Dietary nano-calcium phosphate and hydrolyzed collagen for the inhibition of osteoporosis in calcium-deficient bones

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Abstract. Several studies exhibit that high calcium intake prevents osteoporosis-related bone loss. In this study, the dietary effects of nano-tricalcium phosphate (n-CaP) and hydrolyzed collagen (HC) against bone loss at the peak of the bone growth stage are examined, and the main objectives are to prevent osteoporosis and to induce bone formation. The Sprague Dawley mice (Rattus norvegicus) is observed to be calcium deficient (the average calcium content of serum is ≤ 8%). The mice were randomized into four groups (P1: normal purified diet, P2: HC purified diet, P3: n-CaP purified diet, and P4: n-CaP and HC purified diet). After four weeks, all the groups were euthanized, and their bone formation was analyzed. UV–Vis analysis revealed that the calcium content values were 8.20 (P1), 8.92 (P2), 9.2 (P3), and 10.35 (P4) mg/dL, respectively. The corresponding values obtained by the atomic absorption spectrophotometric analysis are 72.29 (P1), 74.25 (P2), 74.80 (P3), and 76.04 (P4) w/w% respectively. To assess the spinal calcification, the infrared spectra were recorded. The splitting factors were 2.5 (P1), 2.6 (P2), 2.5 (P3), and 2.6 (P4), indicating better absorption of n-CaP for P4. Hence, n-CaP and HC-purified diets are suitable to ensure bone growth.

Keywords: Osteoporosis, nano-calcium phosphate, hydrolyzed collagen

1. Introduction
Bone is a highly specialized form of connective tissue, which is characterized by its hardness, resilience, growth mechanisms, and self-regeneration capability after an injury via the bone healing process. Mature bones predominantly comprise the bone matrix, bone cells, and bone marrow. The bone matrix provides mechanical strength and serves as the storage area of minerals in the human body. The bone matrix is the intercellular substance of the bone tissue, comprising collagen fibers, ground substances (composed of water, glucosaminoglycans, proteoglycan and glycoproteins), non-collagenous proteins, and inorganic bone salts. Bone modeling and remodeling ensures that the old or damaged bones are replaced by new bones. These processes are regulated by various osteotropic cytokines and hormones, which affect the bone cell activity for maintaining the coupling between bone resorption and formation. The imbalance of these interactions causes abnormal turnover cycles, leads to diseases such as osteoporosis (caused by an imbalance between new bone formation and old bone resorption). Osteoporosis-related fractures can decrease the quality of life. Several studies related to the recovery of bone fractures, such as autografts, allografts, and alloplastic materials, still exhibit limitations [1–3].
The 2.3. and crushed For the purpose of further analysis, the specimen were samples were carried out. At the end of the treatment (2.2. dough (w%) were mixed in water. The mixture was obtained by a high milling process (PI 700i Mixer/Mill machine) at 1000 rpm for 3 h. The purified diet-making process was conducted as follows: all the ingredients (w/w%) were mixed in water. The mixture was further stirred at a medium speed until a homogeneous dough was obtained. The dough was shaped into pellets and heated at 60 °C.

Table 1. Formulated diet for rats

| Ingredient         | D   | P1  | P2   | P3   | P4   |
|--------------------|-----|-----|------|------|------|
| Rice Flour         | 25.00 | 25.00 | 22.00 | 25.00 | 22.00 |
| Casein or HC (+)   | 18.00 | 18.00 | 30.00 | 18.00 | 30.00 |
| Corn Oil           | 3.50  | 3.30  | 2.30  | 3.30  | 3.30  |
| Sugar Flour        | 49.00 | 49.00 | 40.00 | 49.00 | 40.00 |
| DL-Methionine      | 0.30  | 0.30  | 0.30  | 0.30  | 0.30  |
| CMC                | 3.00  | 3.00  | 3.00  | 3.00  | 3.00  |
| Ca(OH)             | 0.00  | 0.20  | 2.00  | 0.00  | 0.00  |
| Nano-Ca(OH)        | 0.00  | 0.00  | 0.00  | 0.20  | 0.20  |
| Vitamin Mix        | 0.50  | 0.50  | 0.50  | 0.50  | 0.50  |
| NaCl               | 0.20  | 0.20  | 0.20  | 0.20  | 0.20  |
| Mineral Mix (def.Ca) | 0.50  | 0.50  | 0.50  | 0.50  | 0.50  |
| TOTAL              | 100   | 100   | 100   | 100   | 100   |

Individual studies have reported the implications of high-calcium and special-protein diets on the recovery of bone formations. In this study, the effect of dietary nano-tricalcium phosphate (n-CaP) and hydrolyzed collagen (HC) on the increased bone density of calcium-deficient mice at the peak phase of bone growth was examined. The main objectives of this study included the prevention of osteoporosis and the induction of bone formation. At the end of the treatment, the model animals were euthanized for their serum and skeletal parts (e.g. femur and spines).

2. Materials and methods

2.1. Animals and diet preparation

White female Sprague-Dawley mice (Rattus norvegicus) at their peak phase of bone growth were utilized to examine the effects of n-CaP and HC intake [4,5]. The model animals were approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia with the protocol number 17-08-0841. The formulated diets for mice were denoted as D for tricalcium phosphate deficient, P1 for normal tricalcium phosphate purified diet, P2 for HC purified diet, P3 for n-CaP purified diet, and P4 for n-CaP + HC purified diet (table 1). The raw material used for the diet comprised the purified ingredients of natural diet, which was purchased from the market. HC was obtained from cow bones, and the nanoparticle size was achieved by a high milling process (PI 700i Mixer/Mill machine) at 1000 rpm for 3 h. The purified diet-making process was conducted as follows: all the ingredients (w/w%) were mixed in water. The mixture was further stirred at a medium speed until a homogeneous dough was obtained. The dough was shaped into pellets and heated at 60 °C.

2.2. Collection and bone processing

At the end of the treatment (1 month), the animals were sacrificed. The femur and spines were cleaned from the surrounding soft tissue. Further, the bone samples were baked at 60 °C for 24 h, followed by soaking in hydrazine for 7 days to eliminate the organic substances. Subsequently, immersion of bone samples were carried out for 1 h in alcohol then the sample was rinsed three times with distilled water. For the purpose of further analysis, the specimen were gone through a drying process then weighed, and crushed.

2.3. Material characterization

The calcium and phosphate content of blood serum was analyzed by ultraviolet–visible (UV–Vis) on a Beckman DU-640 spectrophotometer. The bone samples were characterized by atomic absorption spectrophotometry (AAS) to measure the mineral contents (Ca, P, and Mg) of the bones. To assess the
Table 2. UV–Vis spectral analysis results.

| Code | Ca (mg/dL) | P (w/w%) | Mg  |
|------|------------|----------|-----|
| P1   | 8.20       | 4.36     | 2.71|
| P2   | 8.92       | 1.23     | 2.36|
| P3   | 9.02       | 0.54     | 2.94|
| P4   | 10.35      | 0.75     | 1.88|

Table 3. Mineral levels of the right femur sample by AAS (%).

| Code | Ca (w/w%) | P (w/w%) | Mg  |
|------|-----------|----------|-----|
| P1   | 72.29     | 13.39    | 4.01|
| P2   | 74.25     | 13.38    | 4.14|
| P3   | 74.80     | 16.96    | 2.02|
| P4   | 76.04     | 12.09    | 2.77|

Figure 1. FTIR spectra of spine

The chemical structure of the spine. Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer system, which aided in the identification of the phosphate and carbonate groups of bones as well as for the determination of the splitting factor, which mainly corresponded to the $\nu_4$ in case of the PO$_4^{3-}$ bending vibrations.

3. Results and discussion

The UV–Vis spectral analysis results is summarized in Table 2. The blood serum comprised Ca, P, and Mg (Table 2). Clearly, the content of Ca in P3 and P4 sera were greater than that in the P1 and P2 sera, indicating that the particle size of n-CaP positively affected the absorption of particles in the bloodstream.
The Ca, P, and Mg contents in bones that were analyzed by AAS were summarized in table 3. As observed, the contents of Ca in P4, P3, and P2 were greater than that in P1. This result indicated that the n-CaP and HC diets positively influence of the bone resorption. The FTIR spectrum of rat spines is depicted in figure 1. IR spectroscopy measurement of the rat spinal samples revealed the presence of phosphate groups (560 to 607 cm⁻¹ and 982 to 1058 cm⁻¹) and carbonate groups (~881 cm⁻¹ and 1400 cm⁻¹ to 1450 cm⁻¹). This result reconfirms the formation of carbonate apatite. The spectrum revealed the presence of three phosphate absorption bands and carbonate absorption bands. The phosphate absorption band comprised symmetric stretching (ν1, 982 cm⁻¹), asymmetric stretching (ν3, 1058 cm⁻¹), and bending vibration (ν4, 560 and 607 cm⁻¹), whereas the carbonate absorption band comprised symmetric stretching (ν2, 881 cm⁻¹), two degenerated stretching bands (ν3, 1400 and 1450 cm⁻¹), and bending (ν4, ~650 cm⁻¹). Both the groups were predominantly observed in the range of 1650 to 400 cm⁻¹, which can be observed in figure 1, in cases of spinal samples [6,7].

The calcium phosphate crystals were characterized by the presence of ν4 phosphate absorption bands, which were observed as two peaks with maxima at ~564 and 602 cm⁻¹. A continuous band with maxima at approximately 559 cm⁻¹, corresponding to amorphous calcium phosphate, was observed. The splitting factors of all the bone samples are summarized in table 4. The splitting factors were obtained from the peak at ~564 and 602 cm⁻¹ [8]. Although the splitting factor of P4 was less than those of P2 and P3, it was still greater than that of P1. This result probably indicated that a special diet containing n-CaP and HC, either simultaneously or individually, positively affected the increase in bone density.

Table 4. Splitting factor

| No. | Treatment | Splitting Factor |
|-----|-----------|-----------------|
| 1   | P1        | 2.4             |
| 2   | P2        | 2.6             |
| 3   | P3        | 2.7             |
| 4   | P4        | 2.5             |

4. Conclusions

Serum analysis revealed that calcium phosphate nanoparticles were easily absorbed into the blood vessels. Furthermore, AAS and FTIR analysis of bones revealed that nano-tricalcium phosphate (n-CaP) and hydrolyzed collagen (HC) intake positively affected the increase in the bone mass and bone structure. Thus, nano-tricalcium phosphate and hydrolyzed collagen diet at the peak stage of bone growth can prevent osteoporosis.

Acknowledgements

This research was funded by PITTA grant year of 2017 No: 624/UN2.R3.1/HKP.05.00/2017. The authors would like to thank Einago (www.einago.com) for the English language review.

References

[1] Sommerfeldt D W and Rubin C T 2001 *Eur. Spine J.* 10 S86–95
[2] Rodan G A and Martin T J 2000 *Science* 289 1508–14
[3] Russell G, Mueller G, Shipman C and Croucher P 2001 *Novartis Found Symp.* 232 251–67
[4] Brecevich A T, Kiely P D, Yoon B V, Nguyen J T, Cammisa F P and Abjornson C 2017 *Spine J.* 17 855–62
[5] Bagi C M, Berryman E and Moalli M R 2011 *Comp. Med.* 61 76–85
[6] Petibois C, Wehbe K, Belbachir K, Noreen R and Déléris G 2009 *Acta Phys. Pol. A* 115 507–512
[7] Granados-Correa F, Bonifacio-Martinez B and Serrano-Gómez J 2010 *Rev. Int. Contam. Ambient.* 26 129–34
[8] Surovell T A and Stiner MC 2001 *Journal of Archaeological Science* 28 633–42.