Characterization of intrinsic bone properties by nanoindentation: influence of dietary regime

Relevant for: Bone, nanoindentation

The strength of bones is determined on one side by its architecture (geometry, porosity, cortical thickness, et.) and on the other side by its intrinsic properties (mechanical properties of the bone tissue). While the effect of architecture can be determined by macroscopic testing (compression tests), the intrinsic properties have to be investigated locally on a microscopic scale. The nanoindentation technique is a suitable tool for this type of characterization because it can measure mechanical properties on the microscale level. This technique has already been used to detect differences between cortical and trabecular bone, time dependent behavior and gradient in mechanical properties in various areas on the bone.

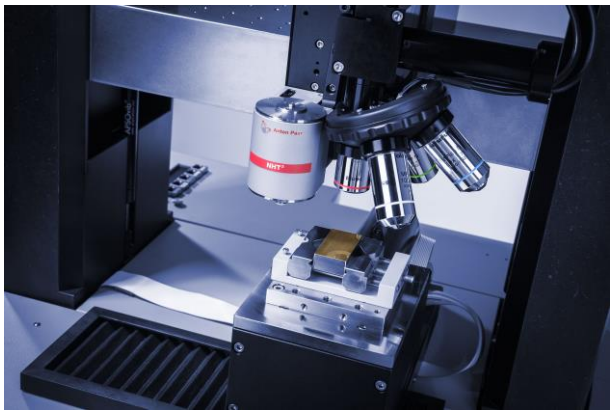

Figure 1 - The Anton Paar Nanoindentation Tester NHT^3 on the STeP 4 platform.

1 Introduction

Measurements of mechanical properties of bones present a major challenge in orthopedic medicine [1–3]. This is because the mechanical properties of bone are determined on one side by its architectural variables such as geometry, porosity and cortical thickness and on the other side by the intrinsic properties of the bone tissue. While macroscopic testing methods for characterization of bone as a bulk material (compression, bending testing, etc.) are established, the intrinsic properties of the bone have to be probed on a microscopic level. Furthermore, macroscopic analysis may not be sensitive enough to identify the differences in local properties between two similar samples. Hence, micro- and nanoscale studies are desirable for resolved characterization of these complex materials. In addition, nanoscale methodologies are useful when the volume of material available is too small for larger scale analyses (for example bone tissue formation in critical-sized defects and rat models).

The nanoindentation technique [4,5] is one of the suitable candidates for these measurements since it offers micrometric spatial resolution and has the capability of measuring hardness, elastic modulus and energy of indentation simultaneously. This is advantageous especially in cases when studying the effects of nutrition on intrinsic properties of the bone, which are not always reflected by change in the overall mechanical properties (that are measured by macroscopic tests). Nanoindentation on the other hand can easily detect differences between cortical and trabecular bone, bone anisotropy, time dependent behavior and gradient in mechanical properties. Also local variations of mechanical properties due to mineral content can easily be measured by the nanoindentation technique. The nanoindentation technique therefore presents an important tool when understanding the changes in the bone tissue and bone strength after a nutrition diet.

To demonstrate the utility of the nanoindentaiton technique, nanoindentation tests were done at the level of the vertebral cortex of adult rats after various dietary and hormonal manipulations. The nano-indentation results were also correlated with the results of compression tests of the whole vertebra.

The results of the present study indicate that besides geometry and microarchitecture, intrinsic bone tissue property is an important determinant of the mechanical competence of rat vertebrae after ovariectomy and low protein intake.

This application report is from a great part based on the publication by Hengsberger et al [6]. For more information and details concerning the additional experimental procedure on macroscopic scale refer there to.

2 Materials and methods

2.1 Nanoindentation

Nanoindentation characterizes the intrinsic (local) mechanical properties of bone tissue. This technique acquires force-displacement data of a pyramidal diamond indenter that is pressed into a material. Figure 2 shows the resulting curve that consists of three parts. During loading, the indenter tip is pressed into the sample that results in a combination of elastic and plastic deformation.

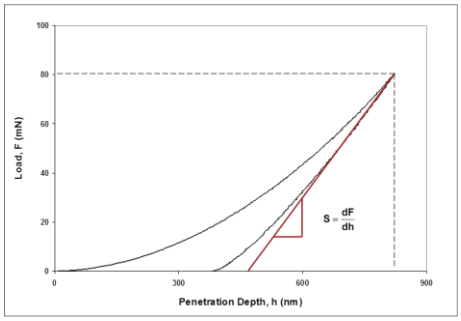

Figure 2 – An example of NHT[3] indentation force-displacement curve showing loading and unloading. The initial slope of the unloading part (S) is used to derive the hardness and elastic modulus of the indented material. The area between the loading and unloading curves corresponds to dissipated (plastic) indentation energy.

At maximum force, the load is usually held constant for a short while so that the creep of the material can be observed. After this hold period the indenter is progressively unloaded. The slope at the point of initial unloading (S in Figure 2) is used when deriving elastic properties and hardness of the sample. The reduced modulus E_r is calculated using:

$$E_r = \frac{S\sqrt{\pi}}{2\beta\sqrt{A_p(h_c)}}$$

Where h_c is the contact depth, A_c is the indenter projected area and β is a geometric factor. Knowing the elastic modulus of the diamond indenter, the elastic modulus E of the indented material (bone) can be calculated. The hardness of the material (bone) can be calculated as

$$H = \frac{F_{max}}{A_p(h_c)}$$

The dissipated (plastic) energy of indentation is calculated as the area of the hysteresis loop (see Figure 2) between the loading and unloading parts of the indentation curve.

2.2 Samples and nutrition diets

Female Sprague-Dawley rats were used in all experiments; all animals were strictly pair-fed isocaloric diet provided by Novartis Nutrition. 6-month-old female rats underwent transabdominal ovariectomy (OVX) or sham operation (SHAM group) under anesthesia. SHAM were maintained on the 15% casein containing diet, whereas OVX rats received a 2.5% casein (low protein, LP) isocaloric diet. Combining a low protein (LP) diet and ovariectomy was shown to cause a more pronounced decrease in bone strength than either manipulation alone. Ten weeks after OVX the rats were randomly allocated to two groups receiving either 2.5% casein (OVX/LP) or 2.5% casein and 5.0% essential amino acids (OVX/LP/EAA) for the next 16 weeks (Table 1). After 52 weeks, the bones were excised. The three groups provided samples with a large range of bone tissue microarchitecture and bone strength alterations. This allowed for investigation of the respective roles played by the determinants of bone strength and to assess the sensitivity of the method to detect the modifications of the intrinsic tissue quality.

Table 1 - Groups of animals tested.

Group	Treatment
SHAM	Sham operation, no diet
OVX/LP	Ovariectomy and low protein (LP) diet
OVX/LP/EAA	Ovariectomy and low protein diet (LP)+ essential amino acids (LP/EAA)

For the nanoindentation tests, the L5 vertebral body of each rat was dissected at the level of the intervertebral disks. The bone specimens were kept frozen until preparation for the mechanical tests. The vertebra was cut transversally in the middle of the approximately 8 mm high body. The samples were embedded in PMMA and the face of the transverse cut was polished with a 0.25 µm diamond solution. After these preparation steps, the specimens were dried for 24 h at 50°C. The mechanical tests included indents on the cortical shell of each vertebral body: three indents at the posterior, three indents at the lateral and three indents on the anterior site.

On each site, three indents were done on the periosteal, the central, and the endosteal location of the bone matrix (Figure 3). The indents were done with 900 nm maximum depth by applying strain rate of $d\varepsilon/dt = 0.066\ s^{-1}$ for both loading and unloading. A five second hold period was kept at maximum load.

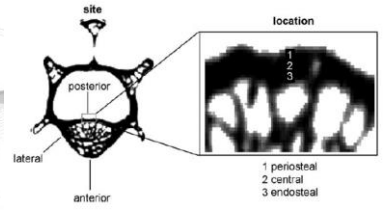

Figure 3 – Schematic representation of the indented areas. On transversal slices of lumbar vertebral body, three sites were chosen: anterior, posterior, and lateral sites (figure on the left). On each site, three locations were defined: the periosteal, central and endosteal locations (figure on the right).

In the present study, only cortical bone was tested, since major deterioration and destruction of the

trabecular structure was observed in OVX rats fed an LP diet.

3 Results and discussion

3.1 SHAM (control) group – effects of the site

The results of nanomechanical characteristics measured in different sites of the vertebral body cortex (anterior, posterior and lateral) on SHAM animals (no OVX) are shown in Figure 4-6. All three mechanical parameters (elastic modulus, hardness, and dissipated energy) were lower on the anterior site.

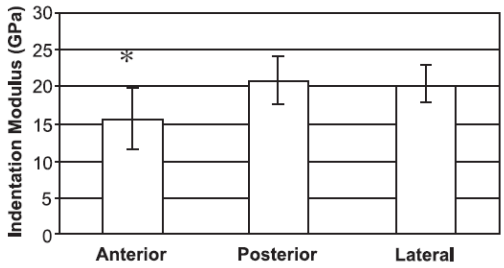

Figure 4 - Elastic modulus in different sites on the vertebra (SHAM).

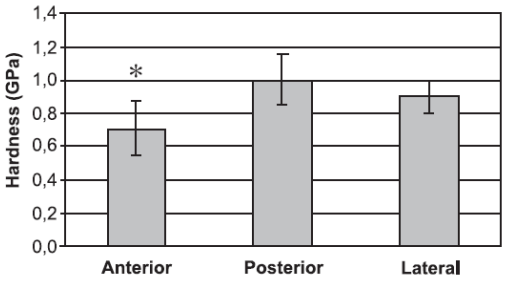

Figure 5 - Hardness in different sites on the vertebra (SHAM).

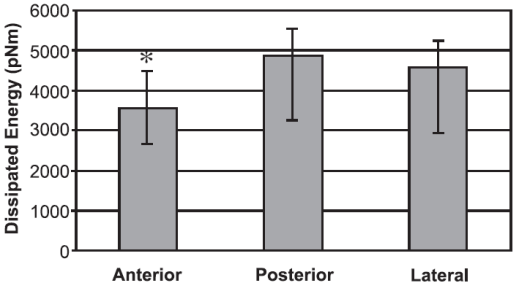

Figure 6 - Dissipated energy in different sites on the vertebra (SHAM). **Denotes significant difference at P=0.05 in all figures.*

3.2 Effect of protein intake on the mechanical properties of the vertebra

The effect of the location (periosteal, endosteal, central) was moderately significant (P = 0.029) and the treatment was not globally significant (P = 0.65). However, the interaction between treatment and site was close to the significance level (P = 0.06). The mechanical properties at the posterior site for the different locations are separately presented in Figure 7-9. Detailed comparison between the two treatment groups and all locations is given in Table 2.

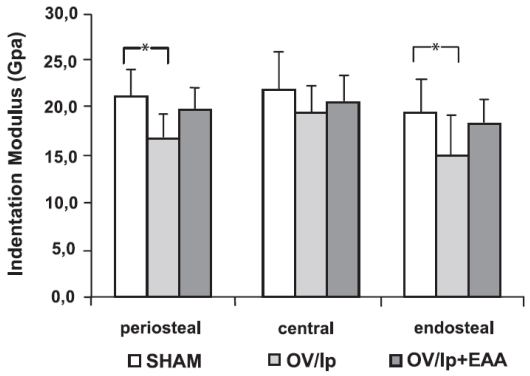

Figure 7 - Elastic modulus as a function of diet and location.

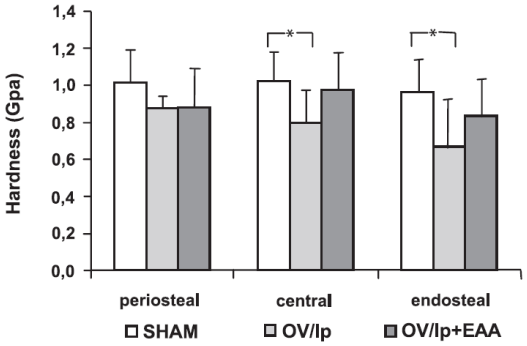

Figure 8 - Hardness as a function of diet and location.

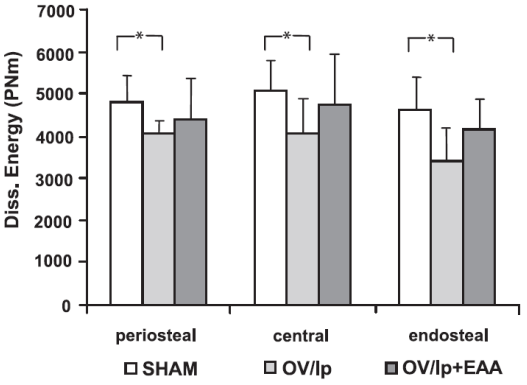

Figure 9 – Dissipated energy as a function of diet and location.

**Denotes significant difference at P=0.05 in all figures.*

A post hoc analysis showed significant decrease of mechanical properties (P < 0.05) at the endosteal location in OVX rats fed the low protein diet compared to the SHAM group. This difference was detectable for all three mechanical parameters. In the central part of the posterior vertex, hardness and dissipated energy were significantly reduced (P = 0.02 and P = 0.03, respectively) in response to ovariectomy and the low protein diet. In the periosteal location, significant alteration of elastic modulus and energy dissipation between SHAM and OVX rats with the low protein diet was also detected (P = 0.01 and P = 0.02,

respectively). The positive effect of essential amino acids (EAA) supplements on the indentation modulus and dissipated energy was not significant ($P < 0.1$) at the endosteal location. Similar effect of the EAA supplemets on the hardness was observed also in the central location ($P < 0.1$). Concerning the elastic modulus at the periosteal location, the effects of EAA supplements were almost significant ($P = 0.06$).

3.3 Correlation with macroscopic compression tests

Mechanical properties from nanoindentation experiments were obtained at the level of a bone volume 10^8 smaller than the whole vertebra. Comparison of indentation elastic modulus with stiffness obtained from compression tests showed poor correlation. However, hardness and ultimate strength as well as indentation dissipated energy and compression energy to failure were significantly correlated [6]. Especially the correlation between the macroscopic energy to failure and the indentation dissipated energy represents an important result of this study. Macroscopic energy to failure includes elastic as well as plastic deformation while the indentation dissipated energy only concerns plastic work. The potential strength of this relationship is the ability to predict how much of mechanical work can be absorbed by a vertebral body before failure occurs.

4 Conclusions

The present study showed the application of nanoindentation for measurement of intrinsic bone properties of the rat vertebral body. The local mechanical properties varied in respect to location and protein intake. For the SHAM group the hardness, elastic modulus and dissipated energy were found to be significantly lower at the anterior site while they were the highest at the posterior site. Pure low protein intake associated with ovariectomy (OVX/LP) lead to decrease in hardness, elastic modulus and dissipated energy in all locations (periosteal, central and endosteal). The OVX/LP group with EAA showed higher values of hardness and elastic modulus compared the OVX/LP group – but these values were still lower compared to the SHAM group. These differences in mechanical properties have not been observed after macroscopic tests and thus underline the advantages of the nanoindentation technique, which can investigate the local changes in mechanical properties of bone tissue. Morever, a correlation between macroscopic mechanical results (ultimate strength and energy) and nanomechanical tissue properties (hardness and dissipated energy) suggests that macroscopic post-elastic behavior is related to material fragility detected at the tissue level. The studies on bone tissue show the utility of this method which has already been applied to other mineralized biological materials such as dentine or tooth enamel.

5 References

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Table 2 - Comparison of mechanical properties of SHAM group, low protein (LP) and low protein with essential amino acids (LP/EAA) group.

		SHAM			OVX/LP			OVX/LP/EAA		
		Endosteal	Central	Periosteal	Endosteal	Central	Periosteal	Endosteal	Central	Periosteal
Posterior	Elastic modulus [GPa]	19.28 ± 1.46	21.81 ± 1.6	21.07 ± 1.13	14.82 ± 2.08*	19.3 ± 1.22	16.74 ± 1.14*	18.21 ± 1.01	20.52 ± 1.15	19.01 ± 0.99
	Hardness [GPa]	0.961 ± 0.07	1.021 ± 0.061	1.019 ± 0.07	0.676 ± 0.108	0.795 ± 0.079*	0.876 ± 0.029*	0.835 ± 0.082	0.967 ± 0.085	0.864 ± 0.079
	Dissipated energy [pJ]	4639 ± 306	5085 ± 291	4801 ± 257	3426 ± 338*	4058 ± 387*	4051 ± 132*	4128 ± 312	4784 ± 472	4306 ± 382
Lateral	Elastic modulus [GPa]	20.52 ± 1.15	19.9 ± 1.43	19.83 ± 1.01	19.42 ± 1.48	21.3 ± 2.38	19.38 ± 1.9	17.74 ± 2.32	19.68 ± 2.37	18.1 ± 2.26
	Hardness [GPa]	0.93 ± 0.034	0.916 ± 0.038	0.86 ± 0.048	0.876 ± 0.114	0.864 ± 0.077	0.813 ± 0.121	0.779 ± 0.141	0.927 ± 0.081	0.826 ± 0.089
	Dissipated energy [pJ]	4695 ± 267	4606 ± 286	4419 ± 253	3925 ± 545	4324 ± 287	3786 ± 576	4008 ± 582	4794 ± 332	4190 ± 284
Anterior	Elastic modulus [GPa]	16.57 ± 1.87	15.41 ± 2.19	15.06 ± 1.34	15 ± 1.88	16.06 ± 1.37	12.32 ± 2.2	16.86 ± 1.55	15.85 ± 2.22	13.8 ± 2.54
	Hardness [GPa]	0.771 ± 0.066	0.6 ± 0.073	0.742 ± 0.05	0.642 ± 0.144	0.615 ± 0.115	0.5 ± 0.11	0.802 ± 0.087	0.741 ± 0.098	0.626 ± 0.087
	Dissipated energy [pJ]	3819 ± 361	3213 ± 547	3558 ± 162	3033 ± 615	2921 ± 478	2494 ± 445	4143 ± 348	3900 ± 470	3298 ± 433