Effects of Ovariectomy and Dieting with Nano Calcium Phosphate on Bone Quality in White Rats *Rattus norvegicus*

Neng Nenden Mulyaningsih[1,*], Ariadne Lakshmidevi Juwono[2], Djarwani Soeharso Soejoko[2] and Dewi Apri Astuti[3]

[1]Department of Physics Education, Faculty of Mathematics and Natural Sciences, Universitas Indraprasta PGRI, Jakarta 13760, Indonesia
[2]Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia
[3]Department of Nutrition and Feed Technology, Faculty of Animal Sciences, Bogor Agricultural University, Bogor 16680, Indonesia

([*]Corresponding author's e-mail: neng_nenden@unindra.ac.id)

Abstract

Calcium supplements are widely used to prevent osteopenia and osteoporosis and can be used as a treatment along with other drugs. The effect of this supplement can be seen from the improved bone quality. The focus in this study was to analyze the quality of femur bone based on the mineral maturity and the degree of crystallinity from ovariectomized *Rattus norvegicus* where the treatment of various nano calcium phosphate diets (1.0×; 1.5× and 2.0× normal needs, respectively called A, B and C diets) also given. In the amount of 40 white female rats age 12 weeks were ovariectomized and 5 sham rats were nurtured. The group of sham was given euthanasia treatment at age 13 weeks, while ovariectomized group was treated euthanasia at age 17 and 21 weeks. At the age of 25 weeks after ovariectomy, the rats were treated with a nano calcium phosphate diet where analysis was performed when they were 31 and 40 weeks old. In each harvest, proximal femur part was collected and characterized by using FTIR Spectroscopy. Rat bone quality shows osteoporosis trait for the first time at age 21 weeks or after 9 weeks of ovariectomy with the mineral maturity and degree of crystallinity decreased in the amount of 1.16 and 2.27 %, respectively compared to femur bone from sham rat group. The dietary treatment study conducted showed that the group of rats fed the A diet had not a significant changes ($p < 0.05$) in the mineral maturity and the degree of crystallinity, while giving B or C diet had a significant increase in the mineral maturity at 40 weeks of age or after 15 weeks on a diet. The increase in the degree of crystallinity of the rats fed B diet (6 weeks) was faster than the group fed C diet (15 weeks). Therefore, diet B is recommended because it is more efficient and effective based on the time it increases the degree of crystallinity and the cost of producing the diet.

Keywords: Ovariectomy, *Rattus norvegicus*, Bone quality, Mineral maturity, Nano calcium phosphate

Introduction

One of the factors which determines life quality of someone is their bone strength. Bone strength can become the factor of fracture risk and osteoporosis [1-3]. General parameter used to analyze bone strength is based on bone mineral density (BMD) and bone quality. Bone mineral density is the number of mineral in bone tissue, but BMD measurement is indirect showing as indicator of fructure risk occurance and osteoporosis [4,5]. Bone quality shows a composition combination and the bone structure which cover bone, turnover, mineralization, microarchitecture, microdamage, and the composition of bone matrix and mineral [6]. These parameters are difficult to be analyzed by using clinical measurement. Some new techniques to analyze the bone quality still continues to be developed.

Fourier Transform Infrared (FTIR) is a powerful technique for analyzing the traits of material and structural property distributions. Therefore, FTIR spectra can be used to analyze bone quality. FTIR spectroscopy in bone shows typical peaks such as the vibration of PO_4^{3-}, CO_3^{2-} and OH^- band. The band absorption of PO_4^{3-} in reach 700 - 500 cm^{-1} is group PO_4^{3-} vibration *bending* (v_4) and the reach 1200 - 900 cm^{-1} is PO_4^{3-} group with symmetry stretching vibration mode (v_1) and asymmetry stretching (v_3). Vibration spectroscopy of PO_4^{3-} at estimation of interval number between 653 - 495 cm^{-1} can give information of

bone crystallinity degree from its splitting factor (SF). Likewise with absorption band of CO_3^{2-} consisted of bending vibration mode (v_2) at the interval of wavenumber 900 - 850 cm^{-1} and asymmetry stretching vibration (v_3) at interval of wavenumber 1600 - 1300 cm^{-1}. Meanwhile OH$^-$ appears at wavenumber 3500 cm^{-1}. The band of CO_3^{2-} can replace PO_4^{3-} band generating apatite B-type CO_3^{2-}, beside that CO_3^{2-} band can also replace OH$^-$ band producing A-type CO_3^{2-} [7]. The parameter for measuring bone quality can be determined from crystallinity at ratio of wavenumber 1030/1020 or by counting the value of its SF at wavenumber between 650 - 450 cm^{-1}, mineral maturity at ratio of wavenumber 1030/1110, B-type CO_3^{2-} at wavenumber 871 cm^{-1}, A-type CO_3^{2-} at wavenumber 878 cm^{-1}, CO_3^{2-} band at wavenumber 890 - 850 cm^{-1}, PO_4^{3-} band at wavenumber 1200 - 900 cm^{-1} and CO_3^{2-} to PO_4^{3-} ratio [8,9].

Even though there have been many previous research that analyzed the bone by using FTIR Spectroscopy, however it is still difficult to find the research which discusses the process of osteoporosis occurance periodically. The previous research had used FTIR in analyzing the mineral content of cow bone [10], the function group on synthetic hydroxyapatite [11], and bone structure that was given collagen to it [12,13]. Therefore, this research will observe the characteristic of femur bone quality by using FTIR of ovariectomized rat until showing osteoporosis trait for the first time. After the rats were confirmed to have osteoporosis, the nano calcium phosphate diet treatment was started. The renewal of this research is the analysis carried out during the stage leading to osteoporosis where the recovery process was conducted step by step. Provision of nano calcium phosphate in the form of nano also has advantages in the absorption process by the body. In this study, we focused on analyzing the mineral maturity and the degree of crystallinity of ovariectomied rats and osteoporosis rats treated with nano calcium phosphate diet conducted periodically. The degree of crystallinity, splitting factor and Ca/P ratio obtained from Energy Dispersive Spectroscopy (EDS) were also analyzed where the results were then used as supporting data in drawing conclusions.

Materials and methods

Bone sample preparation

In this study, 45 female rats (*Rattus norvegicus*) aged 12 weeks were used. The amount of 40 female white rats were ovariectomized and 5 sham rats were nurtured as the comparison. In this study, 9 treatment groups were analyzed, namely the sham group at 13 weeks of age, the ovariectomized group of rats at 17 and 21 weeks of age, and the osteoporosis group of rats treated with A, B and C diets at the age of 31 and 40 weeks, respectively. By using Federer's formula [14], the number of samples for each group was at least 3, in this study 5 rats were used for each group. Ovariectomy or sham was peformed as previously described [15]. The rats used in this research were obtained from The National Agency of Food and Drug Control (BPOM) Jakarta. The usage of rat as the model animal has obtained agreement from the ethic committee of Medical Faculty of Universitas Indonesia with protocol number 17-05-0421. Sham rat group was treated euthanasia at age 13 weeks and their femur bone part was taken, this group was given code FS13. The ovariectomized rat group was also treated euthanasia at age 17 and 21 weeks, then their femur bone parts were also collected and each given code FO17 and FO21. During the stage leading to osteoporosis, the rats were given a standard diet. Starting at 25 weeks of age, the ovariectomized group of rats were given a varied nano calcium phosphate diet (diets A, B and C). Furthermore, the rats were euthanized at the age of 31 and 40 weeks and were coded FDA31/FDB31/FDC31 for the group of rats euthanized at 31 weeks of age and FDA40/FDB40/FDC40 for the group of rats euthanized at 40 weeks of age.

Rats were housed under standard conditions of temperature (27.75 ± 0.66 °C) and relative humidity (57.37 ± 1.48 %) on 24 h light with *ad libitum* access to tap water. Rats were placed in individual cages measuring 40×30×15 cm^3 made of plastic with a cover made of wire and covered with rice husks or sawdust. The replacement of sawdust or rice husks was carried out once a week along with cleaning the cages, eating areas and drinking places for rats.

During the bone preparation, the rats were anesthetized with ketamine (40 - 80 mg kg^{-1}) and xylazine (5 mg kg^{-1}) until the heart rate stopped. The femur was removed after the rat died. The muscles in this part of the bone were also removed/dissected were then dried at 60 °C for 24 h. Deproteinization of femur bone was conducted by immersion of femur bones in hydrazin hydrate (100 % N_2H_5OH) for 14 days in room temperature (28.41 ± 0.50 °C). After soaking with hydrazine hydrate, the bones were soaked using 50 % alcohol for 1 h and rinsed using ethanol and distilled water 3 times. Next, the heat-treated bone were performed in the furnace at a temperature of 110 °C for 24 h. Then, the proximal part of femur bone was particularly characterized using FTIR Spectroscopy.

Diet preparation

The diet for this study was made into 2 types, namely the standard diet and the nano calcium phosphate diet. The composition of the standard diet and nano calcium phosphate diet was made by referring to the method used by Mulyaningsih *et al.* [16]. The nano calcium phosphate diet was varied based on its nano calcium content, namely diets with nano calcium content of 1.0×, 1.5× and 2.0× normal requirements and named A, B and C diets respectively. Calcium requirement for normal growing rats is 0.4 - 0.5 % w/w [17]. All diets were made in pellets with an average length of 1.0 cm and a diameter of 0.5 cm.

Assessment by FTIR spectroscopy

For the purposes of an FTIR spectrophotometer, the bone samples were crushed using a mortar until they became powder and then separated by a weight of 2.5 mg in which 250 mg of potassium bromide (KBr) was also added. This sample was then homogenized using a mortar and pestle. After the sample was homogeneous, the sample was molded into pellets under pressure up to 8 tonnes and analyzed. The measurement was conducted at reach of wavenumber between 4000 until 400 cm^{-1} on a IR Prestige-21 by Shimadzu. The OriginLab 9.1 software was used for all FTIR data manipulations.

Referring to Kimura-Suda method [18], the mineral maturity was calculated from the ratio of PO_4^{3-} band absorption at wavenumber 1030 cm^{-1} with PO_4^{3-} band absorption at wavenumber 1110 cm^{-1}, while the crystallinity was determined by distributing the absorption of PO_4^{3-} band at wavenumber 1030 cm^{-1} with absorption of PO_4^{3-} band at wavenumber 1020 cm^{-1}. From the result of characterization with FTIR, its splitting factor (SF) was also calculated and compared to Ca/P ratio obtained from Energy Dispersive Spectroscopy (EDS).

Splitting factor as an approach to the value of the degree of crystallinity was measured by referring to the method proposed by Sasso *et al.* [19], by adding the height of the 2 anti-symmetric phosphate peaks (wavenumbers range 603 and 563 cm^{-1}) and dividing that value by the height of the trough between the 2 crests. All heights were measured above the *baseline* and drawn from a wavenumber of 650 to 450 cm^{-1}. Meanwhile, to obtain the value of the Ca/P ratio, this was done by capturing and processing the signal from X-ray fluorescence emitted by a small volume of the specimen surface known as the Energy Dispersive Spectroscopy (EDS) technique. The EDS used in this study was the JED-2300 analysis station JEOL brand with measurement specifications as follows: System resolution 61 eV, geometry (degrees) tilt = 0.00, ED geometry elevation = 18.00, azimuth 0.00, entry angle 0.00, accelerating voltage 20.00 kV and quantitative the ZAF method.

Statistics analysis

Data analysis were expressed as means ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Duncan multiple comparisons test for post hoc analysis. All statistical analyses were performed using the statistical package for social science (SPSS) (IBM SPSS statistics for windows, software version 24.0). The value of $p < 0.05$ was considered statistically significant.

Results and discussion

Bone quality based on PO_4^{3-} band at wavenumber 1200 - 900 cm^{-1}

As mentioned beforehand that there have been many research that analyzed the bone quality by using FTIR. Some parameters that had been measured were the mineral-to-matrix ratio, crystallinity, collagen cross-links, and orientation of collagen fibers [20]. However, what has been done in this study is never been conducted before which reviews the process of osteoporosis occurance that was done periodically from ovariectomized rat. Therefore, it is important to analyze and evaluate the characteristics of FTIR Spectra for determining the quality of bone from time to time. In this study, either bone from ovariectomized rat bone or bone from rats treated with the different diets in femoral proximal bone has been characterized by using FTIR and PO_4^{3-} band, regarding the mineral component.

The quality of femur bone from ovariectomized rats and treated with the different diets have been characterized by using FTIR spectroscopy. The trailer of FTIR spectra for PO_4^{3-} band at wavenumber 1200 - 900 cm^{-1} are normalized as showed in **Figure 1**. Based on **Figure 1**, it can be analyzed the difference between mineral maturity, crystallinity, shape and height of PO_4^{3-} band from rats developing osteoporosis and rats treated with the different diets. There is difference in the height of bands at 1110, 1030 and 1020 cm^{-1} between sham rat and ovariectomized rat. The width of the PO_4^{3-} band of the ovariectomized rats (FO17 and FO21) were wider than that of the sham rat (FS13) as showed in **Figure**

1(a) PO_4^{3-} bandwidth absorption at FO21 was also higher when compared to FO17. This causes the ovariectomized rats to experience a decrease in mineral maturity (1030/1110 cm^{-1}). The mineral maturity of the sham (FS13), FO17 and FO21 groups were 1.119 ± 0.003, 1.110 ± 0.003 and 1.106 ± 0.004, respectively. In the FO17 and FO21 group, the decline in mineral maturity were 0.80 and 1.16 %, respectively compared to the sham group. The mineral maturity in bones has dynamic properties, but it cannot be greater than 1 % [21]. If the decrease is more than 1 %, then the bone quality has decreased which is the beginning of osteoporosis. The decrease in mineral maturity is caused by variations in the composition of calcium phosphate and crystal size [22,23]. In the FO17 group the decline in mineral maturity was still below the threshold. Therefore, in this group, there is no mention of osteoporosis. As calculation from the time of ovariectomy at 12 weeks of age, one of the characteristics of osteoporosis would be observed in the FO21 group is after 9 weeks of ovariectomy.

There was no difference in the height of the bands at 1030 and 1020 cm^{-1} between the FS13 and FO17. However, there was a difference in the FO21 group, where the peak of FO21 was steeper, so the degree of crystallinity at FO21 was lower (1030/1020 cm^{-1}). The decrease in the degree of crystallinity in the FO21 group compared to FS13 was 2.27 %. A low degree of crystallinity makes bones more brittle because bones tend to be amorphous.

The bandwidth of PO_4^{3-} at wave numbers 1200 - 900 cm^{-1} of FTIR spectra from ovariectomized rat group was slightly wider than the normal rats, this occurs because there are calcium phosphate variations and crystal size [24,25]. The loss of phosphate group v_I for FO21 group caused the bone fragile. The peak of PO_4^{3-} is lower than ovariectomized rat which also shows sensitive mineral bone properties either towards crystallinity (1030/1020 cm^{-1}) or towards mineral maturity (1030/1110 cm^{-1}). At this FTIR measurement, the case of determining the crystallinity and mineral maturity were done separately, however the result obtained was proportional with crystallinity index and mineral maturity in synthetic apatite [26,27]. Crystallinity is an important trait based on the crystal size [28-30], while mineral maturity relates to the change of non-apatite mineral becoming apatite [31]. The absorption process of infrared spectra can only happen if there is change of value and direction of molecul dipole moment, therefore FTIR spectra is influenced by this composition change of phosphat and calcium during the apatite transformation of crystal of non-stoichiometric to be apatite stoichimetric. In short, the longer the time taken for ovariectomy, then the mineral maturity and cristallinity will get decreased.

The group of rats that treated with nano calcium phosphate diet showed the opposite result, compared with a group of rats that were on their way to osteoporosis. The bandwidth of PO_4^{3-} from FDA40 rat group was tighter than that of the FDA31 rat. These results indicate that the longer the nano calcium phosphate diet is given, the osteoporosis bone will experience improvement through increased mineral maturity. However, in the group of rats that were given diet A (**Figure 1(b)**), after the osteoporotic rats were fed a diet until the age of 40 weeks the PO_4^{3-} bandwith was wide, so that the increase in mineral maturity had not returned to normal (sham group). Likewise, the degree of crystallinity was still lower than the control group and still showed a significant difference at $p < 0.05$. This shows that in the recovery of osteoporosis, the nano calcium phosphate diet that is consumed cannot be compared to the diet consumed by rats under normal conditions. This is different from the group of osteoporotic rats that were given diet B or diet C (**Figures 1(c) and 1(d)**) where at the age of 31 weeks, the bandwith of PO_4^{3-} was still wide, but at the age of 40 weeks the bandwidth of PO_4^{3-} was almost in contact with the PO_4^{3-} bandwith of control group (FS13). At the age of 40 weeks, both of the groups of rats that were given diet B or diet C had no significant difference ($p < 0.05$) in mineral maturity and crystallinity levels compared with the control group. In conclusion, at the age of 40 or 15 weeks after being given nano calcium phosphate diet, ovariectomized osteoporosis rats have experienced bone repair based on the increase in mineral maturity which back to normal. Likewise, the degree of crystallinity also increased significantly as shown in **Table 1**.
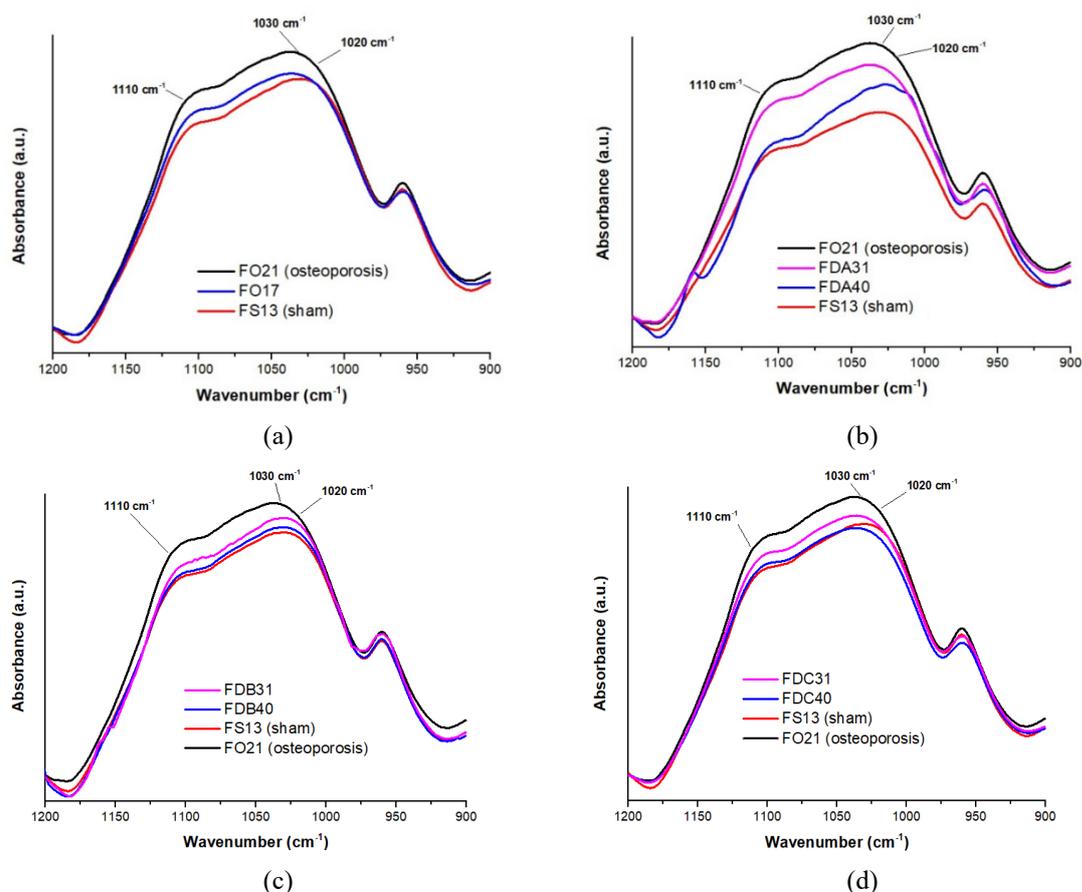
Figure 1 Spectral region of the PO_4^{3-} bands from femur of sham rat and ovariectomized rat. (a) rats developing osteoporosis and (b) - (d) rats treated with the different diets. FS13: Rat femur control (sham group) at age 13 weeks, FO17 and FO21: Femur bone at age 17 and 21 weeks (ovariectomy group), FDA31/FDB31/FDC31 and FDA40/FDB40/FDC40: Femur bone at age 31 and 40 weeks (treated with the A/B/C diet).

Table 1 The mineral maturity and crystallinity of femur bone from rats grouped by osteoporosis status and diet treatment stage (mean ± SD, SD = standard deviation, n = 5).

Group	Parameter	
	Mineral maturity (1030/1110 cm^{-1})	**Crystallinity (1030/1020 cm^{-1})**
FS13	1.119 ± 0.003^a	1.055 ± 0.002^a
FO17	1.110 ± 0.003^b	1.053 ± 0.003^a
FO21	1.106 ± 0.004^c	1.031 ± 0.002^b
FDA31	1.106 ± 0.002^c	1.040 ± 0.005^b
FDA40	1.113 ± 0.003^b	1.042 ± 0.003^b
FDB31	1.107 ± 0.003^c	1.053 ± 0.003^a
FDB40	1.119 ± 0.004^a	1.056 ± 0.004^a
FDC31	1.103 ± 0.003^c	1.037 ± 0.003^b
FDC40	1.119 ± 0.003^a	1.058 ± 0.002^a

FS13: Rat femur control (sham group) at age 13 weeks, FO17 and FO21: Femur bone at age 17 and 21 weeks (ovariectomy group), FDA31/FDB31/FDC31 and FDA40/FDB40/FDC40: Femur bone at age 31 and 40 weeks (treated with the A/B/C diet). Different lowercase superscripts in the same column show significant differences ($p < 0.05$).

Table 1 shows that the group of rats treated with nano calcium phosphate types A, B, and C at the age of 31 weeks each had mineral maturity values of 1.106 ± 0.002, 1.107 ± 0.003, and 1.103 ± 0.003, these 3 values showed different values significant ($p < 0.05$) compared to the mineral maturity of the control rat group 1.119 ± 0.003. This means that at the age of the rats 31 weeks or 6 weeks given the diet, for all types have not been able to restore bone mineral maturity to normal conditions. At the same age, namely, 31 weeks, the degrees of bone crystallinity of the rats fed A, B, and C diets were 1.040 ± 0.005, 1.053 ± 0.003, and 1.037 ± 0.003, respectively. When compared with the control group, only the rat's given B diet showed no significant difference in values to the control ($p < 0.05$). This means that 6 weeks of B diet was able to restore the degree of crystallinity, but giving A or C diet was not able to restore the degree of crystallinity during that time.

At the age of the rats 40 or 15 weeks of diet, mineral maturity (1.113 ± 0.003) and crystallinity (1.042 ± 0.003) of the rats consuming A diet, were also not able to increase their values like the normal group. Therefore diet A is not recommended for consumption for the recovery process of osteoporosis due to ovariectomy. Meanwhile, in the group of rats that were given B or C diet, at the age of 40 weeks each showed mineral maturity values (1.119 ± 0.004) and (1.119 ± 0.003) which were not significantly different from the mineral maturity of the control group (1.119 ± 0.003). Likewise, the value of the degree of crystallinity, namely 1.056 ± 0.004 and 1.058 ± 0.002, respectively, from the group of rats treated with B and C diets. These values also did not show a significant difference ($p < 0.05$) with the crystallinity degree values of the control group of 1.055 ± 0.002.

In short, a comparison of PO_4^{3-} bands in FTIR spectra at the wave number 1200 - 900 cm^{-1} from rats developing osteoporosis and rats treated with the different diets revealed a difference in mineral maturity and crystallinity. Ovariectomy causes mineral maturity and crystallinity to decrease, while the nano calcium phosphate diet can increase mineral maturity and crystallinity again. However, giving A diet until the rats were 40 weeks old has not been able to restore the mineral maturity and crystallinity condition to a normal state. Meanwhile, the provision of B or C diet until the age of the rats were 40 weeks was able to restore the mineral maturity and crystallinity of the femoral bone condition to a normal state. The nano calcium phosphate diets types B and C have the same effect at 40 weeks, but diet B more rapidly increased the degree of crystallinity. Therefore, if it is considered in terms of the osteoporosis recovery process, B diet is recommended because it is more efficient and effective based on the cost of producing the diet. Previous researchers have also shown that giving a nano calcium phosphate diet with a level of $1.5\times$ (B diet) the normal requirement results in calcium absorption in the body of 68.07% [16]. This value was higher than the nano calcium phosphate diet $1.0\times$ or $2.0\times$ the normal requirement.

In this study, a sample of 5 rats was used for each group, as stated in the research method, the number of samples used met Federer's formula. Although the samples were dynamic living things, the homogeneity in each group was quite high. That is could be seen from the standard deviation value which is quite low so that the repetition of each experiment was relatively accurate and the data was statistically reliable.

Bone quality based on its splitting factor
Besides determination the ratio of PO_4^{3-} bandwith absorption at wavenumber 1030 cm^{-1} with PO_4^{3-} bandwith absorption at wavenumber 1020 cm^{-1}, crystallinity index can also be determined based on the value of the splitting factor (SF) [32]. The crystallinity that was analyzed based on its SF at wavenumber between 650 - 450 cm^{-1} showed that ovariectomized rat experienced the decrease of SF value. This low SF value showed that the crystallinity also decreased. The low crystallinity index in the ovariectomized group of rats was caused by the immature crystal size [33]. It has been known that the FTIR spectrum in bone is influenced by changes in bone mineral composition, such as calcium phosphate. Thus, the crystallinity index is also actually related to the Ca/P ratio. The value of the Ca/P ratio is the ratio of the mineral calcium (Ca) and phosphorus (P) which is the parameter for the crystal phase of the hydroxyapatite compound in the bone. The crystal phase of pure hydroxyapatite has a Ca/P ratio of 1.67 [34]. If the Ca/P ratio is higher than 1.67, it means that the P level is low and the bone crystals are large. The large crystal sizes tend to be more porous [35]. The correlation between SF and Ca/P obtained from the analysis by using Energy Dispersive Spectroscopy (EDS) method overall shows contradictory value as showed in **Figure 2**. When SF value decreases, the Ca/P comparison increases. Comparison score of Ca/P obtained for sham rat (FS13) is 1.47 and for ovariectomized rat increases 1.55 until 1.76. At the ninth week since ovariectomy (FO21), its Ca/P score is 1.76. The higher value of Ca/P ration indicates that *p*-value is low. This condition was powerfully supported by the decrease of the phosphate absorption band at the osteoporosis rat group (FO21) as shown in **Figure 3(a)**, namely FTIR spectra samples at a wavenumber between 930 - 480 cm^{-1}. The low *p*-value is due to a decrease of the phosphate group (PO_4^{3-})

accompanied by the appearance of a carbonate group (CO_3^{2-}). The presence of carbon in the form of the carbonate ions could replace phosphate ions becomes apatite crystals of type B, resulting in a decrease the levels of P. **Figure 3(a)** shows that the carbonate group at the wavenumber 723 cm^{-1} was clearly visible in the FO21 group, namely the v_4 CO_3^{2-} type. This carbonate group is metastable [36], in addition, the presence of carbonate ions in the bone can be as an inhibitor, which can interfere with the bone remodeling process.

Giving nano calcium phosphate diet until age of 40 weeks, the SF value thoroughly could thoroughly increase. However, the SF value of the rat group given A diet was still low if compared to the control one (**Figure 2(a)**). But the rat group given diet B or C shows that at the age of 40 weeks, their SF value achieved towards normal score. Likewise with the Ca/P ratio, in the rat group given diet A the value is still fluctuative, meanwhile the Ca/P ratios of the rat groups supplemented by B or C diet evidently decreased again. The decrease of Ca/P value occurs because phosphate group increases/acccumulates again. The absorption of PO_4^{3-} band in FTIR spectra is influenced by the composition change and size of phospate calcium in the bone. Therefore, FTIR spectroscopy sufficiently represents in analyzing bone quality especially mineral maturity.

Likewise, the data for carbonate and phosphate bands in the wavenumber range 930 - 480 cm^{-1} supports the Ca/P scores of the diet-treated group rats. **Figure 3(b)** shows the FTIR spectra of the group of rat that were given A diet, the v_4 CO_3^{2-} the band was still clearly visible in both the FDA31 and FDA40 groups so that more phosphate ion was substituted. This is different from the groups of rats given diet B or C as shown in **Figures 3(c)** and **3(d)**, at the age of 40 weeks the v_4 CO_3^{2-} the band had decreased even though it did not completely disappear phosphate absorption band increased again. These results reinforce the claim that calcium phosphate in bone is present in the form of apatite carbonate. The v_4 PO_4^{3-} absorption band for the FDB40 group was higher than that of the FDC40 group. This is another reason recommending the B diet. The C diet is less effective because the calcium levels in the diet were higher than diet B but the effect was the same. The excess calcium consumed will be excreted through feces as has been shown by previous researchers.

(a)

(b)

(c)
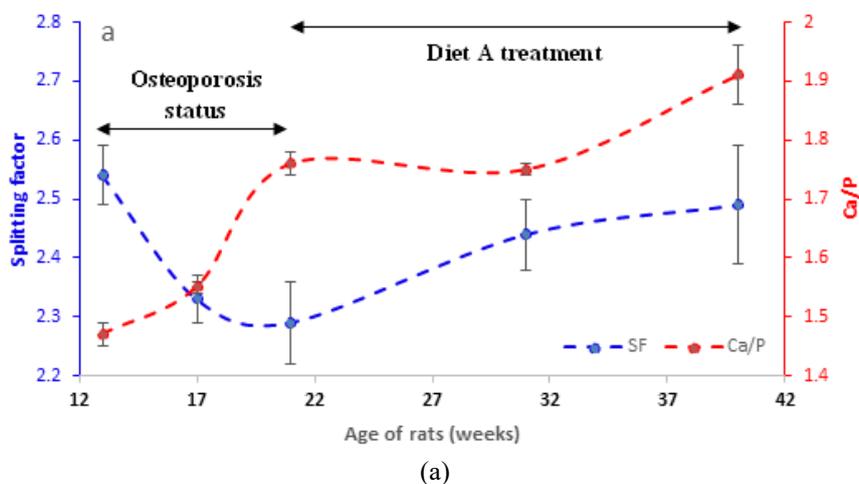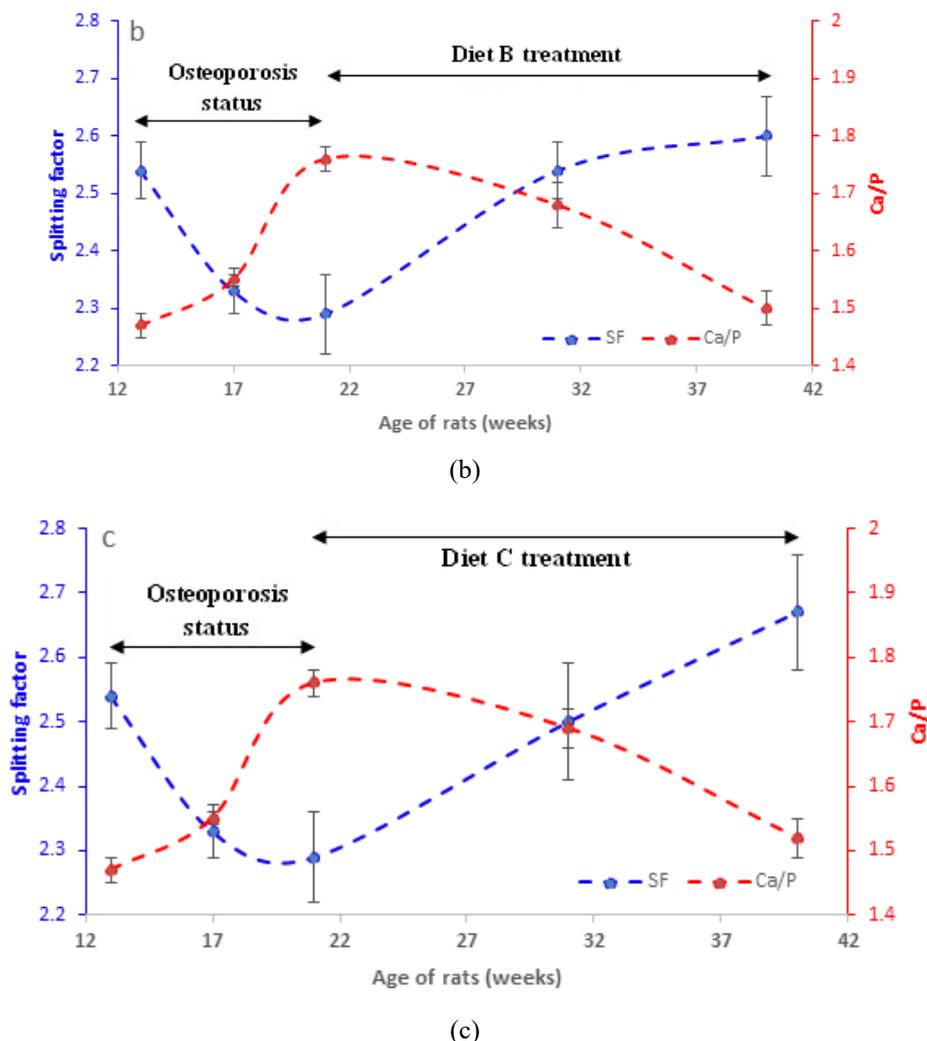
Figure 2 The correlation between splitting factor (SF) and Ca/P ratio of femur bones from rats defined by osteoporosis status and diet treatment stage (a) diet A, (b) diet B, and (c) diet C.

The v_2 CO_3^{2-} groups in the range of wavenumber 875 cm^{-1} for all groups, both the ovariectomized group of rats on the way to osteoporosis and the group of osteoporosis rats treated with diet showed no significant difference. This peak is the carbonate absorption band in the bending vibration mode (v_2) which causes changes in the bond angle between the 2 bonds. The v_2 CO_3^{2-} group is labile and is always present in the FTIR spectrum of bone. Over time, v_2 CO_3^{2-} can enter the crystal lattice through the mineral crystal growth and the carbonate substitution to phosphate, hydroxide, and labile lattice sites in existing minerals [37].
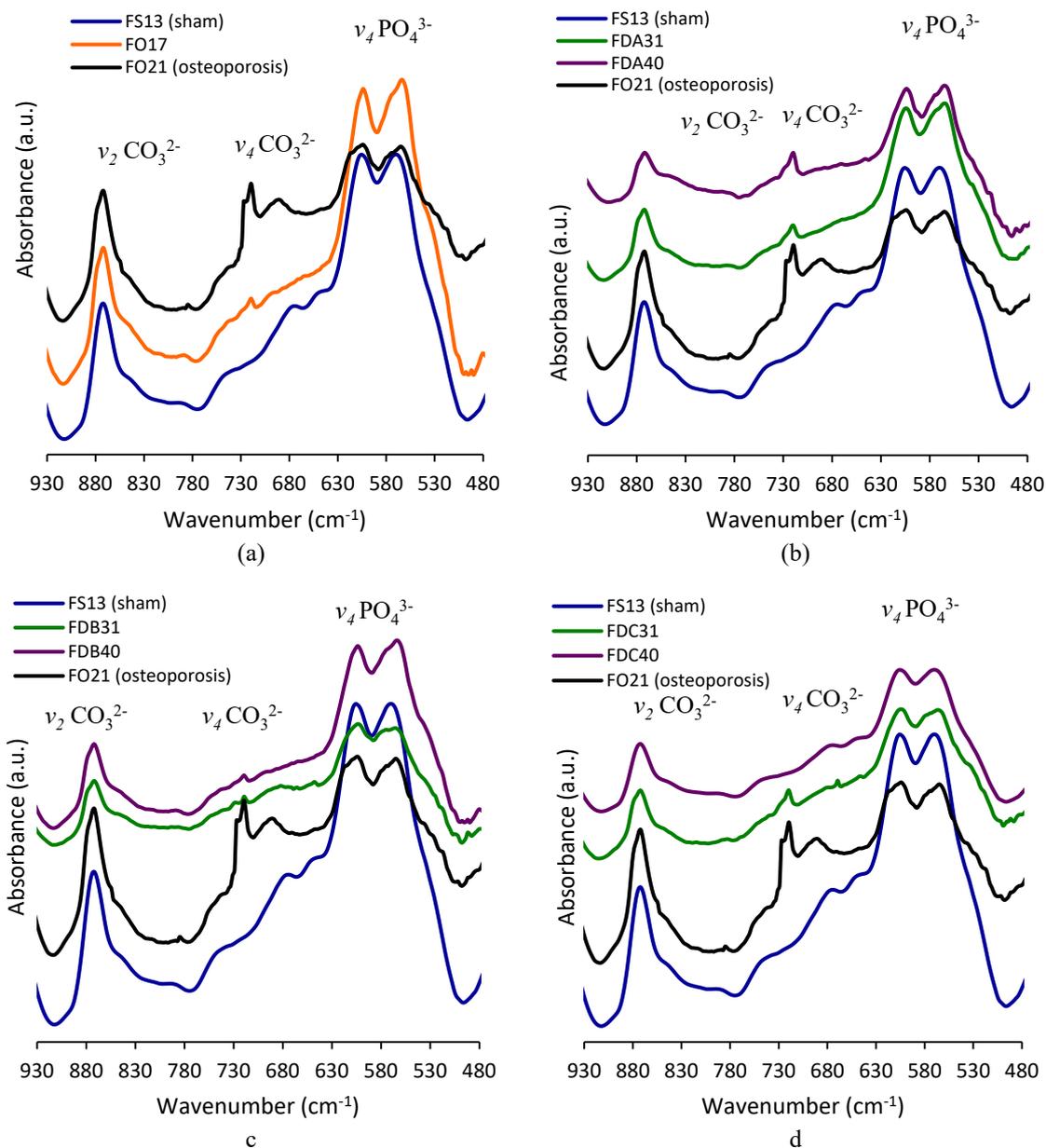
Figure 3 Spectral region of the PO_4^{3-} and CO_3^{2-} bands from femur of sham rat and ovariectomized rat. (a) rats developing osteoporosis and (b) - (d) rats treated with the different diets. FS13: Rat femur control (sham group) at age 13 weeks, FO17 and FO21: Femur bone at age 17 and 21 weeks (ovariectomy group), FDA31/FDB31/FDC31 and FDA40/FDB40/FDC40: Femur bone at age 31 and 40 weeks (treated with the A/B/C diet).

Conclusions

The results of this study have succeeded in analyzing bone quality, especially based on the parameters of bone mineral maturity and the degree of crystallinity from FTIR spectra in ovariectomized rats and treated with nano calcium phosphate diet which was carried out periodically. The mineral maturity or the degree of crystallinity is a complex outcome that reflects both crystal size and the presence of phosphate or carbonate groups in bone. The quality of femur rat bone, based on the parameter of mineral maturity and the degree of crystallinity, firstly shows the signs of osteoporosis after 9 weeks of ovariectomy. As a result, the mineral maturity and the degree of crystallinity decreased 1.16 and 2.27 %, respectively compared to femur bone from the sham rat group. Provision of nano calcium phosphate diet with the nano calcium content of at least 1.5× of normal requirement can increase mineral maturity and the degree of crystallinity back to normal after osteoporotic rats consume the diet for at least 15 weeks.

Acknowledgements

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