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Brief Report

Quantitative X-ray microradiography for high-throughput phenotyping of osteoarthritis in mice

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SUMMARY

Objective: To investigate and validate digital X-ray microradiography as a novel, high-throughput and cost-effective screening approach to identify abnormal joint phenotypes in mice.
Method: Digital X-ray microradiography was used to quantify the subchondral bone mineral content (BMC) in the medial tibial plateau. Accuracy and reproducibility of the method were determined in 22 samples from C57BL/6(B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$) wild-type mice. The method was then validated in wild-type mice that had undergone surgical destabilisation of medial meniscus (DMM) and in a genetically modified mouse strain with an established increase in trabecular bone mass.
Results: The measurement of subchondral BMC by digital X-ray microradiography had a coefficient of variation of 3.6%. Digital X-ray microradiography was able to demonstrate significantly increased subchondral BMC in the medial tibial plateau of male mice 4 and 8 weeks after DMM surgery and in female mice 8 weeks after surgery. Furthermore, digital X-ray microradiography also detected the increase in subchondral BMC in a genetically modified mouse strain with high trabecular bone mass.
Conclusion: Quantitation of subchondral BMC by digital X-ray microradiography is a rapid, sensitive and cost-effective method to identify abnormal joint phenotypes in mice of both genders at several ages.

Introduction

Osteoarthritis is the commonest joint disorder; it causes inexorable joint dysfunction, pain and disability as no drugs are available to prevent or delay disease progression. Patients are asymptomatic in the early stages of osteoarthritis and develop problems only after significant cartilage erosion has occurred. The aetiology of osteoarthritis is complex and multi-factorial with major genetic and environmental contributions[1]. Its heritability is estimated to be between 40 and 65% but currently identified disease susceptibility loci account for only a fraction of this heritability[2]. Thus, there is an urgent need to advance understanding of the pathogenesis of osteoarthritis and define new molecular pathways that facilitate the development of novel treatments.

Animal models are essential for *in vivo* study of disease mechanisms, drug targets and treatment responses[3]. A limited number of mouse models of spontaneous cartilage disorders have been described, including the STR/ort and STR-1N mouse. These models reinforce the importance of genetics in disease susceptibility but their use is limited by variable disease penetrance[4]. Surgically induced models of osteoarthritis are also used to investigate disease pathogenesis and response to treatment. However, this approach requires significant expertise and experience, and requires large numbers of mice at considerable expense. The best characterised and most reliable surgical model requires destabilisation of the medial meniscus (DMM), resulting in a high incidence of osteoarthritis in male mice 8 weeks following surgery[5]. Nevertheless, there is now a clear need to develop robust and rapid screening approaches to identify joint abnormalities in mouse models of spontaneous osteoarthritis.

The International Knockout Mouse Consortium and Medical Research Council *N*-ethyl-*N*-nitrosourea mutagenesis programmes aim to disrupt each of the >20,000 protein-coding genes[6]. These initiatives provide a unique opportunity to identify novel genetic determinants and *in vivo* models of osteoarthritis and define the cellular and molecular basis of disease. Nevertheless, to capitalise on these resources and identify mice with outlier joint phenotypes,

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new high-throughput, sensitive, specific and cost-effective joint phenotyping methods are needed.

Established osteoarthritis is characterised by articular cartilage destruction and subchondral bone sclerosis. Articular cartilage degradation progresses from surface fibrillation to formation of fissures and erosions, and finally cartilage loss with exposure of underlying bone. These changes are associated with increased chondrocyte hypertrophy, cartilage calcification and osteophyte formation[1]. Subchondral bone distributes load-bearing compressive forces and comprises the cortical plate underlying the articular cartilage and the adjacent trabecular bone. Early osteoarthritis is characterised by thinning of the cortical plate due to accelerated remodelling, whereas subchondral sclerosis is the hallmark of established disease[7].

The gold standard histological assessment of articular cartilage type, architecture and integrity in accordance with Osteoarthritis Research Society International (OARSI) recommendations[4] is laborious, expensive and unsuitable for high-throughput screening. Micro-computed tomography (CT) analysis of osteoarthritis in mice is also time consuming; suitable high-resolution imaging equipment is expensive and not widely available and the vast quantity of data generated requires an extensive archive[8].

We previously established that quantitative digital X-ray microradiography is a sensitive and specific method for determining bone mineral content (BMC)[9] and also demonstrated its suitability for high-throughput phenotyping[10]. Thus, we generated reference data for BMC in C57BL/6(B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$) wild-type mice and subsequently screened 100 knockout strains from the IKMC to identify nine outlier strains with either high or low BMC[10]. Since subchondral bone sclerosis is a consistent and established feature of both human osteoarthritis and surgically induced mouse models of osteoarthritis[11,12], analysis of subchondral BMC represents a suitable target for high-throughput joint phenotype screening approaches.

We hypothesised, therefore, that digital X-ray microradiography can be used to quantify subchondral BMC and identify osteoarthritis phenotypes in genetically modified mice. We, therefore, studied knee joints from mice that had undergone DMM surgery[5,13] and genetically modified mice with increased trabecular bone mass[14,15].

Methods

Animals

Studies were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and recommendations of the Weatherall report, and were approved by the Imperial College Ethical Review Committee. All wild-type mice used for DMM surgery were of the same C57BL/6(B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$) genetic background[10]. Additional studies were performed using TR$\alpha^{0/0}$ and control mice originally derived in a mixed C57BL/6 and 129Sv genetic background[14,15], and subsequently backcrossed onto C57BL/6N for more than 20 generations.

DMM surgery

Transection of the right medial meniscotibial ligament was performed at 10 weeks of age and sham surgery was performed on the left knee[5]. Males were sacrificed 4 weeks ($n = 6$ per group) or 8 weeks ($n = 12$ per group) post-surgery. Females were sacrificed 8 weeks post-surgery ($n = 6$ per group).

Sample preparation

Lower limb samples were fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol. Soft tissue was removed but integrity of the knee capsule and patella was preserved [Fig. 1(A)].

Histological analysis

Knees were decalcified in 10% Ethylenediaminetetraacetic acid (EDTA) for 10 days and embedded in paraffin. 5 μm coronal sections were cut at 80 μm intervals at 16 levels (10 sections per level) through the entire thickness of the joint, stained with safranin O and fast green, according to OARSI recommendations[4]. Qualitative analysis was performed to confirm the expected presence of cartilage degradation that co-localised with subchondral bone thickening in the medial tibial plateau following DMM surgery[11].

X-ray microradiography

Knee joints were held in a flexed position of 105° using synthetic rubber adhesive within a 37 mm diameter plastic ring. This resulted in consistent location of the apex of the knee joint 12 mm above the mount with the tibial growth plate in a vertical plane. The patella was used as a landmark to ensure correct orientation of the joint [Fig. 1(B)].

Anterior–posterior projection X-ray images were recorded at 10 μm pixel resolution using a Faxitron MX-20 variable kV point projection X-ray source and digital image system (Qados, Cross Technologies plc, Berkshire, UK) operating at 26 kV for 15 s and 5× magnification. Images were calibrated by X-raying a digital micrometer. The relative medial tibial plateau subchondral BMC was determined by comparison with standards included in each image (1 mm diameter steel, aluminium and polyester wires) [Fig. 1(B)]. The 2368 × 2340 16 bit DICOM images were converted to 8 bit TIFF using ImageJ (http://rsb.info.nih.gov/ij/) and the grey scale histogram was stretched from the polyester (grey level 0) to steel (grey level 255) standards. Increasing gradations of mineralization density were represented in 16 equal intervals by a pseudocolour scheme[9,10].

Image analysis

A 1000 μm × 250 μm area of the medial tibial plateau was selected as representative of subchondral bone and defined as the region of interest (ROI) [Fig. 1(C)]. A single montage was generated from the individual ROIs from all mice within one experimental group. The relative and cumulative frequency distributions of grey levels were determined and compared between groups as indicated in the figures[9,10] [Fig. 1(D)].

Statistics

As grey level frequency distributions were not normally distributed [Fig. 1(D)], the median value was determined for each sample. To define the variation in median BMC within the population of 22 female, 16-week-old wild-type mice [Fig. 1(D)] the mean and standard deviation of the median BMC values were determined. The Kolmogorov–Smirnov test was used to compare grey level cumulative frequency distributions between DMM and sham-operated mice, and between TR$\alpha^{0/0}$ and control mice. P values for the D statistic were $D \geq 6.01$, $P < 0.05$; $D \geq 7.20$, $P < 0.01$; and $D \geq 8.62$, $P < 0.001$[9].

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