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Analysis of Collagen Fiber Orientation in Bone of Different Aged Rats Using FTIR Imaging

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Bone is mainly composed of type I collagen, hydroxyapatite, and water. Bone strength is increased in bone formation and reflects 70% bone mineral density (BMD) and 30% bone quality; however, BMD is clinically used to assess bone strength. In this work, we analyzed collagen fiber orientation in femur of different aged rats using infrared (IR) dichroism image due to assess consequences of collagen fiber orientation for bone strength. IR dichroism images indicated that the degree of collagen fiber orientation in rat femur was increased until 33 weeks old at least, and it can affect bone strength.

Keywords bone; FTIR imaging; IR dichroism; collagen fiber orientation; collagen maturity

Introduction

Bone is mainly composed of approximately 65% mineral (hydroxyapatite and carbonated apatite), 10% water, and 20–25% organic materials consisting principally type I collagen [1]. The mineral in bone is located primarily within the collagen fibril, and during mineralization the fibril is formed first and the water within the fibril is replaced with mineral [2]. The bone strength, which is a barometer of bone health, is increased in bone formation and reflects 70% BMD and 30% bone quality [3]. The bone quality is derived from a combination of everything that contributes to the following four qualities at least: (1) the rate of bone turnover; (2) properties of the mineral/collagen matrix; (3) microdamage accumulations; (4) architecture/geometry of trabecular and cortical bone [4]. The following analytical methods are used to assess bone: quantitative CT, high-resolution MRI, and micro-CT for bone geometry and microarchitecture analysis; scanning electron microscopy (SEM), spectroscopic techniques such as Fourier transform infrared spectroscopy (FTIR), Raman, mass spectroscopy, and nuclear magnetic resonance (NMR) for measuring tissue composition [5].

FTIR can provide molecular structure information, and FTIR imaging can represent the distribution of chemical compositions. In recent years, FTIR imaging is receiving attention for characterization of bone quality such as the mineral to matrix ratio, the collagen maturity,

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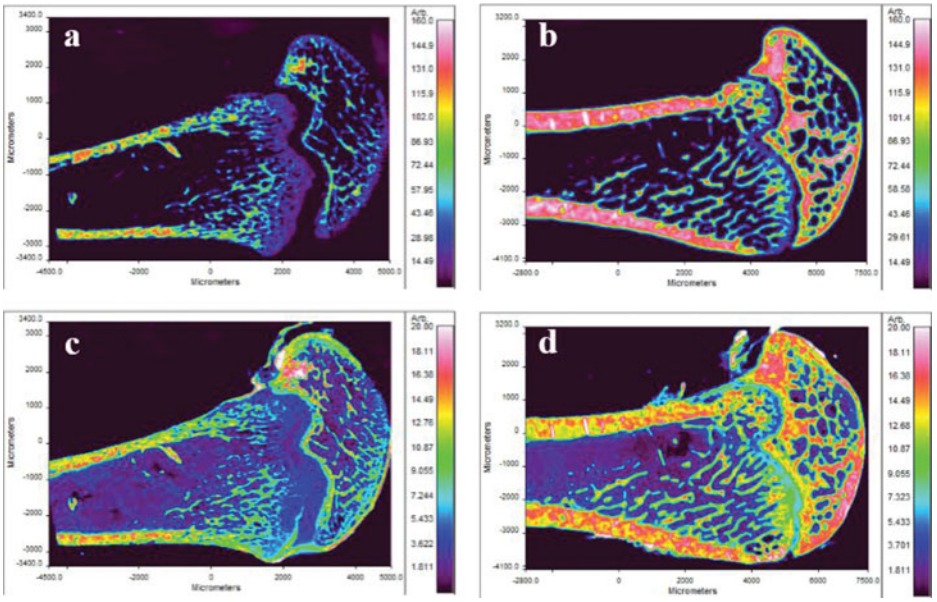

Figure 1. FTIR images of bone matrix distribution in rat femur: PO_4^{3-} derived from hydroxyapatite in 6 weeks old (a) and 33 weeks old (b); amide I derived from collagen in 6 weeks old (c) and 33 weeks old (d).

the carbonate to phosphate ratio, and the crystallinity of hydroxyapatite [6–11]. We have previously characterized bone quality in rats with osteoporosis or chronic kidney disease (CKD) using FTIR imaging [11]. The alteration of bone quality assessed by FTIR imaging was not necessarily coincident with that of bone strength; however, the strength of bone with those diseases was weaker. It has been reported that collagen fiber orientation affects mechanical properties of bone [12]. The bone strength and bone quality including collagen fiber orientation are changed in bone formation. In our previous works, hydroxyapatite and collagen in bone of different aged rats were characterized due to investigate bone formation by FTIR imaging and light microscopy, and we demonstrated that the calcification of rat femur was observed until 33 weeks old at least [9]. In this work, we analyzed collagen fiber orientation in femur of different aged rats using IR dichroism image due to assess consequences of collagen fiber orientation for bone strength.

Experiment

Femurs were harvested from male rats of different ages (16 days, 6 weeks, 12 weeks, and 33 weeks), washed by phosphate buffered saline (PBS), fixed with 70% ethanol, and then embedded in poly methyl methacrylate (PMMA). The PMMA blocks were sliced to prepare 3 μm longitudinal sections by a microtome, and the sections were mounted on BaF_2 windows for FTIR imaging measurement.

FTIR images of the specimens were collected by a FTIR imaging system (Spotlight400, Perkin-Elmer Inc., Waltham, MA, USA) coupled to a FTIR spectrometer (Spectrum400, Perkin-Elmer Inc., Waltham, MA, USA) with a mercury-cadmium-telluride (MCT) linear

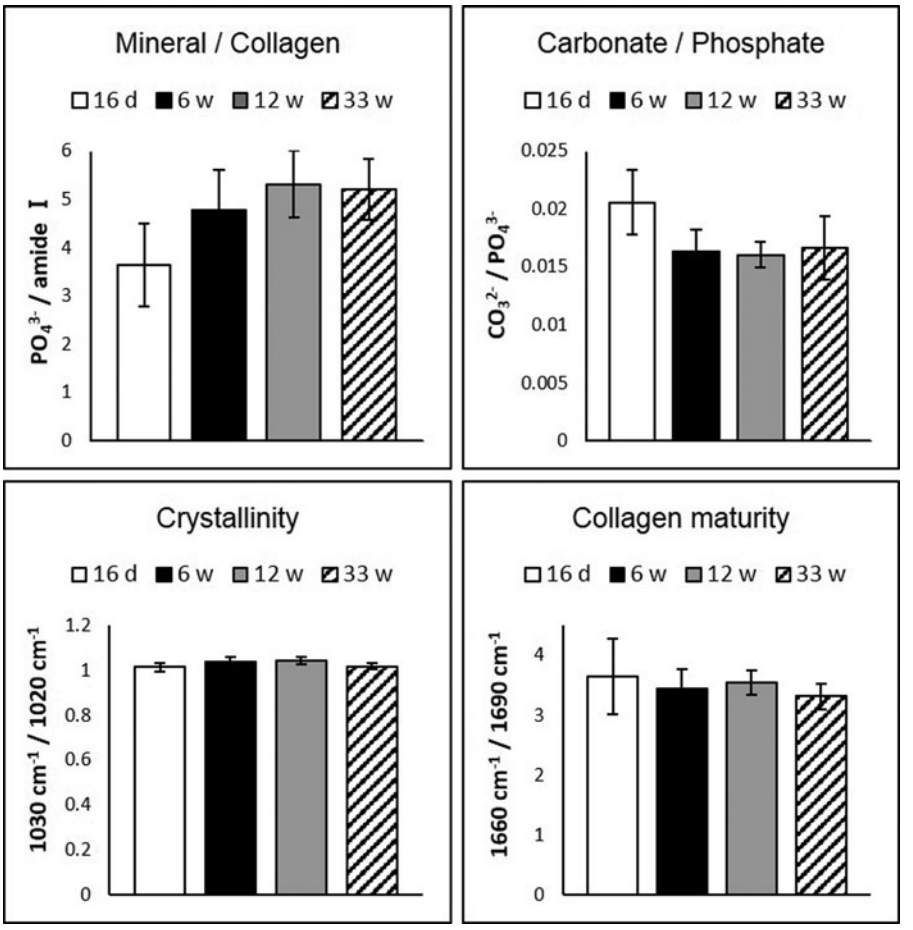

Figure 2. Histogram displaying the average of mineral to collagen matrix ratio (PO_4^{3-}/amide I), carbonate to phosphate ratio (CO_3^{2-}/PO_4^{3-}), crystallinity (1030 cm^{-1}/1020 cm^{-1}), collagen maturity (1660 cm^{-1}/1690 cm^{-1}) of cortical bone in the different aged rat femurs (16 days, 6 weeks, 12 weeks, and 33 weeks). Values are the mean ± SD for n = 60 spectra from each specimen.

array detector, using transmittance mode at a spectra resolution of 4 cm^{-1}, a spatial resolution of 25 μm^2, and frequency region from 4000 to 680 cm^{-1}. A wire grid polarizer (ST Japan Inc., Tokyo, Japan) was mounted on a special holder that allows rotation and placed in the infrared path before the specimen due to obtain IR dichroism image. FTIR images for IR dichroism images were collected using transmittance mode at a spectra resolution of 8 cm^{-1}, a spatial resolution of 6.25 μm^2 and frequency region from 4000 to 680 cm^{-1}.

The mineral to collagen matrix ratio (PO_4^{3-}/amide I) was calculated by integrating the area of the phosphate band (PO_4^{3-}, 1200-900 cm^{-1}) and dividing by the area of the amide I band (1750-1600 cm^{-1}). The carbonate to phosphate ratio (CO_3^{2-}/PO_4^{3-}) was calculated by integrating the area of the carbonate band (CO_3^{2-}, 900-850 cm^{-1}) and dividing by the area of the PO_4^{3-} band. The crystallinity was calculated by dividing the height of the PO_4^{3-} band at 1030 cm^{-1} by the height of the PO_4^{3-} band at 1020 cm^{-1} (1030 cm^{-1}/1020 cm^{-1}). The collagen maturity was calculated by dividing the height of the amide I band at

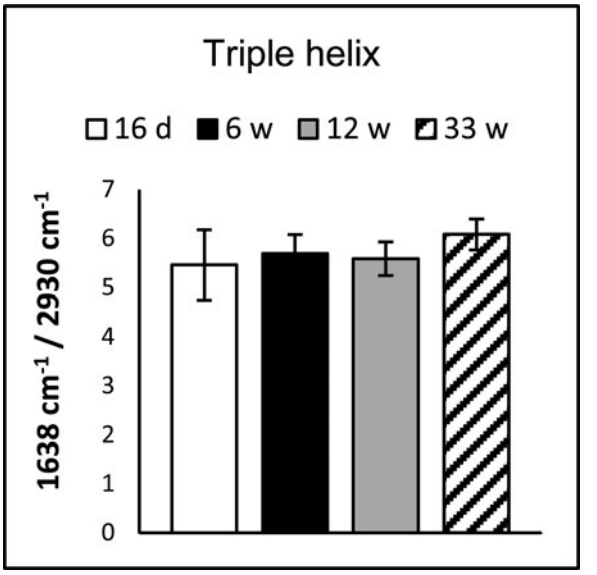

Figure 3. Histogram displaying the average of collagen triple helix to alkyl chain ratio of cortical bone in the different aged rat femurs (16 days, 6 weeks, 12 weeks, and 33 weeks). The alkyl chain hand (CH_2) height at 2930 cm^{-1} was used to normalize the height of the collagen triple helix band. Value is the mean ± SD for n = 60 spectra from the specimen.

1660 cm^{-1} by the height of the amide I band at 1690 cm^{-1} (1660 cm^{-1}/1690 cm^{-1}) [10].

Results and Discussion

Bone quality of femur in different aged rats (16 days, 6 weeks, 12 weeks, and 33 weeks) were accessed by FTIR imaging. Figure 1 shows FTIR images of bone matrix distribution in rat femurs: PO_4^{3-} derived from hydroxyapatite in 6 weeks old (a) and 33 weeks old (b); amide I derived from collagen in 6 weeks old (c) and 33 weeks old (d). Distribution of hydroxyapatite was not always coincident with that of collagen; however, both hydroxyapatite and collagen in femur were increased from 16 days to 33 weeks.

Figure 2 shows histograms displaying the average of mineral to collagen matrix ratio (PO_4^{3-}/amide I), carbonate to phosphate ratio (CO_3^{2-}/PO_4^{3-}), crystallinity (1030 cm^{-1}/1020 cm^{-1}), collagen maturity (1660 cm^{-1}/1690 cm^{-1}) of cortical bone in the different aged rat femurs. The mineral to collagen matrix ratio was increased from 16 days to 12 weeks; however, the crystallinity and the collagen maturity were almost no changed. The carbonate to phosphate ratio of cortical bone was higher in 16-day-old rat femur compared to the matured femur. These results indicated that carbonate content in the cortical bone was higher in early bone formation, and both crystallinity and collagen maturity of the cortical bone were almost no changed form 16 days to 33 weeks. However, it is reported that collagen cross-link affects bone strength [13]; therefore collagen triple helix content in cortical bone was accessed using collagen triple helix band at 1638 cm^{-1} [14]. Figure 3 shows histogram displaying the average of collagen triple helix to alkyl chain ratio of the cortical bone in the different aged rat femurs. The alkyl chain hand (CH_2) height at 2930 cm^{-1} was used to normalize the height of the collagen triple helix band. The result

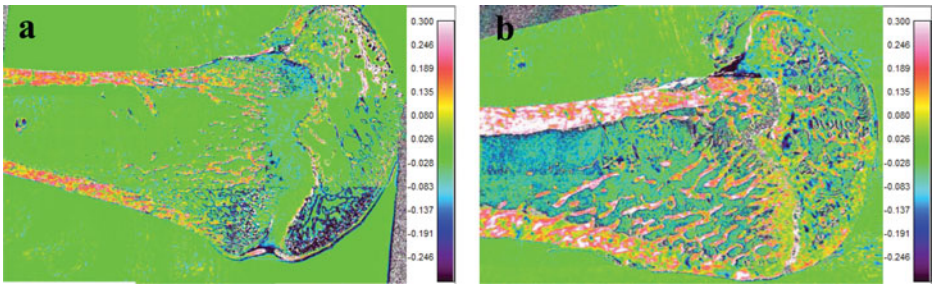

Figure 4. IR dichroism images showing collagen fiber orientations in 6-week-old rat femur (a) and 33-week-old rat femur (b). White and red show parallel to the cortical bone, and blue shows perpendicular to the cortical bone.

indicated triple helix content in the cortical bone was almost no changed from 16 days to 33 weeks. Collagen fiber orientation in rat femur was characterized using FTIR imaging with a polarizer due to obtain IR dichroism image showing collagen fiber orientation. Figure 4 shows IR dichroism images showing collagen fiber orientation in 6-week-old rat femur (a) and 33-week-old rat femur (b). The collagen fiber was oriented approximately parallel to the cortical bone, and the degree of collagen fiber orientation was increased until 33 weeks old at least. The collagen fiber orientation in the cortical bone may affect bone strength stronger than the collagen cross-link in the cortical bone.

Conclusion

Bone quality of femur in the different aged rats was characterized by FTIR image and IR dichroism image. Carbonate content in the cortical bone was higher in early bone formation, and the crystallinity, the collagen maturity and the triple helix content of the cortical bone were almost no changed form 16 days to 33 weeks. The collagen fiber was oriented approximately parallel to the cortical bone, and the degree of collagen fiber orientation was increased until 33 weeks old at least. The collagen fiber orientation in the cortical bone may affect bone strength stronger than the collagen cross-link in the cortical bone.

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