Original Article

# The potential calcium content of anchovy *(Stolephorus sp.)* on mandibular bone growth through osteoprotegerin expression analysis

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## Abstract

**Background:** Anchovy *(Stolephorus sp.)* is a commonly used food ingredient due to its high calcium content, which supports craniofacial growth. Calcium stimulates the formation of osteoblasts, which produce osteoprotegerin (OPG). OPG binds to RANKL, blocking RANKL–RANK bonding and limiting osteoclast development.

**Objective:** The objective of this study was to analyze OPG expression in mandibular bones to assess the potential calcium content of anchovies.

**Methods:** Three groups of 27 male Wistar rats were created: control, anchovy, and milk. After 40 days, the rats were decapitated, and their mandibular bones were surgically extracted, decalcified, and prepared for microscopic examination. The results showed a significant difference in OPG expression of rat mandibles between control, anchovy, and milk groups ($P < 0.05$), as determined by one-way analysis of variance (ANOVA). Tukey's HSD test revealed a significant difference in the average quantity of rat mandibular OPG expression between the control group and each of the anchovy and milk groups, with probability values of 0.00 and 0.003 ($P < 0.05$), respectively. However, the average level of OPG expression in anchovy and milk groups did not differ significantly, as indicated by the probability value of 0.064 ($P > 0.05$).

**Conclusion:** The mandibular development increases after feeding anchovies compared to the control group. Anchovy is not statistically superior to milk in terms of increasing mandibular development. However, as a high-calcium food, anchovy is well-suited to support children's craniofacial development and growth.

**Keywords:** Anchovy, calcium, growth, mandible, osteoprotegerin

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## INTRODUCTION

Calcium is crucial for bone mineralization. Inadequate intake contributes to osteoblast dysfunction and insufficient mineralization,[1] affects maxillofacial growth,[2,3] causes malocclusion.[4] One study found that a low-calcium diet for 4 weeks reduced craniofacial bone growth.[5]

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Calcium stimulates osteoblast formation and activity,[6] enhancing osteoprotegerin (OPG).[7] OPG binds to the receptor activator of nuclear factor kappa-B ligand (RANKL) to suppress osteoclast formation, inhibit mature osteoclast development, and induce apoptosis.[8]

Anchovy *(Stolephorus sp.)* contains high calcium. This fish is approximately 6–9 cm long, affordable, commonly consumed, and available throughout Indonesia.[9,10]

The aim of this study was to evaluate the potential for calcium content of anchovy on mandibular bone growth by examining OPG expression.

## METHODS

The investigation was conducted in an experimental laboratory using 27 male Wistar rats (*Rattus norvegicus*) aged 3 months and weighing 200 g.

Sampling was determined using the Federer formula as follows:

**(n – 1) (k – 1) > 15**

n ≥8,5

n = 9

In this context, "n" represents the number of samples to be analysed, whereas "k" is the total number of groups that will be used in the study.

The rats were randomly assigned to three groups of nine each. After a 1-week acclimatisation period, each group received a different treatment. Group 1 consumed only regular rat food (as control), Group 2 received anchovy, and Group 3 received milk. Group 1 received standard rat diets twice daily and water. Group 2 were given standard rat diets twice daily, supplemented with anchovy pollen (up to 0.828 g per day) and water. Group 3 received standard rat diets twice daily supplemented with milk (up to 1.8 g per day) and water. The therapy was administered regularly until the 40$^{th}$ day.

Before treating the animals, the calcium content of the anchovy powder to be used was tested, which was 1933 mg of calcium in 100 g of anchovy powder based on the calcium content test results of the chemical testing laboratory. The milk used contained 227.5 mg of calcium (35% of calcium recommended dietary allowance [RDA] of 650 mg) based on dairy product information of the factory.

After 40 days, the experimental animals were euthanised with a lethal dose of 0.9 mL of ether. Subsequently, surgery was performed to collect mandible bone specimens from mice. The specimens were washed and placed in a 10% neutral buffer formalin solution and 10% ethylene diamine tetra acetate acid (EDTA) solution for 7 days. Once the mandibular bone had been decalcified, microscopic preparations were made. The slides underwent immunohistochemical examination and were observed under a microscope (Olympus BX-53, Japan) at magnifications of 100×, 400×, and 1000×.

In this study, an immunohistochemical procedure was employed, which involved the use of an enzyme-labelled antibody to visualise interactions between proteins and antibodies. Subsequent to this, the enzyme was reacted with a chromogen substrate, allowing for observation via light microscopy.[11]

The slides were initially washed with phosphate-buffered saline (PBS), pH 7.4, for 5 min. They were then subjected to an endogenous peroxidase blockade with 3% hydrogen peroxide for 20 min. This was followed by three further washes with PBS, pH 7.4, for 5 min, each. Following a three-time, five-minute PBS pH 7.4 wash, the slides underwent non-specific protein blocking using a 5% fetal bovine serum (FBS) solution containing 0.5% Triton X-100. A subsequent three-time, five-minute PBS pH 7.4 wash was then conducted. The slides then proceeded to be incubated with the primary antibody (monoclonal anti-OPG) (SantaCruz) for 60 min, followed by three more five-minute, five-minute PBS pH 7.4 washes. Subsequently, the slides were incubated with an anti-mouse horseradish peroxidase (HRP)-conjugated antibody for 40 min, followed by a three-minute wash in PBS pH 74. The slides were then treated with diamino benzidine (DAB) and incubated for 10 min. Following this, the slides were rinsed in PBS pH 74 for a further 3 min. Finally, the slides were rinsed in distilled water for 5 min, before being left to air-dry. The next step was to perform counterstaining using Mayer's haematoxylin. The slides were incubated for 10 min, washed with tap water, and rinsed with $dH_2O$. They were then air-dried at room temperature. Finally, mounting was conducted using a mounting medium, and the slides were covered with a cover glass. Finally, the slides were observed under a light microscope.[11]

A haematoxylin–eosin staining procedure was carried out on the slides for structural comparison. To facilitate calculation, the slides were anonymised and given new random numbers. Osteoblasts expressing OPG were observed under a microscope, and the presence of brown

cytoplasm indicated that these cells were present.[12,13] Once all data had been returned to its original code and were available for analysis, statistical techniques were employed.

For statistical analysis, the data normality was tested using the Shapiro–Wilk test, followed by Levene's test for homogeneity. The groups were compared using the one-way analysis of variance (ANOVA) and Tukey's HSD tests. A probability value under 0.05 indicated a significant difference at a 95% confidence level. The data were processed using the SPSS application and presented as tables, graphs and figures.

## RESULTS

The study resulted in mean values for OPG expression in the mandibular bones of male Wistar rats from all three treatment groups, as shown in Table 1.

Table 1 shows that the mean value of mandibular bone OPG expression in male Wistar rats ranged from the lowest to the highest in the following order: control group, milk group and anchovy group [Figures 1 and 2].

Additionally, statistical tests were conducted using the one-way ANOVA test, as shown in Table 2, followed by Tukey's HSD test, as shown in Table 3. The results of one-way ANOVA test on OPG expression of the mandibular bone in male Wistar rats showed significant variations in OPG expression of the mandibular bone in three groups ($P < 0.05$, probability value = 0.000).

Tukey's HSD test yielded probability values of 0.000 and 0.003, respectively, indicating a significant difference ($P < 0.05$) in the average OPG expression value of mandibular bones of male Wistar rats between the control group to both the anchovy and milk groups. The probability value between the anchovy and milk groups was 0.064 ($P > 0.05$), indicating no significant difference in the OPG expression rate of the mandibular bone of male Wistar rats between the anchovy and milk groups.

**Table 1: Average value of osteoprotegerin (OPG) expression in the mandibular bones of male Wistar rats in the control, anchovy and milk groups for 40 days**

| Variable | Group | *N* | Average | Standard deviation | *P** |
|---|---|---|---|---|---|
| OPG | 1 (Control) | 9 | 2.67 | 1.323 | 0.545 |
| | 2 (Anchovy) | 9 | 7.55 | 1.810 | 0.712 |
| | 3 (Milk) | 9 | 5.67 | 1.871 | 0.552 |

**Shapiro–Wilk* test, *P*>0.05=normal distributed data

**Table 2: The study tested the average OPG expression value of mandibular bones in male Wistar rats in the control group, anchovies and calcium milk for 40 days using a one-way ANOVA test**

| | Sum of squares | df | Mean square | *F* | Sig.* |
|---|---|---|---|---|---|
| Between groups | 109,407 | 2 | 54,704 | 19,244 | 000 |
| Within groups | 68,222 | 24 | 2,843 | | |
| Total | 177,630 | 26 | | | |

**One-way ANOVA*, *P*<0.05=significantly different

## DISCUSSION

The findings of the study indicate that the calcium of anchovies stimulates the synthesis of OPG in the mandibular bone, promoting mandibular growth. The control group had the lowest mean value of OPG expression, followed by the milk group, whereas the anchovy group had the highest average mandibular bone OPG expression value [Figures 1 and 2].

The OPG expression of the control group was lower because this group did not consume anchovies or milk. Calcium, which is essential for bone formation, is found in anchovy and milk. Calcium deficiency reduces osteoblast development and OPG synthesis, leading to increased osteoclast resorption and inhibited bone growth.

The study found that both the anchovy and milk groups had significantly higher levels of OPG expression compared to the control group. This resulted in higher osteoblast proliferation and greater OPG production, as evidenced by the increased OPG expression in the anchovy and milk groups compared to the control group. Additionally, calcium supplementation in the treatment group led to enhanced extracellular and intracellular calcium levels, as well as increased osteoblast growth. The rats in the control group were calcium deficient because they only received regular water with no calcium supplementation. Udagawa *et al.* (2000)[14] found that the treatment group had higher OPG expression compared to the control group. This prevented osteoclast resorption and increased

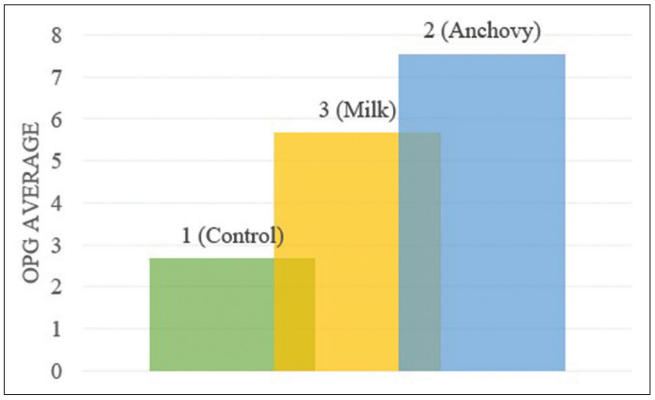


**Figure 1:** The graph dispalys the mean OPG expression in the mandibular bones of male wistar rats from the control, anchovy, and milk groups

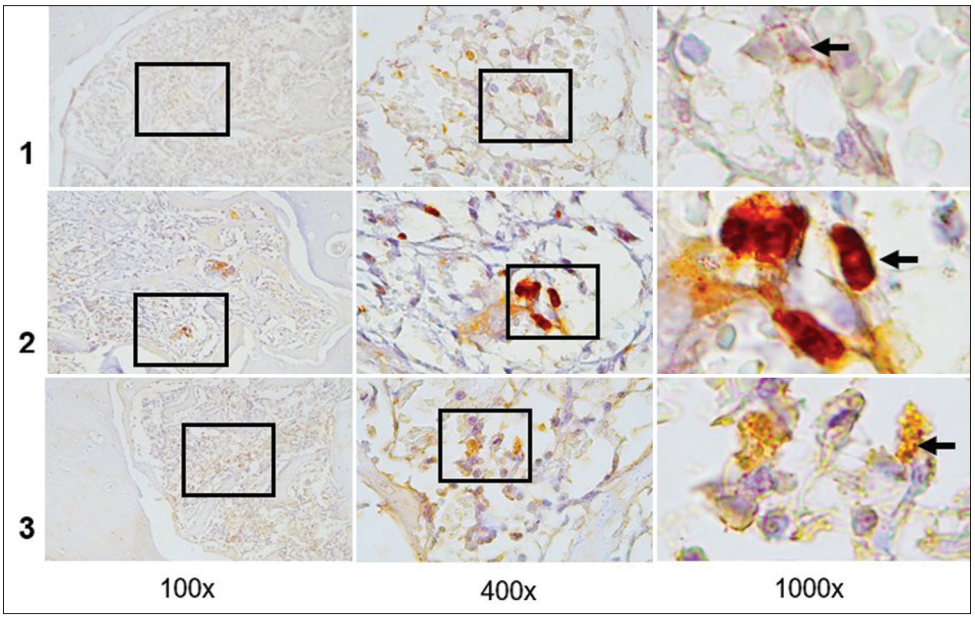


**Figure 2:** Osteoprotegerin (OPG) expression of male wistar rats mandible after 40 days, (1) control group, (2) anchovy group, (3) milk group. Arrows indicate OPG expression in osteoblast cells (Examination using Olympus BX-53 Japan microscope; magnification 100x, 400x and 1000x)

**Table 3: Significant Tukey's HSD difference test on the average OPG expression value of mandibular bones of male Wistar rats in the control, anchovy and milk groups for 40 days**

| (I) GROUP | (J) GROUP | Mean difference (I-J) | Std. error | Sig. | 95% Confidence interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound |
| 1 (Control) | 2 (Anchovy) | -4.889* | .795 | .000 | -6.87 | -2.90 |
| | 3 (Milk) | -3.000* | .795 | .003 | -4.98 | -1.02 |
| 2 (Anchovy) | 1 (Control) | 4.889* | .795 | .000 | 2.90 | 6.87 |
| | 3 (Milk) | 1.889 | .795 | .064 | -.10 | 3.87 |
| 3 (Milk) | 1 (Control) | 3.000* | .795 | .003 | 1.02 | 4.98 |
| | 2 (Anchovy) | -1.889 | .795 | .064 | -3.87 | .10 |

*The mean difference is significant at the 0.05 level

OPG formation, promoting osteoblast activity. *In vitro*, the OPG administration completely prevented enhanced osteoclastogenesis and bone loss in animals deficient in OPG.

OPG is a receptor for RANKL/factor osteoclast differentiation that suppress osteoclast differentiation and function.[15] RANKL, an element of the tumour necrosis Factor (TNF) superfamily, is a strong inducer of osteoclast proliferation and bone resorption. During normal bone remodelling, stromal marrow cells and osteoblasts produce RANKL. The RANKL signal enhances osteoclast differentiation and activation by binding to the receptor activator of nuclear factor kappa-B (RANK) transmembrane receptors, which are present on osteoclasts. This results in the synthesis of osteoclast-specific molecules.[15,16]

OPG and RANKL are factors that regulate osteoblast cell activity and osteoclastogenesis. OPG cytokines reduce bone resorption and osteoclastogenesis by blocking the binding of RANKL to RANK receptors on osteoclasts.[17] The OPG/RANKL ratio in osteoblasts may impede bone resorption metabolism when the ratio rises. This was confirmed by Bae *et al.* (2010),[18] who found that under adequate calcium conditions, the OPG/RANKL proportion and serum OPG values increased in relation to the low calcium group. Calcium consumption affects both bone mineral state and bone metabolism regulatory indicators, such as OPG and RANKL.

Calcium absorption occurs in the duodenum through both extracellular and intracellular pathways. Budiatin *et al.* (2022)[19] reported that increasing extracellular calcium levels significantly enhances osteoblast proliferation and chemotaxis, leading to a significant increase in bone formation. Tantral *et al.* (2004)[20] found that increasing calcium levels to 20 mM induced osteoclast apoptosis via caspase-3, caspase-9 and calcium-sensing receptor (CaR), resulting in the release of intracellular calcium stores that are phospholipase C (PLC)-dependent in the inositol

trisphosphate (IP3) signaling pathway. The activation of IP3 induced nuclear translocation of NF-κB, which in turn resulted in osteoclast apoptosis and inhibition of bone resorption.

Anchovy powder contains 500 mg of calcium per 100 g.[21] According to the nutritional adequacy recommendations of The Food and Agriculture Organization (FAO)/World Health Organization (WHO) 2001, 35 g of milk provides 350 mg of calcium, which is 35% of calcium recommended dietary allowance (RDA).[22] In this study, there was no statistically significant difference in mandibular OPG expression between male Wistar rats in the anchovy and milk groups, although anchovies and milk have significantly different calcium contents. The difference in OPG expression is believed to be due to the effects of hormone regulation. OPG expression is regulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (calcitriol), vitamin D3, estrogens, cytokines, and prostaglandins.[23,24]

OPG is synthesized by osteoblast progenitor cells and has biological effects by binding to its receptor, osteoclast differentiation and activation receptor (ODAR)/RANK, in osteoclast progenitor cells. It can either dissolve or bind to the membrane, with the latter requiring contact with other cells. Calcitropic hormones and cytokines such as PTH, dexamethasone, 1,25-dihydroxyvitamin D3 (1,25-(OH) 2D3), interleukin (IL)-1, IL-11, TNF-α, and prostaglandin E2 (PGE2) modify OPG stable state messenger ribonucleic acid (mRNA) levels in osteoblastic progenitor cells. OPG has various biological effects on bone cells, including preventing the terminal stage of osteoclast growth, decreasing mature osteoclast activation, and inducing apoptosis. Concentrations ranging from 1 to 40 ng/mL inhibit osteoclast differentiation, with half-maximal effects achieved at 4–6 ng/mL. In assays investigating the susceptibility of osteoclastogenesis to OPG, constant administration from days 5 to 11 was required for osteoclast reduction. Discontinuation after day 3 or periodic supply at days 7 and 8 had no effect. OPG inhibits osteoclastogenesis induced by PGE2, PTH, IL-1 or IL-11 treatment.[25] Additionally, to compensate for lower serum calcium levels, PTH regulates excessive calcium resorption from bones. However, serum calcium levels remain abnormally low. A calcium-deficient diet can hinder bone growth over time.[26]

Mendes *et al*. (2019) demonstrated in their study that PTH concentrations were significantly linked to total bone mineral density (BMD), particularly in cases of hyperparathyroidism with vitamin D deficiency or insufficiency.[27] Olmos *et al*. (2015)[28] found that a blood level of 30 ng/mL of 25-hydroxyvitamin D is necessary to prevent secondary hyperparathyroidism and osteoporosis, regardless of milk calcium consumption. Similarly, Chaitou *et al*. (2011)[29] linked sufficient calcium intake to lower PTH levels, which in turn improves bone microarchitecture and strength.

In contrast to PTH, 1,25(OH) 2D3 has varying effects on the expression of OPG and receptor activator of RANKL mRNA in rat osteoblasts. Tian and Huang (2004) found that the addition of 1,25(OH) 2D3 significantly decreased OPG mRNA expression in osteoblasts. After 48 h of culture with 1,25(OH) 2D3,[30] RANKL mRNA expression in osteoblasts increased significantly. Additionally, the 1,25(OH) 2D3 activator protein-1 (AP-1) binding region suppressed *OPG* gene expression by accelerating mRNA degradation and lowering promoter activity. Therefore, 1,25(OH) 2D3 affects both short-term bone resorption and long-term osteoblast proliferation and differentiation.[31]

Piri *et al*. (2016)[32] found that calcium, vitamin D and oestrogen intake increased OPG and RANKL levels, which improved bone mineral density. Taking calcium and vitamin D supplements during puberty promoted calcium crystal formation and enhanced bone mineral density.[33] Stern *et al*. (2007)[34] found that when oestrogen levels decrease, RANKL levels increase, leading to lower BMD and osteoporosis. Peacock *et al*. (2010)[35] also found that inadequate calcium intake reduces bone mineral density and increases the risk of fractures.

Although the calcium content of anchovies and milk may differ, there is no significant difference in the expression of OPG. The levels of phosphorus, protein, PTH, (1,25-(OH) 2D3) and vitamin D were not measured in either group. Further research is needed to investigate the factors that affect calcium absorption and metabolic rate through OPG expression.

No previous research has examined the potential for calcium content through OPG expression in anchovies. This study used anchovy as a local marine resource that can be consumed from head to tail. This fish is inexpensive and abundantly available throughout Indonesia. This research exploited the local marine resources by maximizing the use of anchovies, which contained 1933 mg/100 g. This research suggests that anchovy optimization, due to its high calcium content, may contribute to the mandibular growth of children, through OPG expression on the mandible.

The limitation of this study is that no testing of anchovy content other than calcium was conducted. Consequently, it is unknown what other factors may influence calcium absorption and metabolism in the body, including in terms of OPG expression.

## CONCLUSION

The research suggests that

1. Anchovy *(Stolephorus sp.)* is a high-calcium nutrient that has the potential to stimulate mandibular growth.
2. Anchovy *(Stolephorus sp.)* is more effective than milk at stimulating mandibular growth. However, the analysis of OPG expression is statistically meaningless.
3. Further research is required to investigate the relationship between anchovy (*Stolephorus sp.*) consumption and its impact on calcium absorption and metabolism, specifically in relation to mandibular bone growth and OPG expression analysis.

### Ethical clearance

This research was conducted under ethical approval recommendation, number 0059/PL.09/KEPK FKG-RSGM UNHAS/2023.

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### Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Hayati AW, Ridwan H. The benefits of midmorning snack to combat stunting: A longitudinal panel study in the Riau Province of Indonesia. Curr Nutr Food Sci 2022;18:677-88.
2. Watanabe K, Imamura H, Uchikanbori S, Fujita Y, Maki K. Effects of restricted calcium intake on bone and maxillofacial growth. Angle Orthod 2008;78:445–52.
3. Engström C, Linde A, Thilander B. Craniofacial morphology and growth in the rat. cephalometric analysis of the effects of a low calcium and vitamin D-deficient diet. J Anat 1982;134:299—314.
4. Achmad H, Tahir H, Adam M, Ramadhany YF. Increased overjet in growing child, problem solving in pediatric dentistry. J Int Dent Med Res 2017;10:374–9.
5. Goto S, Fujita Y, Hotta M, Sugiyama A, Maki K. Influence of differences in the hardness and calcium content of diets on the growth of craniofacial bone in rats. Angle Orthod 2015;85:969-79.
6. Henry JP, Bordoni B. Histology, Osteoblast. Treasure Island: StatPearls Publishing; 2024.
7. Drake TM, Gupta V. Calcium. Treasure Island: StatPearls Publishing; 2024.
8. Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, *et al*. Receptor activator of nuclear factor κb ligand (rankl)/ osteoprotegerin (opg) ratio is increased in severe osteolysis. Am J Pathol 2003;163:2021–31.
9. Wirakusumah ES. Prevention of Osteoporosis. Jakarta: Penebar Swadaya; 2017.
10. Astawan M. Healthy with Animal Dishes. Jakarta: Penebar Swadaya; 2008.
11. Fatchiyah. Molecular Biology: The Fundamental Principles of Analysis. Jakarta: Erlangga; 2011.
12. Soini YM, Pääkkö P, Lehto VP. Histopathological evaluation of apoptosis in cancer. Am J Pathol 1998;153:1041–53.
13. Pižem J, Cöer A. Detection of apoptotic cells in tumour paraffin sections. Radiol Oncol 2003;37:225-32.
14. Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, *et al*. Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 2000;141:3478–84.
15. Leibbrandt A, Penninger J. RANK/RANKL: Regulators of immune responses and bone physiology. Ann N Y Acad Sci 2008;1143:123–50.
16. Boyce BF, Xing L. The RANKL/RANK/OPG pathway. Curr Osteoporos Rep 2007;5:98–104.
17. Chun EH, Kim H, Suh CS, Kim JH, Kim DY, Kim JG. Polymorphisms in neuropeptide genes and bone mineral density in Korean postmenopausal women. Menopause 2015;22:1256—63.
18. Bae Y, Kim MH. Calcium and magnesium supplementation improves serum opg/rankl in calcium-deficient ovariectomized rats. Calcif Tissue Int 2010;87:365–72.
19. Budiatin AS, Khotib J, Samirah S, Ardianto C, Gani MA, Putri BRKH, *et al*. Acceleration of bone fracture healing through the use of bovine hydroxyapatite or calcium lactate oral and implant bovine hydroxyapatite-gelatin on bone defect animal model. Polymers (Basel) 2022;14:4812.
20. Tantral L, Malathi K, Kohyama S, Silane M, Berenstein A, Jayaraman T. Intracellular calcium release is required for caspase-3 and-9 activation. Cell Biochem Funct 2004;22:35–40.
21. Aryati EE, Dharmayanti A. Benefits of fresh anchovies (stolephorus sp) on bone and tooth development. Odonto Dent J 2014;1:52–6.
22. Kartono D, Hardinsyah H, Jahari A, Sulaeman A, Astuti M, Soekatri M, *et al*. Summary-recommended daily allowance (RDA) for Indonesians 2012. In: Formulation of Widya Karya Nasional Food and Nutrition X 2012. Jakarta: Bogor; 2012.
23. Fohr B, Dunstan CR, Seibel MJ. Clinical review 165: Markers of bone remodeling in metastatic bone disease. J Clin Endocrinol Metab 2003;88:5059—75.
24. Hofbauer L, Schoppet M. Clinical implications of the osteoprotegerin/ rankl/rank system for bone and vascular diseases. JAMA 2004;292:490–5.
25. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 2000;15:2–12.
26. Fujita Y, Goto S, Ichikawa M, Hamaguchi A, Maki K. Effect of dietary calcium deficiency and altered diet hardness on the jawbone growth: A micro-CT and bone histomorphometric study in rats. Arch Oral Biol 2016;72:200–10.
27. Mendes M, Hart K, Lanham-New S, Botelho P. Association between 25-hydroxyvitamin d, parathyroid hormone, vitamin d and calcium intake, and bone density in healthy adult women: A cross-sectional analysis from the D-Sol study. Nutrients 2019;11:1267.
28. Olmos JM, Hernandez JL, Garcia-Velasco P, Martinez J, Llorca J, Gonzalez-Macias J. Serum 25-hydroxyvitamin D, parathyroid hormone, calcium intake, and bone mineral density in Spanish adults. Osteoporos Int 2016;27:105-13
29. Chaitou A, Boutroy S, Vilayphiou N, Varennes A, Richard M, Blaizot S, *et al*. Association of bone microarchitecture with parathyroid hormone

concentration and calcium intake in men: The STRAMBO study. Eur J Endocrinol 2011;165:151–9.
30. Tian QX, Huang GY. Effects of 1,25dihydroxyvitamin D3 on the expressions of osteoprotegerin and receptor activator of NFkappaB ligand in mouse osteoblasts. Acta Acad Med Sinicae 2004;26:418-22.
31. Kondo T, Kitazawa R, Maeda S, Kitazawa S. 1α,25 Dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. J Bone Miner Res 2004;19:1411–9.
32. Piri F, Khosravi A, Moayeri A, Moradipour A, Derakhshan S. The effects of dietary supplements of calcium, vitamin D and estrogen hormone on serum levels of OPG and RANKL cytokines and their relationship with increased bone density in rats. J Clin Diagn Res 2016;10:AF01—4.
33. Sadat-Ali M, Al Elq AH, Al-Turki HA, Al-Mulhim FA, Al-Ali K. Influence of Vitamin D levels on bone mineral density and osteoporosis. Ann Saudi Med 2011;31:602-8.
34. Stern A, Laughlin G, Bergstrom J, Barrett-Connor E. The sex-specific association of serum osteoprotegerin and receptor activator of nuclear factor kappaB legend with bone mineral density in older adults: The Rancho Bernardo study. Eur J Endocrinol 2007;156:555–62.
35. Peacock M. Calcium metabolism in health and disease. Clin J Am Soc Nephrol 2010;5(Supplement 1):S23–30.