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EFFECT OF HEATING PROCESS ON MICRO STRUCTURE LEVEL OF CORTICAL BONE PREPARED FOR COMPOSITIONAL ANALYSIS

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At the structural level, the bones are composed of inorganic and organic compounds and water, actually being considered a composite biomaterial, each component contributing to the bone remarkable mechanical properties. The investigation presented in this paper aims to highlight the morphological and compositional changes that occur in human bone structure during the preparation of bulk samples for subsequent analysis by X-ray fluorescence spectrometry and atomic absorption methods. We applied a method for hard tissue (cortical bone) processing by thermal treatment, in order to obtain homogenous particles (concerning both their shape and size) having elemental compositions as close as possible to the "real" physiological values. This homogeneous material will be used for *in vitro* XRF analyses, in order to avoid the artefacts induced by the *in situ* matrix effect. Using scanning electron microscopy techniques we characterized the morphological changes occurring during samples processing. By heat treatment, below 300°C, the Ca/P stoichiometric ratio was maintained between 1.65 and 1.69 for all investigated samples. Above this temperature, the Ca/P ratio decreased below its physiological value.

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1. Introduction

At the structural level, the bones are composed of organic (e.g., collagen) and inorganic (e.g., hydroxyapatite) compounds and water, being actually a composite material, each component contributing to the remarkable properties of bone. The organic part consists mainly in a network of collagen and proteins, while the inorganic component is mainly hydroxyapatite (HA), and a small percentage of other elements incorporated into the structure, such as magnesium and sodium carbonate [1]. Compact bone apatite crystals entering in the organic matrix give stiffness to the bone. The HA crystals are flat and fill the gaps between collagen macromolecules. This design confers a great mechanical strength of bone along the loading direction.

Related works have highlighted the important role of the organic matrix on the mechanical properties of the whole biocomposites [2,3]. Therefore, there is a great interest in understanding the interactions between organic and inorganic components of bone and highlight the mechanisms that contribute to its remarkable mechanic properties. To understand bone mechanical properties and to predict and assess its behaviour, depending on the concentration of its elements, it is necessary firstly to understand the nanoscale bone organization [2–4]. The organic matrix is a key element for bone mechanical strength. Water is the third major constituent of bone, collagen

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hydration playing an important role in the mechanical properties of bone. Collagen lateral spaces are different in dry and wet bones. Water can serve as a link between collagen and mineral phases, this link being weakened by temperature increase [5]. With temperature increase, the molecular volume decreases and the hydrogen bonds become stronger [6–8].

Bone investigations can be focused on three structural levels: *mesostructural* (i.e., porous network of trabecular bars), *microstructural* (collagen fibril in trabecular packet arrangements, collagen fibers being rotated, twisted, and orthogonally arranged plaques but including also atypical arrangements), and *nanostructural* levels (collagen fibril and apatite crystals) respectively [9].

Due to the significant amounts of water and collagen, the heat treatment, applied for their removal, may change the trabecular bone microfracture behaviour (important when it is aimed to obtain particles with controlled morphology and characteristics). By the application of heat treatment, bone particles can be obtained, that are extensively used for various surgical treatment of bone defects. It was proven that in the heat treated bones, the crystalline phase composition is similar to that of the natural bones. Heat treated bone (within certain limits) is an inter-connective porous structure with a porosity of about 70 % by volume [4, 10,11].

The temperature effect on bone morphology and composition was previously studied [3, 8,12–16] through SEM (Scanning Electron Microscopy) and EDS (Energy Dispersive Spectroscopy) combined methods, the results being confirmed by X-ray diffraction method.

The experimental study presented in this paper aims to highlight the morphological and compositional changes that occur in human bone structure during the preparation of bulk samples for subsequent analysis by X-ray spectral and atomic absorption methods. By scanning electron microscopy techniques we characterized the morphological changes that occur during sample processing for subsequent XRF analyses. Using the heat treatment procedure, temperature below 300°C, the Ca/P stoichiometric ratio was maintained between 1.65 and 1.69 for all analyzed samples. Above this temperature this ratio decreased below the physiological value.

The present study represents the beginning of a complex investigation regarding the determination of heavy element concentration, in human hard tissues. An important application of the method is the evaluation of the heavy elements concentration in the environmental ambient from different areals. From this point of view the sample preparation and the morpho-compositional study of the particles for compaction are important in order to obtain homogenous and reproducible samples.

2. Experimental

The bones used to perform the experiments (part of the femoral head and femoral compact bone) were collected from local hospitals, following the surgical implantation of the coxo-femoral prostheses (according to agreed procedures on patient privacy and medical ethics), and were frozen immediately after sampling. All femoral bones were placed in individual containers. As a first step, in order to remove the tissue, blood and proteins, macroscopic impurities and adhered substances (including salts, ligaments and tissues stuck to the bone) the samples were cleaned with a surgical blades and forceps, then treated with a jet hot water, steams (t =100°C and p = 1 atm) and solvents. Cortical bone samples were dried by placing them in a dessicator. The dessicator was aerated every 24 hours to release moisture or gaseous emissions, and then, the samples were cut into pieces of quadratic form (5 mm × 5 mm × 5 mm) using a jig saw with a diamond blade. After these preliminary operations, the samples were grounded in a mortar before heat treatment, in order to remove all the organic components. Subsequently, coarse ground samples were heated in air atmosphere, at 300°C, at a rate of temperature rise of 5 °C/min. The temperature was maintained for 2 hours to remove the organic matrix [17–19]. In all cases, the oven was vented to ensure a complete combustion of organic components. Thermally treated bones were crushed using porcelain balls 10 and 60 minutes. Samples were then rinsed in deionized water to remove any organic materials and were degassed in a vacuum furnace. Strict preserving conditions were respected overnight ($p = 10^{-3}$ mm Hg and t = 30°C).

A number of 16 bone samples were investigated, all prepared in the same mentioned conditions. For analysis by SEM microscopy and EDS microanalysis, the samples were examined

in a scanning electron microscope (Philips XL 30 ESEM TMP, from *Politehnica* University of Bucharest, Materials Science and Engineering Faculty), equipped with a secondary electron detector in low vacuum and a solid state detector with two BSE diodes, plus an auxiliary micro analytic EDS system (EDAX Sapphire, UTW, 128 eV resolution). EDS method involved qualitative and quantitative microanalysis. The microscopy and/or micro analytic operating conditions were as follows: 0° tilt angle, 35° TOA, 25 kV accelerating voltage, and 10 mm working distance. Samples were mounted on aluminium stubs coated with double adhesive carbon tape and subsequently analyzed. Due to the special performance of the microscope, in neither case was necessary to cover the samples with a conductive material.

3. Results and discussion

Images shown are the most representative for the characteristics observed in several areas (from all 16 analyzed samples) collected from living humans, male and female. After the first crushing stage, different particles morphologies with different distribution (size 50-1000 μm) were detected. Due to the difference in atomic numbers (the organic component contains mainly light elements) it was easy to distinguish parts containing inorganic particles from the parts covered by the organic product. The detailed image inset from Figure 1, highlights the irregular morphology of the particles and the different compo aspect (different gray levels shows different chemical compositions, due to backscattered electrons signal).

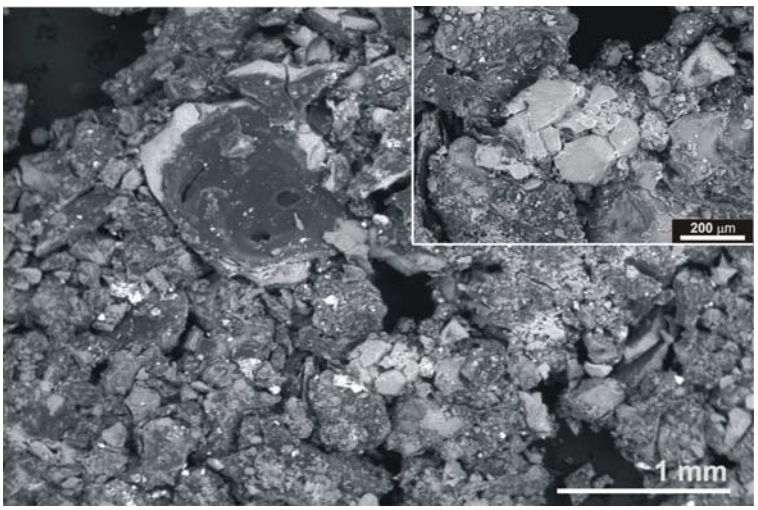

Fig.1. Bone sample after surgical removal of the major organic components

To assess the accuracy of results, additional test was conducted by analyzing a standard sample of HA, with a ratio Ca/P of 1.67 given by the manufacturer. EDS spectral results presented in Figure 2, confirm the stoichiometric composition of the standard sample and provide a ratio to the Ca/P of the studied sample close to the ideal value, with the presence of other chemical elements characteristic for a normal bone composition (Na, Mg, Si, Cl, C).

In the case of investigated sample, the following values were obtained (in atomic percentage): 59.26 % C, 22.28 % O, 0.48 % Na, 0.39 % Mg, 6.38 % P, 0.77 % Ca and small amounts of Al, Si, Cl (Ca/P ratio 1.688) (presented in Table 1), compared with the values obtained for standard HA samples: 50.55 % O, 18.56 % P , 30.90 % Ca (Ca/P ratio 1.664) (presented in Table 2).

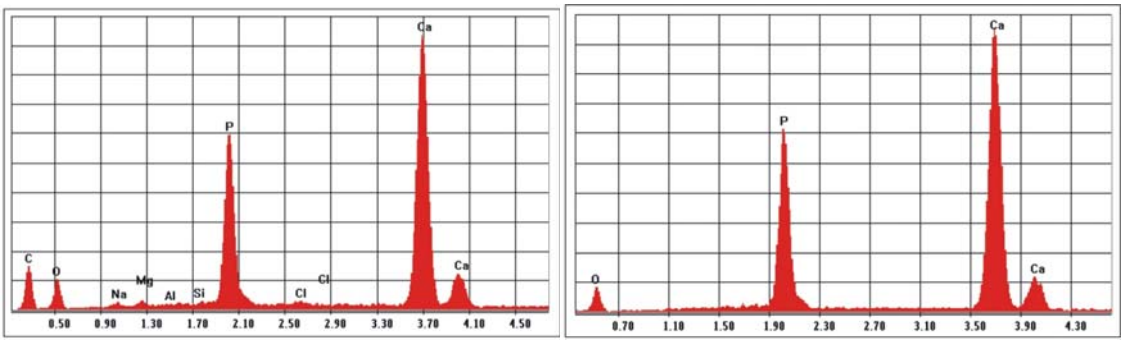

Fig.2. EDX spectra of bone mineral component from figure 1, with a Ca/P ratio = 1.688 (left) compared with analysis of standard samples of hydroxyapatite with a Ca/P ratio = 1.664 (right)

Figure 3 shows the bone particles (of irregular size) fractured, probably at tension concentrating points, and put in evidence the micro-cracks and particle morphology after primary crushing of the samples, required for the next step of water and organic components' removal.

Table 1. The chemical composition of bone mineral component from figure 1 with a ratio Ca/P=1.688; (wt%-weight percents; at%- atomic percents)

Element	Wt%	At %
C	17.48	30.85
O	27.75	36.78
Na	0.57	0.52
Mg	0.81	0.71
Al	0.50	0.39
Si	0.39	0.30
P	17.07	11.68
Cl	0.21	0.13
K	0.20	0.11
Ca	35.03	18.53
Total	100.0	100.0

Table 2. Analysis of standard samples of hydroxyapatite with a ratio Ca/P=1.664

Element	Wt %	At %
O	30.85	50.55
P	21.92	18.56
Ca	47.23	30.90
Total	100.0	100.00

Directions of major part of the cracks are parallel with the lines of equal mechanical tensions, resulting in a delaminating rather than a normal crack of cortical surface. At high magnification (×2000) microfilaments in the spaces between individual pieces can be distinguished. Similar organic filaments have been previously reported by other authors, this type of formations being discovered in some micro cracks of the cortical bone [3,20]. Normal untreated bone contains small pores, but overall gross bone microstructure is very dense due to the presence of organic matter associated with inorganic mineral-impregnated bone.

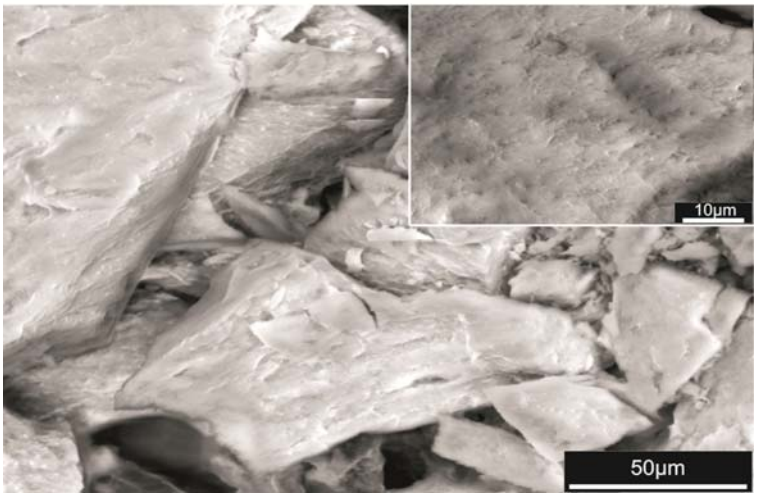

Fig.3. Sample after soft tissue removal - bone surface free of muscle, fascia, tendons, etc.

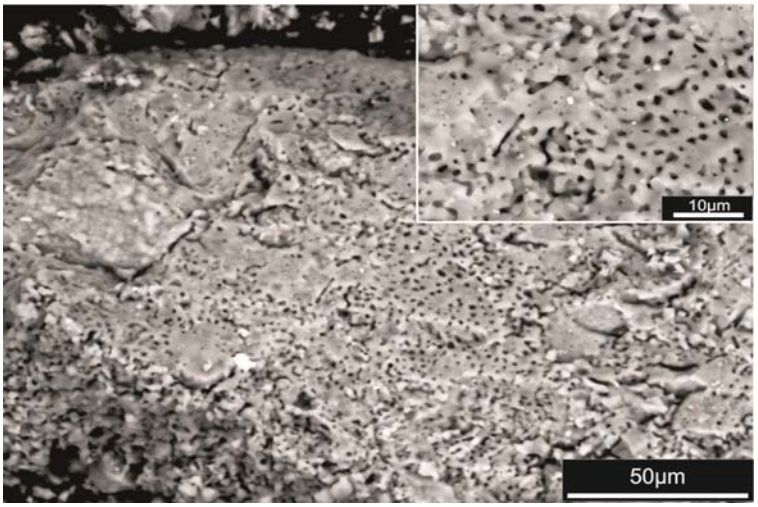

Fig. 4. Sample after heat treatment at 300°C - most organic components from the constitution bone were removed (without water and collagen).

Figure 4 reveals only inorganic component resulting from the application of heat treatment, which shows a significant pore architecture. The surface morphology clearly indicates that the pores are interconnected, further embrittlement being still possible, inherently leading to the possibility of breakage in using the ball mill to produce the powder required further composition analysis [11, 14, 19, 21].

In the detailed images from Figures 3 and 4, a total different aspect of the natural bone microstructure (containing water, proteins and collagen) can be observed, compared with thermally treated bone (from which the organic components were removed).

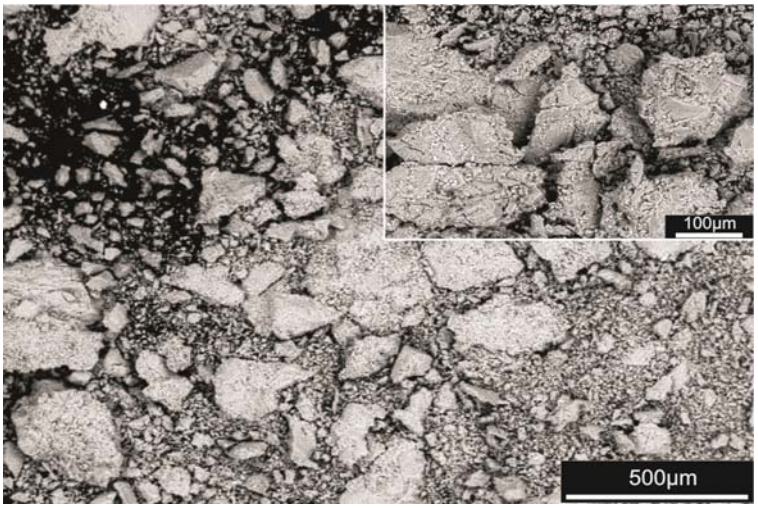

Fig. 5. Bone powder after grinding for 10 minutes in ball mill

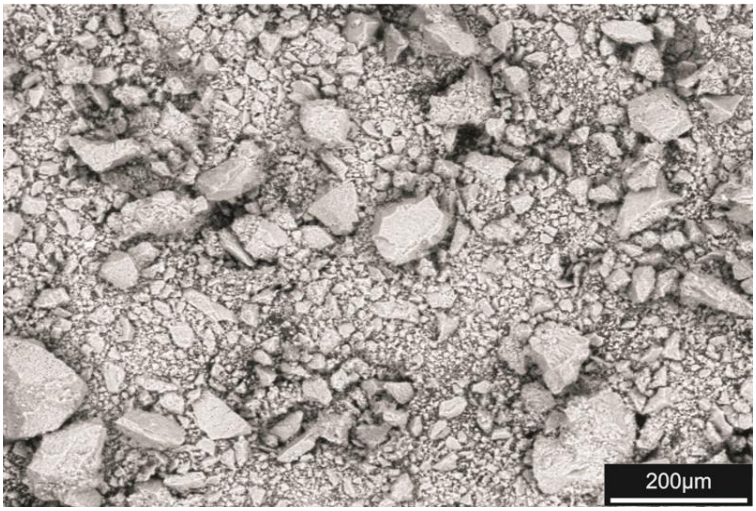

Fig. 6. Bone powder after grinding for 60 minutes in ball mills

Granulometric analysis of particles after their grinding for 10 minutes (Figure 5) allowed the observation of two classes of particles, with sizes at 50 µm level, and another of about 400 µm respectively. Similarly to the large differences, due to variations in manual crushing process, the relative values of these classes are significantly changed after application of heat treatment, large amounts of small particles being detected, compared with the untreated samples. This can be attributed to the embrittlement caused by temperature, due to organic binder and water disposal [13, 22, 23].

In Figure 6 one can see that the heat treated particles at 300°C and grounded 60 minutes are considerably smaller than the other (untreated or short ground time), maintaining the character of irregular form, particle size distribution being almost homogeneous. It is noted that the calcination temperature does not significantly influence the composition of bone particles, but only led to a change in particle size [16, 24]. Comparing the detailed image from Figure 5 with results from Figure 6 one can notice the reduction of particles grain size by heat treatment and the increase of the grinding time. The powder obtained by the method outlined in the previous section has a dark grey colour and their particle sizes are small enough to ensure a reduced matrix effect due to particle shape and size. After the bone was heated to a temperature of about 300°C, for 2 hours, the microstructure has changed as a result of water disposal and organic matter content as collagen [12].

The observed cracks, in the treated bone, are different than those present of the untreated ones. Fracture surfaces are not smooth, but rough and branched. Fractures did not occur mainly because of delamination, but the cracks are noticed in all directions. Heat treatment not only affects the fracture behaviour, but also the aspect of bone surfaces [20]. Degeneration of the organic matrix under the influence of temperature has a great impact on the mechanical properties of bone [25, 26]. In healthy bone, the main failure mechanism is delamination of the cortical contour, while in the bone with organic component removed by heat treatment, the type of fracture is propagated mainly not straightly [23,27].

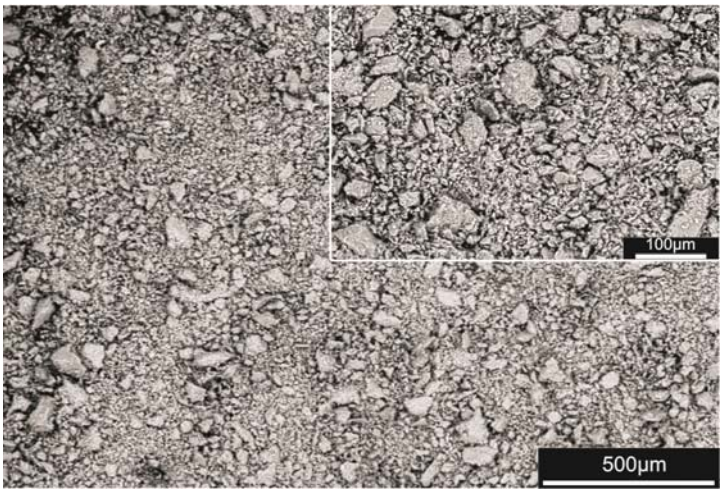

Fig.7. Bone powder after final grinding, sorted by size before compaction

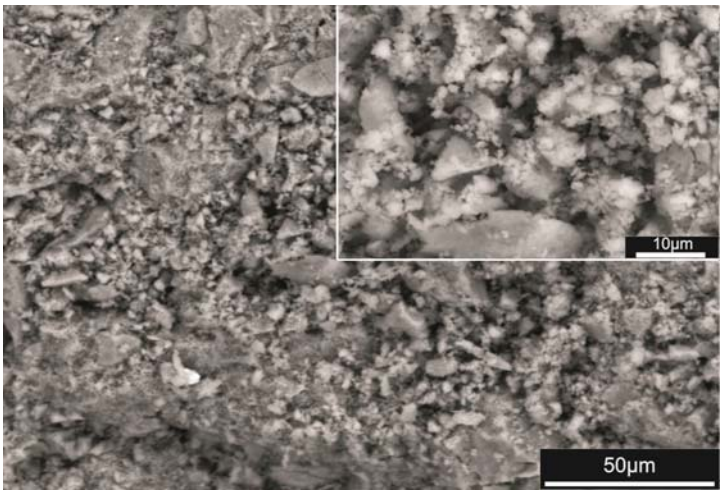

Fig.8. Compacted sample area for compositional analysis averaged over the surface

The detailed images in Figures 7 and 8 highlight the particles granulation and random distribution of particles (Figure 7), respectively the compacted bone samples aspect, designed for compositional XRF and AAS analyses.

Electron-microscopic images revealed a significant structural difference in bone architecture of the femoral bone according to treatment. The particles change their size, being not uniform in diameter. The meso-structure is responsible for the differences in bone mineral density. SEM technique shows the bone structure, but offers only a two-dimensional image of bone structure trabeculae, which only leads to a qualitative analysis of mesostructure [9]. The results of qualitative and quantitative EDS compositional analysis, performed on samples subjected to heat treatment at 400 ºC, in order to remove organic components and water, show a slight modification of the Ca/P ratio, in terms of reducing its value below 1.6. We obtained, in atomic percentage, a

composition of 18.35 % Ca, 11.68 % P, 0.71 % Mg, 36.78 % O, 0.52 % Na and small amounts of Al, Si, K, Cl with a Ca/P ratio of 1.586 (values presented in Table 3).

Table 3. Chemical composition of mineral component of bone, with a ratio Ca/P=1.586, after the heat treatment

Element	wt %	at %
C	41.11	59.26
O	20.59	22.28
Na	0.63	0.48
Mg	0.55	0.39
Al	0.21	0.13
Si	0.23	0.14
P	11.41	6.38
Cl	0.33	0.16
Ca	24.93	10.77
Total	100.0	100.0

The data presented in literature were confirmed by X-ray diffraction analysis, which reveals that the heat treatment does not significantly affect inorganic phase, all the diffraction peaks being almost identical in width. It can be observed only a slight variation related to the relative height of peaks, probably due to a difference in orientation [7, 27].

Analysis of samples of treated and untreated bone showed that the preliminary preparation of the samples, by surgery followed by steam removal of organic components (temperature of about 100°C) does not result in a change in the fraction of organic material which is removed only above 300°C. Fracture behaviour, microscopically studied, changes significantly with the pretreatment of organic matrix [28].

4. Conclusions

Studies carried out on bone samples taken from human subjects have shown that, depending on location, some of the variability in bone particles fracture mechanism is likely to appear, which is essential for understanding the contribution of these factors on bone fragmentation comportment [9]. This makes difficult the bone structure and morphology studies, because the constitution of bone varies greatly from person to person, bone having a hierarchical structure [16].

All the analysis techniques require the tissue processing, each one being subjected to some limitations. Heat-treated human bone differs from normal bone at meso-structural level by the three-dimensional formations, which lead to an increased bone porosity.

Considering that the temperature has a significant effect on the compact bone fractures, the bone testing should be performed at physiological temperatures, to avoid modification of the Ca/P ratio.

Lighter or darker contrast of SEM-BSE images of bone particulates indicates a lower average atomic number of the target, identifying structures both in the HA crystals and organic formations [15,29].

SEM techniques could help to characterize the morphological changes that occur during processing of samples. Electron beam, primarily in the SEM, is the main source of emissions of backscattered electrons BSE in the sample. Scattered electrons can be used to detect contrast between areas with different chemical composition. They can be seen especially when the average atomic number of neighbouring components vary significantly.

Given that BSE signal intensity depends on the average atomic number of the sample components, SEM technique serves not only to distinguish inorganic characteristics, but also offers the possibility to identify heavy metal components and micro morphological details. SEM

imaging method has the advantage that can provide analysis of a wide range of bone particles, and to characterize large sample areas, at the same time.

Acknowledgments

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