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Microindentation in bone: Hardness variation with five independent variables

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Abstract Microindentation is an investigational tool often used to determine hardness and other derived material properties of the material bone. This study explored the variation of microindentation hardness results with five independent variables. The variables were: applied mass, dwell time, drying time, time between indentation and measurement, and distance between the center of an indentation and the edge of other indentations and pores. These variables were selected because they represented a reasonable range of specimen investigational steps. We also investigated the cross sections of typical indentation residual impressions to determine the degree of material pile-up at the edges of the impressions. We found that microindentation hardness varied with applied mass and with distance between the indentation and neighboring indentations and pores but not with the other variables. Our recommended minimum applied mass is 0.10 kg versus a previously published value of 0.05 kg. We also found no discernable material pile-up at the residual impression edges, in contrast to reports of others.

Introduction

The objective of the study was to investigate microindentation hardness results as a function of several independent variables. As part of ongoing work with bovine bone, specifically the bovine metacarpus (MC), we questioned the effect microindentation independent variables had on hardness results. The question came up because we were concerned whether the current *de facto* standard for applied mass [1–3] was appropriate for our fresh wet specimens, and the fact that previous investigators [2] used dry, embalmed human bone with all but one of their specimens silver plated.

Indentation at the micro scale is an often used and effective tool in materials research [4–7]. It is used to derive various material properties including hardness, elastic modulus, and fracture toughness. More specifically indentation has become a method of choice for deriving the hardness and elastic modulus of bone and other hard tissue such as tooth dentin and enamel [8–10].

Bone hardness and elastic modulus are directly related to material microstructure and composition at the indentation site [8]. Bone is an anisotropic and inhomogeneous composite at the micro and nano scale. The degree of anisotropy can vary and has been simply described as transversely isotropic or orthotropic [11]. The constituents at the nano and micro scales are hard calcium mineral crystals and a softer collagen matrix. Because there are observable macro, micro, and nano structures, estimation of material properties is far from straightforward and requires a range of tools to elicit the material properties at the different scales.

The research reported here revisits microindentation as a method of determining hardness. Hardness measurements are carried out with an indentation machine (Fig. 1). The fundamental process involves automatic placement of a discretely selectable mass on the upper end of a pointed stylus. The point that contacts the specimen can be spherical, pyramidal, or other shape depending on the application. The mass is applied to the specimen through the stylus for a predetermined duration (dwell time), then automatically removed. The dimensions of the residual impression are then measured and used to derive hardness at the indentation site. Customary

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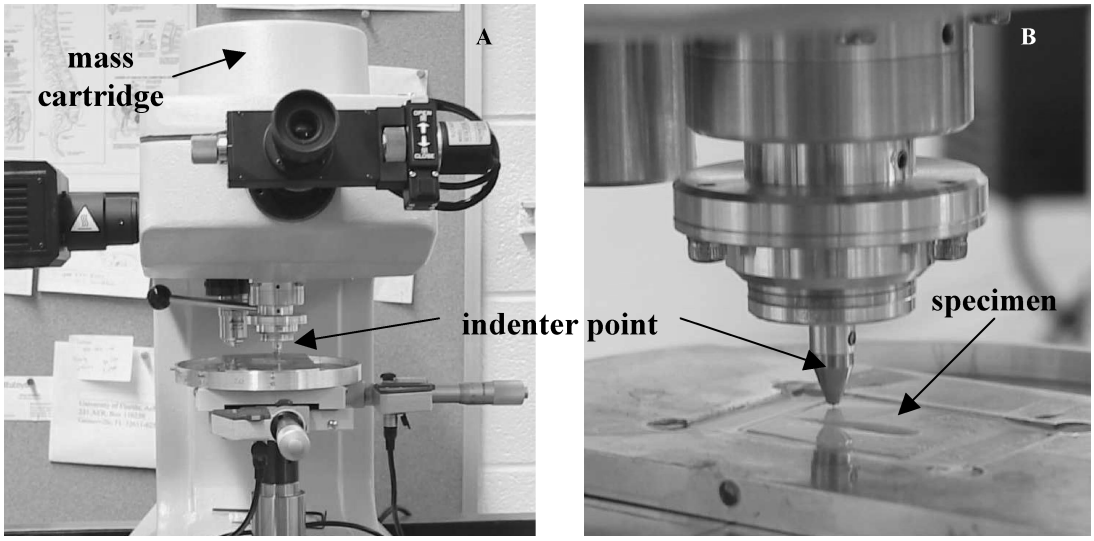

Fig. 1 (A) Microindentation machine, Mitutoyo model HM-112. (B) Detail of indenter point and specimen position

units of hardness are mass per unit projected area, typically kg/mm^2.

In addition to hardness variation with applied mass, we were interested in hardness variation with the following microindentation independent variables: dwell time; residence time on the instrument stage out of liquid (drying time); duration of time between indentation and residual impression measurement; and distance between the indentation site and pores. We also investigated the interaction effect on hardness of applied test mass and dwell time. These two parameters are independently selectable on the microindentation machine.

Choice of appropriate values for the independent microindentation variables is of importance in assuring reproducibility and measurement precision. Differences between sets of measurements on the same specimen could be caused by time domain phenomena such as creep and relaxation. Differences between sets of measurement on the same specimen could also arise from the spatial domain due to pores. Testing machine setup, calibration, and compliance (which can vary between indentation machines) is significant at low applied mass values [12]. Testing machine compliance acts in series with the specimen compliance. If the machine compliance is relatively low it can add to the compliance of the specimen producing a measurement error. In our case these variables were not adjustable once the machine had been set up. Only those variables over which we had control were chosen for investigation.

1. Applied Mass: A previous study on bone showed no variation of hardness with applied mass [13], yet a later study found a value of applied test mass (0.05 kg) below which hardness measurements were not reliable [2]. The later value had acquired the status of a *de facto* standard through handbook reference [1] and use by other bone researchers [3].
2. Dwell Time: The length of time the indenter point is in contact with the specimen could affect residual impression measurement results. Dwell time of the microindentation machine we used is adjustable between 5 and 99 s.
3. Drying Time: Because of our interest in wet specimens, the duration of time a specimen spends out of a water-based liquid was important. The duration of time out of the storage liquid could affect the residual impression measurements because the specimen was drying out. Rho and Pharr [14] reported increased hardness with time out of liquid.
4. Time between Indentation and Measurement: Indentation site material relaxation, between the time the indentation impression is made and when it is measured, could also affect the measurement of residual impression dimensions. We were particularly concerned about the relaxation effect because our specimens were wet. We hypothesized that the collagen component of bone would relax more wet than dry because collagen behaves somewhat like a sponge, when wet it recovers more than when dry.
5. Distance between Indentation and Pores: The distance between the indentation residual impression and nearby pores, predominately Haversian and Volkmann's canals, could affect results because material properties change spatially in the vicinity of pores.

Materials and methods

Specimens and preparation

Specimens used were a right bovine metacarpus (MC); a right bovine femur; and a right first molar from a monkey. The array of specimens were available to us and presented a range of hard biological material for assessment of their

hardness variation with one or more of our chosen microindentation independent variables. All procedures involving animal tissue use were conducted under the approval and auspices of the University of Florida Institutional Animal Care and Use Committee.

Bovine metacarpus

One specimen from a previously fresh frozen bovine right MC was rough cut twice with a 10′ band saw (Delta Machinery; Jackson, TN) across the distal diaphysis to produce a ring of bone approximately 30 mm in length. Additional fine cuts were made (ISOMET Low Speed Saw; Buehler; Lake Bluff, IL) longitudinally along the distal dorsal aspect. The specimen was approximately 25 mm by 45 mm by 1 mm thick with the long dimension parallel to the bone long axis. The length and width dimensions were dictated by the polishing system capability. The specimen was polished after cutting, using a semi-automated polishing system (Minimet 1000, Buehler). Polishing started with a 6 μm diamond slurry and finished with a 0.05 μm alumina and colloidal silica suspension. After polishing a transverse cut was made to produce a specimen approximately 25 mm by 10 mm by 1 mm thick. The bovine MC was supplied by the University of Florida College of Veterinary Medicine from a donor of unknown age and sex whose death was unrelated to this study.

Bovine femur

A specimen from a previously fresh frozen bovine right femur (Animal Technologies Inc., Tyler, TX) was rough cut twice with a 10′ band saw (Delta Machinery) across the mid diaphysis to produce a ring of bone approximately 30 mm in length. Subsequent longitudinal cuts were made with the low speed saw to produce a specimen approximately 25 mm by 25 mm by 1 mm thick. The specimen was polished in the same manner as the bovine MC specimen.

Monkey tooth

A tooth and accompanying mandible from a small monkey (M*acaca fascicularis*), obtained from the Southwestern Foundation for Biomedical Research (San Antonio, TX), were cut with the low speed saw in the buccolingual plane on the right side, between the premolar and the first molar. An additional cut was made on the centerline of the first molar also in the buccolingual plane. The cuts produced a tooth cross section specimen approximately 2 mm thick. After cutting, the specimen was manually polished using the same polishing sequence as the bovine specimens. The monkey tooth specimen was only used for determination of hardness variation with applied mass.

Microindenter

A microindenter (Model HM-112, Mitutoyo, Japan) fitted with a Vickers indenter point was used for measuring: hardness variation with applied mass; hardness variation with dwell time; and hardness variation with residence time out of solution on the bovine metacarpal specimen. That indenter point was chosen because previous research by others [2] used the Vickers indenter point and its use provided a basis for results comparison. A Knoop indenter point was chosen for all other investigations of hardness variation with microindentation independent variables on the bovine femur and monkey tooth specimens. We chose the Knoop indenter point because it allows investigation of hardness [3] and elastic modulus anisotropy [15].

The Vickers and Knoop indenter points are both four-sided pyramids. The major difference is that the Vickers point is a regular pyramid with equal diagonals and the Knoop point has diagonals of two different lengths (Fig. 2). The ratio of the Knoop diagonals is approximately 7 to 1 [16]. Both indenter points exhibit sensitivity to elastic anisotropy but the Knoop indenter point is much more sensitive than the Vickers [3]. The increased sensitivity is due to the ratio of diagonals and the corresponding apex angles. The long Knoop diagonal has an acute angle at its ends while the short diagonal angles are obtuse. During indentation the long diagonal does not change its length [4, 17] when the indenter point is removed from the specimen. However, the short diagonal acts to spread or push the material away from the apex. That dimension does change when the indenter point is removed. In fact the degree to which the residual impression dimension departs from the actual point dimension can be used as a measure of the indentation site material elastic modulus [4, 6, 7, 9].

Indentation procedures

Hardness variation with applied mass

A series of five indentations was made on the wet bovine MC specimen for each of five available masses (0.01 kg, 0.025 kg, 0.05 kg, 0.1 kg, 0.2 kg). A set of 3 indentations was made in monkey tooth dentin at the same five available masses. The indentations in the bovine MC were made with the Vickers indenter point while the indentations in monkey dentin were made with the Knoop indenter point. An arbitrarily selected dwell time of 10 s was used for the indentations. The interaction of applied mass and dwell time was subsequently investigated and 10 s was found to be acceptable. We monitored the time out of solution for each specimen to assure it did not exceed 30 minutes. We held the time between indentation and residual impression measurement to less than 20 s

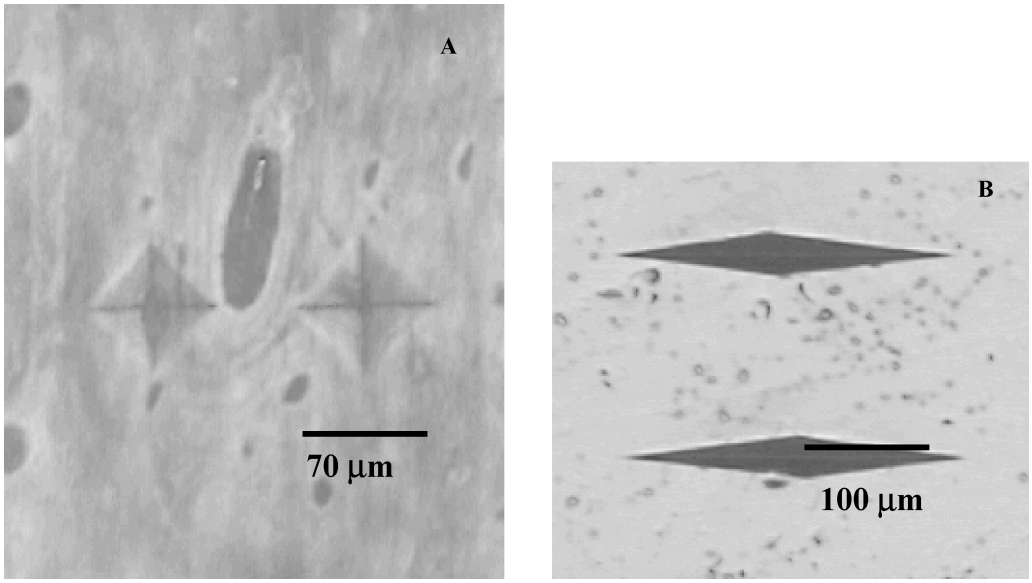

Fig. 2 (A) Typical Vickers microindentation in wet bovine bone. (B) Typical Knoop microindentation in wet bovine bone

for all indentations. We also insured that each indentation was at least 100 μm from pores and previous indentations.

Hardness variation with dwell time

One set of 5 indentations with the Vickers indenter point, at 5 dwell times (5 s, 10 s, 15 s, 20 s, 30 s), was made in the bovine MC. An additional set of 5 indentations with the Knoop indentation point each at 4 dwell times (5 s, 10 s, 15 s, 30 s) were also made in bovine MC. The Knoop indenter point long diagonal was oriented parallel to the specimen longitudinal direction. We compared the 5 sets of Vickers hardness with the analysis of variation (ANOVA) procedure as well as the 4 sets of Knoop hardness results (StatView, SAS Institute, Cary, NC).

For all the indentation sets we used an indentation applied mass of 0.1 kg. We monitored the time out of solution to assure it did not exceed 30 minutes. We held the time between indentation and residual impression measurement to less than 20 s for all indentations. We also insured that each indentation was at least 100 μm from pores and previous indentations.

Interaction effect of applied mass and dwell time

A series of sixty indentations were made in the longitudinal aspect of the bovine femur. They were made with the Knoop indenter point short diagonal parallel to the bone longitudinal direction. Applied masses of 0.01 kg, 0.05 kg, 0.1 kg, 0.2 kg, 0.3 kg and dwell times of 5 s, 10 s, 20 s, 40 s were used. An ANOVA procedure was performed on the resulting interaction data and then a *post hoc* Scheffe's test was preformed (StatView).

Hardness variation with drying time

Seven sets of five indentations each were made in the longitudinal aspect of bovine femur. We recorded the time each indentation was made over a period of 1.75 hours. We made the 5 residual impression measurements approximately every 15 minutes during the period. We used an applied mass of 0.1 kg and a dwell time of 10 s. We also assured that the distance between the indentations and pores and other indentations was at least 100 μm. We compared all 7 sets with the ANOVA procedure.

After the residence time tests the specimen was allowed to equilibrate with the laboratory environment for 47 hours. After 47 hours, we made five additional Vickers indentations and recorded derived hardness. The mean hardness of the previous 35 indentations was compared with the mean hardness of the 5 indentations performed after 47 hours using the ANOVA procedure.

Hardness variation with time between indentation and measurement

One indentation was made in bovine MC with the Knoop indenter point long diagonal perpendicular to the specimen long axis. The specimen was maintained in a bath surrounded by water solution. Based on results from hardness variation with applied mass and hardness variation with dwell time, we used an applied mass of 0.1 kg and a dwell time of 10 s. We also assured that the distance between the indentations and pores and other indentations was at least 100 μm.

We repeatedly measured the indentation starting 5 minutes after the indentation event and repeated the measurement about every 15 minutes 3 additional times. The total elapsed

time between making the indentation and measuring it the last time was 57 minutes. We selected about 60 minutes because we did not expect measurements of subsequent indentation sets to require more time.

Hardness variation with distance between indentation and pores

A series of 176 indentations were made on the bovine MC with the Knoop indenter point short diagonal perpendicular to the bone longitudinal axis. The indentations were made on the bovine bone in a regular pattern without regard to the location of pores. The pattern consisted of 8 equally spaced rows of 22 equally spaced indentations each. The spacing between the rows, measured between the center of adjacent indentations, was 240 μm. The spacing between indentations, measured between the center of neighboring indentations, was 110 μm.

The distance between the center of the indentation and the edge of the closest pore was recorded for each indentation. The derived hardness and measured distance from the indentations were plotted and analyzed with commercially available software (Excel, Microsoft Corporation, Redmond, WA).

Results

1. Applied Mass: Hardness variation with applied mass and data variability was greatest at a low value (0.01 kg) of applied mass for both specimens. We found hardness decreasing with increasing applied mass. The hardness and applied mass curves for both specimens reach a reasonably stable value at different applied masses (Fig. 3). Range bars on the figures were computed by taking the absolute value of the difference between the mean and the greatest and least value.

   An ANOVA for both specimen data sets resulted in statistical significance ($p < 0.05$) between hardness results at 0.01 kg and other results. However, we found no statistical significance ($p > 0.05$) between derived hardness values at 0.05 kg and higher for bovine bone and 0.025 kg and higher for the monkey dentin.
2. Dwell Time: Hardness variation with dwell time up to 30 seconds is not significant (ANOVA $p > 0.05$) for either the Vickers or the Knoop indenter points (Fig. 4).
3. Residence Time: The duration of time the specimen was out of the solution was not significant (Fig. 5). One-way ANOVA results between the first data set taken at about 5 minutes after indentation and the last data set taken at about 1.6 hours later were not significant ($p > 0.05$).

   The mean derived hardness of the specimen after 47 hours out of solution was 9% greater than that measured within 1.75 hours. The mean derived hardness was 48.8 kg/mm^2 with a standard deviation 1.5. One way ANOVA results were significant between the 2 hour hardness results and the hardness at 47 hours ($p < 0.05$).
4. Time between Indentation and Measurement: The mean derived hardness was 42.7 kg/mm^2 with a standard deviation of 0.3.
5. Indentation and Pores: Microindentation distance between the center of the indentation and the edge of a pore shows an effect at a distance of 73 μm and below (Fig. 6). Two linear regression lines were constructed to form a bi-linear plot. The lines reached the same value at 73 μm. A bi-linear fit to the data resulted in:

$$H_K = \begin{cases} 0.15D + 28.4 & 0 \le D \le 73\,\mu\text{m} \\ 39.5 & D > 73\,\mu\text{m} \end{cases}$$

   where D is the distance between the center of the subject indentation and the edge of a pore expressed in μm, and H_K is the Knoop hardness expressed in kg/mm^2.
6. Applied Mass and Dwell Time Interaction: There was a significant difference in the applied mass by dwell time in-

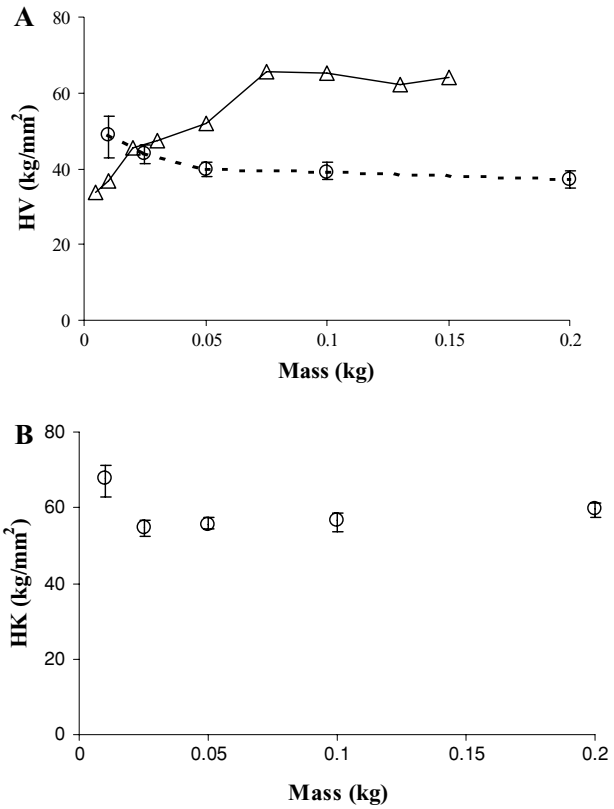

Fig. 3 (A) Hardness variation with applied test mass in bone. Shown are Ramrakhiani et al. (1979) data from dry embalmed human rib (plotted from their tabular data), solid line (triangles); and our data from wet bovine metacarpus, dashed line (open circles). Range bars are also shown for our data. A Vickers indenter point was used for all indentations. (B) Hardness variation with applied test mass in monkey tooth dentin. A Knoop indenter point was used for the microindentations

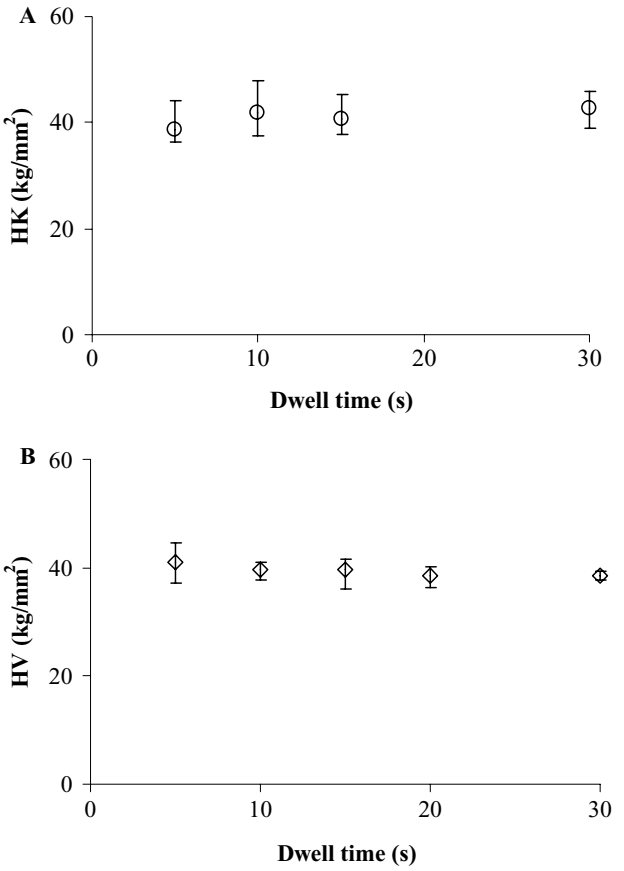

Fig. 4 (A) Hardness variation with dwell time in wet bovine metacarpus showing average values and range of results, Knoop microindentation point. (B) Hardness variation with dwell time in wet bovine metacarpus showing average values and range of results, Vickers microindentation point

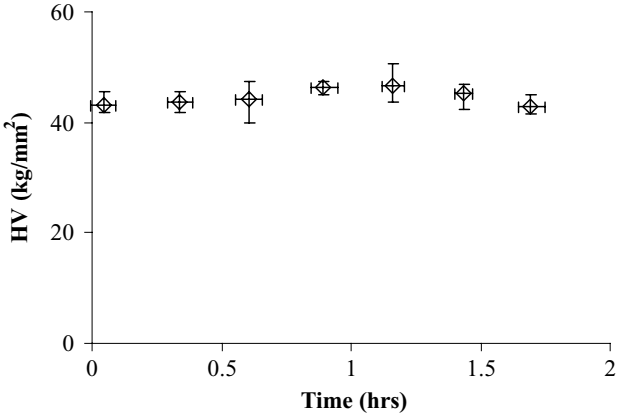

Fig. 5 Hardness variation with residence time (time out of solution) for bovine metacarpus, Vickers microindentation point. Shown are the average values and ranges for each data set

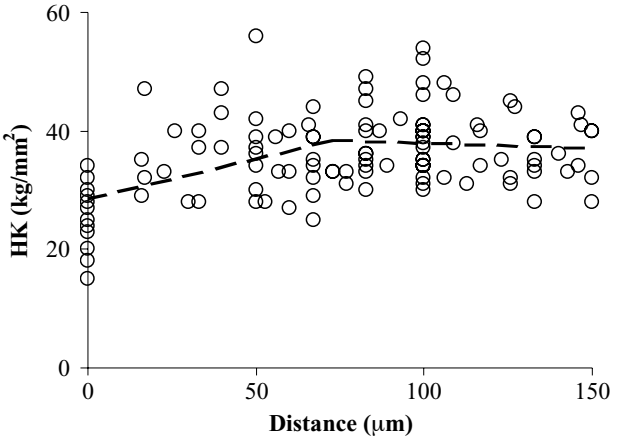

Fig. 6 Hardness variation with distance measured between the center of the microindentation and the edge of pores on wet bovine metacarpus. Bi-linear plot lines have a common value at 73 μm. Bi-linear function described by the dotted lines is: $0 \leq D \leq 73\mu\text{m} : H_K = 0.15D + 28.4$

teraction ($p < 0.05$). Subsequent multiple comparison using Scheffe's *a posteriori* test showed that the significance was limited to applied mass of 0.01. All other interactions were not significant at the 0.05 level.

Discussion

1. Applied Mass: Our results had some similarity to previous work by Ramrakhiani et al. [2] although it presented some differences. The similarity was that the curves leveled off at intermediate loads (Fig. 3 A). The difference was that the previous work reported increasing hardness with applied mass while we report decreasing hardness with increasing applied mass. The other most striking observation was that one set of Ramrakhiani et al. [2] results did not support their own conclusion (Fig. 3 A). Inclusion of that data would have suggested a minimum applied mass value above about 0.07 kg. The excluded data was from a non silver plated specimen. Their conclusion was based on only silver plated specimens. Hardness of their silver plated specimens at low mass values equaled the hardness of silver. Ramrakhiani et al. [2] reported specimen Vickers hardness of 23.8 ± 5.3 kg/mm^2 at 5g applied mass while the published value of Vickers hardness for elemental silver is 25 kg/mm^2 [18].

   For bovine MC hardness the results were not significantly different at applied mass of about 0.05 kg and above. The hardness results for the monkey tooth dentin were not significantly different at applied mass of about 0.025 kg and above. Those results suggests that for our Mitutoyo microindenter the applied mass values for bovine bone could be as low as 0.05 kg for bovine and 0.025 kg for monkey dentin. However, taking Ramrakhiani et al. [2] data into consideration a higher minimum applied mass seems appropriate. Hypothetically, if we used a minimum applied mass of 0.05 kg for indentation of wet bovine

bone, Ramrakhiani and colleagues would not be able to reproduce our test results.

An additional consideration is the size of the indentation residual impression for measurement. One of the advantages of microindentation is the ability to interrogate small regions. The residual impression also needs to be large enough to measure with precision. At low applied mass (0.01 kg) the long diagonal in a Knoop residual impression is about 50 μm. At an applied mass of 0.05 kg the residual impression long diagonal is about 140 μm. While at an intermediate applied mass of 0.1 kg, the long diagonal is about 200 μm. Applied mass selection is governed by: the size of the region of interest; the size of the residual impression; and the variation of hardness with applied mass.

We conclude that a minimum applied mass of 0.1 kg is appropriate for bovine bone in order to assure reproducible results among different microindentation machines. Furthermore, noting the difference in hardness results between bovine specimen and the monkey dentin we also conclude that a hardness variation with applied mass study be performed for each new material being tested.

2. Dwell Time: Values from 5 s up to a limit of 60 s do not have statistical significance. We have adopted 10 s as a typical dwell time in subsequent research for convenience.
3. Drying Time: Derived hardness values did not significantly change with residence time out of solution up to 1.75 hours. That result provided confidence that handling specimens out of the water solution could be done for periods up to 1.75 hours. The result is important because microindentation methods, such as the Elastic Recovery Method for deriving elastic modulus, require time to process. Subsequent to the finding we have adopted a maximum time of 30 minutes out of water solution for similar bone specimens in subsequent work.

   A 9% increase in hardness values after 47 hours was similar to values obtained by Rho and Pharr [14]. They used nanoindentation on bovine femur and reported results on a much finer scale. They reported a 12.2% hardness increase for interstitial lamellae and 17.6% increase for osteonal lamellae. Their specimen had been dried for 14 days while ours was dried for 2 days.
4. Time between Indentation and Measurement: Because there was no significant difference in derived hardness with the time between when the indentation was made and the long diagonal was measured, up to 30 minutes, we arbitrarily chose 10 minutes as a standard.
5. Distance between Indentation and Pores: Distance between the center of the subject indentation residual impression and the edge of pores is significant with an effect at distances closer than about 70 μm. In subsequent microindentation work we have adopted the value of 100 μm between the indentation center point and the closest pore edge or any neighboring indentation edge.
6. Applied Mass and Dwell Time Interaction: Significant interaction between applied mass and dwell time was limited to applied mass of 0.01 kg. Results from the hardness variation with applied mass suggested that use of applied mass of 0.01 kg is not appropriate. The interaction results confirm that finding.

Based on the interaction result we chose not to investigate interactions between the other microindentation variables. The only variability in any of the microindentation independent variables is in derived hardness variation with applied mass. As long as the minimum applied is greater than 0.05 kg for bone and 0.025 kg for dentin, there can be little interaction between variables.

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