Analysis of Nano Ca3(PO4)2 on Bone’s Calcium Deficiency at Peak Age

N N Mulyaningsih¹,², A L Juwono³*, D S Soejoko¹ and D A Astuti³
¹Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Kampus UI Depok 16424, Indonesia
²Department of Physical Education, Faculty of Engineering, Mathematics and Natural Sciences, Universitas Indraprasta PGRI Jakarta 12530, Indonesia
³Department of Ilmu Nutrisi dan Teknologi Pakan, Fakultas Peternakan, Institut Pertanian Bogor, Bogor 16680, Indonesia

*email: ariadne.laksmidevi@ui.ac.id

Abstract. In this research, we studied how the nano Ca₃(PO₄)₂ uptake in bone mineral influenced Rattus norvegicus (Sprague Dawley) calcium deficiency status. Association of Official Analytical standard method was used for proximate analysis, meanwhile calcium and magnesium compositions were determined by atomic absorption spectrophotometry. In addition to that, ultraviolet spectrophotometry and fourier transform infrared were used to determine the content of phosphorus and infrared spectrum of transmittance, respectively. The results showed that administration of nano-sized Ca₃(PO₄)₂ purified diet gives a higher uptake than the regular-size, suggesting that the application of nano Ca₃(PO₄)₂ to rats suspected in a bone mass peak age with calcium deficiency may potentially prevent the occurrence of osteoporosis.

1. Introduction
Bone strength is influenced by bone mass, microarchitecture, macrogeometry, and bone turnover. Human bones are composed of minerals and organic constituents; and the composition ratio is associated with age and health [1-5]. Due to dietary factors, genetics, age, lifestyle as well as some other diseases, bones can experience a low density that can lead to thinning. Dietary factors have also been known to influence bone remodeling and fragility [6-7]. Before the occurrence of osteoporosis, bone firstly experience the process of osteopenia, which is a condition of loss of bone mass due to various circumstances [8-10]. This disease is dubbed the Silent Epidemic Disease, because it attacks silently without any special signs, until the patient suffered a broken bone. The incidence of both osteoporosis and osteopenia fractures increases with age. As age increases over the peak age of bone mass, the balance between formation and bone resorption begins to be impaired. Above this age the resorption process is more active.

Human bones can be modelled by rat bones because biologically close resemble those humans [11-12]. The mouse has many similarities to humans in terms of anatomy, physiology and genetics [13-14]. Old rats have also decreased bone mass. The loss of bone mass or bone mineral density and the increase in bone marrow adiposity are common hallmarks of the aging process. Age related osteoporosis is a common type of osteoporosis that occurs with aging, so to maintain the quality of bone mass need to get more intake of calcium, which is more effectively absorbed by the bone [15-19].
Calcium is an essential nutrient that is the most abundant cation in the body, of which approximately 99% occurs in bone, contributing to its rigidity and strength [20-24]. There is a lack of study in providing calcium intake for rat osteoporosis bones. So that the aim of the current study was to provide purified diets of nano and regular-sized \( \text{Ca}_3(\text{PO}_4)_2 \) to old rats that were osteoporosis conditioned by providing calcium deficiency feed and after being in osteoporosis. The bones were analyzed using atomic absorption spectroscopy (AAS) and fourier transform infrared (FTIR).

2. Materials and Method

The experiment was carried out on female Sprague Dawley rats, \( \text{Rattus norvegicus} \) species at the age of seven months from Biofarmaka Laboratories and were approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia with protocol number 17-05-0421.

Animals were habituated to laboratory conditions for one week prior to use. During the habituation, the rats were fed with a standard diet (S) from commercial diet product (PT. Charoen Pokphand Indonesia Tbk., with a diet phase grower 512). After this period, the animals was fed with deficiency diet (P1: 0.00% \( \text{Ca}_3(\text{PO}_4)_2 \)) for ten weeks and was identified as D group (deficiency group). According to previous research [25-26], mice were in a calcium deficiency state after being fed with deficiency diet for two months. The rats that were already in a calcium deficiency were randomly divided into 2 groups: D1 group was fed with normal purified diet (P2: 0.50% \( \text{Ca}_3(\text{PO}_4)_2 \)) and D2 group was fed with nano purified diet (P3: 0.50% nano \( \text{Ca}_3(\text{PO}_4)_2 \)). After one month, rats were euthanized for bone removal and analyzed. The list of ingredients for the diet can be found in Table 1. The diet was made by mixing all ingredients in a blender at medium speed to form dough-like textures. The homogenized diet formula was then formed into pellet. Pellets were then put into an oven (60 °C) to avoid mushy textures.

At the end of the experiment, rats were then sacrificed; the femur and vertebrae were cleaned from surrounding soft tissue. Thereafter, bone samples were heated at temperature 60 °C for 24 hours then soaked with hydrazine for seven days to remove the organic substances. The bone samples were then soaked with alcohol for one hour and rinsed with distilled water three times. The specimens were dried, weighed and crushed for later analysis.

The mineral compositions of diet and femur, including calcium (Ca), fosfor (P) and magnesium (Mg) were analyzed by atomic absorption spectroscopy (AAS). The other component compositions such as dry ingredients, ash, fat, protein and crude fiber was evaluated by proximate analysis based on the standard of procedures as described by Association of Official Analytical (AOAC) [27]. Each

| Ingredient            | P1  | P2  | P3  |
|-----------------------|-----|-----|-----|
| Rice Flour            | 25.00 | 30.00 | 30.00 |
| Casein                | 18.00 | 21.00 | 21.00 |
| Corn Oil              | 3.50  | 2.50  | 2.50  |
| Sugar Flour           | 49.00 | 41.50 | 41.50 |
| DL-Methionine         | 0.30  | 0.30  | 0.30  |
| CMC                   | 3.00  | 3.00  | 3.00  |
| \( \text{Ca}_3(\text{PO}_4)_2 \) | 0.00 | 0.50  | 0.00  |
| Nano \( \text{Ca}_3(\text{PO}_4)_2 \) | 0.00 | 0.00  | 0.50  |
| Vitamin Mix           | 0.50  | 0.50  | 0.50  |
| NaCl                  | 0.20  | 0.20  | 0.20  |
| Mineral Mix           | 0.50  | 0.50  | 0.50  |
| **TOTAL**             | **100** | **100** | **100** |

P1 : Deficiency diet  P2 : Normal purified diet  P3 : Nano purified diet
analysis was carried out in duplicate. While the presence of certain functional groups in vertebrae was analyzed by Fourier transform infrared (FTIR).

3. Results and Discussion
The results of AAS and proximate analysis are shown in Table 2. Nutritional parameters such as mineral compositions, dry ingredients, ash, fat, protein and crude fiber were determined. Our results indicate that standards diet has the highest calcium content than other diet formulas. The particular standard diet (S) but was given only when rats were in habituation phase.

| Sample Code | Ca  | P   | Mg  | Dry Matter | Ash | Fat | Protein | Crude Fiber |
|-------------|-----|-----|-----|------------|-----|-----|---------|-------------|
| S           | 1.35| 0.48| 0.14| 9.89       | 5.94| 8.75| 19.45   | 2.02        |
| P1          | 0.28| 0.04| 0.02| 93.35      | 2.05| 2.01| 15.80   | 0.33        |
| P2          | 0.94| 0.60| 0.14| 90.89      | 2.57| 2.01| 16.96   | 0.00        |
| P3          | 0.81| 0.28| 0.01| 93.40      | 2.80| 1.67| 16.32   | 0.05        |

S: Standard diet, P1: Deficiency diet, P2: Normal purified diet, P3: Nano purified diet

The P1 sample has the lowest calcium content (0.28%), this value was only half the normal calcium requirement for mice. Thus continuous consumption of P1 diet might cause risk of osteoporosis. On the other hand, P2 and P3 samples relatively had similar amount of calcium content due to addition of Ca₃(PO₄)₂. It is worth noting that the particle size of those P2 and P3 samples were different, as P2 contained regular-sized Ca₃(PO₄)₂ and P3 contained nano-sized Ca₃(PO₄)₂.

P1-P3 samples had similar percentages of dry matter which was 90%, while the dry matter of S sample is below 10%. The corresponding high dry matter content in the diet affected the water consumption of mice. The provision of drinking water in mice during the study was given indefinitely (ad libitum). The highest contents of ash, fat, protein and crude fiber was found in S samples, suggesting that S samples had the lowest organic content since the ash content was inversely proportional to the amount of organic matter and fat required by the body as food reserves. The protein content in the sample refers to the organic material contained in the sample, because the organic material contained in the sample consists of proteins and organic materials without nitrogen. Protein in the food functions for cell regeneration process or builds new body cells to replace damaged body cells.

Table 3 shows the content of minerals on the femur bone. The calcium level of control were determined high (74.63%), however, after feeding of calcium deficiency P1 for 10 weeks, the calcium level decreased for nearly 54.62% (D sample). Our results found that rats were in calcium deficiency conditions following P2 and P3 diet treatment for 1 month, as calcium bone resurgence occurred. D1 and D2 group showed relatively normal value of elemental minerals, yet, D2 was closer to that control treatment. This data indicates that the recovery from calcium deficiency conditions in female mice in best peak age of a given nano-sized Ca₃(PO₄)₂ purified diet.

| Sample Code | Ca  | P   | Mg  | Ash | Fat | Protein |
|-------------|-----|-----|-----|-----|-----|---------|
| C           | 74.63±3.22 | 14.02±1.02 | 2.92±0.23 |
| D           | 40.76±2.43 | 1.58±0.04  | 13.64±1.87 |
| D1          | 72.29±5.40 | 13.39±1.33 | 4.01±0.09  |
| D2          | 74.80±4.23 | 16.96±1.20 | 2.02±0.18  |

C: Control rat with S diet, D1: Deficiency rat with P2 diet, D: Deficiency rat with P1 diet, D2: Deficiency rat with P3 diet
Figure 1. FTIR spectra of (D) deficiency rat with normal diet, (D1) deficiency rat with normal purified diet and (D2) Deficiency rat with nano purified diet.

Figure 1 shows the infrared spectrum of transmittance of back bone samples. The functional groups appearing at the wave numbers of 400 cm\(^{-1}\) to 4000 cm\(^{-1}\) are phosphate groups (PO\(_4^{3-}\)), carbonates (CO\(_3^{2-}\)) and hydroxyl (OH\(^-\)). This fits with previous research conducted previously [28-30].

Based on Figure 1, the emergence of phosphate and carbonate groups indicates that calcium phosphate in bone was present in the form of apatite carbonate. Our data indicates that the FTIR clusters of that D1 and D2 groups were not significantly different. However, the occurrence of hydroxyl group in sample D, between the wave numbers 1500-1750 cm\(^{-1}\), likely showed that the division which indicated that the crystallinity of sample D was higher than the sample D1 and D2. The occurrence of a division at the peak of the FTIR shows a high level of crystallinity and low calcium content [31]. This suggests that samples D1 and D2 begin to improve from the condition of calcium deficiency.

The usefulness of AAS/UV and FTIR studies for the analysis of nano Ca\(_3\)(PO\(_4\))\(_2\) on bone’s calcium deficiency at peak age was confirmed. Application of both experimental methods suggested that nano Ca\(_3\)(PO\(_4\))\(_2\) purified diet can prevent the occurrence of osteoporosis.

Female mice at peak age could be conditioned to osteoporosis by feeding calcium deficiency for ten weeks. The calcium levels of that mice in calcium deficiency could be recovered by providing a diet high in calcium. Dietary administration with a calcium source in regular-sized and nano-sized of Ca\(_3\)(PO\(_4\))\(_2\) for one month showed that the nano-sized Ca\(_3\)(PO\(_4\))\(_2\) in diet was better than the regular-sized Ca\(_3\)(PO\(_4\))\(_2\) diet.
4. Conclusion
Female mice at peak age can be conditioned to osteoporosis by feeding calcium deficiency for ten weeks. The calcium levels of those mice in calcium deficiency could be recovered by providing a diet high in calcium. Dietary administration with a calcium source of Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} in the regular-sized and nano-sized for one month gave the result that the nano-sized Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} diet was better than the regular-sized Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} diet.

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