

Evaluation of Mineral Composition in the Area of the Cortical Osteoporotic Bone: a Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electronic Microscopy (SEM) Study

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Osteoporotic bone is a composite material consisting of a mineral phase (hydroxyapatite), an organic matrix (collagen), in combination with noncollagenous proteins, lipids and water. Patients with postmenopausal osteoporosis experience often bone fractures due to defective mineralization, altered bone turnover and poor remodeling rate. The aim of this case-control experimental study performed on 44 eligible women was to highlight the mineral component variability in the cortical bone by testing the hypothesis that fracture fixation osteoporotic bone has an increased crystallinity and reduced heterogenic texture when compared with the non-osteoporotic one. Qualitative and structural information relating to biological (poorly crystalline) hydroxyapatite was revealed by FTIR spectroscopy in thin sections obtained from osteoporotic bone biopsies. Correlations were noted between spectral parameters and crystal size as determined by SEM. The applications of these structural complementary methods revealed specific variations in the cortical area based on change of the phosphate i_1 and i_2 absorbances in the 900-1200 cm^{-1} most interesting spectral region. The results of this study performed on cortical osteoporotic bone reveal structural and compositional information of the newly deposited mineral bone component in osteoporotic patients. These data allow the correlation of the cortical mineral properties with bone quality and have implications on osteoporotic treatments.

Keywords: *postmenopausal osteoporosis, cortical mineral bone, hydroxyapatite, crystal size, spatial distribution, Fourier Transform Infrared Spectroscopy (FTIR); Scanning Electron Microscopy (SEM)*

Osteoporotic bone is a composite material consisting of a mineral phase (hydroxyapatite), an organic matrix (collagen), in combination with noncollagenous proteins, lipids and water [1]. Patients with postmenopausal osteoporosis experience often bone hip fractures due to defective mineralization, altered bone turnover and poor remodeling rate. In previous studies, we highlighted that FTIR spectroscopy is a valuable research tool, which may improve the knowledge of the bone mineral content, as well as the changes in mineral structure and composition, which occur in osteoporotic postmenopausal women with forearm fractures [2].

Experimental and observational surveys highlighting the mineral component variability in the cortical bone reveal that the mineral osteoporotic bone has an increased crystallinity when compared with the normal one [3-5]. However, these data are still limited and controversial because the lack of pointing out the spatial variations of matrix components and mineral heterogeneity, and therefore the Scanning Electronic Microscopy (SEM) is a valuable tool when used complementary with FTIR [6]. The method of FTIR spectroscopy allows the monitoring of skeletal changes that interfere with the osteoporotic disease owing to therapeutic intervention, and its value to the practitioner lies in the fact that spectral data may be

correlated with the microscopic and clinical data. Significant progress concerning "bone quality" has been performed in the last decade by using FTIR and SEM. The bone remarkable properties as maintaining the phosphocalcic homeostasis and promoting locomotion are due to the main calcium phosphate mineral component, namely the calcium hydroxyapatite [7,8].

Table 1 highlights the properties of hydroxyapatite and its precursors [9,10].

The aim of this study was to highlight the mineral component variability in the cortical bone by testing the hypothesis that fracture fixation osteoporotic bone has an increased crystallinity and reduced heterogenic texture when compared with the non-osteoporotic one.

Experimental part

A longitudinal, randomized, 4 year experimental and case-control survey was conducted by our interdisciplinary team during July 1, 2012 - August 31, 2015. A number of 44 eligible postmenopausal women (BMD T-score spine (-2.6 ± 0.5); BMD at the femoral hip (-2.7 ± 0.6)), treated with bisphosphonates, were matched by age, weight, body mass index, co-morbidities and the type of hip fracture. The patient's age ranged between 56 and 75 years old. Fracture-free controls were represented by samples of

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BCP Type	Chemical Formula	Total Ca:P	Crystals Phosphate nmol/g ⁻¹	K _{sp}	Calcium Balance mol
Hydroxyapatite (HyA)	Ca ₁₀ (PO ₄) ₆ (OH) ₂	1.67 : 1	5970	2.34x10 ⁻⁵⁹	4.36x10 ⁻⁷
	Ca ₅ (PO ₄) ₃ OH	1.67 : 1	5970	2.34x10 ⁻⁵⁹	4.36x10 ⁻⁷
	3Ca ₃ (PO ₄) ₂ ·Ca(OH) ₂	1.67 : 1	5970	2.34x10 ⁻⁵⁹	4.36x10 ⁻⁷
Tricalcium Phosphate	Ca ₃ (PO ₄) ₂	1.50 : 1	6450	2.83x10 ⁻³⁰	1.45x10 ⁻⁶
Octacalcic phosphate	Ca ₈ (HPO ₄) ₂ (PO ₄) ₄ ·5H ₂ O	1.33 : 1	6000	2.00x10 ⁻⁴⁹	1.08x10 ⁻⁶
	Ca ₄ H(PO ₄) ₃ ·3H ₂ O	1.33 : 1	6000	2.00x10 ⁻⁴⁹	1.08x10 ⁻⁶

Legend: BCP = Basic Calcium Phosphate; K_{sp} = Solubility Product Constant; HyA=Hydroxyapatite.

Table 1
THE PROPERTIES OF
HYDROXYAPATITE AND ITS
PRECURSORS

cortical femoral neck samples, collected from cadavers with similar age and nutritional status, assessed by height, weight, and body mass index (mass/height² [kg/m²]). Exclusion criteria: cancer, use of drugs known to affect bone metabolism, systemic diseases.

All patients included in the study provided intraoperative biopsies from the cortical femora - at the Plastic and Reconstructive Surgery Clinic "Austria House" and Orthopedics Clinics, belonging to the Emergency County Hospital "Pius Brînzeu" from the city of Timisoara, Romania. Cadaver femoral neck samples were obtained from the Department of Anatomy, belonging to the "Victor Babes" University of Medicine and Pharmacy, Timisoara.

In order to participate in this research study, patients filled out a medical form including written consent, in accordance with current medical ethics [11].

Bone samples

Horizontal biopsies from the cortical femoral neck (diameter=5.5 mm; height=10 mm, thickness 5 to 10 mm), were taken when performing hip intraoperative fracture fixation. The bone samples obtained from osteoporotic women or cadavers were fixed in 80% ethanol for at least 48 h and thereafter embedded in polymethylmethacrylate (PMMA) [12].

Fourier- transform infrared spectroscopic analysis (FTIR)

Infrared absorption spectra were traced with a JASCO FT-IR 4200 device (with automatic reading of the absorption bands, interferometer Michelson 45°), at the Institute of Chemistry of the Romanian Academy Timisoara. The measuring range was set to 4000-600 cm⁻¹. Spectra were taken at the normal temperature and pressure existing in the laboratory. The smoothness of the line of 100 % T was 100 ± 1.0 % T (repeating the continuous measurement).

The samples were prepared after KBr pelleting, by mixing the ground bone with KBr (1 mg sample/100 mg KBr) under vacuum, as usually applied for for FTIR studies [13].

The crystalline hydroxyapatite was obtained from amorphous calcium phosphate at pH 8.5; preparation was done by the method of Boskey and Posner [14].

Scanning Electronic Microscopy (SEM) and Scanning X-Ray Microradiography

In order to complete the data obtained by FTIR, one performed a SEM study concerning the bone microscopic and petrographic textures. The microscopic images were obtained by examination of bone samples embedded in polymethylmethacrylate (PMMA), then polished, corroded on surface with hydrochloric acid 0.1 N and finally covered with a thin film of silver. In order to reveal the homogenous or heterogeneous cortical status of the mineral matrix, the

petrographic views were completed with X-Ray microradiographs [15].

Measurement of bone mineral density

Bone mineral density (BMD) in the lumbar spine region (L₂ through L₄) and at femoral sites was measured with dual-energy X-ray absorptiometry (DXA HOLOGIC). All scans were taken using general scan software at a scan speed of 10 mm/s and with pixel size 1.0x1.0 mm². The scan width was 5 cm and the total effective dose remained <1μSv.

Results concerning spectral data collection

All FTIR spectra were referred to hydroxyapatite - Ca₁₀(PO₄)₆(OH)₂ - because it represents the most significant mineral phase of the mineral part of teeth and bones. The standard spectra of hydroxyapatite is revealed in figure 1.

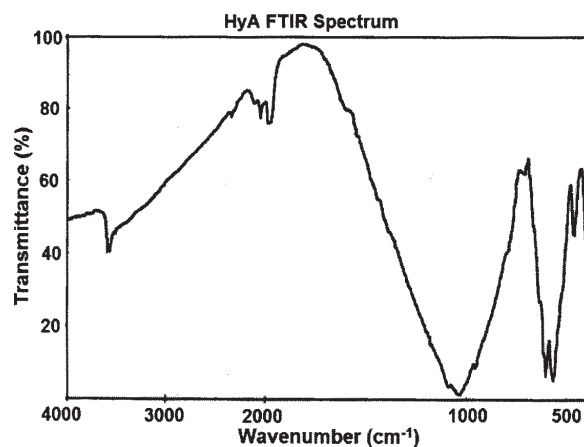


Fig.1. Standard FTIR spectrum of hydroxyapatite

All FTIR spectra - obtained intraoperative from femoral neck samples - pointed out different regions of interest - detailed by vibrational bands which reveal compositional information regarding inorganic and organic components of cortical osteoporotic bone tissue.

A typical FTIR spectrum of a postmenopausal osteoporosis case, obtained from the left fracture fixation femoral neck of patient B.M., 69 years old, is represented in figure 2.

Results related to Scanning Electronic Microscopy (SEM) and Scanning X-Ray Microradiography

Petrographic textures and X-Ray microradiographs from cortical fracture fixation femoral neck samples are suggestively presented in figures 4A and 4B:

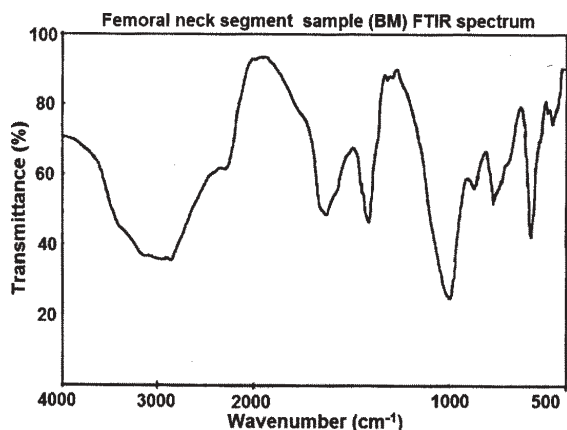


Fig. 2. FTIR spectrum from femoral neck segment taken intraoperative from postmenopausal patient B.M., 69 years old, with fracture of the left hip (T score = - 3.2)

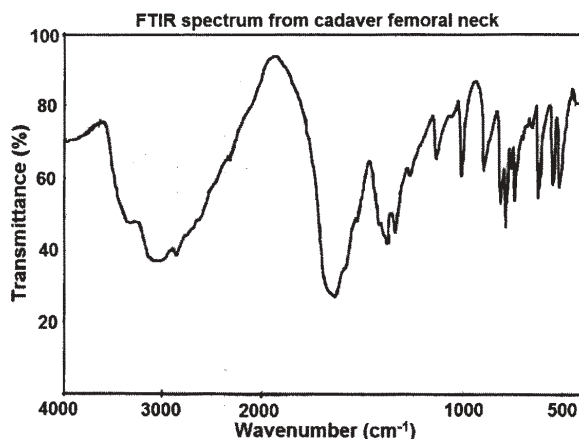


Fig. 3. FTIR spectrum from cadaver femoral neck sample

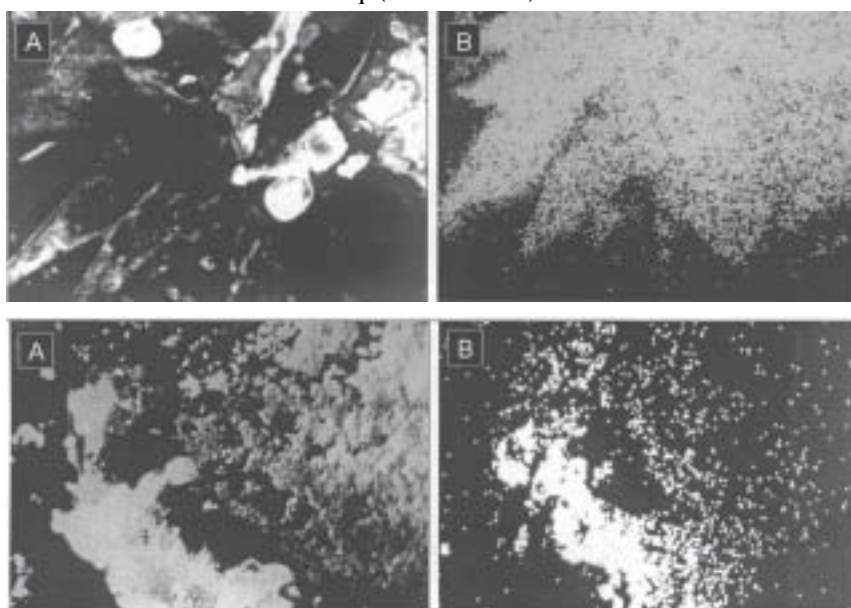


Fig. 4. Cortical femoral neck sample taken intraoperative from postmenopausal patient B.M., 69 years old, with fracture of the left hip (T score = - 3.2). A- SEM examination; B - Scanning X-Ray microradiography

Fig.5. Cortical cadaver femoral neck sample. A- SEM examination; B - Scanning X-Ray microradiography

Results and discussions

FTIR is a very important study method of osteoporotic bone because it allows to reveal the difference between different forms of calcium phosphate. Therefore, this modern spectral method makes a difference between „proper apatites”, „hydroxyapatites” and carbonate-apatites”. In osteoporosis, the difference of crystallinity between poorly crystalline apatite (with crystal sizes of 100-190 Å) and well crystallized ones (200-450 Å) is very important for monitoring the fracture risk [16].

The main characteristic of apatites is the stoichiometric formula; the proportion between main ions (Ca^{2+} , PO_4^{3-}), and supplementary ions (HO , HPO_4^{2-}), varies inside the crystal lattice [17]. When considering the apatite group, the intense HO -stretching is corresponding to the area of interest between 3600 and 3100 cm^{-1} [18]. The stretching of PO_4^{3-} anion, reflects the changes of mineral phase in the osteoporotic bone, and is characterized by an asymmetric and large band, at 1095 cm^{-1} , and intense in the area between 1035 and 1025 cm^{-1} . This important aspect was revealed in all the 31 cases of osteoporosis treated with alendronate (70.45%) (fig. 2).

In comparison, when examining fracture-free cadaveric control samples, changes of the phosphate ν_1 and ν_2 absorbances in the 1200-900 cm^{-1} spectral region have been revealed (fig. 3). Biological (poorly crystalline hydroxyapatite) is revealed by the PO_4^{3-} stretching region of the FTIR spectra in the range of 602-568 cm^{-1} , and is connected with changes of mineral crystallinity (fig. 1).

Qualitative and structural information relating to biological (poorly crystalline) hydroxyapatite was revealed by FTIR spectroscopy correlated with SEM analysis in thin sections obtained from osteoporotic bone biopsies (fig. 4A) and was suggestive for a rapid depositions of small crystals (fig. 4B).

The relationship between crystal size, bone turnover and remodelling is the most important aspect for monitoring postmenopausal osteoporosis, as revealed in our previous studies [2,6]. In the intraoperative fracture samples, petrographic SEM revealed smaller crystals, which are general more soluble and are suggestive for newly formed bone; they are also relevant for a heterogenic mineralization [19]. We revealed this important aspect in 31 patients previously treated with alendronate. In comparison, large crystals in the femoral cortical region (fig. 5A), are linked to lower mineralization level (fig. 5B), accelerated bone turnover and fragility [20].

However, the results obtained by these instrumental techniques will be further investigated by other analytical methods such as thermal analysis (TG/DTG/HF) [21-25] and as well kinetic studies [26-28].

Even if we conducted a case-control study and used bone samples taken *postmortem*, our study needs further research, firstly because of the small sample size; quantitative research based on mineral-to-matrix and carbonate-to-phosphate ratios is needed in order to reveal

unknown aspects in the complex field of postmenopausal osteoporosis.

Conclusions

The results of this study performed on fractured cortical osteoporotic bone indicate that the newly deposited mineral component has reduced heterogeneity and is less crystalline/mature than the normal one. The knowledge of the crystalline texture determined priority by different types of apatite, allows the correlation of the cortical mineral properties with bone quality and can guide the osteoporotic treatments in postmenopausal women.

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