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A comparative study of young and mature bovine cortical bone $\stackrel{\scriptscriptstyle \, \ensuremath{\scriptstyle \simeq}}{}$

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ABSTRACT

The mechanical properties and microstructure of young and mature bovine femur bone were investigated by optical microscopy and compression testing in the longitudinal and transverse directions for untreated, deproteinized and demineralized cases. Optical microscopy revealed that mature bone has a more established and less porous microstructure compared to young bone. Mature bone was found to be stronger in both directions for the untreated and deproteinized cases. Mature untreated bone was also found to be stiffer and less tough compared to young bone in both directions. These results are related to the increase in mineralization of mature bone and significant microstructural differences. Young bone was found to be stronger in both directions for the demineralized case, which is attributed to alterations in the collagen network with age.

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1. Introduction

Bone is a composite material made out of 65 wt.% minerals (carbonated hydroxyapatite), 25 wt.% proteins (mostly collagen-I, with a small amount of non-collageneous proteins) and 10 wt.% water [1]. The main biological functions of bone include mineral storage, protection of internal organs, skeletal support for the body, sites for muscle attachments and shock absorption. Bone is not only a lightweight, tough, strong and stiff material, but also has the ability to sense mechanical stimuli, and can regenerate defected areas in order to maintain its structural and biological integrity [2,3]. These outstanding properties are due to its complex hierarchical structure. There are two main types of bone: cortical and trabecular. Cortical bone is denser and forms the outer sheath, while trabecular bone is present in the areas that need to absorb energy (skull, ribs, vertebra).

Cortical bone further consists of three subtypes, osteonal, interstitial and plexiform. The osteonal bone is made up of cylindrical structures (osteons) that span throughout the bone in the longitudinal direction. Osteons support nutritional needs and regeneration processes. The space in between the osteons is filled with interstitial bone, which consists of bone remnants after remodeling. The plexiform bone is made up of lamellar bone sheets that are perforated by a plexus of blood vessels. Plexiform bone is found in large, fast growing animals and is an indicator of non-human bone [4]. Katz and Yoon [5] showed that plexiform areas are significantly stiffer than Haversian (osteonal) regions. Several studies that related the microstructure, strength and porosity of bone found that remodeled osteons are weaker and softer [6–9].

Several groups have investigated mineral contents of young and mature bovine and human bones [10-12]. Bovine and human bones reach their maturity level at different ages. According to Carter et al. [13], humans achieve full growth by the age of 16 years, while bovines are fully grown in 2 years. Therefore, the rate of bone growth is much higher for bovine bones than for human ones. This factor is extremely important in analyzing the structure and mechanical properties of bone. In bovines and humans, the bone mineral density increases significantly with age, resulting in corresponding changes in elastic properties, toughness and risk of fracture [14,15]. Furthermore, Currey and co-authors [12,16] demonstrated that bones from several species become more mineralized with increasing age, leading to greater stiffness and less toughness.

In addition, other factors, such as collagen deterioration with age, were found to influence age-related bone mechanical properties in humans [17,18]. Studies on bone of different species, including humans, have shown strong dependence of bone strength on collagen alignment and collagen content [19]. Research on osteogenesis imperfecta, a bone protein deficiency disease found in cattle and humans, also showed that a deficiency of proteins decreases bone strength and durability [20]. Several interesting results concerning the age related changes of bone microstructure and its influence on bone toughening mechanisms were reported by Nalla et al. [21] and Ritchie et al. [22]. They attributed the fracture sensitivity of aged human bones to an increasing density of Haversian systems and changes in collagen cross-linking at the nanolevel. A similar study by Zioupos and Currey [23] showed that, in human bones, an increase in stiffness with age leads to a de-

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crease in work of fracture and critical stress intensity factor, which is required to initiate a macrocrack.

Bone deproteinization and demineralization are powerful methods used to separate the two main constituents of bone and allow detailed investigation of properties of the mineral and protein phases separately. Compressive mechanical properties of bone and its main constituents were recently studied for mature bovine cortical bone [24] and mature bovine trabecular bone [25]. It was shown that both bone types are interpenetrating composite materials with mineral and protein constituents [1,4,24–26].

Mature cortical bone and its main constituents were found to have anisotropic mechanical properties [19,24,27–31]. The longitudinal direction was found to be the strongest for demineralized and deproteinized bone due to the preferential collagen fibers orientation in the former case and the mineral crystals preferential orientation in the latter case [24]. Skedros et al. [31] used acoustic microscopy to evaluate the elastic modulus of untreated, demineralized and deproteinized cortical bone of wild deer calcanei. They found that the anisotropy ratio (AR), calculated as the ratio between the longitudinal and transverse elastic coefficients, was significantly different from isotropy (where AR = 1) not only for untreated bone, but also for demineralized and deproteinized bones. This demonstrated that both the mineral and collagen phases behave in an anisotropic manner, along with the whole bone.

To the best of the authors' knowledge, there is no side-by-side investigation of the anisotropic properties of mature and young bones and their main constituents. Such an investigation is the main goal of this study.

2. Materials and methods

2.1. Sample preparation

Mature and young bovine femur bones from the mid-diaphysis region were purchased from a local butcher's shop (La Jolla, CA). The slaughter age was about 18 months for the mature bone samples and about 6 months for the young ones. All the bones were either kept frozen or refrigerated (4 °C) in Hank's balanced saline solution. Cross-sectional samples were first roughly cut with a band saw, then precisely shaped with a diamond blade under constant water irrigation into rhomboid parallelepipeds with dimensions $5 \times 5 \times 7.5$ mm³ for compression testing [32]. The samples were cut in two anatomical directions. The longitudinal direction coincided with the direction of bone growth, and the transverse direction to be perpendicular to the longitudinal one (Fig. 1). The samples came from one mature and one young bovine femur bone. Sixty bone samples were prepared (30 for mature and 30 for young bone). Five mature and five young samples from each group (longitudinal and transverse) were demineralized, and five mature and five young samples from each group (longitudinal and transverse) were deproteinized.

Additionally, samples for optical imaging, which consisted of the entire mid-diaphysis cross-section (1 cm thick), were prepared using four separate grinding papers and two additional polishing papers. A total of four cross-sectional samples (two for mature and two for young bones) were prepared. The compression testing and optical analysis were performed on different bone samples (from the same mature and young bovine animals) due to the difficulties of obtaining mechanical properties and optical analysis data from the same bone samples.

2.2. Mineral content

The ash content of young and mature bovine samples was determined by heating the samples in an oven for 4 h at 105 °C first



Fig. 1. Bone sample orientations. L = longitudinal, T = transverse. Samples are not shown to scale.

to evaporate the water, then for 24 h at 550 °C to eliminate the collagen content. The weights of the individual samples were measured before and after the heating processes. The weight percent of mineral was calculated by dividing the weight after heating by the weight before heating. The mineral volume percent was calculated according to equation:

$\mathrm{vol.\%} = \mathrm{wt.\%} \rho_{\mathrm{app}} / \rho_{\mathrm{HA}}$

where ρ_{app} is the apparent density of mature or young bone samples and ρ_{HA} is the density of hydroxyapatite (3.15 g cm⁻³) [37].

2.3. Deproteinization and demineralization processes

Deproteinization was performed by aging the samples in 5.25 wt.% NaOCl (bleach) at 37 °C [33]. The bleach solution was replaced daily for 2 weeks. Previous reports on deproteinized bone showed that the amount of protein left in the solution after subsequent demineralization of previously deproteinized samples was less than 0.001 wt.% [24,25,34]. At the end of the deproteinization process, samples were left overnight under running water to wash away the bleach solution to avoid any undesirable chemical side effects. Demineralization was performed by aging the samples in 0.6 N HCl solution at room temperature [25]. The acid was replaced daily for 10 days. Complete demineralization was verified by the absence of mineral in the solution, according to the procedure outlined in Castro-Ceseña et al. [35]. At the end of the demineralization process, samples were left overnight under running water to wash away the acid solution to avoid any undesirable chemical side effects. The sample sizes used in the present research were similar to those used in previous studies [24,25,34,35].

2.4. Structural characterization

Mature and young bone samples from all three groups (untreated (UT), demineralized (DM) and deproteinized (DP)) were analyzed by optical microscopy using a Zeiss Axio imager equipped with a CCD camera (Zeiss Microimaging Inc., Thornwood, NY). Entire cross-sections were analyzed along axes of major angles (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°), with 0° corresponding to lateral (outer) side of the femur (Fig. 2). Five photos were taken across each angle of the mature cross-sectional sample and approximately three images across each angle of the young cross-sectional sample. Fracture surfaces of the specimens were examined by scanning electron microscopy (SEM) using a microscope equipped for energy-dispersive spectroscopy (EDS) (FEI-XL30, FEI Company, Hillsboro, OR). DM samples were subjected to critical point drying before SEM imaging to avoid excessive shrinkage. All samples were



Fig. 2. Photographs of cross-sections of (a) mature and (b) young bovine cortical bone.

mounted on aluminum sample holders, air dried and sputter coated with chromium before imaging. The UT and DP samples were observed at 20 keV accelerating voltage, the DM ones at 5 keV.

2.5. Image processing

An image processor, ImageJ, was used to analyze the porosity of the bone samples, similar to the porosity analysis by Zioupos et al. [6]. Optical images at \times 5 and \times 10 magnifications and 150 dpi were examined individually. Vascular channels, Volkmann's canals and lacuna spaces were the pore types used for the porosity calculations. Porosity values were calculated by dividing the sum of the areas of the pores by the total area of the image. ImageJ was also used to calculate the toughness, defined as the area under the stress–strain curve until the fracture point. ArcSoft Panorama Maker Pro Images was used to stitch together the images taken of the cross-sections into a continuous image, which allowed better analysis of the micro- and macrostructures, as well as structural changes with respect to a single position (Fig. 2).

2.6. Compression testing

Five samples for compression testing were prepared for each of the UT, DP and DM samples for both young and mature bones in the longitudinal and transverse directions. UT samples were tested with a universal testing machine equipped with a 30 kN load cell (3367 Dual Column Testing System, Instron, Norwood, MA). DP and DM samples were tested with a universal testing machine equipped with a 500 N load cell (3342 Single Column Testing System, Instron, Norwood, MA) [24,25,36]. A SATEC model I3540 external deflectometer (Epsilon Technology Corp., Jackson, WY) was used to measure the displacements [24]. Samples were stored in Hank's balanced saline solution for 24 h prior to testing and were tested in a hydrated condition. All samples were tested at a strain rate of 10^{-3} s⁻¹, and were loaded until failure.

3. Results and discussion

Table 1 summarizes the volume fraction of minerals, compressive strength, Young's modulus, anisotropy ratio and toughness for UT, DP and DM mature and young bones in both the longitudinal and transverse directions. Compressive strength was defined as the maximum stress of the stress-strain curves. Young's modulus was estimated as the steepest portion of the stress-strain plots for all samples. Anisotropy ratios were calculated as the ratio between the longitudinal and transverse Young's moduli.

Microstructural analysis was performed on the entire cross-sections of mature and young bones (Figs. 2 and 3). It was found that the medial site of mature bone was made up of layers of plexiform bone, with a very few osteons (Fig. 3a), and the lateral side was composed entirely of osteonal bone (Fig. 3a). The lateral and medial sides of young bone were composed of a disorganized mixture of developing osteonal and plexiform bones (Fig. 3b). It was previously shown that different bovine femur bone quadrants have a range of microstructures, related to the rate of bone remodeling [38,39]. The distribution of mechanical stress and muscular activity are the most relevant reasons for the differences in the remodeling rate [38].

More detailed microstructural analysis with a porosity evaluation was performed on the samples taken from the lateral sites of both mature and young bones. Fig. 4 shows optical microscopy images and images processed by ImageJ software, which reveal the porosity (red areas). The amount of porosity for mature bone was found to be ~5%, whereas for the young bone it was ~8%, since young bone needs more pores for nutrients to pass through in order to support its fast-growing tissue. Porosity is one of the main factors contributing to the mechanical properties of bone (along with taxa, hydration conditions, anatomical direction and load distribution). Since porosity has adverse effects on strength, the higher porosity of young bone is in agreement with our results (Fig. 6a and b), demonstrating that mature bone is stronger than young bone.

Comparisons of young and mature bone microstructures also reveal a more undeveloped structure for the young bone (Fig. 4b). Fig. 4a shows that mature bone consists of larger secondary osteons (Haversian systems) that are \sim 150–250 µm in diameter. The secondary osteons are uniformly spread throughout the mature bone. Fig. 4b shows that young bone consists of primary osteons that are \sim 70–90 µm in diameter. The microstructure of young bone appears to be under construction and in the developmental stage. In contrast, the overall structure of mature bone is well developed and more uniform. Mature bone has undergone remodeling, which is clearly seen by the presence of well-developed secondary osteons and interstitial bone regions (Fig. 4a).

More detailed porosity analysis of untreated mature and young bones is shown in Fig. 5 for longitudinal sections (a and b) and

Table 1

Volume percent of minerals, compressive strength, Young's modulus (E), anisotropy ratio (E_L/E_T) and toughness for UT, DP and DM mature and young bovine femur bones in the longitudinal (L) and transverse (T) directions.

Sample	Orientation	Vol.% minerals	Compressive strength, MPa	Young's modulus (E), GPa	Anisotropy ratio (E_L/E_T)	Toughness, MJ/m ³
UT mature (<i>n</i> = 5)	L T	43 ± 1	184.1 ± 14.7 156.5 ± 5.3	20.5 ± 2.3 13.0 ± 2.3	1.58 ± 0.21	1.24 ± 0.07 1.42 ± 0.10
UT young (<i>n</i> = 5)	L T	39 ± 1	113.3 ± 39.4 121.0 ± 3.2	6.6 ± 1.9 5.3 ± 0.1	1.25 ± 0.29	1.87 ± 0.49 2.12 ± 0.03
DP mature $(n = 5)$	L T	100	11.6 ± 1.1 10.0 ± 2.1	2.5 ± 0.7 1.9 ± 0.3	1.32 ± 0.32	0.12 ± 0.01 0.07 ± 0.02
DP young (<i>n</i> = 5)	L T	100	6.1 ± 3.1 3.7 ± 1.6	1.5 ± 0.6 0.5 ± 0.2	3.00 ± 0.57	0.05 ± 0.01 0.05 ± 0.01
DM mature (<i>n</i> = 5)	L T	0	11.1 ± 2.1 8.9 ± 2.7	0.043 ± 0.015 0.041 ± 0.008	1.05 ± 0.06	N/A N/A
DM young $(n = 5)$	L T	0	12.7 ± 3.9 9.9 ± 3.0	0.104 ± 0.032 0.084 ± 0.031	1.24 ± 0.48	N/A N/A



Fig. 3. Optical micrographs of (a) mature and (b) young bovine cortical bones in the medial and lateral quadrants.



Fig. 4. Cross-sectional optical micrographs along with porosity analysis by ImageJ for (a) mature and (b) young bones. Interstitial bone regions, surrounded by secondary osteons, are enclosed in (a). An area with primary osteons is enclosed in (b).



Fig. 5. Porosity analysis of untreated (a) mature longitudinal, (b) mature transverse, (c) young longitudinal and (d) young transverse bovine femur optical images.

transverse sections (c and d). The porosity of the transverse sections of both the mature and young bones was found to be $\sim 2\%$ higher than that of the longitudinal sections. This is one of the reasons for the anisotropy of the Young's modulus for both mature and young bones in the present study (Table 1).

Representative compressive stress-strain curves for UT mature and young bones in the two anatomical directions are shown in Fig. 6a and b. The compressive strength of mature bone is significantly higher than that of young bone in both directions (Table 1). Moreover, the toughness of the young bone was found to be significantly higher than that of the mature bone for the UT case in both longitudinal and transverse directions (Fig. 7). This demonstrates that young bone has a greater ability to absorb energy and to deform plastically without fracturing. These results can be attributed, in part, to the change in the mineralization of the bone as it ages. Young bone was found to have a lower mineral content $(39 \pm 1 \text{ vol.}\%)$ compared to mature bone $(43 \pm 1 \text{ vol.}\%)$, in agreement with Ref. [40]. Currey et al. [16] accounted the decrease in toughness of aging human femora to the increase in bone mineralization. Moreover, according to Skedros et al. [41], hypermineralized lamellae surrounding primary osteons help improve toughness by deflecting the cracks that propagate throughout the bone. The toughness values were slightly larger for the transverse direction (Fig. 7b) compared to the longitudinal one (Fig. 7a) for both mature and young bones (Table 1). The porosity gradient along with a preferential orientation of osteons in the longitudinal direction contribute to the anisotropy.

Representative compressive stress-strain curves for DP mature and young bones in both directions are shown in Fig. 6c and d. In both directions, mature bone exhibits a higher compressive strength and Young's modulus. The toughness values were found to be close to each other (Table 1) for the two anatomical directions for both mature and young bones, suggesting that the cumulative toughness of bone is a result of a sophisticated interaction between two main bone constituents. The strength and stiffness were found to be higher in the longitudinal direction (Fig. 6c and d) for both mature and young bones due to the preferential orientation of mineral crystals, in agreement with Ref. [24].

Representative compressive stress-strain curves for DM bones in both directions are shown in Fig. 6e and f. The compressive strength and Young's modulus were higher for the young bone. These results suggest that collagen is stiffer and stronger in young bone and that the elasticity and strength become progressively degraded as the bone ages. These results are in agreement with other studies on human bone [17,18], which reported that the deterioration of collagen lowers the overall toughness of the bone by weakening the bridges that connect the collagen framework [21,22].

The anisotropy ratio for UT bone was found to be larger for mature compared to young bone, while it was the opposite for the DP case. There was little difference between the AR values of DM mature and young bones. Overall, AR values are in a good agreement with the previous study by Skedros et al. on deer calcanei [31].

SEM images of the fracture surfaces of DP and DM young and mature bovine femur bones are shown in Fig. 8. In both cases, mature bone (Fig. 8a and c) consists of thicker fibers that are similar to the thick collagen fibrils created during the secondary stage of bone formation, a characteristic most closely related to mature bone. Collagen fibrils direct the organization of crystals, creating intrafibrillar minerals embedded within the collagen fibrils and extrafibrillar minerals that form on the surfaces of and between the collagen fibrils [1]. The thicker mineral fibers (Fig. 8a) of mature DP bone are intertwined more than the fibers of DP young bone (Fig 8b). This is a possible reason for the greater strength and stiffness of mature DP bone (Table 1). Additionally, more closely packed collagen fibrils in DM young bone (Fig. 8d) could be a possible reason for the greater strength and stiffness of this bone phase compared with mature DM bone (Fig. 6e and f).



Fig. 6. Representative compressive stress-strain curves. Untreated mature (dashed) and young (solid) bones for (a) longitudinal and (b) transverse directions. Deproteinized mature (dashed) and young (solid) bones for (c) longitudinal and (d) transverse directions. Demineralized mature (dashed) and young (solid) bones for (e) longitudinal and (f) transverse directions.

It is also interesting to note that the compressive strengths for the DM and DP samples in both directions were very similar for both mature and young bones (Fig. 6c–f). The difference is larger for the young bone, however, due to constant remodeling and reformation in its development. The similarity in the strengths of the collagen and mineral constituents of mature bone could be



Fig. 7. Toughness calculations for untreated mature and young bone in the (a) longitudinal and (b) transverse directions.



Fig. 8. SEM images of fracture surfaces of (a) DP mature, (b) DP young, (c) DM mature and (d) DM young bovine femur bones.



Fig. 9. Compressive stress-strain curves of untreated young bone in the (a) longitudinal and (b) transverse directions. Two distinct, visible regions were observed: undeveloped (yellow) and more organized (white). L1 and L2 samples were composed of both regions and L3–L5 consisted only of the yellow regions.

attributed to the strength of the collagen network due to the organization of its collagen fibers in the former case and the highly close-packed arrangement of its mineral fibrils in the latter case (Fig. 8c and a, respectively). The fracture surfaces in Fig. 8 also reveal the brittle and elastic nature of the mineral and collagen constituents, respectively. The fibrils in Fig. 8a and b appear to have been broken off from the other surface, suggesting a brittle fracture, while the fibers in Fig. 8c and d seem to have been pulled from each other, suggesting fracture with plastic deformation.

Detailed compression test results for UT young bone in the longitudinal and transverse directions are shown in Fig. 9. It is clear that the compressive strengths vary significantly among the five longitudinal samples (Fig. 9a), while it is much more uniform for the samples in the transverse direction (Fig. 9b). Longitudinal samples with similar compressive strengths were grouped accordingly: L1 and L2 with the higher compressive strengths and L3-L5 with the lower compressive strengths. More detailed observations of the samples show that the first set of samples (L1 and L2) have a visible striped pattern of two different colors (white and yellow), while the color of the samples from the second set (L3-L5) was more uniform (yellow). All transversely oriented samples were striped and behaved consistently (Fig. 9b). EDS analysis showed that the white areas contain Mg and Na, characteristic of more mature and well-developed bone material, while the yellow areas lacked these elements, suggesting that these areas are less developed.

Many factors, including those explored in this study, affect the mechanical performance of aging bovine femur bone. The decrease in toughness and increase in stiffness and strength of untreated mature bone can be attributed to the increase in mineralization as the bone ages. This increase in mineralization can also account for the increase in strength of mineral framework in mature bone. In addition, the greater strength of untreated mature bone can be related to its more developed microstructure and lower porosity. The decrease in collagen strength in mature bone can be attributed to the deterioration of the collagen network, as seen in previous studies [17,18,21,22]. Since bone is a complex and ever-changing material, more careful and systematic analysis should be done in future studies. Mature and young bone samples for mechanical testing should always be taken from the exact same parts of the

bone cross-section in order to obtain mechanical properties based solely on a single variable.

4. Conclusions

The mechanical properties and microstructure of untreated, deproteinized (mineral) and demineralized (protein) young and mature bovine cortical femur bones were investigated in compression in the longitudinal and transverse directions. The main findings are as follows:

- Untreated, demineralized and deproteinized mature and young bones show anisotropy in the Young's modulus and strength between the longitudinal and transverse directions.
- Untreated mature bone is stiffer, with greater compressive strength and less toughness, compared with young bone.
- Deproteinized mature bone is stiffer and stronger than young bone in both directions.
- Demineralized young bone is stiffer and stronger than mature bone in both directions.
- Young bone is more porous and less mineralized than mature bone.
- Mature bone has a more developed microstructure compared to young bone.
- Untreated young bone samples with a higher percentage of developed bone were found to have greater strength than samples with a lower percentage of developed bone.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–7 and 9, are difficult to interpret in black and white. The full colour images

can be found in the on-line version, at http://dx.doi.org/10.1016/ j.actbio.2012.08.040.

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