Investigation of the composition, turnover, and thermal properties of ruptured cranial cruciate ligaments of dogs

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Objective—To assess different components of the extracellular matrix with regard to their thermal properties, composition, and turnover in ruptured cranial cruciate ligaments (CCLs) of dogs, compared with components of intact CCLs from a breed predisposed to CCL failure.

Sample Population—Ruptured CCLs obtained from 8 dogs of breeds predisposed to ruptured CCLs and intact CCLs from 12 cadaveric Labrador Retrievers.

Procedure—Ruptured and intact CCLs were analyzed for water content; collagen content and collagen cross-links were evaluated via hydroxyproline and amino-acid analyses, respectively. Glycosaminoglycan (GAG) content was analyzed via dimethylmethylene blue and uronic acid assays. Matrix metalloproteinases (MMPs)-2 and -9 and the tissue inhibitors of metalloproteinases (TIMPs)-1 and -2 were detected via gelatin SDS-PAGE zymography and reverse gelatin zymography. Thermal analysis of ligaments was performed by use of differential scanning calorimetry.

Results—Ruptured CCLs had significantly higher lamounts of immature cross-links, total and sulfated GAGs, and water content, compared with that of the intact ligaments. Compared with intact CCLs, concentration of pro–MMP-2 was significantly higher in ruptured CCLs; the maximum temperature of collagen denaturation was significantly lower in the ruptured CCLs.

Conclusions and Clinical Relevance—The extracellular matrix of ruptured CCLs had an increased matrix turnover indicated by increased collagen and GAG synthesis, compared with that of intact CCLs. Although the extracellular matrix changes may have occurred before ligament rupture, it is possible that these observed changes may be part of a reparative process after rupture. (*Am J Vet Res* 2004;65:1136–1141)

The **cranial cruciate ligament** (**CCL**) is an important primary restraint for stability in the stifle joint in dogs, and its dysfunction contributes a major role in the pathogenesis of osteoarthritis of that joint.[1] Failure of the CCL in dogs is believed to be either traumatic (acute) or more commonly degenerative (chronic).[2,3] The etiopathogenesis of the chronic degenerative disease is unknown but is believed to be multifactorial; factors contributing to the development of disease include age-related changes,[4,5] immobilization,[6,7] presence of a stenotic intercondylar notch,[8] abnormal tibial plateau slope,[9] and breed predisposition.[4,10,11] Results of recent studies[10-12] have indicated higher incidence of CCL rupture in breeds such as Rottweiler and Labrador Retriever, with infrequent occurrence in breeds of a similar size such as Greyhound and Old English Sheepdog.

Most skeletal ligaments contain approximately 60% to 80% water, whereas nearly 70% to 80% of the dry weight of ligaments is collagen.[13] As much as 90% of the ligamentous collagen is type I collagen (the principal tensile-resistant fiber), but smaller quantities of types III, V, and VI are also present.[14] Intra- and intermolecular cross-links between collagen molecules confer mechanical strength and stability to tissues, and the ratio of immature to mature cross-links can provide an indication of the relative rates of collagen turnover of that tissue.[15] **Glycosaminoglycans** (**GAGs**) and proteoglycans compose 1% dry weight of the total ligamentous tissue and are involved in the lubrication of ligaments.[16,17] Compared with concentrations in healthy tendons, proteoglycan and GAG concentrations are increased in injured and healing tendons in rabbits and humans,[18,19] but little is known about those concentrations in ruptured anterior cruciate ligaments or canine CCLs.

The primary mechanism of all ligament degradation involves active proteinases, such as cysteine proteases (eg, cathepsin K) and **metalloproteinases** (eg, **MMPs**-2 and -9) that contribute to the turnover of collagen and proteoglycans in the extracellular matrix.[20] Matrix degradation in ligaments is balanced by collagen synthesis and the inhibitors of the active proteinases (eg, **tissue inhibitors of metalloproteinases** [**TIMPs**]), but excessive MMP-2 concentrations may lead to degradation. Ruptured anterior cruciate ligaments in humans and ruptured CCLs in dogs have been studied in terms of their histologic appearance [5,21,22] by determination of grades of rupture and examination of ligament fibroblast viability. The ultrastructural appearance of ruptured ligaments has been extensively studied via scanning electron microscopy to examine the rupture ligament fascicles and collagen fibers.[23-25] However, to date, few studies have examined extracellular matrix turnover and metabolism of ruptured CCLs, particularly with regard to biochemical changes present at

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the end stage of ligament disease. At a molecular level, the expression of collagen and MMPs in ruptured anterior cruciate ligaments in humans have been examined.[26] Results of a recent investigation[27] indicated increased activities of tartrate-resistant acid phosphatase and cathepsin K in ruptured CCLs, compared with those in intact CCLs, which suggests that proteinases may be involved in the collagenolytic remodeling of canine CCLs. However, in that study, control specimens from Beagles (a breed that is not predisposed to CCL disease) were used.

The purpose of the study reported here was to assess different components of the extracellular matrix with regard to their thermal properties, composition, and turnover in CCLs of dogs, compared with components of intact CCLs from a breed predisposed to CCL failure. We hypothesized that increased turnover and degradation of the major extracellular matrix components in ligament (ie, collagen type I and proteoglycans) would be evident in ruptured CCLs, compared with that of intact CCLs.

Materials and Methods

Animals—Specimens of ruptured CCLs were obtained from affected dogs during a standard surgical procedure completed according to guidelines compiled by the Royal College of Veterinary Surgeons, UK, and therefore our study was not approved by an institutional animal care and use committee. Specimens of ruptured CCLs were obtained from 8 dogs (2 males and 6 females) during surgery for CCL reconstruction with extracapsular stabilization techniques.[28] For inclusion in the study, dogs had to be adult (of any age) and have a ruptured CCL identified at stifle arthrotomy; the dogs' weight and sex were unimportant. The exclusion criterion was other musculoskeletal conditions such as osteoarthritis of the hips. Data on the exact duration of lameness were not available for these dogs; however, all dogs had lameness in the affected hind limb that was localized to the stifle joint at least 1 month prior to evaluation. The presurgical diagnosis was made on the basis of findings of physical examination; detection of cranial drawer, stifle joint effusion, and new bone formation on the medial aspect of the joint (medial buttress); radiographic evidence of new bone formation in the stifle joint; and presence of a ruptured CCL at stifle arthrotomy. The samples of ruptured CCLs were obtained from 5 Labrador and Golden Retrievers (breeds susceptible to CCL rupture), 2 Springer Spaniels, and 1 German Shepherd Dog. These dogs were 0.7 to 11 years old (mean ± SD age, 4.56 ± 3.69 years) and weighed 25 to 35 kg (mean weight, 31.13 ± 3.40 kg).

Specimens of intact CCLs were obtained from macroscopically normal-appearing stifles in a group of 11 Labrador Retriever cadavers (6 males and 5 females). These dogs (control group) had been referrals for a second opinion and were evaluated at the University of Bristol; they were euthanatized (with an IV injection of phenobarbitone) for reasons unrelated to musculoskeletal disease according to approved guidelines from the Department of Clinical Veterinary Science regarding use of animal material for research. Owners provided written consent prior to sample removal. Dogs in this group were 0.5 to 11 years old (mean age, 7.25 ± 3.33 years) and weighed 19 to 36 kg (mean weight, 31.25 ± 6.06 kg).

Sample collection and storage—Each stifle joint was examined for evidence of osteoarthritis by use of standard radiographic views, prior to opening the joint postmortem or intraoperatively. The presence of osteophytes on the femoral trochlear ridges was used as an indicator of osteoarthritis of the stifle joint, which was confirmed at arthrotomy. Samples were obtained from the midsection of the intact ligaments and of the ruptured ligaments, but usually only CCL remnants were obtained. The CCL remnants obtained were not directly adjacent to their origin or insertion. Samples (wet weight, 5 to 10 mg) were collected in a sterile container with no medium and stored immediately at –20°C.

Collagen cross-links analysis—The preparation and quantitation of collagen cross-links were performed, as described by Sims and Bailey.[29] Quantitation of the intermediate cross-links requires their reduction with borohydride (wet weight, approx 1%) to stabilize them prior to acid hydrolysis. The mature cross-links (eg, hydroxypyridinoline) are stable to acid hydrolysis. Briefly, 20 to 50 mg of wet CCL tissue was thawed and suspended in 500 µL of PBS solution prior to borohydride reduction and acid hydrolysis. After reduction with potassium borohydride in 1mM NaOH, the samples were hydrolyzed for 24 hours in 6N HCL at 110°C and a 400-µL aliquot was fractionated on CF1-cellulose.[a] After fractionation, the effluent containing the adsorbed amino acids was freeze-dried and reconstituted in 150 µL of 0.01N HCL, filtered, and applied to an amino acid analyzer[b]; the separated cross-links were quantified by use of ninhydrin and known leucine equivalents. The data were processed by use of appropriate software.[c]

Total collagen content—Hydroxyproline was determined via analysis of an aliquot of the acid hydrolysate on a continuous-flow hydroxyproline analyzer. The collagen content of the samples was then calculated by assuming a 14% hydroxyproline content for collagen and multiplied accordingly. The method was based on that described by Woessner.[30] Standards of 1 to 5 µg of hydroxyproline/mL were used to calculate a standard curve and the sample solutions appropriately diluted to within this range.

Gelatin substrate SDS-PAGE zymography for detection and quantitation of MMP-2 and MMP-9—The method of gelatin substrate SDS-PAGE zymography used was based on a method that has been described.[31] In brief, tissue samples were weighed and fragmented (at –80°C in liquid nitrogen) by use of a freeze mill. The powdered tissue was weighed, and extraction buffer (0.1% polyoxyethylene monolauryl ether in 20mM triethanolamine [20 µL of buffer/mg of tissue]) was added. The soluble protein was extracted at 4°C with constant agitation for 12 to 18 hours. The remaining insoluble material was removed by centrifugation (at 7,500 × *g* for 20 minutes) and the supernatant stored at –20°C until required.

Supernatant samples were thawed at 21°C and reconstituted in sample buffer (0.06M Tris-HCL [pH, 6.8], 2% SDS, 10% glycerol, and 2 mg bromophenol blue). These samples, MMP-2 human standards,[d] and rainbow protein molecular weight markers[e] were separated in a 10% gelatin resolving gel by use of a standard mini-electrophoresis system.[f] After electrophoresis, the gels were washed in 2.5% Triton for 30 minutes to displace the SDS and then incubated for 16 to 20 hours at 37°C in MMP proteolysis buffer (500mM NaCl, 50mM $CaCL_2$, and 50mM Tris-HCL [pH, 7.8]). After staining with Coomassie brilliant blue R250[g] (0.25%), then destaining for 1 hour, zones of proteolysis were revealed. The relative quantities of proteolytic enzymes were analyzed via scanning densitometry[32] and values expressed as a percentage of activity of the pro- and active MMP-2 standard (2 ng/gel) used as an internal standard of gel clarification.

Reverse gelatin zymography for the detection and quantitation of TIMP-1 and TIMP-2—The method of reverse gelatin zymography was based on a method described by Oliver et al.[33] Gelatin was incorporated into a 12.5% resolv-

ing gel at a final concentration of 2.25 mg/mL. Recombinant human MMP-2[h] was also copolymerized into the gel at a final concentration of 0.5 µg/mL. The samples (diluted 1:1 in sample buffer), standard,[i] and molecular weight markers[j] were loaded onto the gel, and SDS-PAGE was performed under nonreducing conditions. The gels were washed in 2.5% Triton for 90 minutes and incubated in MMP proteolysis buffer for 48 hours, after which they were stained and destained at 21°C, as described. The areas of inhibition (mainly TIMP-2) were marked as dark staining areas on a clear background. Duplicate gels without substrate were run in parallel to identify protein bands. The relative values were calculated as previously described for gelatin zymography.

GAG assays—Sulfated GAG content was assayed by use of an assay described by Farndale et al.[34] The wet weight of samples of CCLs was 10 to 50 mg. Briefly, samples underwent a papain[k] digest for 18 hours at 60°C and were reacted with the color reagent 1,9–dimethylmethylene blue[l] (polychromatic dye). The optical density was recorded after 1 minute at a wavelength of 535 nm by use of a spectrophotometer.[m] Any sample with an optical density that was not within the linear part of the standard curve (0 to 40 µg/mL) was rediluted and remeasured.

Total GAG (uronic acid) content was assayed via a method based on that described by Bitter and Muir.[35] A sodium tetraborate and carbazole mixture was added to each well of a microtiter plate and stored at –80°C for 2 to 3 hours. Thirty microliters of standard (glucuronic acid lactone [10 to 100 µg/mg]) and sample (taken from the papain digest described) were added to the prepared plates in triplicate and baked at 80°C for 30 minutes. The plates were cooled and read in a plate reader[n] at a wavelength of 490 nm.

Thermal analysis via differential scanning calorimetry—The thermal properties of collagen in the CCLs were assessed via **differential scanning calorimetry** (**DSC**).[36] Thermal analysis of collagen can provide information about the state of collagen in collagenous tissues such as tendon and ligament[36,37] by monitoring changes in the **maximum temperature of denaturation** (T_{max}) and the energy (or enthalpy) involved in denaturation. Approximately 5 to 15 mg of CCL specimens that had been stored at –20°C were thawed at 21°C. The collagen was heat denatured by use of a calorimeter[o] fitted with intracoolers and a computer controller. Each aliquot was sealed in aluminium pans, weighed, and scanned from 5 to 120°C at 10°C/min. The pans were placed in an oven at 100°C for 18 hours, and the collagen content of the specimens was measured by determining hydroxyproline concentration, as described. The enthalpy of denaturation and T_{max} of the DSC thermograms were determined in the specimens of ruptured CCLs and compared with those of specimens of healthy, intact CCLs obtained from Labrador Retrievers. The DSC thermogram peak properties of these ligament specimens were also determined (peak height, width, and the height-to-width ratio); changes in both the T_{max} and enthalpy (area under the thermogram peak) can provide evidence of an alteration in the state of collagen fiber, such as partial denaturation and fiber packing.[36]

Assessment of water content—The percentage of water content in both ruptured and intact CCLs was determined by dividing the freeze-dried weight by the wet weight of the ligaments.

Statistical analyses—The data were statistically analyzed with standard software.[p] Data did not satisfy the hypothesis of normality and therefore, nonparametric tests were applied. Data from ruptured and nonruptured ligaments were compared by use of a Mann Whitney *U* test. Values of $P < 0.05$ were considered significant. The relationships among background variables (age, weight, and sex) on the extracellular matrix parameters examined were determined via multiple regression tests. Results are presented as mean values ± SD.

Results

Sample population—All ruptures in the CCLs of affected stifle joints were complete, and there was no evidence of meniscal damage. Osteoarthritis was confirmed by the presence of osteophytes on the distal femoral trochlear ridges. Radiographic evidence of osteoarthritis was confirmed by the presence of joint effusion and new bone formation on the femur, tibia, patella, or fabellae. The stifle joints from the control group had intact CCLs and no anatomic or radiographic evidence of osteoarthritis, according to the criteria described.

Collagen content—The collagen content (% dry weight) of the ruptured CCLs was 66.49 ± 21.94%, compared with 58.73 ± 6.73% in the unruptured CCLs. There were no significant ($P = 0.41$) differences in the collagen content between the 2 groups of ligaments.

Collagen cross-links—The collagen cross-links detected in the ruptured and intact CCLs were the mature cross-link hydroxypyridinoline and the immature cross-links, dihydroxylysinorleucine and hydroxyly sinorleucine (**Figure 1**). There were significantly higher amounts of both types of immature cross-links (dihydroxylysinorleucine, $P = 0.03$; hydroxylysinorleucine, $P = 0.02$) in the ruptured ligaments, compared with amounts in the unruptured CCL specimens from Labrador Retrievers. The ratio of immature to mature

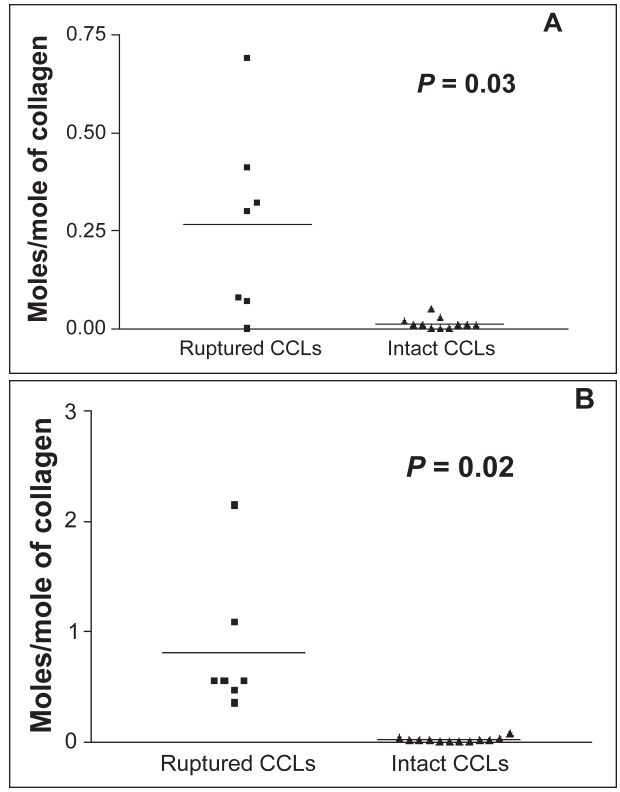

Figure 1—Dot diagram of the concentration of immature collagen cross-links dihydroxylysinorleucine (A) and hydroxylysinorleucine (B) in 7 ruptured and 11 intact cranial cruciate ligaments (CCLs) from 18 dogs. Horizontal lines represent the mean values of each data set.

cross-links was 4:96 of total cross-links in control tissue and 57:43 of total cross-links in ruptured ligaments.

Total and sulfated GAGs contents—The sulfated GAG content was 0.27 ± 0.30% and 0.06 ± 0.007% wet weight in ruptured and intact CCLs, respectively; the value for the ruptured CCLs was significantly (P = 0.002) higher than that of the intact CCLs (**Figure 2**). The total GAG content was 0.42 ± 0.22% and 0.11 ± 0.03% wet weight in ruptured and intact CCLs, respectively; the value for the ruptured CCLs was significantly (P = 0.002) higher than that of the intact CCLs.

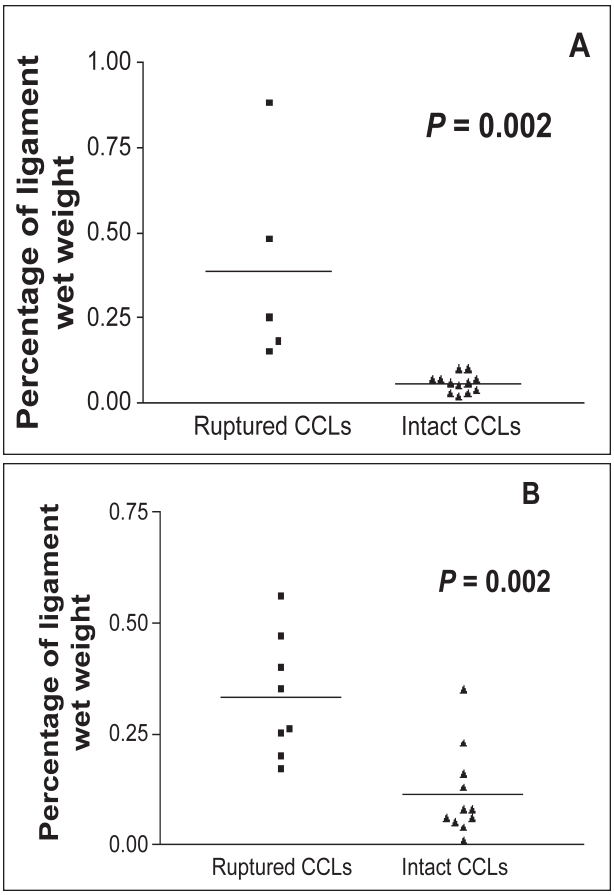

Figure 2—Dot diagram of sulfated (A) and total (B) glycosaminosglycan concentrations in 8 ruptured and 11 intact CCLs from 19 dogs. Horizontal lines represent the mean values of each data set.

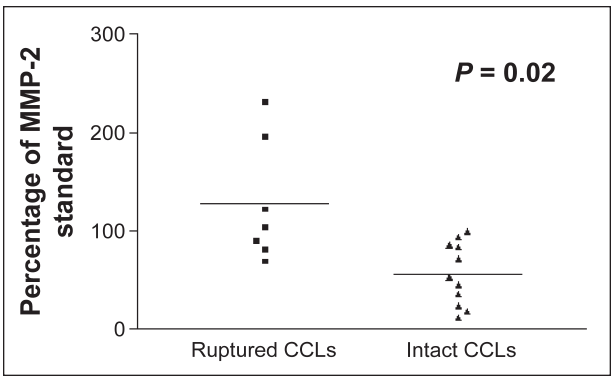

Figure 3—Dot diagram of the pro-form of matrix metalloproteinase 2 (pro–MMP-2) as a percentage of the MMP-2 standard indicative of collagen remodeling in 7 ruptured and 11 intact CCLs from 18 dogs. Horizontal lines represent the mean values of each data set.

Results of zymography to detect MMP-2, MMP-9, and TIMPs—Both pro- and active MMP-2 were detected in ruptured and intact CCL specimens, but pro–MMP-9 was only detected in 2 ruptured ligaments, which precluded further statistical analysis of the amounts of this enzyme. The relative concentrations of pro–MMP-2 were significantly (P = 0.02) higher in the ruptured CCL specimens (127.37 ± 23.37% MMP-2 standard) than in intact CCLs (56.48 ± 9.61% MMP-2 standard; **Figure 3**). There were no significant differences in the concentrations of activated MMP-2, TIMP-1, and TIMP-2 between ruptured and intact CCL specimens.

Thermal properties of ruptured CCLs—The T_{max} was significantly (P = 0.004) lower in the ruptured CCL specimens, compared with that of the intact CCLs (**Figure 4**). There were no significant differences in enthalpy of denaturation, peak height, peak width, or peak height-to-width ratio between the ruptured and intact CCL specimens.

Water content—The water content of the ruptured and intact CCL specimens was 84.28 ± 7.45% and 42.30 ± 12%, respectively. The difference in water content between groups was significant (P < 0.001; **Figure 5**).

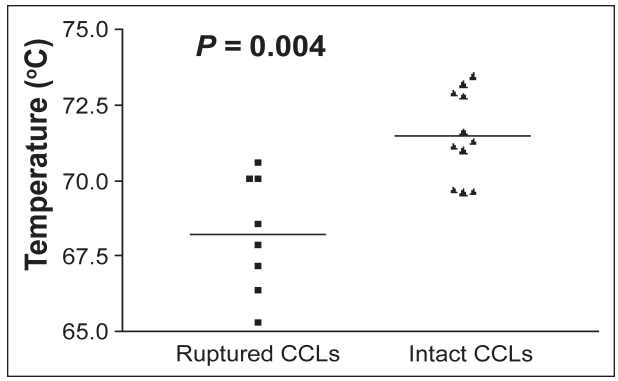

Figure 4—Dot diagram of the maximum temperature of denaturation determined via differential scanning calorimetry in 8 ruptured and 11 intact CCLs from 19 dogs. Horizontal lines represent the mean values of each data set.

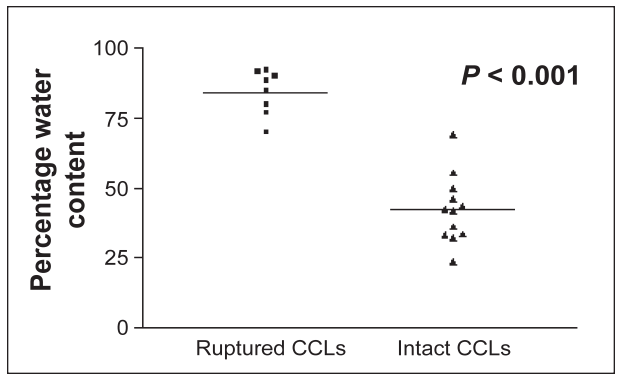

Figure 5—Dot diagram of the percentage water content in 8 ruptured and 11 intact CCLs from 19 dogs. Horizontal lines represent the mean values of each data set.

Effects of age, weight, and sex on the biochemical properties of ruptured CCLs—Age and sex did not have any relationship to the biochemical properties of the ruptured CCL specimens. Body weight did have a positive correlation with the concentration of the immature cross-link, dihydroxylysinorleucine ($r = 0.84$, $P = 0.02$).

Discussion

Our data have indicated that the turnover of the collagen of ruptured CCL ligaments is significantly higher than that of intact CCL ligaments and that the composition of the ruptured and intact ligaments is different. Compared with intact CCL specimens, ruptured CCL specimens had greater extracellular matrix synthesis as indicated by higher total collagen content, higher proportion of the immature collagen cross-links (implying the presence of new collagen), and higher GAG content. Higher collagen turnover in ruptured CCL ligaments was also indicated by the increased content of the degradative enzyme pro–MMP-2, compared with that detected in intact CCL specimens.

The authors of this report have had previous experience with DSC in the investigation of the integrity of the collagen fiber in collagenous tissues, such as equine tendons. The decreased denaturation temperature (T_{max}) detected in diseased tendons, compared with that of normal equine tendons, may be caused by the inadequate packing of the collagen molecules in the ligament fibers, and the lower enthalpy is indicative of the presence of denatured collagen.[36,38] To the authors' knowledge, no similar studies have been previously performed involving canine ligaments. In the study of this report, we detected a significantly lower T_{max} of the ruptured CCL specimens, compared with that of the control samples, but there was no significant difference in the enthalpy of denaturation between the 2 groups. A relationship between high water content of collagen fibers and low T_{max} has been demonstrated.[39] Therefore, the lower T_{max} of the ruptured CCLs suggests sparse packing of the newly synthesized collagen molecules as a result of the higher water content or disorganized alignment of the molecules in the fiber, compared with that of the control samples. The lack of a significant difference in enthalpy between the ruptured and intact CCL specimens indicates the absence of substantial amounts of degraded collagen retained in the fiber after rupture. Denatured collagen is rapidly cleared by MMP-2, and the concentrations of pro–MMP-2 were high in the ruptured CCL specimens. The DSC results therefore corroborate the biochemical results.

During normal collagen turnover, synthesis is balanced by degradation, but any imbalance can lead to fibrosis or tissue destruction. The extracellular matrix is generally under mechanical load, and the metabolism of the collagen is affected by changes in loading, such as stretching of skin, bone deformation,[40] or modification of the joint mechanics in osteoarthritis leading to subchondral bone sclerosis.[41] In previous investigations,[42,43] a sustained high turnover of collagen in the palmar aponeurosis in Dupuytren's disease was detected and tension on the palmar aponeurosis increased the production of active degradative enzymes, which ultimately resulted in the relaxation of the collagen fibers. Results of a subsequent controlled in vitro study[44] indicated that there was a direct correlation between tension applied and the amount of MMPs.

In the study of this report, the high concentration of pro–MMP-2 was consistent with increased collagen turnover in the ruptured ligaments. Although not significantly different, the collagen content of the ruptured CCLs was greater than that of the intact specimens, which may indicate that the balance of turnover is toward collagen deposition. This may in part be a consequence of the low level of activation of MMP-2 in ruptured ligaments.

Rupture is an endstage of ligament degeneration; in the study dogs of this report, it was not possible to stage the disease or determine the time of rupture of the CCLs. The inability to determine the timing of pathologic changes and rupture of the CCLs is one of the major limitations of our study; it was not possible to conclusively ascribe the extracellular matrix changes detected to the period before or after rupture of the CCLs. Other investigators have highlighted problems with the staging of CCL rupture in dogs at initial evaluation[8] because, at the time of surgery, most dogs with CCL rupture will also have notable osteoarthritis.[45] The other limitations of this study include small sample population sizes and the single-point evaluation of all biochemical data. In the study of this report, we used intact ligaments from breeds of dog that are predisposed to CCL rupture because, in our experience, there is a higher turnover of collagen in the CCLs in these breeds, compared with that in the CCLs of breeds that are not predisposed to rupture.[q] If it were possible to analyze partially ruptured CCLs, we would anticipate that results would indicate high levels of both collagen synthesis and degradative enzyme activity in those ligaments, with an imbalance in favor of degradation. In ligaments exposed to spatial differences in mechanical loading, greater tensile stress is associated with high collagen content and lower tensile stress with high GAG content.[46] Compared with the intact CCL specimens in the study of this report, the higher GAG content detected in the ruptured ligaments may reflect the release of tensile loading; the collagen changes in the ruptured ligaments may be an indication of attempted repair of the tissue. The extracellular matrix of the ruptured CCLs appears to have a balance of collagen turnover that promotes GAG production and collagen deposition, compared with that of nonruptured CCLs. However, this greater extracellular matrix turnover in ruptured CCLs may lead to random organization of the fibers of the CCL, which we suggest is an initial attempt at repair. The data collected in the study of this report may provide further insight into the biochemical changes involved in the pathogenesis of CCL disease and rupture.

[a]Whatman, Maidstone, Kent, UK.
[b]Alpha Two plus, Pharmacia, Camberley, Surrey, UK.
[c]Dionex AL450, Dionex plc, Tadworth, Surrey, UK.
[d]MMP-2, Biogenesis, Poole, UK.
[e]Mr=14-200kDa, Amersham, Buckinghamshire, UK.
[f]Mini Protean II, Bio-Rad, Hemel Hempstead, Herts, UK.
[g]Aldrich Chemical Co, Reading, UK.
[h]Oncogene Research Products, Calbiochem, Beeston, Nottinghamshire, UK.
[i]TIMP-2, Oncogene Research Products, Calbiochem, Beeston, Nottinghamshire, UK.
[j]Prestained SDS-PAGE Precision Standards, Bio-Rad, Hemel Hempstead, Herts, UK.

[k]Sigma, Reading, Berks, UK.
[l]DMMB, Sigma, Reading, Berks, UK.
[m]CE 2292, Cecil Instruments, Cambridge, UK.
[n]MCC/340 Labsystems Multiskan, Manchester, UK.
[o]Perkin Elmer DSC-2, Beaconsfield, Bucks, UK.
[p]Instat version 3.0, GraphPad Software, San Diego, Calif.
[q]Comerford EJ. *Evaluation of extracellular matrix composition, meta bolism, joint mechanics and joint conformation as potential predisposing factors of cranial cruciate ligament rupture in three dog breeds*. PhD Thesis, Department of Clinical Veterinary Science, University of Bristol, Bristol, UK, 2002.

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