Synchrotron radiation micro CT

Osteoporosis

Femoral head

Subchondral bone

Osteoporosis

Femoral head

Subchondral bone

Keywords:

Microcallus

Subchondral bone

Femoral head

Osteoporosis

Synchrotron radiation micro CT

ABSTRACT

Trabecular bone microfracture pathogenesis and associated healing processes are not well understood. We analyzed the microcalluses that form subsequent to microfractures in patients with osteoporosis (OP) using synchrotron radiation micro CT (SRCT).

Subchondral bone columns were extracted from the femoral heads of 11 female patients with a femoral neck fracture. SRCT scanning was performed with 5.9 × 5.9 × 5.9 μm³ voxel size and the microcallus number was measured in a 5-mm cubic subchondral bone region. The trabecular bone microstructure was measured and its relationship to the microcallus number was analyzed. In addition, the degree of mineralization of the microcallus region and that of the rest of the trabecular bone were measured and compared.

Microcallus formations were detected in all cases, with a mean microcallus number of 4.9 (range, 2–11). The microcallus number had a significantly negative correlation with bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and degree of mineralization, and had a positive correlation with specific bone surface (BS/BV). The degree of mineralization of the microcallus region was lower than that of the rest of the trabecular bone and had a wider range of values.

Microcallus formations were frequently detected in patients with OP, and more prevalent in the bone with thinner trabeculae, suggesting microfractures might occur due to activities of daily living as the OP progresses. The degree of mineralization of microcallus might represent the process of bone healing from immature woven bone to mature trabecular bone.

© 2014 Elsevier Inc. All rights reserved.

Introduction

Patients with bone fragility due to bone diseases such as osteoporosis (OP), rheumatoid arthritis, and hyperparathyroidism sometimes have bone fractures by normal impacts of daily living, which is called insufficiency fracture. Insufficiency fractures usually occur at the vertebral body, pelvis, and femoral neck. Generally, the fracture line or deformation is not clearly visible by plain radiography, and can only be detected by computed tomography (CT) or magnetic resonance imaging (MRI). Such fractures sometimes occur in the subchondral bone of the femoral head, and this is now receiving attention as a “subchondral insufficiency fracture of the femoral head” [1–3]. This condition can result in femoral head collapse, producing a rapidly progressing deformity [4,5].

Fractures that occur in trabecular bone at a microscopic level are referred to as microfractures. A callus that subsequently forms at a microfracture site as part of the healing process is referred to as a microcallus [6,7]. Microfractures and microcalluses cannot be detected by conventional clinical imaging equipment. It is postulated that microfractures occur and are repaired by microcalluses even in normal bones without any symptoms. If this bone healing is not successful, the trabecular bone connectivity will be lost at the site of the microfracture, resulting in trabecular structural weakness. If such microfractures occur extensively, it can eventually cause clinical signs, which can be related to osteoporotic pain and insufficiency fracture.

Synchrotron radiation micro CT (SRCT) has significant advantages when compared with standard micro CT. The SRCT possesses a high resolution and a high quantitative capability and can identify three-dimensional (3D) trabecular bone mineralization in addition to the bone microstructure [8–10]. Synchrotron radiation is generated by passing electrons accelerated to the speed of light in a magnetic field. The resulting X-ray beam is highly intense, strongly collimated, and practically monochromatic. Artifacts such as beam hardening and noise rarely occur with the synchrotron radiation X-rays used for micro CT, whereas they are certainly present in conventional micro CT. Therefore, a high quantitative performance can be achieved with SRCT. It can be used to identify the 3D distribution of trabecular mineralization and to visualize the trabecular structure [11–13].
The degree of trabecular mineralization is equivalent to the density of the mineral deposited in collagen, and is regarded as an index of bone turnover as well as an index of the mechanical property of the bone [8,9]. Highly mineralized bone generally means that the bone is mature and aged, and has low bone turnover with hard material. Poorly mineralized bone indicates that there is high bone turnover and soft material. Therefore, SRCT is one of the few ways to perform 3D analysis of the trabecular bone microstructure and metabolism at the same time.

Whereas microcalluses have been detected using optical microscopy, electron microscopy, histological techniques, and radiographic techniques, there has been no published report of 3D analysis using SRCT. Although the degree of mineralization of microcallus has been analyzed histologically [14], there has been no study which analyzed microcallus mineralization quantitatively using SRCT. It might be possible to understand more about microfracture pathogenesis and their healing processes by performing such an analysis. A study of microcalluses in the subchondral bone of the OP femoral head might also lead to greater understanding of subchondral insufficiency fractures of the femoral head.

In this study, we analyzed microcallus formations that occurred in subchondral trabecular bones of OP femoral heads using SRCT to investigate: (1) the prevalence and number of microcallus formations; (2) the relationship between microcalluses and the trabecular bone microstructure; and (3) the characteristics of the degree of mineralization of microcallus.

Materials and methods

Subjects

The subjects were 11 patients with femoral neck fracture who had undergone bipolar hip arthroplasty at Nishiisahaya Hospital (mean age, 76.2 ± 7.7; range, 65–92 years, all female). Those who were male, patients with any symptoms in the hip joints or bone and joint diseases such as rheumatoid arthritis, or patients with medications that affect bone metabolism such as bisphosphonate were excluded. Eleven femoral heads were obtained in surgery, and subchondral bone samples were extracted from the loading area of each femoral head (Fig. 1). Subchondral bone columns (10 mm in diameter and 10 mm in height) were extracted using a coring reamer at a position 15 mm outside the lateral edge of the fovea of the capitis femoris. The samples were preserved in an acrylic case filled with physiological salt solution, and maintained in a freezer.

The ethics review board of our institute approved the study protocol, and all patients were provided informed consent to participate. The study protocol complied with the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects.

Imaging

SRCT scanning was performed at beamline BL20B2 in the synchrotron radiation facility SPring-8 (Hyogo, Japan). A 30-keV X-ray beam and a 4000 × 2624-pixel charge-coupled device (CCD) camera were used. The voxel size was 5.9 × 5.9 × 5.9 μm³. The samples were thawed gradually at room temperature, and maintained at the same temperature while being scanned. Each sample was placed on the table, and a 1-s exposure was performed every 0.1° of rotation for a total of 180° and 1800 radiographic projections. The total scanning time for each sample was approximately 2.5 h. The raw data were reconstructed as Tag Image File Format (TIFF) images.

Image analysis

The (1) microcallus number, (2) trabecular bone microstructure, and (3) degree of mineralization of the microcallus region were measured using the bone microstructure measurement software TRI/3D-

![Fig. 1.](image-url) a, b: A subchondral bone column 10 mm in diameter and 10 mm in height was extracted from the loading area of the femoral head. c, d: The microcallus number, microstructural parameters, and the degree of mineralization of the subchondral trabecular bone were measured in a 5-mm cube region located 1 mm beneath the subchondral bone plate at the center of the column.
BON (Ratoc System Engineering, Tokyo, Japan). The measurement region was a 5-mm cube region located 1 mm beneath the subchondral bone plate at the center of the extracted column (Fig. 1).

First, two observers counted the microcallus number. Discontinuous bone-like tissues surrounding a trabecular bone circumferentially were defined as microcalluses, as shown in Figs. 2B and C. If the bone-like tissues were formed partially on one side, they were not considered to be microcalluses. All images were investigated carefully and each microcallus was labeled in all three planes of the multi-planar reconstruction.

Next, the trabecular bone microstructure was measured in the same region where the microcallus number was counted. The images were binarized with a fixed threshold. The threshold between bone and background in a histogram of the image was determined by discriminant analysis, and the mean value of the threshold for an arbitrary five samples was set as the fixed threshold. The measurement parameters were the bone volume fraction (BV/TV) (%), the specific bone surface (BS/BV) (mm²/mm³), the trabecular thickness (Tb.Th) (μm), the trabecular number (Tb.N) (1/mm), the trabecular separation (Tb.Sp) (μm), the structure model index (SMI), the degree of anisotropy (DA), and the connectivity density (ConnD) (1/mm³) [15]. The SMI is an index for evaluating whether trabecular bone is rod-like or plate-like. SMI = 0 indicates an ideal plate-like structure, while SMI = 3 indicates a rod-like structure; therefore, a smaller value means a more plate-like structure. The DA is a parameter indicating the direction of the trabecular bone, which is determined by the mean–intercept–length (MIL) method, and a higher value indicates higher anisotropy [16]. The ConnD is a parameter describing trabecular connectivity, and a higher value means greater connectivity [17].

Finally, the degree of mineralization was measured. The CT value was converted to the degree of mineralization using a calibration curve obtained from a BMD phantom. A cylindrical stack of BMD phantoms made of epoxy resin with hydroxyapatite (Kyoto Kagaku, Kyoto, Japan), which were 6 mm in diameter and 1 mm in height, ranging from 200 to 800 mg/cm³, was scanned. The degree of mineralization of whole trabecular bone in the measurement region was measured. Then, as shown in Fig. 2D, microcalluses and trabecular bone in the center of the microcallus were extracted manually, and defined as the microcallus region. The microcallus cavities were not included in the microcallus region.

In addition, we defined the trabecular bone excluding the microcallus region as the trabecular region. Thus, the whole trabecular bone was divided into two parts, which included both the microcallus region and the trabecular region. The degree of mineralization of whole trabecular bone, that of the microcallus region, and that of the trabecular region were then measured.

Statistical analysis

Statistical analysis was performed using SPSS ver. 16.0 (SPSS, Chicago, IL, USA). The correlations among microcallus number, age, trabecular bone structure parameters, and degree of mineralization were analyzed with Spearman’s rank correlation coefficient test. The degree of mineralization of the microcallus region and that of the trabecular region were compared using a Mann–Whitney U test. The level of statistical significance was established at p < 0.01 for all analyses.

Results

As shown in Table 1, microcallus formations were detected in all 11 cases (100%), with a mean microcallus number of 4.9 (range, 2–11) in a 5-mm cube region. The mean BV/TV was 18.7%, and the mean Tb.Th was 147.0 μm. The mean SMI was 1.31, which indicated that the trabecular bone structure was rather plate-like.

Table 2 shows the rank correlation coefficients between microcallus number and age, trabecular bone structure parameters, and the degree of mineralization. The microcallus number had a significantly negative correlation with BV/TV and Tb.Th (r = −0.744, p = 0.009 and r = −0.878, p < 0.001, respectively), and had a positive correlation with BS/BV (r = 0.850, p = 0.001). This means that patients with a higher microcallus number have lower BV/TV and Tb.Th, and higher BS/BV. The microcallus number also had a significantly negative correlation with the degree of mineralization of whole trabecular bone (r = −0.767, p = 0.006), so that for patients with a higher microcallus number, the degree of mineralization of whole trabecular bone was significantly lower. In addition, the patients with a higher microcallus number tended to have lower degrees of mineralization of trabecular region (r = −0.665, p = 0.026).

The mean microcallus region mineralization was significantly lower than that of the trabecular region, as shown in Table 1 and Fig. 3 (966.5

Fig. 2. a: 3D SRCT microcallus image. b, c: The discontinuous bone-like tissue surrounding trabecular bone circumferentially was defined as a microcallus. d: Microcallus and repaired trabecular bone in its center were defined as a microcallus region, and its degree of mineralization was compared to that of the trabecular region. The microcallus cavities were not included in microcallus region.
Table 1
Data of the microcallus, trabecular bone structure parameters, and the degree of mineralization.

<table>
<thead>
<tr>
<th>Number of microcallus</th>
<th>BV/TV (%)</th>
<th>BS/BV (1/mm)</th>
<th>Tb.Th (μm)</th>
<th>Tb.N (1/mm)</th>
<th>Tb.Sp (μm)</th>
<th>SMI</th>
<th>DA</th>
<th>ConnD (1/mm²)</th>
<th>TMD All (mg/cm³)</th>
<th>TMD Mc (mg/cm³)</th>
<th>TMD Tb (mg/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.9</td>
<td>18.7</td>
<td>17.6</td>
<td>147.0</td>
<td>0.82</td>
<td>568.3</td>
<td>1.31</td>
<td>2.31</td>
<td>5.11</td>
<td>1032.6</td>
<td>966.5</td>
</tr>
<tr>
<td>SD</td>
<td>3.7</td>
<td>3.7</td>
<td>2.0</td>
<td>21.4</td>
<td>0.10</td>
<td>995.5</td>
<td>0.19</td>
<td>0.33</td>
<td>1.13</td>
<td>33.0</td>
<td>66.3</td>
</tr>
<tr>
<td>Min</td>
<td>2</td>
<td>12.6</td>
<td>14.0</td>
<td>115.8</td>
<td>0.66</td>
<td>456.3</td>
<td>0.99</td>
<td>1.63</td>
<td>2.50</td>
<td>966.6</td>
<td>798.2</td>
</tr>
<tr>
<td>Max</td>
<td>11</td>
<td>25.5</td>
<td>21.2</td>
<td>185.1</td>
<td>0.97</td>
<td>834.2</td>
<td>1.61</td>
<td>2.80</td>
<td>6.79</td>
<td>1081.0</td>
<td>1085.9</td>
</tr>
</tbody>
</table>

And 1033.1 mg/cm³, respectively, p = 0.002). The mean distribution of the degree of mineralization is shown in Fig. 4 and 3D color image of the degree of mineralization and cross-sectional image are shown in Fig. 5. The mode values were 1092.4 mg/cm³ and 1165.5 mg/cm³, the median values were 1001.2 mg/cm³ (interquartile range, 824.7 to 1121.2 mg/cm³) and 1084.1 mg/cm³ (interquartile range, 927.2 to 1178.6 mg/cm³) for the microcallus region and trabecular region, respectively. The degree of mineralization of the microcallus varied more widely than that of the trabecular region.

The rank correlation coefficients between the degree of mineralization of whole trabecular bone, TMD Mc: degree of mineralization of microcallus region, TMD Tb: degree of mineralization of trabecular region.

Table 2
The rank correlation coefficient between the number of microcallus formations and age, trabecular bone structure parameters, and the degree of mineralization.

<table>
<thead>
<tr>
<th>Age</th>
<th>BV/TV</th>
<th>BS/BV</th>
<th>Tb.Th</th>
<th>Tb.N</th>
<th>Tb.Sp</th>
<th>SMI</th>
<th>DA</th>
<th>ConnD</th>
<th>TMD All</th>
<th>TMD Mc</th>
<th>TMD Tb</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>0.005</td>
<td>−0.744</td>
<td>0.650</td>
<td>−0.787</td>
<td>−0.268</td>
<td>0.388</td>
<td>−0.476</td>
<td>0.259</td>
<td>−0.05</td>
<td>−0.767</td>
<td>−0.231</td>
</tr>
<tr>
<td>p</td>
<td>0.989</td>
<td>0.009**</td>
<td>0.001**</td>
<td>&lt;0.001**</td>
<td>0.426</td>
<td>0.238</td>
<td>0.139</td>
<td>0.442</td>
<td>0.092</td>
<td>0.006**</td>
<td>0.494</td>
</tr>
</tbody>
</table>

Spearman’s rank correlation coefficient test.

**p < 0.01.
*p < 0.05.

Previously, Freeman et al. [21] and Watson [22] observed femoral heads in OP patients with femoral neck fractures using an optical microscope, detecting microcallus formations in all cases.

We acknowledge that patients with the greater numbers of microcallus formations had significantly lower BV/TV and Tb.Th, higher BS/BV, and a lower degree of mineralization (Table 2). The correlation between microcallus formation and lower bone volume or thinner trabeculae suggests that a greater microcallus number is correlated with greater bone structure fragility. However, the reduced bone volume also produces concavities and through-holes in the trabecular bone, which increases the surface area. The degree of mineralization on the surface of trabecular bone is lower than that of the center. There may be a tendency for the degree of mineralization to be calculated lower because of the increase in surface area within the trabeculae. Thus, the microcallus number also had a significantly negative correlation with the degree of mineralization of whole trabecular bone. Furthermore, microcallus occurred at the trabecule with low mineralization because bone strength was correlated to the degree of mineralization.

Hahn et al. evaluated 37 vertebrae using a histological technique, and reported that microcallus formations were located mostly in the lower thoracic and lumbar vertebrae [14]. Microcallus formations can be frequently found where there is a trabecular bone volume of less than 11% and higher values of trabecular bone pattern factor (TBPf). Cheng et al. assessed microcallus formations in the 4th lumbar vertebra (L4) and femoral neck taken from 50 cadavers, and analyzed the relationship between the number of microcallus formations counted by a microscope and the trabecular bone structure measured by micro CT [23]. The results indicated that the prevalence of L4 vertebrae microcallus formation was 82%, and that of the femoral neck was 11%. The L4 vertebrae with a lower bone mineral density also had a greater number of microcallus formations. However, there has been no prior published report that analyzed the relationship between the occurrence of microcalluses and the trabecular bone microstructure in the femoral head.

The degree of mineralization of the microcallus region was lower than that of the rest of the trabecular bone (Figs. 3, 5). This suggests that a microcallus consists of immature woven bone, where mineralization does not progress. There has been no published report on the detailed analysis of the degree of mineralization of microcalluses.
The process of microcallus formation has been described as follows [14]: When a trabecular fracture occurs, (1) connective tissue proliferates within a few days (fibrous phase). Mineralization can already be seen at the center. (2) This tissue develops into a loosely structured cal- lus of woven bone (fibrous-bony phase). (3) The microcallus reaches its maximum size (bony phase). (4) The microcallus undergoes modeling or remodeling processes (modeling phase). (5) Finally, the fracture crevice is closed by mineralization a few weeks after the trabecular frac- ture (later stage). We consider that the microcallus formations observed in this study were in the fibrous-bony phase, the bony phase, and the modeling phase.

The degree of microcallus mineralization ranged widely (Fig. 3), suggesting that the onset time was different for each microfracture. It also suggests that the repair speed might vary depending on the microfracture. It is expected that microfractures occur continuously in daily living and are successively repaired by microcalluses. A subchondral insufficiency fracture of the femoral head is a relative- ly new disease concept proposed in 1996 [1]. It is defined as a fracture in the subchondral bone of the femoral head due to bone fragility, such as in OP. Patients with OP may have asymptomatic microfractures due to activities associated with daily living. Subchondral insufficiency frac- tures might develop clinically when microfractures occur extensively during the same period and exceed a certain level.

Blackburn et al. performed a mechanical test revealing that the micrOhardness of a microcallus was similar to that of a normal trabecular bone [24]. Cheng et al. considered that microcalluses might help maintain the strength of bone tissue [23]. Thus, microcallus formation is considered to maintain bone strength in elderly OP patients. Even if microfractures occur, if the number is small and the normal repair pro- cess works, there would be no harm biomechanically. In contrast, in- complete repair with microcallus formation might also be a factor that promotes the development of subchondral insufficiency fractures of the femoral head.

In this study, we analyzed only a part of the femoral head loading area. We would like to investigate the predilection site of microcallus by extending the area of analysis or investigate the difference in the microcallus number corresponding to the depth from the subchondral bone plate in the future. Subchondral insufficiency fracture of the fem- oral head occurs near a subchondral plate, and the shape is usually con- vex superiorly and parallel to a cartilage surface in T1 MRI weighted images [2,25,26]. We might be able to better understand the relation- ship of the femoral head microcallus and insufficiency fracture by ex- tending the area of analysis. Furthermore, it might be possible to understand more about the characteristics of the pathological condi- tions, by comparing the differences of the microcallus formations in various secondary OP cases caused by glucocorticoid medications, diabetes mellitus, and rheumatoid arthritis, etc.

This study has several limitations. First, we had a very limited number of cases. The opportunity to use SRCT is generally limited, and scanning is a lengthy process. Moreover, SRCT is a comparatively extensive technique in comparison with conventional micro CT. As a result, the
number of cases was also small in previously published studies using SRCT. To accommodate for this statistical limitation, we only used defined data with a significance of p < 0.01 in this study. As there were no healthy control subjects, we could not assess whether the number of 4.9 microcalluses in a 5-mm cube region was considered numerous or few. In addition, there was no control sample other than the loading area. Therefore we could not assess whether this phenomenon occurred only in the loading area, in the whole femoral head or on the acetabular side. In Japan, it is very difficult to obtain the femoral head of a cadaver for any purpose other than medical education due to religious issues. We did not perform measurement of dual-energy X-ray absorptiometry (DXA) of the proximal femur and the lumbar spine of the subjects. Osteoporosis is defined as the presence of low-impact hip fracture [27, 28]. We manually performed measurement of the microcallus number and extracting of the microcallus region. The definition of a microcallus was visually clarified, incomplete formations were strictly excluded, and two observers performed these processes in order to make a precise and specific assessment of the microcallus. It was difficult to identify microcalluses in the fibrous phase, where mineralization had not yet progressed, and in the late stage, where fracture healing was almost completed. We considered that the microcallus formations we detected with wide-ranging degrees of mineralization would be in the fibrous-bony phase, the bony phase, or the modeling phase of healing. As this was a cross-sectional microcallus study, we could not provide an in-depth description or analysis of the onset and progression of microfractures.

In conclusion, microcalluses were detected in the subchondral bone of the femoral heads in all patients with OP, with a mean of 4.9 microcalluses in a 5-mm cube region. This indicates that fractures at the trabecular bone level had occurred asymptotically due to activities or impacts associated with daily living.

Cases with greater numbers of microcallus formations showed lower BV/TV, narrower Th.Th, higher BS/BV, and lower degrees of mineralization. Greater fragility of the bone structure is definitely correlated with a greater microcallus number.

The degree of mineralization of the microcallus region was lower than that of the rest of the trabecular bone, suggesting that microcalluses may consist of immature woven bone. The degree of mineralization of the microcallus region varied widely, and might represent stage in healing process of each microcallus, the time of microcallus onset, and the speed of healing for each microcallus.

Authors' roles

Study design: NO and KC. Data collection: NO, KC, KT, NN and SK. Data analysis: NO and KC. Data interpretation: NO, KC, KT, MI and MO. Drafting manuscript: NO and KC. Revising manuscript content: MI, and MO. Approving final version of manuscript: NO, KC, KT, NN, SK, MI, and MO. NO and KC takes responsibility for the integrity of the data analysis.

Acknowledgments

The authors would like to thank Kentaro Usugi and Naoto Yagi (Spring-8, Japan Synchrotron Radiation Research Institute, Hyogo, Japan) for SRCT imaging. This research was partially supported by a Grant-in-Aid for Scientific Research for Young Researchers (B) from the Japan Society for the Promotion of Science (JSPS) (20791046) and Hip Joint Foundation of Japan, Inc.

Disclosures: All authors state that they have no conflicts of interest.

References

[19] Megancik JA, Kozloff KM, Thornton MM, Broski SM, Goldstein SA. Beam hardening artifacts in micro-computed tomography scanning can be reduced by X-ray beam filtration and the resulting images can be used to accurately measure BMD. Bone Dec 2009;45(6):1104–16.