Time Course of Osteonecrosis in Rabbit Articular Intercalated Bone: Line Scan Spectroscopic Imaging and Correlation with Histology

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Purpose: Magnetic resonance (MR) imaging offers the highest sensitivity for detecting bone necrosis. We evaluated osteonecrosis in rabbit models by calculating the percentage of fat to (fat + water) [F/(F+W)] on MR spectroscopy (MRS) and compared MR spectroscopy and imaging findings with corresponding histological results.

Methods: To model the natural course of articular osteonecrosis, we removed the fourth tarsal bone in 45 rabbits, froze it for 5 min in liquid nitrogen to produce complete cellular necrosis, and then replaced the bone into the knee joint. We performed Carr-Purcell-Meiboom-Gill proton spectroscopic imaging to assess necrotic bone at 3 days and one, 2, 3, 4, 8, 12, 16, and 20 weeks after osteonecrosis and calculated the percentage of F/(F+W) of each bone. We also performed conventional T1- and T2-weighted imaging and compared all data to histological findings to analyze the natural course of necrosis.

Results: T1-weighted MR imaging demonstrated obvious low signal intensity at 2 to 8 weeks and recovery at 12 to 20 weeks, whereas T2-weighted imaging demonstrated inconsistent intensities throughout the period. The postoperative percentage of F/(F+W) measured using line scan MRS decreased to 8.88% at 3 weeks, 6.22% at 8 weeks, and 34.40% at 20 weeks results that were mostly consistent with MR imaging findings. Histological findings demonstrated complete absence of osteocyte nuclei and loss of osteoid-osteogenesis at 3 to 8 weeks. Recovery of bone marrow was identified as an increase in the area of fat after 12 weeks.

Conclusion: Osteonecrosis delineated by T1-weighted MR imaging demonstrated fat content in the bone marrow that correlated with histology. The present MRS modality can be used to calculate the percentage of F/(F+W) of osteonecrosis to enable objective assessment of recovery and quantification of osteonecrosis to provide a numerical value for osteonecrosis.

Keywords: animal model, line scan spectroscopic analysis, magnetic resonance imaging, magnetic resonance spectroscopy, osteonecrosis

Introduction

Magnetic resonance (MR) imaging, the most sensitive and noninvasive tool for detecting osteonecrosis, has been used widely in clinical settings since the mid-1980s.¹⁻⁴ The high signal intensity of normal cancellous bone on T1-weighted imaging and moderately high signal on T2-weighted imaging reflect the content of fat tissue. Ischemia causes avascular necrosis of cancellous bone, which leads to fibrosis as a result of the loss of fat tissue. Consequently, ischemic bone often appears with low intensity on T1-weighted imaging. However, in the early stages of osteonecrosis, T2- and even T1-weighted images demonstrate a variety of signal changes because of variations in the degrees of bleeding and edema, which complicate the objec-
tive assessment of bone status. 

To resolve this problem, MR spectroscopy (MRS) has been used to diagnose diseases of the bone marrow. Several studies have objectively assessed the signal intensity of normal joints and bone marrow using Carr-Purcell-Meiboom-Gill (CPMG) proton spectroscopy. In particular, for necrotic intercalated bone, which indicates lunatomalacia (so-called Kienböck disease), fat signals could be analyzed for early diagnosis and objective assessment. However, no reports have discussed the time course for the recovery of vascularization on MRS.

We examined the time course of MR imaging signal intensities of necrotic bone using rabbit models of osteonecrosis and then assessed the relationships among MRS, MR imaging, and histological findings.

Materials and Methods

Animal preparation and experimental model

Our institutional animal care committee approved the protocol, and animals received humane care during the study. We used 45 female Japanese white rabbits (weight 1.2 to 1.3 kg) and housed them at a constant temperature (22 ± 3°C).

The animals were anesthetized with intramuscular injection of ketamine at a dose of 100 mg/kg body weight and xylazine at a dose of 20 mg/kg body weight, respectively. We removed the fourth tarsal bone of each rabbit through an incision and closed the wound, immersed the bone for 5 min in liquid nitrogen to ensure complete cellular necrosis, and then placed it inside the intra-articular space through a longitudinal incision in the knee to reproduce intra-articular osteonecrosis, as seen in conditions such as Kienböck disease. At 3 days and one, 2, 3, 4, 8, 12, 16, and 20 weeks after bone immersion in liquid nitrogen, rabbits were sacrificed by intravenous injection of pentobarbital sodium at a dose of 200 mg/kg body weight to remove the tarsal bones for MRS, MR imaging, and histological study. At the same time, the contralateral intact fourth tarsal bone of each rabbit was also obtained as a control. Assessments were performed using 5 animals at each period of sacrifice.

MR imaging and spectroscopy

We obtained conventional MR imaging of the necrotic tarsal bone on coronal section using a 1.5-tesla superconductive imager (Signa, GE Medical Systems, Milwaukee, WI, USA). A 3-inch transmit/receive extremity coil using a linearly polarized radiofrequency pulse was used for this study to delineate osteonecrotic bone. Fast spin-echo T1-weighted imaging (repetition time [TR], 300 ms; echo time [TE], 10 ms) and fast spin-echo T2-weighted imaging (TR, 3000 ms; TE, 96.5 ms) were performed. Other imaging parameters were: field of view (FOV), 8 cm; matrix, 256 × 192; number of excitations, 2; and echo train length, 12 for T1-weighted imaging and 3 for T2-weighted imaging.

We compared changes in signal intensity observed on T1- and T2-weighted imaging to controls at each period of osteonecrosis. One radiologist (H.S., with more than 20 years of experience in musculoskeletal MR imaging) and 2 orthopedists (N.O. and T.N., each with more than 10 years of experience in musculoskeletal MR imaging) independently assessed the signal intensity of osteonecrosis on T1- and T2-weighted images compared with control bone (homogeneous or heterogeneous, and low, intermediate, or high). If assessments differed, results were discussed until a consensus was reached. Assessments were performed using 5 bones at each period of sacrifice. When signals varied between individuals in one group, the majority signal was adopted. Figure 1 shows representative samples. All spectroscopic imaging was performed using the same 1.5-T Signa imager just after the MR imaging study. The same radiologist and a radiological technologist (T.W., with more than 20 years of experience in musculoskeletal MR imaging) selected a column of tissue measuring 5 × 5 mm² within the tarsal bone on the sagittal scout images. The column was visualized with a rapid acquisition relaxation enhanced (RARE) type of sequence (Fig. 2). The line scan spectroscopic CPMG sequence was then applied along this tissue column. Three echoes with echo spacing of 25 ms (TE, 25, 50, 75 ms) were generated with a CPMG sequence using orthogonal slice-selective 90° and 180° pulses as in classic inner-volume imaging methods. These settings result in the confinement of echo signals to a tissue column that represents the intersection of the 2 planes. The echoes are read out in the absence of gradients, so that spectroscopic information is retained. Phase encoding along the column was performed using 64 phase encodes and an 8-cm FOV, which resulted in a 1.25-mm along-column spatial resolution. The same phase-encoded gradient strengths were applied to each echo so that individual spectroscopic images of the column were generated for each TE. The technical details of the line scan spectroscopic CPMG sequence are described elsewhere.

Data analysis of MRS

We reconstructed raw data in magnitude mode on
the Signa imager and transferred the reconstructed data to a personal computer for further analysis. We used software developed in-house to extract spectra from selected locations along the column including 5 voxels. The same radiologist determined the extracted spectra to include the target area (osteonecrosis) with reference to T1- and T2-weighted imaging. We used custom-made software to analyze magnitude spectra with 2-peak Gaussian functions to obtain areas of fat and water peak at 3 separate TEs. The dependence of the peak area on TE theoretically provides data suitable for making T2 corrections to obtain the extrapolated peak area at zero TE. T1 correction is unnecessary because this method is largely free from T1 relaxation due to the relatively long TR (2000 ms).

We also analyzed ratios of T2 values for fat and water in necrotic bone marrow. The logarithm of peak area versus TE was fit using a linear least-squares method to estimate individual water and fat T2 values and to extrapolate peak area at 0 for both water (pw) and fat (pf). The relative percentage of fat within the voxel was then estimated using the following formula:

$$\text{Percentage of Fat} = \left(\frac{pf}{pw+pf}\right) \times 100.$$ 

**Histological analysis**

Immediately after MRS and MR imaging, we examined the tarsal bone histologically. We embedded in paraffin and decalcified the tissue specimen, sliced the tarsal bone in the specimen in the same plane as the MR imaging frontal plane, stained the specimen using hematoxylin and eosin (HE) and Villanueva-Goldner (VG) stains, and performed light microscopic analysis. One pathologist (H.S., with more than 20 years of experience in musculoskeletal pathology) evaluated pathological findings of osteonecrosis. Two orthopedists (Y.O. and T.N., each with more than 10 years of experience in musculoskeletal pathology) performed additional checks. Assessments were performed using 5 bones at each period of sacrifice. In terms of reproducibility, histological findings among the groups did not differ significantly in evaluated parameters. Figure 3 shows representative samples.
Statistical analysis
We calculated mean values and standard deviations of spectral parameters averaged over voxels for 5 necrotic bones in each period of sacrifice. We used one-way analysis of variance with the Tukey post-hoc test to compare the percentage of F/(F+W) for each period after immersion of the tarsal bone into liquid nitrogen. All statistical analyses were performed using SigmaPlot version 12.1 software (SYSTAT, San Jose, CA, USA). P < 0.01 was considered statistically significant.

Results
Progression of necrotic intercalated bone on MR imaging
The marrow of normal fourth tarsal bone in the sagittal plane was delineated as areas of homogeneously high intensity on T₁-weighted imaging and heterogeneously high intensity on T₂-weighted imaging. At 3 days and one week, the bone marrow demonstrated a mixture of extremely high and low intensity on T₁-weighted imaging. Signal intensities of bone marrow subsequently began to decrease...
from 2 to 8 weeks on T1-weighted imaging and displayed areas of low intensity compared with controls. At 12 to 20 weeks, signal intensities of bone marrow were increased on T1-weighted imaging compared to those at 2 to 8 weeks but remained relatively lower than those in controls.

At 3 days and one week, bone marrow displayed spots of extremely high and low intensity on T2-weighted imaging. Subsequently, signal intensities of bone marrow began to decrease from 2 to 20 weeks on T2-weighted imaging compared with controls. T2-weighted imaging showed heterogeneous bone marrow with coexistence of areas of iso-, hypo- and hyperintensity. T2-weighted imaging demonstrated inconsistent intensities throughout the study period (Fig. 1).

MRS of necrotic intercalated bone

According to line scan MR spectra, fat signals were always greater than water signals in controls. The mean (± standard deviation) percentage of F/(F+W) in bone marrow of normal tarsal bone was 82.67 ± 1.75%. The mean percentages of F/(F+W) postoperatively were 18.97 ± 15.71% at 3 days, 16.35 ± 4.71% at one week, 15.93 ± 2.51% at 2 weeks, 8.88 ± 4.51% at 3 weeks, 9.27 ± 5.45% at 4 weeks, 6.22 ± 1.86% at 8 weeks, 18.60 ± 12.08% at 12 weeks, 14.35 ± 12.83% at 16 weeks, and 34.40 ± 17.76% at 20 weeks (Table 1).

During the course of this study, fat signals were always smaller than water signals in necrotic bone (Fig. 4). At 3 days, one week, and 2 weeks, we could barely detect fat signals. In particular, at 3 to 8 weeks, fat signals were even weaker than water signals and lower than signals in the first 2 weeks. After 12 weeks, fat signals began to increase and showed identical strength to water signals at 20 weeks. The mean percentage of F/(F+W) postoperatively decreased significantly at 3 days and one, 2, 3, 4, and 8 weeks compared with controls (P < 0.01). The percentage of F/(F+W) recovered at 12 and 16 weeks, though a significant decrease was evident compared with controls (P < 0.01). The percentage of F/(F+W) against water finally increased at 20 weeks, representing a significant increase compared with 3, 4, and 8 weeks (P < 0.01) but was still significantly lower than in controls (P < 0.01) (Fig. 5).

Histological analysis

Histological findings of the control fourth tarsal bone stained with HE demonstrated an osteocyte nucleus in each lacuna of cortical bone and the massive presence of adipose tissue in the bone marrow. The trabecular layer was thick and obvious, and no fibrous proliferation was apparent in the bone marrow. VG staining indicated a thick osteoid layer as a red line at the internal border of trabecular bone. Active osteocyte nuclei were also stained red (Fig. 3a).

At 3 days, one week, and 2 weeks (Fig. 3b) after immersion of the bone in liquid nitrogen, both HE

<table>
<thead>
<tr>
<th>Follow-up interval</th>
<th>Control</th>
<th>3 days</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>16 weeks</th>
<th>20 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/(F+W) (%)</td>
<td>82.67</td>
<td>18.97</td>
<td>16.35</td>
<td>15.93</td>
<td>8.88</td>
<td>9.27</td>
<td>6.22</td>
<td>18.60</td>
<td>14.35</td>
<td>34.40</td>
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<td>Standard deviations</td>
<td>1.75</td>
<td>15.71</td>
<td>4.71</td>
<td>2.51</td>
<td>4.51</td>
<td>5.45</td>
<td>1.86</td>
<td>12.08</td>
<td>12.83</td>
<td>17.76</td>
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</tbody>
</table>

Fig. 4. Magnetic resonance spectroscopy (MRS) of each period of sacrifice, with the lipid line to the left (asterisk) and the water line to the right (double asterisk). In line scan MRS, fat signals were always greater than water signals for the control. At 3 days, one week, and 2 weeks after immersion in liquid nitrogen, fat signals were barely evident. At 3 to 8 weeks, fat signals were even weaker than the water signals at 3 days, one week, and 2 weeks. At 12 and 16 weeks, fat signals again appeared, and fat signals were identical to water signals at 20 weeks.
and VG staining demonstrated obvious fibrosis in the bone marrow and deformity of osteocyte nuclei, indicating osteonecrotic changes. At 3, 4, and 8 (Fig. 3c) weeks, complete absence of osteocyte nuclei, loss of adipose tissue, and proliferation of fibrous tissue were clearly evident in HE-stained sections and loss of osteoid-osteogenesis was noted in VG-stained sections. Blood vessels had penetrated slightly into surrounding necrotic bone. At 12 weeks (Fig. 3d) and 16 weeks, recovery of adipose tissue in the bone marrow and angiogenesis were noted in HE-stained sections. In addition, new osteogenesis restoring the osteoid layer was obvious on trabecular bone with VG staining. However, osteocyte nuclei were still absent from trabecular bone. Many blood vessels had penetrated surrounding necrotic bone. At 12 weeks (Fig. 3d) and 16 weeks, recovery of adipose tissue in the bone marrow and angiogenesis were noted in HE-stained sections. In addition, new osteogenesis restoring the osteoid layer was obvious on trabecular bone with VG staining. However, osteocyte nuclei were still absent from trabecular bone. Many blood vessels had penetrated surrounding necrotic bone from 8 weeks. At 20 weeks, the bone marrow with adipose tissues was seen and contained many lipoblasts. Active osteocyte nuclei and prominent adipose tissues were detected in HE-stained sections and clear osteoid layers were visualized in VG-stained sections (Fig. 3c).

**Discussion**

**Progression of necrotic intercalated bone on MR imaging**

In the present study, we observed partially decreased signal of the bone marrow of the fourth tarsal bone with T1-weighted imaging starting 3 days after the procedure. Tarsal bone displayed hypointensity up to 8 weeks. From 12 to 20 weeks, gradually increasing signal intensity of T1-weighted imaging corresponded with histological recovery from osteonecrosis. Conversely, T2-weighted imaging indicated almost identical findings to T1-weighted imaging in the early period, but signal intensity of the tarsal bone varied and mostly showed areas of both low and high signal intensity in the middle and late periods, possibly due to residual bone marrow edema or increased blood flow. These findings suggest that in the early stages of osteonecrosis, both T1- and T2-weighted imaging are useful for assessing and confirming the condition of osteonecrosis. T1-weighted imaging may offer a more useful, validated indicator than T2-weighted imaging in both middle and late periods.

MR imaging is a strong diagnostic tool for osteonecrosis.\textsuperscript{14–16} T1- and T2-weighted imaging demonstrate necrotic changes in bone as lesions of low intensity. However, in Kienböck disease, signal may sometimes vary on T2-weighted imaging. T2-weighted imaging demonstrates increased signal intensity during the recovery phase following surgery, likely because of the increased blood flow inside ischemic bone.\textsuperscript{15} However, T2-weighted imaging is also influenced by other conditions, such as residual bone marrow edema and increased blood flow, as shown in the middle and late periods of the present study. Such findings may mislead the clinician if T2-weighted imaging is considered only to demonstrate increased blood flow inside necrotic bone.\textsuperscript{17}

**MRS of necrotic intercalated bone in comparison with histology**

Line scan spectroscopic analysis permits the acquisition of spectroscopic imaging in a short time because it was developed with a view to clinical applications.\textsuperscript{6–10} The frequency resolution and sensitivity are inferior to those of single-voxel techniques but appear sufficient for measurements of fat/water imaging. We used 3 echoes and magnitude-mode spectra.\textsuperscript{6} The use of more echoes with CPMG spectroscopy always involves a tradeoff between spectral resolution and loss of signal with T2 decay, so using 3 echoes with the same settings would have made the later echoes heavily T2-weighted and lacking in signal-to-noise ratio.\textsuperscript{6} These magnitude-mode spectra are broadened compared to pure absorption-mode phased spectra, but traditionally with this sequence, we have simply used magnitude spectra with Gaussian fits to capture the primary water and combined methyl/methylene resonances. Magnitude spectra are the easiest to reconstruct using scanner software and are useful for simple estimates of the percentage of F/(F+W) when raw data are not collected. Of note, the natural line widths in bone marrow are often broadened by susceptibility differences between bone and tissue.
The percentage of F/(F+W) corresponds to histological findings of changes in chronological osteonecrosis.

so even with higher spectral resolution and use of absorption mode spectra, it is difficult to separate the different lipid resonances.5–10 The settings we employed were sufficient for estimating the primary water resonance and the combined methylene/methyl lipid peaks, even though water resonance would have been slightly saturated at the TR of 2000 ms, thus overestimating the percentage of F/(F+W) compared to true values.

MRS-targeted fat components (–CH₃ and water (–OH) in bone marrow were measured under both intact and experimental osteonecrotic conditions. The mean percentage of F/(F+W) in normal tarsal bone, 82.67%, suggested a relatively higher fat content in bone marrow, which was consistent with histological and radiological findings of areas of high intensity on T₁ and T₂-weighted imaging. The percentage of F/(F+W) soon decreased at 3 days, one week, and 2 weeks (early osteonecrosis period), even though histological examination revealed only a slight decrease in adipose tissue. Bone marrow changed to partially edematous tissue. Adipose tissue decreased and fibrous structure increased. The percentage of F/(F+W) further decreased until 8 weeks (mid-osteonecrosis period) where loss of adipose tissue was seen with proliferation of fibrous tissue on histology. At 12 to 20 weeks (late osteonecrosis recovery period), the percentage of F/(F+W) gradually increased and was consistent with histological findings showing regenerating adipose tissue. The percentage of F/(F+W) at 20 weeks remained lower than in controls, whereas histological findings indicated almost complete recovery of adipose tissue. This may be due to the influence of increased water, likely attributable to angiogenesis in bone marrow or residual bone edema. Signal intensity on T₁-weighted imaging was decreased in the osteonecrosis model compared to controls, and that on T₁-weighted imaging was increased in 12- to 20-week models compared to 3- to 8-week models. The change in signal intensity on T₁-weighted imaging tended to reflect the change in the percentage of F/(F+W) in the target bone. Agreement of our radiological findings with pathological findings for all periods suggests the possibility for identifying both prognosis and immediate condition of osteonecrosis from examinations of percentage of F/(F+W) (Table 2).

We classified osteonecrosis into 3 periods, early (up to 2 weeks), middle (3 to 8 weeks) and late (12 to 20 weeks), based on histological findings. Comparison of the percentage of F/(F+W) of the target bone in each period demonstrated significant differences between the early and mid-osteonecrosis periods and between the mid- and late osteonecrosis recovery periods (Table 2).

**Limitations**

We did not assess osteonecrosis in the long term, so we could not demonstrate complete recovery of osteonecrotic bone, and because we chose a relatively traumatic method to create the osteonecrosis, results may differ from avascular osteonecrosis in clinical situations, such as Kienböck or Preiser disease.17

Although a relatively old technique, line scan spectroscopic analysis is simple and easy and so can be used at any facility.6–10 Variation of the percentage of F/(F+W) in both early (3 days) and late (12, 16, and 20 weeks) stages with large standard deviation may indicate early-stage osteonecrosis and the time for recovery from osteonecrosis. The smaller standard deviation in the percentage of F/(F+W) at 8 weeks indicated complete osteonecrosis. Line scan spectroscopic analysis in this animal model offered high reproducibility of complete osteonecrosis. MRS analysis of the percentage of F/(F+W) appears to allow objective, straightforward,

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<thead>
<tr>
<th>Phase</th>
<th>Normal</th>
<th>Early osteonecrosis period</th>
<th>Mid-osteonecrosis period</th>
<th>Late osteonecrosis recovery period</th>
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</thead>
<tbody>
<tr>
<td>Term after immersion in liquid nitrogen</td>
<td>F/(F+W) (%)</td>
<td>3 days to 2 weeks</td>
<td>3 to 8 weeks</td>
<td>12 to 20 weeks</td>
</tr>
<tr>
<td>Histological findings (trabecular layer)</td>
<td>82.67</td>
<td>15.93 to 18.97</td>
<td>6.22 to 9.27</td>
<td>14.35 to 34.40</td>
</tr>
<tr>
<td>Histological findings (bone marrow)</td>
<td>osteocyte nucleus in each lacuna</td>
<td>massive presence of adipose tissue</td>
<td>change to edematous tissue; adipose tissue decreases and fibrous structures increase</td>
<td>absence of osteocyte nuclei in each lacuna</td>
</tr>
</tbody>
</table>

**Table 2. Comparison of histological findings and percentage of fat to (fat + water) [F/(F+W)]**
and noninvasive evaluation of osteonecrosis to provide clinically useful information.

**Conclusion**

The present MRS modality can be used to calculate the percentage of $F/(F+W)$ of osteonecrosis. We can achieve objective assessment of recovery for osteonecrosis using Line-scan MRS.

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**References**