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Analysis of Pelvic Bone of Ovariectomized Rats using Infrared and Raman Spectroscopies

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Abstract

Osteoporosis is a bone disease that generally occurs in postmenopausal women. Osteoporosis can be studied using an animal model such as rats because rat bone structure is similar to human bone structure. This research aimed to analyze pelvic bones of ovariectomized rats that showed osteoporosis signs for the first time. In this study, 20 *Rattus norvegicus* were given ovariectomy at 12 weeks of age and were used as an animal model for menopausal women. The rats were euthanized every 2 weeks from 13 to 21 weeks of age. In addition, the pelvic bones of ovariectomized rats will be analyzed using FTIR and Raman spectroscopy to show the first osteoporosis signs periodically. Results of FTIR and Raman spectroscopy indicated that the pelvic bones of the ovariectomized rats showed the first osteoporosis signs at 17 weeks of age with changes in phosphate and carbonate contents, increased hydroxyl functional groups, reduction of mineral maturity about 7.47%, and reduction of crystallinity about 29.26%. In addition, morphological changes from fine fibers to coarser fibers and reduction of the crystal size and crystallinity index at the angle of $2\theta \cong 25°$ about 9.29% and 25.18%, respectively, were observed using SEM-EDS and XRD.

Keywords: osteoporosis, ovariectomy, Rattus norvegicus, rat pelvic bones

Introduction

Osteoporosis is a bone disease with reduced bone mass, which increases the risk of fractures and changes in bone composition that can reduce bone strength [1]. Some factors such as gender, age, race, hormonal factors, body weight, heredity, and lifestyle can increase the risk of osteoporosis [2]. Osteoporosis often occurs in women who experienced menopause, in which estrogen hormone decreases in their bodies. The effects of the loss of estrogen in the body increase bone resorption and reduce bone formation because it plays a role in bone remodeling [3]. Osteoporosis in postmenopausal bone can be analyzed using an animal model such as white rats, which are given ovariectomy. This treatment is used to remove the ovaries from the body of an animal model; thus, it can be studied when it first shows osteoporosis in the bone. World Health Organization has reported that white rats with osteoporosis are a good representation of osteoporosis that occurs in the human bone because the rat bone structure is similar to the human bone structure by anatomy and bone conditions [4].

In previous research, the pelvic bone of ovariectomized rats was rarely used to determine osteoporosis. In addition, rats were used to determine the bone quality of rats with chronic kidney disease and characterized using Fourier transform infrared (FTIR) and Raman spectroscopies to analyze the functional groups of the bone. Changes in bone quality were not studied because the rats were only given a treatment in one time of period, and no rats were given a placebo for comparison [5]. In similar research, rats were used to determine the effects of standard dietary and nano-calcium dietary on the femur and tibia of ovariectomized *Rattus norvegicus* with osteoporosis [6]. The results showed that at the age of 21 weeks, the femur and tibia were in osteoporosis condition after being given ovariectomy, but this finding was not shown by Raman spectroscopy results compared with FTIR results. In addition, the pelvic bone was not used in previous studies to analyze the first osteoporosis signs caused by ovariectomy. Therefore, in this study, the pelvic bones of ovariectomized *Rattus norvegicus* were used to observe the changes in pelvic bones, which was considered as an active bone that hold the upper body

after being given ovariectomy. The novelty of this study was to analyze rat's pelvic bone with ovariectomy using two different spectroscopies, namely, FTIR and Raman spectroscopies, to show the first osteoporosis sign periodically. This study aimed to analyze the characteristics of the pelvic bones of ovariectomized rats with regard to functional groups, mineral maturity, morphology, and crystallinity using FTIR spectroscopy, Raman spectroscopy, scanning electron microscopy–energy-dispersive spectroscopy (SEM-EDS), and X-ray diffraction (XRD).

Methods

In this research, the use of an animal model of rats and treatment such as ovariectomy and euthanasia was approved by the Health Research Ethics Committee of the Faculty Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital with protocol number 17-05-0421. Twenty white (*Rattus norvegicus*) Sprague–Dawley rat strains were obtained from the Indonesian Food and Drug Authority (Indonesian FDA) at the age of 6 weeks. This age was the weaning age of rats, which was maintained until 12 weeks where the reproductive organs were mature. Rats were given standard dietary F1-Spectra-3, and enough water was placed on a bottle hung on the cage. Rat pelvic bone preparation and breeding were referred to as the modified bone preparation from a previous study [6]. In addition, rat pelvic bones were obtained from four rats without ovariectomy as control rats (non-OVX rats) at 13 weeks of age. The samples were identified as PK. Furthermore, 16 rats (OVX rats) were ovariectomized at 12 weeks of age and euthanized every 2 weeks from 13 to 21 weeks of age [6]. These samples were identified as PO15, PO17, PO19, and PO21.

The pelvic bones of rats were cleaned from bone tissues and dried at 60 °C for 24 h. Dried pelvic bones were soaked in hydrazinium hydroxide for 7 days to remove organic substances that were stuck on the bones. After 7 days, pelvic bones were soaked in alcohol for 1 h and then rinsed with Aquades three times. Afterward, the pelvic bones were dried again at 60 °C for 24 h [6]. The pelvic bones were then crushed with a mortar and pestle until smooth to be characterized.

The pelvic bones of rats were characterized using FTIR (Nicolet™ iS50 FTIR Spectrometer with NIR Module, Universitas Indonesia ILRC Laboratory) and Raman spectroscopy (Micro Confocal Hyperspectral 3D Imaging Raman Spectrometer, Universitas Indonesia ILRC Laboratory) to observe the changes in wavenumbers, mineral maturity, and crystallinity in pelvic bones with ovariectomy. The mineral maturity and crystallinity were analyzed using FTIR spectroscopy with a ratio of wavenumbers of 1030/1110 cm^{-1} and splitting factor (SF) of v_4 phosphate. Furthermore, the mineral maturity and crystallinity were analyzed using Raman spectroscopy with a full width at half maximum (FWHM) of v_1 phosphate. In addition, SEM-EDS (Hitachi SU-3500 Scanning Electron Microscope) and XRD (Rigaku SmartLab) were used in this study to analyze the morphology, mineral contents, and crystallinity such as crystal size, crystallinity index, and lattice parameter of pelvic bones with ovariectomy. Moreover, a t-test was used in this study to analyze the significant values of rat bones with and without ovariectomy. Statistically, $P < 0.01$ was considered as significant values.

Results and Discussion

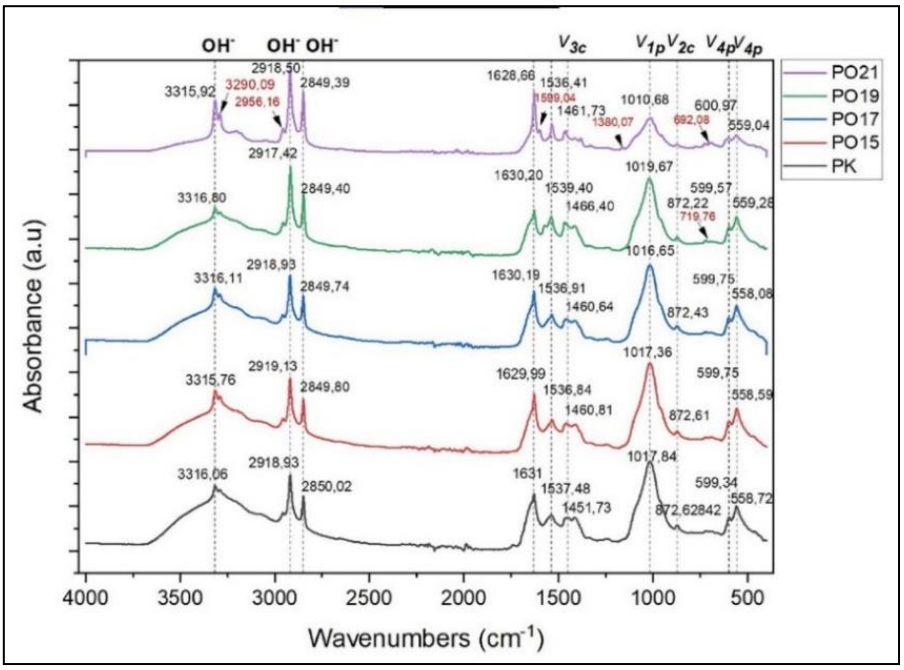
FTIR spectra showed inorganic and organic contents of bones (Figure 1.a). The inorganic contents are v_4 phosphate at a wavenumber of 599 and 558 cm^{-1}; v_2 carbonate at 872 cm^{-1}; v_1 phosphate at a range of 1017 cm^{-1}; v_3 carbonate at a range of 1451–1466 cm^{-1}; and hydroxyl groups at 2850, 2918, 3290, and 3316 cm^{-1}. Furthermore, amide II and amide I show organic contents at wavenumbers of 1537 and 1631 cm^{-1}, respectively.

The splitting peak of v_4 phosphate at wavenumbers of 559 and 599 cm^{-1} decreased by about 3.45% in the PO21 bone. These changes indicate that calcium phosphate is in crystal form because those peaks are linear to crystallinity [7]. The v_2 carbonate intensity also decreased at wavenumbers of 872 cm^{-1} by about 2.01%, indicating that the crystallinity of the bone is low [8]. The appearance of $v3$ carbonate showed that calcium phosphate was in the form of carbonate apatite, which is a type-B carbonated apatite [7]. The $v1$ phosphate band shifted to the right of the spectra, which indicated a loss in the vibrational band [9]. The increase and narrower peaks of the hydroxyl groups by about 40.8% showed a reduction in crystallinity and crystal size of the bone. Moreover, the appearance of amide I and amide II in bones indicated that bones still contained organic content in the form of protein. The changes in pelvic bones without and with ovariectomy were analyzed using a t-test. The FTIR result showed that the wavenumbers of the PK bone with PO15, PO17, PO19, and PO21 were significantly different ($P < 0.01$). In a previous study, the wavenumbers of osteoporosis are significantly different from normal bones because of the loss of carbonate (P=0.007) [10].

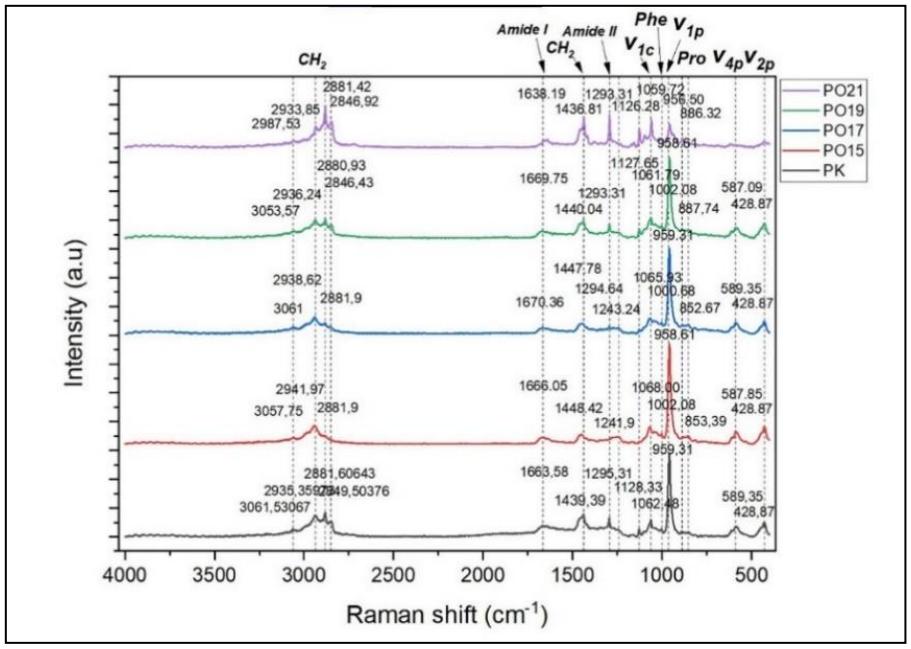
Mineral maturity was calculated on the basis of the absorption band v_1 phosphate deconvolution with a ratio of wavenumber peaks at 1030/1000 cm^{-1} (Table 1). The mineral maturity of pelvic bone toward osteoporosis decreased by about 7.47% and showed an imbalance of bone remodeling. Mineral maturity is related to bone remodeling because it is used to balance bone remodeling [11]. The SF of v_4 phosphate had a fluctuating pattern (Table 1). When the crystallinity decreases, the bone density also decreases, and bone becomes brittle. The SF

values of rat's bone did not show consistent changes because FTIR was not sensitive to determine the crystallinity of bone samples [5].

Raman spectra showed inorganic and organic contents of bones (Figure 1.b). The inorganic contents are v_2 phosphate at 428 cm^{-1}, v_4 phosphate at 589 and 587 cm^{-1}, v_1 phosphate at the range of 959 cm^{-1}, and v_1 carbonate at a wavenumber of 1062 cm^{-1}. Furthermore, the organic contents that were found were phenylalanine at 1002 cm^{-1}; proline at 853 and 887 cm^{-1}; amide I at the range of 1663–1666 cm^{-1}; amide II at 1295 cm^{-1}; and CH_2 and CH_3 at 2849, 2881, 2935, and 3061 cm^{-1}.



(a)



(b)

Figure 1. Rat's Pelvic Bones toward Osteoporosis Spectra in the Range of 4000–400 cm^{-1} using (a) FTIR and (b) Raman Spectroscopy

Table 1. Comparison between Crystallinity and Mineral Maturity using FTIR and Raman Spectroscopy

Sample Code	Crystallinity		Mineral Maturity	
	FTIR	Raman spectroscopy	FTIR	Raman Spectroscopy
	Splitting Factor (v_4 phosphate)	FWHM (v_1 phosphate)	Ratio 1030/1110 cm^{-1}	Proportion with Crystallinity using Raman Spectroscopy
PK	3.43	17.60	3.88	17.60
PO15	3.47*	17.42*	3.81*	17.42*
PO17	3.48*	16.72*	3.79*	16.72*
PO19	3.39*	16.53*	3.65*	16.53*
PO21	3.14*	12.45*	3.59*	12.45*

(*) shows that pelvic bones without ovariectomy (PK) were significantly different with pelvic bone with ovariectomy ($P < 0.01$)

In Raman spectroscopy, the sharp and narrow peak is related to uniform and well-arranged crystals [12]. The intensity peak of v_2 and v_4 phosphate bands decreased, which indicates the reduction of crystal arrangement. By contrast, the v_1 carbonate band at 1062 cm^{-1} increased, which is a type-B carbonated apatite. In addition, it influences the intensity of v_1 carbonate to increase and replace the phosphate band [13]. Phenylalanine and proline, which are proteins of bone from type I collagen, decreased. Protein plays a role in maintaining bone strength [14]. Moreover, the appearance of amide I, amide II, CH_2, and CH_3 showed that pelvic bones still contained organic contents of bone after characterization using Raman spectroscopy [15]. The changes in pelvic bones without and with ovariectomy were analyzed using a t-test, and Raman spectroscopic results showed that PK bones with PO15, PO17, PO19, and PO21 bones were significantly different ($P < 0.01$). In a previous study, the risk of osteoporosis was affected significantly by rats' age because of the shift of phosphate and carbonate on the spectra [16].

Crystallinity values also determine the mineral maturity of bone because these values are proportional to the crystallinity in Raman spectroscopy [17]. Crystallinity was calculated using the FWHM of the v_1 phosphate band at approximately 959 cm^{-1} in the range of 1000–900 cm^{-1}. The crystallinity of PO21 bones was lower than that of PK bones (Table 1). Crystallinity was decreased with the mineral maturity about 29.26%. In this research, phosphate, carbonate, and hydroxyl were found in pelvic bone toward osteoporosis by FTIR, but in Raman spectroscopy, only phosphate and carbonate were found in the spectra. This result indicates that FTIR is sensitive to analyze the inorganic contents of the bone. However, Raman spectroscopy had a good resolution to analyze organic contents of the bone, such as amide I, amide II, CH_2, CH_3, phenylalanine, and proline, compared with FTIR spectra, which could only analyze amide I and amide II. In addition, mineral maturity is more precisely determined using FTIR. It has constant values compared with Raman spectroscopy that uses crystallinity values. However, crystallinity is more precisely determined using Raman spectroscopy because it has constant values compared with FTIR, which has fluctuating values.

The morphology with 2000× magnification of pelvic bones toward osteoporosis was analyzed using SEM-EDS, and the result shows that fibers form with some granules that are not uniform. The morphology of the PK and PO15 bones (Figure 2.a and Figure 2.b) was smooth, and these bones had fine fibers. However, the morphology of the PO17, PO19, and PO21 bones (Figure 2.c–2.e) was rough, and these bones had coarse fibers. A previous study [18] showed a coarse fiber in the morphology of bone with osteoporosis. The changes in bone and the large granules that appeared on the morphology of the PO21 bone indicated osteoporosis after ovariectomy.

Mineral contents that were obtained using EDS are presented in Table 2. This table shows calcium, phosphorus, magnesium, oxygen, carbon, alumina, silicone, and natrium of pelvic bones toward osteoporosis. All the variations of rat's pelvic bones toward osteoporosis contained calcium, phosphorus, and magnesium, which are the dominant minerals of bone [19]. The bone mineral contents, particularly calcium, phosphorus, and magnesium, had fluctuating values because they depend on the spectrum areas. Based on previous studies [2], in human bones, osteoporosis can be influenced by other factors other than the changes in mineral contents, such as heredity, hormonal activity, lack of exercise, and less vitamin D intake.

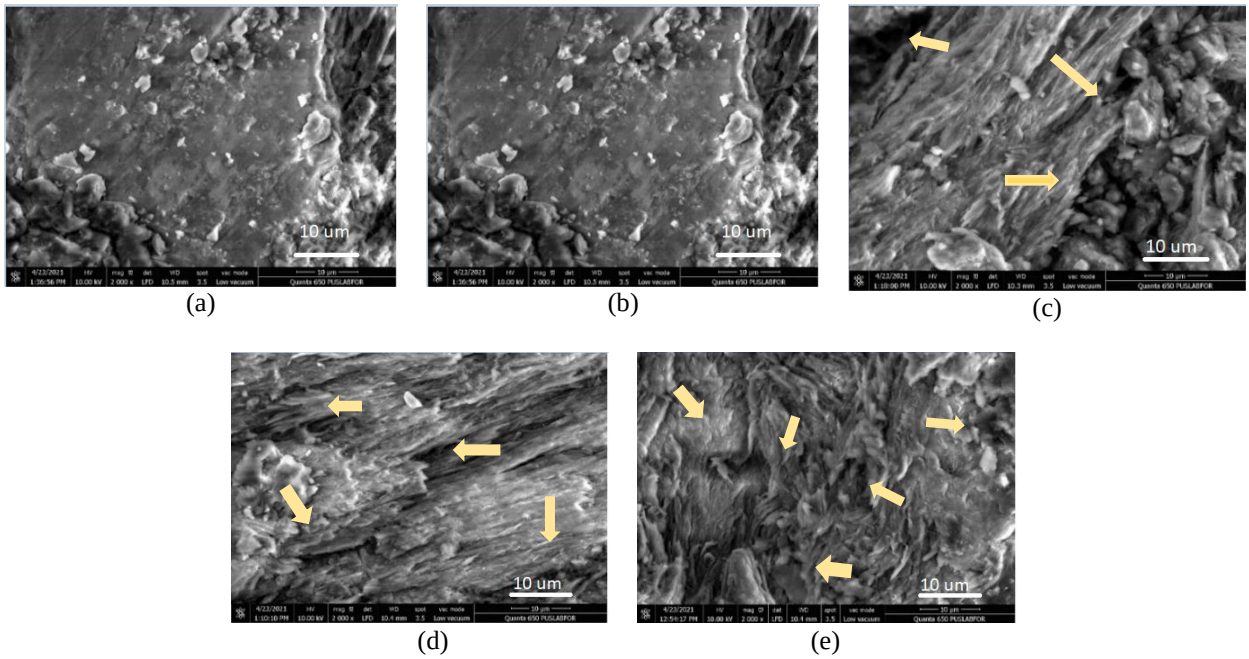


Figure 2. Morphology with 2000× magnification of rat pelvic bones toward osteoporosis: (a) PK, (b) PO15, (c) PO17, (d) PO19, and (e) PO21

Table 2. Bone Mineral Contents of Rat's Pelvic Bones toward Osteoporosis using EDS

Sample Code	Ca	P	Mg	O	C	Al	Si	Na
	Wt%							
PK	18.2	10.4	0.4	39.7	28.1	2.7	0.3	0.3
PO15	21.1*	10.6*	0.5*	39.1	26.6	1.2	0.5	0.4
PO17	27.8*	12.6*	0.6*	30.9	27.8	0.2	-	0.2
PO19	21.3*	12.0*	0.3*	41.0	24.5	0.4	-	0.4
PO21	19.6*	10.6*	0.3*	39.1	28.8	1.2	-	0.4

additional: the gray table shows the dominant mineral of bones
(*) shows that pelvic bones without ovariectomy (PK) were significantly different from pelvic bones with ovariectomy $P < 0.01$)

Ca/P ratio values were obtained from rat's pelvic bones with and without ovariectomy (Table 3). The values were higher than the stoichiometry Ca/P ratio, which is 1.67. This result indicates the possibility to form other compounds such as CaO, which could reduce bone mineral strength and increase carbonate ion [20]. Therefore, the increase in the Ca/P ratio of rat's pelvic bones toward osteoporosis showed a decrease in bone mineral strength.

XRD analysis focused on scattering angles (2θ) between 20° and 40° (Figure 3). Pelvic bone toward osteoporosis showed a (002) plane on 2θ angles at 25°. The (002) peak of ovariectomized pelvic bone shifted to the right (Figure 3), thereby decreasing the distance between planes. Moreover, the diffraction peak of the pelvic bone with ovariectomy was wider than that of PK bones, which indicated a reduction in crystal size. The t-test of the XRD pattern showed that PK bones (non-OVX rats) were significantly different ($P < 0.01$) from PO17, PO19, and PO21 (OVX rats). However, PO15 bones were not significantly different from PK bones without ovariectomy. A previous study explained that the patterns of the bone with and without ovariectomy were significantly different using XRD [21].

Table 3. Ca/P Ratio of Rat's Pelvic Bones toward Osteoporosis

Sample Code	Ca	P	Ca/P ratio
	Wt%		
PK	18.2	10.4	1.75
PO15	21.1	10.6	1.99*
PO17	27.8	12.6	2.21*
PO19	21.3	12.0	1.77*
PO21	19.6	10.6	1.85*

(*) shows that pelvic bones without ovariectomy (PK) were significantly different from pelvic bones with ovariectomy ($P < 0.01$)

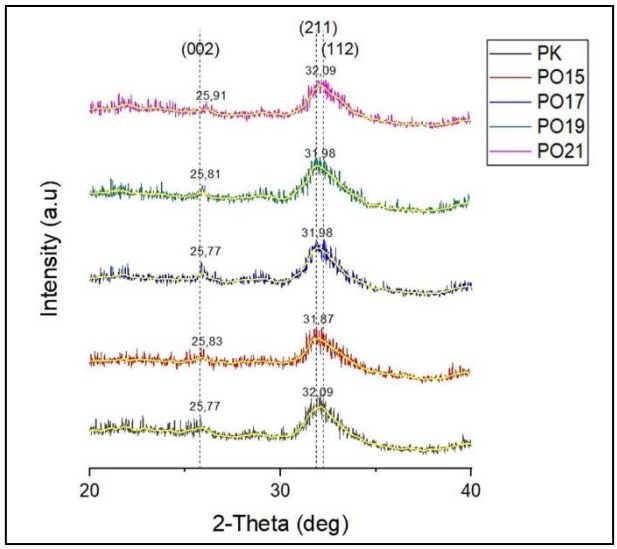


Figure 3. XRD Pattern of Rat's Pelvic Bones toward Osteoporosis in the Range of 2θ Angles between 20° and 40°

Table 4. Crystal Size, Index Crystallinity, and Lattice Parameter of Pelvic Bones toward Osteoporosis using XRD in 2θ Angles at 25°

Sample Code	2θ (deg)	FWHM	Crystal Size (nm)	Crystallinity Index	Lattice Parameter (Å)	
					a = b	c
PK	25.77	1.28	12.05	6.50×10^{-3}	9.39	6.88
PO15	25.83	1.35	11.45	5.59×10^{-3}	9.39	6.87
PO17	25.77	1.40	11.03*	4.99×10^{-3}*	9.41	6.87
PO19	25.81	1.40	11.01*	4.97×10^{-3}*	9.39	6.89
PO21	25.91	1.41	10.93*	4.87×10^{-3}*	9.39	6.87

(*) shows that pelvic bones without ovariectomy (PK) were significantly different with pelvic bone with ovariectomy ($P < 0.01$)

Crystallinity from XRD determines the crystal size, crystallinity index, and lattice parameter. The decrease of crystal size and crystallinity index is shown in Table 4. Crystal size in osteoporosis is smaller than that of normal bone without osteoporosis [22]. The decrease in crystal size about 9.29% indicates osteoporosis. When the crystal size decreases, bone density also decreases because the crystal size affects bone density [23]. The crystallinity index was obtained using FWHM of the (002) plane because it has a good resolution to determine the bone during maturity. The crystallinity index slowly decreased in the PO21 bone (Table 4). The decrease of

the crystallinity index about 25.18% indicates that the bone gradually develops osteoporosis [24].

The crystal structure of hydroxyapatite is hexagonal with lattice parameters, a = b = 9.432Å, c = 6.881 Å, and α = β = 90°, γ = 120°. The lattice parameters were obtained using *Highscore Plus* software (a = b ≠ c and α = β ≠ γ, Table 4). This table shows that the crystal structure of pelvic bones toward osteoporosis was hexagonal.

Conclusion

Rats with ovariectomy started to show first osteoporosis signs in the pelvic bone of rats that were euthanized at 17 weeks of age as indicated by the reduction and changes in phosphate and carbonate content about 3.45% and 2.01%, respectively. Moreover, the increase of hydroxyl functional groups about 40.8%, the reduction of mineral maturity about 7.47%, and the reduction of crystallinity about 29.26% were compared with those of the pelvic bone of non-ovariectomized rats. Furthermore, morphological changes from fine fibers to coarse fibers were observed in the pelvic bones of ovariectomized rats, and the crystal size and crystallinity index at the angle of 2θ ≅ 25° decreased by 9.29% and 25.18%, respectively.

Acknowledgements

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