Experimental Study on Phase-contrast Imaging With Synchrotron Hard X-ray for Repairing Osteonecrosis of the Femoral Head

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abstract

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Synchrotron radiation light is 1 of 4 artificial light sources, the others being electric light, X-ray, and laser. Phase-contrast imaging with hard X-ray has achieved wide application in many scientific fields, such as biomedicine and material science. This article compares the effectiveness of nano-hydroxyapatite/collagen (nHAC) and autologous mesenchymal stem cell for the repair of defects in a rabbit model with osteonecrosis of the femoral head under the monitoring of phase-contrast imaging with synchrotron hard X-ray. We established models of bilateral osteonecrosis of the femoral head defect using New Zealand rabbits and divided them into 3 groups. Imaging techniques such as phase-contrast imaging and diffraction enhanced imaging with synchrotron hard X-ray were applied to assess the degradation and repair process of nHAC and mesenchymal stem cell at 4, 8, and 12 weeks postoperatively. We found phase-contrast imaging with synchrotron hard X-ray displayed the reparative process of the bone defect, degradation of nHAC, and osteocyte substitution. There were significant differences in the repair of the bone defect and osteogenesis in groups B and C compared with group A (control). Osteogenesis was more significant in group C. We provided experimental data for the development and application of synchrotron hard X-ray imaging techniques and concluded that phase-contrast microimaging with synchrotron hard X-ray displays the reparative process of bone tissue at a micro-level and plays an important role in the development of tissue engineering.

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With the high luminance, high collimation, and high spatial coherence, the light source of synchrotron radiation may develop the theoretical basis of X-ray image towards wave optics, which is initially geometrical optics. Phase-contrast imaging with hard X-ray has achieved wide application in many scientific fields, such as biomedicine and material science.1-5 This is attributed to its ability to obtain microstructural information of weakly absorptive contrast samples (such as early tumor, vessels, and high polymer materials) by means of high spatial resolution (at micrometer) and high density resolution (the phase term was 1000 times as high as absorption term in the optical constants of light elements including C, H, and O). Thus, synchrotron radiation offers a novel research approach for the material sciences, biology, medicine, and environmental science, as well as medical diagnosis and treatment owing to the high spatial resolution and electron flux of synchrotron radiation light.

Synchrotron radiation light is the fourth artificial light source, which previously include electric light, X-ray, and laser. Phase-contrast imaging including interferometry, diffraction method, and in-line holography is an imaging technique based on a phase-shift distribution due to X-ray refraction effect. It can record different structural information in weakly absorptive substances, and clearly displays X-ray two-dimensional (photography) or three-dimensional (computed tomography [CT]) micro-image, which consists of the unrecorded information by the traditional absorption image.

Large specimens are always observed at the minimal level of 10 to 25 μm, and density resolution of the boundary between different structures was set at 0.0003 to 0.002 g/cm³, while the spatial resolution for the small specimen is 0.2 nm (soft X-ray microscopy, holography at 10 nm resolution of the wavelength in the water window).

The phase-contrast imaging is superior to traditional absorption imaging in readability and resolution. Moreover, the synchrotron radiation light has the following characteristics:
- **Strong radiation power:** The energy spectrum is 103 to 104 as bright as the characteristic line of X-ray pipes, leading to shorter data collection times;
- **Wide spectrum:** Phase-shift information of any structure can be selected because the spectrum covers the infrared, visible, ultraviolet, and X-ray wave bands;
- **Polarizability:** Both plane polarization and circular polarization are observed;
- **Collimation and strong directionality:** Suppose competition with laser. Thus, synchrotron radiation offers a novel research approach for the material sciences, biology, medicine, and environmental science, as well as medical diagnosis and treatment owing to the high spatial resolution and electron flux of synchrotron radiation light.

We evaluated the effectiveness of nano-hydroxyapatite/collagen (nHAC) and autologous mesenchymal stem cells in the repair of rabbit osteonecrosis of the femoral head defect with phase-contrast imaging of synchrotron hard X-ray.

### Materials and Methods

#### Modeling of Osteonecrosis of the Femoral Head Defect and Culture and Identification of Mesenchymal Stem Cells

Forty-five New Zealand white rabbits, both genders, weighing 2.5 to 3.0 kg, were used for the establishment of osteonecrosis of the femoral head models. Under 3% pentobarbital anesthesia, the rabbits bilateral hip joints were incised anterolaterally to expose the femoral head and anterior superior femoral neck, in which Light Bulb5,7 operations were performed. The anteromedial femoral head was drilled with a 5-mm electric drill bit, and cancellous bone and subchondral bone were scraped out, accounting for 60% to 70% of the total femoral head. After osteonecrosis simulation and focal debridement were performed, the carboxyl acid was used to cauterize the tissues surrounding the bone defect to induce necrosis of osteocytes and bone marrow cells.

The rabbits were divided into 3 groups, each containing 15 animals. Group A was induced only with osteonecrosis of the femoral head without filling, Group B was filled with nHAC (provided by Department of Materials Science and Engineering, Tsinghua University, Beijing, China), and Group C (control) was filled with nHAC and mesenchymal stem cells.

Rabbits in Group C were anesthetized with 3% pentobarbital and sterilized with 1% iodophors. After iliac crest was punctured with a 16 G needle, the bone marrow was extracted at anticoagulation with an injector filled with 3 mL heparin solution (1000 U/mL), 3 mL from each side and 6 mL from each rabbit. Next, bone marrow cells were agitated to prepare cell suspension and centrifuged. Mesenchymal stem cells were isolated using Ficoll method, rinsed and counted. Cells at a density of 1×105/mL, were cultured in a 25-cm² culture flask (containing 5×105 cells) at 37°C with 0.05 volume fraction of CO2. Culture medium consisted of RPMI 1640, 0.10 volume fraction of fetal bovine serum, 10 mM sodium glycerophosphate, 100 IU/mL penicillin, and 100 IU/mL streptomycin (pH 7.2). The medium was changed 5 days later, and then changed every 2 to 3 days after supernatants were removed. At the 10th day, as expected, cells covered the bottom of the culture flask. Cells were subcultured by mixing with 0.9% saline, alone with digestion with 0.05% pancreatin and 0.01% EDTA, resuspension, centrifugation at a diameter of 11.18 cm (1500 turns every 7 minutes), removal of supernatants, addition of culture fluid, and swinging. The prepared cell suspension (1×106/mL) was dropped onto the material surface, 1 mL for each hole that was added with 1 mL culture fluid 4 hours later. Cellular growth was observed under inverted phase contrast microscope.

Cells were assayed with alkaline phosphatase staining before and after culture, and flow cytometry was used to detect...
CD34, CD68, and CD105. Samples were observed under confocal microscope and environmental scanning electron microscope. After mesenchymal stem cells were co-cultured with the material for 2 weeks, osteonecrosis of the femoral head model was induced in animals. Rabbits of each group were sacrificed at 4, 8, and 12 weeks postoperatively, and the harvested samples were fixed with 4% formalin.

**Phase-contrast Imaging Observation of Synchrotron Hard X-ray**

Synchrotron radiation system used was provided by the Laboratory of Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences.

Refracted by 2 crystals, synchrotron radiation light projected on the samples and then imaged on the posterior monitor. Synchrotron radiation broadband hard X-ray, which was emergent from Beijing Synchrotron Radiation Facility 4W1 single-cycle electromagnetic undulator, served as the light source; energy 5 to 18 KeV, electron energy of synchrotron light 2.202 2 GeV, critical energy of synchrotron radiation 5.8 KeV, beam intensity 60.4 to 70 mA, and size of electron beam group was sx = 0.786 mm and sy=0.279 mm. A 4W1A beam line, 43 m long, was introduced to guide the synchrotron light into the chamber, with two four-blade slits fixed on the line to block stray light and collimate synchrotron light. Photon shutter on the beam line was applied to control exposure time. Broadband hard X-ray projected into CWOX ray scintillating crystal through the samples, yielding to the inversion of X-ray image to visible image. The final image was obtained by 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 >10 μm.

Here, synchrotron radiation broadband light was introduced to carry out in-line holography phase-contrast imaging and diffraction enhanced imaging according to different rotation angles (mean, 30) in all the femoral head samples at the anteroposterior position, contended with the phase-contrast imaging terms. All the samples were imaged by the same synchrotron radiation operator at the same energy, beam intensity and luminance, so as to compare the bone repair.

**Statistical Analysis**

All the images of the 3 groups at different times were graded according to Lane-Sandhu bone grafting imagology approach and the results were analyzed with SPSS11.0 statistical software using variance analysis. Differences with \( P < 0.05 \) were considered statistically significant.

**RESULTS**

Images were obtained by both in-line holography phase-contrast imaging (Figures 1-4) and diffraction enhanced imaging (Figures 5-8).

In Group A, cartilage and trabecular bone were imaged with synchrotron radiation light in the rabbit femoral head at all time points, and bone defects were clearly observed (Figures 1, 3).
In Group B, filled with nHAC, the density of bone repair area was discordant while that of unrepaird area was low 4 weeks postoperatively, indicating nHAC could promote the repair of the bone defect. At 8 weeks, even density was observed between nHAC and surrounding tissues, and was relatively high in the central part, without the presence of osteosclerosis (Figures 4, 5). At 12 weeks, bone density of the defect area was identical with the surrounding tissue or the defect area was indistinguishable, and trabecular bone was in disorder (Figure 7).

In Group C, filled with nHAC plus mesenchymal stem cells, the boundary between the defect area and surrounding tissues was clear, and parts of trabecular bone disordered at 4 weeks postoperatively. At 8 weeks, even density was observed in the defect area with surrounding tissues, and the boundary was still clear (Figure 6). At 12 weeks, no obvious difference was observed in the defect area compared with surrounding tissue, but trabecular bone was abnormal (Figures 2, 8). The Statistical results were shown in the Table.

**DISCUSSION**

In recent years, a novel method of X-ray imaging, phase-contrast imaging of X-ray, has attracted more attention in research of the Beijing Synchrotron Radiation Facility image. Synchrotron radiation light is the one of four artificial light sources, the remainders of which previously includes electric light, X-ray, and laser. The synchrotron electromagnetic radiation of high energy is created by the movement of a charged particle in the magnetic field, approaching light velocity motion. Produced by light source, X-ray contrast resolution is >1000 times higher than the density resolution of X-ray absorption. Phase-contrast imaging of X-ray plays an important role in medicine and life science, and its use will be applied widely. Medical techniques for live imaging have progressed from X-ray radiography, discovered by Rontgen in 1895, to CT, discovered by Hounsfield in the late 20th century. However, the medical imaging techniques used now, such as CT and positron emission tomography, only have a spatial resolution at millimeter and sub-millimeter levels, whereas the fifth (multi-slice helical) CT imaging with its high precision has achieved 0.35 mm spatial resolution. At present, magnetic resonance imaging (MRI) is widely applied in clinical practice, expensive, time-consuming and with a 5-mm plane scanned. Therefore, MRI is ineffective for some diseases, and both its precision and utility need to be improved. Live microscopy and functional imaging techniques have become key elements that restrict biomedical study.

Here, the samples were isolated and fixed with formalin, and some research has proved that 4% formalin has no influence on the phase-contrast imaging of synchrotron hard X-ray. Our results revealed a high spatial resolution of synchrotron hard X-ray phase-contrast imaging. Neither matter tissue engineering technique nor stem cell transplantation is effective enough for observing the effect of transplants on the repair of tissue organs in clinical practice.

Researchers have used synchrotron hard X-ray phase-contrast imaging on the mammary gland, and found that synchrotron hard X-ray is superior to molybdenum radiography for comparison of mammary gland tissues resulting from different lesions.

Muehlemann et al applied synchrotron radiation diffraction enhanced imaging on the imaging of knee joint cartilage in live rabbits, and proved that diffraction enhanced imaging is advantageous in the diagnosis of cartilage degeneration and is deemed to be more widely used. Previous research also indicates the crucial application value of synchrotron radiation in the imaging of cartilage defects.

In regards to bone imaging research, Salomé initially reported the imaging of trabecular bone with synchrotron radiation light in 1999, and then progressed through a series of investigations. For the first time, Weiss observed the differences in osteocyte growth in different biomaterials with synchrotron radiation light, and obtained satisfactory images. Mollenhauer has suggested that, with future technical modifications, more practical light sources, even modified X-ray, it may expand the application of X-ray phase imaging, although synchrotron radiation light is now dominant in synchrotron hard X-ray phase-contrast imaging.

The effect of nHAC and mesenchymal stem cells in the repair of femoral head defects was observed by in-line holography and diffraction enhanced imaging, in which the boundary between materials and surrounding tissue was found, as well as bone formation and substitution. Trabecular bone formed on both Groups B and C at 12 weeks postoperatively, but more obviously in Group C. We have provided experimental data for the develop-

<table>
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<th>Group</th>
<th>N</th>
<th>Weeks</th>
<th>4</th>
<th>8</th>
<th>12</th>
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<td>A</td>
<td>10</td>
<td></td>
<td>0.1±0.316</td>
<td>0.8±1.033</td>
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<tr>
<td>B</td>
<td>10</td>
<td></td>
<td>4.3±0.949*</td>
<td>7.4±0.516*</td>
<td>11.0±0.667*</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td></td>
<td>6.5±0.707*</td>
<td>10.2±0.422*</td>
<td>11.9±0.316*</td>
</tr>
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*P<0.01 vs A group _ 3 P<0.01 vs B group.
ment and application of synchrotron hard X-ray imaging techniques.

The current imaging techniques such as CT and positron emission tomography, have not been useful in detection, but synchrotron X-ray phase-contrast imaging may be advantageous. First, its penetrating capacity is suitable for gross observation. Second, its high spatial resolution at a millimeter scale is useful for the observation of cells and tissues. Third, due to strong coherence, the contrast resolution of phase-contrast imaging is 1000 times as high as the density resolution of X-ray absorption, which enables the observation of the internal structure of cells and tissues. Synchrotron radiation imaging is inferior to a micrometer resolution, which limits its application and will be solved by the enhancement of synchrotron radiation. Synchrotron hard X-ray phase-contrast imaging has an irreplaceable role in the development of tissue engineering.

REFERENCES