Evaluation of surface blood flow in intact and ruptured canine cruciate ligaments using laser Doppler flowmetry

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Abstract: Objective: To evaluate the usefulness of laser Doppler flowmetry (LDF) to measure surface blood flow in canine cruciate ligaments, compare measurements in different sites of intact and partially ruptured canine cranial cruciate ligaments (CrCL) and intact caudal cruciate ligaments (CaCL), and investigate any association between surface blood flow in partially ruptured CrCL and synovitis or duration of clinical signs. Study design: Case-controlled clinical study. Animals: Sixteen dogs with partially ruptured CrCL and five dogs with intact CrCL. Methods: Blood cell flux (BCF) readings during three measurement cycles using LDF at two sites in each ligament (mid-substance and the distal portion of the CrCL, and mid-substance and the proximal portion of the CaCL) were recorded. Synovial changes were graded grossly and histologically using the Osteoarthritis Research Society International histopathology scoring system. Results: The within-run coefficients of variation (CV) for a single BCF measurement cycle were 12.2% and 12.7% in the ruptured and intact CrCL groups, respectively. The between-run CV for three measurement cycles was 20.8% and 14.8%, respectively. The intraclass correlation coefficient (ICC, absolute agreement) was 0.66 for a single measurement cycle and 0.86 for the average of three cycles. No difference in average BCF readings was found between any two sites in either group, but BCF readings in both CrCL sites were significantly higher in the ruptured CrCL group than the intact CrCL group. No associations between BCF and synovial grades or duration of lameness were identified. Conclusions: Laser Doppler flowmetry can be used to assess surface blood flow in intact and partially ruptured canine cruciate ligaments with acceptable precision. Using this method, surface blood flow appears greater in partially ruptured canine CrCL than intact CrCL. Further studies are required to determine if this is a sequela of trauma or synovitis.

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Evaluation of Blood Flow in Intact and Ruptured Canine Cruciate Ligaments using Laser Doppler Flowmetry

Abstract

Objective: To evaluate the usefulness of laser Doppler flowmetry (LDF) to measure surface blood flow in canine cruciate ligaments (CCL), compare measurements in different sites of intact and partially ruptured CCL, and investigate any association between surface blood flow in partially ruptured cranial cruciate ligaments (CrCL) and synovitis or duration of clinical signs.

Animals: 16 dogs with partially ruptured and 5 dogs with intact CrCLs.

Methods: Blood cell flux (BCF) readings during three measurement cycles using LDF at two sites in each ligament (midsubstance, distal portion of the CrCL, midsubstance, proximal portion of the caudal CaCL). Synovial changes were graded grossly and histologically using the OARSI histopathology scoring system.

Results: The within-run coefficients of variation (CV) for a single BCF measurement cycle were 12.2% and 12.7% in the ruptured and intact CrCL groups, respectively. The between-run CV was 20.8% and 14.8%, respectively. The intraclass correlation coefficient was 0.66 for a single and 0.86 for the average of three cycles. No difference in BCF was found between any two sites in either group, but BCF in both CrCL sites were significantly higher in ruptured than intact CrCLs. No associations between BCF and synovial grades or duration of lameness were identified.

Conclusions: LDF can be used to assess surface blood flow with acceptable precision. Using this method, surface blood flow appears greater in partially ruptured than intact canine CrCLs. Further studies are required to determine if this is a sequela of trauma or synovitis.

Key words: Cruciate ligament, Blood cell flux, Dog, Laser Doppler flowmetry, Stifle joint
Introduction

Cruciate disease is one of the most frequent causes of lameness in dogs, most commonly associated with a midsstance rupture of the cranial cruciate ligament (CrCL) (1). Findings of previous studies suggest that the CrCL is subjected to progressive degeneration of the extracellular matrix associated with both quantitative and qualitative cellular changes (2, 3). The initial ligament degeneration progresses to partial and then to complete rupture causing joint instability in most dogs (1). The precise aetiology of CrCL rupture remains unclear, but is generally thought to be a multifactorial process, components of which may include genetic predisposition (4-6), abnormal stifle conformation (7-10), aging (11), and joint inflammation (12), as well as predisposing factors, such as obesity and inactivity (13). In addition, recent studies suggest that hypovascularisation and/or hypoxia may be involved in the pathophysiology of CrCL rupture, and that hypoxia may be involved in the cartilaginous transformation in ligaments (14, 15). Histologic studies have shown the CrCL to be a hypovascular tissue in which the epiligamentous and core regions have similar vascularisation, but the middle portion of the CrCL has fewer vessels than the proximal and distal portions (14, 16, 17). Perfusion to the cruciate ligaments arises predominantly from the supporting soft tissues, particularly the infrapatellar fat pad and the synovial sheath that surround the ligament (16, 18, 19), and vascular proliferation following partial CrCL transection or rupture has been demonstrated (14, 16).

Although vascularization of canine CrCLs has been investigated (16, 17, 20), little information is available about blood flow in normal cruciate ligaments and no information exists on blood flow in ruptured canine CrCLs. Laser Doppler flowmetry (LDF) provides a relative measure of blood flow in the microvasculature at a depth of penetration of 1.0–1.5 mm using a low-power laser light, and has previously been used to assess blood flow in a variety of tissues, including human and canine ligaments (19-21). Using LDF, photons incident on tissue are scattered by moving red blood cells and stationary tissue cells. Those
interacting with moving cells are Doppler-shifted, whilst those colliding with stationary tissue
retain their original frequency. The backscattered photons are returned to a photodetector,
which converts energy into electrical signals. The output signal, the blood cell flux (BCF), is
expressed in arbitrary units of perfusion (AU), and is proportional to blood flow (22). In spite
of the fact that increased vascularization has previously been shown in ruptured canine CrCLs
(16), it remains unclear if this is a response to injury, associated with inflammation, or the
result of a hypoxic environment. Although LDF only assesses superficial blood flow due to
the low depth of penetration, it may hold potential as a tool to investigate alterations in blood
flow in canine CrCL disease, objectively score synovitis in dogs with ruptured CrCLs or
evaluate an association between genetic predisposition and blood flow in dogs with intact
CrCLs.

The purpose of the present study was to evaluate the usefulness of LDF to measure
surface blood flow in intact and partially ruptured canine cruciate ligaments and compare real
time blood flow in intact and ruptured CrCLs. A secondary purpose was to perform a
preliminary investigation into associations between BCF and synovitis or duration of clinical
signs in dogs with partially ruptured CrCLs.

Materials and Methods

Dogs

Sixteen client-owned dogs (ruptured CrCL group), presented to xy for tibial plateau levelling
osteotomy (TPLO) for partial CrCL rupture, and 5 beagle dogs (intact CrCL group) with no
history of orthopaedic disease, provided by the zy, were included in the study. Study inclusion
criteria for the ruptured CrCL group were a diagnosis of a partial CrCL rupture confirmed by
probing and observation during arthroscopy, and unremarkable results of routine
haematologic and serum biochemical analyses. Dogs were excluded if there was a recent
history of illness other than hindlimb lameness or if they had undergone previous intra-
articulation of any substance or previous surgery on the affected limb. Data collected included the breed, age, body weight and gender of the dogs, and duration of lameness prior to surgery. Informed client consent was obtained for all dogs in the ruptured CrCL group. The study was approved by YX N°……

**Anaesthesia**

A standard anaesthesia protocol was followed in all dogs. This included premedication with acepromazine\(^a\) (0.03 mg/kg intramuscularly), induction with propofol\(^b\) (2–8 mg/kg intravenously [IV] to effect), and maintenance with isoflurane in an air-oxygen mixture, delivered using a rebreathing system (end-tidal concentration, 1.2–2%). An ultrasound-guided femoral and sciatic nerve block was performed with 0.3 ml/kg 0.5% ropivacain\(^c\) for each nerve. Intra-operative fluids consisted of a balanced electrolyte infusion\(^d\) administered IV at a rate of 10 ml/kg/h. Dogs were allowed to breathe spontaneously. Continuous anaesthesia monitoring consisted of electrocardiography, SpO\(_2\) measurement, gas monitoring (end-tidal CO\(_2\) and isoflurane), body temperature and invasive blood pressure (metatarsal artery) measurements. End-tidal CO\(_2\) concentrations were maintained between 35–45 mmHg by positive pressure ventilation. Body temperature was maintained between 36–38°C using a forced-air warming blanket. Mean blood pressure was maintained above 60 mmHg using a crystalloid\(^d\) bolus (10 ml/kg delivered IV over 10 min) and, if necessary, a colloid\(^e\) bolus (2 ml/kg delivered IV over 10 min). Persistent hypotension was treated with a dopamine\(^f\) infusion, starting at a rate of 0.005 mg/kg/min IV, with incremental steps of 0.0025 mg/kg/min IV every 10 minutes until a mean blood pressure \(\geq\)60 mmHg was reached. Bradycardia, defined as a heart rate lower than 35 beats per minute, was treated with glycopyrrolate\(^g\) (0.01 mg/kg IV).
Postoperative care

Dogs in the ruptured CrCL group received carprofen (4 mg/kg IV, q24h) and buprenorphine (0.02 mg/kg IV, q12h) starting from weaning off the block up to 48h postoperatively as necessary. Dogs were discharged from hospital with carprofen (4 mg/kg PO, q24h) for one week.

Laser-Doppler flowmetry

The LDF used in this study has an infrared helium-neon laser (780 nm) directed at tissue using an 8 cm x 1.5 mm optical fibre probe. Measurements were performed in all dogs after mean blood pressure, body temperature and heart rates were stable. Exposure of the cruciate ligaments was obtained with minimal dissection and retraction to avert iatrogenic soft tissue trauma that may affect blood flow. The probe sites selected were the midsubstance and distal portion near the tibial insertion for the CrCL, and the midsubstance and proximal portion near the femoral origin for the CaCL (Figure 1). The BCF measurements provided by the LDF monitor software were the mean and standard deviation (SD) of at least three noise-free BCF recordings of 10s duration for each measurement cycle of approximately one minute. The data acquisition rate was set at 20 Hz with an integration time of 0.1 s for averaging and comparable readings. In total, three measurement cycles were performed at each site, lifting the probe off the ligament and repositioning the probe prior to each measurement cycle.

Intact CrCL dogs

For dogs in the intact CrCL group, a medial parapatellar arthrotomy was performed on both stifle joints by a single board-certified surgeon (xy). This approach was selected because the size of the stifles in the intact CrCL group did not allow consistent placement of the probe under arthroscopic assistance. Following LDF measurements, the CrCLs and CaCLs were harvested from both stifles for histologic examination. The dogs were then immediately euthanised for reasons unrelated to this study.
**Ruptured CrCL dogs**

Dogs in the ruptured CrCL group were positioned in dorsal recumbency. A trocar and sleeve were gently inserted into the stifle joint with the arthroscope portal lateral to the patellar tendon midway between the distal end of the patella and proximal to the tibial crest. The stifle joint was held at 30° flexion to minimize tension on the ligament. The LDF probe was inserted into the stifle joint through a 3.5-mm modified sleeve, positioned medial to the patellar tendon at the same level as the scope portal, shortened by 2 cm and coupled with a 22-mm long and 10-mm diameter plastic cylinder bored in its centre to exactly fit the diameter of the grip of the probe. The cylinder was also bored perpendicularly at its distal end to allow fluid escape from the joint. The probe was applied perpendicular to the surface of the ligament without exerting pressure on the ligament. During LDF measurements, no lavage fluid was instilled into the stifle and effluent fluid was allowed to freely flow out of the joint to avoid hydrostatic pressure on the ligament. In addition, the light of the endoscope was switched off. Following LDF measurements, biopsies of synovial membrane were harvested from the lateral and medial femoro-tibial joint compartments and the femoro-patellar joint (craniolateral, craniomedial and axial to the optic port located lateral to the patellar ligament and halfway between the patella and tibial tuberosity (Figure 1)). Debridement of the CrCL was not performed. Following stifle arthroscopy, dogs were treated by TPLO, performed by a single board-certified surgeon (yx).

**Grading of synovitis alterations**

Synovial alterations were graded during arthroscopy or arthrotomy using the macroscopic scoring system described by the Osteoarthritis Research Society International (OARSI), which assesses synovial thickening, discolouration and vascularity (23). Haematoxylin and eosin stained sections from each synovial biopsy site, taken in the ruptured CrCL group dogs, were graded in a blinded fashion by two observers (zy, yz) using the microscopic scoring system described by the Osteoarthritis Research Society International (OARSI), which
assesses synovial lining cell layer thickness, villous hyperplasia and inflammatory cellular infiltrates (23).

**Histologic examination of cruciate ligaments**

Ligaments from the intact CrCL group dogs were fixed in 10% formalin for 24h and embedded in paraffin. Longitudinal sections of 5 µm were cut from the epiligament and the subjacent core ligament, mounted for histologic examination and stained with haematoxylin and eosin. Sections were assessed for signs of cruciate ligament degeneration according to criteria previously described (3) and scored from 0–3 by the same two blinded observers (zy, yz).

**Statistical Analysis**

Statistical analysis was performed using commercial software. Summary statistics was performed and normality assessed using D’Agostino-Pearson tests. As some data were normally distributed but others were not, nonparametric statistics were used. Variability in BCF measurements was assessed as the coefficients of variation (CV) within each measurement cycle (within-run CV) as well as of the triplicate cycles (between-run CV) at each site. The intraclass correlation coefficient (ICC) was assessed for the triplicate cycles using a two-way random model with absolute agreement. Differences in CVs between different ligaments and between the ruptured and intact CrCL groups were assessed using Kruskal-Wallis and Mann-Whitney tests, respectively. For ICC, a coefficient above 0.7, meaning that 70% of the observed variance is real variance, was considered desirable.

For all subsequent analyses, the average of the mean BCF values of the three measurement cycles at each site was used and, in the intact CrCL group, the average of recordings in both stifles at each measurement site was used. Differences in BCF between the two groups at each site were evaluated using a Mann-Whitney test. Differences in BCF between sites within the groups were evaluated using a Wilcoxon signed-rank test. In the
ruptured CrCL group, associations between BCF and gross and microscopic synovitis scores were evaluated using Kruskal-Wallis tests. A Spearman’s rank correlation was used to examine the relationship between BCF and duration of lameness and between gross and microscopic synovitis scores. Significance was set at $p < 0.05$ throughout.

**Results**

**Ruptured CrCL group**

Dogs were between 1.9–9.2 years old (median, 6.0 years) and included 10 females (9 spayed, 1 intact) and 6 males (3 castrated, 3 intact). Breeds represented were 3 mixed breed dogs, 2 Malinois, 2 Golden Retrievers, 1 Labrador Retriever, 1 Flat Coated Retriever, 1 Boerboel, 1 Doberman, 1 German Shepherd, 1 Australian Shepherd, 1 Newfoundland, 1 Dogue de Bordeaux, and 1 Boxer. Their median body weight was 34.2 kg (range, 23.5–67 kg). The duration of lameness prior to surgery was 3–104 weeks (median, 12 weeks). Partial rupture of the CrCL was confirmed during arthroscopy in all 16 stifles (7 left, 9 right). The degree of rupture varied from minor fibre tearing up to rupture of just under one half of the ligament. In all cases, sufficient intact lament was present to position the head of the LDF probe. Synovitis was identified in all stifles with a median gross synovitis score of 4/5 (95% CI, 2.6–5.0) and a median histologic synovitis score of 7.5/18 (95% CI, 5.6–9.0).

**Intact CrCL group**

The intact CrCL group consisted of 5 intact male Beagles between 1.1–4.1 years old (median, 3.8 years) and weighing between 9.7–13.9 kg (median, 12.1 kg). The BCF data from one right stifle joint was discarded due to a technical error (data acquisition rate was inadvertently set to a different rate as all other measurements). As a result, data from 9 stifle joints (5 left, 4 right) were included for data analysis. Gross examination of the stifle joints revealed no evidence of joint pathology and all joints received a gross synovitis score of 0/5. Histologic examination
revealed some degree of ligament degeneration in at least one ligament in 4/5 dogs with a median score of 0.7/3 (range, 0–2) for the CrCL and 0.25/3 (range, 0–2) for the CaCL.

Blood cell flux: measurement variability

The LDF monitor provided BCF readings in all ligament sites. The median within-run CV of BCF measurements (variation based on at least three measurements within a single cycle) was 12.2% (interquartile range (IQR), 10.0–15.9%) for dogs in the ruptured CrCL group, and 12.7% (IQR, 10.2–15.5%) for dogs in the intact CrCL group (Figure 2). No significant difference in CVs was found between ligament sites in the ruptured CrCL group (p = 0.441) or the intact CrCL group (p = 0.648), or between the ruptured and intact CrCL groups (p = 0.458).

The median between-run CV (variation based on triplicate measurement cycles) was 20.8% (IQR, 12.9–30.2%) in the ruptured CrCL group and 14.8% (IQR, 8.0–20.8%) in the intact CrCL group (Figure 3). No significant difference in CVs was found between ligament sites in the ruptured CrCL group (p = 0.301) or in the intact CrCL group (p = 0.387), but a significant difference was found between the two groups (p = 0.033), where the CV was significantly higher in the ruptured group for the midsubstance CrCL (p = 0.032) and for the proximal CaCL (p = 0.034) (Figure 3).

The reliability of measurements based on ICC was 0.66 (95% CI, 0.57–0.75) for a single measurement cycle, and 0.86 (95% CI, 0.80–0.90) for the average of three measurement cycles.

Blood cell flux: mean measurements

No difference in BCF was found between any two ligament sites within either group. Significantly higher BCF readings were found in the ruptured CrCL group compared to the intact CrCL group for midsubstance CrCL and for distal CrCL, but no significant difference was found for midsubstance CaCL or proximal CaCL (Table 1, Figure 4).
In the intact CrCL group, no association was found between BCF readings in the midsubstance CrCL and gross synovitis scores ($r_s = 0.29$; 95% CI, -0.24 to 0.69; $p = 0.278$), microscopic synovitis scores ($r_s = -0.14$; 95% CI, -0.59 to 0.39; $p = 0.613$), or duration of lameness ($r_s = 0.08$; 95% CI, -0.43 to 0.55, $p = 0.77$). In addition, no significant relationship was found between the gross and microscopic synovitis scores ($r_s = -0.22$; 95% CI, -0.65 to 0.31, $p = 0.41$) in these dogs.

**Discussion**

A variety of methods have been used to investigate the vascular distribution and blood flow in cruciate ligaments (16, 17, 19-21, 24). The advantage of LDF compared to other techniques, such as microsphere studies, radioactive ion uptake, and washout techniques, is a real-time application without sacrificing tissue integrity (16, 17, 19-21, 24, 25). However, LDF measurements are only possible at a tissue depth of 1.0–1.5 mm and only relative blood cell flux values are obtained. In consequence, the BCF values measured in the present study reflect blood flow only in the superficial vasculature of the ligament enveloped by synovial membrane.

Measurement of BCF using LDF in this study was possible in all sites with a relatively low median within-run CV. This is similar to previous studies that reported CVs varying from 4% to 17% using LDF on other tissues (26-29). However, the between-run CVs in the present study were highly variable and ICC for single measures was relatively low. This suggests that measurements varied considerably from cycle to cycle. This was also found in other studies investigating BCF in the skin or retina (28, 30). Whether this is due inherent measurement imprecision or heterogeneous surface blood flow reflected by placement of the probe in slightly different positions between cycles is unclear. In one study, a low CV was obtained using a probe holder, ensuring a stable location and position of the probe (28). Some of the imprecision observed in the present study may therefore be due to the limited space to adjust
probe angulation through the port and repositioning of the probe. Nonetheless, the high ICC for average measures suggests reliable measurements are obtained when the average of three cycles is determined. However, as all BCF readings were performed by a single operator, intra-operator variation was not evaluated in the present study.

The higher between-run CV observed in midsubstance CrCLs of partially ruptured ligaments compared to intact ligaments may be due to heterogeneous surface blood flow in the damaged ligament. However, given differences in measurement methods (arthrotomy versus arthroscopy), breed and age of the dogs, further studies are required to confirm this finding.

No difference in BCF was found between the ligaments or between the different sites of the ligaments within either group. Similar findings were observed in healthy canine cruciate ligaments evaluated using LDF (20) and a microsphere technique (21). These findings are in contrast to the greater number of vessels observed in the proximal portion of the CrCL than in the central portion using immunohistochemistry or microangiography (14, 16). It appears therefore that vascularisation itself may not reflect blood flow (or at least not surface blood flow) although the reason for these apparently discrepant findings is unclear. A higher BCF was found in both sites of the CrCL in the ruptured compared to the intact CrCL group. This finding would appear to corroborate previous studies that demonstrated increased vascularisation in transected canine CrCLs (16). However, given that no difference in BCF was found between sites in the ruptured CrCL group and that LDF measures only surface blood flow, this finding suggests that higher BCF in the ruptured CrCL group may merely reflect a greater degree of synovitis. In addition, a greater decrease in perfusion of the ligaments due to arthrotomy in the intact CrCL group cannot be ruled out (20, 24).

Gross and microscopic synovitis were identified in all dogs in the ruptured CrCL group although no correlation was found between the scores. In the intact CrCL group, no gross synovitis was found but mild microscopic ligament alterations were frequently detected,
corroborating findings of previous studies that demonstrate microscopic age-related
degenerative changes in apparently healthy dogs (1, 3, 31). No correlation was observed
between BCF and duration of lameness or gross or histologic synovitis scores in the ruptured
CrCL group. However, the present study evaluated a relatively low number of dogs with
highly variable duration of lameness and disparate degrees of CrCL rupture. Moreover,
grading of synovitis is subjective. Evaluation of BCF in larger numbers of dogs may therefore
be necessary to confirm this finding.

Although LDF may hold promise as a tool to investigate blood flow in intact and
ruptured cruciate ligaments, there are several limitations to the method. Firstly, LDF provides
only superficial measurements in a very small area and does not evaluate overall blood flow
across the diameter of the ligament. However, a large proportion of the blood supply to the
ligaments is restricted to the surface of the ligaments, with a similar density of capillaries in
the epiligamentous and core regions. Secondly, general anaesthesia required to access the
ligaments may affect blood pressure and vascular tone. Although these effects can be
mitigated using a standardised anaesthesia protocol and controlling blood pressure, they
cannot be eliminated entirely. Finally, although BCF measurements are proportional to blood
flow, reading is in arbitrary flow units and absolute flow is not known. In addition, an
important limitation to our study is that the surgical approach was not identical between the
groups, resulting in possible differences in stress on the ligament and injury to surrounding
tissue.

In conclusion, precision of BCF measurements in intact and ruptured canine cruciate
ligaments varied somewhat between cycles but measurements were found to be reliable using
the average of several cycles. Results of this study suggest that partial rupture is associated
with increased surface blood flow in the CrCL. Further studies are necessary to determine the
extent to which alterations in surface blood flow occur prior to rupture or are associated with
joint inflammation, and whether BCF can be used to assess ligament changes in dogs prone to
rupture, assess the need for ligament debridement or evaluate synovitis or the effect of inflammation-modifying articular therapy.

Footnotes:

a Preqillan: FATRO S.p.A, Ozzano Emilia, Italy
b Propofol 1% MCT Fresenius Kabi AG
c ROPivacain, Fresenius
d Plasma Lyte A: Baxter
e Voluven 6% balanced, free flex, Fresenius
f Dopamin Sintetica, Fresenius
g Robinul, Riemser Pharma GmbH,
h Rimadyl: Pfizer AG
i Temgesic: Reckitt Benckiser
j Moor DRT4, Moor Instruments Ltd., Devon, UK
k VP3, Moor Instruments Ltd.
l MedCalc version 16.2.1, MedCalc software bvba, Ostend, Belgium.
References


Figure legends

**Figure 1**  Diagram of cruciate ligament sites and arthroscopic image of the midsubstance of a partially ruptured cranial cruciate ligament, showing the sleeve used for laser Doppler flowmetry measurements in the canine stifle joint. A1: midsubstance CrCL, A2: distal portion CrCL, B1: midsubstance CaCL, B2: proximal portion CaCL. X denotes sites of synovial biopsies.

**Figure 2**  Box plots showing coefficients of variation of within-run BCF readings at different sites in the cruciate ligaments of dogs in both groups.

**Figure 3**  Box plots showing coefficients of variation of between-run BCF readings at different sites in the cruciate ligaments of dogs in both groups. * denotes a significant difference.

**Figure 4**  Box plots showing average BCF readings at different sites in cruciate ligaments of dogs in both groups. * denotes a significant difference.