



Assessment of microarchitecture and crystal structure of hydroxyapatite in osteoporosis

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ABSTRACT

Osteoporosis is characterized by lower bone mineral density (BMD) and microarchitectural degeneration, which tends to increase bone fragility and fracture risk. Bone microstructure depends on interactions between the mineral atoms, which may perform substitution or incorporation into bone crystals, and may dynamically take over the function of calcium or may become a complementary part. The mineral atoms may also become a composite in the hydroxyapatite crystals. The aim of this study was to find an association between the bone microstructure and hydroxyapatite crystal structure in osteoporosis, in comparison to normal bone. The subjects of this study were surgery patients at the Department of Orthopedics of Ulin General Hospital in Banjarmasin and other centers. Inclusion criteria consisted of the presence of fracture of trabecular bone, normal or osteoporotic BMD values, and no past history of chronic disease. Bone was obtained from fracture patients during surgery. The characteristics of the hydroxyapatite crystals were analyzed by X-ray diffraction (XRD) and the microarchitecture by scanning electron microscopy (SEM). SEM showed degeneration of the microarchitecture of osteoporotic bone, in comparison with normal bone. On XRD there was a peak of hydroxyapatite crystals only and no other crystal phases. Diffraction patterns showed a larger crystal size in osteoporotic bone as compared to normal bone, indicating higher porosity. It may be concluded that there is a difference in crystal size and morphologic distribution of hydroxyapatite in osteoporotic bone, determining bone microarchitecture.

Keywords: Microarchitecture, hydroxyapatite, osteoporosis

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Univ Med 2011;30:29-35.

INTRODUCTION

The characteristic features of osteoporosis are low bone mineral density (BMD) and microarchitectural degeneration, which increases the fragility of bone and the risk of fractures.⁽¹⁻³⁾ In developing or less developed countries the prevalence of osteoporosis is still not known with certainty, due to a lack of studies. In Asian population segments of those over 50 years old, osteoporosis of the lumbar spine has a prevalence of 11%-37% in women and of 5.4%-37.4% in men; osteoporosis of the femoral neck accounts for 2% of women and 6.3%-11.4% of men; and osteoporosis of the hip affects 16% of women and 3.8%-24.3% of men.⁽⁴⁻⁹⁾ According to the white paper issued by the Indonesian Osteoporosis Society (*Perhimpunan Osteoporosis Indonesia, PEROSI*), the prevalence of osteoporosis in 2007 was 28.8% in men and 32.3% in women.⁽¹⁰⁾ These percentages were consistent with the results of an analysis of osteoporosis risk by the Nutritional Research and Development Center (*Pusat Penelitian dan Pengembangan Gizi, Puslitbang Gizi*) of the Department of Health (*Depkes*) in cooperation with Fonterra Brands Indonesia, in 2005 2 in 5 Indonesians were at risk for osteoporosis.⁽¹¹⁾

The paradigm assumed in taking measures for improving BMD is an adequate intake of calcium, although field data show inconsistent results. The study by Prentice et al.⁽¹²⁾ revealed that osteoporosis was rarely found in the Gambian population, although having a low daily dietary calcium intake. A similarly low fracture rate is found in calcium-deficient Asian populations. On the other hand, a high incidence of fractures was found in Western populations, which have a high calcium consumption. Therefore it must be concluded that bone strength and elasticity does not solely depend on bone density but also on bone quality.⁽¹³⁾

Mammalian bone is a mineralized tissue comprising solid phases of calcium phosphate mineral and organic matrix, with a crystal

structure similar to that of the geological mineral hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Biological hydroxyapatites have multiple substitutions and deficiencies at all ionic sites.⁽¹⁴⁾

The microstructure of a material strongly affects its physical characteristics, such as strength, ductility, hardness, corrosion resistance, and behavior at low or high temperatures. The microstructural development of bone is determined by the role of the mineral atoms. Each single atom may be substituted in the bone crystals or may be incorporated into them, and may dynamically replace the function of calcium or may complement calcium and simultaneously affect the elasticity and strength of bone. Bone microstructure has frequently been studied in experimental animals as well as in humans,⁽¹⁵⁻¹⁷⁾ but there have been few studies of the microstructure of osteoporotic bone.⁽¹⁸⁾

Substitution and incorporation determines the pattern and quality of growth of the bony matrix, which ultimately affects the atomic configuration of the hydroxyapatite complex and interaction of collagen with hydroxyapatite, thus affecting the characteristics of the bone. In the study of Ren et al.⁽¹⁹⁾ it was found that substitution of Ca^{2+} ions by Zn^{2+} ions in the hydroxyapatite structure significantly decreased the crystal size proportionally with increasing zinc concentrations. The implication was that zinc inhibited crystallization and crystal growth in hydroxyapatite, which was consistent with the findings of Miyaji et al.⁽²⁰⁾ This phenomenon is believed to be due to the difference in size of Ca^{2+} and Zn^{2+} ions. Zn^{2+} ions have an ionic radius of 0.74 Å, which is significantly smaller than the ionic radius of Ca^{2+} of 0.99 Å, thus the substitution of Ca^{2+} by Zn^{2+} results in a decrease in the lattice parameters and reduction in crystal lattice volume, leading to impaired crystal growth.⁽²¹⁾

The current study on the crystallization profile of hydroxyapatite in osteoporotic bone, in comparison with normal bone, is the first of its kind in Indonesia. The aim of this study was

to evaluate the relationship between the crystallization profile of hydroxyapatite and the microstructural profile of osteoporotic bone, compared to normal bone, leading to an understanding of the role of mineral atoms as the basic components of bone composite in the course of osteoporosis.

METHODS

Research design

A laboratory observational study using cross-sectional design was conducted to assess the microstructure and crystal profile in osteoporotic bone.

Study subjects

Patients undergoing surgery at the Department of Orthopedics of the Ulin Regional General Hospital in Banjarmasin and at other centers. The inclusion criteria for this study were: i) presence of fracture of trabecular bone (in patients undergoing surgery); ii) normal or osteoporotic BMD values; and iii) no past history of chronic disease.

Tools for bone measurements

During the surgery bone samples were taken for the following investigations/assessments: i) bone microstructure by means of scanning electron microscopy (SEM); ii) characteristics of hydroxyapatite crystals in bone by X-ray diffraction (XRD); and iii) BMD. On the basis of the BMD scores, the subjects were assigned to two groups, i.e. the normal group and the osteoporosis group. According to calculations performed with Epi Info version 6, the minimum sample size was 32 for $\hat{\alpha}=95\%$ and $\hat{\alpha}=80\%$. Assessment of BMD was performed at Ulin Regional General Hospital in Banjarmasin and Syaiful Anwar Regional General Hospital in Malang. SEM and XRD were conducted in the Physics Laboratory and the Central Laboratory of the Faculty of Mathematics and Natural Sciences (FMIPA), Malang State Hospital. XRD was

also performed on standard hydroxyapatite crystals.

Characterization of the X-ray diffraction results was performed by means of PANanalytical X'Pert PRO-MPD, for osteoporotic and normal bone. Subsequent analysis was by means of the software programs High Score Plus, Crystal Maker and DDVIEW, complemented with the latest version of PDF2. Diffraction spectra were recorded at an angle of 2θ , from 20° to 60° , with a Cu-K_α radiation source (wave length = 1.54056 \AA , 40 mA, 40 kV) and step size of 0.05° . Mean apatite crystal size in osteoporotic and normal bone was evaluated by means of the Scherrer equation.⁽²²⁾ Rietveld refinement analysis was obtained from spectrum details of osteoporotic and normal bone apatite samples.

Rietveld refinement analysis was performed by means of the High Score Plus program. Space groups, cell parameters, atomic positions, and hydroxyapatite thermal parameters were introduced in an initial structural model. Rietveld refinement was performed in several stages. In the first stage, scale factors and background were refined, followed by refinement of other parameters, respectively comprising profile parameters, zero shift, asymmetric parameters, cell parameters, preferential orientation, atomic coordinates, thermal parameters, and occupancy factors.

Ethical clearance

The study was approved by the Health Research Ethics Committee Faculty of Medicine University of Brawijaya. All the subjects were informed of the purpose of the study and were requested to sign an informed consent form.

Statistical analysis

Data were analyzed by analysis of variance followed by Tukey when appropriate. A p value < 0.05 was considered significantly.

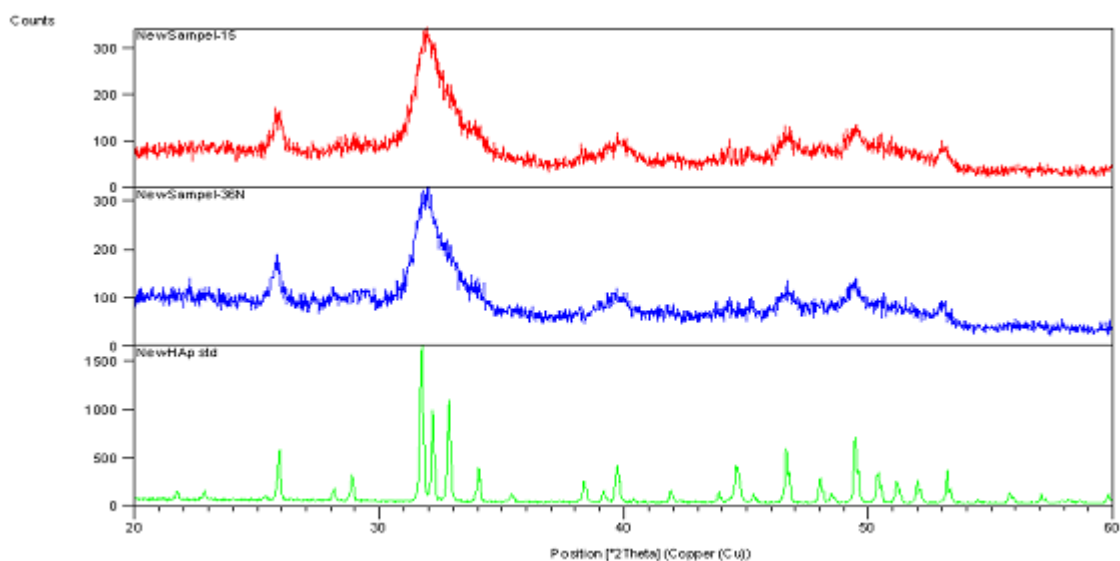


Figure 1. XRD patterns of osteoporotic bone sample (red), normal bone (blue) and standard hydroxyapatite crystals (green)

RESULTS

The results of XRD characterization are presented in Figure 1, indicating that only the hydroxyapatite crystal peak was present and that no other crystal phases were detected.

From search and match tests it was found that all bone samples, both normal and osteoporotic, had a crystal phase with a hexagonal structure and space groups $P63/m$ and 176. Application of the Crystal Maker program yielded the crystal structure depicted in Figure 2.

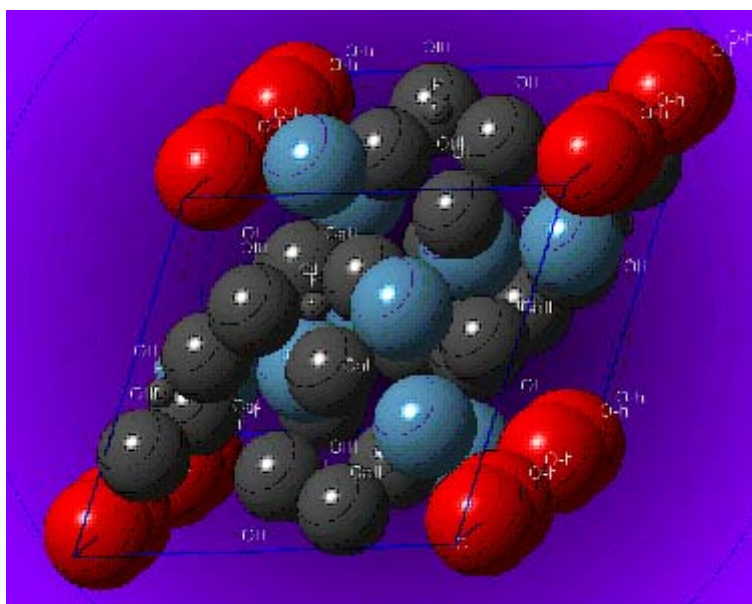


Figure 2. Crystal structure of bone
Legend: O-H = red; Ca = gray (large); P = gray (small); O = blue

Table 1. Crystal sizes of standard hydroxyapatite, osteoporotic bone and normal bone

Sample	FWHM (degree)	Intensity (counts)	Average crystal size (nm)
Hydroxyapatite	0.0984	1628.57	190.98
Osteoporotic bone	0.5510	232.78	33.98
Normal bone	0.6298	192.16	19.01

From Figure 2 it is apparent that O-H groups are located at the corners of the crystal lattice, whereas Ca, P, and O atoms are located within the crystal lattice space or volume, in a highly regular manner. The hexagonal crystal structure has the crystal lattice parameters $a = b \neq c$, with angles $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$. According to crystal geometry, this structure possesses a relatively high stability. Crystal sizes are presented in detail in Table 1. Anova test concluded that there is significantly different of crystal size in all groups ($p=0.000$). Post hoc test concluded there is significantly different of crystal size in all groups ($p=0.000$).

The microarchitecture of osteoporotic bone is significantly different from that of normal bone. Figure 3 depicts SEM images at 200x magnification, and Figure 4 depicts SEM

images at 3000x showing trabeculae with large perforations surrounded by resorption cavities. In the trabecular structure there are stump structures and the inner surface of the remaining cavities appear flat and thin (A). In normal bone the resorption cavities have not yet formed stump structures and the trabecular walls are still thick with a crumpled surface (B).

DISCUSSION

In connection with the abovementioned results, the mechanical properties of bone should not be viewed as a table of constants, but rather as a function of various factors.⁽²³⁾ Osteoporosis is a process progressing to the amorphous that is difficult to characterize.⁽²⁴⁾

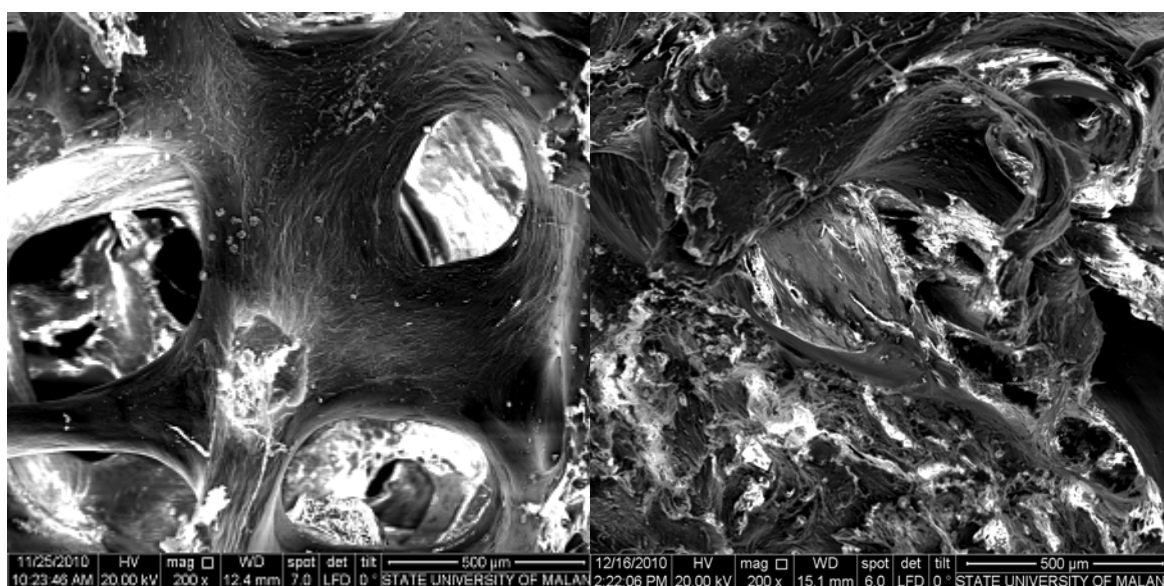


Figure 3. Trabeculae in osteoporotic (A) and normal (B) bone at 200x magnification

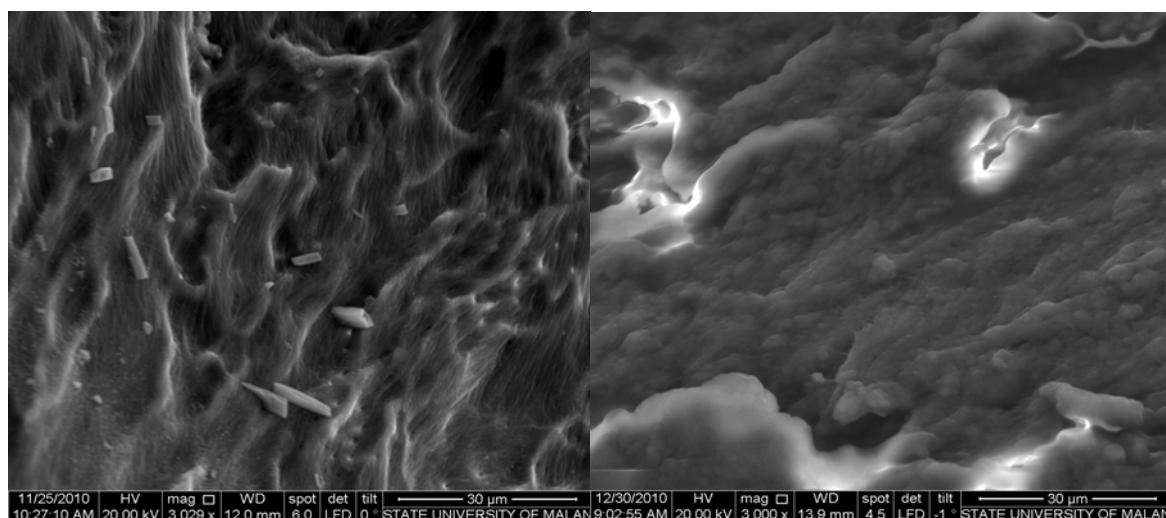


Figure 4. Trabeculae in osteoporotic (A) and normal bone (B) at 3000x magnification
 At 3000x magnification, osteoporotic bone consists of parallel strands of collagen fibrils with intervening furrow-like resorption cavities, and also broken fibrils (A). Normal bone lacks strands and presents predominantly closely packed hill-like structures (B)

The larger crystal size in osteoporotic bone is a result of the atomic make-up of the composite. The porosity of bone is proportional to the crystal size, where with a larger crystal size there is increased porosity. This is the reason why normal bone in this study exhibits a smaller crystal size (has a lower porosity) in comparison with osteoporotic bone. This phenomenon may be explained by the fact that within the bone structure the larger crystal size also leads to larger-sized interstitial cavities. The larger-sized cavities within the bone increase the total number of voids, such that ultimately the void ratio also increases. It is the void ratio that increases the porosity, as shown by the agreement between XRD and SEM results.

According to physical fact and logic, materials with a higher porosity level have poorer mechanical properties, possessing greater fragility, lower hardness, and lower flexibility, in consequence of the weaker bonds between the bone crystals. The present study found a reduction in bone mass and deterioration of its microarchitecture, which is comparable with the findings of Shen.⁽¹⁸⁾


In summary, the atomic mineral make-up of the bone composite determines the crystal size, which is the basis for the development of bone microarchitecture. Furthermore, the results of this study also indicate that although the crystal structure in osteoporotic bone and normal bone may be of similar hexagonal geometry, at the most basic level the crystal size and bone porosity are also important in determining bone quality, either physical, mechanical, or otherwise.

A limitation of this study is that SEM only reveals bone structure in two dimensions, necessitating further studies using micro-CT reconstruction as a three-dimensional analytical tool for osteoporotic bone as compared to normal bone. The crystal size also needs to be investigated three-dimensionally by means of atomic force microscopy.

CONCLUSION

Crystal size and morphologic distribution of hydroxyapatite in osteoporotic bone differs from those of normal bone and determine the microarchitecture of bone.

ACKNOWLEDGEMENT

The author thank Mr. Abdulloh Fuad and Ms. Zulaikha in Malang State of University for sample preparation. 

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