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The Analysis of Blood Cockle *(Anadara granosa)* Flour Supplementation on The Concentrations of Zinc, IGF-I, And Ephiseal Plate Width of Femur Malnourished Male Rats (Rattus Norvegicus)

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Abstract. Blood cockle *(Anadara granosa)* is one of the foods and it is economically and culturally acceptable to the community, but has not been fully utilized in Gorontalo. Blood cockle contains high of zinc and protein, so that it has the potential of supplementation therapeutic for malnourished children, particularly stunting. The objective of this study is to analyze the supplementation of blood cockle flour in increasing the levels of plasma zinc, IGF-I serum, and epiphyseal plate width, in malnourished male Wistar strain rats *(Rattus norvegicus)*. The design of this research was The Separate Sample Pre - Post Test Design. The 48 Wistar male rats, 6 weeks age, 115-120 g body weight were randomly grouped into 2 groups: 12 rats in normal control (NC) and 36 rats in the malnourished group (Kkg0). After eight weeks, 4 rats from each group were sacrificed as initial data. Eight rats of normal control were standard fed (NC), whereas 32 malnourished rats were randomly grouped into 4 groups, namely malnourished (Kkg1), malnourished supplemented with blood cockle flour in the amount of 2.5 g (Pkg1), 5 g (Pkg2), and 10 g (Pkg3) for 8 weeks. Rats were sacrificed after treatment. Data analysis was performed with One Way ANOVA, LSD test, Kruskal - Wallis and Mann-Whitney test. The results of this study indicate that supplementation of blood cockle flour can improve the growth of malnourished rats evidenced by the increasing plasma zinc level, IGF-I serum level, and the width of the epiphyseal plate of malnourished rats. Thus, it can be concluded that zinc and protein derived from blood cockle flour improve growth through a mechanism of change in the IGF-I level. which further improve bone metabolism, demonstrated growth of chondrocytes in epiphyseal plate and epiphyseal plate width.

Key-Words: *blood cockle (Anadara granosa), zinc, IGF-I, epiphyseal plate, malnourished, rats*

1. Introduction

Malnutrition in toddlers is still a major nutritional problem in Indonesia. Malnutrition which occurs in long term or it is chronic will interfere height growth, causing the child to grow shorter (stunting). The nutritional status problem with the index of H/A in Indonesia is still frequently found. This is evidenced by the prevalence of stunting toddlers in Indonesia, which is as much as 36.8 % [1]. Health Research Data 2010 show that stunting toddlers in 2010 amounted to 35.6% [2]. Gorontalo Provincial Health Office reported that by using the index of H/A, it was obtained that the percentage of stunting toddler 41,52% in 2009 was and in 2010 it was 38.06%. The data show a decrease in the percentage of stunting toddlers nationally and in Gorontalo. However, the prevalence of stunting toddler is still above the threshold (cutoff) that has been universally agreed, in which, if the stunting problem is above 20%, it is still a public health problem [2]**.** Various government programs have been undertaken to address stunting problems, such as a society nutritional improvement program, namely the provision of vitamin A for toddlers in order to provide sufficient vitamin A in the body. The government also conducted a program of providing appropriate complementary feeding. The government is currently focusing on first 1000 days program, namely the care of nutrition since pregnancy until the baby is two years old **(3)**. If the nutritional improvement program is terminated, the problem of stunting toddler will reappear. This is because the purchasing power of most families of malnourished patients is low

Malnutrition in stunting toddlers is mostly related to macronutrient deficiency. This macronutrient deficiency is usually followed by a deficiency of micronutrient deficiency such as the deficiency

of zinc, thus worsening the body's defense against infectious diseases and causing growth retardation. Zinc deficiency and malnutrition are interrelated and both suppress activity and synthesis of Insulin-like growth factor-I (IGF-I). The initial clinical manifestations of zinc deficiency can cause growth retardation and hypogonadism [4].

The results of the study conducted by Adriani (2009) [5] show that the zinc provision in vitamin A supplementation may improve toddler's linear growth significantly through the process of increasing IGF-I. It proves that zinc also has a regulatory function, where 'zinc finger protein' regulates gene expression by acting as a transcription factor (binding to DNA and affect specific gene transcription) that can affect the production of the IGF-I hormone.

Zinc plays a role in synthesis, secretion and action of the growth hormone (GH) in the production of IGF-1. Gluckman *et al.,* 1987 [6] states that the target of GH directly or through IGF-I is to stimulate anabolic processes, such as cell division, bone growth, and protein synthesis. IGF-I can initiate proliferation and maturation of chondrocytes [7]. The chondrocytes proliferation in epiphyseal plate causes the elongated growth of a long bone. The growth of long bone contributes to determine a person's final height [8]. At the end of the growth, cartilage in epiphysiswill entirely be replaced with bone so that the epiphysis is fused with diaphysis (fusion) which is characterized by the formation of epiphyseal line [9].

Given the need for efforts to reduce the prevalence of stunting toddlers, it is necessary to find an alternative nutritional improvement by utilizing the local potential available in nature which is affordable as well as containing nutrition and effective to promote growth. One of the local potentials found in Gorontalo, which can be used as an alternative in addressing malnutrition is selfish.

Shellfish is one of the foods that are economically and culturally acceptable to the community. In addition, shellfish contain 75 mg/100 g of zinc. The content of zinc in shellfish is higher than the content of zinc in egg white which is only 0.02 mg/100 g and in chicken which is only 1mg/100 g [10]. A variety of shellfish that is often consumed by the community is blood cockle *(Anadara granosa).*

The results of the proximate analysis of blood cockle flour from Gorontalo show that it contains 27.26 % total protein, 2.54 % total fat, 9.74 % water, and 10.62% ash. It also contains amino acid, namely 10.56 % aspartic acid, 15.47 % glutamic acid, 7.01% serine, 0.84 % histidine, 10.39 % glycine, 6.96 % arginine, 13.49 % alanine, 2.3166 % tyrosine, 1.39 % methionine, 5.50 % valine, 2.92 % phenilalanin, 4.36 % isoleucine, 7.86 % leucine, and 3.46 % lysine. The mineral content in blood cockle is: 81.16 ppm, zinc, 1720.46 ppm Fe, 4.26 ppm Cu, and 318.67 ppm Ca.

Blood cockle contains high zinc and protein so that it has the potential of therapeutic supplementation for malnourished children particularly stunting ones. Zinc derived from animal foods is more easily absorbed than those derived from vegetable food [11]. Moreover, the content of proteins in blood cockle helps zinc absorption and increases the protein intake in the body.

Based on the nutritional composition of the blood cockle, blood cockle has a potential to be developed as an alternative source of zinc and protein. However, blood cockle has not been optimally utilized in Gorontalo. Blood cockle is used only as a substitute for fish if fishermen do not get fish.

Based on the above introduction, laboratory experimental research has been conducted in order to the supplementation of blood cockle flour at zinc level, IGF-I hormone, and the width of the epiphyseal plate femur of malnourished male Wistar strain rats *(Rattus norvegicus).* Rat is selected as a test animal because its gastrointestinal and absorbing capability are similar to human's. In addition, nutrients necessary for rat growth are similar to the ones necessary for human growth, namely carbohydrates, oils/fats, proteins, minerals and vitamins [12].

2. Methods and Materials

2.1. Experimental Design

This research was conducted in the Laboratory of the Department of Biochemistry, Faculty of Medicine, Airlangga University, Surabaya. This is of laboratory experimental research with Separate Sample Pre - Post Test Control Group Design [13]. The samples in this research were *Rattus norvegicus,* male Wistar strain, aged ± 6 weeks and weighed 115-120 grams. Rats were in healthy physical condition characterized by a lively movement, thick fur, and shining eyes. Prior to treatment, 48 rats were acclimatized for a week. Then, the rats were randomly grouped into two

groups consist namely 12 rats in the normal control group (NC) and 36 rats in the malnourished group (Kkg0). After eight weeks, 4 rats of each group were sacrificed as initial data. Eight rats of normal control group remained fed with standard feed (NC), whereas 32 rats of the malnourished group were randomly grouped into 4 groups, namely malnourished (Kkg1), malnourished supplemented with blood cockle flour in a dose of 2.5 g (Pkg1), 5 g (Pkg2), and 10 g (Pkg3) for 8 weeks. At the end of 8^{th} week, data collection was performed. The observed data included the plasma zinc level, serum IGF-I level, and the width of the epiphyseal plate.
The feed used to create malnourished condition was *karak* (dry rice) containing 8.46 % protein. Supplementation Feed was blood cockle flour containing 81.16 ppm zinc. The blood cockle flour supplementation given were 2.5 g, 5 g, and 10 g. Calculation of blood cockle flour supplementation was based on the need of zinc in children, namely 10 mg [14], which was converted to the need of the tested rats. This dose was converted based on a rat with a body weight of 200 grams. Human-to-rat conversion rate was 0.018 [15]. Drinking water was tap water. Food and drinks were provided in *ad libitum.*

2.2. Measurement of Plasma Zinc Level

The plasma zinc level was measured using the Absorbent Atomic Spectrophotometer (AAS) of Zeenit 700. The plasma zinc level was measured in parts per million (ppm). The measurement was conducted in the Hall of Health Laboratory Surabaya.

2.3. Measurement of Serum IGF-I level

Measurement of serum IGF-I level was conducted with Enzyme Linked Immunosorbent Assay (ELISA) method [16], Brand Medicine Kid, Lot number*:* 201212. IGF-I level was stated in ng/ml. The measurement of the IGF-1 hormone level was conducted in the Laboratory of Clinical Pathology of Dr. Soetomo Hospital.

2.4. Measurement of the width of femoral epiphyseal plate

The measurements of the width of the right femoral epiphyseal plate were conducted using the femoral histologic preparation with *Hemaxtosilin-Eosin* staining, observed with a NIKON Eclipse Ci Light microscopes. The width of the epiphyseal plate is the width of chondrocytes in proliferation zone and hypertrophic zone (maturation). Measurements were conducted at the central and the edge area [17]. The width of epiphyseal plate is expressed in micrometers (μm). The observation of the width of epiphyseal plate was conducted with 100 times magnification. Measurements were performed four times and then the average results were calculated. The level of growth of epiphyseal plate was also microscopically calculated in the observation of the width of the femoral epiphyseal plate. Measurement of the width of the right femoral epiphyseal plate was conducted in the Laboratory of Histology by Faculty of Veterinary, Airlangga University, Surabaya.

2.5. Statistical Analysis

Quantitative data (post-test data) obtained were level of zinc, level of IGF-1 hormone, and the width of the femoral epiphyseal plate. The data were obtained through normality test using Kolmogorov-Smirnov test and homogeneity test using a Levene's test. When the data resulted from the tests were normally distributed and homogeneous, variance test using parametric statistical test namely One Way ANOVA was performed. Statistical test was performed at the confidence level of 95 % and the variance was said to be significant if $\rho < 0.05$. When significant variance was resulted, the test was followed by statistical test using the Least Significance Difference (LSD) [18]. When the data resulted from the test were not normally distributed and not homogeneous, variance test using a different non-parametric statistical test namely the Kruskal – Wallis was performed. When significant variance was resulted, the test was followed by statistical test using the Mann-Whitney.

3. Result

3.1. Effect of Supplementation of Blood Cockle Flour on the Level of Plasma Zinc of Malnourished Rats

Based on one-way ANOVA test, the p obtained is 0.002, indicating that the supplementation of blood cockle flour significantly (p = 0.05) affects the level of plasma zinc of malnourished rats. It means that the supplementation of blood cockle flour may increase the level of plasma zinc of malnourished rats.

Results of LSD test (Figure 1) show that the average of the levels of plasma zinc of the control group and malnourished group is significantly different (p = 0.05). It indicates that the malnourished group had lower levels of plasma zinc compared to the control group. Similarly, the group of malnourished rats and the groups of rats supplemented with blood cockle flour in a dose of 2.5 g/feed/day, 5 g/feed/day, and 10 g/feed/day showed significantly different (p = 0.05) level of plasma zinc. Meanwhile, the comparison between the group supplemented with blood cockle flour in a dose of 2.5 g/feed/day and the one supplemented with blood cockle flour in a dose of 5 g/feed/day and the comparison between the group supplemented with blood cockle flour in a dose of 2.5 g/feed/day and the one supplemented with blood cockle flour in a dose of 10 g/feed/day show that the average of the level of plasma zinc is not significantly different (p = 0.05). It can also be seen in the group supplemented with blood cockle flour in a dose of 5 g/feed/day and the one supplemented with blood cockle flour in a dose of 10 g/feed/day.

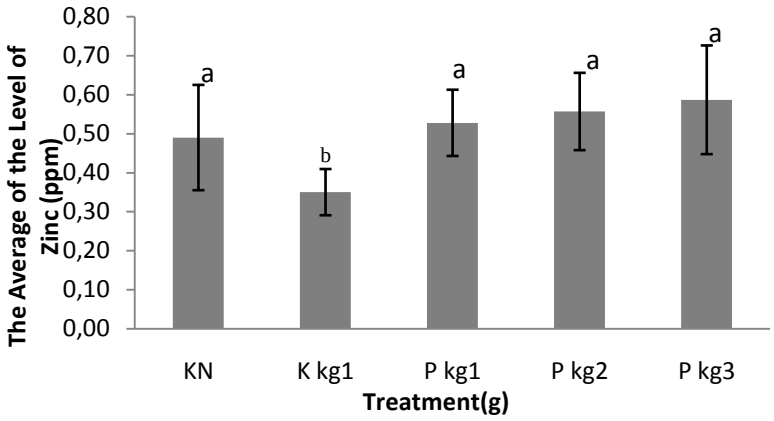

Figure 1. The Average of the Level of Plasma Zinc of Tested Rats Supplemented with Blood Cockle Flour.

Description a, b = the same notation indicates not significantly differently (p = 0.05).
KN: Normal Control (NC), Kkg1= Malnourished Control
P kg 1: *karak* + blood cockle flour in a dose of 2,5 g
P kg 2: *karak* + blood cockle flour in a dose of 5 g,
P kg 3: *karak* + blood cockle flour in a dose of 10 g.

This suggests that the supplementation of blood cockle flour in a dose of 2.5 g/ feed / day is enough to increase the average of the level of plasma zinc of malnourished rats. Furthermore, the comparison between the control group and the group supplemented with blood cockle flour in a dose of 2.5 g/ feed / day, 5 g/feed/day, and 10 g/feed/day, shows no significant difference (p = 0.05). This suggests that supplementation of blood cockle flour may increase the average of the level of zinc of malnourished rats so that they have a level of zinc which is not different from the control group.

3.2. Effect of Supplementation of Blood Cockle Flour on the Level of IGF-I in The Blood of Malnourished Rats

Results of one-way ANOVA analysis showed that the level of IGF-I of malnourished rats supplemented with blood cockle flour was significantly different (p = 0.05), indicated by the value of p = 0.000. It means that the supplementation of blood cockle flour can increase the level of IGF-I of malnourished rats.

Results of LSD test (Figure 2) showed that the average of the level of IGF-I of malnourished rats was significantly different (p=0.05) compared to the control group and the groups supplemented with blood cockle flour in the amount of 2.5 g/feed/day, 5 g/feed/day, and 10 g/feed/day. Meanwhile, the comparison between the group supplemented with blood cockle flour in a dose of 2.5g/feed/day and the group supplemented with blood cockle flour in a dose of 5 g/feed/day shows the significance difference (p = 0.05), as well as the comparison between the group supplemented with blood cockle flour in a dose of 2.5 g/feed/day and the group supplemented with blood cockle flour in a dose of 10 g/feed/day shows a significant difference (p=0.05). However, the comparison between the group supplemented with blood cockle flour in a dose of 5 g/feed/day and the group supplemented with blood cockle flour in a dose of 10 g/feed shows no significance difference (p = 0.05).

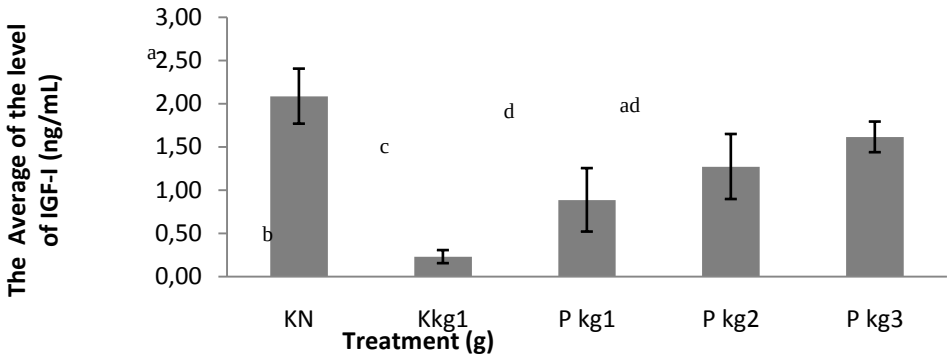

Figure 2. The Average of the Level of IGF-I of Tested Rats Supplemented with Blood Cockle Flour.

Description a,b = the same notation indicates not significantly different (p = 0.05).
KN: Normal Control (NC) Kkg1= Malnourished Control
P kg 1: *karak* + blood cockle flour in a dose of 2,5 g
P kg 2: *karak* + blood cockle flour in a dose of 5 g, P kg 3: *karak* + blood cockle flour in a dose of 10 g.

Furthermore, comparison between the group supplemented with blood cockle flour in a dose of 2.5 g/feed/day and the control group shows a significant difference (p=0.05), the comparison between the group supplemented with blood cockle flour in a dose of 5 g/feed/day and control group also shows a significant difference (p=0.05). However, the comparison between the group supplemented group with blood cockle flour in a dose of 10 g/feed/day and the control group does not show a significant difference (p = 0.05). This suggests that supplementation with blood cockle flour at a dose of 2.5 g/feed/day and 5 g/feed/day may increase the level of IGF-I of malnourished rats, yet the increase of the level of IGF-I has not reached the level of IGF-I of the control group. Meanwhile, supplementation of blood cockle flour at a dose of 10 g/feed/day in malnourished rats can cause the increase of the level of IGF-I to be as much as the level of IGF-I of normal group.

3.3. Effect of Supplementation of Blood Cockle Flour on the Width of Femoral Epiphyseal Plate of Malnourished Rats

Results of Kruskal - Wallis showed that supplementation of blood cockle flour significantly affected (p = 0.05) the width of the femoral epiphyseal plate of malnourished rats. It is shown by the value of p = 0.002. It shows that the supplementation of blood cockle flour improves the width

of femoral epiphyseal plate of malnourished rats. Mann Whitney test results showed that there was a significant difference (p = 0.05) between the width of femoral epiphyseal plate of control group and the one of malnourished group. This suggests that malnutrition can reduce the width of the femoral epiphyseal plate.

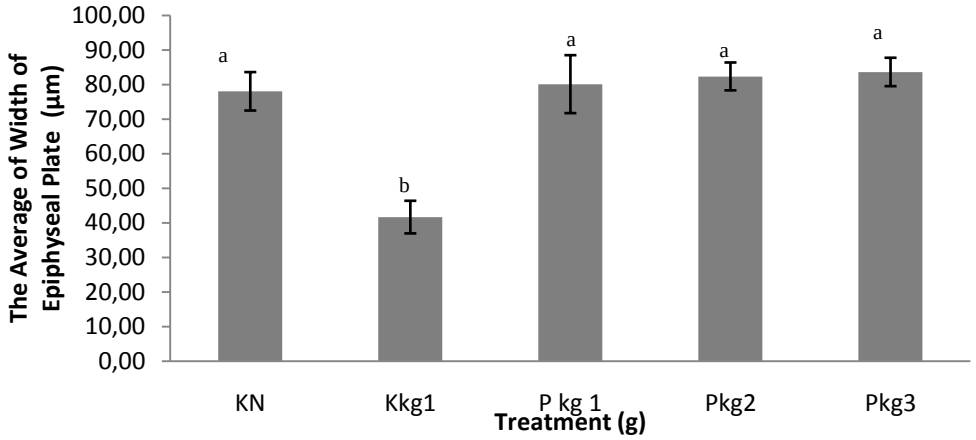

Figure **3.** The Average of The Width of Femoral Epiphyseal Plate of Tested Rats Supplemented with Blood Cockle Flour.

Description a, b = the same notation indicates not significantly differently (p = 0.05).
KN: Normal Control (NC) Kkg1= Malnourished Control
Pkg1: *karak* + blood cockle flour in a dose of 2,5 g, Pkg2: *karak* + blood cockle flour in a dose of 5 g,
P kg3: *karak* + blood cockle flour in a dose of 10 g.

Figure 3 shows that the width of femoral epiphyseal plate of the groups supplemented with blood cockle flour in a dose of 2.5 g/feed/day, 5 g/feed/day and 10 g/feed/day was significantly different (p = 0.05) from the one of the malnourished group. However, the width of femoral epiphyseal plate of the groups supplemented with blood cockle flour was not significantly different (p = 0.05) from the one of the control group. Similarly, among the treatment groups supplemented with blood cockle flour showed no significant difference (p = 0.05). This suggests that the supplementation of blood cockle flour can improve the width of femoral epiphyseal plate of malnourished rats and the supplementation of blood cockle flour in a dose of 2.5 g/feed/day is enough to improve the width of femoral epiphyseal plate of malnourished rats to be as wide as the femoral epiphyseal plate of control rats.

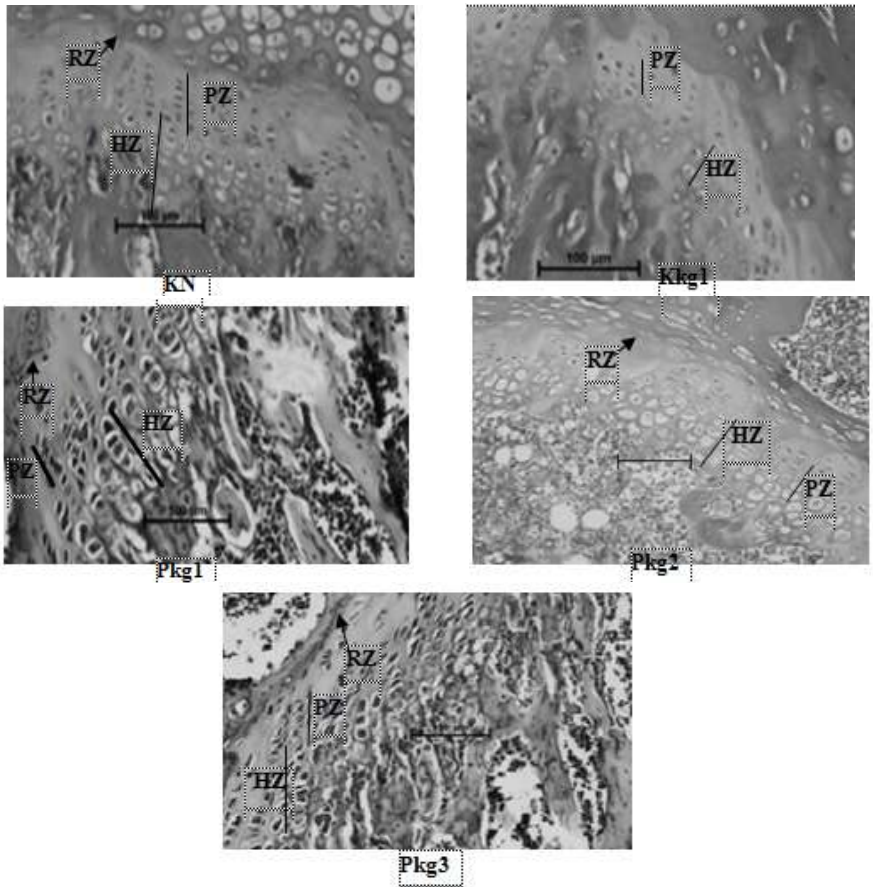

Figure 4. Longitudinal section of femoral epiphyseal plate of rats with 6 μm of section thickness, H-E staining and 100 x magnification.

PZ: Proliferation Zone, HZ: Hypertrophy Zone. RZ: Reserve Zone, KN: Normal Control (NC), Kkg1= Malnourished Control,
P kg 1: *karak* + blood cockle flour in a dose of 2,5 g
P kg 2: *karak* + blood cockle flour in a dose of 5 g, P kg 3: *karak* + blood cockle flour in a dose of 10 g.

The image of longitudinal section of microanatomy structure femoral epiphyseal plate can be seen in Figure 4. Figure 4 shows the femoral epiphyseal plates of normal control (NC) rats, malnourished rats (Kkg1), malnourished rats supplemented with blood cockle flour in a dose of 2.5 g/feed/day (pkg1), malnourished rats supplemented with blood cockle flour in a dose of 5 g/feed/day (Pkg2), and malnourished rats supplemented with blood cockle flour in a dose of 10 g/feed/day (Pkg3).

4. Discussion

4.1. Effect of Supplementation of Blood Cockle Flour on the Level of Plasma Zinc of Malnourished rats.

The results of this study showed a decrease in the level of zinc of malnourished rats compared to the control group. Meanwhile, supplementation of blood cockle flour for malnourished rats could increase the level of plasma zinc of malnourished rats. The increase in the level of plasma zinc of malnourished rats was assumed to be related to the levels of zinc and protein in the feed consumed by the tested rats. Results of research conducted by Everett and Apgar (1979) in Choudhary (2013) [19] showed the feed with low zinc (< 1 ppm) caused test rats to have a lower level of plasma zinc compared to animals whose feed contained sufficient zinc. Pederson and Eggum (1983) in Choudhary (2013) [19] explained that rats whose feed contained marginal protein were unable to efficiently use zinc from the feed. Results of research conducted by Shidu

et al.,(2004) [20] showed that protein deficiency (8 %) could reduce the level of hepatic zinc of test rats.
Meanwhile, feed supplemented with blood cockle flour led to increase in the levels of zinc and protein. The increased is assumed to increase the level of plasma zinc of malnourished rats. This is supported by the results of research by Hamza *et al*., (2012) [21] which showed that supplementation of zinc could increase the level of serum zinc. Choundhary (2013) [19] reported that zinc deficiency in rats could decrease the level of zinc and provision of zinc could increase the level of zinc in rats with zinc deficiency.
The increase in the level of plasma zinc of malnourished rats in this study was due to zinc and protein contained in blood cockle flour. This is because zinc derived from blood cockle is more easily absorbed than those from vegetable food [11]. Animal food does not contain phytate so that zinc is easily absorbed compared to zinc contained in vegetable food. This is supported by protein in the blood cockle so that it can increase the absorption of zinc as proposed by Hotz and Brown (2004) [22] who said that high protein would highly absorb zinc in food. Based on above opinions, it is assumed that the trasport and absorption of zinc may be increased due to protein in the blood cockle flour so that it can increase the level of plasma zinc of malnourished rats.

4.2. Effect of Supplementation of Blood Cockle Flour on the level of IGF-I of Malnourished Rats

The level of IGF-I is highly dependent on nutritional status in both human and test animal [23]. Protein [24] and micronutrin intake such as zinc has been found to control the synthesis and release of IGF-I into the circulation (Ninh et al., 1995 in Ninh *et al*., 1996) [25]. Meanwhile, Devine *et al*., (1998) [26] reported that the low level of IGF-I was affected by low zinc intake and the effect of zinc depended on protein intake.
Malnourished group has a lower level of IGF-I. This is indicated by the research results which showed that the level of IGF-I of malnourished rats had decreased compared to a normal control group. The decrease of the level of IGF-I is presumably due to low zinc and protein feed. According to the results of the research conducted by Hazel *et al*., (1998) [27], the level of IGF-I of the group of rats fed with a low-protein feed (6 %) was only 30% of the normal group (21 %) and the group fed with a high protein feed (35 %).
Tirapegui *et al*., (2012) [28] reported that the level of IGF-I of the test rats fed with 12% protein was 573 ± 67 ng / ml and the level of IGF-I of test rats fed with 26% protein increased to 627 ± 11.6 ng/mL. The results of the study conducted by Ninh et al., (1998) [29] showed that zinc deficiency led to low level of IGF-I of rats as a result of the status of GH resistance.
Serum insulin-like growth factor I (IGF-I) of zinc-deficient rats is lower than the one of rats with adequate zinc and the decrease in IGF-I is associated with a decrease in serum zinc [30]. Oner *et al*., (1984) in Kaji and Nishi, (2006) [31] reported that zinc deficiency caused a decrease in the concentration of serum IGF-I of rats. This suggests that zinc is involved in the production of IGF-I. Kurtaglu, (2008) [32] reported that children with low level of zinc also had lower levels of IGF-I compared to those with normal zinc level.
Peisen *et al*., (1995) in Thissen *et al*., (2004) [33] reported that some models of diet manipulation such as zinc deficiency could interfere GH secretion in rats together with growth retardation and a decrease in the level of serum IGF-I. Zinc deficiency can reduce gene expression of IGF-I and GH receptor in rat liver [33]. Zinc deficiency can inhibit the synthesis of IGF-I and the provision of exogenous GH a cannot increase the level of IGF-I of zinc-deficient rats [31].
Meanwhile, Cleg (1995) in MacDonald (2000) [30] stated that zinc deficiency tended to interfere the distribution of IGFBPs which further decreased the level of IGF-I. Hamza et al., (2012) [21] also reported that the levels of IGF-I and IGFBP-3 of zinc-deficient children were low. The research results showed that supplementation of blood cockle flour could increase the level of IGF-I of malnourished rats. Supplementation of 10 g blood cockle flour may increase the level of IGF-I of malnourished rats, in which the level is not significantly different from the level of IGF-I of normal control rats.
Supplementation of 2.5 %, 5 %, and 10 % blood cockle flour may increase the level of serum IGF-I respectively 74.16 %, 81.89 %, and 85.71 % of the level of IGF-I of malnourished rats. This suggests that supplementation of blood cockle flour can improve the regulation of serum IGF-I production. The increase in the level of serum IGF-I is assumed to be associated with zinc supported by the protein in the blood cockle. The increase in the level of IGF-I follows the

increase in blood cockle flour composition. Supplementation of blood cockle flour at a dose of 2.5 g/feed/day has been able to increase the level of serum IGF-I of test rats.
Cossacks (1984) in MacDonald (2000) [30] stated that the low level of IGF-I of zinc-deficient rats could not be increased by the provision of a high protein diet, but the addition of zinc in the low protein diet could increase the level of serum IGF-I. Imamoglu *et al*, (2005) [34] stated that zinc supplementation could increase the levels of IGF-I and IGFBP-3 presumably because (1) zinc supplementation could increase the sensitivity of endogenous GH.It is based on the research results on test animals which showed that ion zinc stimulated hGH specific binding of rat's adipocytes isolates [35]. Further zinc was found to induce dimerization of human GH [36] and affect the hGH bioactive [37];(2) zinc supplementation could improve the physiology of GH secretion, whereas the alternative was zinc directly affected the synthesis of IGF-I and IGFBP-3 without mediated by GH. The results of the study conducted by Adriani (2009) [5] showed that zinc supplementation could increase the level of IGF-I of children with zinc deficiency.
The results of research conducted by Anlar *et al*., (1999) in AstraZeneca, (2012) [38] showed that nutritional status significantly affected the level of IGF-I and the lack of protein and energy could decrease the level of IGF-I. The change in the level of IGF-I does not depend on hipophysis GH secretion but rather is associated with the decrease in expression and hepatic GH receptor signaling. Hayden *et al*., (1994) [39] stated that the decrease in serum IGF-I due to low protein feed was associated with the decrease in the level of hepatic mRNA IGF-I. This suggests that nutrients regulate gene expression of IGF-I at pretranslation stage.
Furthermore, the increase in plasma zinc of malnourished rats due to supplementation of blood cockle flour is assumed to be able to increase IGFBP-3, thereby increasing the level of serum IGF-I. Most of the circulating IGF-I (75- 80 %) binds to IGF-I binding serum protein namely IGFBP-3. This presumably can be explained based on the results of the study which showed that in protein deficiency status, the binding of IGF-I to the circulating complex 150-kD IGFBP-3 and acid-labile subunit decreased and further IGF-I binded to molecule which has a smaller weight, is IGFBPs. This smaller protein can pass through capillaries and tends to lower the concentration of circulating IGF-I [40].
This opinion is also supported by the results of the study conducted by Hamza *et al.,* (2012) [21] which stated that there was a positive correlation between serum zinc, levels of IGF-I and IGFBP-3. Hamza *et al*., (2012) [21] also showed that children with zinc deficiency had lower levels of IGF-I and IGFBP-3 and zinc supplementation could increase the levels of serum IGF-I and IGFBP-3. However, the increase was still below the range of children without zinc deficiency. Therefore, Hamza *et al.,* (2012) [21] concluded that zinc supplementation required longer time.
The ability of the supplementation of blood cockle flour on increasing serum IGF-I in this study is assumed to be related to zinc of feed supported by protein, which in turn may increase the plasma zinc. Therefore, there is an increase in the availability of zinc in the body so that the role of zinc is optimal. Zinc plays a role in hormonal mediation because zinc plays a role in the synthesis, secretion and action of GH in IGF-I production in the liver [4]. Zinc plays a role in intracellular transduction line of several hormones and can activate protein kinase that plays a role in GH signal transduction [20]. Zinc is also a component of zinc-finger protein that functions as a DNA-binding domain in transcription factors [4,41]. Zinc-finger protein gene expression by activating the transcription factors plays an important role in cell transcription and synthesis of IGF-I [4] .

4.3. Effect of supplementation of blood cockle flour on the width of femoral epiphyseal plate of malnourished rats undernourished

The results showed that supplementation of blood cockle flour could increase the width of the femoral epiphyseal plate of malnourished rats (Figure 4). It is based on research results that indicate that the width of the epiphyseal plate of malnourished rats has decreased compared to the one with the control group. The abnormality in the growth of epiphyseal plate of malnourished rats is presumably related to low protein and zinc feed. This is in line with Rossi *et al*., (2001) [42], Lohmann and Beyersmann (1993) [43] who stated that the abnormality in the growth of epiphyseal plate was related to the role of zinc in cell division, differentiation and apoptosis.
Results of research conducted by Follis and McCollum, (1941) in Rossi et al., (2001) [42] showed that zinc-deficient rats experienced a decrease in the width of the epiphyseal plate and the chondrocytes activity in growth plate was also disrupted [44]. Rosi *et al.,* (2001) [42] suggested that the activity of the growth plate of zinc deficient rats was reduced. Growth plate of zinc

deficient rats significant experiences atrophy compared to rats fed with feed with adequate zinc and rats fed with standard feed.

The decrease in the width of the epiphyseal plate of malnourished rats presumably leads to a reduction in proliferating chondrocytes, thereby reducing chondrocytes with hypertrophy. Histologically, the growth of the epiphyseal plate of malnourished rats (Kg1) in this study when compared to the one of normal control showed that chondrocytes were not orderly arranged. Parallel columns of chondrocytes were not visible and the developmental stage of the cells could not be identified (Fig. 4Kkg1), the decrease in staining affinity in the matrix was visible and cells were degenerating. This opinion is based on the results of research conducted by Suwarnasarn and Wallwork (1982) [45] which showed that the epiphyseal plate of zinc-deficient rats contained cells that could not be identified.

The results of the study showed that supplementation of blood cockle flour in a dose of 2.5 g/feed/day has been able to increase the width of epiphyseal plate. This suggests that blood cockle flour supplementation can correct the abnormality of the growth of epiphyseal plate by improving the morphology of chondrocytes to increase the width of the epiphyseal plate of malnourished rats.

The increase in the width of the epiphyseal plate and chondrocytes improvement as in the chondrocytes improvement in normal rats due to supplementation of blood cockle flour was presumably associated with high zinc and protein contained in blood cockle flour thereby increasing the availability of zinc of malnourished rats. Furthermore, it could optimize the role of zinc in the growth of femoral epiphyseal plate. This opinion is in line with the results of the research conducted by Ovesen *et al*., (2004)[46] which showed that zinc supplementation could increase the width of epiphyseal plate of rats. Rodriguez et al., (2001)[47] showed that zinc supplementation could stimulate the proliferation of chondrocytes in the epiphyseal plate.

The increase in the width of epiphyseal plate indicates an improvement of epiphyseal plate that leads to chondrocytes proliferation improvement in the proliferation zone as seen in Fig 4Pkg1, 4Pkg2, and Figure 4Pkg3. The figures show the numbers of proliferating chondrocytes. The figures also show chondrocytes improvement in hypertrophy and matrix surrounding the chondrocytes.

Histologically, no difference was found in the comparison between femoral epiphyseal plate of malnourished rats supplemented with 2.5 g 5 g, and 10 g blood cockle flour and the epiphyseal plate of normal control rats (Fig. 4KN). Epiphyseal plate consists of 3 zones, namely (1) reserve zone (quiet) containing single chondrocyte or chondrocytes in small group with a basophilic matrix, (2) proliferation zone containing chondrocytes that form cell columns parallel to the long axis of bone and cellular matrix that has a strong affinity; (3) hypertrophy zone containing huge chondrocytes and matrix cellular that is clearly stained.

The increase in the width of epiphyseal plate and chondrocytes improvement due to supplementation of blood cockle is presumably associated with the increase in the level of IGF-I of malnourished rats as seen in this study. This opinion is based on results of research conducted by Rossi *et al.,* (2001) [42] which showed that the decrease in the level of IGF-I in the circulation could indicate the disruption mechanism of the growth of the plate and the change in the quantity of growth plate of zinc deficient rats. This is because IGF-I acts on specific membrane receptors to stimulate proliferation or special function of differentiated cell types [48].

Insulin like growth factor-I (IGF-I) is known to affect proliferation and differentiation of various cell types including chondrocytes and oesteblas precursor cells [49,50]. Schlechter (1986) [51] suggested that the infusion of IGF-I could increase the width of epiphyseal plate and the growth of long bones. The results of the research conducted by Ohlsson *et al*., (1992) [52] showed that IGF-I only acted on chondrocytes proliferation. MacDonald (2000) [30] stated that zinc was essential for IGF-I in inducing cell proliferation.

Possible mechanisms to explain the increase in width of epiphyseal plate and microanatomy structure of epiphyseal plate is zinc and protein in blood cockle thus optimizing the role of zinc in improving the growth of epiphyseal plate. This is presumably related to the ability of zinc to participate in the synthesis of DNA and RNA, which in turn related to cell division, chondrocytes differentiation, cells transcription and synthesis of IGF-I. Zinc potentially increases GH action in the liver and further stimulates the synthesis and action of IGF-I in cartilage [4].

The role of zinc in gene expression, endocrine function, and mechanism of action of zinc involved in the synthesis of DNA, RNA and cells division can be explained through the concept of zinc-finger protein [4,41]. Zinc-finger protein provides one of the fundamental mechanisms for regulating gene expression.

The role of zinc in increasing the width of epiphyseal plate is also supported by the existence of zinc on chondrocytes both in proliferation zone and hypertrophy zone. This assumption is based on the results of research conducted by Ovesen, *et al*., (2004) [46] which showed that the ion of zinc was found in chondrocytes in proliferation zone and hypertrophy zone. The highest concentration of zinc was found in chondrocytes in proliferation zone. Furthermore Ovesen *et al.*, (2004) [46] also stated that zinc played an important role in regulating the formation of epifisial cartilage, therefore, it could be related to the growth of long bones. Based on the opinions above, zinc and protein in blood cockle flour may work synergistically to increase the levels of zinc and IGF-I. Insulin like growth factor-I (IGF-I) with zinc spur cells proliferation, thereby increasing the width of epiphyseal plate and improving the structure of epiphyseal plate.

5. Conclusion

Based on the results of this research, it can be concluded that supplementation of blood cockle flour at a dose of 2.5 g/feed/day to malnourished rats can improve the growth of malnourished rats by increasing the levels of plasma zinc, IGF-I and the width of the femoral epiphyseal plate. The results of this research also showed that supplementation of blood cockle flour in malnourished rats could promote growth through changes in the level of IGF-I, which further improve bone metabolism, as seen in the improvement of the growth of chondrocytesand the width of epiphyseal plate.

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