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Characterization of calcium and zinc spatial distributions at the fibrocartilage zone of bone–tendon junction by synchrotron radiation-based micro X-ray fluorescence analysis combined with backscattered electron imaging

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ABSTRACT

Tendon attaches to bone through a functionally graded fibrocartilage zone, including uncalcified fibrocartilage (UF), tidemark (TM) and calcified fibrocartilage (CF). This transition zone plays a pivotal role in relaxing load transfer between tendon and bone, and serves as a boundary between otherwise structurally and functionally distinct tissue types. Calcium and zinc are believed to play important roles in the normal growth, mineralization, and repair of the fibrocartilage zone of bone–tendon junction (BTJ). However, spatial distributions of calcium and zinc at the fibrocartilage zone of BTJ and their distribution–function relationship are not totally understood. Thus, synchrotron radiation-based micro X-ray fluorescence analysis (SR-μXRF) in combination with backscattered electron imaging (BEI) was employed to characterize the distributions of calcium and zinc at the fibrocartilage zone of rabbit patella–patellar tendon complex (PPTC). For the first time, the unique distributions of calcium and zinc at the fibrocartilage zone of the PPTC were clearly mapped by this method. The distributions of calcium and zinc at the fibrocartilage zone of the PPTC were inhomogeneous. A significant accumulation of zinc was exhibited in the transition region between UF and CF. The highest zinc content (3.17 times of that of patellar tendon) was found in the TM of fibrocartilage zone. The calcium content began to increase near the TM and increased exponentially across the calcified fibrocartilage region towards the patella. The highest calcium content (43.14 times of that of patellar tendon) was in the transitional zone of calcified fibrocartilage region and the patella, approximately 69 μm from the location with the highest zinc content. This study indicated, for the first time, that there is a differential distribution of calcium and zinc at the fibrocartilage zone of PPTC. These observations reveal new insights into region-dependent changes across the fibrocartilage zone of BTJ and will serve as critical benchmark parameters for current efforts in BTJ repair.

1. Introduction

Fibrocartilaginous bone–tendon junction (BTJ), such as the patella–patellar tendon complex (PPTC), is connected through a transitional fibrocartilage zone that plays a critical role in minimizing stress concentrations and mediating the load transfer between the bone and the tendon (Fig. 1) [1–3]. Functional fibrocartilage is especially important for physiological musculoskeletal motion and joint stability [4–7]. The biomechanical functionality of the fibrocartilage is rooted in its organized structure, with region-specific extracellular matrices and cell shapes [1,5–8]. Specifically, the fibrocartilage zone of the BTJ is histologically separated by the tidemark (TM), the mineralization front and the boundary between the soft and hard tissues, into two distinct yet continuous tissue regions [3,9]. The first region is uncalcified fibrocartilage (UF), which composes of fibrochondrocytes in a matrix of consisting of type II and III collagens with small quantities of type I, IX, and X collagens as well as proteoglycans aggrecan and decorin. This region marks the beginning of the transition from soft tendinous material to hard bony material [10–13]. The second zone, calcified fibrocartilage (CF), shows a marked transition towards bony tissue. Here, hypertrophic chondrocytes are surrounded by predominantly type II collagen, significant amounts of type X collagen and aggrecan-containing matrix [10,12–14]. These variations in structure and composition give rise

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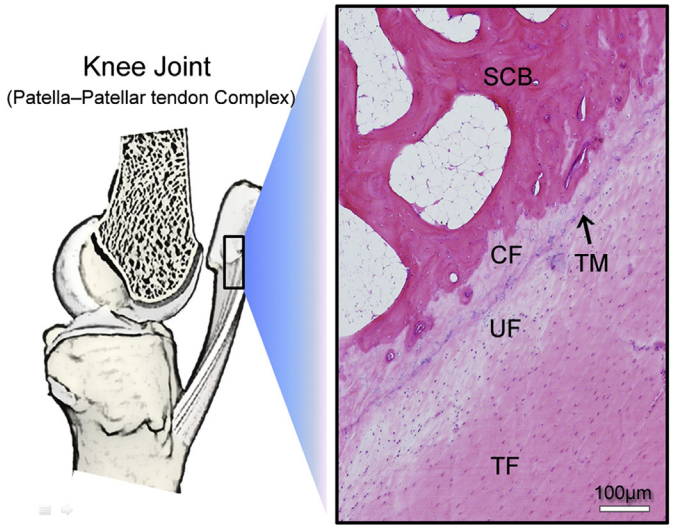

Fig. 1. Tendon attaches to bone across a biomechanically, compositionally, and structurally graded fibrocartilage zone (a H&E-stained section from an adult rabbit patella–patellar tendon complex is shown above); SCB (subchondral bone), CF (calcified fibrocartilage), TM (tidemark), UF (uncalcified fibrocartilage), TF (patellar tendon).

to graded mechanical properties across this complex interface, and these properties contribute to the effective load transfer from tendon to bone [1,5,7,8].

Although some structural features of the fibrocartilage zone has been illustrated, the spatial distributions of the critical elements have not been totally understood. As the element spatial distribution is usually uneven in different organizational structures, the spatial distribution imaging to localize critical elements in specific tissues is important to reveal their metabolic and physiological roles. Thus, the investigation of critical element spatial distribution at the fibrocartilage zone of the BTJ is necessary. Calcium and zinc that exist in the muscle, cartilage and bone are closely related to various biological processes of these tissues. Calcium and zinc are vital for these tissues to fulfill their physiological functions [15–19]. The roles of calcium and zinc mainly rely on the form of metallic ions, metalloproteinases or metallic cofactors [17,18]. Studies have indicated that calcium was involved in the mineralization of cartilage mediated by a family of annexins [15] and the calcium content significantly increased across the bone–cartilage interface [16,17]. In contrast, zinc is widespread in the active site of various enzymes and crucial for cartilage mineralization [19–21]. D.A. Bradley et al. observed an accumulation of zinc around the TM between uncalcified and calcified articular cartilage [21]. In the TM, zinc acts as a significant functional co-partner of the alkaline phosphatase (ALP) that is intimately associated with cartilage mineralization [16,17,21,22]. Above all, studies involving the spatial distribution of calcium and zinc at musculoskeletal tissue have primarily focused on bone and articular cartilage. However, no study investigated in the complex musculoskeletal tissue of the fibrocartilage zone of BTJ.

In general, element analysis of biological samples is based on destructive methods. The information about the element spatial distribution within the tissue is usually lost [23]. Meanwhile, the amount of calcium and zinc in living organisms is generally low, so the distributions of these elements cannot be readily visualized. In contrast to calcium, zinc is a trace element, which is present in minute quantities (<ppm) in living organisms [19,23]. Therefore, it would be even more challenging to investigate the distribution of zinc in biotissue with high spatial resolution and sensitivity. With the advent of third-generation synchrotron radiation (SR) light source, synchrotron radiation-based micro-X-ray fluorescence analysis (SR-μXRF) has become available for the non-destructive detection of calcium and zinc in the femtogram range at the micrometer level [24–26]. In addition, most elements of biological interest can be simultaneously detected and mapped by SR-μXRF [23].

Therefore, the goal of this study is to characterize the spatial distributions of calcium and zinc at the fibrocartilage zone of the PPTC by using SR-μXRF with BEI. BEI is a well-validated and established technique for visualizing the distribution of calcium in calcified tissues with a spatial resolution of <1 μm [20,27]. This technique is suitable to define the distinct tissue regions of the fibrocartilage in the SR-μXRF maps. Findings from this study will lead to new insights into element distribution at this critical musculoskeletal tissue and provide some benchmarks for the evaluation of the BTJ repair.

2. Methods

2.1. Sample preparation

The experimental protocol was approved by the Animal Ethics Committee in accordance with the Animal Care and Use Guidelines of Central South University. Healthy adult male New Zealand rabbits were used in this experiment. Five harvested PPTC samples were split into two parts along the middle sagittal plane of the PPTC. One half of every sample was immediately fixed in 70% alcohol and then dehydrated in a graded series of alcohol. Then, the samples were prepared as undecalcified PPTC tissues in polymethylmethacrylate (PMMA) blocks [20]. Afterwards, the undecalcified PMMA blocks were cut along the sagittal plane of the PPTC by a microtome (Leica Sp1600; Leica Instruments, Nussloch, Germany) and were grinded to a thickness of 100 μm with a grinding machine (Phoenix4000; Wirtz Buehler, Germany). To reduce errors, the entire sample preparation process was tested to be free of detectable calcium and zinc contaminations. Sections of PPTC were then photographed by optical microscopy (Leica M165 FC; Leica Microsystems, Germany) to select and define the regions of interest (ROI) prior to SR-μXRF. The ROIs are the patella, CF, UF and patellar tendon (Fig. 2). The other half of the samples was fixed in 10% neutral buffered formalin for 1 day. These samples were then decalcified with EDTA for 3 weeks and embedded in paraffin. Then, the paraffin blocks of the PPTC samples were used for histology.

2.2. BEI and histology

The BEI was based on the detection of electrons backscattered from 1.5 μm below the surface of the specimen struck by the primary electron beam of a scanning electron microscope (SEM) [27–29]. The gray level of the BEI image was proportional to the concentration by weight of

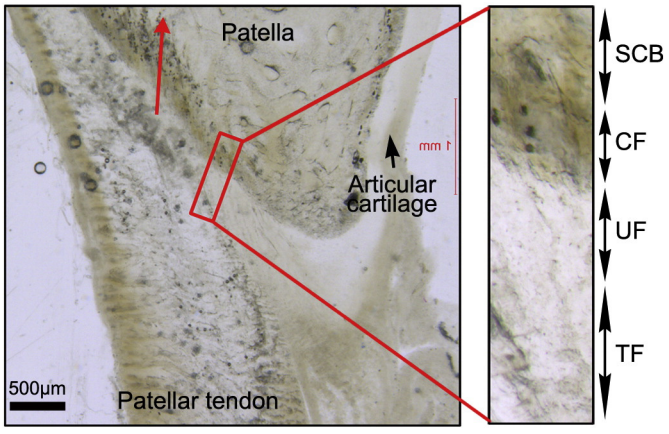

Fig. 2. Histomorphological image of a PMMA-embedded section of the PPTC to define the regions of interest (ROI) prior to SR-μXRF; area scan (red-framed box), line scan (red arrow), SCB (subchondral bone), CF (calcified fibrocartilage), UF (uncalcified fibrocartilage), TF (patellar tendon). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)