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Investigations into Demineralized Cortical Bone

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ABSTRACT

Partially demineralized (DM) bone is of interest due to its promising osteointegrative properties for advanced bone grafts. Structural features of partially DM (35 vol.%, 45 vol.% and 55 vol.% reduction), and untreated cortical bone samples were studied by scanning electron microscopy. Mechanical properties were investigated by compression testing in three anatomical directions at different stages of DM. The radial direction appears to be the stiffest and strongest bone direction for the all DM stages.

INTRODUCTION

Partially demineralized bone is of interest due to the excellent osteointegrative properties for advanced bone grafts [1-4]. Bone is a hierarchically structured composite material consisting mainly of a biopolymer (type-I collagen) and a mineral phase (carbonated hydroxyapatite). Additionally, there is water and some amount of non-collagen proteins [5]. A major structural component in cortical bone is the osteon that consists of thin (about 5-10 μm) lamellar sheets composed of mineralized collagen fibers. Osteons have a cylindrical shape (150-250 μm in diameter) and align parallel with the long axis of the bone.

Bone loss (osteoporosis) and demineralization occur as bones age and are a major cause of bone fractures. The mineral/collagen interaction is important in understanding how this affects bone fractures. Demineralization (DM) studies have been carried by several groups [6-9]. Broz *et al.* [6] investigated properties of partially DM (by ethylenediamine-tetraacetic acid) bone samples in three-point bending. They showed that specimens became less brittle with the increasing demineralization time. Lewandrowski *et al.* [7] provided an electron microscopy study of the DM process. They found that the DM process is described by advancing reaction front theory. Kotha *et al.* [8] summarized different techniques, which affect the bone mineral content, and found that sodium chloride solutions (NaCl) do not affect the mechanical properties of bone, while fluoride treatments (NaF) reduced the mechanical strength by converting some amount of bone mineral into calcium fluoride. The kinetics of cortical bone DM in 0.6N HCl was discussed in detail by Castro-Ceseña *et al.* [9]. The steady state DM reaction was found to be a first order reaction and kinetic parameters (activation energy and rate constant) were calculated. Since there are no systematic studies on mechanical properties of partially DM cortical bone, the main goal of this research is to find a correlation between mineral content during demineralization and the corresponding mechanical response.

MATERIALS AND METHODS

Sample preparation

Bovine cortical femur bone samples were obtained from local butcher (slaughter age ~ 18 months). About 100 samples for compression testing (5mm x 5mm x 7.5mm) were cut out from the same portion of the bone in order to minimize variations in density and mineral content. Samples were prepared for all three anatomical directions (see Fig. 1). The longitudinal direction was chosen as a direction parallel to growth direction of the bone, the radial direction was normal to the bone growth direction, and the transverse one was orthogonal to both.

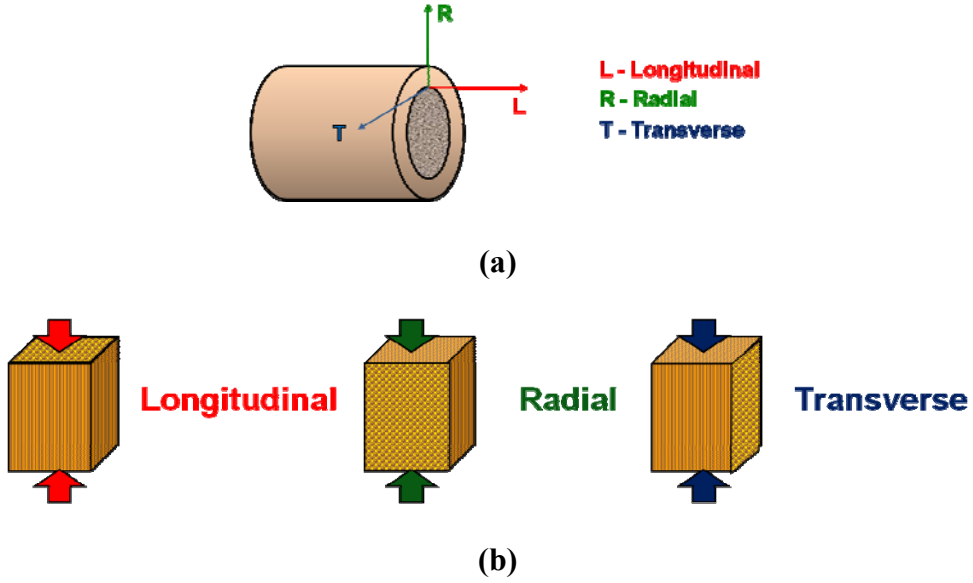


Fig. 1. (a) Schematic diagram of a bovine femur bone showing the three anatomical directions used for testing. (b) Osteon orientation for the three directions.

Demineralization process

Bone samples were demineralized at different times through a controlled process by aging in 0.6N hydrochloric acid (HCl) at room temperature, following the procedure described in [10]. Acid solutions were changed every two hours in order to avoid saturation that can affect the process. The whole demineralization process took about 50 hours. The amount of mineral removed was calculated by measuring the Ca concentration in solutions extracted at set periods of time. The solutions were quantitatively analyzed by the inductively coupled plasma optical emission spectrometry (ICP-OES). Based on these results, master curves for DM were determined for all three anatomical directions (see Fig. 2). Complete DM was verified using the procedure outlined in [9]. Three conditions of DM were tested: 35 vol.%, 45 vol.% and 55 vol.% mineral removal from the untreated (UT) bone.

Compression testing

Five different sets of the samples were prepared: 18 untreated samples (6 for each anatomical direction), 54 partially DM (18 for each degree of demineralization (35 vol.%, 45 vol.%, 55

vol.% minerals removed)), and 18 completely DM specimens. Specimens were submerged in Hank's balanced saline solution for 24 hours before testing, and were tested wet in compression. Compression testing was performed on universal testing machine equipped with 30kN and 500N load cells (Instron 3367 Dual Column Testing Systems, Instron). Specimens were tested with $1x10^{-3}$ s^{-1} strain rate. An external extensometer (Epsilon Series SATEC) was used in order to measure the displacement of the samples.

Structure characterization

Fracture surfaces of the specimens from all five groups were investigated by scanning electron microscope (SEM) equipped with EDS (FEI-XL30, FEI Company, Oregon, USA). The samples were subjected to a critical point drying procedure in order to avoid excessive shrinkage. The samples were mounted on aluminum sample holders, air dried and sputter-coated with chromium before imaging. SEM images were taken at a 20kV accelerating voltage.

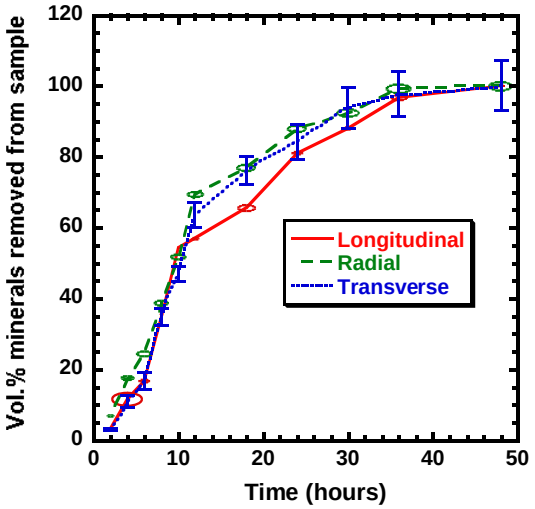


Fig. 2. Master demineralization curves for the three anatomical directions. Demineralization was slightly faster in the radial and transverse directions as compared to the longitudinal direction.

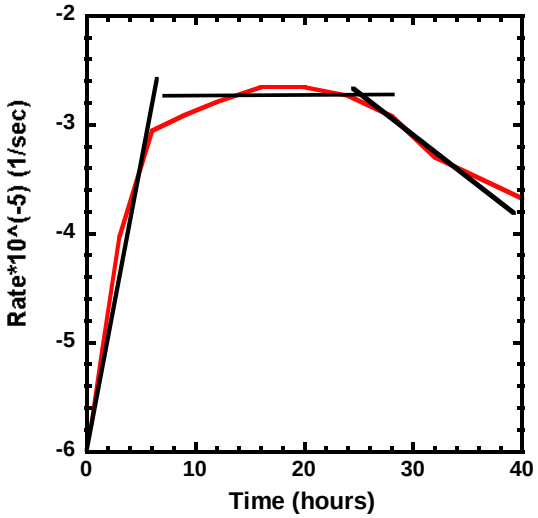


Fig. 3. Rate of demineralization as a function of time. The demineralized samples were from the steady state region (flat region). Modified from [9].

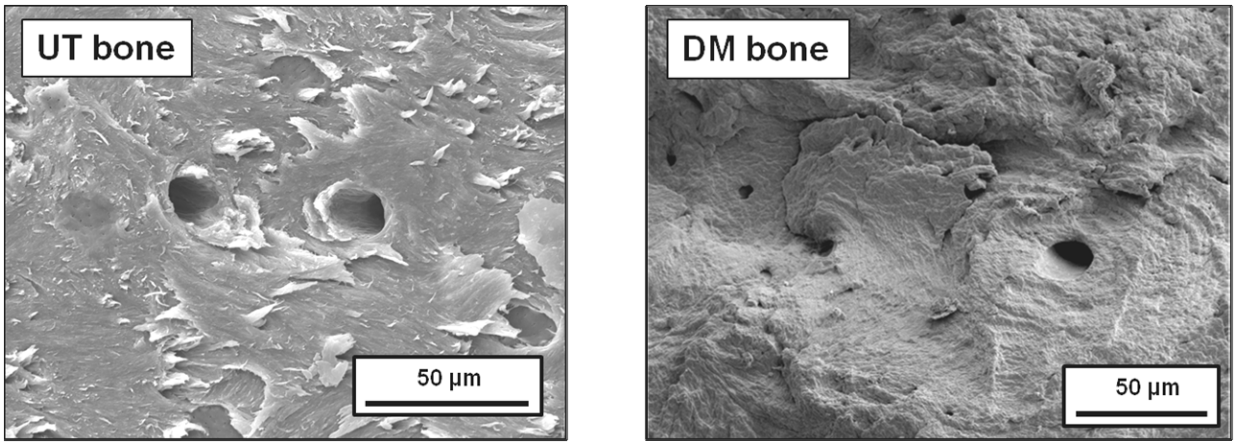
RESULTS

The DM process occurred at a somewhat higher rate for the radial and transverse samples compared with longitudinal ones in the time range of 10 to 36 hours, as can be observed in Fig. 2. The quickest way for the minerals to dissolve from the bone is along the vascular channels in the osteons, therefore, samples with the largest osteon surface area will demineralized slightly faster, as shown at Figs.1 and 2.

It was shown by Castro-Ceseña *et al.* [9] that demineralization process went through three distinctive stages, corresponding to different demineralization rates. During the first stage, the rate constantly increased as acid solution demineralized the peripheral part of the sample. During

the second stage DM occurred on a steady state that corresponds to DM of the central part of the bone sample. At the end of reaction (third stage) rate constant decreased, as mineral concentration in the bone became depleted (see Fig.3). Based on these results, we used samples that were DM uniformly inside the steady state region. Three different degrees of DM were chosen inside this steady state region (35 vol.% DM, 45 vol.% DM, and 55 vol.% DM). Cortical bovine femur bone contains ~ 45 vol.% mineral – thus our samples had total bone mineral contents of ~ 16%, 20% and 25%.

Fig. 4 (a) shows a fracture surface of an UT sample with vascular channels visible. Fig. 4(b) demonstrates that DM bone is a contiguous, stand-alone structure (protein continuous network) that can be mechanically tested. Microscopic features, such as the vascular channels (10-20 μm diameter) and lacuna spaces (5-10 μm in diameter), are preserved, in agreement with [11]. Moreover, well defined osteonal structures are clearly seen.



(a) **(b)**

Fig. 4. Scanning electron microscopy images shown: (a) untreated bone structure and (b) demineralized bone structure (protein continuous network). Images taken from different regions of the sample.

Fig. 5 shows the stress-strain curves for the different stages of DM and the three anatomical directions. For the UT bone, the radial direction has the highest toughness (area under the stress-strain curve) value while the longitudinal one is the stiffest, in agreement with [12]. Removal of the mineral shows a drastic change in the curves. First, what is noted is that there is now a 'toe' region, the low strain region. This indicates the larger influence of a protein phase (as the DM progressed). Usually this region is called physiological region where the tissue is normally functions [13]. Secondly, the maximum stress has been reduced significantly. Third, the elastic modulus – taken as the steepest portion of the stress-strain curve – was significantly reduced, ~10 times, between the untreated state and 35 vol.% DM. It is clearly seen that the radial direction appears to be stiffest and strongest bone direction for all DM stages. Table I summarizes the elastic modulus and the peak stress under the various DM conditions.

One interesting feature is that there is a measurable difference between the radial and transverse directions for the all DM stages. From Fig. 1, it appears that there should be no difference, since the osteon orientation is perpendicular to the loading direction in both cases. However, the difference can be clearly seen at Fig. 5 and Table I for all DM stages. The radial direction is stiffer and stronger than the transverse one. This can be explained by consideration of the bone structure. The outer part of bone consists of a circumferential lamellar structure that is

parallel to the bone surface [14]. This region has a thickness of ~ 200 μm, consisting of 5-10 lamellae. In addition, the mineralized collagen fibers in each lamella are oriented at different angles, forming an outer sheath and thus giving the bone extra strength in the radial direction.

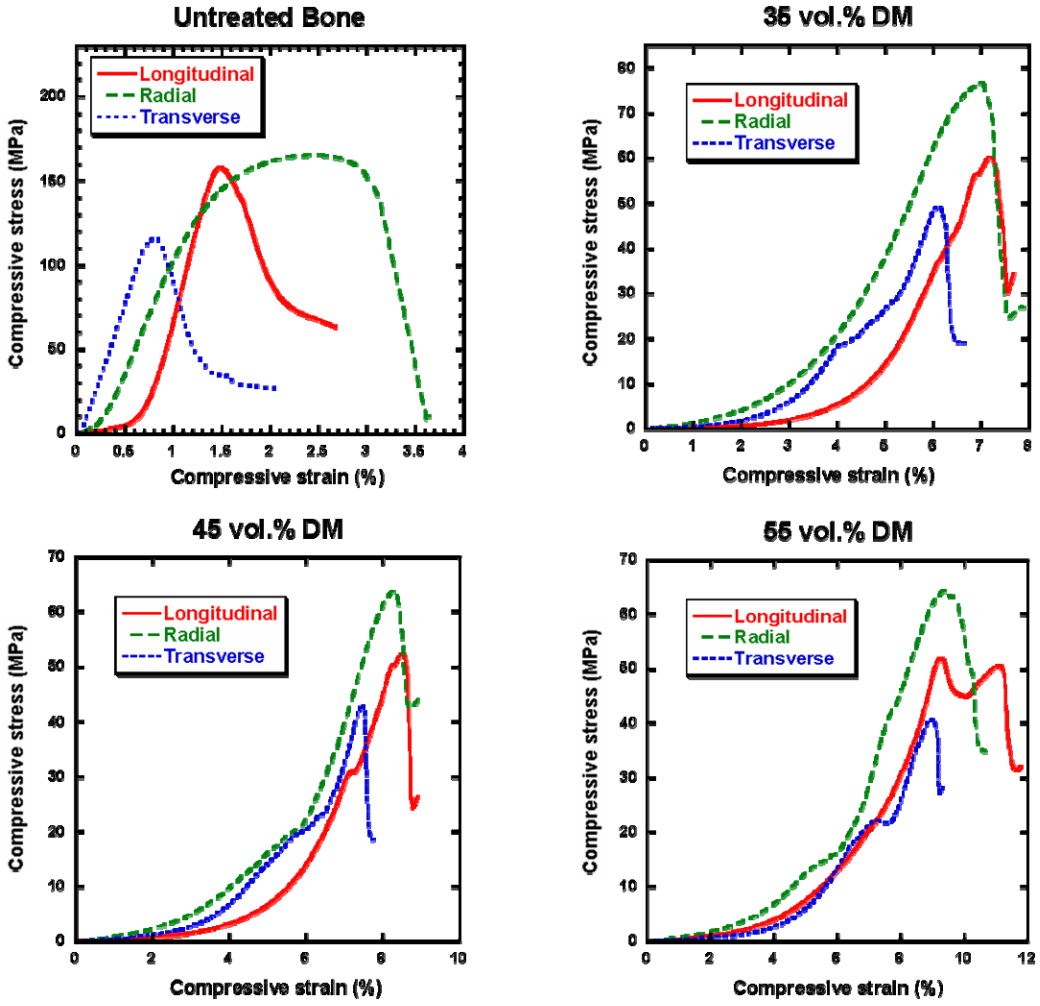


Fig. 5. Stress-strain curves for untreated bone and different degrees of bone demineralization. It is clearly seen that even a small amount of mineral deficiency dramatically changes the stiffness and strength.

CONCLUSIONS

This study is first to evaluate the compressive properties of partially demineralized cortical bone in three anatomical directions. It was found that the radial direction is the strongest and the stiffest for all demineralization stages due to existence of an outer circumferential lamellae sheath with mineralized collagen fibers oriented differently, compared to the interior of the bone. Moreover, even a small mineral deficiency (16 vol.%) dramatically affects strength and stiffness of bone.

Table I. Elastic modulus (GPa) and peak stress (MPa) from compressive stress-strain curves of untreated, 35 vol.% DM, 45 vol.% DM and 55 vol.% DM in the longitudinal (L), transverse (T) and radial (R) directions. *n* is the number of samples tested.

	Untreated		**35 vol.% DM**		**45 vol.% DM**		**55 vol.% DM**	
	E	σ_p	**E**	σ_p	**E**	σ_p	**E**	σ_p
L (*n* = 6)	22 ± 2	138 ± 20	2.3 ± 0.3	51 ± 5	1.6 ± 0.1	45 ± 8	1.5 ± 0.3	40 ± 8
R (*n* = 6)	12 ± 1	145 ± 10	2.4 ± 0.1	75 ± 10	2.0 ± 0.1	69 ± 7	1.8 ± 0.1	64 ± 7
T (*n* = 6)	16 ± 2	124 ± 10	1.5 ± 0.3	50 ± 10	1.0 ± 0.2	42 ± 5	0.9 ± 0.1	29 ± 7

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