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Surface characterization of the raw and cooked bovine cortical metatarsal bone

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Purpose: The purpose of this study was to quantify the elastic properties and evaluate microscopical features of raw and boiled metatarsal bovine bone. *Methods*: The elastic modulus, hardness and microscopic surface of raw and cooked bovine metatarsal bone have been investigated using nanoindentation, SEM/EDX and Panasis microscope. *Results*: Regarding raw bovine bone, the average elastic modulus was 30.515 ± 6,769 GPa, while the average hardness was 0.5683 ± 0.211 GPa. When it comes to boiled bone corresponding values were 22.298 ± 7.0303 GPa and 0.408 ± 0.199 GPa, respectively. The values for investigated parameters were significantly higher ($p < 0.05$) in raw bone specimens. Elastic modulus significantly correlated with hardness ($p < 0.05$). EDX analysis revealed significant decrease in wt% of oxygen in boiled samples ($p < 0.05$) No significant differences could be observed in SEM images particularly when analysing in smaller magnifications. Using higher magnification, additional branching of the existing voids as well as discrete reorganization and smoother edges of nutrient canals could be observed. The surface of boiled specimens was without the presence of crusts and layering, and no microscopical evidence of structural damage could be observed. *Conclusions*: This study provides detailed analysis of hardness, elastic modulus of raw and cooked bovine bone and their relation and changes during exposure to temperature. These results of elastic moduli and hardness could be comparable to similar studies of bovine and human bone tissue, but the careful analysis of experimental design, type of the bone as well as limitations of the employed techniques must be carried out before interpolation of the results to other theoretical, clinical, biomaterial and archeological studies.

Key words: *bone, elastic properties, direct temperature exposure, SEM, nanoindentation*

1. Introduction

Bone research is nowadays focused on progress and novel understanding of all aspects of bone science and bone morphology, histology, pathophysiology and tissue regeneration as well as other significant findings related to bone are widely investigated in different scientific disciplines. Superior mechanical and ultrastructural physical properties of bone are substantially influenced by the complex hierarchical arrangement of the constituting components and their multiple interactions [19], [20]. Adult compact bone consists mainly of osteons, which are column-like structural and functional units with altering directions, but commonly parallel with the longitudinal axis of long bones [11]. Cortical bone is more compact and creates the external portion, whereas trabecular bone is located in the parts of the body that require absorption of substantial amounts of energy [13].

The majority of contemporary studies investigating bone are using the model of adult human bone and the bone is the topic of investigation in tremendous amounts of various disciplines. The adult human bone

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is made of approximately 80% cortical bone and about 20% trabecular bone [10].

Bovine bone is characterized by specific laminar structure with discontinuous stratification of fibered bone in parallel direction and lamellae. Nutritive system is organized in the form of two-dimensional network consisting of blood vessels and surrounding vascular spaces that lie between lamellae. The characteristics of bovine osteons is that they are without any cement lines. Haversian bone is positioned in the proximity to the endosteal surface, and Haversian canals are of medium size and variable in shape [23]. The metatarsal bones present a group of five bones, classified as long bones of the foot, located between the tarsal bones from one side and the phalanges of the toes at the other side. In bovine anatomy, similarly to other mammals the metatarsals, like the metacarpals, are numbered from 1 (MT 1) to 5 (MT 5), according to the five rays of the foot. In addition, each of the tarsals in the distal row articulate with at least one metatarsal base [6].

After the Lower and Middle Palaeolithic Ages, bovine bone was being physically processed and used as tools all over the world, as discussed by Olson [19]. The bone artefact analysis has persisted as the topic of major interest for a small group of scientists, and employment of standardized and strict methods has been developing rather slowly. On the other hand, the amount, distribution and significance of prehistoric bone tools require implementation of contemporary methods with the aim of clarifying and modernization of protocols and techniques in order to achieve better objectivity [19]. For the purposes of the adequate interpretation, specialists involved in bone tools analysis have to adopt ideas, methodological approaches and tools and devices from other disciplines, such as biomaterial science, mechanical science and engineering. Understanding the manufacture procedure, usage pattern, various roles of bone artefacts could give important information significant to a various range of topics related to different aspects of prehistoric societies.

The importance of bone tools has been emphasized by Erdalakiran [7] and it has been clearly underscored that bone tools are among the most significant groups of tools for prehistoric societies, as they were usually prepared from the bones of previously consumed animals, they needed no or very little labour force, and were made from fresh resources which were fairly easily accessible [7], [15].

The effects and influences of temperature on the bone have not been fully clarified, and research has been carried out over last several decades to describe and explain the changes in morphology, structure and mechanical properties of the bones caused by thermal agents. In addition, it has been also clearly emphasized that the type of external agent can significantly affect the mechanical properties of the bone, and that lack of precise description of experimental and specimen preparation can result in huge differences in reported mechanical properties values, up to several hundred percent [14], [17]. The majority of the studies have been performed in the field of forensic medicine addressing questions related to sudden or deliberate burnings [3], and in anthropology when describing cremation processes and mortuary practice [12]. The impact of low temperature (less than 100 °C), linked to cooking, on bones has been investigated in the study conducted by Solari et al. [24]. It has been argued that, although there are macroscopic modifications as a consequence of cooking, these modifications have not been clearly or systematically described. There are attempts to correlate macroscopic features, such as smoothness or light transparency with physicochemical characterization results that could aid in the direction of detecting cooked bones in the archaeological research. But, in-depth analysis of the differences in mechanical properties between raw and cooked bones and their relation to macroscopic features requires additional clarification [24]. It has also been observed that not all cooking methods affect bone in the same way, and due to the lack of substantial work on the topic, researchers currently are not able to reliably differentiate between uncooked bones and cooked ones, much less interpret cooking methods.

To the best of authors' knowledge, there are no studies reporting details about elastic moduli and hardness of raw and cooked bovine cortical metatarsal bone.

2. Material and methods

All the analyses were performed on fresh and deflashed bovine metatarsal bone gotten from a neighbouring butchery. The cortex of the bone in the area between the metaphysis of metatarsus and diaphysis was sliced down up to 3.5 mm long samples with a diamond-impregnated low velocity saw (1000 r/min) (Isomet Low-Speed Saw, Buehler; Lake Bluff, IL, USA) using continuous irrigation with distilled water in order to avoid or reduce formation of the mineral phase at the surface of the specimen as a consequence of temperature increase. A total of 4 samples were stored in distilled water at +4 °C in one bottle prior to the experiment, for a period no longer than 2 weeks.

All analyses have been initially performed in all four raw samples using nanoindenter, SEM/EDX and Panasis microscope without any additional preparation. After that, the same experimental procedures and test has been conducted on same bone samples prepared as follows: the samples were boiled in glass pots at 100 °C (temperature of boiling water) in contact with water for 1 h. The covered pots were allowed to cool to room temperature for 24 h. The bone samples were then air-dried for 24 hours and analysed.

All the nanoindentation analyses were performed using a Nano Indenter G200 (Agilent Technologies®), under dry conditions, using a Berkovich tip (Micro Star Technologies®). The apparatus is enclosed in an insulated cabinet to provide thermal stability and sound noise reduction. Microscope to Indenter calibration is preformed, meaning that the desired location under the microscope was the location of performed measurement with accuracy of 1.5 µm. For each bone sample, same indentations protocols were used.

Examination of samples was performed at room temperature and Poisson's ratio for all samples was set to be 0.3. The number of indentations and the indentation zones are described below. A total of 200 indentations (2 stages: raw and cooked, 4 samples per stage and 25 tests per sample). Concretely, the experimental protocol is composed of three steps. The first step was the loading stage at constant loading time of 15 s and maximum load of 10 mN. This phase was executed after proving that the thermal drift is lower to a value of 0.2 nm/s. The second step involved upholding a hold load plateau following loading. The holding period (1 s) has been selected long enough since there is no substantial so-called "nose" phenomenon of the unloading phase and at the same time short enough in order to minimize the influence of the thermal drift. The third step was the unloading stage until 90 % of the maximum load at constant unloading time of 15 s. Percent to unload was set at 90% because it allowed for a compromise between the creation of a assessable viscoelastic return and to keep a quasi-constant connection zone while unloading. Literature data suggest that unloading ratio beyond 25% causes incorrect fitting of the experimental curves. Any indentations close to the mounting resin and cracks were removed from the data set to minimize the effects of embedding on the measurements.

The surface morphology of both raw and boiled bone specimens was examined at various magnifications, from ×50 up to ×20000. Before testing, the samples were rinsed using deionized water. SEM/EDX examination was performed with table top scanning electron microscope (TM3030, Hitachi, Tokyo, Japan) using samples without coating. The surface analysis of each sample was conducted in three randomly chosen spots. The COMPO mode was consistently used in all samples and in all magnifications. EDX analysis has been carried out in 10 randomly chosen spots and concentrations of the following ions had been observed in raw and cooked specimens: O, C, P, Ca and Mg and expressed in wt % as a mass fraction of the material.

The profiles of the bone samples (i.e., intensity of cut marks, surface smoothness) were analysed by non-contact measurement using profilometer Huvitz HRM300 using magnifications of ×50, ×100, ×200 and ×500. 2D profiles of both raw and boiled specimens are shown in Fig. 1. Dark field filter was used for both raw and boiled specimens and in all magnifications.

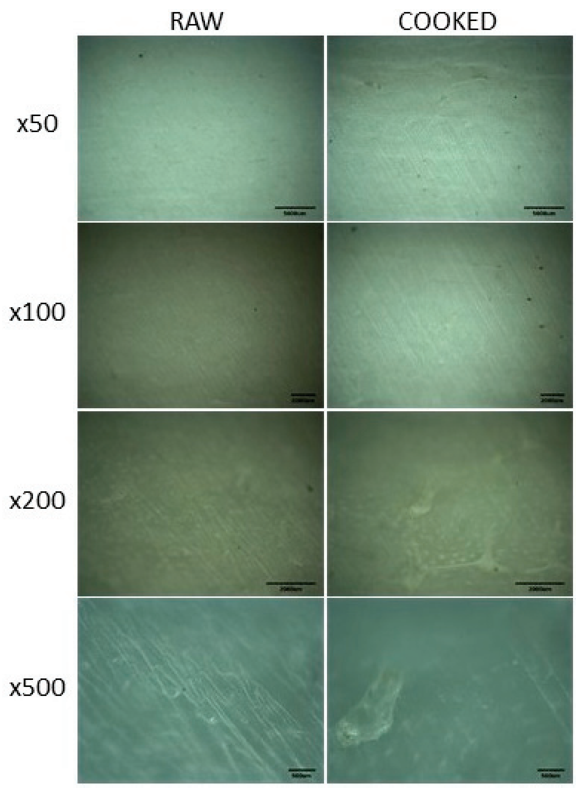

Fig. 1. Profiler graphs of raw and cooked bone surfaces at different magnifications

Statistical analysis was carried by calculating a table of means and standard deviations of both elastic moduli and hardness. All statistical analyses were conducted employing the Student's *t*-test and correlation analysis with the level of significance for intergroup differences set at $p < 0.05$.

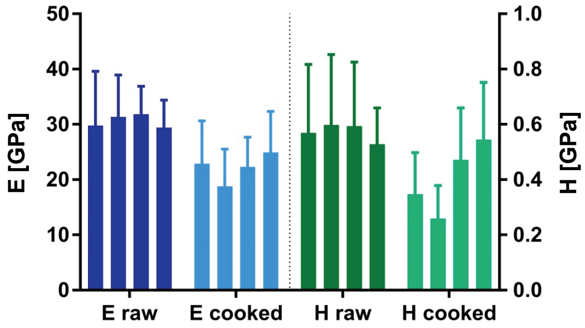

Fig. 2. Bar graph of Young's modulus and Hardness

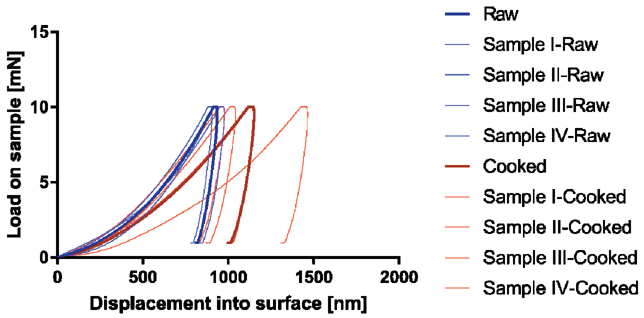

Fig. 3. Average Load vs. Displacement curves for raw and cooked bone

3. Results

Results for Young's modulus (E) and surface hardness (H) determination for all tested specimens are shown in Fig. 2. Force-displacement curves for raw and boiled bone out of which the elastic moduli and hardness of were calculated are plotted in Fig. 3, suggesting a different response of raw and boiled bone.

Regarding raw bovine bone, the average elastic modulus was 30.515 ± 6.769 GPa, (with the range between 29.451 ± 4.945 GPa and 31.810 ± 5.077 GPa) while the average hardness was 0.5683 ± 0.211 GPa (with the range between 0.528±0.131 GPa and 0.598 ± 0.255 GPa). When it comes to boiled bone specimens, corresponding values were 22.298 ± 7.0303 GPa (with the range between 18.830 ± 6.707 GPa and 24.930 ± 7.419 GPa) for elastic modulus and 0.408 ± 0.199 GPa (with the range between 0.258 ± 0.119 GPa and 0.544 ± 0.207 GPa) for hardness, respectively. The values for investigated parameters were significantly higher ($p < 0.05$) in raw bone specimens, compared to boiled

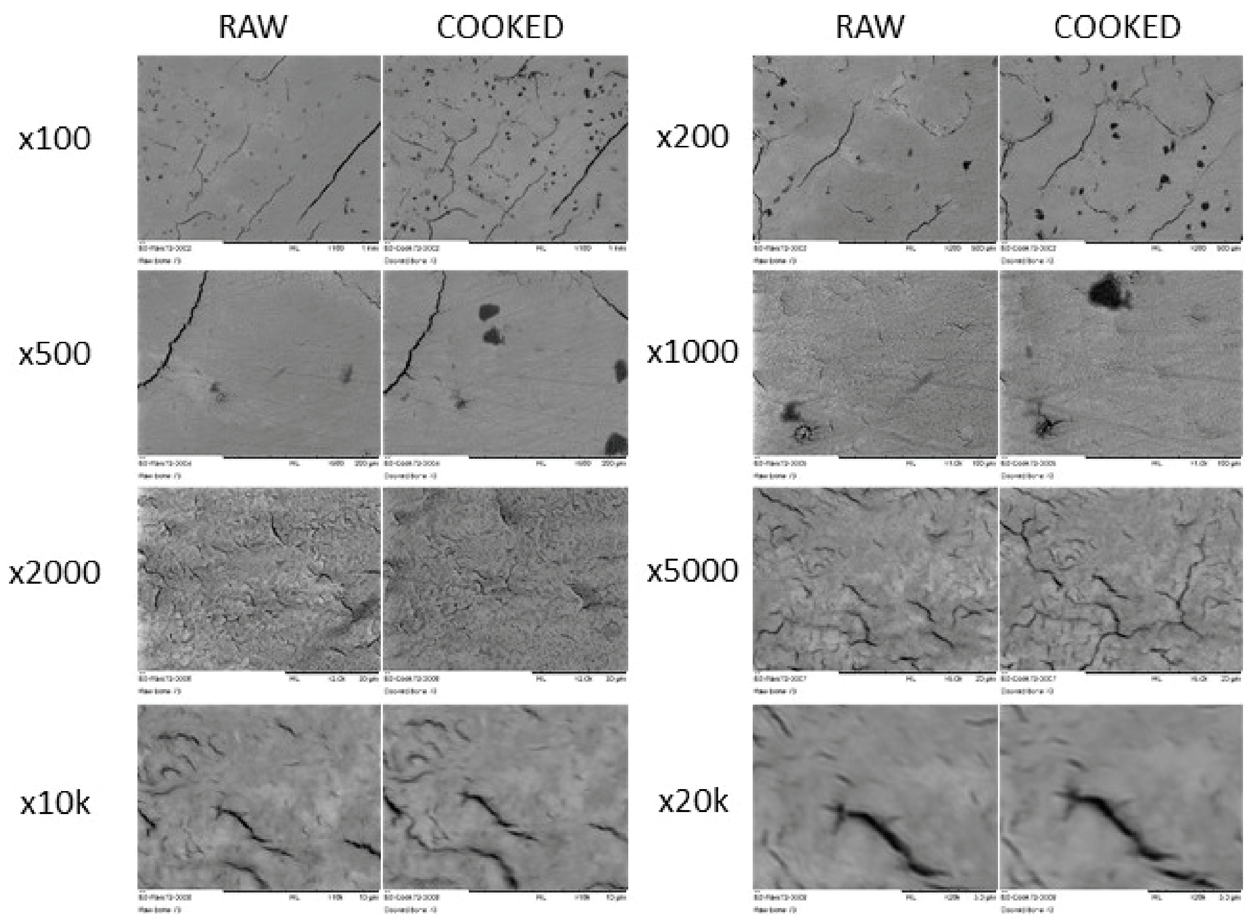

Fig. 4. SEM micrographs

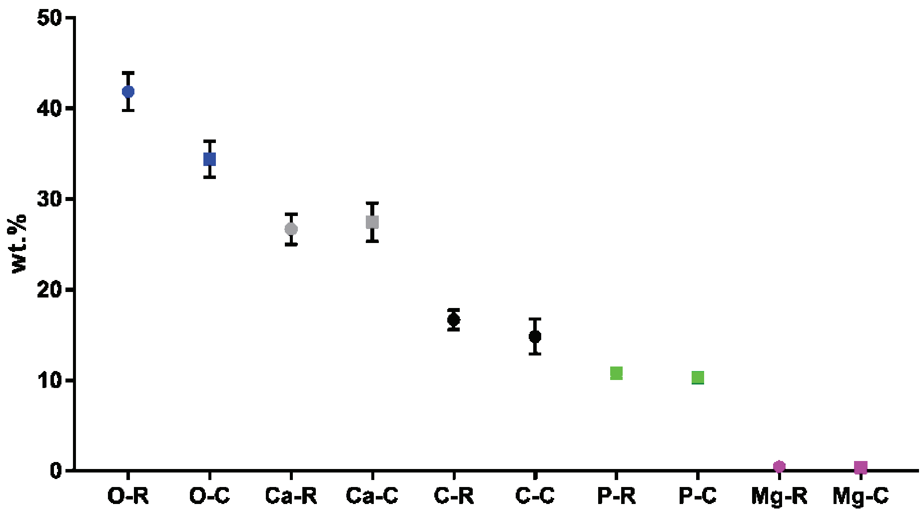

Fig. 5. SEM EDS graph for elemental analysis

samples. For Young's modulus Tukey HSD post-hoc test revealed the p value less than 0.0001 with 95% CI = 265.8060 to 468.1940, while at the same time the p value for hardness comparison was 0.0066 with 95% CI = –500.0451 to –81.9549. When analysing the differences between average values for both hardness and elastic modulus within 4 samples of raw bone, post-hoc t test revealed no statistically significant differences between 4 samples ($p < 0.05$). Similarly, there were no statistically significant differences when intragroup analysis had been carried out with respect to hardness and elastic modulus of average values for 4 samples of boiled bone ($p < 0.05$) (Fig. 2).

Elastic modulus significantly correlated with hardness ($p < 0.05$) in both boiled and raw samples, but the Pearson coefficient of correlation was higher in raw bone samples ($r = 0.937$) in comparison to boiled bone ($r = 0.839$).

No significant differences could be observed at the surfaces of boiled and raw specimens in SEM images, particularly when analysing in smaller magnifications (Fig. 4). Using higher magnification, additional branching of the existing voids could be observed as well as discrete reorganization and smoother edges of nutrient canals. Some of the pore openings appeared to be obturated. The surface of boiled specimens was without the presence of crusts and layering, and no microscopical evidence of structural damage could be observed.

EDX analysis revealed statistical significant decrease in oxygen concentrations in boiled specimens ($p < 0.05$), while, at the same time, there were no statistically significant differences regarding remaining four investigating ions (Fig. 5).

Profile analysis revealed no significant colour alterations in boiled bone specimens. In higher magnifications the form of the surface of the boiled bone was more transparent and the whole structure appeared to be smoother with less distinct and noticeable cut marks.

4. Discussion

In the last four decades the nanoindentation was used to test biological specimens such as bone and teeth [28]. Regarding the macro characterization of the bone, the most frequently reported mechanical properties are the elastic and shear modulus as well as Poisson's ratio. At the micro scale, the evaluations of the microstructural mechanical heterogeneity and their relations with their physico-chemical properties are usually performed at nano scale [10], [29].

The most frequently used method to analyse the nanoindentation data is the Oliver and Pharr mechanical method [18]. It is adapted to isotropic elastoplastic materials and enables computation of a modulus of elasticity and the hardness of bone material with the assumption that it is a quasi-isotropic non-viscous material [8]. It is evident that the majority of the contemporary zooarcheological, anthropological and mechanical studies of bone share many characteristics related to issues they are attempting to resolve, and also share the methodological approach in order to find a "balance between the biological aspects of bone tools and purely culturally determined aspects including manufacturing techniques, function and style [4]". It has been emphasized that from an archaeological and anthropological point of view it is very important to discern how cooking processes changed bone structure, at both macroscopical and microscopical levels of observation [24].

In the comprehensive study regarding the factors affecting mechanical properties of bone tissue conducted by Nikodem and Scigala, it has been clearly pointed out that because of substantial differences in reported values of the majority of the bone mechanical properties there is a need to classify individual experimental and measurement setup together with observations in order to determine research standards, extract the variable of interest and that in that way the obtained data can be interpreted and compared [16].

In order to overcome this obstacles and possible errors in data interpretation and clarify the effect of just one component in the whole system (boiling for one hour), we performed the analysis on the same specimens before and after the experimental procedure.

It should be taken into consideration that physical properties significantly vary according to the type of the bone and to the anatomical source of the specimen. In addition, all physical and mechanical properties could not be completely compared between individuals and various samples derived even from the same organs. Moreover, data obtained from human bones investigations cannot be completely extrapolated to animal bones since there are differences between bovine and human bones due to the fact that they reach their maturity level at different ages. It has been stated by Currey et al. [5] that humans reach full growth by the age of 16 years, while bovine bones are completely grown in 2 years, implicating that the pace of bone development is much more intense for bovine bones, in comparison with humans, and this factor is exceptionally important when analysing and interpreting the structure, physical and mechanical properties of bone.

Nanoindentation evaluation of bones needs a careful choice of the load and of the type of the physical dimension and protocol of indentation as well as a lot of other experimental parameters, including the unloading percentage, number of data points, predefined indentation depth or maximum load and its setup: constant depth, load and strain rates. It has been clearly emphasized by many research groups that specimen preparation and sample storage can significantly affect the mechanical response of bone [8], and there are reports that suggest that mechanical response of bone varies along with the degree of the hydration state of the sample, and values of the elastic modulus and hardness could increase between changing from wet to dry environments from 10% and 17%, [28], [21], and, for example, the storage in open air significantly affects the increase in Young's modulus. Regarding the anisotropy ratio for the elastic properties of the long bone cortical zone, the reported values are between 1.61 and 1.79 [9], [27]. Nevertheless, cortical bone is considered as approximately linear elastic, transversely isotropic, and relatively homogenous [22].

One scientific field which nowadays reveals its great potential possibility is the examining of biomechanical characteristics of materials such as bone, antler, ivory, and horns, with the aim to identify prehistoric preferences for specific types of material for certain assignments, as discussed by Olsen [19].

Elastic modulus has been evaluated using various testing methods during the last several decades, and it has been reported [28] that the mechanical characteristics of bone fluctuate when observed at different organisational levels. It has been reported that the elastic modulus of cortical bone evaluated using nanoindentation method at microstructure rank was in the range of 22–25 GPa [28].

The hardness is defined as the ability of a material to resist a constant indentation, and the tests evaluating the hardness of various materials are extensively employed in order to quantify physico-mechanical characteristics of materials.

Microscopic analyses have been employed in order to describe morphological changes at the bone structure. In the present investigation, no significant differences could be observed at the surfaces of boiled and raw specimens in SEM images, particularly when analysing in smaller magnifications. Using higher magnification, additional branching of the existing voids as well as discrete reorganization and smoother edges of nutrient canals could be observed. Some of the pore openings appeared to be obturated. In the study conducted by Ostrowska et al. [21], after 6 hours of boiling deposits of calcium carbonate were clearly visible at the specimen surfaces, a finding that was not confirmed in the present investigation. This can be attributed to shorter boiling time in our experiment. COMPO mode used during SEM investigations offers tremendous possibilities for precise material composition maps. Using lower magnifications with COMPO mode some of the fine surface topographical features, such as cut marks, surface irregularities and roughness could not be detected. For these reasons, the additional microscopic technique has been used, namely, Huwitz profile-meter that offered the possibilities for in depth analysis of the material surface using lower magnifications giving additional information about colour, colour changes, smoothness and roughness of the specimen. The surface of boiled specimens was without the presence of crusts and layering, and no microscopical evidence of structural damage could be observed.

As discussed by Novitskaya et al. [17] and Nikodem et al. [16], taking the multilevel organization of the bone tissue into consideration, it is not unexpected that values reported for elastic modulus in the literature significantly range from 5 GPa up to 34 GPa, since the modulus of elasticity is strongly related to the origin (human or animal), inorganic content, age, the level of hydration, amount of porosity, and anatomical location. Additionally, a comprehensive review of bone mechanical properties determined by nanoindentation tests was published [26], where the results from the nanoindentation experimentations of various types of human and animal bones were explained. It has been emphasized that despite the fact that nanoindentation is an effective technique for the evaluation of elastic modulus and hardness of the bone, it is at the same time extremely sensitive to specimen preparation procedure and testing environments. Corresponding to findings from the above-mentioned report, elastic modulus of human long bone (middle portion of femur) was in the range of 17–27 GPa, differing in the level of hydration, as well in the maximal forces that were used, indenter tip design together with the actual anatomical direction that was analysed. Because of that, it is of outmost importance to state the precise testing details regarding experimental protocols that was used to prepare bone samples for nanoindentation analysis which is in line with our investigation, where all these data were reported and described.

In the present study the obtained data for raw cortical metatarsal bone indicated that the average elastic modulus was 30.515 GPa, while the average hardness was 0.5683 GPa. Our results completely correspond to the results of the published studies investigating bovine bone mechanical properties. When it comes to elastic modulus of the bovine bone cortex, the reported values were in the range of 19.3–24.7 GPa (observed in the osteon area) and 27.5–30.1 GPa (recorded for the lamellae). At the same time, hardness was in the range of 0.68–0.81 GPa (osteon) and 0.82–0.89 GPa (lamellae). The hardness also varied among the microstructure components in the range of 0.41–0.89 GPa [28]. It has been reported for bovine femoral bone that the elastic properties vary between 8.7 to 30.3 Gpa, depending on the direction of the nanoindentation testing [10]. In-depth analysis of some mechanical properties of bovine bone has been published by Wang et al. [28]. Namely, the elastic modulus and hardness of several microstructure components of dry bovine vertebrae and tibia have been investigated in the longitude and transverse directions using nanoindentation. The elastic modulus for the osteons and the interstitial lamellae in the longitude direction were found to be around 24.7 and 30.1 GPa. In this report it has been pointed out that it is difficult to distinguish osteons from lamellae in some directions. The values for hardness correspondingly fluctuated between 0.41 and 0.89 GPa. Due to substantial differences between investigated parameters, in the present investigation, we used the same samples for data analysis, and first, the raw samples were analysed and then boiled for one hour, and experimental protocol was completely identical in order to rule out the effect of as many variables as possible. The present results regarding Young's modulus partially correspond with the results published by Ostrowska et al. [21], where decrease in Young's modulus of bovine femur was around 19% after boiling for 6 hours. It is evident that with shorter boiling time, in the present investigation a more intensive decrease in Young's modulus was observed [14]. This discrepancy could be attributed to different specimen preparation in terms that in the above-mentioned study transversal sections were analysed, while in the present investigation longitudinally sections of compact bone were analysed.

It is well known fact that bone can be softened substantially by boiling, but the exact effect of temperature on bone properties is still not completely clarified, and some important features regarding bone exposure to high temperatures require additional elucidation. It has been clearly demonstrated in the present investigation that one hour of boiling significantly affects both elastic modulus and hardness of the raw bovine bone, but it is also evident that this effect is not linear (decrease in elastic modulus was more distinct compared to decrease in hardness). On the other hand, circumstances regarding heat exposure can differ substantially (for example, boiling on low temperature for longer period of time vs. dry roasting) and will have a different influence on bone properties. Moreover, it has been reported [2] that bone cooked while still protected from the heat by the muscles and meaty tissue around it acts differently from bone cooked without this so called "cushioning" effect. In similar studies the attempts have been made to interpret the effect of cooking using data about breakage, and compare it to findings from different processes, such as charring and element frequencies [1], [25]. Several possibilities could be opened from these interpretations. If the difference in fracture pattern and bone morphology, as well as physical properties between cooked and raw bones is easily identifiable and actually verified, numerous applications to archaeological studies will become possible: for example, it could be investigated when early fire started to be used for cooking and, in general, more information could be obtained on details of food processing activities. All these factors are of particular interest for researchers trying to understand the practice of bone tool manufacture in prehistory, feeding pattern and feeding preferences, together with substantial implications in the field of mortuary practice and cremated bone.

5.Conclusions

This study provides a detailed analysis of hardness, elastic modulus of raw and cooked bovine bone and their relation and changes during exposure to temperature, using nanoindentation and microscopic techniques. Physical and mechanical properties quantified by nanoindentation technique could provide valuable information in the development of interdisciplinary approach in bone structure analysis. Regarding raw bovine bone, the average elastic modulus was 30.515 GPa, while the average hardness was 0.5683 GPa, and in boiled bone corresponding values were 22.298 GPa and 0.408 GPa, respectively. These results of elastic properties and hardness could be comparable to analogous studies of bovine and human bones, but the careful analysis of experimental design, type of the bone, limitation of the employed techniques must be carried out before interpolation of the results to other theoretical, clinical, biomaterial and archaeological studies.

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