รายงานโครงการวิจัย

(รายงานในช่วงตั้งแต่วันที่ 30 สิงหาคม 2550 ถึงวันที่ 30 สิงหาคม 2555)

โครงการนำร่องเพื่อการพัฒนาเทคนิคการวินิจฉัยโรคข้อเสื่อมในสุนัขโดยใช้สเปกโทรสโกปีอินฟราเรด

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สนับสนุนโดยคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(งานวิจัยยังไม่เสร็จสมบูรณ์ โปรดอย่านำไปใช้อ้างอิง)

Summary

At present 126 synovial fluid samples were submitted for analysis at the Institute of Biodiagnostic, National Research Council of Canada. Demographic data of those 51 control samples were derived from 39 male and 12 female dogs with the mean age and body weight of 4.9 ± 1.7 years and 19.8±3.1 kg (mean±SE) respectively. Breed represents of the control group are crossbreed, golden retrievers and poodle. The samples of cranial cruciate ligament rupture group (CCRL) collected prior the surgical correction procedures are from 41 male and 34 female canine patients with the mean age and body weight of 5.9±1.7 years and 20.9±3.5 kg (mean±SE). Breed represents of CCLR group includes Crossbreed, Golden Retrievers, Poodles, Thai Bangkaew, Rottweiler, American Pit Bull terriers, Bull terriers, Lhasa Apso, Shih Tzu, Yorkshire terriers and Miniature Pinscher.

The mean score of orthopedics grading system of the CCRL group including locomotor ability, weight bearing, joint mobility and pain scores are 2.7±0.7, 2.7±0.8, 3.2±0.8, 2.9±0.6 (mean±SE), respectively. The radiographic evaluations had been carried out (figure 2). Thirty-three canine patients demonstrated radiographic sign of joint effusion without any significant change of the bony component. The radiographic score of 42 canine patients in the CCRL group revealed the changes according to the features of the radiographic grading system (ranging from 0-39) with radiographic score of 10.7± 1.9 (mean±SE). All of the cruciate ligaments were completely torn with varying degree of medial meniscal injury (figure 3). Among those CCLR group, 49 of 75.
patients (65.3%) are patients with normal gross appearance of medial and lateral menisci. The partially torn and severely torn menisci are detected intraoperatively at the proportion of 5.3%, and 29.3% respectively.

The infrared spectra of synovial fluid samples from canine patients reveal the peaks that represent functional group of biological components within the sample. The synovial fluid spectrum composes of the N-H stretching vibration of the protein, CH_2 and CH3 stretching vibration, C-O stretching vibration of the protein, N-H bending vibration of the protein and C-O stretching vibration of hyaluronic acid. The pattern recognition process of the samples from both CCLR and control group are still conducted at the Institute of Biodiagnostics, NRC, Canada by using the genetic algorithm software. Once the pattern recognition step yields the satisfactory result the specificity and sensitivity of the technique will be reported to determine the feasibility of using infrared spectroscopic method as a diagnostic tool for diagnosis of osteoarthritis in canine population.

Development of an infrared-based diagnostic test for canine osteoarthritis associated with cranial cruciate ligament rupture: a feasibility study

Introduction

Osteoarthritis is a disease process characterized by pathological changes in synovial joints which resulted from an imbalance between synthesis and degradation of the macromolecules essential to maintain the biomechanical and functional properties of synovial joints (1). The pathological changes of osteoarthritis include variable degree of cartilage degeneration, joints capsule thickening, subchondral bone sclerosis and osteophyte formation (1). These pathological changes eventually result in typical clinical signs of pain and disability (1-4). Osteoarthritis remains a major public health problem in both humans and animals. Prevalence of knee osteoarthritis in adults based on national survey in United State has been reported as high as 37.4% (5). In Thailand, the prevalence of osteoarthritis of the knee was 59.4% in monk population (6). In a veterinary literature, an estimated 20% of canine population over one year of age is affected by osteoarthritis (2, 7) . In dogs, the hip joint was the most common site for osteoarthritis (42.5%) followed by the stifle (18.5%) and elbow joints (12.8%) (8).

The etiopathogenesis of osteoarthritis is based on two major causes intersection at a final common pathway leading to cartilage breakdown (3). These two major causes are abnormal stresses applied to normal cartilage or normal loading transmitted through abnormal cartilage (2, 3). Conformational abnormalities such as joint incongruity or joint instability result in an abnormal distribution of loading forces that predisposes the joint to damage and interferes with cartilage homeostasis (9). Generally, osteoarthritis is depicted as a disease of articular cartilage, although initial alteration in subchondral bone is also important (4, 10). Even though articular cartilage is naturally designed to be a good shock absorber, a thin layer of cartilage is insufficient to disperse the forces associated with repetitive joint movement. Subchondral bone plays an important role in supporting overlying cartilage as a secondary shock absorber. Excessive stress may lead to microfractures and remodeling of subchondral bone (4, 10). Bone deposits resulting from microfractures cause subchondral bone stiffening and renders it ineffective as a shock absorber, mechanically resulting in articular cartilage damage (4, 10).

Over the past few decades, when scientific communities have embraced the sciences of molecular biology, the etiopathogenesis of osteoarthritis has been moved from fairly mechanical hypothesis towards the biomolecular changes within the main structures of the joints including synovial membrane and cartilage. The mechanical insults to the cartilage can cause the release of wear and tear particle that may stimulate synovial membrane to release proinflammatory cytokines, proinflammatory mediators and protease enzymes into synovial fluid (1, 11). These chemicals may lead to further degradation of the cartilage. Furthermore, inflammatory cascades occurring within an affected joint can stimulate chondrocytes to produce more catabolic enzymes, inflammatory cytokines and inflammatory mediator including nitric oxide and reactive oxygen species into synovial fluid (1;11). These molecules have been reported to be involved in chondrocyte apoptosis and irreversible changes of the articular cartilage (1;11). Timely diagnosis is crucial. Early stage diagnosis of the disease is required in order to provide an appropriate treatment before irreversible stage of osteoarthritis has occurred.

Prevention of further articular damage, alleviation of pain and disability as well as the maintenance of good quality of life are the primary goals of treatment (12).

Currently, there is a lack of practical, economical and reliable method for early detection and objective evaluation of osteoarthritis. Diagnostic modalities for osteoarthritis include radiography, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), nuclear medical imaging, arthroscopy, and routine synovial fluid analysis (13). Although radiography is presently the most practical imaging technique used to aid diagnosis, pathologic changes in articular cartilage cannot be readily assessed (14). Nuclear scintigraphy is an advanced diagnostic tool for musculoskeletal disease with high sensitivity but low specificity (14;15). Factors such as age, breed, and activity of animals can affect the radiopharmaceutical uptake and image interpretation (15). Magnetic resonance imaging generates excellent anatomic and pathoanatomic information on articular structures but the high cost of acquiring and maintaining equipment, the limited availability for use in veterinary clinical practice, and the need for general anesthesia for high resolution images have prevented its widespread use in veterinary medicine (16). None of these tools yield useful biochemical information.

Conventional synovial fluid analyses are not widely used for evaluation of non-infectious joint disease because they rarely provide clinicians with a specific diagnosis (17). Recently, ELISA evaluation of biomarkers within SF has been described (18). Complex multiple assays are required. Individual testing by use of these techniques is expensive. The relationships of the concentrations of the biomarkers to age, breed, sex and circadian rhythms are poorly understood (18). Early results are promising, but further study is required to determine the clinical usefulness of biomarkers for classifying OA (18). Presently the means to objectively identify the level of pathologic progression in most cases of osteoarthritis are not available primarily because no generally accepted objective standards exist. There is a real need for a rapid, economical, practical, and reliable diagnostic test for objective evaluation of joint disease, as well as the unbiased monitoring of responses to treatment in both human and veterinary medicine.

One of the most common joint injuries in both humans and canines that may lead to osteoarthritis is cranial cruciate ligament rupture (19, 20). The cranial cruciate ligament is an intra-articular structure that provides stability to the knee joints (21). Rupture of this ligament either due to single traumatic event or degenerative change overtime within the ligamentous structure leads to joint instability and alters mechanical loading in the knee joint (21). If the diagnosis and treatment cannot provide in timely and appropriate fashion, this condition will lead to inflammation of synovial membrane and degeneration of articular cartilage subsequently osteoarthritis (22, 23). Moreover experimentally induced osteoarthritis by transection of cranial cruciate ligament in laboratory dogs (Pond –Nuki model) is one of the standard models that extensively used in osteoarthritic research (24-26). The Pond-Nuki animal model yields the degenerative changes in cartilage and synovial tissue that resemble those in natural occurring canine and human osteoarthritis (27, 28). Early changes of osteoarthritis have been investigated in Pond-Nuki animal model by using in vivo MRI (25). Subchondral bone edema in the posteromedial tibia, posteromedial surface irregularities of the articular cartilage and progressive degeneration of the posteromedial artilage have been detected after transaction of CCL at 6, 12, 24 weeks respectively (25). Biomarker analyses of synovial fluid demonstrated alterations in concentrations of several biomarkers and biochemical markers in both

experimental induced osteoarthritic animals and natural occurring cruciate ligament deficiency cases (22-24, 27-33). These molecular markers include type II collagen neoepitope, proteoglycan epitopes, fibronectin, cartilage oligomeric protein, keratan sulfate, hyaluronan, proteases enzymes (metalloprotease-2, -9, cathepsin etc.) and prostaglandin E2 (22-24, 27-33). The change in concentrations of these molecules within the affected joint suggesting the alteration in composition of synovial fluid that is at least in part related to degenerative process within the affected joint (22-24, 27-33).

Infrared (IR) spectroscopy is rapidly emerging as a powerful diagnostic probe for biological molecules in humans and other animals (34, 35). Infrared spectroscopy measures IR absorption patterns of molecules when exposed to IR light (35). An IR spectrum is obtained when IR radiation is transmitted through a sample in a Fourier transform IR spectrometer (FT-IR). The fraction of the incident radiation absorbed at a particular wavenumber (cm^{-1}) is determined and displayed as absorption bands on the spectrum (36). These absorption bands correspond to carbon skeletal and functional group vibrations (37). Simple molecules yield simple spectra with well-resolved absorption bands that reflect both structure and concentration (34, 38). In a complex sample, compared with a simple sample, the number of chemical functional groups increases, causing the number of absorption bands and the extent of band overlap to increase (38). The IR spectrum of a biological sample becomes more complex. However, the IR spectrum of body fluids or tissues still reflects both the structure of the individual IR active constituents and their relative abundance (35, 38). The absorption patterns in the IR spectra of biological samples may be viewed as biochemical fingerprints that correlate directly with the presence or absence of diseases (35, 39). For example, IR spectroscopy has been used in diagnosis of human diseases such as diabetes mellitus (40), Alzheimer's disease (41), breast tumors (42) and arthritic disorders (43-47). The advantages of an IR spectroscopic approach in clinical diagnosis are that no reagents are required, and automated repetitive analyses can be carried out at very low cost (35). In addition, because the IR spectrum of biological samples such as synovial fluid reflects the sum of all IR-active components (48), the infrared spectra of such samples may carry infrared signatures of known and unknown biomarkers rather than relying upon a few novel disease markers.

We hypothesized that osteoarthritis associated with natural occurring CCL rupture in dogs leads to the change in synovial fluid composition, altering the IR absorption pattern of synovial fluid samples, and that these spectroscopic changes can be detected and used to differentiate the synovial fluid spectra of joints with osteoarthritis from the spectra of control samples. The objective of the present study is to determine the feasibility and to evaluate the accuracy of IR spectroscopy for diagnosis of osteoarthritis in dogs.

Materials and Methods

Study dogs and sample collection

Synovial fluid samples (n=75) were collected from 75 otherwise healthy dogs presenting to the Kasetsart Veterinary Teaching Hospital for surgical stabilization of natural occurring cranial cruciate ligament rupture. Demographic data including age, breed, sex, and body weight were recorded for each dog. Orthopedic examinations were performed and orthopedic examination score was assigned to classify the clinical sign of

osteoarthritis in 4 criteria 1) locomotor ability, 2) weight bearing ability, 3) joint mobility, and 4) pain perception (see appendix 1). Severity of osteoarthritis in the affected knee joints were graded according to radiographic scoring system (see appendix 2) modified from Johnson et al. 2003 (20). Intraoperative scoring system (see appendix 3) was used with modification from Johnson et al. 2003 to classify the intra-articular lesion (20). Synovial fluid samples were aseptically collected from the affected knee joint prior surgical intervention, centrifuged at 2700 x g for 10 minutes and kept in plain cryovial at -80^{o}C until the batch analysis were performed.

Control synovial fluid sample were collected from 51 dogs presented at Kasetsart University Veterinary Teaching Hospital either for orthopedic evaluation or dead canine patients with non-related musculoskeletal disease. The normalcy (no evidence of cruciate ligament rupture, cartilage erosion and synovitis) of the knee were confirmed by orthopedic and radiographic examinations or necropsy (figure 1). Synovial fluid sample were collected aseptically in normal dogs or immediately after dead. The sample were centrifuged at 2700 x g for 10 minutes and kept in plain cryovial at -80^{o}C until the batch analysis were performed.

At this preliminary state, whole batch of samples were shipped frozen to the Institute of Biodiagnostics , NRC, Canada via express shipping method.

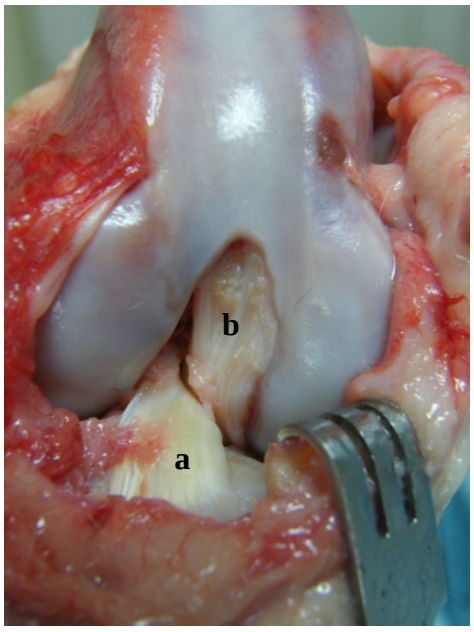

Figure 1 Cranial cruciate (a) and caudal cruciate ligament (b)

Fourier transform infrared spectroscopy (FT-IR)

Synovial fluid samples were thawed at room temperature (approximately 22 ^{o}C) and centrifuged; the supernatants were kept for analyses. Synovial fluid samples were prepared as described previously (47). Triplicate dried films were made for each sample by applying 5 µL of the diluted synovial fluid preparation evenly in a circular motion onto 5-mm-diameter circular islands on a custom-made, adhesive-masked, silicon microplate;

the adhesive mask serves to spatially define and systematically separate the 5 mm islands on the microplate so that sample islands are correctly aligned with the FT-IR detector. The synovial films were left to dry at room temperature. After the films were thoroughly dried, the microplate was mounted in a multisampler interfaced to the FTIR spectrometer to enable acquisition of IR spectra. Infrared spectroscopic analyses of all samples were performed during the same period of time.

Infrared absorbance spectra in the range of 400-4000 cm^{-1} (mid infrared region) were recorded using a FT-IR spectrometer, 512 interferograms were signal averaged and Fourier transformed to generate a spectrum with a nominal resolution of 4 cm^{-1} (47).

Data preprocessing

Triplicate spectra of each sample yielded mean values. By use of spectral manipulation software, differentiation and smoothing procedures were performed on all spectra to resolve and enhance weak spectral features and to remove variation in baselines.

Statistical Analysis

Infrared region selection was performed in order to search for diagnostic features within the spectra using genetic algorithms (47). Classification model was developed using discriminant analysis based on the diagnostic feature within the spectra selected in the previous step. The group membership (cranial cruciate ligament rupture VS control) was predicted and compared to the clinical diagnosis. Clinical diagnosis can be made based on direct visualization of intact (during necropsy in case of a control joint) or rupture of the cranial cruciate ligament (during surgical stabilization in case of a diseased joint). Sensitivity, specificity, and accuracy of the classification will be calculated for this data set.

Result

One hundred and twenty-six synovial fluid samples were submitted for analysis. Of those samples, 51 control samples were derived from 39 male and 12 female dogs with the mean age and body weight of 4.9 ± 1.7 years and 19.8±3.1 kg (mean±SE) respectively. Breed represents of the control group are crossbreed, golden retrievers and poodle. The samples of cranial cruciate ligament rupture group (CCRL) collected prior the surgical correction procedures are from 41 male and 34 female canine patients with the mean age and body weight of 5.9±1.7 years and 20.9±3.5 kg (mean±SE). Breed represents of CCLR group includes Crossbreed, Golden Retrievers, Poodles, Thai Bangkaew, Rottweiler, American Pit Bull terriers, Bull terriers, Lhasa Apso, Shih Tzu, Yorkshire terriers and Miniature Pinscher.

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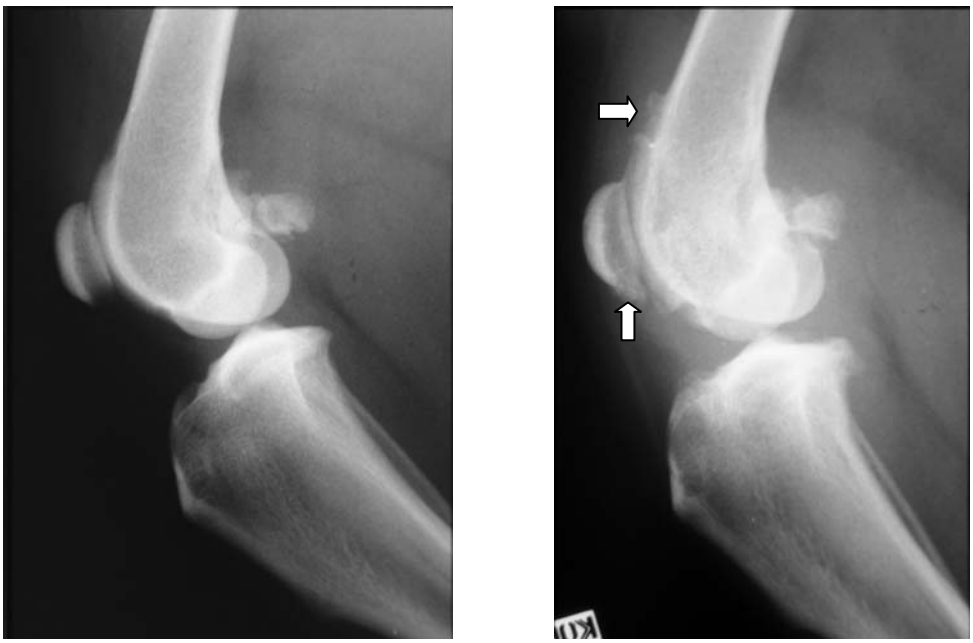

A B

Figure 2 Radiographic appearance of the normal canine stifle (A) and the radiographic changes commonly found in osteoarthritic knee of the canine patients (B); osteophyte formation (white arrow)

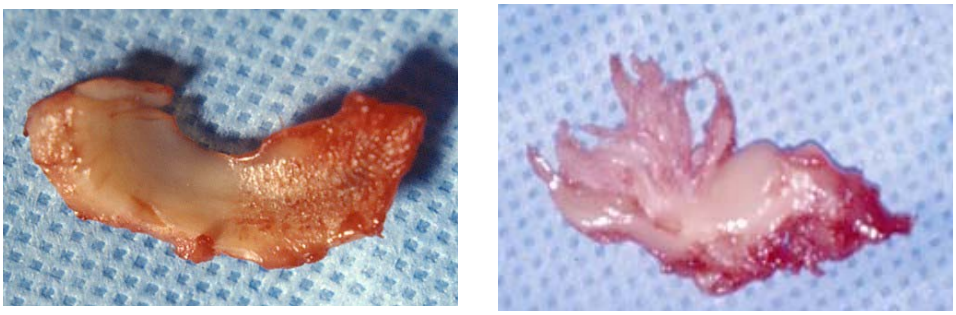

Figure 3 Gross appearance of the medial meniscus after surgically remove from the knee joints in CCLR group

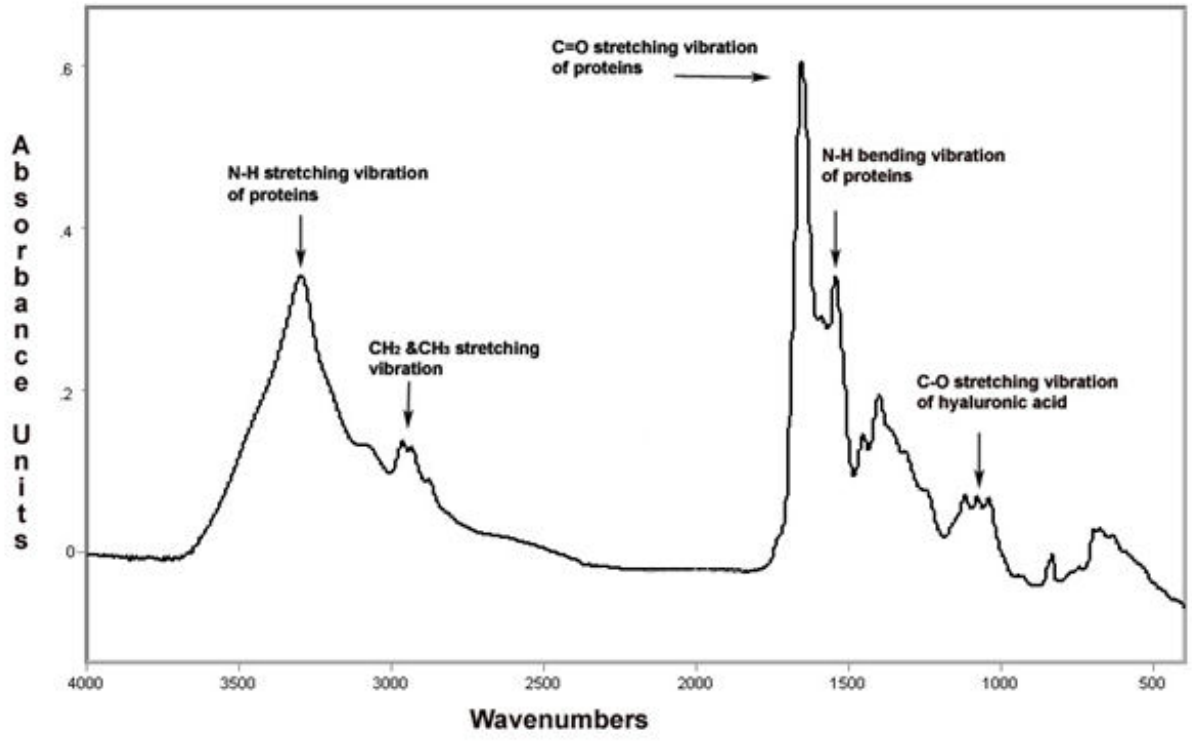

Figure 4 The infrared spectrum of synovial fluid sample composed of multiple peak derived from vibration of the functional groups within the sample

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Appendix 1 Orthopedic examination grading system

Score or grading categories	**Clinical findings**
Locomotor ability	
1	Reluctant to rise and will not walk more than a few strides
2	Abnormal posture when standing, severe lameness when walking
3	Stand normally, severe lameness when walking
4	Stand normally, slight lameness when walking
5	Stand and walk normally
Weight bearing	
1	Non-weight bearing (affected limb) at rest and when walking
2	Partial weight bearing at rest, bear no weight when walking
3	Partial weight bearing at rest and when walking
4	Normal weight bearing at rest, favours affected limb when walking
5	Normal weight bearing on all 4 limbs at rest and when walking
Joint mobility	
1	Not applicable
2	Severe (>50%) decreased range of motion, palpable joint crepitus
3	Moderate (20-50%) decreased range of motion, palpable joint crepitus
4	Mild (10-20%) decreased range of motion, palpable joint crepitus
5	Mild (10-20%) decreased range of motion, no palpable joint crepitus
6	No limitation of joint movement, no palpable joint crepitus
Pain	
1	No pain indicated on palpation of the affected joint
2	Mild pain indicated on affected joint e.g. animal turns head in recognition
3	Moderate pain on palpation of affected joint e.g. animals pulls limb away
4	Severe pain on palpation of affected joint e.g. animal vocalizes or become aggressive
5	Animal will not allow examiner to palpate joint due to pain

Appendix 2 Radiographic grading system

Note: Each features will be assigned a value of 0 (absent), 1 (mild), 2 (moderate) or 3 (severe). Score will be added to produce a cumulative joint score ranging from 0 to 39 (modified from Ref 27).

Compartment and Features	Score			
Femoropatellar				
Apical or basilar patellar osteophytes	0	1	2	3
Cranial apical patella enthesopathy	0	1	2	3
Femoral trochlear groove osteophytes	0	1	2	3
Femoral supratrochlear lysis	0	1	2	3
Femorotibial				
Femoral and tibial periarticular osteophytes	0	1	2	3
Femoral subchondral bone sclerosis	0	1	2	3
Tibial subchondral bone sclerosis	0	1	2	3
Subchondral bone lysis of femur	0	1	2	3
Subchondral bone lysis of tibia	0	1	2	3
Lateral fabellar osteophyte	0	1	2	3
Lateral collateral ligament enthesopathy	0	1	2	3
Medial fabellar osteophyte	0	1	2	3
Medial collateral ligament enthesopathy	0	1	2	3

Appendix 3 Intra-operative score

Score and anatomical structure	**Clinical Findings**
Cranial cruciate ligament	
1	Normal
2	Partially torn
3	Completely rupture with remnants of ligament remaining
4	Ligament rupture and completely absorbed
Medial or lateral meniscus	
1	Normal
2	Partial tear
3	Caudal pole folded or macerated