Tissue level mechanical properties of cortical bone in skeletally immature and mature dogs

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Summary

Objectives: The purpose of this study was to quantify the tissue level mechanical properties of cortical bone of skeletally immature (~five-month-old) Beagle dogs and compare them to data from mature dogs measured in a previous study.

Methods: Eight femoral cross sectional specimens (two bone sections/dog) were obtained from four skeletally immature dogs. A pair of calcine bone labels were administered intravenously to the dogs to mark sites of active mineralization prior to euthanasia. Prepared bone specimens were placed in a nanoindenter specimen holder and the previously identified calcine labeled osteons were located. Labelled (n = 128) and neighbouring unlabelled (n = 127) osteons in skeletally immature femurs were examined by instrumented indentation testing. Indents were made to a depth of 500 nm at a loading rate of 10 nm/s. Indentation modulus (IM) and hardness (H) were obtained.

Results: The overall IM of the cortical bone in the skeletally mature groups was significantly greater than in the immature group (p = 0.0011), however overall H was not significantly different. The differences between the groups in IM were significant for the unlabelled osteons (p = 0.001), but not for the labelled osteons (p = 0.56).

Conclusion: There are differences in the IM of unlabelled osteons in skeletally immature and mature groups of Beagle dogs. In contrast to whole bone mechanical tests, where there are obvious differences between growing and mature bones, there are only small differences in the micro-mechanical properties.

Introduction

It is important to understand the evolution of physical properties of bone during and at the completion of growth, and also as a consequence of aging. Such an understanding will be critical to elucidating alterations in normal physiology and the effect of disease on the skeleton. There is limited information in the literature on the material properties of the bone of skeletally immature animals during the dynamic phases of development. The initial research on material properties of cortical bone established the elastic modulus of bone and emphasised the importance of composition (organic and inorganic phase) in determining elastic modulus (1–3). While there are studies examining whole bone mechanics of cortical and trabecular bone of the developing skeleton of the rat (4), dog (5), pig (6), sheep (7, 8), horse (9), and monkey (10, 11), there are few studies examining the micromechanics of newly formed bone. Most of the above cited studies do not report the tissue level elastic modulus of bone but rather the organ level physical properties. Previously, we reported the mechanical properties of labelled and neighbouring unlabelled cortical bone osteons from the femur of skeletally mature dogs as measured by nanoindentation (12). In addition, we have quantified the intracortical bone formation rate (BFR) in the femurs of skeletally immature and skeletally mature age animals (13, 14). While tissue level properties may be related to factors such as collagen cross-linking (15), a portion of the tissue level properties are likely to be attributed to bone crystal size, orientation and its relative maturation (16). Both the organic and inorganic components of bone may be different in skeletal immaturity and mature age groups. For example, it is known that the mineral apposition rate and the remodelling rate (13) are both higher in skeletally immature dogs.

We believe that a more complete understanding of the metabolic activity and material properties of bone will aid us in understanding bone adaptation in the appendicular skeleton. The objective of this study was to define the micromechanical indentation properties of mammalian bone matrix during growth and development of the skeleton in a canine model. We desired to examine osteonal and interstitial bone in skeletally immature dogs. Instrumented indentation testing, also referred to as nanoindentation, has been used to directly measure the material properties at high levels of resolution (17). Mineralised tissues including bone (18–22), enamel (23) and tooth tissues (24) have been examined by this method. Estimates of modulus and hardness are possible on extremely small volumes of tissue or irregularly shaped bones through nanoindentation. We hypothesise that there is not any difference in the in...
indentation modulus and hardness of newly forming labelled osteons and unlabelled osteons in skeletally immature animals.

Materials and Methods

Materials

Tissue specimens were obtained from another study which had institutional animal care committee approval. Calcein\(^{a}\) bone labels (5 mg/kg body weight) were administered intravenously to four purpose bred skeletally immature male Beagle dogs, each approximately five-months-old, at 16 days and two days prior to euthanasia. Radiographs made of the long bone confirmed that the physis were not fused. The left femurs were harvested immediately after euthanasia and frozen in saline soaked gauze at \(-20^\circ\) C for a brief storage period of less than six months.

Specimen preparation

The method for sample preparation, and osteon identification and localization has been described in detail in a recent publication (12). The only difference between the two studies was the age and maturity of dogs from which the femoral cortical bone cross sections were obtained. Briefly, prior to mechanical testing, the femurs were thawed and approximately 3–4 mm thick bone slices were obtained from the mid-diaphyseal region of the femur on a band saw under water irrigation. The femoral cross sections were examined under an epifluorescent microscope\(^{b}\) to confirm the presence of labelled osteons. For example, if a section did not have any labelled osteons, it could not be used in our study. Among the labelled osteons we choose those that were approximately half way or greater in their bone formation cycle (Fig. 1). An osteon that had just started the formation phase would not be selected as it would not have adequate bone for placement of indents. When using thick bone slices, epifluorescence only detects osteons on the bone surface.

The selected femoral bone surfaces were then lightly polished (18, 26), and care was taken to prevent overpolishing of the specimens (27). The bone block was glued into a well of a custom-made polycarbonate specimen holder on a certified level stage to ensure parallelism. The sectioned specimens were wet-polished on a rotary wheel\(^{c}\) at 120 rpm with 2,400 grit SiC papers. Polishing was done on a napless cloth\(^{d}\) with diluted 0.3 \(\mu\)m and 0.05 \(\mu\)m alumina oxide pastes\(^{e}\). The specimens were sonicated for 2 minutes. The height of each bone specimen within the specimen holder was verified with a digital caliper\(^{f}\) to ensure a uniformly flat surface after polishing.

Multiple perpendicular lines were scribed into the surface of the polished bone specimen with a fine surgical blade (12). The exact location \((x, y)\) (coordinates) of the central Haversian canal of the labelled osteon, from the nearest intersection of two perpendicular lines was located. The indenter optics were moved to the intersection of the two perpendicular lines and \(x, y\) coordinates to the centre of the labelled osteon in microns were entered into the software. The indenter optics was then driven to the specified coordinates.

Once the osteon was located, comparison was made with the colour, epifluorescent photographic images. The osteonal morphology of the labelled osteons and nearby structures confirmed the location of the labelled osteon. In addition, a neighbouring osteon that did not contain any calcein label was also identified (Fig. 1). The above procedure was repeated to locate additional labelled and unlabelled osteons on the bone slice.

Approximately 15 pairs of osteons were located in each bone slice. Each location was flagged into the computer with one set of coordinates for each labelled osteon. After all
osteons that we desired to indent were located, we then programmed the software to place indents on the bone of the osteons (Fig. 1). The indents were located approximately half the distance between the reversal cement line and the central canal for all the osteons (Fig. 1). This is because each osteon does not have the same dimension in each cross section and osteons were sampled at different times during their formation phase. We made five to six indents on each labelled and unlabelled osteon (Fig. 1). The sample size for this study consisted of two bone slices / dog for each of the four dogs.

The identification of all labelled and unlabelled osteons took approximately one hour/bone slice, after which the hydration system was turned on. A hydration fluid containing a mixture (26) of distilled water and 0.5 mg/ml gentamicin sulphate was supplied to the sample tray. The specimens were kept moist through the entire test period. A total of 255 osteons (labelled = 127; unlabelled = 128) were measured. In addition to the osteonal bone, a total of 206 indents were placed in the eight femoral sections on non-osteonal interstitial bone of the femur. Calibration of the machine against a silica standard was achieved prior to bone testing.

Indents were made on the osteonal bone, at a rate of 10 nm/second, at room temperature (12). For each measurement, the bone was loaded to 500 nm with a Berkovich diamond indenter using previously established and well described protocols for bone (12, 19, 28, 29). Surface roughness (30, 31) and demineralization (24, 31) can alter the bone properties at shallow depths (e.g., 100 nm). These effects are diminished at larger depths that were used in this study. A 30 second hold period was imposed at each peak depth. The unloading rate equaled the loading rate.

The software of the nanoindenter ran the prescribed custom test method and provided the indentation modulus and hardness using the Oliver & Pharr method. Oliver and Pharr (32) proposed the application of the Sneddon equation to a pyramidal Berkovich tip used in nanoindentation, to calculate the reduced elastic modulus. The Sneddon equation is based on elastic contact theory (33). The elastic modulus is calculated from the composite response modulus, $E_c$. To obtain $E_c$, the stiffness ($S$) is calculated from the slope of the initial portion of the unloading curve (17). The relationship between contact stiffness and the elastic properties of a sample is given by,

$$ S = \beta \frac{2}{\sqrt{\pi}} \sqrt{AE_c}, $$

(1)

where $\beta$ is an empirical indenter shape factor ($1.034$ for Berkovich tip) and $A$ the contact area. The composite response modulus, $E_c$ takes into consideration that elastic deformation occurs in both the specimen and the indenter tip and is given by,

$$ \frac{1}{E_c} = \frac{(1-v_i^2)}{E_i} + \frac{(1-v_s^2)}{E_s} $$

(2)

The indices $i$ and $s$ refer to the sample and the indenter material, respectively and $v$ is the Poisson’s ratio. For diamond values of $E_i = 1141$ GPa and $v_i = 0.07$ are typically used. Microhardness was calculated a $P_{\text{max}}/A_s$, where $P_{\text{max}}$ was peak load, and $A_s$ was the contact area. Poisson's ratio for bone was assumed to be 0.3. The elastic modulus estimates obtained from indentation testing have been referred to as nano or indentation modulus to distinguish properties obtained by nanoindentation from values obtained by more traditional organ level (three-point bending) and macroscopic level (tensile) tests.

### Statistical analyses

Data from the skeletally mature dogs were compared to the data obtained from the skeletally immature dogs. Since both indentation modulus and hardness are continuous variables and repeated measurements were taken on each animal, linear mixed effects models were used to take the correlation between measurements from the same animal and randomness into account.

Specifically, random intercept for each animal and the compound symmetric variance structure were used for repeated observations within an animal. Histograms of the data were visually analyzed, also residual analyses (residual plots, Q-Q plots and normality tests) were performed to test the model assumptions. Effects from age and type (labelled/unlabelled) of osteon as well as interaction term between age and type were explored at a significance level of 0.05. Tukey’s method was used to adjust for p-values in pair-wise least square means comparison between conditions. Square root transformation was used for hardness since its distribution was slightly skewed.

Animal effects on indentation modulus and hardness of femoral interstitial bone from skeletal immature animals only, were also analyzed by linear mixed effects models. The intercept for each animal and the compound symmetric variance structure were used for repeated observations within an animal. Square root transformation was used for femoral interstitial bone since the indentation modulus distribution is slightly skewed.

### Results

The load/displacement curve of each indent was examined. Some tests did not commence due to inability of the machine to find a surface and occasionally the target depth was exceeded (e.g., greater than 530 nm) and such tests were eliminated. This can happen due to a variety of reasons (e.g., a structure that is under the surface of the bone that is not apparent when selecting site of indentation) and is not uncommon in experiments that are conducted on hydrated biologic specimens. In contrast, testing of metallic specimens does not lead to such errors. Thus ~10% of the attempted indents failed. On one unlabelled osteon all indents failed. For the statistical analyses, a total of 578 indents were made on the 128 labelled osteons and 575 indents on 127 non-labelled osteons were included from the eight bone slices of the four skeletal immature dogs. Similarly, a total of 600 indents were analyzed on 102 labelled osteons, and 606 indents on 101 unlabelled osteons from the nine bone slices of the five skeletally mature dogs (12).

The indentation modulus of all bone types combined was significantly greater for skeletally mature cortical bone than skeletal immature bone ($p = 0.0011$) (Table 1 and 2). Unlabelled osteons had a significantly higher indentation modulus than labelled osteons ($p<0.0001$) irrespective of age of the animals. In addition there was a significant interaction between age and type
for indentation modulus between age and type (labelled/unlabelled) of osteons (p<0.001). Significant differences existed between the two age groups in the indentation modulus of the unlabelled osteons (p<0.0001). However, such differences did not exist between the labelled osteons (p = 0.83).

Hardness was not significantly different (p = 0.56) between the two age groups (Table 1). Unlabelled osteons had a significantly higher hardness than labelled osteons (p < 0.0001) irrespective of age of animals. In addition, there was a significant interaction for hardness between age and type (labelled/unlabelled) of osteons (p < 0.001). There was not any significant difference in hardness between the age groups in both labelled (p = 0.067) and unlabelled (p = 0.55) osteons.

For the non-osteonal interstitial bone, two bone slices from two different dogs had extremely low values of indentation modulus and hardness and were considered as outliers. For the statistical analyses, these outliers from the two slices were eliminated from the 206 indents. The means and standard deviation (SD) of the indentation modulus and hardness of the interstitial bone for the skeletally immature animals were 14.9 (4.2) GPa and 0.43 (0.16) GPa, respectively. Both variables of indentation modulus and hardness, were not different among the four skeletally immature dogs.

### Discussion

The study provides an estimate of the elastic properties of osteonal cortical bone in a group of skeletally immature dogs. We compared these data to cortical bone properties of skeletally mature dogs. Our principle finding is that there was a statistically significant difference in the indentation modulus of the two maturity groups. Indentation modulus of labelled osteons did not differ between the two groups, implying that a newly forming osteon has the same physical properties. However, unlabelled osteons from the two groups had different indentation modulus but not significantly different hardness.

All the values were obtained on wet specimens. Outliers were identified on two of the eight bone slices only for the interstitial bone of the skeletally immature dogs. The femoral interstitial bone was tested at the end of the test cycle for each bone slice. The entire cycle can take up to 10 hours. However, both the labelled and unlabelled osteons in these slices registered values that were not considered outliers. We have tested other specimens in the past without degradation of physical properties over a 10 hour period (34). We analyzed each of the curves in these two bone slices and the curves appeared normal, however the indentation modulus had abnormally low values (means (SD)) [0.4 (0.2) GPa and 3.9 (1.1) GPa]. We reported these outliers, and for the purposes of the statistical analyses, the outliers were eliminated.

In a previous study, we did not measure the properties of interstitial bone in skeletally mature dogs, as studies have demonstrated that the indentation modulus of interstitial bone is higher than that of osteonal bone (35). In the skeletally immature group, the indentation modulus and hardness of the interstitial bone was higher than the osteonal bone, suggesting that a major portion of the mineralization is achieved soon after bone formation.

There is limited information in the literature on the tissue level elastic or material properties of bone in the growing skeleton. Most of the bone measurements have been made at an organ level of biomechanical testing, and such values are influenced by measurement of bone mass and geometry. However, there is general agreement that the material properties of the growing skeleton are different from the adult skeleton for both cortical and trabecular bone.

One study of whole bone torsional properties of the femur and tibia of eight to 44 week old Harrier Hounds found that the stiffness and shear modulus of these bones increased up to age 25 to 30 weeks (5). The stiffness and shear modulus measured from torsional testing increased up to an age of 25 to 30 weeks. The elastic modulus of cortical cannon bone specimens from French saddle horses in-

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**Table 1** Significant main effects and interactions obtained from the mixed model analyses for indentation modulus (IM) and hardness (H). Interactions at the p ≤ 0.01 level were considered significant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM Main Effects</td>
<td>Age (Skeletally immature vs. mature)</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>Type of osteon (Labelled vs. unlabelled)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Interaction</td>
<td>Age*Type of osteon</td>
<td>&lt;0.0001</td>
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**Table 2** Mean and standard deviation (SD) of indentation modulus (IM) and hardness (H) for type of osteons (labelled and unlabelled).

<table>
<thead>
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* Data from Reference (12)
creased by approximately 40% between the ages of one day to two months (9). A study of machined femoral cortical bone specimens derived from humans between the ages of two to 48 years found an increase in elastic modulus with age during childhood (36).

There are also several studies that measured the changes in material properties of trabecular bone with age. Ovine bone from ages three to 80 months was obtained from the proximal tibia (7) and small cubes were machined for mechanical testing. The skeletal maturity group had an elastic modulus almost twice that of the skeletally immature group; however, the differences were not as large when comparing the three and 36 month age groups. A similar study of human bone (37) found an increase in Young’s modulus between the ages of 16 and 40 years but not between 40 and 83 years. In a pig vertebral study, skeletally mature animals had higher apparent modulus than a skeletally immature group (6).

We reported a nearly two-fold increase in indentation modulus via indentation testing of trabecular bone from the dog mandibular condyle in two age groups (~5 month vs. 1 year) (38). This increase was potentially due to the vast amounts of bone formation activity in the trabecular bone of the skeletally immature group, which lowered the indentation modulus.

It is clear from the above studies that modulus increases with skeletal maturity. In the current study, there was a 20% increase in indentation modulus for unlabelled osteons from the skeletally immature to the mature age group. This change cannot be due to modelling activities or changes in trabecular architecture (number and separation of trabecular columns), and represents tissue level changes in material properties.

Hardness was not significantly different between age groups. Hardness is a composite measure of the elastic and plastic behaviour of the material and does not separate the two components. The post-yield behaviour may have a large influence on the hardness and thus may not be different in skeletally mature versus immature bone.

Mineralization consists of a nucleation phase followed by crystal growth (39). With aging, the collagen matrix becomes more mineralised and there is an increase in the mineral size. This increase in mineral to matrix ratio results in an increase in the tissue level elastic properties (40). It has been suggested that the increased mineralization is associated with decreased mobility of the collagen fibres (40), which could impact both indentation modulus and hardness values differentially.

The overall indentation modulus was only higher by one GPa in the adult group [11.4 (4.3) GPa vs. 12.5 (5.0) GPa] and was significantly different. The interstitial bone in the skeletally immature group had a higher indentation modulus than the unlabelled osteons and is similar to the pattern seen in the skeletal mature femoral bone, where the tissue level properties of the interstitial bone are higher than osteonal bone (35). It is unlikely that an indent was directly located over the band calcified labelled bone, which can be 1 µm wide (the mineral apposition rate). It remains unknown if the calcine incorporated into the bone will alter the physical properties of the bone.

In previously reported studies, the mean BFR rates were 71 %/year in skeletal immaturity (13) and 2.5 %/year in the mature group (14). While there is a large difference in BFR, there is a relatively small difference in indentation modulus. One limitation to this interpretation is that the remodelling was not measured on the identical sections from which we evaluated the elastic properties. This is an important consideration as remodelling rates vary through the length of the long bone (41, 42).

It is possible that a major portion of the primary and secondary mineralization is complete within six months with minor additional increments occurring over a longer period of time (40, 43, 44). Tissue level indentation does not demonstrate large differences in the elastic properties between these two age groups. This is supported by two recent studies (16, 22) that demonstrate that the mechanical properties of tissues are achieved rapidly in growing animals and the microstructural properties do not differ between skeletally immature and mature animals.

This study provides data on the tissue level material properties in the femurs of skeletally immature and mature groups of dogs. Whole bone testing reveal a larger increase in elastic properties from a skeletally immature and mature time point, especially for trabecular bone. However, the mechanical properties of microstructural features of bone evaluated by nanoindentation, demonstrate relatively smaller difference than that obtained by whole bone tests.

Acknowledgments
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References