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Contrast enhancement with uranyl acetate allows quantitative analysis of the articular cartilage by microCT: Application to mandibular condyles in the BTX rat model of disuse

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ABSTRACT

Microcomputed tomography (microCT) is well adapted to quantitative analysis of calcified tissues but soft tissues (such as cartilage) are radiolucent and need a contrast enhancement procedure for microCT. We developed a "staining" method allowing microCT imaging of articular cartilage using uranyl acetate (UA). The method was used to see whether adult rats with a botulinum toxin (BTX) injection in masticatory muscles present a change at the condylar cartilage of the mandible in association with a localized trabecular bone loss.

Human femoral head samples were used to develop the enhanced contrast method using UA or lanthanides (recently proposed as a substitute for UA). The method was then applied to the condylar cartilage of rat mandibles. Mature male rats ($n = 11$) were randomized into 2 groups: control (CTRL; $n = 4$) and BTX group ($n = 7$). Rats of the BTX group received a single injection of BTX into the right *M. Masseter* and *M. Temporalis*. Rats of the CTRL group were similarly injected with saline. Rats were sacrificed 4 weeks after injection. Condyles were harvested, fixed in formalin and immersed in UA. MicroCT was performed for bone and cartilage measurements.

After UA impregnation, articular cartilage of human femoral head samples was clearly seen on its full thickness whereas lanthanides produced a much less pronounced contrast, with a faint labeling at the upper layer. In BTX rats, microCT analysis showed a significant bone loss at the right condyles. After UA, the whole thickness of articular cartilage was clearly evidenced. Cartilage thickness measurement showed no difference when comparing the right with the left sides of the BTX group nor between the two sides of the CTRL group.

Contrast enhancement with UA is a simple technique allowing quantitative analysis of cartilage by microCT.290 words.

1. Introduction

Articular cartilage is a highly specialized connective tissue of synovial joints, it is supported by subchondral bone. Its main function is to provide a smooth articular surface in order to facilitate the transmission of loads with a low frictional coefficient (Sophia Fox et al., 2009). It is made of a dense extracellular matrix composed mainly of water, type II collagen and proteoglycans with specialized cells called chondrocytes. Histology is a commonly used method to analyze articular cartilage in pathologic conditions. The main limitation of the technique is that it is destructive and causes tissue alterations. Furthermore, sample positioning is difficult to reproduce, leading to a different section obliquity for each slide. Microcomputed tomography (microCT) allows high resolution 3D imaging making precise quantitative measurement possible (Ruegsegger, 1994; Sasov and Van Dyck, 1998). This technique is challenging for soft tissue imaging, such as cartilage, because X-ray attenuation of a non-mineralized soft tissue is very low (Naveh et al., 2014). Contrast enhancement using a "staining" procedure is therefore mandatory with the use of various metal salts (Metscher, 2009; Pauwels et al., 2013). Several contrasting

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methods allowing cartilage imaging have been described using iodinated contrasting agents for computed tomography or magnetic resonance imaging (such as ioxaglic acid (Hexabrix®) iopromide (Ultravist®) gadolinium), iodine-potassium iodide (IKI), phosphotungstic acid (PTA), phosphomolybdic acid (PMA), or osmium tetroxide (Cockman et al., 2006; Das Neves Borges et al., 2014; Kerckhofs et al., 2014; Metscher, 2009; Nieminen et al., 2015; Pauwels et al., 2013; Renders et al., 2014; Xie et al., 2010; Yoo et al., 2011). However, none of them were fully reproducible in our hands for contrast enhancement of cartilage imaging in 3D using microCT.

The aim of the present study was to describe a new contrast enhancement technique of cartilage for microCT based on electron microscopy procedures allowing a quantitative analysis of cartilage thickness. The technique was first elaborated on the human hip articular cartilage and then applied in an animal study. We have searched if changes in a thin articular cartilage (*e.g.*, the condylar articular cartilage of the mandible) occur in a pathologic condition. We chose to study the changes induced by botulinum toxin (BTX) injected in masticatory muscles. In animal models, BTX injections cause a muscle atrophy associated with a disuse bone loss (Chappard et al., 2001). Whether disuse also acts on this articular cartilage is largely unknown and a microCT method after contrast enhancement of the cartilage matrix is of great interest.

2. Materials and methods

2.1. Human femoral heads

Femoral heads from patients with a recent trans-cervical hip fracture were used in this study to develop the contrast enhancement technique for articular cartilage. The specimens were cut at the top of the head, in the area of the main compressive bundle of trabeculae. On a first femoral head, four samples were harvested and fixed in 10% formalin for 48 h (Merck KGaA, Darmstadt, Germany) (Carson and Hladik, 2009). The first specimen was included undecalcified in poly(methylmethacrylate) for routine histology. The second sample was imaged by microCT while in the fixative. The second sample was immersed for 48 h in uranyl acetate (UA). UA (Merck) was prepared as a 3% solution in 50° ethanol; the solution was filtered on a 0.2 μm syringe filter and stored at 4 °C in the dark. The "stained" specimen was rinsed during 1 h in running tap water, transferred to 10% formalin and scanned in the fixative. The fourth specimen was stained with a lanthanide solution (LFG Distribution, Lyon, France). It comprised 1% lanthanum acetate, 1% gadolinium acetate and 1% samarium acetate and was prepared in ethanol 50°. The specimen was immersed similarly during 48 h in the lanthanide salts solution, rinsed during one hour in tap water, placed in formalin and scanned as above. Additional trials were done with IKI (1% iodine, 2% potassium iodide), PTA (1.5% in water) and Iopamiron 300® (Bracco Imaging France), a contrasting agent for computed tomography containing 300 mg iodine/ml. These three additional reagents were used on pieces coming from another femoral head.

2.2. Animals and experimental procedure

Animal care and experimental protocols were approved by the French Ethical Committee (protocol agreement number 01732.01) and under the supervision of authorized investigators. Eighteen weeks-old male Sprague-Dawley rats ($n = 11$), weighing 587 ± 26 g, were used for the study (Janvier-Labs, Le Genest-Saint-Isle, France). They were acclimated for two weeks to the local vivarium conditions (24 °C and 12 h/12 h light dark cycle) where they were given standard laboratory food (UAR, Villemoison-sur-Orge, France) and water *ad libitum*. Rats were randomized into 2 groups: control group (CTRL, $n = 4$) and BTX-injected group (BTX, $n = 7$). Rats from the BTX group were anesthetized with isoflurane and injected intramuscularly with 2 U (0.4 ml) of type A BTX: 1 U in the *M. Masseter* and 1 U in the *M. Temporalis* (Botox®, Allergan Inc., Irvine, CA, USA) as previously described (Kün-Darbois et al., 2015). Three points of injection for each *M. Masseter* and two for each *M. Temporalis* were necessary. Rats of the CTRL group were similarly injected with equivalent volume of saline. Rats were weighed weekly and were sacrificed 4 weeks after injection by CO_2 inhalation. Hemimandibles were dissected, defleshed and fixed in 10% formalin until use (Carson and Hladik, 2009). Condylar processes were separated from the rest of each hemimandible and scanned in the fixative prior to immersion in UA (Fig. 1A and B). Because the rat condylar cartilage is thinner than the human hip cartilage, each sample was then immersed for 24 h in the UA solution, rinsed in tap water for one hour to remove unlabeled UA, transferred in 10% formalin and scanned as above. After microCT analysis, samples were embedded undecalcified as above for histological analysis.

2.3. Microcomputed tomography

MicroCT of human femoral samples and rat mandibular condyles were performed using a Skyscan 1172 X-ray computerized microtomograph (Bruker microCT, Kontich, Belgium) equipped with an X-ray tube working at 70 kV/100 μA. Bones were placed in plastic tubes filled with formalin. The tubes were fixed on a brass stub with plasticine. Analysis was done with a pixel size corresponding to 13 μm (human femoral heads) and 2.94 μm (rat condyles). The rotation step was fixed at 0.20° with a 0.5 mm aluminum filter. For each sample, a stack of 2D-sections was obtained and reconstructed using NRecon software (Bruker) and analyzed with the CTAn, DataViewer and CTvol softwares (Bruker). MicroCT acquisition parameters, reconstruction and rendering settings were strictly alike between all the concerned specimens and for all images. After microCT analysis, the condyles were embedded in poly(methylmethacrylate) for histological analysis.

2.4. Quantitative analysis of cartilage and bone effects of BTX injections

Cartilage thickness (Cart.Th) measurement of the condyles was performed on 2D sagittal and frontal sections obtained by using the cutting plane facility of the software. For each condyle, 12 measurement points were used in 3 serial frontal sections and 1 sagittal section (Fig. 1C). The measured width (in μm) was comprised between the tide-mark and the external surface of the articular cartilage.

Bone morphometric parameters were obtained from the first scan images obtained before immersion in UA. The absolute bone volume (C.BV/C.TV, expressed in % and representing the percentage of volume occupied by cortical and trabecular bone) was measured in 3D using the CTAn software for each condyle. Trabecular bone volume could only be accurately measured in 2D (B.Ar/T.Ar, expressed in %, representing the fractional area occupied by trabecular bone) as previously described because the delineation of the region of interest is difficult on such a small bone segment (Kün-Darbois et al., 2015).

2.5. Histology on undecalcified bones

Because we wanted to see if the UA location corresponded to specific histological areas, the bones were processed undecalcified. Samples were dehydrated in a mixture of acetone/xylene and embedded undecalcified in poly(methylmethacrylate) as previously described (Chappard, 2014). Sections (7 μm in thickness) were cut dry on a heavy-duty microtome equipped with 50° tung-