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RESEARCH ARTICLE

Effectiveness of vitamin D3 supplementation with protein realimentation in osteoblast and osteoclast of the maxilla of breastfed malnourished rat infants

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ABSTRACT

Child malnutrition can happen because of lack of nutrition during pregnancy. Malnutrition can cause low birth weight in babies. This can cause jaw and alveolar bone abnormalities that can lead to malocclusion, periodontitis, and others. This condition can affect bone cells, such as osteoblast and osteoclast. Vitamin D3 supplementation accompanied by protein realimentation may promote optimum bone growth. This research aims to investigate the effect of protein realimentation and vitamin D3 on osteoblast and osteoclast of malnourished rat pups' maxilla. In this research, the rat pups were divided into 5 groups: a positive control group (K+), a negative control group (K-), and 3 treatment groups (P1, P2, and P3). The subjects of this research were 10 male and 30 female Wistar rats. A condition of malnutrition was induced in the rat pups and they were given vitamin D3 supplementation with protein realimentation. Rat pups in each group were euthanized on the 22nd day to observe the number of osteoclast and osteoblast in the maxilla. The results showed that the number of osteoblasts between normal and malnourished rats was significantly different (p value < 0.05), but the number of osteoclasts between rats in the control and treatment groups was not significantly different. Administration of vitamin D3 may affect the number of osteoblasts and may not affect the number of osteoclasts on bone. The effective dose of vitamin D3 is 0.36 IU/day.

Keywords: osteoblast; osteoclast; protein; vitamin D3

INTRODUCTION

Child malnutrition can happen because of the lack of protein during pregnancy, which can have an impact on physical growth such as the growth of bones and teeth. Malnutrition until the last week of pregnancy causes a low birth weight or a birth weight of < 2,500 g.[1,2] This condition inhibits teeth mineralization, bone calcification, and bone cell differentiation due to disturbance of calcium and phosphor deposit.[3] Low birth weight could increase the risk of osteopenia and inhibit bone growth.[4] The function of the jaw bone is to form the upper and lower jaw, support the teeth, and aid mastication. The maxilla undergoes changes in shape and size through a process of bone remodeling.[5] Jaw bone abnormalities caused by lack of protein may affect the bone mass. It is weaker and smaller than the normal bone because abnormal bone is structurally incompetent.[6]

Osteoblast and osteoclast are the two main types of bone cells. Both have an important role in the bone remodeling process such as resorption and absorption. Remodeling is an important basis of bone growth. The remodeling process allows the bone to adapt to physical signals such as increasing load or hormones in the growth process. Osteoblast produces matrices and will partially calcify to be pre-osteoid and is then perfectly calcified into bone. This cell controls mineralization by regulating the passage of calcium and phosphate ion through the surface membrane and synthesis of most of the protein found in bone. When osteoblasts are activated, mineral matrices will dissolve because of the bone resorption process. Bone synthesis and destruction happen simultaneously with bone formation as the result of both processes, known as coupling of bone resorption and formation. An increase in osteoclast number indicates bone

resorption, while and an increasing number of osteoblast indicates bone formation.[7–9]

Protein intake in pregnant mothers in Indonesia is still inadequate. Based on the Total Diet Study in 2014 in Indonesia, pregnant mothers had protein intake of ≤ 80% from Protein Adequacy Rate of 49.6% in urban areas and 55.6% in rural areas. Protein is needed to form the bone organic matrix that consists of collagen and non-collagenous materials which are the place of deposition of calcium and phosphor in bone calcification.[9,10] Realimentation or improvement of protein intake in sufficient quantities can indirectly increase vitamin D3 synthesis which impacts on the increase of calcium and inorganic phosphate absorption in the intestines and regulates parathyroid hormone (PTH), thus increasing bone strength, bone mass, and decreasing fracture risk.[3] Vitamin D3 plays a role in bone formation regulation by osteoblast and bone resorption by osteoclast to create a coupling system.[11] The results of a survey in Indonesia found that 43% of children in urban areas and 44% of children in rural areas have vitamin D deficiency. Vitamin D deficiency in Indonesia, a country with sufficient sun exposure, is caused by decreased outdoor activities, baby's morning sunbathing, breastfeeding without vitamin D supplementation, keeping all the skin covered, and the use of sunscreen. Indonesian children only consume food naturally rich in vitamin D because foods with vitamin D fortification are rare. These conditions may cause low levels of vitamin D in the blood.[12]

Endocrine Society in the United States of America encourages pregnant women to consume vitamin D at least 600 IU/day. To maintain vitamin D levels in blood above 30 ng/ml, it is recommended to consume vitamin D of 1,500-2,000 IU/day.[13] Another research shows that intake of vitamin D 4,000 IU during breastfeeding will increase vitamin D levels in breast milk which is useful as an intervention to prevent vitamin D deficiency in mother and baby.[14,15] Vitamin D levels in serum are said to be sufficient if they reach 32-100 ng/ml, low when the rate is 11-32 ng/ml, and very low if the rate is < 11 ng/ml.[15]

As discussed above, the balance of bone formation by osteoblasts and bone resorption by osteoclasts determines bone quality in the present or in the future. This research aims to investigate the effect of protein realimentation and vitamin D3 on osteoblast and osteoclast of malnourished rat pups' maxilla. Vitamin D3 supplementation accompanied by protein realimentation may promote optimum bone growth. Vitamin D3 with protein realimentation also becomes an alternative for mothers and babies who are rarely exposed to the sun and consume less food containing vitamin D. Various studies have revealed the effective dose of vitamin D3 to achieve sufficient level of serum and for optimum bone growth. However, to the best of our knowledge no research has been conducted on the effectiveness of vitamin D3 supplementation with protein realimentation on the number of osteoblast and osteoclast in malnourished children. This has inspired us to conduct preliminary research on protective nutrition to maintain maxillary bone quality, especially in malnourished children.

MATERIALS AND METHODS

This research was an experimental laboratory research with a posttest-only control group design. This research was conducted in several places, such as the Center of Food and Nutrition Studies of Universitas Gadjah Mada (UGM) and Pathology Anatomy FKKMK UGM. Ethics approval was obtained from Muhammadiyah Purwokerto University (UMP) (project number: KEPK/UMP/24/XII/2020).

The subjects of this research were 10 male and 30 female Wistar rats (*Rattus novergicus*). They were divided into 5 groups: a positive control group (K+), a negative control group (K-), and treatment groups (P1, P2, and P3). These 40 subjects were adapted for 7 days, and a condition of malnutrition was induced in 30 female rats in early pregnancy. Malnutrition induction was done by administration of 4% low protein diet, which consisted of 4.6% casein, 4.95% corn oil, alphacel, 7.35% non-nutritive bulk, AIN-76 with 4% mineral mixture, 63.1% sucrose, and 15% cornstarch. Low protein diet was given from the first day of

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gestation until the rat mother gave birth on day 21. Malnutrition induction was carried out in K-, P1, P2, and P3 groups.

K-, P1, P2, and P3 groups were then given treatment in the form of a standard and low-protein diet. Standard protein diet was fed on adult rats in accordance with the required protein intake, consisting of 20% casein, 39.7% cornstarch, 13.2% dextrinized cornstarch, 10% sucrose, 7% soybean oil, alphacel, 5% non-nutritive bulk, AIN-93 with 3.5% mineral mixture, AIN-93 with 1% vitamin mixture excluding vitamin D3, 0.3% L-cystine, 0.25% choline bitartrate, and 0.0014% tert-butylhydroquinone (optional).

Healthy rats in K+ group and malnourished rat pups in K- group were breastfed by mother rats which were given standard protein diet without vitamin D3 intake. Malnourished rat pups in P1 group were breastfed by mothers whose diet consisted of standard protein and vitamin D3 0.09 IU, while malnourished rat pups in P2 group were breastfed by mothers given low protein diet and vitamin D3 0.18 IU. Malnourished rat pups in P3 group were breastfed by mothers given standard protein diet and vitamin D3 0.36 IU. The treatment was carried out until one of the rats in each group was euthanized on day 22. Then a procedure for the histological observation of hard palate around the maxillary alveolar bone was developed to determine the number of osteoclasts and osteoblasts present in each group. Osteoblasts have a single cell nucleus, which varies in shape from flat to globose, reflecting a level of cellular activity and at a later stage of the maturation process paralleling the formation of bone on the surface. Osteoclasts are multinucleated cells formed by the fusion of myeloid hematopoietic precursors present in the bone marrow adjacent to the bone surface. The number of osteoclasts and osteoblast was observed by hematoxylin-eosin (HE) staining.

The data obtained was processed with STATA 13. Normality test with Shapiro-Wilk test ($N < 50$) was done to determine the data distribution, and test for homogeneity used the Levene's test. Univariate analysis consisted of data analysis by calculating the average value of the rat's body weight. Data with normal distribution and homogeneity of variance were analyzed by one-way ANOVA test followed by post hoc Bonferroni test. Data with abnormal distribution and non-homogeneous data were analyzed by non-parametric test, the Kruskal-Wallis test, followed by the post-hoc Mann-Whitney test.

RESULTS

The adapted rats were then weighed to determine their drastic weight loss (Figure 1). Based on the diagram, no weight loss was observed in the mother and male groups. Post-adaptation to rat body weight was within the normal limits of their age, which was 150-200 grams. Thus these rats were included in the sample criteria of the research. In this study, a male rat was allowed to mate with 3 female rats. Pregnant rats were characterized

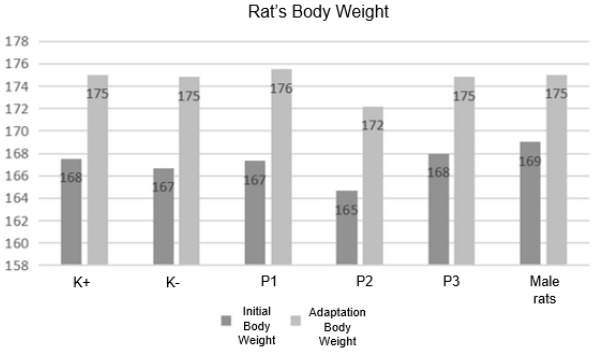

Figure 1. Rat's body weight

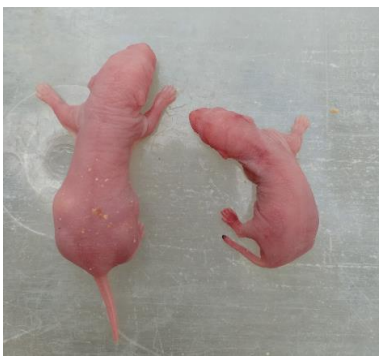

Figure 2. Comparison of normal birth weight rat pup (left) and low birth weight rat pup (right)

Table 1. Birth weight of rat pups from each group

Characteristic	Groups				
	K+ (n = 65)	K- (n = 47)	P1 (n = 50)	P2 (n = 53)	P3 (n = 34)
Birth weight (gram)	8.32 ± 0.47	4.40 ± 0.58	4.18 ± 0.39	4.08 ± 0.27	4.56 ± 0.50

Notes: K+ = positive control group, K- = negative control group, P1 = treatment group Malnourished rat pups breastfed to mother with standard protein diet and vitamin D3 0.09 IU, P2 = malnourished rat pups breastfed to mother with low protein diet and vitamin D3 0.18 IU, P3 = malnourished rat pups in P3 group breastfed to mother with standard protein diet and vitamin D3 0.36 IU.

Table 2. The number of osteoblasts and osteoclasts of each group.

Characteristics	Groups					p
	K+ (n = 6)	K- (n = 6)	P1 (n = 6)	P2 (n = 6)	P3 (n = 6)	
Osteoblasts	62.17 ± 13.08[a]	26.33 ± 10.13[c]	39.83 ± 6.62[b]	40.67 ± 14.87[b]	49.83 ± 19.99[a,b]	0.002*
Osteoclasts	17 (19-14)	14 (8-24)	25 (11-27)	13 (9-25)	17 (8-37)	0.342

Note: a, b, c : Different letter notations shows a significantly different results.
- The number of osteoblasts in K+ group significantly different with K-, P1, and P2 groups.
- The number of osteoblasts in K- group significantly different with P3 group.
- No significant difference between P1, P2, and P3 groups.
- No significant difference between K+ and P3 groups.

*: P value shows a significantly different results.

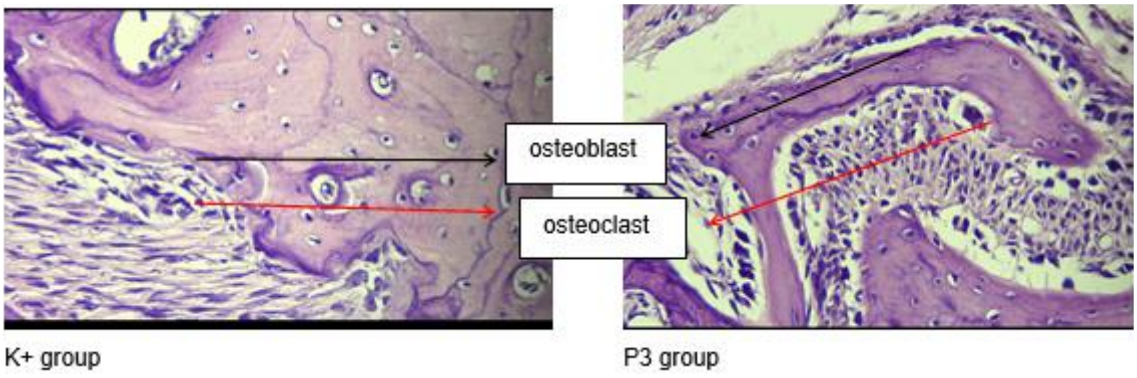

Figure 3. Histological image of osteoblast (black arrows) and osteoclast (red arrows) in positive control (K+) group and 3th treatment group (P3).

by the presence of a vaginal plug and a positive vaginal swab containing sperm. These rats were then divided into 5 random groups: 1 group was given a standard protein diet (K+), and the other 4 groups were given a low protein diet (K-, P1, P2, and P3). Each rat mother was weighed every week to determine the amount of feed which was 10% of the rat's body weight. The rat pups in each group were also weighed. The birth weight of the rats was listed in Table 1, while the number of osteoclasts and osteoclasts of each group was listed in Table 2.

Based on the Table 2, vitamin D3 and protein realimentation treatment during breastfeeding could result in a difference in the number of osteoclasts and osteoblasts of malnourished rat pups. Healthy rat pups (positive control) had the highest osteoblast number (62.17 ± 13.08).

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Malnourished rat pups with mothers given a standard protein diet and vitamin D3 (negative control) had the lowest osteoblast (26.33 ± 10.13). Malnourished rat pups which were breastfed by mothers with standard protein diet and 4,000 IU vitamin D3/day (P3) had the number of osteoblasts that was not significantly different from that of the other 2 treatment groups, but significantly different from that of the malnourished rat pups without vitamin D3. By contrast, the number of osteoclasts in each group was not significantly different.

DISCUSSION

The increase in body weight of mother rats and males during adaptation was within the normal range, which was 150-300 grams in 2-3 months old. Rats' pregnancy in this research lasted for 21 days, which corresponds to Ghasemi et al who found that rat pregnancy lasts for 21-22 days. Under good lighting conditions, 57% of rats were born on the 21st day, while the rest were born on the 22nd day. In this research, the lighting condition within the rat facilities was sufficient.[16]

In Table 1, the birth weight of rat pups in groups K-, P1, P2, and P3 fed a low protein diet was lower than the birth weight of pups in K+ group fed a standard protein diet. The birth rate in rat pups in groups K-, P1, P2, and P3 was categorized into low birth weight because the birth weight was below 5 grams. The result was in line with Ghasemi et al who showed that normal birth weight of rat pups was 5-7 grams.[16] The weight loss was caused by the low protein diet (4%) in the pregnancy period. Xie et al. and Hassan and Hegazy found that protein diet of 5-10%, which was lower than the standard protein diet of 21% (control) in rats during pregnancy, could result in lower birth weight compared to the control group.[17,18] Based on the 2013 Nutritional Adequacy Rate (*Angka Kecukupan Gizi* or AKG), 20 g/day of additional protein during pregnancy and the other macro and micronutrients to support fetal growth is in accordance with IOM that recommends that the addition of protein for pregnant women is about 10%-35% of total daily energy.[19] Protein consumed by pregnant women plays an important role in fetal growth and development, placenta, uterus, breast, and increase in mother's blood volume.[20] Lack of protein intake during pregnancy is related to chronic energy deficiency in the pregnant woman.[21] Pregnant women who suffer malnutrition throughout the last week of pregnancy may give birth to a baby with low birth weight (< 2,500 g).[1,2] Nutrient deficiency such as protein in pregnancy can inhibit fetal growth, cause muscle development disorder, reduce bone density, and lead to metabolic disorders of the fetus.[22]

Based on the results of this research, the group with a standard protein diet (20%) and 4,000 IU/day of vitamin D3 showed an optimum increase in osteoblast. The number of osteoblasts in this group was not significantly different from that in the healthy rat pups. The number of osteoblasts in treatment groups with standard protein diet and 1,000 IU/day vitamin D3 (P1), low protein diet and 2,000 IU/day vitamin D3 (P2), and standard protein diet with 4,000 IU/day vitamin D3 (P3) shows the amounts that are not significantly different from each other. The group with 4,000 IU/day vitamin D3 (P3) has significantly different number of osteoblasts with malnourished rat which are given protein realimentation without vitamin D3 (Table 2).

The number of osteoblasts in treatment groups with a protein diet and vitamin D3 had no significant difference from the number of osteoblasts in healthy rats. This might be caused by the increase in protein intake that could increase the level of insulin-like growth factor-1 (IGF-1). As a result, the synthesis of vitamin D3 which has a role in bone formation would increase. The groups were also given vitamin D3 supplementation which has a role in the growth and differentiation of osteoblasts, so the number of osteoblasts increased due to vitamin D3 and protein intake.[3,22]

In contrast, the number of osteoclasts that has a role in bone resorption, had no significant difference in all groups. It is because all rat pups in all groups had been given protein realimentation. Protein intake can increase the calcium absorption rate and non-organic phosphate in the intestines, so parathyroid hormone (PTH) can decrease and

reduce bone resorption. The limitation of this study is less varied treatment groups. Further research is needed to conduct other histopathological examinations to find out the whole picture of the maxillary bone activity. Further investigations are also required for the examination of other bone minerals and serum calcium and vitamin D to obtain more accurate results with more varied treatment groups.

CONCLUSION

To conclude, there is a significant difference in the number of osteoblasts and no significant difference in the number of osteoclasts between malnourished rat pups and rat pups with mothers treated with protein realimentation and various doses of vitamin D3 (0.09 IU/day, 0.18 IU/day, and 0.36 IU/day). The effective dose of vitamin D3 that affects the number of osteoblast and osteoclast is 0.36 IU/day.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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