Relative quantification of white blood cell mitochondrial DNA and assessment of mitochondria by use of transmission electron microscopy in English Springer Spaniels with and without retinal dysplasia

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Objective—To compare relative amounts of WBC mitochondrial DNA (mtDNA; assessed via real-time PCR assay) and morphology of lymphocyte mitochondria (assessed via transmission electron microscopy [TEM]) in blood samples collected from English Springer Spaniels with and without retinal dysplasia.

Animals—7 and 5 client-owned English Springer Spaniels (1 to 11 years old) with and without retinal dysplasia, respectively.

Procedures—Blood samples were obtained from affected and unaffected dogs via venipuncture. Genomic DNA was extracted from WBCs of the 7 affected and 5 unaffected dogs, and relative quantification of the cytochrome c oxidase subunit 1 gene (COX1) was determined via analysis of real-time PCR results. White blood cells from 3 affected and 4 unaffected dogs were embedded in epoxide resin for TEM; cross sections were examined for lymphocytes, which were measured. The mitochondria within lymphocytes were quantified, and the mitochondrial surface area per lymphocyte cross section was calculated. A masked technique was used to compare mitochondrial morphology between the 2 groups.

Results—Compared with the smallest measured quantity of mtDNA among unaffected dogs, mtDNA amounts varied among unaffected (1.08- to 4.76-fold differences) and affected dogs (1- to 2.68-fold differences). Analysis of lymphocyte measurements and mitochondrial surface area, morphology, and quantity revealed no significant differences between affected and unaffected dogs.

Conclusions and Clinical Relevance—No significant differences were detected in relative amounts of WBC mtDNA or the size, number, or morphology of lymphocyte mitochondria in English Springer Spaniels affected with retinal dysplasia, compared with results for unaffected control dogs. (Am J Vet Res 2010;71:454–459)

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Inherited retinal dysplasia in English Springer Spaniels has been studied, yet the pathogenesis of this disease is unknown. In 1 study, the eyes of English Springer Spaniel fetuses with retinal dysplasia (examined by use of light microscopy and TEM) had a marked decrease in the size of and area occupied by gap junctions within the external limiting membrane, compared with those of mixed-breed dog fetuses without retinal dysplasia. It was proposed that inappropriate expression of cell adhesion molecules or glycoproteins on the neuroepithelium or Müller cells may lead to poor cell adhesion and the inability to form cell junctions or establish proper communication between cells, which results in disorganized proliferation of neuroblasts and causes the typical retinal dysplasia rosettes that are observed via light microscopy.

In another study, investigators detected differences in intramembranous particle density and filipin binding in the retinas of affected English Springer Spaniels, compared with findings in age-matched unaffected control dogs. Analysis of those results suggested that

Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>COX1</td>
<td>Cytochrome c oxidase subunit 1 gene</td>
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<tr>
<td>Ct</td>
<td>Threshold cycle</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase gene</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>T_m</td>
<td>Melting temperature</td>
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abnormalities in the membrane structure of neuroblastic cells may be related to the dysplasia process. Other investigators proposed that the defective development of Müller cells or abnormal glialovascular relationships may result in retinal dysplasia because disorganized Müller fibers and focal defects in the external limiting membrane have been detected histologically within the dysplastic areas. These mechanisms may also cause other aspects of this disease, such as retinal detachment and persistence of the hyaloid vasculature.

Investigators reported in 2006 that the WBCs of Miniature Schnauzers that had retinal dysplasia had decreased amounts of mtDNA (determined via real-time PCR analysis), compared with amounts in WBCs from unaffected Miniature Schnauzers. The study also revealed that the retinas and RPEs of affected Miniature Schnauzers had decreased expression of several mitochondrial genes as well as transcription factor A mitochondrial, a nuclear-encoded gene that controls mitochondrial gene transcription and copy number. The mitochondria of affected Miniature Schnauzers altered morphology and were reduced in size and number, compared with findings in unaffected dogs. Those conclusions led to the postulation that retinal dysplasia in this breed may be attributable to decreased retinal energy supply.

Retinal dysplasia in English Springer Spaniels is similar in several aspects to that in Miniature Schnauzers. In both breeds, retinal dysplasia is inherited as a bilateral, geographic condition that is occasionally associated with retinal detachment or nonattachment. Histologically, the eyes of affected dogs of both breeds have an incomplete external limiting membrane, convoluted outer nuclear layer, and dysplastic outer and inner segments. Therefore, the molecular and morphological findings for Miniature Schnauzers may also apply to other breeds. The purpose of the study reported here was to compare the relative amounts of mtDNA in the WBCs of several English Springer Spaniels with and without retinal dysplasia by use of real-time PCR analysis. Detection of decreased amounts of mtDNA in the WBCs of affected dogs could provide the basis for a potential screening test for the disease. Quantification, measurement, and morphological assessment of lymphocyte mitochondria were also performed to further characterize retinal dysplasia in English Springer Spaniels.

**Materials and Methods**

**Animals**—Twelve client-owned English Springer Spaniels (7 with and 5 without retinal dysplasia; 6 males and 6 females; age range, 1 to 11 years) were included in the study. Ocular examinations were performed by a veterinary ophthalmologist to determine whether dogs were affected with retinal dysplasia. General physical examinations did not detect any systemic abnormalities except for the ocular defects. All dogs were handled according to the standards set by the Canadian Council on Animal Care and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals and Ophthalmic and Vision Research. The study and procedures were approved by the University Committee on Animal Care and Supply Animal Research Ethics Board of the University of Saskatchewan, and owner consent was obtained prior to inclusion of the dogs in the study.

**Experiment design**—For mtDNA relative quantification, samples of WBCs were obtained from the blood of 7 affected (4 male and 3 female) and 5 unaffected (2 male and 3 female) English Springer Spaniels. For mitochondrial evaluation, WBC samples were obtained from 3 affected (2 male and 1 female) and 4 unaffected (all female) dogs.

**Sample preparation and DNA extraction**—Single blood samples were collected via jugular venipuncture from affected and unaffected dogs into EDTA-containing tubes. The RBCs were lysed with Tris ammonium chloride, and the WBC pellets were collected via centrifugation. For TEM, a portion of the WBCs was fixed in 5% glutaraldehyde with 0.2M s-collidine buffer. The fixed cells were embedded in epoxy resin, sectioned at a thickness of 90 to 100 nm, and stained with uranyl acetate. For PCR analysis, the total cellular DNA of WBCs was extracted by use of a phenol-chloroform method after treatment with proteinase K. The DNA was quantified by use of a spectrophotometer, and absorbance was measured at 260 and 280 nm.

**Primer design and real-time PCR conditions**—To estimate mtDNA values, COX1 was selected as a representative mitochondrial gene, and GAPDH was selected as a reference gene to normalize COX1 values. Optimal primers for COX1 and GAPDH were designed and synthesized. Primer design criteria included similar Tm values and avoidance of primer and template secondary structure at the primer Tm. A bioinformatics algorithm search of a genetic sequence database was performed on the primers and the canine genome to ensure specificity.

The designed primer pairs were tested in a standard PCR reaction, and products were detected by the use of ethidium bromide after agarose gel electrophoresis. Those primers that yielded a clean, single product of expected size were selected and further tested for their ability to produce a sharp melting curve peak after successive PCR cycles in a thermal cycler. The nucleotide sequences and Tm of the primer pair selected for GAPDH were as follows: sense strand, 5′-GGTCATCGTTCGCTGAGAT-3′; Tm = 59.6°C; and antisense strand, 5′-TGCCTGACAATCTTGAGGGAT-3′; Tm = 93.9°C. These primers yielded a 184-bp product that had a calculated Tm of 91.8°C and a measured Tm of 86.3°C. The nucleotide sequences and Tm of the primer pair selected for COX1 were as follows: sense strand, 5′-GGATGCTGGTGCTGAGAT-3′; Tm = 59.6°C; and antisense strand, 5′-TGCTGACACACAGCGGTA-3′; Tm = 55°C. These primers yielded a 77-bp product that had a calculated Tm of 82.4°C and a measured Tm of 80.3°C.

The relative quantification of COX1 concentrations was determined for dogs in the unaffected group by comparison with the results for the unaffected dog that had the least amount of mtDNA, and the same analysis was performed for dogs in the affected group. The PCR amplifications were carried out in a total volume of 25 μL, which contained 0.5μM of each primer,
Melting curve analysis—The melting curve analysis to ensure product homogeneity was provided by the system software and was performed after the real-time PCR procedure. The amplified products were incubated at 95°C for 1 minute followed by ramped-up temperatures of 55°C to 95°C at a rate of 0.2°C/s. The fluorescence data were collected continuously on the ramp.

TEM—A minimum of 10 lymphocytes from each dog were examined via TEM and photographed (13,000X). Selected lymphocytes were agranular and had round or oval nuclei and scant cytoplasm. Lymphocytes were selected instead of other WBCs because they were easily identified, and the lack of cytoplasmic granules simplified identification of mitochondria. Images were scanned into a computer and viewed with a graphics program. Mitochondrial assessment was performed in a masked manner; the images were randomly assigned identification numbers, and individuals who interpreted mitochondrial morphology and performed measurements were not informed of the identity or the grouping of dogs from which samples were obtained. The perimeter of each photographed lymphocyte was measured, and the surface area was calculated (µm²). Mitochondria within each lymphocyte cross section were identified and counted. Selection criteria for the mitochondria included double membrane organelles and visible cristae. The perimeter of each mitochondrion was measured, and the mitochondrial surface area (µm²) per lymphocyte cross section was calculated.

Table 1—Mean ± SD Cₜ quantifications from real-time PCR assay of COX1 and GAPDH amplicons in WBCs of English Springer Spaniels (dogs 1 to 5) without retinal dysplasia.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Variable</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cₜ, COX1</td>
<td>19.46 ± 0.13</td>
<td>19.69 ± 0.10</td>
<td>17.86 ± 0.10</td>
<td>21.83 ± 0.16</td>
<td>20.35 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Cₜ, GAPDH</td>
<td>15.55 ± 0.05</td>
<td>15.89 ± 0.05</td>
<td>16.20 ± 0.04</td>
<td>18.50 ± 0.19</td>
<td>18.03 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>∆Cₜ</td>
<td>3.91 ± 0.14</td>
<td>3.80 ± 0.11</td>
<td>1.66 ± 0.11</td>
<td>3.33 ± 0.25</td>
<td>2.23 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>∆∆Cₜ</td>
<td>0 ± 0.14</td>
<td>-0.11 ± 0.11</td>
<td>-2.25 ± 0.11</td>
<td>-0.58 ± 0.26</td>
<td>-1.59 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.00</td>
<td>1.08</td>
<td>4.76</td>
<td>1.49</td>
<td>3.01</td>
</tr>
</tbody>
</table>

∆Cₜ = Difference between mean Cₜ value of COX1 and mean Cₜ of GAPDH for samples from the same dog. ∆∆Cₜ = Difference between ∆Cₜ for samples from a given dog and ∆Cₜ for samples from dog 1 (ie, ∆Cₜ value normalized to dog 1). R = Fold difference of total WBC mtDNA expressed as a ratio relative to unaffected dog 1.
face area values were not significantly ($P = 0.097$) different between the 2 groups. Mitochondria number per lymphocyte cross section ranged from 0 to 9 (median, 3) in unaffected dogs and from 0 to 12 (median, 3) in affected dogs. The median values were not significantly ($P = 0.830$) different between the 2 groups. Mitochondrial surface areas ranged from 0.0199 to 0.3090 µm$^2$ (median, 0.0912 µm$^2$) in unaffected dogs and from 0.0209 to 0.3420 µm$^2$ (median, 0.0954 µm$^2$) in affected dogs. Statistical analysis revealed no significant ($P = 0.310$) difference in mitochondrial surface areas between unaffected and affected dogs. Mitochondrial morphology was assessed by use of a masked technique and did not appear altered between the 2 groups.

**Discussion**

The amount of WBC mtDNA in English Springer Spaniels varied widely in the present study. The values were independent of whether dogs were affected with retinal dysplasia. Additionally, there was no significant difference in lymphocyte surface area or mitochondrial number, size, or morphology between affected and unaffected dogs. In another study,$^9$ it was suggested that the pathogenesis of retinal dysplasia in Miniature Schnauzers may result from a reduced energy supply to the retina and RPE. This was proposed on the basis of decreased WBC mtDNA and decreased mitochondrial number and size in multiple tissues of affected Miniature Schnauzers, compared with findings in unaffected dogs of this breed.$^6$

In the study reported here, the relative quantities of WBC mtDNA in English Springer Spaniels with and without retinal dysplasia were compared via real-time PCR analysis. The values of all dogs were calibrated against those of the unaffected dog that had the least amount of mtDNA (dog 1). Because affected dogs were hypothesized to have less WBC mtDNA than unaffected dogs, mtDNA values of the affected dogs were also calibrated against the value from dog 1 (Table 2). The relative ratios obtained by the Pfaffl method,$^7$ which corrects for differences in amplification efficiency, were similar to those obtained by use of the $\Delta\Delta C_T$ method.$^8$ Homogenity of the accumulated PCR products was confirmed in the assays by dissociation curves, which had single sharp peaks (Figure 2).

TEM—Images from a total of 81 lymphocytes were obtained and measured (48 from unaffected dogs and 33 from affected dogs, Table 3). Lymphocyte surface areas ranged from 8.76 to 28.36 µm$^2$ (median, 17.62 µm$^2$) in unaffected dogs and from 8.79 to 26.48 µm$^2$ (median, 18.94 µm$^2$) in affected dogs. Mean lymphocyte surface area values were not significantly ($P = 0.097$) different between the 2 groups. Mitochondria number per lymphocyte cross section ranged from 0 to 9 (median, 3) in unaffected dogs and from 0 to 12 (median, 3) in affected dogs. The median values were not significantly ($P = 0.830$) different between the 2 groups. Mitochondrial surface areas ranged from 0.0199 to 0.3090 µm$^2$ (median, 0.0912 µm$^2$) in unaffected dogs and from 0.0209 to 0.3420 µm$^2$ (median, 0.0954 µm$^2$) in affected dogs. Statistical analysis revealed no significant ($P = 0.310$) difference in mitochondrial surface areas between unaffected and affected dogs. Mitochondrial morphology was assessed by use of a masked technique and did not appear altered between the 2 groups.

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In the study reported here, the relative quantities of WBC mtDNA in English Springer Spaniels with and without retinal dysplasia were compared via real-time PCR analysis. The values of all dogs were calibrated against those of the unaffected dog that had the least amount of mtDNA (dog 1). Because affected dogs were hypothesized to have less WBC mtDNA than did unaffected dogs, this was a more stringent approach to analysis than a comparison to the mean value of all unaffected dogs would have been. Compared with the mtDNA value for dog 1, the values of unaffected dogs ranged from a 1.08- to a 4.76-fold difference, and the values of affected dogs ranged from a 1.04- to a 2.66-fold difference. The calculated relative ratios of mtDNA
were similar when both the ΔΔCT method\textsuperscript{10} and the Pfaff method\textsuperscript{11} were used, which indicated that the difference in amplification efficiencies of GAPDH and COXI (1.883 and 1.913, respectively) did not have a significant effect on relative quantification.

Mitochondrial biogenesis and mtDNA maintenance depend on coordinated expression of genes in the nucleus and the mitochondria. The abundance of mitochondria and mtDNA necessary to meet a cell's energy needs is determined by a variety of hormonal and second messenger signals and may change when energy demand and physiologic or environmental conditions change.\textsuperscript{12} Thus, a variety of factors can influence the amount of mtDNA in a particular cell. It has been proposed that increased oxidative stress contributes to the abundance of mitochondria in addition to mtDNA content.\textsuperscript{12} This was determined in a study\textsuperscript{13} of human leukocytes, in which elevated oxidative stress led to increased mtDNA amounts. In human patients, hyperlipidemia also results in decreased mtDNA.\textsuperscript{14} The blood samples collected from English Springer Spaniels in our study were not grossly lipemic; however, exact measurements of lipoprotein concentration were not determined.

The effect of age on mtDNA amount is controversial and tissue dependent.\textsuperscript{15} In many tissues (eg, mouse heart, spleen, and kidney\textsuperscript{16} and human lung\textsuperscript{17}), mtDNA content increases with age, whereas there is no age-related pattern in human bone marrow.\textsuperscript{16} Quantifications of mtDNA content in mouse and human brains\textsuperscript{16,18} and skeletal muscles\textsuperscript{16,19–22} in relation to age are inconsistent. To our knowledge, the correlation between age and amounts of WBC mtDNA has not been investigated. In the study reported here, the ages of the dogs did not appear to correlate with WBC mtDNA content. How-ever, on the basis of the results of aforementioned investigations in other tissues, age-matched control dogs should be included if mtDNA content is to be further evaluated in dogs affected with retinal dysplasia.

A study\textsuperscript{20} of mtDNA in aged human muscle revealed that mtDNA concentrations are only weakly correlated to age-adjusted aerobic fitness (maximal oxygen consumption and self-reported levels of physical activity); therefore, aerobic fitness of each dog was not considered an important factor in our study.

Results of the 2006 investigation\textsuperscript{6} of retinal dysplasia in Miniature Schnauzers suggested that the amount of mtDNA is decreased in WBCs of affected dogs. However, considering the results of the present study, this may not be the case in other breeds of dogs. Additionally, the mtDNA content varies widely in different tissues,\textsuperscript{16} and the differences detected may be secondary to tissue-specific energy demands.\textsuperscript{23} Because many dogs affected with retinal dysplasia are otherwise healthy, the relative quantification of mtDNA in the retina and RPE of age-matched affected and unaffected dogs would increase our understanding of the role of mtDNA in the pathogenesis of the condition. Although decreased retinal and RPE mitochondrial transcripts were observed in affected Miniature Schnauzers,\textsuperscript{6} to the authors' knowledge, the correlation between mtDNA quantity and transcript content in the retinas of healthy dogs and dogs with retinal dysplasia has not been evaluated in other breeds. Additionally, mtDNA content and transcript amount do not always have a positive correlation.\textsuperscript{16} Aging has been associated with increased mtDNA content and reduced transcript quantity in human brains.\textsuperscript{18} In liver, heart, and skeletal muscle in rats, decreases in mtDNA values do not correlate with decreases in transcripts, nor do reduced mtDNA values appear to affect enzyme activities.\textsuperscript{19}

Mitochondrial DNA depletion in association with abnormal mitochondria in dogs and mice has been reported.\textsuperscript{6,7,24} Abnormal mitochondria have been detected in mouse pancreatic cells with severe mitochondrial depletion and deficient oxidative phosphorylation\textsuperscript{24} as well as in human mtDNA-depleted myoblasts\textsuperscript{25} and mouse mtDNA-depleted embryos.\textsuperscript{7} The report\textsuperscript{6} of a possible decrease in WBC mitochondrial number and size as well as altered mitochondrial morphology in Miniature Schnauzers affected with retinal dysplasia led to the quantification, measurement, and morphological assessment of lymphocyte mitochondria in English Springer Spaniels in our study. We observed no differences in mitochondrial number, size, or morphology between unaffected dogs and those affected with retinal dysplasia. The observation in our study that the amount of WBC mtDNA is not decreased in affected versus unaffected English Springer Spaniels also supports these TEM findings. However, there are limitations to the use of TEM to evaluate mitochondria. The classic measurement of mitochondrial section by this method is limited by the complex 3-D organization of mitochondria,\textsuperscript{26} and a more accurate method of determining mitochondrial content would be to measure citrate synthase activity.\textsuperscript{23}

Analysis of the results of our study suggests it is possible that the pathogenesis of retinal dysplasia in English Springer Spaniels may be different from that in Miniature Schnauzers. However, evaluation of a greater number of English Springer Spaniels with retinal dysplasia would be beneficial to confirm these negative findings.

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Table 3—Median lymphocyte surface area, number of mitochondria per lymphocyte cross section, and mitochondrial surface area in lymphocytes from blood samples collected from English Springer Spaniels with (n = 7) and without (5) retinal dysplasia.

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Total No. of lymphocytes</th>
<th>Lymphocyte surface area (μm(^2))</th>
<th>No. of mitochondria/lymphocyte cross section</th>
<th>Mitochondrial surface area (μm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected</td>
<td>33</td>
<td>18.94</td>
<td>3</td>
<td>0.0954</td>
</tr>
<tr>
<td>Unaffected</td>
<td>48</td>
<td>17.62</td>
<td>3</td>
<td>0.0912</td>
</tr>
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</table>

Data were obtained from images acquired via TEM (magnification, 13,000×).
References