmological exams were performed biweekly by a board-certified veterinary ophthalmologist. The remaining puppies (97) had at least 2 evaluations that included physical examination, ECG, and echocardiogram between 8 and 16 weeks of age.

The Cardiac Committee of the Portuguese Water Dog Club of America provided postmortem reports and additional pedigree information from litters containing affected animals not evaluated in this study. Matings in which at least 1 puppy had a diagnosis of JDCM, based on the report of an enlarged heart and pulmonary congestion upon postmortem examination or by echocardiographic examination, were included. Additional cases of unexplained sudden death between 7 weeks and 7 months of age, from matings containing a confirmed case, were also assumed to be JDCM. Puppies surviving past 7 months of age were considered to be unaffected. Segregation analysis to test the hypothesis of autosomal recessive inheritance was performed by the singles method to correct for ascertainment bias.[5]

ECGs were performed for 2 minutes with 6 leads (I, II, III, aVR, aVL, and aVF). A portion of the ECG was obtained at a paper speed of 50 mm/s in lead II to permit measurement of relevant variables. All ECGs were obtained with the animal in right lateral recumbency. No animals were sedated for electrocardiographic or echocardiographic examination. All echocardiograms included in this study were obtained from the right parasternal window by the same sonographer. The M-mode measurements were acquired from the short-axis view with measurements obtained from 2 representative cardiac cycles from the same frozen sequence of images.

Echocardiographic variables were plotted versus age. The age at which abnormalities were 1st detected was determined visually as the age at which the plotted shortening fraction (SF) and E point to septal separation (EPSS) began their deterioration for each animal. This age was used as the age of normalization for that animal only to calculate the ratio of age to normalization age, and the data were plotted on this

normalized-age horizontal axis. Descriptive statistics were computed for the age at which abnormalities were detected in each animal.

Blood and myocardial carnitine concentrations were measured by the General Clinical Research Center of the Children's Hospital of Philadelphia (Philadelphia, PA) by established methods.[6] Blood was obtained from 4 affected and 5 unaffected littermates, skeletal muscle was obtained from 1 affected and 1 age-matched unaffected dog from a breed other than PWD (nonPWD), and myocardium was obtained from 2 affected puppies and 1 age-matched unaffected nonPWD for this analysis. Blood (5 affected PWDs, 6 unaffected PWDs, and 1 unaffected nonPWD) and myocardial (4 affected PWDs, 1 unaffected PWD, and 7 unaffected nonPWDs) taurine concentrations were measured by the amino acid analysis laboratory in the Department of Molecular Biosciences, School of Veterinary Medicine, University of California at Davis.[7] Two of the affected dogs in this group received supplementation with taurine. Serum chemistry values were measured in 2 affected puppies, and serum ionized magnesium (iMg) and ionized calcium (iCa) concentrations were measured in 1 litter (3 affected and 5 unaffected puppies). The sensitivity range for iCa was 0.1–2.7 mmol/L, and the sensitivity for iMg was 0.1–2.5 mmol/L. An enzyme-linked immunosorbent assay (ELISA)[a] was used to qualitatively analyze the presence of cardiac troponin I (cTnI; sensitivity of 0.3 ng/mL). Homogenized PWD myocardium (unaffected and affected) was used as a positive control and serum from healthy dogs was used as a negative control. The cTnI was measured in 1 litter (3 affected and 5 unaffected puppies). Screening of urine metabolites in 1 litter (2 affected and 8 unaffected puppies) consisted of 1-dimensional paper chromatography to detect the presence of unusual amino acids or unusual concentrations of amino acids, carbohydrates, and organic acids.[8]

Three affected puppies were supplemented with taurine after values of echocardiographic variables were clearly indicative of JDCM (decreasing SF in conjunction with left ventricular [LV] dilation). The puppies were treated until death (4–30 days). One puppy was supplemented with 500 mg taurine daily and the other 2 were supplemented with 1 g daily. All evaluations continued in these puppies as previously described.

Affected animals were euthanized before the development of overt congestive heart failure when possible (7 of 10 affected pups), but only after definite and sustained deterioration of systolic function had been observed. Three puppies demonstrated signs of early congestive heart failure at the time of euthanasia. Tissue samples were snap frozen in liquid nitrogen for storage and subsequent biochemical analysis. Histopathology was submitted on 4 of the 10 affected puppies, 2 of which were supplemented with taurine (16 and 30 days). Neither of these puppies was in overt congestive heart failure at the time of death.

Mitochondrial DNA (mtDNA) was examined by dot blot analysis to compare affected pups with age-matched control dogs of different breeds. Total RNA was isolated from heart (0.5–1 g of minced tissue) by means of the guanidine thiocynate procedure described previously.[9] Total RNA (30 μg) was analyzed by northern blot hybridization with [^{32}P]-labeled cDNA probes (cytochrome *c* oxidase subunits I, II, IV, and Vb) under standard conditions.[10] Gel-purified, double-stranded DNA probes were labeled with [^{32}P]-deoxycytidine triphosphate (dCTP)[b] by random primer extension with Klenow polymerase. The RNA loading was normalized by hybridizing the stripped blots with a [^{32}P]-labeled DNA probe for 18S ribosomal RNA.[c]

The following enzyme activities were assayed by standard methods as described: complex IV (cytochrome *c* oxidase),[11] complex I (reduced nicotinamide adenine dinucleotide–ubiquinone oxido reductase),[12] complex V (adenosine triphosphate [ATP] synthase),[13] and isocitrate dehydrogenase.[14] Protein concentrations were estimated by the method of Lowry et al.[15] These are methods that have been used previously in our laboratory.[16]

Portions of the left ventricle from 2 unaffected (nonPWD, 12 weeks of age) and 2 affected dogs were frozen in isopentane cooled in liquid nitrogen. Immunohistochemistry was performed on 10-μm-thick sections that were then fixed in situ for 2 minutes with 200–300 μL of Prefer fixative.[d] All washes and rinses were performed in Dulbecco's modified phosphate-buffered saline (DPBS) and were repeated 5 times. After washing for 30 seconds for each rinse, sections were incubated for 30 minutes with a mouse monoclonal antibody specific for sarcomeric α-actinin[e] diluted 1:200 with DPBS. After rinsing, the sections were incubated in 4% normal donkey serum (in DPBS) for 10 minutes. After rinses, the tissue was incubated for 20 minutes with Cyanine Cy5-conjugated donkey-anti-mouse immunoglobulin G diluted 1:200 with DPBS. Sections were then washed and incubated for 1 minute with propidium iodide diluted 1:4,000 with DPBS. After a final wash, the sections were mounted in Fluoromount G.[f] Photomicrographs were recorded from images obtained with a Lieca TCS4D laser scanning confocal microscope.[g] In a similar fashion, additional immunohistochemical staining with the following primary antibodies and their dilutions was performed: dystrophin,[h] α-sarcoglycan,[i] titin,[j] tumor necrosis factor-α,[k] cytochrome oxidase I (1:50), cytochrome oxidase 5B (1:200), cytochrome oxidase 6A (1:100), and F1-ATPase (1:50, gift from P.L. Pederson). All cytochrome oxidase antibodies were obtained from Molecular Probes.[l]

Hearts from 3 affected PWDs (11, 13, and 16 weeks of age) that had abnormal echocardiograms and from 2 unaffected 11-week-old mixed-breed dogs were prepared for electron microscopy. The left anterior descending coronary artery was cannulated and perfused with calcium-free physiological solution (150 mM K-acetate, 20 mM potassium-phosphate buffer, 5 mM ethyleneglycoltetraacetic acid, pH 7.4) followed by 6% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Portions of the left ventricle were cut into 3 × 5 × 10-mm slices and maintained in the fixative. Small bundles of fibers teased from the prefixed myocardium were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer for 1 hour at room temperature, en-block stained in saturated uranyl acetate either at 60°C for 4 hours or at room temperature overnight, and embedded in Epon 812.[m] Thin sections were stained in ~ 4% uranyl acetate in 50% EtOH and in Sato lead stain.[17] Electron microscopy was performed with a Philips EM 410 transmission electron microscope.[n]

Results

Pedigree Analysis

Information was available from matings between 18 different breeding pairs (20 litters) from which at least 1 case of JDCM was confirmed. Confirmation of JDCM in 31 puppies was based on the report of an enlarged heart and pulmonary congestion upon postmortem examination or by echocardiographic examination. Eighteen puppies born of these matings that died suddenly and unexpectedly also were presumed to have JDCM. Deaths in these animals occurred between the ages of 7 and 21 weeks. Of 174 puppies born in these litters, 49 were affected (26 males, 22 females, and 1 gender unknown). After correction for bias of ascertainment, the segregation ratio (proportion of affected offspring) was 0.269 ± 0.04. This is not significantly different than the expectation of 0.25 predicted by fully penetrant, autosomal recessive inheritance ($.6 < P < .7$). The numbers of affected males and females are consistent with the predicted 1:1 ratio (also $.6 < P < .7$). Absence of clinical signs in the parents (3 examined by echocardiography at the University of Pennsylvania) and surviving littermates of affected puppies (26 examined in this study) are also consistent with this mode of inheritance, and strongly argue against a dominant mode of inheritance.

Table 1. Echocardiographic variables and body weight of healthy Portuguese Water Dog puppies. Values are ranges with means in parentheses.

Age (weeks)	No. puppies	Weight (kg)	EDD (cm)	EDS (cm)	SF (%)	EPSS (cm)
7	12	1.4–4.5 (2.1)	1.3–2.1 (1.6)	0.8–1.3 (1.0)	31–48 (39)	0–0.2 (0.1)
8	12	2.3–4.2 (3.2)	1.6–2.4 (2.1)	0.9–1.6 (1.3)	32–44 (38)	0.1–0.4 (0.2)
9	22	2.6–4.8 (3.9)	1.9–2.8 (2.2)	1.0–1.8 (1.5)	21–46 (34)	0.1–0.3 (0.2)
10	21	3.9–6.4 (5.1)	1.9–2.7 (2.3)	1.1–1.9 (1.5)	21–42 (32)	0–0.3 (0.1)
11	70	1.8–6.5 (4.7)	1.9–2.9 (2.4)	1.1–2.2 (1.6)	21–44 (32)	0.1–0.3 (0.2)
12	21	4.7–8.2 (5.9)	1.9–3.0 (2.3)	1.3–2.0 (1.5)	28–44 (36)	0.1–0.3 (0.2)
13	9	4.7–8.0 (6.4)	2.3–2.7 (2.5)	1.4–1.8 (1.6)	26–40 (35)	0.1–0.2 (0.2)
14	10	6.0–8.0 (7.1)	2.3–3.0 (2.6)	1.3–2.2 (1.7)	26–52 (34)	0.2–0.4 (0.3)
15	11	5.5–9.1 (7.9)	2.4–3.1 (2.7)	1.5–2.1 (1.8)	29–42 (35)	0–0.4 (0.2)
16	7	7.5–9.8 (7.9)	2.2–3.0 (2.7)	1.4–2.3 (1.9)	21–36 (32)	0–0.4 (0.2)
17	7	8.0–10.5 (9.3)	2.6–2.9 (2.7)	1.8–2.1 (1.9)	25–42 (31)	0.1–0.4 (0.2)
18	7	8.6–11.4 (10.1)	2.4–3.4 (2.9)	1.7–2.2 (2.0)	28–38 (32)	0–0.4 (0.2)
19	7	9.0–11.5 (10.6)	2.8–3.1 (2.9)	1.8–2.2 (2.0)	29–40 (33)	0–0.4 (0.2)

EDD, end diastolic diameter; EDS, end systolic diameter; SF, shortening fraction; EPSS, E point septal separation.

Clinical Findings

Biweekly ophthalmological examinations of 1 litter including 3 affected puppies did not reveal abnormalities in any pups. Serial echocardiographic examination of 10 affected puppies demonstrated progressively worsening global function until they were euthanized at ages ranging from 72 to 197 days (mean 118 days). No clinical signs were detected in 7 of the affected puppies in this series because euthanasia was performed before development of congestive heart failure, but only after the progression of severe systolic dysfunction on echocardiographic parameters (SF and EPSS). Three of the affected puppies demonstrated signs of early congestive heart failure. These puppies developed S3 gallops and grade 3/6 systolic murmurs consistent with mitral regurgitation in the last 24 hours of life. An increased respiratory rate, crackles on thoracic auscultation, and dyspnea were very late findings and were considered to be signs of congestive heart failure. No clinical or echocardiographic difference was recognized in the progression of JDCM between puppies supplemented with taurine and those without supplementation.

Occasional ventricular premature beats were observed during echocardiography in 1 affected puppy. One unaffected puppy also had intermittent ventricular premature beats detected during only 1 of 12 ECGs. No difference was apparent between unaffected (puppies that survived beyond 7 months were stratified by age retrospectively) and affected puppies in electrocardiographic intervals (PR, QRS, and QT duration). No difference was found in R wave amplitude (lead II) between unaffected and affected pups. A terminal sinus tachycardia (190–210 beats per minute [bpm]) was present in 3 of the affected puppies secondary to congestive heart failure. However, sinus tachycardia was also observed occasionally during the study in affected and unaffected puppies without congestive heart failure (160–190 bpm). Table 1 gives the echocardiographic data in clinically normal PWDs. In affected puppies (72–197 days, mean 118), significant differences were the end diastolic diameter (3.5–5.2 cm, mean 3.9 cm), the end systolic diameter (2.9–4.8 cm, mean 3.3 cm), SF (6–18%, mean 12%), and the EPSS (0.8–1.6 cm, mean 1.1 cm). A trend was observed of decreasing LV wall thickness, but the change was not statistically significant. The time course of the echocardiographic EPSS parameter is presented in Figure 1, in which the age of each animal has been normalized to the age of the beginning of deterioration. The mean normalization age (the breakpoint in the figure) of the 10 affected animals was 104 ± 29 days. All echocardiographic results are shown for each date on which an echocardiogram with measurement of EPSS was performed. In the upper panel, note the time course in affected animals. The lower panel shows that no such deterioration occurred over a similar age range in unaffected littermates and other unrelated, unaffected PWDs. The horizontal axis in the lower panel is the actual age at the date of evaluation, because no appropriate age is available with which to normalize data. Figure 2 shows the SF values over time for the animals studied. The upper panel shows the trend for affected animals, whereas the lower panel shows the data for all unaffected animals studied. The upper panel again shows age as normalized to the age of onset of deterioration in affected animals. The lower panel shows the SF over the same age range with wide variation (21–52%) in unaffected puppies. Clear deterioration in these variables can be seen in affected puppies from 1 to 3 weeks before euthanasia.

Laboratory and Postmortem Findings

The results of various biochemical assays in age-matched, restrospectively identified unaffected and affected puppies, and the number of puppies evaluated for each parameter are summarized in Table 2. Puppies were not matched by gender. Screening of urine from affected and unaffected littermates revealed no differences in crude amino acid, carbohydrate, or organic acid profiles (data not shown). Myocardial histology with hematoxylin and eosin, and trichrome stains revealed histological changes consistent with those in our previous report,[3] including thin, irregular and wavy fibers with loss of cross-striations and obvious intercalated discs.[3] Myofiber clearing and swelling was also noted (not shown). Immunohistochemistry for

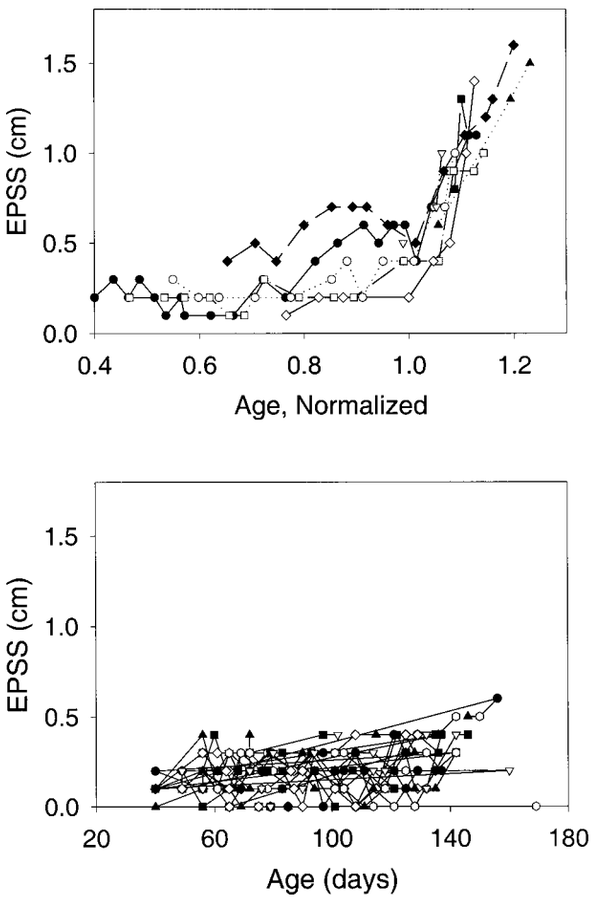

Fig 1. Time course of the mitral valve E point to septal separation (EPSS) in Portuguese Water Dogs (PWDs). **(Upper panel)** EPSS shown in affected dogs versus age, normalized as a ratio of chronologic age to age of initial deterioration. **(Lower panel)** Data from all unaffected PWDs.

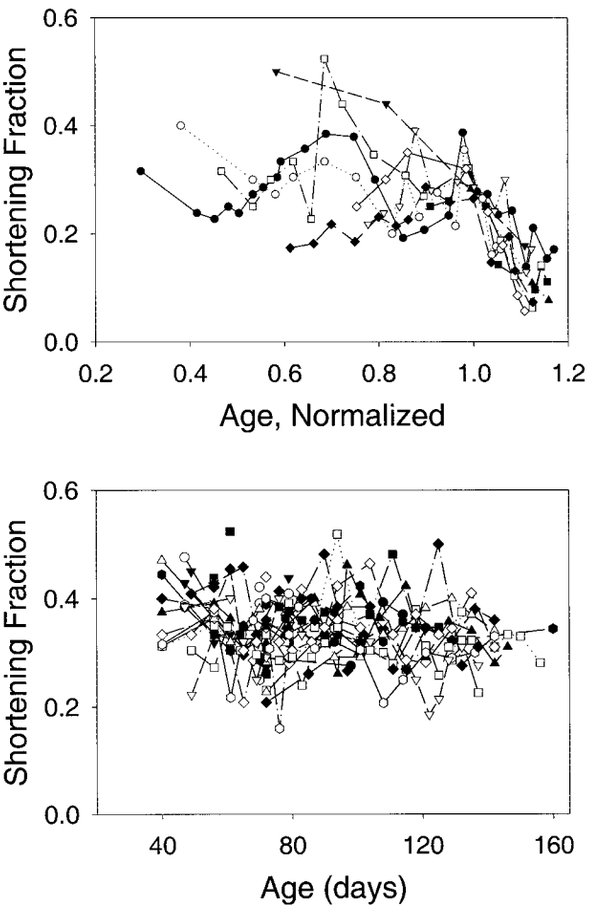

Fig 2. Time course of shortening fraction (SF) in Portuguese Water Dogs. **(Upper panel)** SF in affected animals, with left ventricular SF by M-mode echocardiography shown on the vertical axis. The age on the horizontal axis is the normalized age as in Figure 1A. **(Lower panel)** SF of unaffected animals, shown versus actual age, as in Figure 1B.

Table 2. Candidate biochemical markers of dilated cardiomyopathy in unaffected and affected age-matched Portuguese Water Dog (PWD) puppies.

	Affected			Unaffected		
Marker	No.	Range	Mean	No.	Range	Mean
Carnitine						
Plasma, free (μm)	4	15.1–26.6	21.3	5	17.2–26.1	23.2
Plasma, total (μm)	4	16.8–35.3	26	5	25.4–39.1	30.5
Cardiac, free (μm)	2	53.5–92.7	73.1	1	34.1	
Cardiac, total (μm)	2	79.9–100	90.3	1	55	
Skeletal muscle, free (μm)	1	121	—	1	85.6	
Skeletal muscle, total (μm)	1	130	—	1	104	
Taurine[a]						
Plasma (nmol/mL)	5	56.8–108	82	2	77–175	126
Whole blood (nmol/mL)	5	91–292	207	7	84–370	238
Cardiac (mg/kg wet tissue)	4	1,660–3,420	2,290	5	1,010–2,420	1,660
Cardiac troponin I serum[b]	4	Negative[b]	—	4	Negative[b]	
Ionized Ca^{2+} (mmol/L)	2	1.41–1.49	1.45	4	1.37–1.50	1.42
Ionized Mg^{2+} (mmol/L)	2	0.25	0.25	4	0.24–0.27	0.25

[a] Reference concentrations are 60–120 nmol/mL (plasma) and 300–600 nmol/mL (whole blood).

[b] Homogenized myocardiums from 1 affected and 1 unaffected PWDs were positive (positive control).

dystrophin, α-sarcoglycan, titin, α-actinin (Fig 3), tumor necrosis factor-α, cytochrome oxidase I, cytochrome oxidase 5B, cytochrome oxidase 6A, and F1-ATPase was similar in unaffected and affected dogs. Quantitation of mtDNA by dot blot analysis did not reveal significant alterations in its content between the control and affected puppies. The messenger RNA concentrations for mitochondrial genome-encoded cytochrome *c* oxidase (complex IV) I and II subunits were also found to be nearly normal in affected puppies. No apparent differences in mitochondrial enzyme activities between the control and affected puppies were detected (not shown).

Ultrastructurally, several abnormalities were detected in the hearts of PWDs with JDCM. All the myofibers of failing hearts had myofibrillar profiles that varied greatly in diameter (Fig 4A). Additionally, the myofibrils often had irregular contours and absent myofilaments (Fig 4C). Myofibrils and mitochondria were separated by apparent empty spaces. In sections from tissue that was not stained en-block with uranyl acetate, some of the intermyofibrillar spaces were occupied by glycogen, but others were empty. Mitochondria were noticeably frequent in the cytoplasm because they were surrounded by empty spaces. In longitudinal sections most sarcomeres had normal appearance. Elements of the sarcoplasmic reticulum (SR) and transverse (T) tubules were present and SR-T tubules and SR-surface junctions were well formed. Evidence was found of myofibrillar damage in some areas of each failing heart. Z-Line streaming and disassembly of sarcomeres affected either single myofibrils or groups of closely spaced ones (Fig 4A,F). The defect was particularly obvious at the intercalated discs, where sometimes it affected a relatively large portion of 2 adjacent myofibers. To make comparisons with age-matched unaffected dogs, we performed ultrastructural examinations of the myocardium from mixed Beagle–Basset Hounds of a similar age. Myofibrils and mitochondria were tightly packed within the cells, the myofibril size was more uniform, and the contours of the myofibrils more regular than in affected dogs (Fig 4B,D). No evidence was found of myofibrillar damage. The average surface area of myofibrils in cross section in affected dogs ($0.5 \pm 0.3\ \mu m^2$, mean ± SD, from 612 measurements in 3 affected hearts) was one half of that in 1 unaffected heart ($1.1 \pm 0.6\ \mu m^2$ from 138 measurements). The ratio of mitochondrial and of myofibril area to total fiber area was measured in cross sections of 1 affected and 1 unaffected dog at 11 weeks of age. This value was the same as the ratio of mitochondria and myofibril volume to fiber volume. In the affected heart, myofibrils and mitochondria occupied 39 ± 7% and 27 ± 7% of the fiber volume, respectively, whereas the 2 ratios were 54 ± 6% and 32 ± 6% in the unaffected heart. Thus, myofibrils showed indication of atrophy, but mitochondrial volume appeared normal.

Discussion

In this evaluation of 124 PWDs, including 10 with DCM, all affected dogs remained healthy by clinical examination until the last 3 days of life. This is consistent with a retrospective study[3] and anecdotal reports from dog owners. A previous study suggested an autosomal recessive mode of inheritance,[3] which is supported by analysis of additional litters in this study. In analyzing the segregation ratio, we assumed a diagnosis of JDCM in puppies that died suddenly of unexplained causes, with no obvious illness, if they were siblings of confirmed affected animals. Although no formal attempt was made to rule out other modes of inheritance, the hallmarks of autosomal recessive inheritance are very clear in this disease. These features are that affected puppies are offspring of clinically healthy parents, affected puppies comprise 25% of puppies in litters with affected puppies, no gender predilection is found, unaffected littermates show no detectable signs of disease, and affected puppies share common ancestors.[3] In the absence of test matings to rule out other modes of inheritance, we believe that autosomal recessive inheritance is a reasonable working hypothesis.

The earliest means of detection of JDCM in this study was by echocardiography, which revealed slowly progressive global LV eccentric hypertrophy and poor systolic function approximately 1–4 weeks before clinical deterioration. Also evident from these studies was that considerable variation occurred in values of the clinical variables used to quantify cardiac function in young, unaffected PWDs. For example, although the average SF at 10 weeks was 32%, some unaffected dogs had values as low as 21%. This variability is unlikely to be related to carrier status because it was present in all PWD litters evaluated, many of which contained no affected individuals. This variation in the SF of unaffected puppies did not have any pattern over time. However, in affected puppies, the EPSS gradually increased in conjunction with a falling SF. Given the variation in the echocardiographically determined variables in unaffected PWDs, consecutive evaluations are necessary to detect the trend of progressively worsening cardiac function that precedes the development of clinical signs. The time range of death of affected puppies in this series (72–197 days) was slightly higher than the previously published series,[3] indicating that dogs cannot be determined by echocardiography not to have the disease until reaching 7 months of age.

Irreversibly injured myocardial cells release a number of enzymes into the circulation that can be measured in the blood.[18] Troponin I is a peptide subunit of the globular protein troponin that regulates calcium activation of actomyosin ATPase.[19] After cardiomyocyte injury, cTnI is released into the circulation, making it a useful marker of myocardial cell injury and death in humans with myocardial in-

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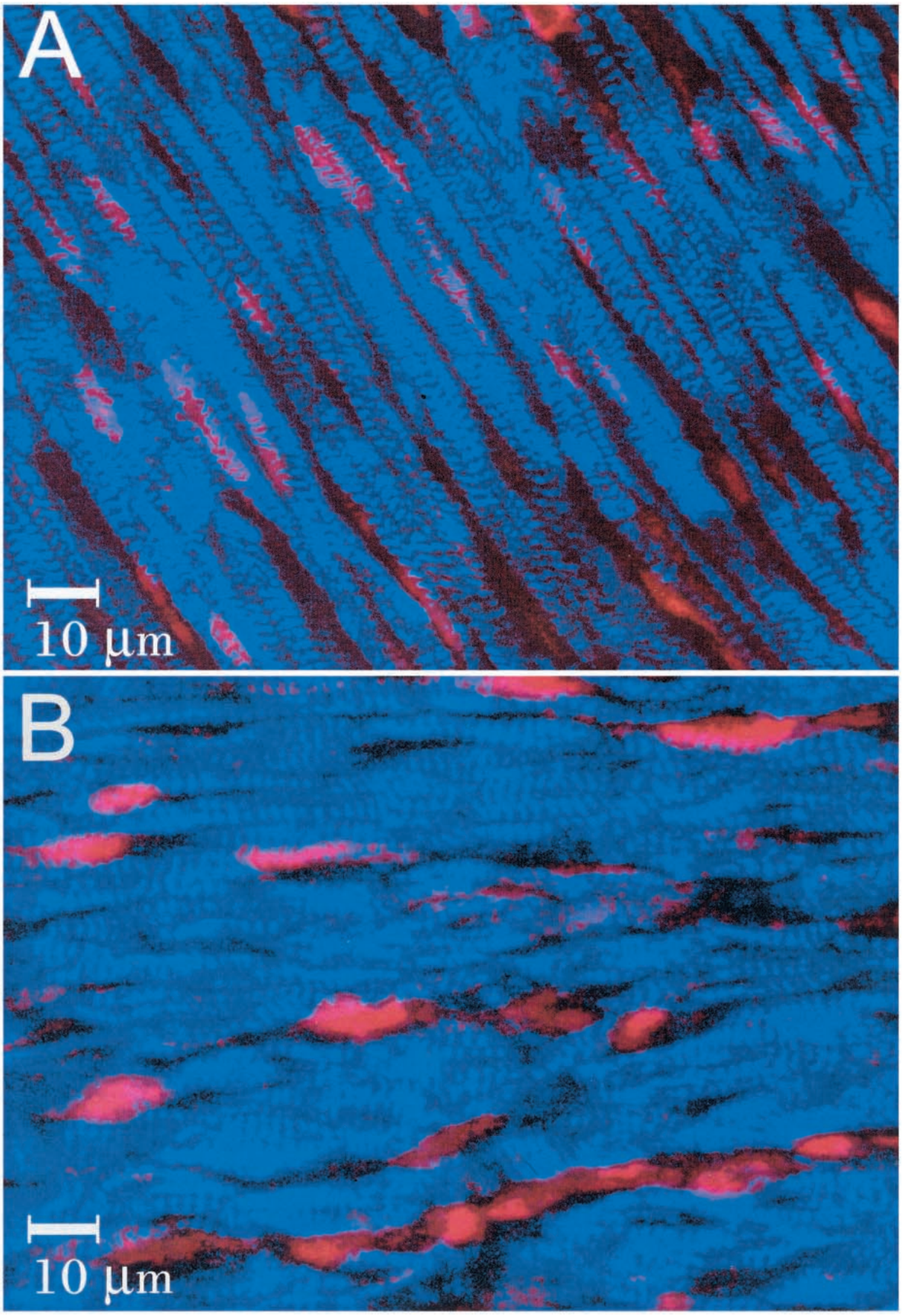
Fig 3. Immunohistochemistry of Portuguese Water Dog myocardium stained with a primary anti-α-actinin antibody, and propidium iodide, with laser scanning confocal imaging. **(A)** Normal myocardium showing antibody staining along sarcomeres. **(B)** Cardiomyopathic myocardium from an affected dog showing similar staining. The cardiomyopathic section shows more nuclei (red) but otherwise is similar. Bar = 10 μm.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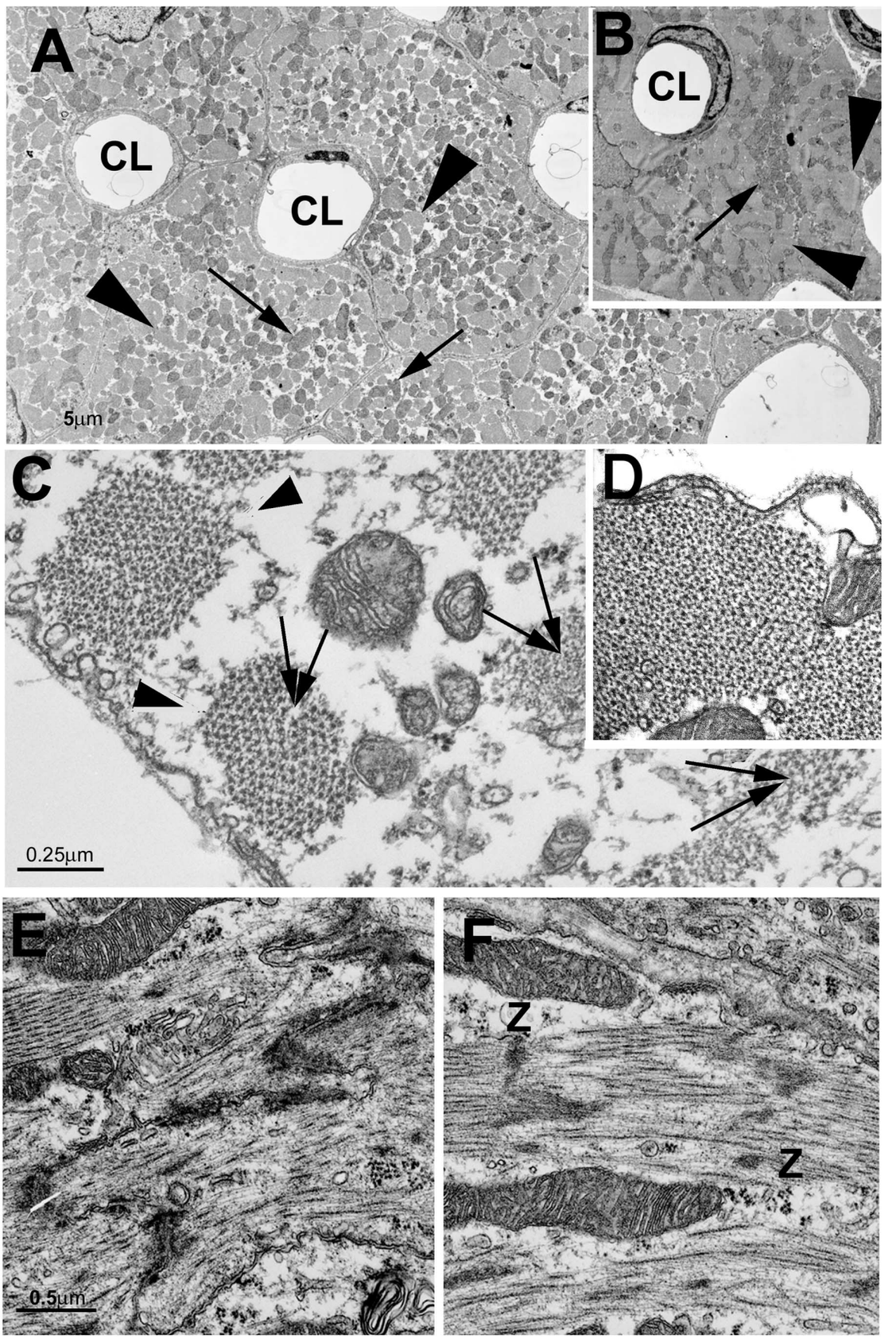
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farction.[20,21] Troponin I is more specific than alpha hydroxy butyrate dehydrogenase and is more sensitive than the cardiac isoform of creatine kinase for detection of myocardial cellular injury in humans, and therefore has been found to be useful in human medicine for the detection of myocardial infarcts, doxorubicin toxicity, and severe congestive heart failure.[22–24] Activity of serum creatine kinase in 2 affected puppies was found to be within the reference range 2 days before euthanasia (compared to the clinical laboratory reference range at the Veterinary Hospital of the University of Pennsylvania). Neither of these pups was supplemented with taurine. Results of an ELISA[a] used to analyze for the presence of cTnI in peripheral blood were negative in 4 affected and 4 unaffected age-matched puppies, which probably indicates that this test is not useful in diagnosis of this disease. Plasma iCa and iMg concentrations were not different between unaffected and affected pups. These concentrations are not indicative of the intracellular electrolyte content,[25] but the results reveal that plasma concentrations are not useful in detecting affected puppies.

Urine analysis is useful for evaluation of inherited metabolic disorders because most abnormal metabolites are filtered through the renal glomerulus, but are not actively reabsorbed by the renal tubules because no specific transport mechanisms exist.[26] Therefore, these compounds are more concentrated in urine than in blood. Because no major abnormalities were found in the urine metabolic screens in the affected PWD puppies that were evaluated, combined with the fact that the disease seems to affect only the heart, we conclude that a defect in a metabolic pathway is probably not responsible for the disease.

No difference was apparent between blood and myocardial carnitine concentrations between affected and unaffected puppies; however, the number of individuals examined was small. In dogs, L-carnitine is concentrated in cardiac and skeletal myocytes. In unaffected animals, plasma and myocardial carnitine concentrations correlate closely, but this relationship is often lost when DCM is present.[27,28] Carnitine is a critical component of 2 mitochondrial membrane enzymes, carnitine acetyltransferase 1 and 2, which transport activated fatty acids across the mitochondrial membrane. One report has documented the efficacy of carnitine supplementation in the therapy of 2 related male Boxers with DCM.[28] Supplementation with L-carnitine resulted in a clinical response in 2 additional studies evaluating its supplementation in DCM patients[29,30]; however, in a group of Doberman Pinschers with DCM, only the dogs that were carnitine deficient and later supplemented with L-carnitine survived significantly longer than did dogs with myocardial carnitine concentrations that were within the reference range.[30] Carnitine transporter defects have also been recognized as a cause of cardiac changes and sudden death in human infants.[31] However, carnitine is unlikely to play a role in JDCM of PWDs, given that myocardial and blood concentrations of the amino acid were within the reference range in the affected puppies evaluated in this study.

Blood and myocardial taurine concentrations were also found to be similar in unaffected and affected littermates. Blood taurine concentrations were on average lower in affected puppies compared to unaffected puppies, but this finding may be secondary to heart failure. None of the affected puppies had plasma taurine concentrations in a range consistent with taurine deficiency cardiomyopathy as seen in other species, although 2 puppies had whole blood concentrations under 200 nmol/mL (166 and 138 nm/mL). Cardiomyopathy in cats secondary to taurine deficiency has been associated with a plasma taurine of less than 30 nmol/mL in 52 of 77 cats with DCM in 1 study.[32] Moreover, in our study, supplementation of taurine was unsuccessful in preventing or slowing progression of myocardial dysfunction in 3 affected PWDs, contrary to feline taurine deficiency cardiomyopathy. Although 1 of the puppies was supplemented for only 4 days, another puppy was treated for 30 days, a time period that resulted in clinical improvement in cats with taurine-responsive cardiomyopathy.[33] In cats, taurine supplementation does not provide survival benefit until 2 weeks of supplementation, but survival for 2 weeks confers a good prognosis, although an echocardiographic response requires 3–16 weeks of supplementation.[33] Central retinal degeneration has been associated with taurine deficiency in cats, but biweekly ophthalmological examinations in 1 affected litter did not reveal abnormalities consistent with taurine deficiency.[34] In contrast to the situation in cats, taurine is not an essential amino acid in the dog. Not all cats fed taurine-deficient diets develop myocardial failure, and 1 author hypothesized that the gradually deteriorating myocardial failure seen over months or years in his study could explain the predominance of middle-aged or older cats with taurine-responsive DCM.[35–37] Thus, taurine-dependent cardiomyopathy seems to develop after chronic taurine deficiency, at least in the cat, and is unlikely to be a factor in JDCM of PWDs.

A higher than normal rate of mitochondrial gene mutations has been found in human (adult) idiopathic DCM.[38] JDCM can also be caused by mitochondrial mutations.[39] Mitochondrial proteins are encoded by genes found both in mtDNA and in the nuclear genome; therefore, mitochondrial diseases can show either mitochondrial inheritance

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Fig 4. Electron micrographs of myocardia from affected Portuguese Water Dogs **(A, C, E, F)** and from an unaffected mixed Beagle–Basset Hound of approximately the same age **(B, D).** CL indicates lumen of capillaries, which appear empty due to perfusion. Affected hearts tend to present wide, apparently empty spaces between myofibrils (arrowheads in **A** and **B**) and mitochondria (arrows). In hearts from unaffected dogs, myofibrils and mitochondria are tightly arranged **(B).** Myofibrils show a loss of sharp contours and often have unusually small diameters (double arrows in **C**). Compared to a portion of a myofibril from an unaffected dog (**D,** at the same magnification), **C** shows 2 defects. One is a fairly diffuse disarrangement of myofibrils in the proximity of intercalated discs. An intercalated disc runs from top to bottom in the center of **E** and the myofibrils in its proximity are disarranged. The other defect is an alteration of sarcomeres along small groups of myofibrils within the cell (**F**; Z indicates Z lines). In both cases, Z line streaming and disruption occur, accompanied by disarrangement of the myofilaments and loss of sarcomere structure.

(transmitted from the mother to all offspring) or nuclear inheritance (Mendelian or complex modes). Diagnosis of mitochondrial defects is usually made based on biochemical analysis. Mitochondrial impairment has been shown to be a major cause in drug-induced and hereditary forms of cardiomyopathy in animals.[40,41] Moreover, marked impairment of respiratory chain function was reported in idiopathic DCM of Doberman Pinschers.[42] In the absence of genetic analysis, most of the studies cited above concluded that mitochondrial changes may be secondary to an underlying genetic defect or may indicate a deficiency of the mitochondrial respiratory chain that predisposes the individual to heart failure. In a recent study that directly addressed the significance of mitochondrial integrity in relation to normal biological function, Li et al[43] demonstrated DCM and neonatal lethality in mutant mice lacking mitochondrial manganese superoxide dismutase. Biochemical analysis of many mitochondrial enzyme activities in this study revealed no significant differences between control and affected puppies. Additionally, electron microscopy showed normal numbers and structure of mitochondria.

Electron microscopy did reveal myofibrillar atrophy. This is likely to develop as chamber dilation and thinning of the heart walls accompanies acute heart failure. A decrease of myofibrillar content has been noted in end-stage DCM in human patients at the time of cardiac transplant.[44] Myofibrillar atrophy also has been seen in individuals who had acute dilation from decompensated pressure overload heart failure[45] and aortic regurgitation.[46] This is in contrast to the usual finding in chronic DCM in humans in whom cardiomyocyte and myofibrillar hypertrophy are seen during the years that chronic dysfunction exists. Therefore, we interpret the myofibrillar atrophy as secondary to the contractile abnormality. We hypothesize that the very rapid deterioration of contractile function allows a similarly rapid progression of dilation in these dog hearts. Ventricular dilation increases the load on the cardiomyocytes and the mechanical load grows very quickly. As was proposed by Scholz et al,[44] protein synthesis likely cannot keep up with the cellular needs to remodel the myocardium once dilation has progressed to some threshold. In a mouse knockout model of cTnI, there was a larger volumetric proportion of mitochondria, and perhaps myofibrillar atrophy.[47] Analysis of the electron microscopic images found no hypertrophy in homozygous knockout mice (Walker, personal communication). Similarly, we believe that the myofibrillar atrophy is unlikely to be the primary genetic defect.

At least 5 protein abnormalities now are known that may individually cause human muscular dystrophy with DCM.[48] These are dystrophin and the dystrophin-associated proteins, which assemble at the sarcolemma. In limb-girdle muscular dystrophies types 2C through 2F, the causative protein is one of the sarcoglycans (α, β, γ, or δ); δ-sarcoglycan causes the myopathy in the cardiomyopathic Syrian hamster. Staining for any of the sarcoglycan proteins, when any protein of the complex is absent, reveals either absent or greatly diminished protein content at the sarcolemma. Therefore, staining for only 1 or 2 is a useful screening technique for the presence of this entire complex of proteins, all of which are required to prevent cumulative damage at the sarcolemma from contraction-induced injury.[49–51] Immunohistochemistry with antibodies to dystrophin and α-sarcoglycan is within reference ranges in affected PWD puppies, arguing against a primary sarcoglycan- or dystrophin-associated protein deficiency in these dogs.

As a general rule, diseases caused by mutations in enzyme genes are inherited in an autosomal recessive manner, whereas diseases due to structural gene mutations show dominant inheritance. This guideline is sufficiently inaccurate (eg, α-sarcoglycan mutations are recessive, and 1 normal dystrophin gene prevents disease in muscular dystrophy carriers) that it is unhelpful in individual diseases. One hypothesis for the type of defect responsible for PWD JDCM is that it involves a developmental switch from the normal fetal isoform of a cardiac gene to a defective adult one. The disease onset occurs at the time of this isoform shift. An example of this scenario is the transgenic mouse with mutated cTnI, which uniformly dies by day 18, whereas heterozygotes are clinically normal.[47] Some of our efforts have been directed toward evaluating a similar mechanism in these dogs.

Thus far, we have been unsuccessful in determining the cause of JDCM in PWD with the candidate protein approach; however, we have made many possibilities unlikely. These include metabolic or mitochondrial defects, sarcoglycanopathies, dystrophinopathies, and abnormalities of most sarcomeric proteins. By closely following 124 PWD puppies, we have characterized normal as well as abnormal echocardiographic parameters in this breed. Additionally, we have found that the previously published SF reference range in young English Springer Spaniels[52] is not appropriate for PWDs. Healthy PWD puppies can have transient drops in the SF to as low as 19% that are not related to changes in systolic function. It is important to remember that SF does not directly reflect contractility, but is altered by acute changes in preload and alterations in afterload.[53] However, a decreasing SF with an increasing EPSS and LV diameter are consistent with poor ventricular contractility as seen in these puppies. Therefore, the SF must not be used in isolation for screening PWDs for JDCM, but must be evaluated in conjunction with other echocardiographic parameters. Echocardiography used serially is still the only way to identify affected puppies; however, affected puppies can appear healthy on echocardiographic exam until 4 weeks before death. It is hoped that additional studies that are underway will find the affected gene, and allow early diagnosis and perhaps effective therapy.

Footnotes

[a] Biocard Troponin I immunochromatographic test, Ani Biotech OY, Helsinki, Finland
[b] [^{32}P]dCTP, 6000 Ci/mmol, Dupont NEN, Boston, MA
[c] Bio-Rad GS-525 Molecular Imager, Bio-Rad, Hercules, CA
[d] Prefer fixative, Anatech, Ltd, Battle Creek, MI
[e] A7811, Sigma Chemical Co, St Louis, MO
[f] Fluoromount G, Southern Biotechnology, Birmingham, AL
[g] Leica TCS4D, Leica Microsystems Inc, Bannockburn, IL
[h] 1:100, DYS2, Vector Laboratories, Burlingame, CA
[i] 1:100, a-SARC, Vector Laboratories, Burlingame, CA
[j] F146.9B9, Vector Laboratories, Burlingame, CA
[k] Tumor necrosis factor-α, R & D Systems, Inc, Minneapolis, MN

[l] Cytochrome oxidase antibodies, Molecular Probes, Eugene, OR
[m] Epon 812, Electron Microscopy Sciences, Fort Washington, PA
[n] Philips EM 410, Philips Electron Optics, Mahwah, NJ

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Characterization of a Coronavirus Isolated from a Diarrheic Foal.

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A coronavirus was isolated from feces of a diarrheic foal and serially propagated in human rectal adenocarcinoma (HRT-18) cells. Antigenic and genomic characterizations of the virus (isolate NC99) were based on serological comparison with other avian and mammalian coronaviruses and sequence analysis of the nucleocapsid (N) protein gene. Indirect fluorescent-antibody assay procedures and virus neutralization assays demonstrated a close antigenic relationship with bovine coronavirus (BCV) and porcine hemagglutinating encephalomyelitis virus (mammalian group 2 coronaviruses). Using previously described BCV primers, the N protein gene of isolate NC99 was amplified by a reverse transcriptase PCR (RT-PCR) procedure. The RT-PCR product was cloned into pUC19 and sequenced; the complete N protein of NC99 (446 amino acids) was then compared with published N protein sequences of other avian and mammalian coronaviruses. A high degree of identity (89.0 to 90.1%) was observed between the N protein sequence of NC99 and published sequences of BCV (Mebus and F15 strains) and human coronavirus (strain OC43); only limited identity (<25%) was observed with group 1 and group 3 coronaviruses. Based on these findings, the virus has been tentatively identified as equine coronavirus (ECV). ECV NC99 was determined to have close antigenic and/or genetic relationships with mammalian group 2 coronaviruses, thus identifying it as a member of this coronavirus antigenic group.