Effects of Moisture Content and Loading Profile on Changing Properties of Bone Micro-Biomechanical Characteristics

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: Departmental sources

Background: Our study explored the influences of hydration conditions and loading methods on the mechanical properties of cortical bones and cancellous bones.

Material/Methods: Elastic modulus and hardness of human cortical bones and cancellous bones that contained different moisture levels (20%, 30%, 40%, 50%, and 60%) were measured with nanoindentation with different peak loads and loading rates. Cortical bones with 20% and 60% moisture were tested with 30 nm, 40 nm, and 50 nm peak loads at 6 nm/s, 8 nm/s, and 10 nm/s loading rates, respectively. Cancellous bones with 5% or 40% moisture percentages were tested with 600 µN, 750 µN, and 1000 µN peak loads at 200 µN/s, 250 µN/s, and 333 µN/s loading rates, respectively.

Results: Under the same loading condition, specimens with higher moisture contents showed decreased elastic modulus and hardness. Under different loading conditions, the loading modes had little influence on elastic modulus and hardness of cortical bone and cancellous bone with low moisture, but had significant influence on specimens with higher moistures.

Conclusions: The elastic modulus and bone hardness were affected by the moisture content and the loading conditions in cortical and cancellous bones with high hydration condition but not in those with low hydration condition.

MeSH Keywords: Bone and Bones • Microarray Analysis • Micromanipulation

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/906910
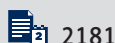
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Background

A mature bone consists of 2 types of tissue components: cortical and cancellous bone tissues [1,2]. Cortical bone tissue accounts for 80% of bone mass [3]. Its basic unit is osteon, which is composed of concentric layers, or 3-µm- to 7-µm-thick lamellae [4]. Cancellous bone consists of bone trabeculae around 1000 µm long and 0.2 µm thick [1].

Nanoindentation technique was originally used in the field of material science. In recent years, with the deepening of research, nanoindentation technique has been widely used to analyze the relationship between elastic modulus and hardness in cancellous bone [5]. When measured with nanoindentation, the resolution of load and displacement can be accurate to 0.3 µN and 0.16 nm, respectively [6,7]. Many factors, such as temperature, humidity, moisture content, shape of probe, surface roughness, loading method, and surrounding noises, can affect the test results [8,9]. For example, high humidity can increase the viscoelasticity of bone tissues and give a smaller test value. An early study by Hoffler et al. [10] showed that elastic modulus and hardness of dry human bone specimens had higher values than those of wet specimens. Dry or humid environments can also affect the microscopic properties of bovine femur and bone enamel [11–13]. Currey et al. showed that the mechanical properties of dehydrated bone were restored to the levels of fresh tissues if it was rehydrated [14]. The loading rate also plays an important role in estimating the behavior of bone tissues under different conditions of hydration. In addition, different loading rates were associated with different press depths, and a high press depth was critical to eliminating the effects caused by the surface roughness [15]. These studies suggested that the results of bone microbiology tests were affected by the moisture content and the changes of loading rate and peak load [9]. However, how the above variables affected the test results needs further study.

Our study demonstrates how the moisture content and loading methods affect the elastic modulus and hardness of femoral bones. The results indicate that higher moisture content decreases the elastic modulus and hardness of cortical bone and cancellous bone, which provides a relevant reference for numerical analysis of the further nanoindentation measurement.

Material and Methods

Specimen preparation

Bilateral femoral and lumbar specimens were taken out from the same corpse provided by the Department of Anatomy of the Fourth Military Medical University. Eight cortical bone specimens and 8 cancellous bone specimens with a thickness of 5 mm were processed by a saw machine (SP1600, Leica, Germany) (Figure 1A). The specimens were kept in a freezer at −20°C after being polished and washed in an SB25-12D ultrasonic cleaning machine (Ningbo Scientz Biotechnology Company, Ningbo, Zhejiang, China). The specimens were stored in a 50–70% humidified environment at 20°C. Moisture analysis was conducted using infrared trace moisture spectrometry, and the cortical bone specimens with different moisture contents (20%, 30%, 40%, 50%, and 60%) were prepared. The moisture content decreased by 10% in the first 10 min and then successively decreased by 5% every 10 min when the bones were dried at 50°C. The same method was used to analyze the moisture contents of lumbar cancellous bones. The cancellous bone specimens with different moisture contents (5%, 15%, 25%, 35%, and 40%) were prepared (Figure 1B).

Nanoindentation test of different moisture contents

All the bone specimens were tested using a TI-900 Hysitron TriboIndenter (Hysitron, MN, USA) with a Berkovich indenter. When cortical bones were tested with different moisture contents, the indent depth was 500 nm and the loading rate was 10 nm/s. When unloaded to 15% of the maximum load, the indenter was maintained for 1 min and then uploaded at the same rate. We chose 6–8 pressure points in the symmetric plane of the specimens. The distance between the pressure points was around 20 µm. To test cancellous bones with different moisture contents, the peak load was 1000 µN and the loading rate was 333 µN/s. When loaded to the maximum load of 1 mN, the indenter was maintained for 5 s, and then unloaded at the same rate. We chose 6–8 pressure points in the symmetric plane of the specimens. The distance between the pressure points was around 10 µm. Unrelated factors, such as temperature, humidity, shape of probe, surface roughness, and surrounding noises, were kept unchanged during the experiment.

Nanoindentation test of different loading methods

The loading mode was first set as the loading rate of 6 nm/s and the peak load of 300 nm. When the cortical bones with 20% and 60% moisture contents were tested, the peak load was 300, 400, and 600 nm, and the loading rate was set at 6, 8, and 10 nm/s. The indenter was maintained for 1 min, and then uploaded at the same rate. We chose 6–8 pressure points in the symmetric plane of the specimens. The distance between the pressure points was kept at 20 µm. Similarly, cancellous bones with 5% and 40% were tested at loading rates of 200 µN/s, 250 µN/s, and 333/s, and peak loads of 600 µN, 750 µN, and 1000 µN. When unloaded to 15% of the maximum load, the indenter was kept for 1 min, and then uploaded at the same rate. We chose 6–8 pressure points in the middle of the specimen's symmetric plane. The distance between the pressure points was kept at 10 µm. Unrelated factors, such as temperature,
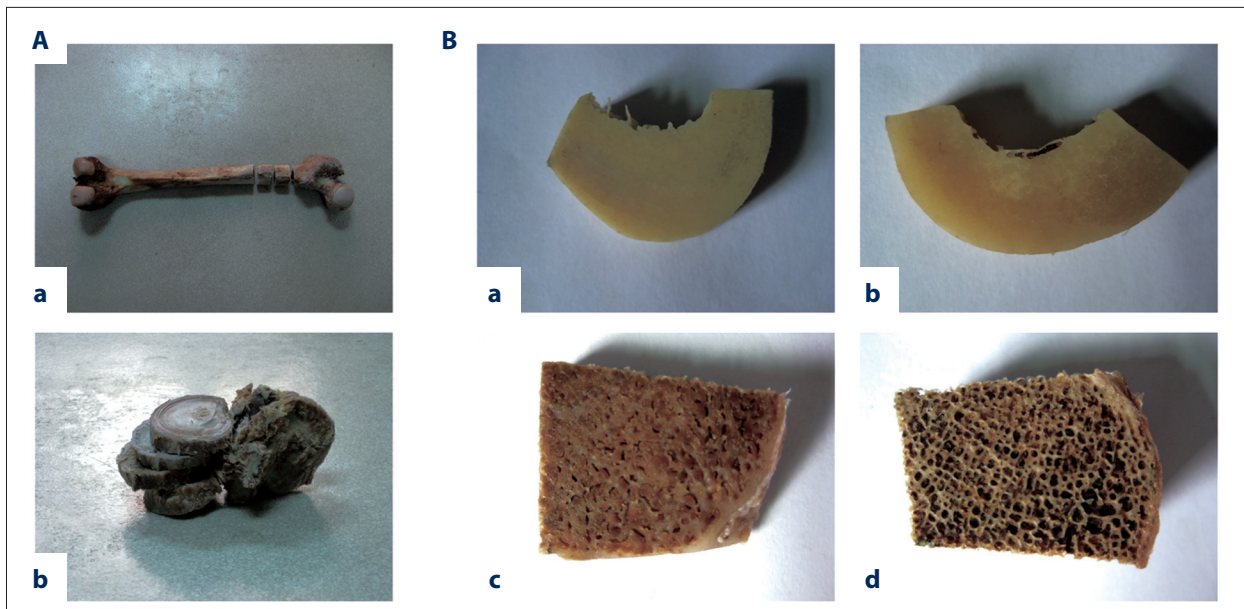
Figure 1. Preparation of the specimens required for experiments. (**A**) Representative cortical bone specimens (**a**) and cancellous bone specimens (**b**) with a thickness of 5 mm were photographed and are presented here. (**B**) The change in moisture content in cortical bone (**a, b**) and cancellous bone (**c, d**) after heating.

humidity, shape of probe, surface roughness, and surrounding noises, were kept unchanged during the experiment.

Elastic modulus and hardness measurement

Firstly, the contact stiffness was calculated by the equation: $S = \frac{dP}{dh}$ (P is the load, it can be calculated by the equation: $P = a(h - h_f)^m$, a and m are mechanical constants, h is the pressure depth, and h_f is the corresponding depth). Then, the effective elastic modulus was calculated by the equation: $E_r = \frac{1}{\beta} \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A}}$ (β is the geometry constants of the probe (Berkovich probe β=1.034); A is the effective indentation area, which can be calculated by $h_c = h_{max} - 0.75 P_{max}/S$). Finally, according to the relationship between local material and probe $\frac{1}{E_r} = \frac{(1-V_i)}{E_i} + \frac{(1-V^2)}{E}$, the elastic modulus could be calculated with the formula $E = \frac{(1-V^2)}{((1/E_r) - ((1-V_i)/E_i)}$ (V_i is the probe's Poisson's ratio, E_i is the elastic modules of the probe, V is the Poisson's ratio of the sample, the Poisson's ratio of the bone V=0.3. The hardness was calculated as $H_c = \frac{P_{max}}{A}$).

Statistical analysis

SPSS 21.0 (SPSS Inc., USA) was utilized to perform statistical analyses and all data are presented as mean ± standard deviation (SD). The Levene variance equality test was applied to test homogeneity of variance. One-way ANOVA was used for comparison between groups. The least-significant difference (LSD) method was used if variance was homogeneous, otherwise, the Welch method was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased moisture content decreased elastic modulus and hardness of cortical bone and cancellous bone

The ranges of elastic modulus and hardness of cortical bones were 5.47–21.27 GPa and 185–560 MPa, respectively. High moisture contents had inhibitory effects on elastic modulus and hardness of cortical bone (Figure 2A, 2B). The elastic modulus of cortical bone tissues with 40% moisture content were significantly different from those with other moisture contents ($P<0.05$), and no difference was found between cortical bone tissues with 20% and those with 30%, or those with 50% and 60%. Similarly, the hardness of cortical bone tissues with 30% and 40% moisture contents were significantly different from those with other moisture contents ($P<0.01$), and no difference was found between the tissues with 50% and 60% moisture in terms of hardness (Table 1). The ranges of elastic modulus and hardness of cancellous bones were 3.89–18.81 GPa and 0.16–0.58 GPa, respectively. Elastic modulus and hardness of cancellous bones also decreased as the moisture content increased (Figure 2C, 2D). The elastic modulus and hardness of cancellous bones with 5% and 15% moisture contents were significantly different from those with other moisture contents. There was no significant difference between those with 25%, 35%, and 40% moisture in terms of elastic modulus, whereas there was a significant difference between bones with 25%, 35%, and 40% in terms of bone hardness ($P<0.05$, Table 2). Hence, as moisture content increased, elastic modulus and hardness of cortical bone and cancellous bone decreased.
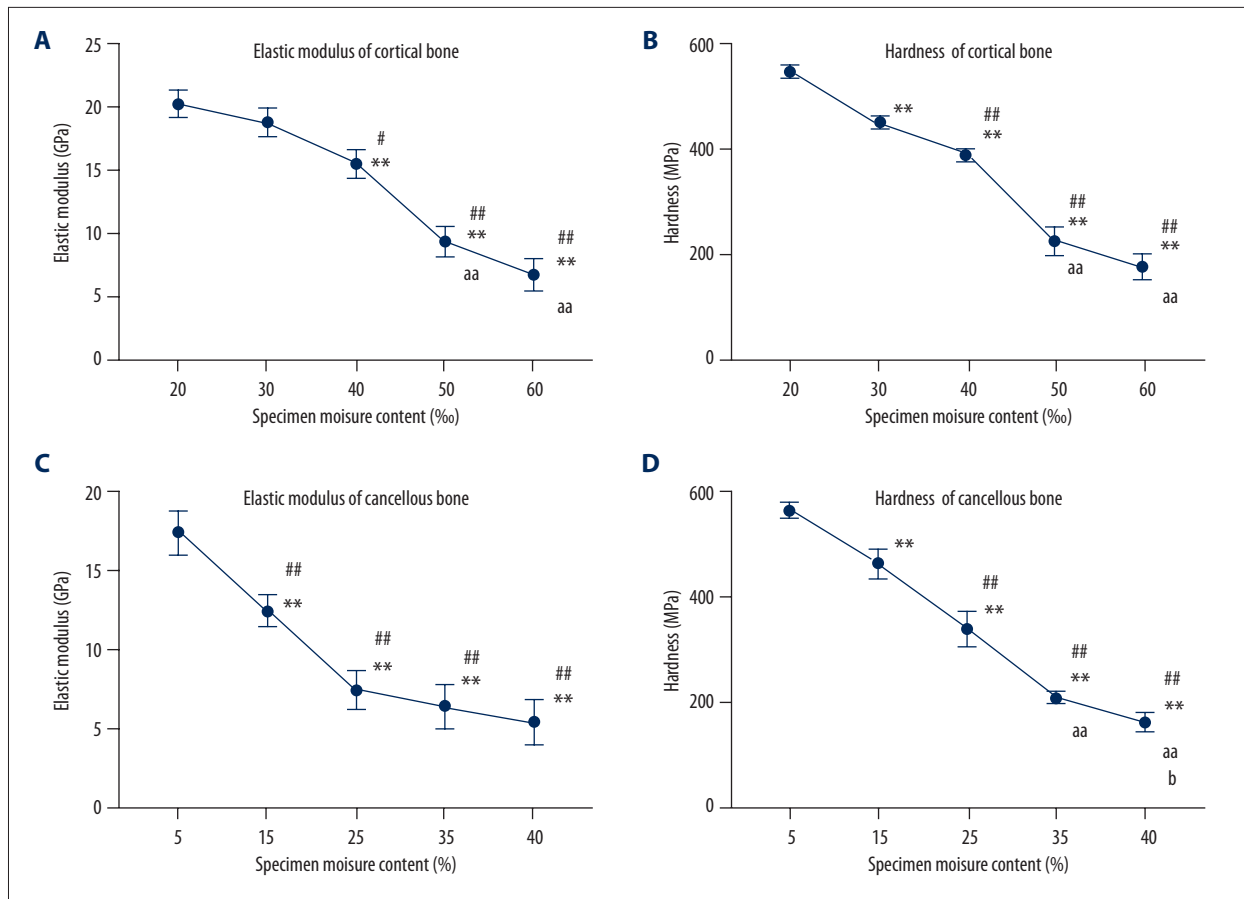
Figure 2. Increased moisture content decreased elastic modulus and hardness of cortical bone and cancellous bone. (**A, B**) Elastic modulus and hardness of cortical bone decreased with increased moisture content. ** $P<0.01$, compared with the group with 20% moisture; # $P<0.05$, ## $P<0.01$, compared with the group with 30% moisture; aa $P<0.05$, compared with the group with 40% moisture. (**C, D**) Elastic modulus and hardness of cancellous bone decreased with increased moisture content. ** $P<0.01$, compared with the group with 5% moisture; ## $P<0.01$, compared with the group with 15% moisture; aa $P<0.01$, compared with the group with 25% moisture; b $P<0.05$, compared with the group with 35% moisture.

Table 1. Elastics and hardness of cortical bones with different moisture percentages and same loading profiles.

Moisture (‰)	Peak load (nm)	Loading rate (nm/s)	Elastic modulus (GPa)	Hardness (MPa)
20	500	10	20.22±1.05	548.76±12.19
30	500	10	18.76±1.17	451.52±11.58**
40	500	10	15.52±1.18**,#	390.99±8.62**,##
50	500	10	9.36±1.22**,##,aa	225.25±27.87**,##,aa
60	500	10	6.74±1.27**,##,aa	176.30±24.47**,##,aa

** $P<0.01$, compared with the group with 20‰ moisture; # $P<0.05$, ## $P<0.01$, compared with the group with 30‰ moisture; aa $P<0.01$, compared with the group with 40‰ moisture.

Table 2. Elastics and hardness of cancellous bones with different moisture percentages and same loading profiles.

Moisture (%)	Peak load (µN)	Loading rate (µN/s)	Elastic modulus (GPa)	Hardness (MPa)
5	1000	333	17.35±1.46	565.31±15.16
15	1000	333	12.42±1.06**	463.54±28.43**
25	1000	333	7.44±1.23**,##	340.76±33.54**,##
35	1000	333	6.36±1.41**,##	211.18±11.25**,##,aa
40	1000	333	5.38±1.49**,##	164.43±18.32**,##,aa,b

** $P<0.01$, compared with the group with 5% moisture; ## $P<0.01$, compared with the group with 15% moisture; aa $P<0.01$, compared with the group with 25% moisture; b $P<0.05$, compared with the group with 35% moisture.

Table 3. Elastics and hardness of cortical bones with 20‰ moisture percentages under different loading modes.

Moisture (‰)	Peak load (nm)	Loading rate (nm/s)	Elastic modulus (GPa)	Hardness (MPa)
20	300	6	20.06±1.41	540.33±37.74
20	400	8	20.65±1.67	545.21±37.33
20	500	10	21.29±1.58	568.34±27.66

Table 4. Elastics and hardness of cortical bones with 60‰ moisture percentages under different loading modes.

Moisture (‰)	Peak load (nm)	Loading rate (nm/s)	Elastic modulus (GPa)	Hardness (MPa)
60	300	6	4.48±0.79	145.3±11.59
60	400	8	5.40±0.86	159.1±18.65
60	500	10	7.76±0.97*,#	188.7±14.09*

* $P<0.05$, compared with the group with 300 nm peak load; # $P<0.05$, compared with the group with 400 nm peak load.

Incremental loading methods increased the elastic modulus and hardness of cortical bones and cancellous bones with different moisture contents

The 3 different loading modes had little effect on the elastic modulus and hardness of cortical bones with 20% moisture content (Table 3). However, the same 3 different loading modes significantly affected the elastic modulus of cortical bones with moisture content of 60%. In terms of hardness of cortical bone tissues, difference was only seen between 300 nm peak load group and 500 nm peak load group. The load peak and load rates were positively correlated with elastic modulus and hardness values. (Table 4). Similarly, the 3 loading modes had little effect on the elastic modulus and hardness of cancellous bones with 5% moisture (Table 5). A statistically significant difference was seen in cancellous bones with 40% moisture between 1000 µN and the other 2 loading

modes in terms of both elastic modulus and hardness, whereas there was no significant difference between the 600 µN and 750 µN groups (Table 6). Therefore, when cortical bones and cancellous bones were high-moisture, the increase of peak load and loading rate significantly increased the elastic modulus and hardness.

Discussion

The micro-mechanical properties of cortical bone and cancellous bone were tested by nanoindentation experiments. The results demonstrated that the mechanical properties of both bone types were influenced by the moisture content and loading method. As the moisture content of a specimen increased, the bone's elastic modulus and hardness were reduced.

Table 5. Elastics and hardness of cancellous bones with 5‰ moisture percentages under different loading modes.

Moisture (‰)	Peak load (µN)	Loading rate (µN/s)	Elastic modulus (GPa)	Hardness (MPa)
5	600	200	15.56±1.04	491.21±33.91
5	750	250	15.98±1.59	502.74±26.12
5	1000	333	16.62±0.77	513.23±24.92

Table 6. Elastics and hardness of cancellous bones with 40% moisture percentages under different loading modes.

Moisture (‰)	Peak load (µN)	Loading rate (µN/s)	Elastic modulus (GPa)	Hardness (MPa)
40	600	200	3.24±0.66	103.42±17.47
40	750	250	4.01±0.59	114.01±10.42
40	1000	333	6.29±0.79**,#	168.43±13.23**,##

** $P<0.01$, compared with the group with 600 µN peak load; # $P<0.05$, ## $P<0.01$, compared with the group with 750 µN peak load.

Bone is a connective tissue mainly composed of mineral and type I collagen, which determines the hardness and strength of bones [16,17]. Micro-indentation and ultrasound techniques have been used to determine the hardness and strength of bones [18,19]. Nanoindentation, as well, has been applied in bone analyses. Nanoindentation can accurately estimate the mechanical properties and the tissue anisotropy [20–22]. For instance, Zysset et al. used nanoindentation to determine elastic modulus and hardness of cortical and trabecular bone lamellae, reporting that the nanostructure of bones differed significantly among lamellae and donors [23]. Indeed, the biomechanical properties of bones are determined by composition and micro-/nano-structures, which can be dependent on factors such as anatomical locations and donor conditions [24–27]. Otherwise, the loading method was also an influence factor of bone micro-biomechanical characteristics [22,28]. In the present study, we applied nanoindentation technique to determine the elastic modulus and hardness of cortical and cancellous bones. We found that the effects of different levels of moisture content, indentation rate, and peak load influenced the elastic modulus and hardness of cortical and cancellous bones. In a study by Lee et al., dry, moist, and fully hydrated bones were tested at 3 different peak loads (600, 800, and 1000 µN at loading/unloading rates of 60, 80, and 100 µN/s, respectively). Elastic modulus and hardness of dry bones were not found to be significantly different between the different loading profiles. However, in both the moist and fully hydrated bones, the elastic modulus and hardness were significantly different under almost all loading profiles [28]. They did not mention the moisture contents of most condition, but a trend can be inferred in which different loading conditions influence moist and saturated bones more easily than dry bones.

Similarly, it was shown that there were no significant differences in the mechanical properties between 3 different peak loads and loading rates on cortical bone with a moisture content of 20% and cancellous bone with a moisture content of 5%. In addition, Lee et al. also found that the hydration conditions of bones were negatively correlated with the elastic modulus and hardness [28]. This is consistent with our findings in the present study that the elastic modulus and hardness were both negatively associated with the moisture contents in cortical bones and cancellous bones.

We explored the dynamic process of cortical bone and cancellous bone mechanical properties, as well as the effects on bone strength, but a larger-sized study cohort is needed to make the conclusion convincing, since our study was based on samples from a single corpse. Other factors influencing the nanoindentation test could be included in further studies as well. For instance, indentation rate has been reported to be critical in nanoindentation testing [22], which could be taken into consideration when conducting nanoindentation testing. Lastly, trabecular bone tissue properties have been reported to be strongly correlated with degree of mineralization [21]. Thus, mineralization could also be further studied in terms of femoral bone mechanical property research.

Conclusions

Two conclusions can be drawn from our study. Firstly, the elastic modulus and the hardness of dry femoral cortical bone was neither sensitive to peak load, nor to loading rate with the present loading profiles, whereas that of more hydrated

bone tissues were significantly affected by both peak load and loading rate. Our results should contribute to understanding of factors that affect bone micro-biochemical properties, which could improve control of diseases such as osteoporosis.

Conflict of interests

None.

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