Int J Clin Exp Med 2015;8(11):20086-20091
www.ijcem.com /ISSN:1940-5901/IJCEM0014978

Original Article

Phase-contrast imaging with synchrotron hard X-ray of micro lesions of the cartilage of the femoral head in rabbits

Wei Sun[1*], Yong Zhang[2*], Fuqiang Gao[1], Zirong Li[1], Gang Li[3], Lin Pan[4]

[1]Centre for Osteonecrosis and Joint-Preserving & Reconstruction, China-Japan Friendship Hospital, Beijing 100029, China; [2]Department of Cardiovascular Surgery, Jinan Command General Hospital of PLA, Jinan 250031, China; [3]Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Beijing 100039, China; [4]China-Japan Friendship Institute of Clinical Medical Science, Beijing 100029, China. [*]Equal contributors.

Received August 24, 2015; Accepted October 25, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Background: To observe micro lesions on the cartilage of the rabbit femoral head using phase-contrast imaging with synchrotron hard X-ray and to prove that this method can be useful in the study of the degeneration of cartilage. Methods: New Zealand white rabbits were used in a micro lesion model of rabbit femoral head cartilage. Bilateral femoral heads were excised from rabbits, and micro lesions were made on one side with a specially made knife with a blade 20 μm in width. The other femur was left intact to serve as the control. Phase-contrast imaging with synchrotron hard X-ray and conventional X-ray imaging were used to observe the cartilage. Histological changes were investigated using modified Golden tri-color staining. Results: Phase-contrast imaging with synchrotron hard X-ray clearly showed the 20 μm lesions on the cartilage on the heads of rabbit femurs. These lesions were not visible with conventional X-ray imaging. Histological observation confirmed the presence of the microscopic lesions. Conclusion: Phase-contrast imaging with synchrotron hard X-ray can detect microscopic lesions on cartilage that cannot be detected by conventional absorption-contrast X-ray. This provides an unequivocal, non-invasive alternative to histological examination in the diagnosis of joint disease. It should be considered a new tool in osteoarthritis and cartilage research.

Keywords: Phase-contrast, synchrotron, femoral head, cartilage

Introduction

Osteoarthritis (OA), also known as degenerative arthritis, degenerative joint disease, or osteoarthrosis, is a kind of disease characterized by loss of cartilage caused by many factors such as increased age, trauma, obesity, strain, joint congenital abnormality and so on. Symptoms may include joint pain, tenderness, stiffness, locking, and sometimes an effusion. Unfortunately, OA is poorly understood. Until now, there is no satisfactory tool to detect OA progression, especially the early stage cartilage degeneration and micro lesions. Diagnosis of OA is based mainly on clinical symptoms, signs and imaging examinations such as radiography, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (UI), biomarker detection [1]. The lack of sufficient image quality and resolution and other limitations for an adequate visualization of cartilage has called for the development of new methods sensitive to micro lesions in cartilage that has not yet been damaged irreversibly [2]..8

Phase-contrast imaging (PCI) with hard X-ray has been used to fill the gap by facilitating the early and precise visualization of OA through the analysis of both cartilage and subchondral bony details. Currently, PCI with hard X-ray has seen widespread use in many scientific fields, such as biomedicine and material science. This is attributed to its ability to produce microstructural information of weakly absorptive contrast samples (such as early tumors, vessels, and high-polymer materials) by means of high

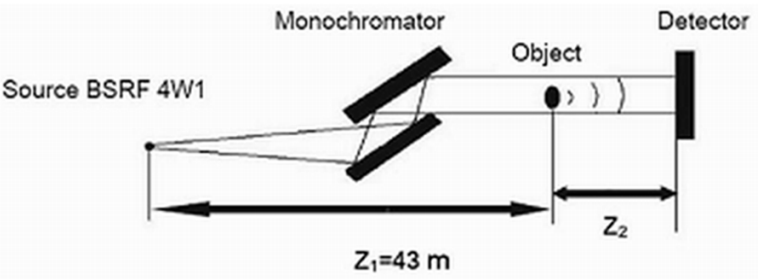

Figure 1. Schematic representation of the phase-contrast imaging with synchrotron hard X-ray. The synchrotron radiation light projected on the samples was refracted by two crystals, and then imaged on the posterior monitor.

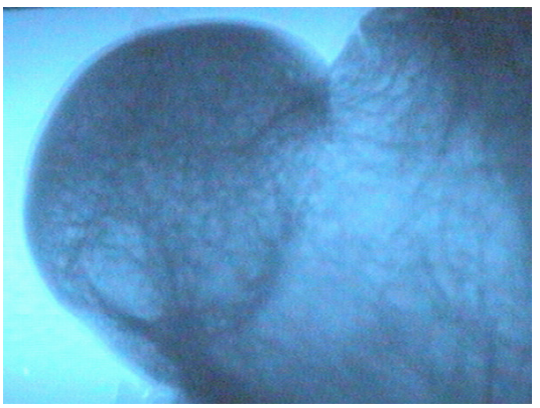

Figure 2. PCI with synchrotron hard X-ray of rabbit femoral head, overview. Full-thickness cartilage of rabbit femoral head can be clearly observed.

spatial resolution (at micrometer) at high-density resolution (the phase term was 1,000 times as high as the absorption term in the optical constants of light elements including C, H, and O). The light source of synchrotron radiation may be used to develop the theoretical basis of X-ray image towards wave optics, which initially uses geometrical optics for the high luminance, high collimation, and high spatial coherence. In this way, synchrotron radiation offers a novel approach to materials science, biology, medicine, environmental science, and medical diagnosis and treatment [3].

The effectiveness of PCI was evaluated using synchrotron hard X-ray to detect micro lesions in cartilage that cannot be detected by conventional absorption-contrast X-ray.

Materials and methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the ethics committee of China-Japan Friendship Ho-spital before implementation (Permit Number: 2012-KY-29), and the information was stored in the hospital database and for use in research. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Experimental animals and taxidermy

Twenty-five New Zealand white rabbits (from Japan Friendship Clinical Medicine Institute of Animal House), male and female, weighing 2500-3000 g, were killed by air embolism to the ear vein after anesthesia with 3% sodium pentobarbital at 30 mg/kg doses. Vertical incisions were made upon the greater trochanter to the distal femur. After separating the muscle tissue around the joints of the hip and knee, the femurs were removed. Cartilage lesions of the femoral head were made with a specially made knife whose blade was just 20 μm in width. The contralateral femurs were kept intact and served as the control group. Specimens were kept in 4% formalin for the analysis of conventional X-ray imaging and PCI with synchrotron hard X-ray.

X-ray observations

All specimens were examined on conventional X-ray imaging and used to observe contrast imaging of cartilage.

The femoral heads were shot using a single same batch of X-ray film with the following radio piece conditions: time 0.03 s, current 80 m, and voltage 45 kv. Images were saved in tagged image file format (tff).

PCI with synchrotron hard X-ray

The synchrotron radiation system used here was provided by the Laboratory of Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences.

The synchrotron radiation light projected on the samples was refracted by two crystals, and

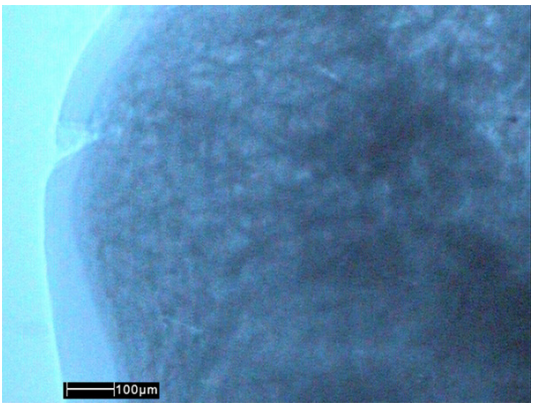

Figure 3. PCI with synchrotron hard X-ray of cartilage lesion of the femoral head. The 20 μm cartilage lesion could be distinguished clearly.

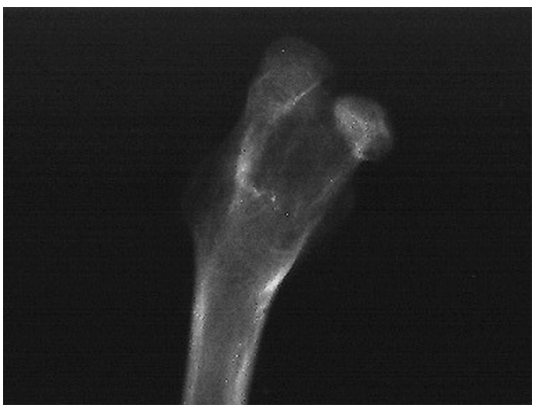

Figure 4. Conventional X-ray imaging of a rabbit femoral head. Not all of the cartilage is visible.

then imaged on the posterior monitor (**Figure 1**). The synchrotron radiation broadband hard X-ray from the BSRF 4W1 single-cycle electromagnetic undulator served as the light source; energy 5-18 KeV, electron energy of synchrotron light 2.202 2 GeV, critical energy of synchrotron radiation 5.8 KeV, beam intensity 60.4-70 mA, and size of electron beam group sx = 0.786 mm and sy = 0.279 mm. A 4W1A beam line 43 m long was used to guide the synchrotron light into the chamber, which had two four-blade slits fixed on the line to block stray light and collimate synchrotron light. A photon shutter on the beam line was applied to control exposure time. Broadband hard X-ray was projected into CWOX ray scintillating crystal through the samples, converting the inverted X-ray image to a visible image. The final image was obtained using the visible image and CCD monitor. ACdWO3 crystal with 450°C of the incident light was located behind the sample, serving as a fluorescence target, while the visible microscope and visible CCD served as the imaging monitor. Image format was set as BMP. The spatial resolution of the system was accurate than more than 10 μm.

Synchrotron radiation broadband light was then introduced to carry out in-line holography PCI according to different rotation angles (mean 30) in all the femoral head samples at the anteroposterior position, as in the PCI terms. All the samples were imaged using the same synchrotron radiation operator at the same energy level, beam intensity, luminance, and the micro lesions on the cartilage were compared.

Histological observation

Animal specimens were fixed in 10% formalin solution for more than 48 h after PCI observation, decalcified by hybrid technology (mixed decalcifying solution: formaldehyde 100 ml, formic 80 ml, hydrochloric acid 70 ml, aluminum chloride 60 g, glacial acetic acid 25 ml, saline 1000 ml) at room temperature for 2 days, then directly into 70% ethanol for 10 h, 80% ethanol for 14 h, 95% ethanol for 10 h, ethanol I for 10 h, ethanol II for 10 h, clove solution for 10 h, butanol for 14 h, xylene I for 1 h, xylene II for 1 h, then in dipping wax for 10h, and finally embedded in paraffin for coronal slicing (6 μm). Modified Golder tri-color staining was used after slicing. The sample was washed in distilled water and sliced while wet. Excess water was removed with hematoxylin and ferric chloride at 50:50, stained for 3 min, rinsed twice with warm water, stained with Ponceau-Acid magenta dye for 12 min, rinsed with 0.5% acetic acid I, stained with 0.5% phosphomolybdate for 3 min, rinsed with 0.5% acetic acid II, stained with brilliant green dye for 3-5 min, quickly rinsed with 0.5% acetic acid, air-dried, and xylene sealed. Then the lesions on the cartilage were observed.

Statistical analysis

All the images of the 2 groups were graded according to Kellgren-Lawrence (KL) grading scale and the results were analyzed with SPSS17.0 statistical software. The Student's T test was used to determine the KL grading scale difference. Differences with $P<0.05$ were considered statistically significant.

Table 1. Imageology grading of different groups

Group	N	KL (MEAN Rank)
PCI	25	34.2*
X-ray	25	0.33

*Statistical difference was found between PCI and X-ray group. P<0.01.

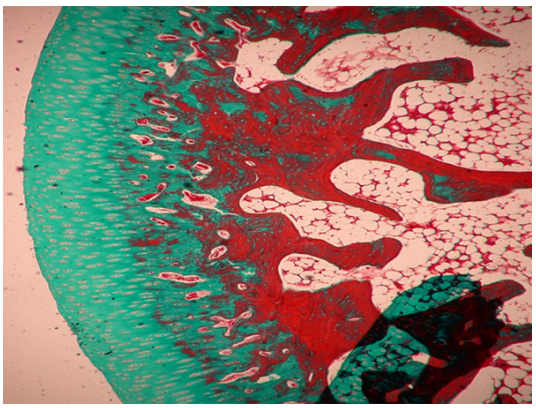

Figure 5. Pathological observation: modified Golder tri-color staining of the rabbit femoral head. The cartilage was stained green, bone tissue red, and bone marrow light pink (×400).

Results

Full-thickness cartilage from the femoral heads of rabbits can be clearly observed with synchrotron hard X-ray (**Figure 2**) which has a resolution of only about 100 μm. However, the 20 μm lesions were clearly distinguished here (**Figure 3**). Not all of the lesions on the cartilage of the specimens were visible under conventional X-ray imaging (**Figure 4**; **Table 1**).

Pathological observation showed cartilage to have been stained green by modified Golder tri-color staining (**Figure 5**), but bone tissue was stained red and bone marrow light pink. The 20 μm lesion of rabbit femoral head cartilage was observed under a light microscope (**Figure 6**). This confirmed that the cartilage micro lesion model had been created successfully and the accuracy of detection technology of PCI with synchrotron radiation hard X.

Discussion

Synchrotron radiation light is the fourth type of artificial light. The others are electric, X-ray, and laser light [4]. Phase-contrast imaging is superior to traditional absorption imaging in readability and resolution [5]. In this way, synchrotron radiation is a suitable for use in material sciences, biology, medicine, and environmental science, as well as in medical diagnosis and treatment. It has high spatial resolution and electron flux of synchrotron radiation light. Large specimens are always observed at least 10 to 25 μm. Here, the density resolution of the boundary between different structures is set to 0.0003-0.002 g/cm^3. For small specimens, the spatial resolution was 0.2 nm (soft X-ray microscopy, holography at 10 nm resolution of the wavelength in the water window) [6].

Articular cartilage is a soft tissue composed of chondrocytes and cartilage matrix, chondrocytes divided into three layers, shuttle-type chondrocytes at the surface, pillar-shaped in the middle, and hypertrophic chondrocytes in the deepest later. These are separated from the rest of the body by a narrow calcified cartilage matrix layer. Cartilage matrix was secreted primarily from chondrocytes. The matrix was composed of water, type II collagen, and proteoglycan. There were no vessels or lymphatic vessels. Normal cartilage was light blue or white in color, transparent, smooth, and elastic. In the event of a disease, the cartilage can undergo breakage, erosion denudation, thinning, and other problems. Conventional X-ray and CT imaging of cartilage are not currently used in imaging studies of cartilage damage and degeneration can involve. Ultrasound is used for diagnosis of articular cartilage lesions, but because it can only provide information regarding the surface of the cartilage, its usefulness is limited in clinical settings. Clinically, MRI is the gold standard in the detection of lesions on cartilage, but it also has many limitations. These include its high cost and long imaging time. Clinical MRI scans are usually 5 mm thick, so smaller degenerative cartilage lesions and early cartilage disease are difficult to get diagnose. Its accuracy and utility are needed to improve [7, 8]. However, in recent years, studies showed PCI with synchrotron radiation to be uniquely capable of visualizing cartilage. Previous research has shown the value of synchrotron radiation in the imaging of cartilage defects [9, 10]. Muehleman et al. used synchrotron radiation diffraction to enhance the imaging of joint cartilage, and proved that diffraction enhanced imaging is

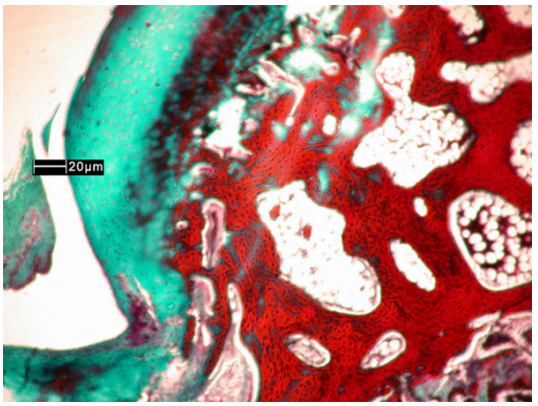

Figure 6. The 20 μm lesion on the cartilage of the rabbit femoral head as viewed under a light microscope (×400).

advantageous in the diagnosis of cartilage degeneration and is deemed to be more widely used [11-15]. The results suggest that PCI may be of use in the study of osteoarthritis and cartilage.

Researchers have used synchrotron hard X-ray phase-contrast imaging on the mammary gland. They found synchrotron hard X-ray was superior to molybdenum radiography for comparison of mammary gland tissues with various lesions [16, 17]. Salomé initially reported the imaging of trabecular bone with synchrotron radiation light in 1999 and then progressed through a series of other investigations [18]. Weiss was the first to observe the differences in osteocyte growth in different biomaterials using synchrotron radiation light and obtained satisfactory images [19]. In a previous study, the current team also assessed the effectiveness of PCI on bone tissue repair at a microscopic level on rabbit model with osteonecrosis of the femoral head [20].

Researchers have suggested that, although synchrotron radiation light is now dominant in synchrotron hard X-ray phase-contrast imaging, future technical modifications and more practical light sources, such conventional X-ray sources, may also be utilized, which may expand the range of application of X-ray phase imaging [21, 22]. However, currently, the most promising technology for transporting phase contrast imaging into a clinical environment is the table-top synchrotrons, which are currently under development [23]. In the current study, the samples were isolated and fixed with formalin. Studies have shown that 4% formalin has no influence on the PCI with synchrotron hard X-ray [15]. Pilot experiments were used to preliminarily assess the feasibility of in vivo studies with PCI. Results revealed that the PCI with synchrotron hard X-ray had high resolution. The lesions on the cartilage of the femoral heads were only 20 μm in size, but they were still clearly visible. This means that PCI has great significance in the early diagnosis and treatment of bone and joint arthritis.

The limitations of this study were first that suitable MRI facilities could not be found for such small samples, second that it was not easy to confirm that the micro lesions had indeed been made on the cartilage. Finally, it was not possible obtain the exactly matched PCI images and corresponding histology sections because of the consummate slicing skill required to create these micro lesions. This may be improved in the next study.

In short, micro cartilage lesions can be observed by PCI with synchrotron hard X-ray. This may facilitate the discovery of early osteochondral lesions. In this way, it has great clinical value for the early diagnosis of degenerative diseases of the bones and joints. It is a clear and non-invasive method suitable for clinical use in osteoarthritis and cartilage research. We are confident that this method will supplement MRI even in pathology and it may become an alternative approach to evaluation and diagnosis and so influence the treatment of cartilage disease.

Acknowledgements

We acknowledge the support of the Laboratory of Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences. This study was supported by the National Natural Science Foundation of China (81372013), the Research Fund of China-Japan Friendship Hospital (2013-MS-27) and China-Japan Friendship Hospital Youth Science and Technology Excellence Project (2014-QNYC-A-06).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei Sun, Centre for Osteonecrosis and Joint-Preserving & Reconstruction, 2 Yinghuadongjie, Chaoyang District, Beijing

100029, China. Tel: 00861084205009; E-mail: sun887@163.com

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