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Morphological and Morphometrical Analyses of Fracture-Healing Sites of an Atypical Femoral Fracture in Patients with and without Long-Term Bisphosphonate Treatment for Osteoporosis: A Report of Two Cases

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Bisphosphonates have been the first drug of choice for osteoporosis in the recent years because of their known ability to suppress osteoclast activity. The adverse effect of long-term bisphosphonate administration in the fracture-healing process is controversial. The aim of our study was to observe not only morphology but also morphometry of the fracture site of atypical femoral fracture with and without long-term bisphosphonate administration, in a case study of two difficult-to-obtain human samples. The patients with insufficient healing of atypical femoral fracture were treated with valgus wedge osteotomy. Histomorphometrical analysis was performed in bone samples of fracture sites harvested during osteotomy. The thickness of the femoral cortex was measured in the fracture site and the adjacent, non-fracture site. A comparative analysis of the content of hypertrophic osteoclasts in fracture sites, shape and size of osteons, mass, and ratio of the woven bone to the total bone mass was performed, comparing bisphosphonate-treated and untreated samples. In bisphosphonate-treated samples, we observed femoral cortex thickening at the fracture site; the appearance of hypertrophic osteoclasts; decreased bone resorption surface, decreased osteoclast numbers on the bone resorption surface, and increased ratio of multinuclear osteoclasts; osteons were misshapen and thin; and the mass and ratio of the woven bone to the total bone mass were higher. This study demonstrated that long-term bisphosphonate administration can alter the morphological features of the fracture site compared to its physiological state.

Keywords: atypical femoral fracture; bisphosphonates; bone morphology; fracture healing; long-term therapy
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Introduction

Bisphosphonates are widely used drugs for osteoporosis, since they increase bone mass by suppressing osteoclasts, which are bone resorbing cells important for maintaining normal turnover, but are relatively overdriven in the osteoporotic state. Bisphosphonates have been administered to hundreds of millions of patients as the first drug of choice worldwide to date (Bone et al. 2004, Hagino et al. 2009). Despite their remarkable effects on increasing bone mass and decreasing potential fracture risk, atypical femoral fracture has arisen as one of the severe complications in patients undergoing long-term treatment with bisphosphonate agents. Atypical femoral fracture is defined as a femoral fracture triggered by a minor trauma and occurring between the lower end of the lesser trochanter and just above the femoral condyle (Shane et al. 2010, Rizzoli et al. 2011). A previous study demonstrated that 94% of patients with atypical femoral fracture had been treated with bisphosphonate (Shane et al. 2010). Bisphosphonates were

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reported to cause unusual mid-shaft fracture of long bones due to prolonged suppression of bone turnover causing accumulation of microdamage (Odvina et al. 2010). Regarding the fracture-healing process, some animal models demonstrated that bisphosphonates delayed the turnover of woven bone and remodeling in the fracture site, but did not affect its mechanical stability (Matos et al. 2010, Saito et al. 2010, Hegde et al. 2016). Normally, bisphosphonates do not delay the fracture-healing process in humans (Xue et al. 2014). However, several reports indicate that long-term bisphosphonate administration negatively influences the bone healing process of a human atypical fracture (Edwards et al. 2013, Egol et al. 2014, Miyakoshi et al. 2015, Kates and Ackert-Bicknell 2016). Also, a report of histological assessment of bone biopsy specimens of 8 atypical femoral fracture patients demonstrated delayed healing of the microfracture gap (Schilcher et al. 2014). The specific objective of our study was to observe not only the morphology, but also the morphometrical appearance of the fracture site of atypical femoral fracture, comparing patients with and without long-term bisphosphonate administration. We expected to find the morphometrical differences between bisphosphonate-treated and untreated human bone samples from the fracture sites, in terms of the length of bone resorption surface, the osteoclast numbers, osteon thickness and so on.

Materials and Methods

An 84-year-old Japanese female patient was admitted to our hospital due to insufficient healing of atypical femoral fracture after 16 years of bisphosphonate administration for osteoporosis (bisphosphonate patient). She was treated with retrograde intramedullary nail insertion combined with valgus osteotomy, including the fracture site to correct the laterally convexed femur 8 days after the onset. The bone piece removed via the valgus osteotomy, including the fracture site and adjacent bone, was harvested (bisphosphonate sample; Fig. 1a), and prepared for histopathological analysis. An 83-year-old Japanese woman with insufficient healing of atypical femoral fracture, but without treatment history for osteoporosis (non-bisphosphonate patient) was also surgically treated by valgus wedge osteotomy 19 days after the onset of severe thigh pain. The bone piece of the fracture site was excised and retrieved (non-bisphosphonate sample; Fig. 1b). Demographic data of the cases are shown in Table 1. Both patients were informed of the study purpose and signed the consent form. This study was approved by the Ethical Committee of Hiraka General Hospital, Yokote, Akita, Japan (Approval number: 179).

The harvested bone samples were decalcified in a 10% EDTA solution in phosphate-buffered saline on a rocking incubator at room temperature for 1 week, following which

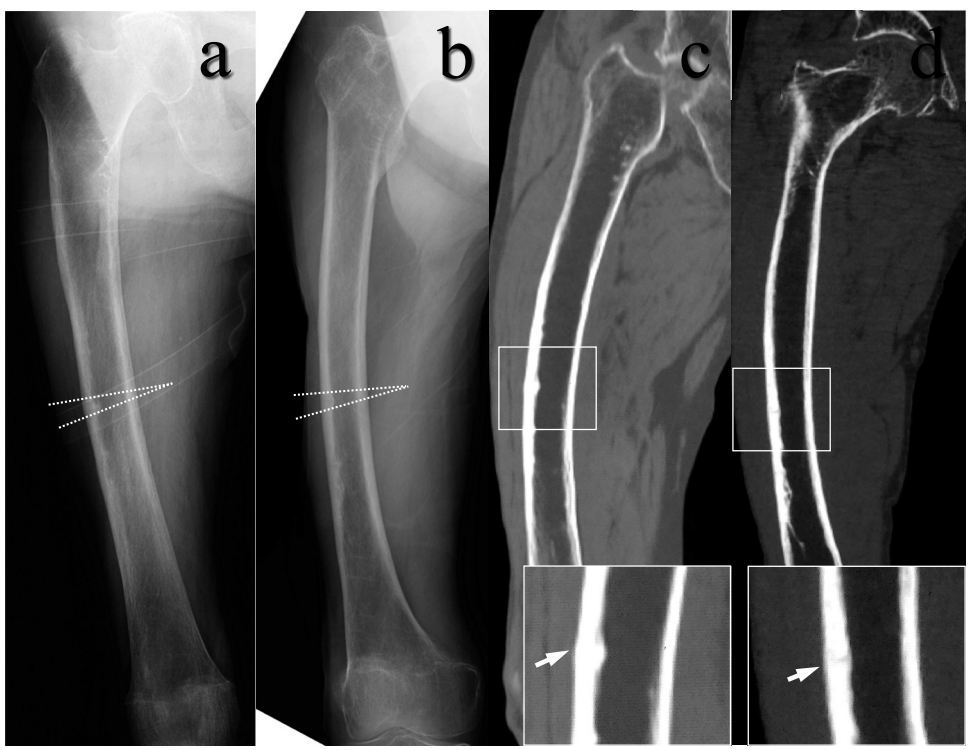

Fig. 1. Radiographs and computed tomograms (CT) of the femur.
Radiographs of femoral fracture site of the patients with (a) and without (b) bisphosphonate administration, and coronal sections of CT from the patients with (c) and without (d) bisphosphonate administration are shown. Broken lines indicate the site and the shape of wedge osteotomy (bone samples). Boxed areas were magnified in the windows at the bottom of each image. Arrow heads indicate fracture lines.

Table 1. Demographic data of the cases.

	Case 1 Bisphosphonate patient	Case 2 Non-bisphosphonate patient
Age / Sex	84 / Female	83 / Female
Height (cm) / Body weight (kg)	129 / 35	130 / 25.8
BMI	21.0	15.3
Comorbidities	Hypertension	None
Medication for osteoporosis	Oral Alendronate 5 mg/day × 16 yrs	None
BMD (g/cm^2) / YAM		
Lumbar Spine	0.345 / 32.8%	N/A
Femoral neck	0.206 / 24.0%	N/A
Laboratory data (serum)		
Calcium (mg/dL)	9.4	9.1
IP (mg/dL)	2.7	4.2
Albumin (g/dL)	4.3	4.2
BUN (g/dL)	11.4	20.6
Creatinine (mg/dL)	0.55	0.49
eGFR (mL/min/1.73m^2)	77.3	88.0
NTx (nmolBCE/L)	358	N/A
BAP (μg/L)	18.5	N/A
Bone morphometry of the femur		
Neck-shaft angle (degrees)	117	127
Lateral bowing angle (degrees)	10.4	20.2
Grade of the anterolateral femoral bowing (Park et al. 2017)	Mild (Grade I)	Severe (Grade III)

BMI, body mass index; BMD, bone mineral density; YAM, young adult mean; IP, Inorganic phosphorus; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; NTx, N-terminal telopeptide; BCE, bone collagen equivalent; BAP, bone alkaline phosphatase; N/A, not available.

they were fixed with 10% formalin neutral buffer solution and embedded in paraffin. Samples were cut into 4-μm sections and deparaffinized in Histo-Clear® (Cosmo-Bio, Tokyo, Japan), a xylene substitute, and dehydrated in sequentially diluted ethanol, from 100% to 70%. Hematoxylin and eosin (HE) staining was performed to evaluate the morphology of bone structure.

Histological analyses of HE-stained preparations were performed using an incident-light fluorescence polarizing microscope (OLYMPUS BX-53, Tokyo, Japan) with CSS-840 Histometry RT digitizer software (System Supply, Ina, Japan). First, changes in porosity of the femoral cortices from non-fracture sites were observed and compared between bisphosphonate and non-bisphosphonate samples. Then, the content and the morphology of osteoclasts in bisphosphonate and non-bisphosphonate samples were examined in the bone-resorbing area of the fracture site. For bone morphometry, the ratio of the length of bone resorption surface to the total bone surface, and the number and content (mononuclear or multinuclear) of osteoclasts on bone resorption surfaces were analyzed in a 6.25 mm^2 field at × 200 magnification in fracture sites of both bisphosphonate and non-bisphosphonate samples. Also, the shape and size of osteons, the bone mass of the woven bone, and the ratio of the woven bone to the total bone mass were analyzed in a 5.75 mm^2 field at × 200 magnification with a polarized filter in fracture sites of both bisphosphonate and non-bisphosphonate samples.

Results

The femoral cortex in the bisphosphonate sample was obviously more porous compared to non-bisphosphonate sample (Fig. 2a, b). In the bisphosphonate sample, the number of osteoclasts on the bone resorption surface was decreased compared to non-bisphosphonate sample (Fig. 2c, d). In the bone resorption surface of the fracture site, multinuclear, hypertrophic osteoclasts were observed in bisphosphonate samples only (Fig. 2e). The histomorphometrical data of bisphosphonate and non-bisphosphonate samples is shown in Table 2. The ratios of the length of bone resorption surface to the total bone surface in fracture sites of bisphosphonate and non-bisphosphonate samples were 2.9% and 21.9%, respectively. The average osteoclast numbers on the bone resorption surface were 4.84 and 8.59 /mm with 94% and 74% ratios of multinuclear osteoclasts in bisphosphonate and non-bisphosphonate samples, respectively.

The osteon was misshapen in the bisphosphonate sample, but smooth and round or oval in the non-bisphosphonate sample (Fig. 3). The mean width of osteons at the fracture site was 24.5 ± 1.5 and 60.8 ± 1.8 μm in bisphosphonate and non-bisphosphonate samples, respectively.

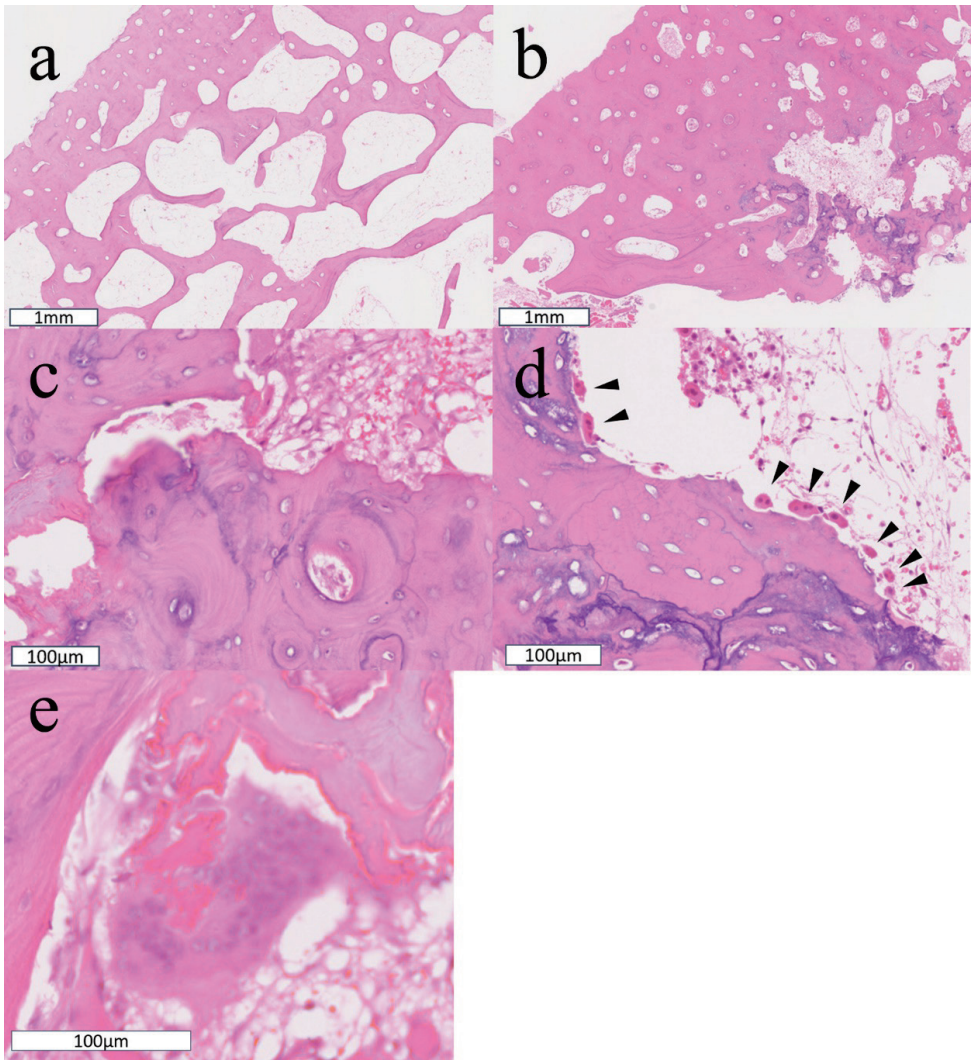

Fig. 2. Hematoxylin and eosin (HE) staining of bone specimens from sites of atypical femoral fracture in patients with and without bisphosphonate administration.
a, b: Transverse sections of non-fracture sites at the lateral femoral diaphyseal cortices in patients with (a) and without (b) bisphosphonate administration. The change in porosity of the cortex is obvious in the sample with bisphosphonate treatment compared to the non-bisphosphonate sample.
c, d: Fracture sites of atypical femoral fracture in patients with (c) and without (d) bisphosphonate administration. The number of osteoclasts is fewer in the bisphosphonate-treated sample compared to the non-bisphosphonate-treated sample. Black arrows indicate osteoclasts.
e: The multinucleated, hypertrophic osteoclasts at the fracture site of atypical femoral fracture in a patient with bisphosphonate administration.

The areas of the woven bone in bisphosphonate and non-bisphosphonate samples were 2.33 mm^2 and 0.80 mm^2 in 5.75 mm^2 field at × 200 magnification. The ratios of the woven bone to the total bone were 52.2% and 22.4% in bisphosphonate and non-bisphosphonate samples, respectively.

Discussion

Bisphosphonates have been regarded as the first drug of choice for osteoporosis due to its efficacy in fracture prevention. In recent years, however, it is well recognized that atypical femoral fracture is one of the major bisphosphonate-induced complications. The long-term use of bisphosphonates was reported to increase the risk of atypical femoral fracture by 25.65 times compared to non-bisphosphonate users (Lim et al. 2018). However, its therapeutic benefit is regarded to exceed the risk of bisphosphonate-induced atypical femoral fracture because the incidence of atypical femoral fracture in patients administered with bisphosphonate is reported to be relatively as low as 0.22% (Park-Wyllie et al. 2011). In our study, we also observed atypical femoral fracture in a non-bisphosphonate user. Park et al. (2019) reported that the anterolateral bowing of the femur affects the distal location of atypical femoral fracture. Also, Somford et al. (2017) described histological microdamage in the diaphysis at the fracture site of atypical femoral frac-

Table 2. Histomorphometrical assessment of the fracture sites.

	Bisphosphonate patient	Non-bisphosphonate patient
The ratio of the length of bone resorption surface / total bone surface	2.9%	21.9%
The average number of osteoclasts on the bone resorption surface (cells/mm)	4.84	8.59
The ratio of the number of multinuclear osteoclasts / total osteoclasts	94%	74%
The thickness of osteons at the fracture site (μm; mean ± SD)	24.5 ± 1.5	60.8 ± 1.8
The space of the woven bone (mm^2; in 5.75 mm^2 field at × 200 magnification)	2.33	0.80
The ratio of woven bone / total bone	52.2%	22.4%

SD, standard deviation.

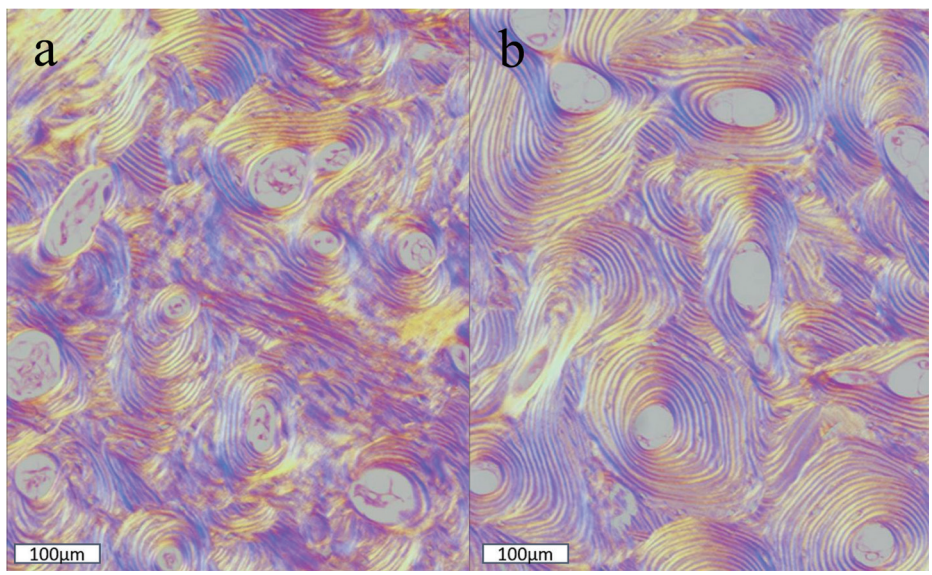

Fig. 3 Polarized microscopic images of femoral cortices at the adjacent part of fracture sites in patients with and without bisphosphonate administration.
The bisphosphonate-treated sample (a) demonstrated small and misshapen osteons with intervening woven bones, compared to the large, well-shaped, and densely composed osteons with less woven bone in the non-bisphosphonate-treated sample (b).

ture in an undecalcified specimen. A prominent anterolateral femoral bowing in our non-bisphosphonate patient was presumed to have caused and accumulated microfractures in the middle of the femoral diaphysis, resulted in atypical femoral fracture. Our histological assessment of a decalcified bone sample, regrettably, could not demonstrate microcracks of the femoral cortex at the fracture site, as shown in the previous study of the undecalcified specimen (Somford et al. 2017).

Regarding the effects of bisphosphonate on the human fracture-healing process, Xue et al. (2014) reported that bisphosphonate administration does not delay human bone healing in their meta-analysis of 8 randomized controlled trials (RCTs), including 2,508 cases. Adami et al. (2012) conducted an RCT including 7,808 patients to evaluate the effects of denosumab, an anti-receptor activator of nuclear factor-κB ligand (anti-RANKL) antibody agent that, similar to bisphosphonates, inactivates the osteoclast function during fracture healing. This study concluded that the fracture-healing process was not significantly delayed by an anti-RANKL antibody. In contrast, Lim et al. (2018) reported the higher rate of delayed union and nonunion of atypical femoral fracture in patients with long-term bisphosphonate administration, compared to those without bisphosphonate use, in their retrospective study of 196 patients with atypical femoral fracture, including 6,644 hip and femoral fractures. Edwards et al. (2013) also reported the delayed bone healing in 26% of patients with atypical femoral fracture with long-term bisphosphonate use.

The normal fracture-healing process starts with callus and woven bone formation based on the induction of undifferentiated mesenchymal cells, osteoblast precursor cells, and angiogenesis to the fracture site. Once the fracture site is filled with the woven bone, it is remodeled by osteoclasts, followed by replacement of the woven bone with a lamellar bone through the newly developed osteoblasts. In other

words, as bone healing progresses, the ratio of the woven bone to the lamellar bone decreases. Bisphosphonates can inactivate the function of osteoclasts almost irreversibly. In addition, some reports have claimed that long-term bisphosphonate administration delays callus turnover and bone remodeling in rat fracture models, although mechanical properties of the callus were similar to those in control animals (Manabe et al. 2012, Fu et al. 2013). To date, only a small number of reports are available demonstrating histological analysis of the human fracture-healing process in its early stage, because of the difficulty in obtaining samples from human fracture sites due to ethical issues. In our study, precious human bone samples obtained via valgus osteotomy for atypical femoral fracture with and without bisphosphonate administration could be retrieved. Especially, our report has a novelty in terms of focusing not only on morphological, but also on morphometrical aspects of the fracture sites in patients with atypical femoral fracture with or without long-term bisphosphonate administration.

In the fracture site of the bisphosphonate-treated patient, the ratio of the bone resorption surface to the total bone surface, and the number of osteoclasts on bone resorption surfaces were decreased compared those in samples from the non-bisphosphonate patient. Moreover, multinucleation of osteoclasts was remarkable in bisphosphonate samples. The decrease in bone resorption surface induced by bisphosphonate administration has been demonstrated in animal experimental models (Odvina et al. 2010, Kates and Ackert-Bicknell 2016) and is regarded to be due to the prevention of osteoclast induction. Furthermore, osteoclasts are known to become hypertrophic as their bone-absorbing activity decreases due to long-term exposure to bisphosphonate, rather than shrinking and decreasing in number as they resorb the bone (Weinstein et al. 2009). Kondo et al. (2015) reported a case with a histological analysis of the delayed union site of bisphosphonate-related atypical femoral fracture, and a histomorphometrical analysis of the ilium obtained by biopsy. In the fracture site, callus formation and multinucleated osteoclasts were observed, as shown in our study. Also, an examination of an iliac bone specimen demonstrated that the ratio of the bone resorption surface to the total bone surface was low compared to the normal iliac bone samples. Our results, therefore, support the findings of the previous report of bisphosphonate-related atypical femoral fracture (Kondo et al. 2015).

In our study, the amount and ratio of the woven bone to the total amount of the bone was higher in a bone sample from a patient after a long-term bisphosphonate administration, compared with a patient not treated with bisphosphonate. Regarding the healing process of the fracture site, the woven bone generally resorbed by osteoclasts is replaced by newly formed bone by osteoblasts. Presumably, the resorption of the woven bone was suppressed by malfunction of osteoclasts due to long-term bisphosphonate administration in the fracture site in bisphosphonate samples, which resulted in the higher ratio of the woven bone to total bone mass than in the non-bisphosphonate sample. As regards the shape and size of osteons, they were misshapen and thin in the bisphosphonate sample, but nicely oval and thick in the non-bisphosphonate sample. In a cadaveric study comparing the size of the osteon in the proximal femur of untreated osteoporotic and bisphosphonate-treated women without fracture, the osteon was thicker in bisphosphonate-treated patients (Bernhard et al. 2013). On the other hand, in the fracture site where repetitive microdamage-healing cycles of the cortical bone had occurred, bisphosphonate-induced disturbances of osteoclast-osteoblast coupling (Pederson et al. 2008) might have evoked non-physiological restoration of osteons, resulted in misshapen osteons in the bisphosphonate sample, as observed in our study. Although radiological bone union is not delayed by osteoclast-inactivating agents in the human fracture-healing process, our study demonstrated that long-term bisphosphonate administration alters the histological features of the fracture site from its physiological state.

This study is limited because of the human samples obtained from only one patient in each case with or without a long-term bisphosphonate administration, the difference in the timing of sample harvesting from the onset of fracture, the cross-sectional observation without a time course, and the possible impact on the fracture-healing process due to differences in the physical status of each patient. With careful considerations of these factors, we still believe that this study, using difficult-to-obtain human samples, adds to the body of knowledge accumulating on the histological features of fracture sites from human atypical femoral fracture and changes due to bisphosphonate administration.

In conclusion, this study analyzed the histological features of fracture sites of atypical femoral fracture in patients with or without bisphosphonate treatment for osteoporosis. In the fracture site of the bisphosphonate patient, the ratio of bone resorption surface to the total bone surface and the number of osteoclasts in bone resorption surface were smaller than those in the sample from the non-bisphosphonate patient. Moreover, the multi-nucleation of osteoclasts was remarkable in the bisphosphonate sample. The amount and ratio of the woven bone to the total amount of the bone were higher in a bone sample after a long-term bisphosphonate administration compared to those without bisphosphonate treatment. The shape of the osteon was misshapen in the bisphosphonate sample, but nicely oval in the non-bisphosphonate sample. Our study demonstrated that long-term bisphosphonate administration can alter the histological features of fracture sites from their physiological state.

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Conflict of Interest

The authors declare no conflict of interest.

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