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Original Contribution

HISTOLOGICAL CHANGES IN SPONTANEOUS RUPTURE OF THE CRANIAL CRUCIATE LIGAMENT IN THE DOG

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ABSTRACT

Introduction and aim of the work: The rupture of the cranial cruciate ligament (CrCL) is among the most common causes of pelvic limb lameness in dogs. The objective of this study was to investigate the histological changes in dogs with cranial cruciate ligament rupture and to compare the result using three different score systems

Materials and Methods: The study cohort comprised of 30 dogs of various breeds, ages, genders and body weights with spontaneous cranial cruciate ligament rupture divided into 3 groups (1 week, 2 weeks and 3 weeks' post-rupture). All tissue samples from CrCL were collected after open medial or lateral arthrotomy and histology slides were prepared and stained with hematoxylin-eosin. Three different score systems were used for grading the histological changes-synovitis score modified Bonar and modified Vasseur score.

Results: Our results showed that no inflammatory cells were observed in groups II and III and a very limited number in group I. We also found the highest erythrocytes deposition and larger lumen of blood vessels, uneven arrangement of the collagen fibers as well as fibrocytes transformation into chondrocytes in group I. In group II multiple blood vessel were also filled with erythrocytes and the number of chondrocytes is increased in comparison to group I. In group III blood vessels size and number were reduced in comparison to group I and II and many sections of the collagen fibers showed hyalinization.

Conclusion: In summary, degenerative changes were seen in all samples examined of cranial cruciate ligaments with different grades of chondroid metaplasia, increased cellularity and vascularity. Inflammatory changes were rarely detected by lymphoplasmacytic infiltrates with or without lymphoid aggregate deposition. No correlation was noted between age and all histological scores.

Key words: Dog, cruciate ligament, histological changes, score system

INTRODUCTION

Rupture of the cranial cruciate ligament (CrCL) in dogs is one of the most commonly reported orthopedic diseases in veterinary medicine (1, 2). Partial or complete disruption of CrCL causes hind limb lameness, joint inflammation and instability, meniscal injury, leading to secondary degenerative changes such as osteoarthritis (3).

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Cruciate ligaments are the primary stabilisers of the canine stifle joint. The two components are defined as cranial (CrCL) and caudal (CaCL). Canine CLs are considered to function as a unit, hence the term CL complex (4). Within the CLs, groups of collagen fibres are referred to as bundles (divided by interbundle regions), which are grouped as fascicles, divided by interfascicular regions (5).

The base unit of both cruciate ligaments is collagen which also contains many wavy fascicular subunits. Each fascicle usually consists of one up to 10 subfascicles, containing bundles of collagen fibers (6). As the collagen

subunits repeat, fibrils are formed, which in turn are organized into fibers (7). Their complex structure is a combination of differently oriented parallel, planar, twisted or spiral networks. The centrally located collagen fibrils are nearly straight, whereas those at the periphery are arranged in a helical wave pattern (8). This typical structure of CCL is important for their functioning, with the striated surface also determining their role in the biomechanics of the knee joint in the dog (9).

The cranial cruciate ligament is a complex structure consisting of an extracellular matrix (ECM) and a diverse population of cells. Histological features are typical of dense connective tissue and its anatomical elements such as ligaments and tendons. The ECM proteins in are mainly composed of type I collagen. Bundles of collagen fibers are longitudinally oriented, mostly running parallel to one another. Normal CrCL collagen fibers have a recurrent undulating wave or crimped structure (10). The matrix of the CrCL represents a complicated regulatory network of proteins, glycoproteins, viscoelastic fibers and glycosaminoglycans with multiple functional interactions. The most commonly observed cells in the CrCL are fibroblasts. They are arranged in long parallel rows between collagen fiber bundles. However, some authors reported that there is a zone where the tissue resembles fibrocartilage, making the histological texture inhomogeneous (11).

It was investigated, that the canine stifle joint is incompletely divided in the sagittal plane by a quite uniform fold of synovial membrane which covers both cruciate ligaments (12). These enveloping epiligamentous membranes consist predominantly of dense connective tissue, small fibroblasts, and some adipocytes (6). Scanning electron microscopy examination by Kobayashi et al. (13), discovered that cruciate ligaments are also supplied with nutrients due to the presence of multiple little openings in the synovial membrane that covers them.

Compared with other ligaments, the canine cranial cruciate ligament has poor intrinsic healing capacity and it has been suggested ligament healing in breeds at low risk of cranial cruciate ligament disease may be superior to those at higher risk (14).

Despite the clinical importance of cruciate ligament disease in dogs, few studies have investigated histological findings of cranial cruciate ligament rupture in relation to the duration of history. We therefore propose that a comparison of the histological changes after CCL rupture by three different scores will be a useful and valuable tool for better evaluation of its inflammation and degeneration.

The objective of this study was to determine and compare the histologic changes in spontaneous rupture of the cranial cruciate ligament in the dog focusing on the time of rupture by using three different histological scores.

MATERIALS AND METHODS

All dogs included in the present study were referred to the Small Animal Clinic of the Faculty of Veterinary Medicine, Stara Zagora, Bulgaria between September 2017 and March 2020 with a history of unilateral pelvic limb lameness. After confirmation of the tentative diagnosis, dogs with CCL rupture dating back to 3 weeks' maximum were used in the survey. All patients with bilateral CCL rupture, radiographic signs of osteoarthritis, medical treatment and body weight under 15 kg were excluded.

The study comprised 30 dogs (30 stifle joints) of different breeds, sex (15 males and 15 females), 68.76±25.65 months of age (mean±SD), weighing 33.72±5.65 kg (mean±SD).

Thirty tissue samples from CrCL were collected after open medial or lateral arthrotomy depending on the surgical technique and divided into three groups with 10 dogs in each group. Group I consisted of 10 dogs with a history of CrCL rupture up to 1 week, group II up to 2 weeks and group III up to 3 weeks. The resulting tissue samples were fixed in a 10% aqueous formaldehyde solution (Merck KGaA, Darmastadt, Germany). After fixation, they were washed under running water, dehydrated in ascending ethanol series, clarified in xylene and embedded in paraffin. Sequential cuts with a thickness of 4 μm were made using a rotary microtome Leica RM2235 (Germany). The preparations were stained with hematoxylin and eosin and slides were subsequently stored in the dark at room temperature. After all samples were obtained, slides were coded with random numbers, and interpreted by a single, experienced clinical pathologist who was blinded to sample identities and time points (RS).

The obtained preparations were observed with a trinocular microscope– Kern OBN 135 (Germany), and the results were documented using a digital HD camera Autofocus (Euromex, Netherlands). The results were interpreted in accordance with the histological terms.

All tissue samples were assessed for degenerative changes of the CCL by use of a modified Bonar score (15), modified Vasseur score (16) and synovitis score (17). Modified Bonar score was on a scale of 0 to 20, Modified Vasseur score was on a scale of 0 to 24 and synovitis score was on a scale of 0 to 18.

Statistical analysis. Data were presented as mean, standard deviation and range (for body weight and age) and as median and range (for clinical scores). Statistical analysis of data was done by the non-parametric Mann-Whitney method at level of significance $P<0.05$. Pearson correlation test was used to evaluate the relationships between the studied parameters.

RESULTS

Group I (mean ± SD age 72.3±26.06 months; range 41 to 110 months) comprised 6 males (1 castrated and 5 intact) and 4 females (3 spayed and 1 intact) consisting of 1 each of Chow Chow, Cane Corso, Labrador Retriever, German Wirehaired Pointer, German Shorthaired Pointer, and mixed breed; there were 2 Pit Bulls and Golden Retrievers **(Table 1).**

Group II (mean ± SD age, 78.2±25.92 months; range 27 to 120 months) comprised 6 females (2 spayed and 4 sexually intact) and 4 males (all sexually intact) consisting of 2 Central Asian Shepard Dogs, and 1 each of Siberian Husky, Bulgarian Shepard Dog, Bulgarian Scenthound, German Shepherd Dog, Golden Retriever, Labrador Retriever, Pit Bull, and Rottweiler **(Table 1)**.

Group III (mean ± SD age, 55.8±21.67 months; range 30 to 100 months) comprised 5 females (2 spayed and 3 sexually intact) and 5 males (3 castrated and 2 sexually intact) consisting of 2 English Bulldogs, and 1 each of Pit Bull, Labrador Retriever, Samoyed and Cane Corso; there were 3 mixed-breed dogs **(Table 1).**

Mean ± SD body weight was 33.2±6 kg (range 27.6 to 48.1 kg) for group I, 36.69±5.59 kg (range 28.8 to 44.6 kg) for group II, and 31.29± 4.35 kg (range 24.5 to 38.4 kg) for group III.

In general, degenerative changes were seen in all samples examined of cranial cruciate ligaments. Most ruptured CCLs had mild to moderate regenerative responses with different grades of chondroid metaplasia, increased cellularity and vascularity. Some of the samples had focal areas of poorly organized matrix loss of demarcation of collagen bundles, separation, disorganization and loss of collagen bundles. We also observed, synovial hyperplasia characterized by stacked plump synoviocytes, ligamentocytes with conspicuous cytoplasm and round nuclei, chondroid changes of ligamentocytes, as well as mucoid or cartilaginous changes of the extra-cellular matrix ground substance. Inflammatory changes were rarely detected by lymphoplasmacytic infiltrates with or without lymphoid aggregate deposition.

In group I (up to 1 week after the rupture) multiple areas of erythrocytes deposition and clusters of capillaries were observed in all specimens with a moderate number of inflammatory cells **(Figure 1)**. Most ruptured CCLs had mild regenerative responses with mild increases in vascularity and epiligamentous proliferation. An uneven arrangement of the collagen fiber was also found. Instead of the normal longitudinal or wave pattern, their position is jagged and uneven **(Figure 2)**. Part of the fibrocytes (spindle cells) have been transformed into chondrocytes (ovoid cells) **(Figure 3)**.

Table 1. *Characteristics of all 3 groups (30 joints in 30 dogs) 1 week, 2 weeks and 3 weeks after CCL rupture*

Group	Sex	Age, months	Body weight, kg	Side of stifle joint	Synovitis score	Modified Bonar score	Modified Vasseur score
1 week n=10	F-1, FS-3 M-5, MC-1	72.3±26.06 (41 to 110)	33.2±6 (27.6 to 48.1)	L-8, R-2	10* (6-15)	10.5 (7-17)	13 (9-20)
2 weeks n=10	F-4, FS-2 M-4, MC-0	78.2±25.92 (27 to 120)	36.69±5.59 (28.8 to 44.6)	L-4, R-6	8 (5-12)	9.5 (6-11)	11.5 (6-21)
3 weeks n=10	F-3, FS-2 M-2, MC-3	55.8±21.67 (30 to 100)	31.29± 4.35 (24.5 to 38.4)	L-5, R-5	7 (4-10)	8 (6-13)	13.5 (8-19)

Values reported for sex and side of the stifle joint are counts and for other variables are mean ± SD (range). Synovitis score was on a scale of 0 to 18. Modified Bonar score was on a scale of 0 to 20. Modified Vasseur score was on a scale of 0 to 24.

F = Female, sexually intact. FS = Female, spayed. L = Left. R = Right. M = Male, sexually intact. MC = Male, castrated. *P<0.05 for Synovitis scores between 1 week and 3 weeks.

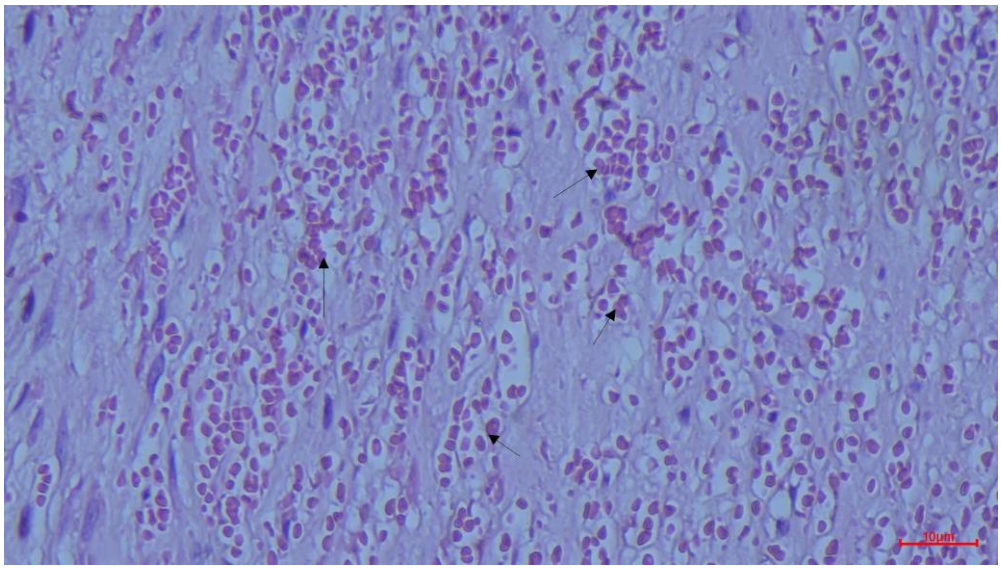

Figure 1. Presence of many erythrocytes (arrows) and moderate degree of inflammatory process. Hematoxylin/Eosin staining

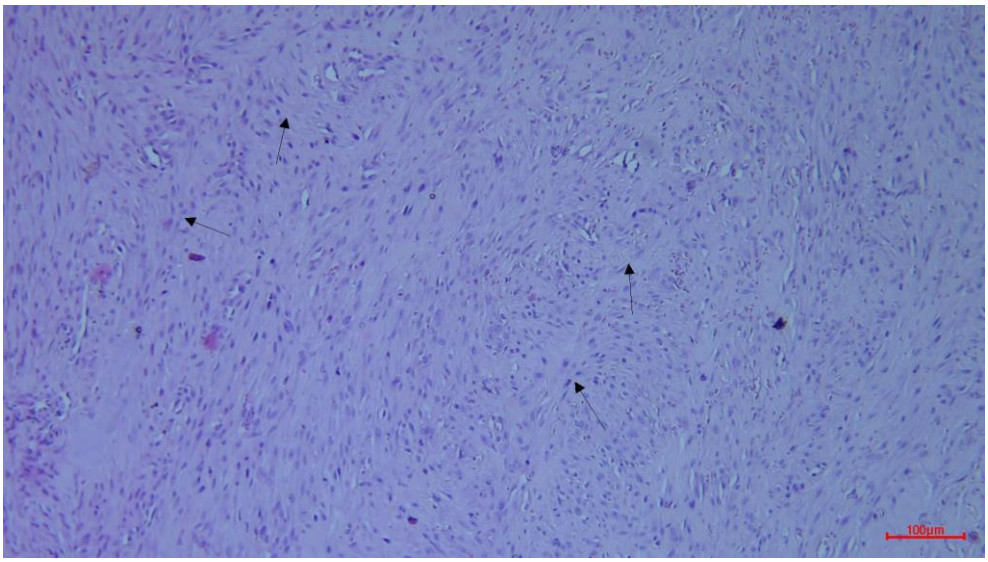

Figure 2. Uneven arrangement of collagen fibers (arrows). Hematoxylin/Eosin staining

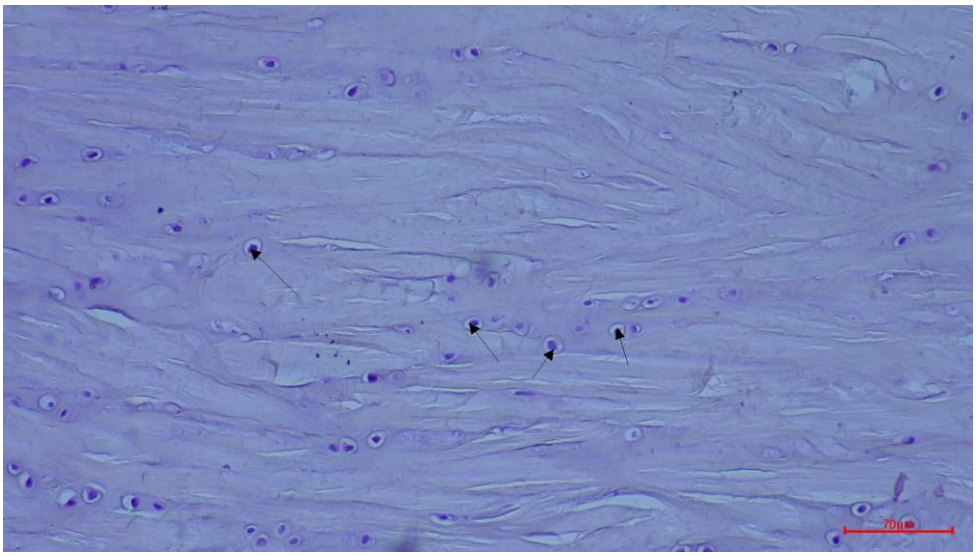

Figure 3. Transformation of fibrocytes into chondrocytes (arrows). Hematoxylin/Eosin staining

In group II (up to 2 weeks after the rupture) limited number of inflammatory cells were observed and a large number of blood vessels (neovascularization) filled with erythrocytes were seen **(Figure 4)**. Most ruptured CCLs from group II had focal areas of moderate regenerative response interspersed throughout a generally poorly organized matrix with mild increases in cellularity. Synovial hyperplasia characterized by stacked plump synoviocytes was commonly seen and there was little chondroid metaplasia and no evidence of matrix degeneration. However, there were some areas where ligament fibroblasts had a slightly more ovoid than fusiform appearance in association with a pronounced regenerative response. The number of chondrocytes is increased in comparison to group I **(Figure 5)**. In certain areas, hyalinization of the collagen fibers was also observed.

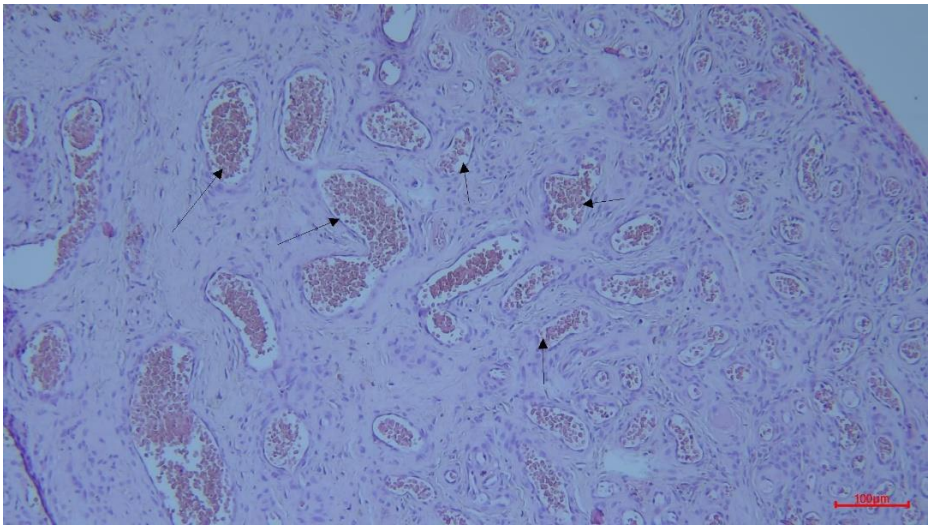

Figure 4. Presence of multiple blood vessels filled with erythrocytes (arrows). Hematoxylin / Eosin staining

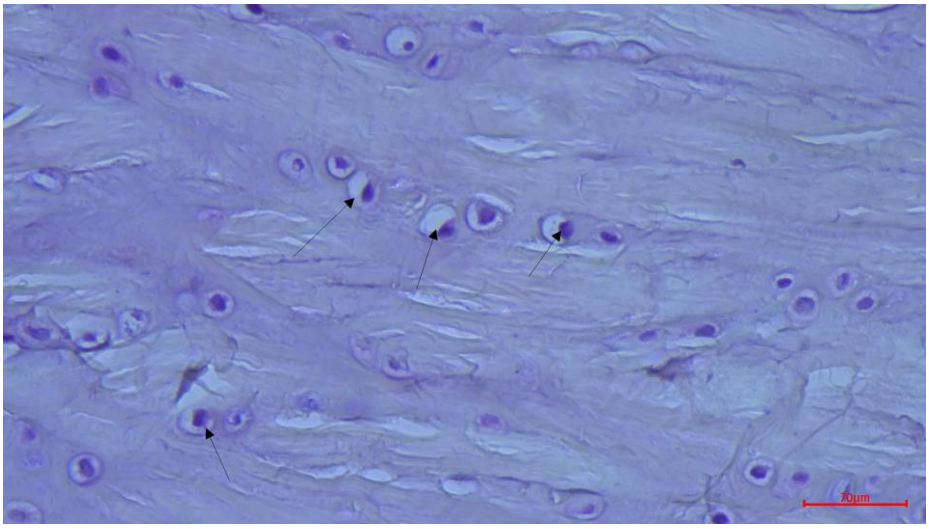

Figure 5. Increased number of chondrocytes (arrows) in comparison to the group I. Hematoxylin / Eosin staining

In group III (up to 3 weeks after the rupture) low number of inflammatory cells were also observed and the blood vessels were indiscernible and reduced in size in comparison to group II **(Figure 6)**. In this group, ligamentocytes with conspicuous cytoplasm and round nuclei were often seen, as well as chondroid changes of ligamentocytes, mucoid or cartilaginous changes of the extracellular matrix ground substance, separation, disorganization and loss of collagen bundles. The number of fibrocytes (arrows) is increased in comparison to chondrocytes **(Figure 7)**. In many samples of this group, hyalinization of the collagen fibers was a common feature and often observed in multiple sections (arrows) **(Figure 8)**.

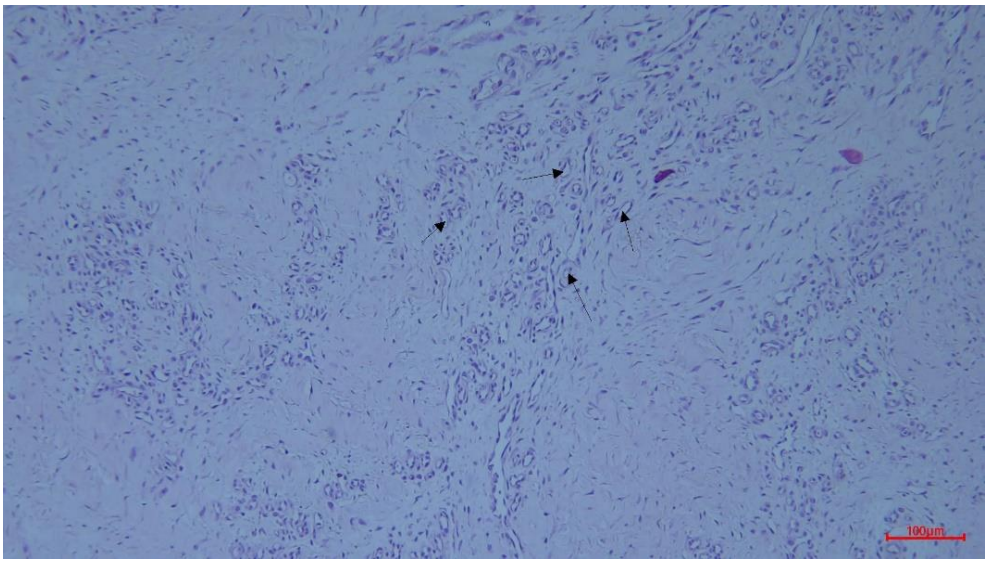

Figure 6. Reduction of blood vessels in comparison to group II (arrows). Hematoxylin /Eosin staining

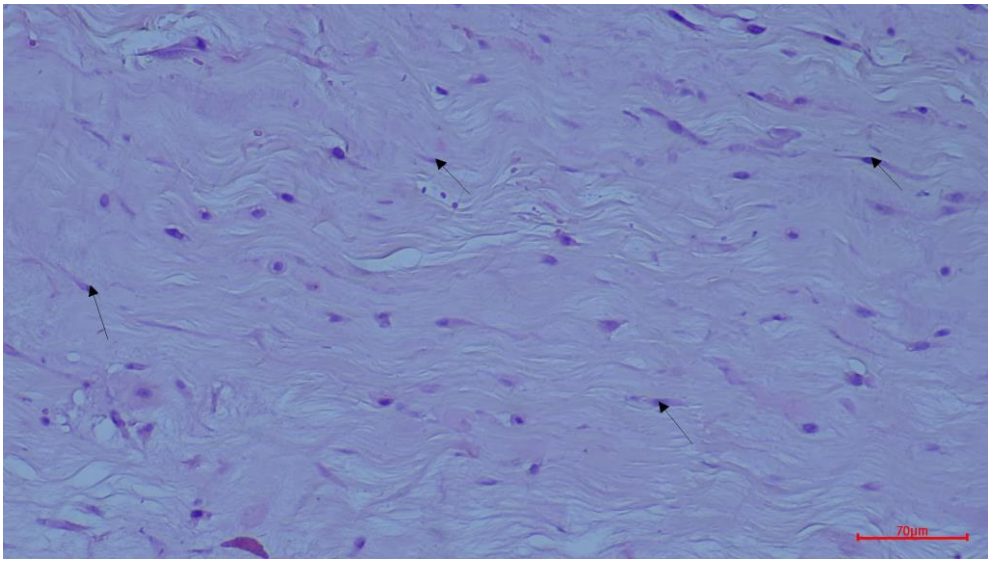

Figure 7. The number of fibrocytes (arrows) is increased in comparison to the number of chondrocytes. Hematoxylin /Eosin staining

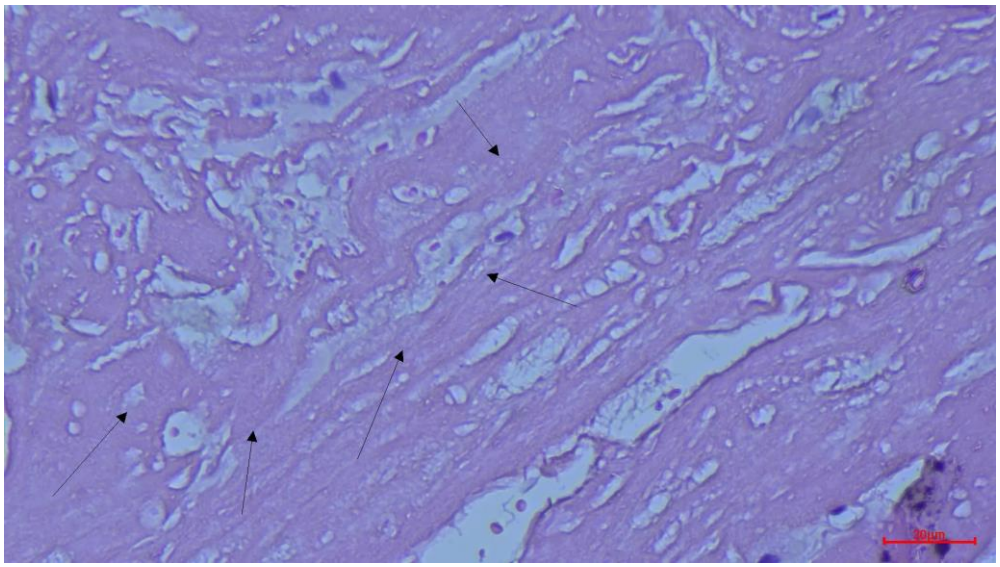

Figure 8. Hyalinization of collagen fibers. Hematoxylin /Eosin staining

Synovitis score in group I ranges from 6 to 15, in group II from 5 to 12 and in group III from 5 to 10. Modified Bonar score in group I ranges from 7 to 17, in group II from 6 to 11 and in group III from 6 to 13 **(Table 1).** Modified Vasseur score in group I ranges from 9 to 20, in group II from 6 to 21 and in group III from 8 to 19. The highest recorded Synovitis (15) and modified Bonar (17) scores were in group I and the highest recorded modified Vasseur score was in group II (21). Using the same methodology, the lowest values were recorded in both II and III groups (Synovitis score of 5; Modified Bonar score of 6 and modified Vasseur score respectively 6 and 8).

The median Synovitis score of group I was statistically significantly different from that of group III (10 vs 7; $p<0.05$). For the two other scores, between-group differences were inconsistent.

The three scores were not correlated to either the age or the body weight of the thirty dogs. The comparison of the Synovitis score, Modified Bonar score, and Modified Vasseur score showed a strong positive correlation between the first two indices ($r=0.757$; 95% CI 0.545-0.878; $p<0.0001$). The relationship between the Modified Bonar and Modified Vasseur scores was also positive but moderate ($r=0.449$; 95% CI 0.106-0.697; $p=0.013$) (Table 1).

DISCUSSION

Cell morphology was studied in normal cruciate ligaments from disease-free stifle joints from dogs with a high (Labrador Retriever) and low (Greyhound) risk of CR (18). Extracellular matrix degeneration was found in every sample examined, consistent with previous studies on canine cruciate ligaments. Advancing degeneration has been associated with mechanical weakness of the canine cranial cruciate ligament (19, 20).

A histological study indicates that in the CrCL of dogs over 15 kg, permanent microscopic changes of degeneration were evident by 5 years of age (19). These changes are characterized by a loss of ligament fibroblasts, metaplasia of surviving fibroblasts to chondrocytes, and failure to maintain collagen fiber bundles, which progress in severity with age. The CrCL in dogs weighing less than 15 kg generally had less severe alterations than those in heavier dogs, and the onset of the degenerative process was delayed by several years. In ruptured CrCL, more severe changes can occur such as hyalinization, mineralization, and appearance of chondrocyte-like cells. Inflammatory or reparative responses are rarely seen (10), although the remarkable loss of fibroblasts from the core region of ruptured CrCL occurs. In contrast, cell number densities are nearly the same in ruptured and intact CrCL in the epiligamentous region. It is established that, in ruptured CrCL, the numbers of typical ligament fibroblasts (fusiform and ovoid cells) are decreased, while the numbers of cells exhibiting chondroid transformation (spheroid cells) are increased in the core region. (10, 21).

The canine CCL is considered to be subject to multiaxial stresses (22) and local variation in cell morphology may reflect variation in stresses and strains. Some authors (19-20) described areas of acellularity in the canine CCL and have been considered early degenerative changes. Other researchers (22, 23), ascribed this loss of cellularity to apoptosis but suggested that it is not known whether the decreased cellularity leads to degenerative change in the matrix.

Some authors' histological studies (24, 25) revealed that there is no difference between cell population, the morphology and the state of the extracellular matrix in the cranial cruciate ligament and other ligaments in the body of the dog. Typical CCL fibroblasts have a prolonged oval shape, and they are occasionally found in lacunae. The structural feature of the CCL was considered abnormal by some researchers (26), while others described it as a physiological condition typical of intra-articular ligaments (20).

Generalised changes were described in both the cranial cruciate ligament and the caudal cruciate ligament in the greyhound, a breed at very low risk of canine cruciate ligament rupture, leading to the suggestion that these low-grade changes are not, in fact, degenerative but adaptive (16, 20). In a study, conducted by Kuroki et al., 2019 (27) of juvenile and adult dogs with intact CCLs and dogs with CCL rupture, concluded that synovitis scores were not significantly different among groups. There was a significant negative relationship between modified Bonar scores and vascularity scores for juveniles and adults and for adults and dogs with ruptured CCL when controlling for age, but there was not a significant relationship between modified Bonar scores and synovitis scores. These authors also suggested that poor blood supply to the core

region could be an important underlying condition for spontaneous degeneration of the CCL in at risk dogs may be a precursor to rupture.

Joint inflammation, mechanical loading, ligament microinjury, and ischemia may influence cellular metabolism, resulting in matrix changes. Progressive mechanical overload diminishes the typical crimped structure of the collagen fibrils seen in intact CCLs, and further tensile loading causes disruption of the ligament fascicles (10,27). Our results support the hypothesis that increased forces acting through ligamentous tissues lead to chondroid metaplasia and the development of hyalinization of collagen fibers within the ligament. Our data are consistent with the study of Comerford et al. (20), in which fibrocartilage development was considered as a physiological feature of the CCL under tensile stress. Our observations one week after the CCL rupture also confirmed the partial transformation of the fibrocytes into chondrocytes in group I.

Dogs with CCL rupture typically have inflammatory changes in the synovial membrane and the CCL epiligament, as well as in the synovial fluid (10, 27, 28). Our study revealed similar changes within the ligament and showed a high mean score in all three scoring systems one week after the CCL rupture, but also a decrease in score after 2 and 3 weeks, except in the Modified Vasseur score. The present study indicated a various number of inflammatory cells in all groups, but high erythrocyte deposition and multiple blood vessels as well as their reduction in groups II and III, which may suggest that degenerative changes continue after the initial CCL rupture. Although further studies are needed to evaluate whether this inflammation precedes actual CCL rupture, Muir et al., (2005) (29) hypothesized that the inflammation develops in the early phase of cruciate disease and before the development of stifle instability. The degree of these inflammatory changes is linked to the degree of degenerative changes within the CCL, characterized by largely mononuclear chronic synovitis which progressively affects the entire joint (30). Because we found strong evidence of inflammation and degeneration in all three scoring systems, our findings are consistent with those reported by Muir et al., (2005) and Döring et al., (2017). We also suggest that variations within the population of studied ligament specimens and the lack of a control group with intact CCL should be considered a main limitation in our investigation. Nevertheless, the exact role of the stifle joint synovitis in dogs with CCL rupture remains to be elucidated.

CONCLUSION

In general, degenerative changes were seen in all samples examined of cranial cruciate ligaments with different grades of chondroid metaplasia, increased cellularity and vascularity. The obtained results showed diverse inflammatory changes in all groups by lymphoplasmacytic infiltrates with or without lymphoid aggregate deposition. Our data also showed that up to 1 week, the number of chondrocytes, erythrocytes, blood vessels and their lumen is the greatest and gradually decreases till 21 days after CCL rupture.

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