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Research Article
Serum Mineral Status and Long Bone Morphometry of Ovariectomized Rats Fed a Nano-Calcium Phosphate Diet

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Abstract

Background and Objective: Serum calcium (Ca) is maintained at a balanced level under normal circumstances by a homeostatic system. When the serum Ca level is high, the excess will be deposited in the bone; however, if the serum Ca level is low, Ca will be resorbed from the bone and Ca absorption in the intestine will be increased. As a result, serum Ca metabolism can affect bone morphometry characteristics and Ca status in the body. The aim of this study was to investigate the effect of a nano-calcium phosphate diet on osteoporotic rats after ovariectomy. Experiments were conducted to analyse the serum response to Ca in rats that were ovariectomized and treated with a nano-calcium phosphate diet; additionally, the relationship between the diet and both the bone morphometry characteristics and Ca status in the body was examined. **Materials and Methods:** Female rats (*Rattus norvegicus*) aged 12 weeks were ovariectomized or acted as a control (non-ovariectomized rats). Euthanasia was carried out on 13-week-old control rats and ovariectomized (OVX) rats at the ages of 15, 17, 19 and 21 weeks. The 21-week-old OVX rats were divided into 3 groups and were given a nano-calcium phosphate diet containing a nano-Ca content of 0.10% (diet A), 0.40% (diet B) or 0.70% (diet C). At the ages of 27, 29, 31, 33 and 40 weeks, euthanasia was carried out for the collection of the serum, femur and tibia. The serum mineral levels [calcium (Ca), phosphorus (P) and magnesium (Mg)], morphometric characteristics (mass, mass density, length and diameter) of the long bones and Ca status in the body were analysed. **Results:** The results showed that in the ovariectomized rats, serum Ca and P levels decreased at week 7, while the Mg levels fluctuated. Treatment with the 0.40% nano-Ca diet could increase serum Ca levels from the 6th week of diet administration (age 27 weeks). The overall femoral morphometry and tibia characteristics, in addition to the mass density data, showed values that increased with age. The highest Ca absorption was shown by the OVX rats that consumed the 0.40% nano-Ca diet. The difference between the consumed and absorbed amounts of Ca was shown by the Ca content in the faeces, which averaged 68.07% for calcium intake. **Conclusion:** The female *Rattus norvegicus* white rats exhibited osteoporosis based on serum mineral status seven weeks post-ovariectomy. The effects of the nano-calcium phosphate diet were first observed in the sixth week of diet administration.

Key words: Femur, minerals, morphometry, nano-calcium, *Rattus norvegicus*, serum calcium, serum, tibia

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many studies have discussed the effects of reduced oestrogen hormone levels in the body as a result of ovariectomy (OVX). Reduced oestrogen levels can cause changes in gene expression[1], risk of fracture[2], cancer[3], sarcopenia and dynapenia[4] and tumours[5]. Moreover, the decrease in oestrogen levels causes a decrease in calcium absorption in the intestine, increases calcium excretion by the kidney[6-8], increases bone resorption by osteoclasts, inhibits osteoblast activity[9-11] and increases bone calcium resorption and the loss of bone mass[12,13]. The decline in oestrogen levels during menopause is often associated with increased bone resorption[14], decreased bone density[15,16] and a high risk of fracture[17]. These conditions cause the bones to become increasingly fragile because of the decreased bone density. As a result, bone density decreases and the risk of bone fractures increases, resulting in osteoporosis.

Decreasing oestrogen levels can also occur at a young age and one of the causes of this decrease is OVX. Oestrogen regulates the menstrual cycle[18] and bone remodelling balance[19]. Therefore, OVX also triggers osteoporosis.

However, how soon the symptoms of diseases, such as osteoporosis, begin as a result of a lack of oestrogen after OVX is still unclear. Some researchers have previously shown that OVX results in osteoporosis at least 18 weeks after surgery[20]. Other researchers have shown that in the 6th week after OVX, osteoporosis occurs in the form of a cortical bone vascular cavity, the proximal tibia tissue mineral density and bone volume fraction decrease and the number of trabeculae in the proximal tibia also decrease[21]. In another study, rats showed characteristics of osteoporosis in the form of a decrease in bone mineral density (BMD) in the maxilla and femur12 weeks after OVX[22]. Although, the long-term effects of OVX have been studied, the results vary.

In addition, OVX-associated treatment of osteoporosis varies and research on the treatment of osteoporosis with calcium carbonate has been conducted. Calcium carbonate can increase calcium levels in the bone but causes the bones to become crystallised and results in a disturbance in the digestive tract[23]. Other researchers have treated osteoporosis with bisphosphonates and hormone replacement therapy (HRT) and the results showed that administration of bisphosphonates and HRT can treat osteoporosis but can cause breast dysplasia[24]. Recently, researchers have recommended a diet containing 700-1200 mg of calcium per day[25]. However, whether dietary intervention can treat osteoporosis has not been reported due to a lack of strong evidence; therefore, this approach is not generally approved. Similarly, the mechanism of absorption of calcium from the diet has not been explained. The time needed for osteoporosis to develop after OVX remains unclear and no studies have used a nano-calcium phosphate diet as a supplement to treat OVX-associated osteoporosis while measuring calcium absorption. Therefore, in this study, the time needed for rats to develop osteoporosis after OVX was investigated. The OVX rats were used as an oestrogen-deficiency model that resembles menopausal women[26]. In addition, the effect of the nano-calcium phosphate diet on osteoporosis caused by OVX and the percentage of absorption in the body were assessed. In the present study, ultraviolet-visible (UV-vis) spectroscopy was used to assess both the decline in serum mineral levels in OVX rats as a result of osteoporosis and the serum mineral levels in rats that were given the nano-calcium phosphate diet treatment. The current study thus contributes to the discovery of possible nano-calcium phosphate levels that are effective and efficient for the treatment of osteoporosis.

MATERIALS AND METHODS

Animals: The main research animals in the study were 12-week-old (40) female white rats (*Rattus norvegicus*), which were obtained from the Food and Drug Administration (BPOM) in Jakarta. Ten rats were given a standard diet and 30 rats were given nano-calcium phosphate diet each of 10 animals for A, B and C diets. The use of rats as model animals was approved by the ethics committee at the Faculty of Medicine, Universitas Indonesia, with protocol number 17-05-0421. After OVX, the rats were allowed to adapt for 1 week and then were housed individually at room temperature (26.5°C) under 24 h lighting. Normal (non-OVX) 13-week-old rats were used as a control. Every two weeks after the adaptation period (starting at the age of 15 weeks), the OVX rats were analysed for decreasing levels of calcium, phosphorus and magnesium in the serum and long bone morphometry measurements were performed in terms of mass, mass density, length and diameter. The long bone samples used were obtained from the femur and tibia.

Research procedure: The study was divided into two stages: Process of osteoporosis development and diet treatment. The animals were divided into two groups: Un operated control rats (non-OVX rats) and ovariectomized rats (OVX rats). The non-OVX rats were sacrificed at 13 weeks of age and their serum and long bones were dissected. The rats ovariectomized at 12 weeks of age were sacrificed at 15, 17,

19 and 21 weeks and the group stage of osteoporosis development was identified. During the osteoporosis development stage, the rats were given a standard diet (S). The remaining OVX rats were divided into 3 groups at 21 week of age and were given various nano-calcium phosphate diet treatments. At the ages of 27, 29, 31, 33 and 40 weeks, each group was euthanised and the diet treatment group was identified. Euthanization was carried out for the collection of the serum, femur and tibia. All rat treatments were carried out in accordance with the standards of the ethics committee of Universitas Indonesia.

The analysed parameters were serum mineral levels (calcium, phosphorus and magnesium), which were measured with UV-vis spectroscopy; conventional bone morphometry (mass, mass density, length and diameter), which was assessed with callipers and an analytic balance; and calcium absorption in the bodies of rats, which was calculated based on the difference between calcium consumed and calcium found in the stool. Calcium intake was calculated as the amount of dietary dry matter consumed (g day^{-1}) multiplied by the percentage of dietary calcium levels from the analysis. Dry matter consumption was calculated daily by weighing the provided food, subtracting the weight of the remaining food (g day^{-1}) and multiplying the resulting value by the percentage of dietary matter from the proximate analysis. Stool calcium was analysed by the dry dewatering method and the results were obtained using atomic absorption spectrometry (AAS) to determine the calcium concentration. The sample preparation method used for mineral analysis was based on the Association of Official Analytical Chemists[27].

The compositions of the standard and nano-calcium phosphate diets are shown in Table 1 and 2, respectively. The standard diet was obtained commercially[28], while the nano-calcium phosphate diet was made with the dietary modifications described by Astuti[29]. The mineral content of the diet was measured by AAS and UV-vis, while the nutritional content was measured by proximate analysis. Three types of nano-calcium phosphate diets were prepared, namely, 0.25% w/w nano-calcium phosphate (diet A), 1.00% w/w nano-calcium phosphate (diet B) and 1.80% w/w nano-calcium phosphate (diet C). Based on atomic comparison, the nano-calcium contents of the three diets were 0.10% (diet A), 0.40% (diet B) and 0.70% (diet C). In accordance with the recommendations of the National Research Council, the maximum daily amount of feed provided to each rat was 14 g[30].

Table 1: Composition of a standard diet

Composition	Percentage (% w/w)
Protein	13-15
Fat	3-7
Fibre	8
Ash	12
Calcium	1-3
Phosphorous	0.7-1.5
Humidity	12

Table 2: Composition of the nano-calcium phosphate diet in every 100 g

	Composition percentage (% w/w)		
Composition	A	B	C
Rice flour	25.06	25.00	25.00
Casein	18.05	18.00	18.00
Corn oil	3.51	3.50	3.50
Glucose	49.12	48.50	47.70
DL-methionine	0.30	0.30	0.30
Carboxymethyl cellulose	3.01	3.00	3.00
Nano-calcium phosphate	0.25	1.00	1.80
Vitamin mix	0.50	0.50	0.50
NaCl	0.20	0.20	0.20
Total	100.00	100.00	100.00

A: Diet with 0.10% nano-calcium, B: Diet with 0.40% nano-calcium, C: Diet with 0.70% nano-calcium

Statistical analysis: Statistical analysis was performed using one-way ANOVA followed by a t-test. A p value of <0.01 was considered statistically significant. All research results are presented as mean values ± standard deviations.

RESULTS

Characterisation of the Nano-Calcium Phosphate Diet: Table 3 shows the percentage of calcium (Ca), magnesium (Mg) and phosphorus (P) in samples of diets A, B and C. The total calcium contents for diets A, B and C were 0.40, 0.62 and 0.99%, respectively. In addition to calcium, phosphorus and magnesium are also required minerals for rats. The levels of these two minerals were proportional to the calcium levels: when the calcium content in the diet was increased, the phosphorus and magnesium contents also increased.

The results of the above characterisations indicate that the diets contained total calcium contents of 1.0, 1.5 and 2.0× that of the normal requirements. The contents of other nutrients in the diets were determined based on proximate analysis results, which show that the percentages of dry matter in the three types of diets were almost the same, at above 90% (Table 4). Moreover, the dry matter contents of the three diet types did not differ significantly (p>0.01). The dry samples had a longer shelf-life because mould did not grow easily. The dryness of the diet also affected the drinking water requirements of the animals; therefore, the provision of drinking water for the rats during the study was *ad libitum*.

Table 3: Average results of the Ca, P and Mg analysis of the nano-calcium diet

Diet	Ca	P	Mg
	----------% (w/w)----------		
A	0.40±0.01	0.03±0.01	0.19±0.00
B	0.62±0.05	0.07±0.00	0.24±0.02
C	0.99±0.02	0.08±0.01	0.47±0.02
Normal feed	0.40-0.50[29]	0.05[30]	0.01[30]

A: Diet with 0.10% nano-calcium, B: Diet with 0.40% nano-calcium, C: Diet with 0.70% nano-calcium

Table 4: Proximate analysis of the nano-calcium diet

Diet	Dry matter	Ash	Fat	Protein	Crude fibre
	----------% (w/w)----------				
A	92.92±0.56	3.02±0.21	2.11±0.05	15.07±1.05	0.12±0.04
B	93.08±0.06	3.45±0.22	2.05±0.19	16.84±0.56	0.19±0.07
C	91.92±0.10	4.45±0.22	2.42±0.08	16.59±0.86	0.26±0.09

A: Diet with 0.10% nano-calcium, B: Diet with 0.40% nano-calcium, C: Diet with 0.70% nano-calcium

Total ash is defined as the residue produced in the combustion process of organic matter in the form of inorganic compounds, such as oxides, salts and minerals. The percentages of ash for all three types of diets (A, B and C) varied and tended to increase. Specifically, the ash content in the diet, which represents dietary mineral levels, ranged from 3.02-4.45%. This result was consistent with the results of the AAS and UV-vis analysis, which showed that diets A to C had increasing contents of Ca, P and Mg.

The average fat content of the three types of diets was approximately 2%. The reason for this value is that the basic fat-containing ingredients, namely, rice flour, casein, corn oil and carboxymethyl cellulose, were added at the same ratio for diets A, B and C. This fat was needed by the body as an energy reserve.

The percentages of protein determined from the proximate analysis of the three diet samples were all approximately 16%. The similar protein content reflects the organic ingredients found in the diet because these ingredients consist of protein and organic matter without nitrogen. Protein in food functions to regenerate cells in the bone or build new body cells, replacing damaged cells.

The crude fibre contents of the three types of diets (A, B and C) showed differences. In the manufacturing of large-scale feed, the crude fibre content of the three diets is increasingly clear. Diet C had twice the crude fibre content than did diet A. This fibre is insoluble and cannot be absorbed by the body; however, fibre has the ability to bind water, cellulose and pectin and can help speed up the excretion of food debris through the digestive tract.

Serum minerals: At the age of 13 weeks, the control rats (non-OVX) had average calcium, phosphorus and magnesium values of 11.72±0.75, 6.92±0.10 and 2.96±0.06 mg dL^{-1}, respectively (Fig. 1). The group of ovariectomized rats

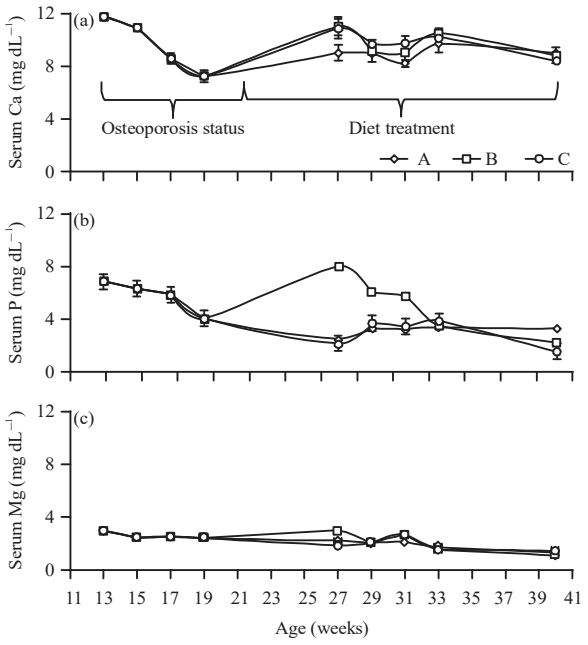

Fig. 1(a-c): Mineral contents of (a) Calcium, (b) Phosphorous and (c) Magnesium from rats grouped by osteoporosis status and diet treatment stage

experienced a decrease in calcium, phosphorus and magnesium levels at the age of 19 weeks (7 weeks after OVX). The calcium, phosphorus and magnesium contents at 19 weeks were 7.23±0.46, 4.07±0.12 and 2.42±0.25 mg dL^{-1}, respectively (Fig. 1). Based on the t-test results, the calcium and phosphorus levels in the OVX rat group decreased significantly ($p<0.01$), while the decrease in the magnesium level was not significant ($p = 0.02$).

During the diet treatment stage, analysis was performed from the age of 27 weeks to 40 weeks. The serum calcium levels of the 27-week-old rats for diets A, B and C showed an increase compared to the levels of the

19-week-old rats (Fig. 1a). However, the increase in serum calcium content in rats fed diet A was not significant ($p = 0.01$), whereas rats fed diets B and C had significant results ($p<0.01$). At the ages of 29 and 31 weeks, there was a decrease in calcium levels in all dietary treatment groups. There was an increase in serum calcium levels at the age of 33 weeks and a decrease at the age of 40 weeks. Fluctuating calcium levels in the serum reflect an attempt to maintain serum homeostasis. However, even though the serum calcium levels of rats in the diet treatment group fluctuated, these levels tended to increase. The serum calcium levels of 40-week-old rats for all diet groups were higher and significantly different from the levels at 19 weeks.

At the beginning of the analysis stage for diet B (27 weeks old), the phosphorus (8.06 ± 0.04 mg dL^{-1}) levels increased significantly ($p<0.01$) compared to the levels in 19-week-old rats (4.07 ± 0.12 mg dL^{-1}). However, at the same age (27 weeks), the group of rats fed diets A and C still had lower levels of phosphorus, which increased at the age of 29 weeks (Fig. 1b). At the end of the observation period (40 weeks old), the phosphorus level of rats fed diet A tended to remain constant beginning at the age of 29 weeks, while the groups of rats fed diets B and C still showed a significant pattern of decline at the age of 19 weeks ($p<0.01$).

The magnesium contents in rats in the diet treatment group showed a significant decrease ($p<0.01$) at 40 weeks of age compared with the levels among 19-week-old rats. At the age of 40 weeks, the average magnesium contents of rats fed diets A, B and C were 1.28 ± 0.05, 1.12 ± 0.03 and 1.46 ± 0.21 mg dL^{-1}, respectively (Fig. 1c). The three diets did not have a significant effect on serum magnesium levels.

Morphometry characteristics of the femur and tibia: The average length of the femur and tibia during the observation period (from the age of 13 weeks to 40 weeks) showed increasing values (Fig. 2). At the osteoporosis development stage, the average mass of the femur bone (non-OVX) in the control group was 0.34 ± 0.05 g and that of the tibia bone was 0.29 ± 0.05 g. At the age of 21 weeks, the average femoral bone mass was 0.36 ± 0.05 g and that of the tibia bone was 0.34 ± 0.05 g. Both the femur and tibia showed a significant increase in mass ($p<0.01$) compared to the mass of the control group. At the diet treatment stage, the three types of diets did not show significant differences in the mass of the femur or tibia. At the age of 40 weeks, the femur bone masses of rats fed diets A, B and C were 0.50 ± 0.05, 0.51 ± 0.04 and 0.51 ± 0.05 g and the tibial bone masses were 0.45 ± 0.04, 0.48 ± 0.05 and 0.46 ± 0.05 g, respectively.

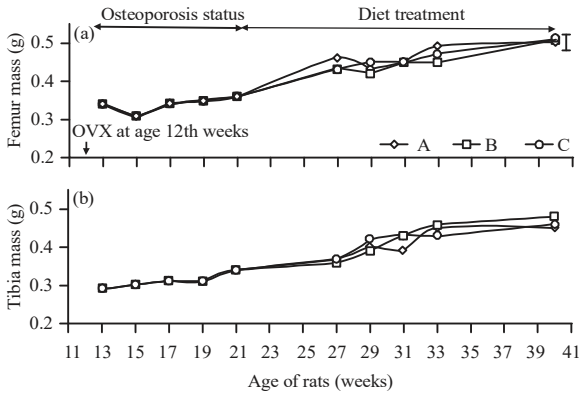

Fig. 2(a-b): Average mass of the (a) Femur and (b) Tibia in rats developing osteoporosis and rats treated with the different diets

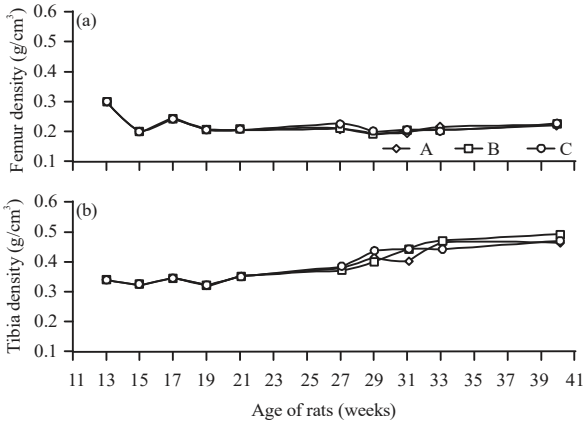

Fig. 3(a-b): Average mass density of the (a) Femur and (b) Tibia in rats developing osteoporosis and rats treated with the different diets

The average mass density of the control femur bone was 0.29 ± 0.03 g/cm^3 and at the end of the osteoporosis development stage (age of 21 weeks), the average density was 0.20 ± 0.02 g/cm^3 (Fig. 3). During the process of osteoporosis development, there was a significant decrease in mass density in the femur bone ($p<0.01$). Initially, the mass density of the control tibia bone was 0.34 ± 0.03 g/cm^3; at 21 weeks, the density was 0.35 ± 0.03 g/cm^3. During the osteoporosis development process, the change in mass density was not significant ($p = 0.56$). While the rats were treated with diets A, B and C, a significant increase in femoral bone density was observed ($p<0.01$) but the mass density of the tibia did not increase significantly.

The average length of the femur and tibia during the observation period increased from 13 weeks to 40 weeks. The length of the control femur was initially 33.64 ± 2.59 mm and

Table 5: Dry matter consumption, Ca consumption, faecal Ca content, Ca absorption and percentage of Ca absorption (Mean±SD, n = 5)

Groups	Dry matter consumption (g day^{-1})	Ca consumption	Faecal Ca (mg day^{-1})	Ca absorption	Ca absorption (%)
Normal rats fed a standard diet	11.59±1.22	62.13±2.22^{a}	23.88±3.26	38.25±7.31^{a}	61.56^{a}
OVX rats fed diet A	11.63±1.23	47.98±5.86^{c}	18.05±1.65	29.93±5.84^{c}	62.38^{a}
OVX rats fed diet B	11.00±1.99	68.00±12.36^{b}	21.71±6.97	46.29±12.36^{b}	68.07^{b}
OVX rats fed diet C	11.44±1.58	70.83±9.77^{b}	23.30±6.17	47.53±9.77^{b}	67.10^{b}

Different lowercase superscripts in the same column show significant differences (p<0.01)

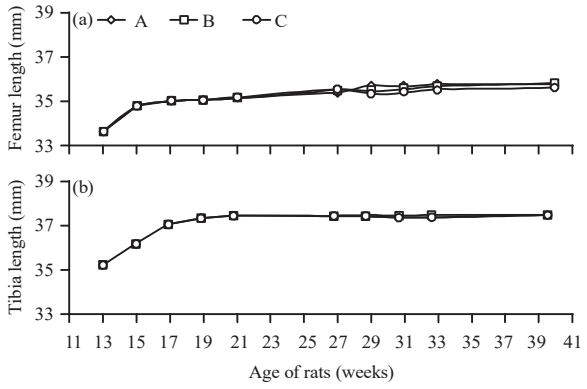

Fig. 4(a-b): Average length of the (a) Femur and (b) Tibia in rats developing osteoporosis and rats treated with the different diets

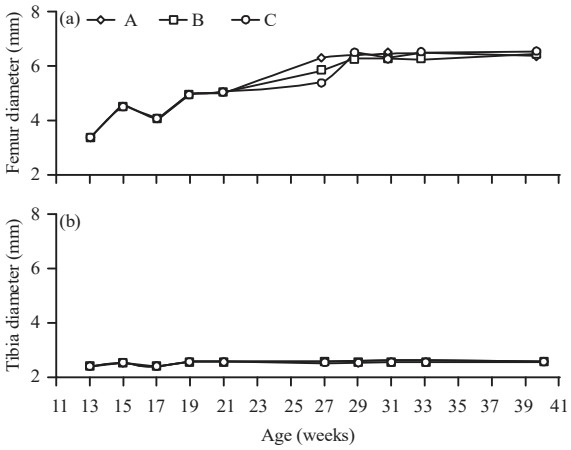

Fig. 5(a-b): Average diameter of the (a) Femur and (b) Tibia in rats developing osteoporosis and rats treated with the different diets

at the age of 21 weeks, the length reached 35.20±0.73 mm; however, this increase was not significant (p = 0.37). At the treatment stage, the three diets did not show significant differences in the length of the femur. At the age of 40 weeks, the femur bones of rats fed diets A, B and C were 35.79±1.56, 35.78±1.67 and 35.65±1.88 mm, respectively (Fig. 4a). The three diets did not show a significant difference either in the control rats or in rats aged 21 weeks (osteoporosis). Likewise, in the tibia, the length increased starting from the age of 13 weeks to the age of 17 weeks and the increase tended to be constant (Fig. 4b).

The average femoral bone diameter increased during the osteoporosis development stage and continued to increase significantly at the dietary treatment stage until the age of 29 weeks (p<0.01). The average control femur bone diameter was 3.38±0.76 mm and the femur diameter at 29 weeks was 6.33±1.02 mm. Furthermore, until 40 weeks of age, the average diameter did not change significantly (p<0.01) (Fig. 5a). The diameter of the control tibia was 2.41±0.75 mm; at 21 weeks, the average diameter was 2.57±0.66 mm; at the end of the observation age of 40 weeks, the average diameter was 2.57±0.83 mm (Fig. 5b). The diameter of the tibia did not increase significantly in any stage.

Calcium absorption: Table 5 shows the measurements of dry matter consumption, calcium consumption, calcium in the faeces, calcium absorption and percentage of calcium from the four groups of rats: normal (non-OVX) rats fed a standard diet, OVX rats fed diet A, OVX rats fed diet B and OVX rats fed diet C. The measurement results showed that the dry matter consumption of all groups did not differ significantly (p<0.01). The average consumption of dry matter ranged from 11.00±1.99 to 11.63±1.23 g day^{-1}.

The calcium consumption of normal rats fed a standard diet was 62.13 ± 2.22 mg day^{-1}. This value was higher than the consumption of calcium in OVX rats fed diet A (47.98±5.86 mg day^{-1}) but was lower than the consumption of calcium in OVX rats fed diets B (68.00±12.36 mg day^{-1}) and C (70.83±9.77 mg day^{-1}).

The group of rats that consumed the standard diet had higher faecal calcium values (23.88±3.26 mg day^{-1}) than the group of rats that consumed the nano-calcium phosphate diet. The faecal calcium levels of rats that consumed nano-calcium phosphate diets with higher nano-calcium levels exhibited an increasing trend. The average faecal calcium contents of rats that consumed diets A, B and C were 18.05±1.65, 21.71±6.97 and 23.30±6.17 mg day^{-1}, respectively.

The percentage of calcium absorption among rats fed a nano-calcium diet tended to be higher than that of the group

of rats fed a standard diet. OVX rats fed a nano-calcium diet showed percentages of calcium absorption between 62.38 and 68.07%. Among the three types of diets, diet B showed the highest percentage of calcium absorption, which was 68.07%.

DISCUSSION

The results showed that the serum calcium and phosphorus levels in ovariectomized rats decreased significantly ($p<0.01$) seven weeks after OVX (19 weeks) and that the magnesium levels decreased but the decrease was not significant ($p = 0.02$). The control rats (non-OVX) used in the study had a serum calcium content of 11.71 ± 0.75 mg dL^{-1}. This value confirmed that the initial condition of the rats was normal. Previous studies have reported that the serum calcium levels of normal rats were 9.20-10.40[31], 10.09[32] and 11.89-14.86[33] mg dL^{-1}. The calcium levels of rats with osteoporosis should be lower than 8.35[23] mg dL^{-1}. Therefore, 7 weeks after OVX (19 weeks), the rats had serum calcium levels of 7.23 ± 0.46 mg dL^{-1} and were deemed osteoporotic.

Based on the serum calcium analysis, the mechanism of homeostasis was not sufficient to explain the decrease in blood calcium levels. This condition triggered the occurrence of osteoporosis and caused the resorption of calcium from the bones to meet the calcium needs of the blood. If calcium is continuously resorbed from bone for a long time, then the bone will lose a large amount of calcium and become porous. Previous research confirmed that OVX causes a decrease in serum calcium levels up to 7.26 ± 0.09 mg dL^{-1} 8 weeks after ovariectomy[23].

Whereas serum mineral status was not checked periodically in previous research, in this study, serum minerals were analysed every 2 weeks beginning from when the rats were ovariectomized. *Rattus norvegicus* white rats have an average oestrus cycle of 8 days[34], so the 2-week interval was sufficient to analyse changes in serum.

In addition to analysing the calcium levels, the levels of phosphorus and magnesium minerals were also measured. Similar to the calcium levels, the serum phosphorus level also decreased due to OVX. The serum phosphorus level of normal rats was 6.92 ± 0.10 mg dL^{-1} and seven weeks after OVX, the phosphorus level was 4.07 ± 0.12 mg dL^{-1}. A previous study[35] reported normal serum phosphorus levels of 2.7-4.5 mg dL^{-1}. Other researchers measured serum phosphorus levels of 3.24 ± 0.49 mg dL^{-1} in normal rats[23]. The serum phosphorus level obtained from the current research results was still higher than the results of previous research. This phenomenon reflects the maintenance of the homeostatic system; because the calcium levels are low, the intestinal absorption of phosphorus is increased[36]. The role of phosphorus in blood ranks second most important after calcium. When the blood levels of phosphorus are deficient, phosphorus is taken from the bone and if this occurs continuously, bone loss will result. The serum levels of phosphorus among the ovariectomized rats showed that serum blood phosphorus levels were influenced by time: the more time passes since OVX, beginning from 15 weeks to the age of 19 weeks, the lower the serum phosphorus levels in the rats.

The magnesium serum content of the OVX rats was not stable, as seen from the fluctuating data. The magnesium content obtained in this study ranged between 2.42 and 2.48 mg dL^{-1} for ovariectomized rats and 2.96 mg dL^{-1} for normal rats (control serum). Previous research has shown that normal levels of serum magnesium range from 2-4[37], 1.8-3.0[38] and 3,58[23] mg dL^{-1}; therefore, the magnesium contents of the rats in this study were still in the normal range. This result is because magnesium plays an important role in maintaining calcium homeostasis in the serum. When there is a decrease in calcium levels, the magnesium level will be adjusted so that the body's physiological condition remains stable. As such, magnesium and phosphorus play a role in the absorption of calcium in the body and an important role in mobilising calcium from the bone extracellular fluid[39].

Overall, the serum mineral levels were affected by time: the more time passes since OVX, the lower the serum mineral levels of the rats. A clear reduction in calcium levels was followed by a decrease in phosphorus levels, while magnesium levels tended to fluctuate with insignificant changes.

The administration of the nano-calcium diet for 19 weeks (from week 29 to week 40) significantly increased the serum calcium level compared to the levels of the osteoporosis group (week 19). Similarly, the phosphorus content exhibited an increasing trend. However, the effect of diet on serum magnesium levels were not significant. Previous research has suggested that providing a calcium diet to rats with osteoporosis can increase the calcium, phosphorus and magnesium levels after 2 weeks of diet administration[40]. Other researchers have found that serum calcium and phosphorus levels of ovariectomized rats increased after the rats consumed a calcium carbonate diet for 4 weeks[23]. Overall, after diets A, B and C were given to rats with OVX-induced osteoporosis, serum mineral levels were not affected. The whole diet increased the serum calcium and phosphorus levels and had a constant influence on the magnesium levels.

Long bone (femur and tibia) morphometry was assessed in terms of mass, mass density, length and diameter. The average mass, length and diameter of the femur and tibia increased with increasing age of the rats, at both the osteoporosis development and diet treatment stages. This phenomenon occurred because the rats used in the study were in the growth phase, as diets consumed in the growth phase affect bone size. In the initial growth phase, the rats consume nutrients for growth and development, which results in increased mass, bone length and diameter. If the rats used in this study had passed the growth phase, then the amount of feed consumed would have been less; therefore, the mass, length and diameter of the bones would have been constant and might even have decreased[41].

The average mass and diameter of the femur were greater than those of the tibia. However, the femur bone has a shorter length than that of the tibia. With respect to bone morphometry, diets containing different calcium levels had no significant effect ($p>0.01$) on either type of bone between rats with osteoporosis and rats fed diets A, B and C. The mass, length and diameter of rats treated with diet B tended to be greater than those of rats treated with diet A. This occurred because the nano-calcium diet is more easily absorbed than the usual diet. However, rats treated with diet B had almost the same mass, length and diameter as those of rats treated with diet C. This result showed that diet B and diet C had the same effect on femoral and tibia morphometry.

The mass density of the femoral bone decreased significantly ($p<0.01$) at the osteoporosis development stage and increased significantly ($p<0.01$) during the administration of the nano-calcium phosphate diet. In the tibia, the mass density during the osteoporosis stage also decreased but the decrease was not significant. Additionally, diet regulation increased bone density in the tibia.

The largest percentage of calcium absorption was identified in the group of OVX rats that consumed diet B (68.07%). The percentage of calcium absorption depends on the calcium content in the diet because nanosized calcium is easier to mix with a homogeneous diet and there is more nanosized calcium than larger particles in the same volume. As a result, when consumed, the amount of calcium will increase.

Calcium that is excreted in the faeces cannot be absorbed by the intestines. This phenomenon is in line with the results of previous studies in which the calcium levels in faeces did not increase significantly even though calcium consumption increased[42]. Factors that affect faecal calcium contents include the amount of crude fibre in the diet. Diets A, B and C have increasing crude fibre contents (Table 4), which caused faecal calcium contents to increase in rats that consumed diets A, B and C, respectively.

The higher the crude fibre contents in the diet are, the more calcium particles will be attached to the fibre. Without the help of fibres, faeces with a low water content will stay longer in the intestinal tract and will be difficult to excrete because the large intestinal peristaltic movements become slower[43]. The time to absorb nutrients in the intestine will be shorter, so the amount of calcium absorbed in the intestine will also be low. The data showed that diets B and C were absorbed in the same amount. As a result, diet C, which has a higher nano-calcium content than that of diet B, became ineffective. Calcium is absorbed in the body at an approximate rate of 30-50%[44]; however, some researchers have stated that the rate of calcium absorption in the body can be 60%[45]. In the current study, the percentage of nano-calcium absorption was 71.52±3.96% and that of normal calcium absorption was 63.51±4.78%[46]. The calcium absorption rate among OVX rats that consumed the 0.39% nano-calcium diet (diet B) was 68.07%. These values are different from the findings of previous studies and some of the reasons for these differences include the differences in age and sex of the rats and in the type of diet. Further research is needed to clarify this problem by varying the age of the rats when OVX is performed, serum oestrogen levels and dietary intakes based on sex.

This study has several limitations. The diet used in the study contained non-nano-calcium from other ingredients. In addition, the homeostasis system always maintained the mineral balance in the serum, so measurements of mineral levels in bone are also needed.

Based on the results of this study, rats with OVX-induced osteoporosis were recommended to consume diet B, which contained 0.40% nano-calcium, 0.07% phosphorus and 0.24% magnesium. Consuming diet B resulted in the best absorption. The calcium requirement for normal rats is 0.40-0.50% for non-nano-calcium[29]. The requirement for normal phosphorus and magnesium among rats is 0.05 and 0.01%, respectively[47]. The phosphorus data for diet A (0.03%) revealed that the amount consumed was less than normal; therefore, to meet the body's need for phosphorus, additional phosphorus was taken from the bones. Diets B (0.07%) and C (0.08%) had higher phosphorus levels than needed and the excess would be discharged in the faeces. Similarly, excess magnesium levels would also be removed via the faeces. The high phosphorus and magnesium levels in the diet originated from the rice flour. In total, 100 grams of pure rice flour contained 5 mg of calcium, 140 mg of phosphorus and 35 mg of magnesium[48].

CONCLUSIONS AND SUGGESTIONS

OVX can result in a decrease in calcium, phosphorus and magnesium levels in the serum of white rats (*Rattus norvegicus*). As soon as seven weeks after OVX, the serum calcium level was already out of the normal range. This condition defines the starting point of osteoporosis. Providing a nano-calcium phosphate diet to rats with OVX-induced osteoporosis was effective for a diet with a 0.40% nano-calcium content (diet B). The effects of the diet were first observed in the sixth week of diet administration. Diet B increased the serum calcium levels (51.87%), femur bone mass density (2.95%) and tibia bone mass density (1.66%) and resulted in a high percentage of calcium absorption (68.07%). However, the nano-calcium diet did not have a significant effect on the mass, length or diameter of the femur and tibia ($p>0.01$).

SIGNIFICANCE STATEMENTS

Knowledge of the status of serum minerals and bone mass density can be used as a reference to estimate osteoporosis risk after ovariectomy. New theories regarding the time required for osteoporosis to develop after ovariectomy have not been widely explored by previous researchers. Additionally, the results of this study can help medical practitioners design a diet for osteoporotic patients based on their osteoporosis status so that treatment is effective and efficient.

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