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Blood cockle (*Anadara granosa*) supplementation to increase serum calcium level and femur growth of low-protein diet rat

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Keywords: *Anadara granosa*, calcium, femur, low-protein diet, rat

INTRODUCTION

Chronic low-protein diet can cause disruption of linear growth or disruption of height growth (stunting). In 2013, the prevalence of infants stunting in Indonesia reached 37.2%. The prevalence rate above is over the cut-off rate which has been universally agreed upon, namely 20%, so it is still a public health problem (Mofit 2010). The intake of energy and nutrients such as carbohydrates, proteins, fats, zinc, and calcium affect the prevalence of stunting. Toddlers having a shortage of protein intake have the risk of becoming stunting 3.46 times higher than normal toddlers (Hidayati et al. 2010). Results of research by Dewi and Adhi (2016) showed that toddlers who lack protein consumption have odds 11.26 times higher than toddlers with adequate protein intake to experience stunting.

Meanwhile, stunting is also influenced by economic factors so that the necessary efforts to harness the potent of food containing nutrients, having relatively affordable price, as well as being culturally acceptable by society. The blood cockle was a food containing proteins, minerals such as zinc, iron, and calcium, as well as the complete amino acid (Nurjanah 2005; Solang 2014). The blood cockle could be used as an alternative protein source (Broom 1985). The protein content was about 9.64% (Aziz et al. 2007), while the blood cockle flour contained of approximately 27.29% protein, 81.16 ppm of zinc, 1720.46 ppm of Fe, 4.26 ppm of Cu, and 318.67 ppm of Ca (Solang 2014). Proteins play a role in helping the absorption of calcium. Calcium could influence the

proliferation of osteoblasts, thus affecting bone growth in humans (Huang et al. 2001). The growth of long bones contributes in determining the final height a person (van der Eerden 2003).

Protein, zinc, and calcium derived from blood cockle works together in repairing bone growth by improving serum calcium levels as well as the weight and length of rat femurs on low-protein diet. Rat femurs are used as test animals because they had similar digestive tract and absorption abilities with human (Smith and Mangkokowidjono 1987). This study aimed to evaluate the effect of supplementation of flour of blood cockle to serum calcium, length and weight of male rat femurs (*Rattus norvegicus*) Wistar strain which were on a low-protein diet.

MATERIALS AND METHODS

Design and sample

The blood cockles are taken from Puhuwato, Gorontalo Province, Indonesia. The treatment on animals test is performed at the Laboratory of Biology, Faculty of Mathematics and Sciences, Universitas Negeri Gorontalo, Gorontalo, Indonesia. The study used *The Separate Sample Pre-Post Test Control Group Design* (Campbell and Stanley 1963). Forty-eight male Wistar rats age 6 weeks weighing 115-120 g were randomly divided into 2 groups: 12 rats of normal control group were fed with standard and 36 rat group was fed a low-protein (6%) (Kintore 2010). After eight weeks, four rats from the control group and

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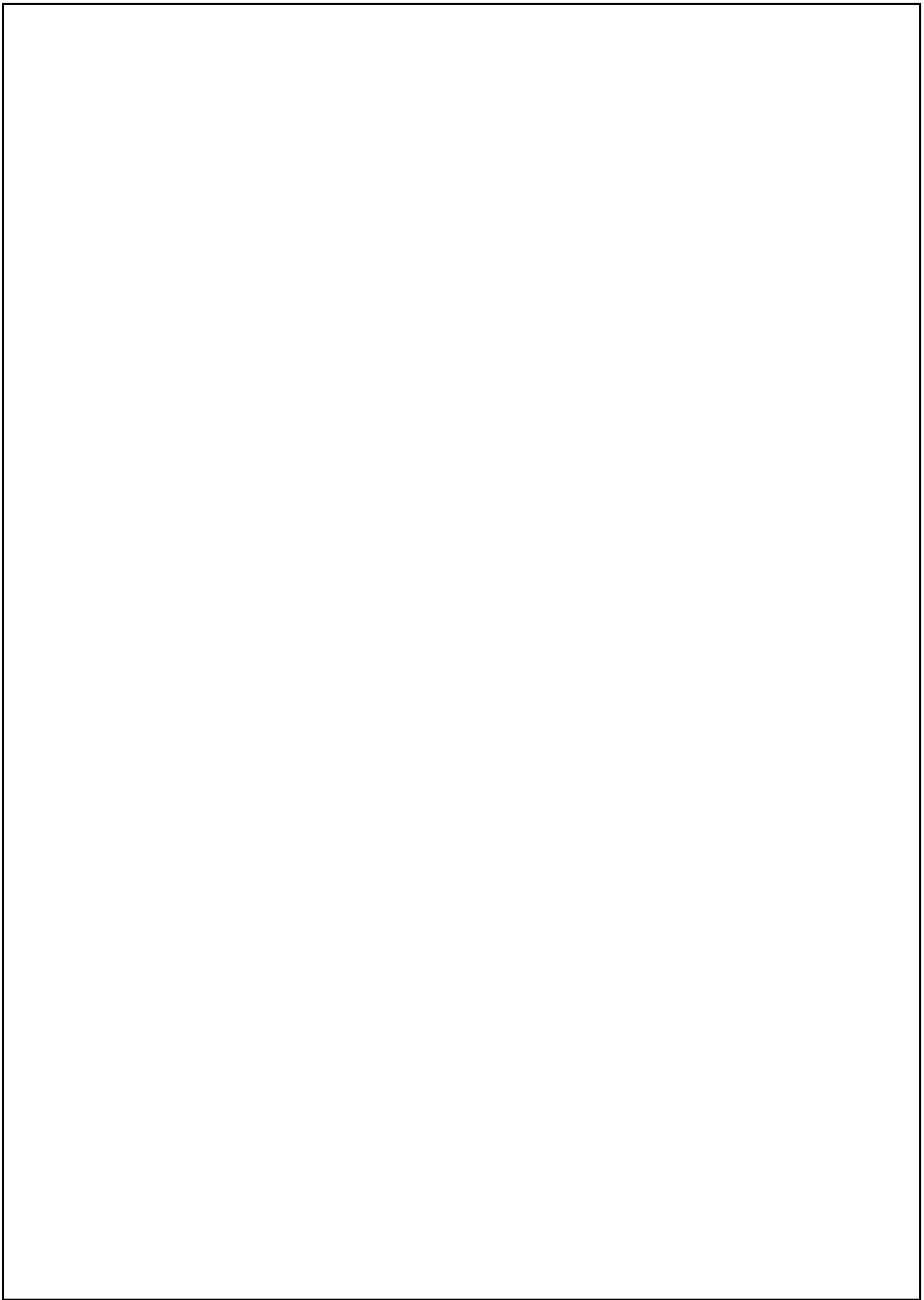
proliferation of osteoblasts, thus affecting bone growth in humans (Huang et al. 2001). The growth of long bones contributes in determining the final height a person (van der Eerden 2003).

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from the low-protein diet group are sacrificed to assay albumin. Rats are categorized malnourished if the results of the test of albumin serum levels are less than 3.3 mg/dL (Giknis 2008; Susanto 2011). Eight rats of normal control remains to be fed by the standard food, while the other 32 rats on low-protein diet were randomly divided into four groups which were fed on *karak* and given blood cockle flour with quantity of 12.5% /kg of food, 25%/kg of food, and 50%/kg of food for 8 weeks.

Animal test feed

Normal control group were fed by standard feed produced by PT. Charoen Pokpan Indonesia. The standard pellet feed contains 13-15% protein. Meanwhile, the feed used for conditioning malnutrition is feed containing 8.46% protein, 81.16 ppm of zinc and 27.26% protein. The composition of blood cockle flour substituted in *karak* is respectively 12.5% kg/feed, 25% kg/feed, and 50% kg/feed. Drinking water is provided water taps from PDAM. Feed and water were given in the way of *ad libitum*.

Ethics test

Ethics test was conducted at the Faculty of Public Health, Universitas Airlangga, Surabaya, East Java, Indonesia. Researchers have presented before the ethics committee and have earned ethics eligibility certificate from those institution.

Procedures

Blood samples were taken from the rat heart. 2 ml of blood is collected in the blood collection tube-no additive and another 3 ml of blood is collected in the tube of blood collection tube - EDTA. To get the blood serum, blood samples were then centrifuged at 3000 rpm for 15 minutes. Serum was separated into an Eppendorf tube. The conditions of low-protein diet can be concluded by the value of albumin of rats having pre-treated test. The results showed that the control rats had higher levels of albumin of about 3.2 g/dL, while rats given *karak* had levels of albumin of 2.7 g/dL. Albumin levels were measured using Bromocresol Green (BCG) method (Doumas et al. 2009) by automatic chemical analysis tool Prestige 24i.Cat. No. 4-238.

The serum calcium levels were measured using Atomic Absorbant Spectrophotometer (AAS) with tool Zeenit 700. The calcium levels were expressed in mg/dL. Femur length was measured with a digital caliper Einhill with a precision of two digits after the decimal point and femur was weighed with digital scales (g) Camry Model EHA401 with an accuracy of two digits after the decimal point.

Data analysis

The data of serum calcium levels, femur length, and weight of femur were tested using parametric statistical tests One Way ANOVA at 95% confidence level and Least Significance Difference (LSD). Data analysis was performed using SPSS.

RESULTS AND DISCUSSION

Calcium level

The serum calcium levels in the low-protein diet group decreased 31.40% of the normal control group. The giving of blood cockle flour to low-protein diet group showed an increase in serum calcium levels. This group was given blood cockle flour of 12.5%, 25% and 50% respectively and it resulted an increase in serum calcium levels at 35.72%, 19.88% and 17.60%.

The giving of blood cockle flour 12.5% showed the highest increase in calcium levels among the treatments. ANOVA test results showed that administration of blood cockle flour increase serum calcium levels of rats with a low-protein diet ($p = 0.03$). Furthermore, LSD test results showed that the average serum calcium levels of rats with a low-protein diet differ significantly from the low-protein diet rats group given by blood cockle flour.

The low-protein diet group had lower serum calcium levels. This is presumably caused by low protein diet which leads to the decrease in the availability of proteins in the body, such as the availability of albumin. As the results of previous studies showing that the conditions of the low-protein diet can lead to the decrease levels of albumin (Solang 2014), and albumin is a means of transportation for calcium. Therefore, low-protein diet can lower serum calcium levels. This means that the absorption of calcium is related to protein intake; therefore, low-protein diet causes absorption of calcium is lowered, so that the level of calcium in the blood decreases.

The metabolism of calcium in the blood is maintained by homeostatic mechanisms of the body, involving the role of parathyroid hormone and calcitonin (Ganong 2012), so that the low levels of blood calcium in rats on a low-protein diet thought to be responded by the pituitary by stimulating the parathyroid glands to produce parathyroid hormone (PTH). This can mean that a low-protein diet will induce the changes in the binding of calcium in the intestine and bone (Giannini et al. 1999; Kerstetter et al. 2003).

The group of low-protein diet rats given by blood cockle flour significantly increase its serum calcium levels. The increase in the serum calcium levels is due to the giving of blood cockle flour to rats whereas the flour contains quite high protein and calcium, namely 318.67 ppm, so it helps to improve the availability of calcium for the body. The repair of serum calcium levels in the test rats is presumably related to calcium homeostasis involving parathyroid hormone. Parathyroid will stimulate the kidneys to perform reabsorption of calcium and also to produce specific vitamin D in the form of 1.25-dehidroksikalsiferol protein in the jejunum to increase calcium absorption in the active transport way (Murray et al. 2003).

1.25-dehidroksikalsiferol protein causes the formation of calcium-binding protein in the epithelial cells of the jejunum. The proteins on epithelial cells of the small intestine serves to absorb calcium in the form of apatite or tricalcium phosphate from blood cockle flour, then the protein transports calcium into the cytoplasm of cells (intracellular), then calcium is released into the blood

passing through the basolateral membrane which is found on epithelial cells of small intestine by the way of facilitated diffusion. Calcium in the form of apatite or tricalcium phosphate is ready to be absorbed by the small intestine to its maximum capacity in the amount of 60.70% (Orias 2008), so the calcium levels in the blood of test rats will increase.

However, the results of this study indicate that the increased concentrations of blood cockle flour lowers serum calcium levels (Table 1). It is thought to be related to the protein content of the feed which becomes high as the increase in the percentage of supplementation of blood cockle flour. Feed analysis results indicate that the feed supplemented by blood cockle flour 25% and 50%/kg feed each will have a protein content of 18.74% and 26.39%. This protein content exceeds the protein needed by the rat which is equal to 12%. The high protein content of feed consumed by test rats could be expected to increase calcium absorption by the intestine, and furthermore, increases urinary calcium (Kerstetter et al. 2005). Some research indicates that in rodents fed on a high-protein diet, the absorption of calcium in the small intestine is affected and calcium excretion in the urine is increased (Bennett et al. 2000).

Length of femur

The average length of femurs in low-protein diet group decreased about 11.87% compared to those in the normal control group. The average length of the rat femurs in a low-protein diet group which were given by blood cockle flour made an increase compared to the control group. The length of femur in low-protein diet group which were given by blood cockle flour 12.5%, 25% and 50% respectively increased by approximately 8.39%, 9.17%, and 9.96% (Table 2).

ANOVA test results showed that administration of blood cockle flour significantly affected ($p = 0.000$) the length of the rat femurs in low-protein diet. These results indicate that the administration of blood cockle flour can increase the length of the rat femurs in low-protein diet.

This means that the provision of blood cockle flour can improve the growth of the rat femurs in low-protein diet (Figure 1). The increase of femur length by the administration of blood cockle flour is suspected to be caused by the content of protein, calcium, and zinc which are high in blood cockle flour. Protein can increase the absorption of calcium and zinc thus increasing the availability of calcium and zinc in the body. High protein will absorb zinc in the feed which is also high (Hotz and Brown 2004). Thus, the provision of blood cockle flour can increase the availability of zinc in the body so as to optimize the role of zinc in boosting the growth of the femur, especially in rats with low-protein diet.

Mechanisms that could explain the increase in the length of the femur as a result of the provision of blood cockle flour that the availability of zinc in the body due to the provision of flour blood cockle, thus optimizing the role of zinc in the growth of the femur of rats low diet protein. The role of zinc on the growth of the femur allegedly associated with the participation of zinc in the synthesis of

DNA. Zinc is an essential component of the structure of the zinc-finger protein. The presence of zinc in the protein is essential in binding the specific DNA sites and gene expression. The role of zinc is supported by the presence of zinc in the bones that have higher zinc concentration than those in other tissues (Ovesen et al. 2004). Zinc is present in bone osteoid, synovial membrane and cartilage. Zinc supplementation may improve bone metabolism in rats. The increased bone metabolism is indicated by an increase in the width of the epiphysis, an increase in femur length and weight (Ovesen et al. 2004).

In addition, the increase in femur length is allegedly associated with elevated levels of calcium in the blood of test rats which were given by blood cockle flour. The increased level of calcium in the blood of test rats will be responded by the body with the production of the calcitonin hormone by the thyroid organ. The calcitonin hormone stimulates osteoblasts in bone formation. Osteoblasts produce osteonectin to bind calcium in the blood in the form hydroxyapatite, and then produce osteocalcin for bone mineralization process. Calcium could influence the proliferation of osteoblasts, thus affecting bone growth (Huang et al. 2001). The results showed that the blood cockle flour increases the growth of long bone of rats in low-protein diet, so that the blood cockle can be developed into food supplements to overcome the interference of linear growth.

Weight of femur

The average weight of femur in low-protein diet control group decreases 30.14% of the weight of the femur in normal control group. The average weight of the rat femur in low-protein diet fed on blood cockle flour increases compared to those in low-protein diet group. The weight of femur in the group given by blood cockle flour 12.5%/kg of feed, 25%/kg of feed, and 50%/kg of feed respectively increases by 15.00%, 16.39%, and 22.73% (Table 3). Kruskal-Wallis test results showed that the blood cockle flour had significant effect ($p = 0.002$) to the weight of the rat femurs in a low-protein diet. This means the provision of blood cockle flour can increase the weight of the rat femurs in a low-protein diet.

Mann Whitney test results showed the average weight of the femur between the normal control group and the control group of low protein diet differed significantly ($p < 0.05$). These results indicated that the administration of blood cockle flour 12.5% /kg of feed can enhance the weight of rat femurs in low protein diet. The results showed that the weight of rat femur in low protein diet decreases, and it is suspected due to the feeding of *karak* containing zinc and low protein, respectively 0.791 ppm and 8%. Zinc deficiency can lead to bone loss and inhibit bone metabolism (Oner et al. 1984).

Meanwhile, the provision of blood cockle flour to rats in low protein diet can increase the weight of the femur. This represents an improvement in femur growth by the administration of blood cockle flour, particularly the weight of rat femurs in low diet protein. The repair of femur bone growth is thought to relate to the activity of osteoblasts, the bone-forming cells.

Table 1. Average serum calcium levels in the rats which were given by blood cockle flour

Treatment	Average of serum calcium level (mg/dL)	Decrease of serum calcium level (%)	Increase of serum calcium level (%)	P -value
Normal control	11.18±0.20 ^a	-	-	
Low-protein diet	7.67±1.31 ^c	31.40	-	
Blood cockle flour 12.5%/kg feed	10.41±0.79 ^a	-	35.72	
Blood cockle flour 25%/kg feed	9.19±0.84 ^b	-	19.88	0.003
Blood cockle flour 50%/kg feed	9.02±0.37 ^b	-	17.60	

Note: The average value followed by different letters indicate significant difference based on LSD test at 5% error level

Table 2. Average length of test rat femurs which were given by blood cockle flour

Variable of femur length	Average length of femur (mm)	Decrease length of femur (%)	Increase length of femur (%)	P value
Normal control	33.96±0.62a	0	-	
Low-protein diet	29.93±1.38b	11.87	-	
Blood cockle flour 12.5%/kg of feed	32.67±1.04c	-	8.39	
Blood cockle flour 25%/kg of feed	32.95±1.04ac	-	9.17	0.000
Blood cockle flour 50%/kg of feed	33.24±0.93ac	-	9.96	

Note: The average value followed by different letters indicates significant differences based on LSD test at 5% error level

Table 3. Average weight of test rat femur administered by blood cockle flour

Variable of femur weight	Average weight of femur (g)	Decrease weight of femur (%)	Increase weight of femur (%)	P value
Normal control	0.73±0.13a	0	-	
Low-protein diet	0.51±0.06b	30.14	-	
Blood cockle flour 12.5%/kg of feed	0.60±0.04a	-	15.00	0.002
Blood cockle flour 25%/kg of feed	0.61±0.04a	-	16.39	
Blood cockle flour 50%/kg of feed	0.66±0.06a	-	22.73	

Note: The average value followed by different letters indicates significant differences by Mann Whitney test at 5% error level

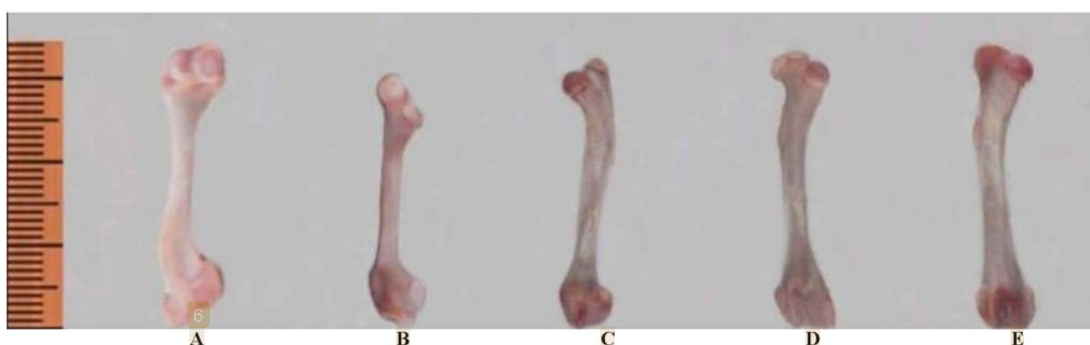


Figure 1. Morphology of rat femurs with low-protein diet which were fed on blood cockle flour. A. KO = normal controls, B. K1 = low protein diet, C. P1 = fed on blood cockle flour 12.5%/kg feed, D. P2 = fed on blood cockle flour 25%/kg feed, E. P3 = fed on blood cockle flour 50%/kg feed

The increase in weight of the rat femur in low protein diet which is the result of the provision of flour blood cockle is allegedly associated with the zinc and protein in blood cockle flour. The presence of zinc and protein in

blood cockle flour can increase the availability of zinc in the body which is indicated by the increase in plasma zinc levels (Solang et al. 2013). Increased plasma zinc levels can support the role of zinc in bone growth, particularly in

the weight of femur. This conjecture is based on the research results by Rossi et al. (2001) which showed that the weight of the rat femur with zinc deficiency decreases compared to the weight of rat femurs fed on standard feed and on feed with the content of zinc. Research results of Yamaguchi et al. (2004) showed that zinc in yeast can improve serum zinc, liver zinc and femur tissue zinc, calcium, alkaline phosphatase in the diaphysis, and metaphysical tissue of femur, and can induce stimulatory effect on bone calcification.

Zinc can stimulate proliferation, differentiation, and protein synthesis in osteoblasts (Hashizume and Yamaguchi 1994). Zinc is needed to activate the alkaline phosphatase (ALP). The enzyme produced by osteoblasts plays a role in calcium deposition in bone diaphysis (Hall et al. 1999). Zinc can increase the osteogenic effect by stimulating cell proliferation, activity of alkaline phosphatase (ALP), and collagen synthesis in osteoblasts (Seo et al. 2010). Synthesis and ALP activity as well as collagen synthesis increase during the osteoblast differentiation on early stage of osteogenic. ALP contains zinc as part of metallo-enzymes homodimeric and zinc is its components (Bremner and Beattie 1995). The synthesis of collagen is also known as a sign of osteoblast differentiation. Zinc directly activates the aminoacyl-tRNA synthesis in osteoblasts cells which and synthesizes cellular proteins. However, zinc inhibits osteoclasts which are benefit to bone reabsorption by suppressing the former of osteoclast cells from marrow cells. The ability of zinc in suppressing the formation of osteoclasts can increase bone mass.

Based on these descriptions, it is allegedly thought that zinc and protein from blood cockle can stimulate the activity of osteoblasts that play a role in bone formation by activating alkaline phosphatase (ALP), thereby increasing the proliferation of osteoblasts and deposition of calcium. The proliferation fixation of osteoblasts and deposition of calcium is thought to be able to stimulate bone formation and calcification of the bones, especially the bones of femur.

Based on these results, it can be concluded that the administration of blood cockle flour can improve the growth of malnourished rat femur caused by low protein diet by improving serum calcium levels, femur length, and weight of the femur. The supplementing of blood cockle flour 12.5% /kg of feed has been able to improve growth due to low protein diet. Based on these results, it is necessary to conduct further research relating to the development of blood cockle as a product of food to improve the malnutrition condition.

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