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Analysis of T Lymphocytes (CD4 $^+$), Serum Zinc and the Histology of Thymus of Malnourished Rats Supplemented with Blood Cockle (*Anadara granosa*)

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Abstract: Blood cockle (*Anadara granosa*) is invertebrates containing high protein and zinc. Malnutrition is characterized by the decreasing number of T lymphocytes (CD4 $^+$) and serum zinc level as well as organ dysfunction such as the atrophy of thymus. This study aims to analyze the blood cockle supplementation in increasing the number of T lymphocytes (CD4 $^+$), serum zinc level and improving the thymus of malnourished rats (*Rattus norvegicus*).
Experimental study with Randomized Post Test Only Control Group Design was conducted to 30 female *Spraque Dawley* rats. Samples were selected using simple random sampling, divided into five treatment groups: (1) normal control (KN), (2) and (3) groups of malnourished rats supplemented with non protein food as positive control K.kg (+), and 20% casein as negative control K.kg (-), and (4), (5) groups of malnourished rats supplemented with 10% casein combined with 10% blood cockle flour (P.kg1), and (P.kg2) 20% blood cockle flour. The treatment was conducted for 45 days. Malnutrition is characterized by the albumin level <3.0 g/dL. At the end of the treatment, the rats were sacrificed for blood and tissue collection, followed by the examination on the number of T lymphocytes (CD4 $^+$) and serum zinc. The examination on the histology of thymus was conducted with Hematoxilin-Eosin staining (HE). Data were analyzed using Anova and Duncan's test.
The results that blood cockle supplementation is proved to increase the number of T lymphocytes (CD4$^+$) and serum zinc level to normal, and to improve the cortex and medulla structure of malnourished rats significantly (p <0,05).
Keywords: Blood Cockle (*Anadara granosa*), T lymphocytes (CD4 $^+$), serum zinc, thymus, malnourished rats.

I. Introduction

Cockle is a commodity with high economic value. Production volume of cockles in Indonesia in 2010 reached 8321 tons. Cockle is widely consumed due to its high nutrition and its protein value categorized as complete protein because it contains high amino acid level, having complete profile and approximately 85%-95% of it is easily digested by our body. Cockle is known as the source of zinc as it contains more zinc compared to egg white(0.02 mg/100 g), and chicken(1 mg/100 g) [1].

The high content of protein and zinc makes blood cockle potential for supplementation therapy for malnourished children. The conjugation of protein and zinc is a compound component that plays a role in cell metabolism regulation in the body. Protein helps the process of zinc absorption and subsequently affects the process of the formation of the immune system.

The mechanism of the roles of zinc and protein is shown in zn-finger whose mechanism is reversible at sufficient zinc level, the increasing zinc pool can trigger the synthesis of the methalotionin of intestinal cells that can bind intracellular zinc excess. After entering erythrocytes, zinc is bound by cysteine rich intestinal protein (CRIP) which transfers zinc to methalotionin or across the serous side of enterocytes to be bound with the plasma proteins (albumin) entering the portal of circulation and concentrated in the liver. Other plasma components which contain zinc are alpha-2 macroglobulin, transferin, and amino acids, particularly cysteine and histidine {2].

Thymus gland is a source of lymphocytes known as T lymphocytes (lymphocytes derived from thymus gland). T lymphocytes ($CD4^+$) are very sensitive to the incoming antigens (derived from viruses, bacteria or other substances). One of the reactions of lymphocytes in thymus gland when antigens get into the body is proliferating or multiplying themselves through division. The T lymphocytes control immunity. The growth of thymus gland is highly influenced by age, hormones and disease. Thymus with rapid atrophy is a reaction to stress, thus an animal that is dead after a long illness may have a very small thymus[3], this can be found in malnourished.

This study aims to analyze the number of T lymphocytes ($CD4^+$), serum zinc level and thymus improvement through measuring the cortical thickness and the diameter of medulla with hematoxylin-eosin (HE) staining in the thymus tissue of malnourished rats supplemented with blood cockle flour. The results of this research are expected to be used as a scientific basis in an attempt to improve the immune system, especially in the nutrition managerial by utilizing blood cockle that contains complex nutrition, especially protein and zinc to address the issue of malnourished.

II. Research and Methods

Location and Time of Research

The amount of threty (30) rats (*Rattus norvegicus*) of Spraque Dawley strain were kept in *Seafast* laboratory of Food and Nutrition by Bogor Institute of Agriculture. Analysis of the number of T lymphocytes ($CD4^+$) and serum zinc were conducted at the Laboratory of Clinical Pathology of Cipto Mangunkusumo Hospital. The process of the histology of thymus was carried out at the Laboratory of Primate Center in Bogor.

Research Design

This study is a laboratory experimental research with Randomized Post Test Only Control Group Design using rats as *ad libitum* test subjects.

Making Blood Cockle Flour

Materials used were the whole flesh of blood cockle *Anadara granosa* species. Blood cockles were obtained from the Coast of Tomini Gulf of Gorontalo Indonesian. The process of making blood cockle flour includes: washing, steaming, taking the whole flesh out of the shell, draining, drying and grinding. The dough was dried under the sunlight to reduce the water content. The grinding was performed using 40 meshdisc mill, resulting blood cockleflour.

Analysis of Blood Cockle Flour

Analysis performed on blood cockle flour includes:

a. Proximate analysis (water content, ash level, fat, protein and carbohydrate)[4]

b. Analysis of the mineral content of zinc (Zn) with *Atomic Absorption Spectrofotometer* (AAS) method.

Creating malnourished rats

The creation was based on the concept proposed through the provision of non-protein food with the composition listed on table 1 for 45 days. Rats were affirmed to be malnourished when their albumin was below 3,0 g/dL or at least at the range of approximately 2,7 g/ dL.

Food composition, animal-test treatment, and sampling

Table 1 The Food Composition of Experimental Rats

Component	Normal Control (KN)	MalnourishedNegative Control K.kg (-)	Malnourished Positive Control K.kg (+)	Treatment (P.kg1)	Treatment (P.kg2)
Protein (%) Fat (%) Carbohydrate (%) Minerals (%) Vitamin (%) CMC (%) Water (%)	20.039 6.999 53.508 2.627 1 5 8.992	0.212 6.844 74.529 2.627 1 5 10	20.039 6.999 53.508 2.627 1 5 8.992	20,114 6.167 58.100 2.627 1 3.58 9.052	20.036 6.996 57.877 2.627 1 2.160 9.052
Total	100 (98,165)	100 (100,212)	100 (98,165)	100 (99,64)	100 (99,974)
Energy (Kal)	367,93	369,305	367,93	376,54	376,128

Note:

KN : NormalControl (20% casein standard)
K.kg(+) : Malnourished (non protein)
K.kg(-) : Malnourished (20% casein standard)
P.kg 1 :Malnourished (10% casein + 10% Blood Cockle Flour)
P.kg 2 : Malnourished (20% Blood Cockle Flour)

Analysis of the Number of T-lymphocytes ($CD4^+$)

The samples of rats' blood were taken from all treatment groups and put into micro tainer tubes and stored in a cooler box. The samples were then prepared and analyzed at the Laboratory of Clinical Pathology of RSCM Jakarta. Results examination and perusal follow laboratory procedures using FACSCalibur.

Analysis of Serum Zinc Level

The samples were then centrifuged to obtain serum, and proceeded with the analysis of serum zinc level using Atomic Absorption Spectrofotometer (AAS) method.

Processing and StainingThymus Tissue (Hematoxylin-eosin)

Upon the sampling, the thymus tissue of the five groups of rats were fixed in faraformaldehide solution for 24 hours, proceeded with dehydration process with compound alcohol prior to embeddin in paraffin. Tissue cuts (± 5μm) were then processed with hematoxylin eosin (HE) common staining. HE staining[5] was performed to determine the general morphology of thymus tissue which includes the cortical thickness and the diameter of the medulla of thymus using light microscope at 100 times magnification.

Observation and Data Analysis

The results of the analysis of the number of T lymphocytes ($CD4^+$), serum zinc level and the cortical thickness and the diameter of the medulla of thymus of five treatment groups were arranged as a complete random design analyzed using ANOVA. If the treatment showed a significant effect ($p < 0.05$), the treatment would be proceeded with Duncan's significant difference test to determine the difference of each treatment[6].

III. Results And Discussion

Chemical Analysis of Blood Cockle Flour

Chemical analysis of the Blood Cockle Flour and casein in the study are presented in TABLE 2.

Table 2.The Results of the analysis of the Blood Cockle Flour and casein in the research

Nutrition Component	Content (%)	
	Casein (*Alacid acid casein)*	Blood Cockle Flour
Protein (%)	86, 46	27,26
Fat (%)	0,66	2,54
Water content	4,36	9,47
(%)	3,78	10,62
Ash content (%)	4,74	48,01
Carbohydrate (%)	-	81,16
Zinc (mg/L)		

The results of the analysis of the number of T lymphocytes (CD4 $^{+}$) and serum zinc level Cortical Thickness and the Diameter of Medulla of the Thymus of Experimental Rats in all treatment groups are presented in Table 3.

Table 3. The Mean of the Number of T lymphocytes (CD4 $^{+}$), Serum Zinc, Cortical Thickness and the Diameter of Medulla of the Thymus of Experimental Rats

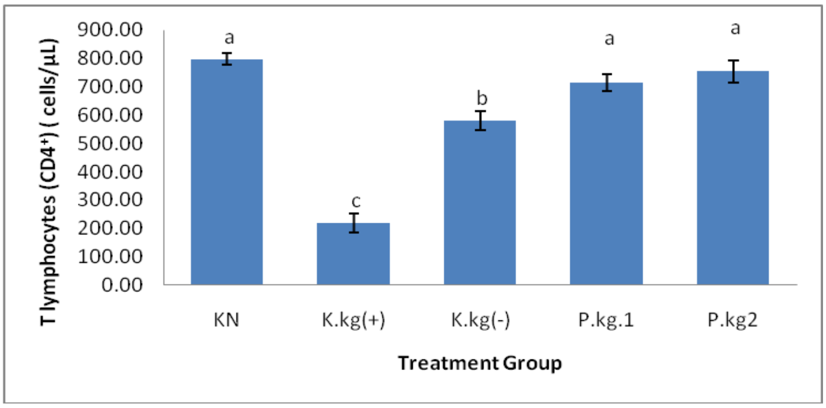
Component	Normal Control (KN) ±SD	Malnourished Negative Control K.kg (-)±SD	Malnourished Positive Control K.kg (+)±SD	Treatment Treatment (P.kg1) ±SD (P.kg.2) ±SD
T lymphocytes (CD4^{+})(cell/µL)	797,667±21,17	219,000±23,46	579,833±22,98	713,500±110,06 754,333±108,428
Serum Zinc (mg/L)	0,990±0,07	0,458±0,05	0,902±0,06	1,012±0,09 1,093±0,07
Cortical thickness of the thymus (µm)	284,30±20,15	217,88±17,49	237,31±9,93	232,57±15,28 264,67±20,93
Diameter of medulla of the thymus (µm)	290,049±40,54	503,322±26,61	381,504±37,82	333,646±24,36 354,508±25,71

P (significant)* 0,05

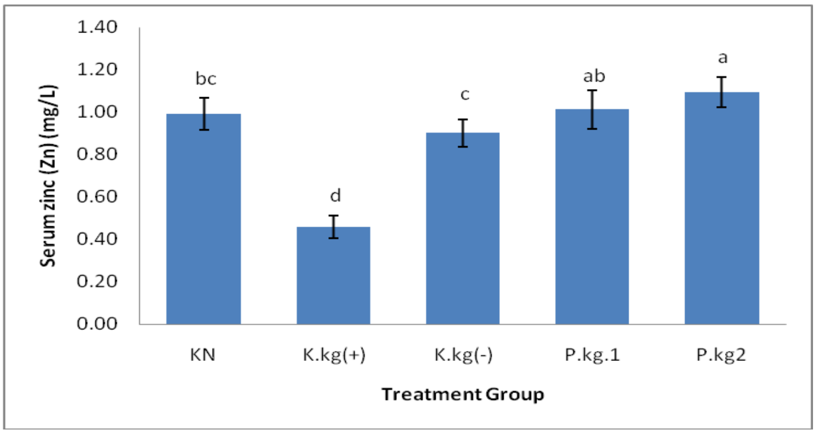
The results of statistical analysis the supplementation of blood cockle flour in the food given to experimental rats has a significant effect ($p < 0.05$) on the increasing number of T lymphocytes (CD4^{+}) of malnourished rats. The results of Duncan's test KN does not have a significant difference compared to P.kg1 and P.kg2. The positive control K.kg (+) has a significance difference compared to the K.kg (-) whose number of T lymphocytes (CD4^{+}) is increasing slightly lower than KN, P.kg1 and P.kg2. This suggests that the increasing number of T lymphocytes (CD4^{+}) is caused by the supplementation of blood cockle flour or its combination with casein.

The mean of the number of serum zinc level of malnourished groups was 0.473 mg/L, whereas that of the normal group was 0.866 mg/L. Plasma and serum contain approximately 10-20% circulation zinc. Zinc level in the plasma is normally 80-110 µg/dL, blood contains twenty fold due to the anhydrase carbonic enzyme in erythrocytes, hair contains 125-250 µg/g, musculus contains 50 µg/g[7].

The results of statistical analysis show that the supplementation of blood cockle flour into experimental rats feed has a significant effect ($p < 0.05$) on the increasing serum zinc level of malnourished rats. Duncan's test results indicate that P.kg1 does not show a significant difference from P.kg2 and KN. Similarly, KN does not show a significant difference from K.kg (-) and P.kg1. In contrast to the positive control K.kg (+), the effect of blood cockle flour supplementation is very significant. This suggests that blood cockle flour supplementation can increase serum zinc level of malnourished rats.



(a) T lymphocytes ($CD4^+$)



(b) Zinc Serum

Results of the Observation on the Histology of Thymus

The results of statistical analysis show that Blood Cockle Flour supplementation into rats feed has a significant effect on the cortical thickness at $p = 0.042$ and on the diameter of medulla at $p = 0.004$ of malnourished rats, at the level of confidence of $p < 0,05$.

Duncan's test results indicate that KN and P.Kg2 show a significant difference from K.kg (+) with the treatment of non-protein feeding. KN does not show a significant difference from K.kg (-), malnourished group provided with casein and P.kg1, malnourished group provided with a combination of casein and blood cockle flour. Similarly, K.kg (+) does not show a significant difference from K.kg (-), although the value is slightly lower. It is concluded that the supplementation of Blood Cockle Flour or its combination with casein into the feed of experimental rats can improve cortical thickness of the thymus of malnourished rats as indicated by KN.

Duncan's test results indicate that K.kg (+) shows a significant difference from P.kg2 and normal control (KN). KN does not show a significant difference from the malnourished group fed with casein, K.kg (-), and the malnourished group fed with a combination of casein and blood cockle flour, P.kg1.

Results of photomicrograph on the preparations of the histology of thymus with HE staining are presented in

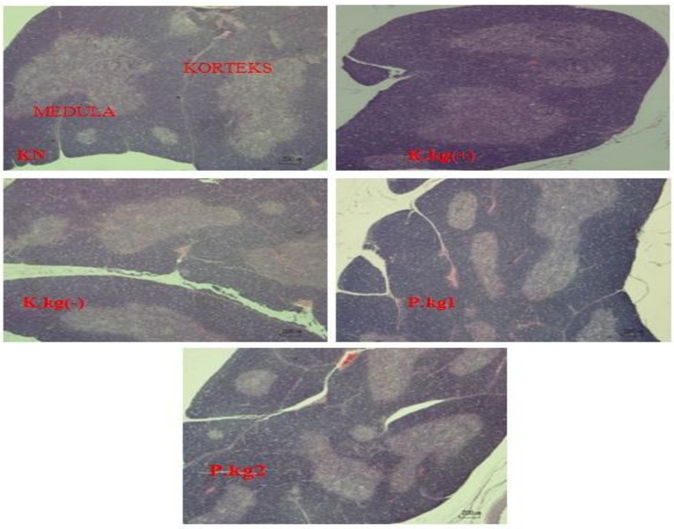


Figure 1. Results of Microscopic Observation on the Preparations of the Histology of Thymus of Rats (Rattus norvegicus) with HE Staining, scale: 100µm to: (KN) Normal Control Rats, K.kg (+) malnourished control rats fed with non protein, K.kg (-) Malnourished control rats fed with Standard Casein, P.kg1 malnourished Rats fed with Casein and 10% Blood Cockle Flour and (P.kg2) malnourished Rats fed with 20% Blood Cockle Flour.

Discussion

The Number of T lymphocytes ($CD4^+$)

Based on the results of this study and the statistical analysis, the supplementation of blood cockle flour into the feed of the experimental rats has a significant effect ($p < 0.05$) on the increasing T lymphocytes ($CD4^+$) level of malnourished rats.

The increase in the number of T lymphocytes ($CD4^+$) of the groups of malnourished rats upon the supplementation of blood cockle and its combination with casein indicates that blood cockle contains adequate protein to increase the number of T lymphocytes in the body.

A lymphoid organ associated with the production of T lymphocytes is thymus. The maturation of T lymphocytes occurs in the thymus. Thymus gland contains thymocytes. A study on the role of thymocytes and their connection to the content of leptin in the thymus has been conducted by Yablonski, *et al.*,[8]. It is suggested that in malnutrition, the leptin level decreased stimulating adrenal axis, pituitrin and the hypothalamus, resulting in an increase in the glukokorticoid hormone and ultimately an increase in apoptosis of thymocytes. Furthermore, it is also suggested that the apoptosis of thymocytes can be prevented and can be reversed by the administration of adequate protein as long as the leptin is still at adequate level. If leptin level decreased, thymus will experience atrophy, and further the production of T lymphocytes decreased. The mechanism of the role of leptin in lymphoid organ is to build the energy balance of the organism through the stimulation in the hypothalamus and stimulate lymphocytes production and spur growth hormone in thymus (thymulin hormone) in the presence of leptin.

Another study conducted by Nasser, *et al.*,[9] suggests that children with PEM experience a decline in the number of T lymphocytes as a result of a decline in thymic hormone synthesis, which is responsible for the development of cell-mediated immune response. It is also suggested that lymphoid tissue, primarily thymus, found stops developing, resulting a decline in the production of T lymphocytes.

The decreased level of T lymphocytes ($CD4^+$) in the groups of malnourished rats supplemented with non-protein feed may be caused by the declining absorption of nutrients from the small intestine due to the declining in digestion motion rate. It is suspected that infection may occur in malnutrition, and if it is the case, it will further increase reactive oxygen species (ROS). Increasing ROS will further increase epithelial necrosis, causing an increase in Hsp70.

Serum Zinc Level

Albumin proteins can bind zinc in serum and together they play a role in protein synthesis in the body. The results of the study show an increase in serum zinc level upon the supplementation of blood cockle flour or its combination with casein. This is evidenced with the significant value of = 0.0001 which means that the treatment has an effect on the increase in serum zinc level at $p < 0.05$.

If it is reviewed based on the results of Duncan's test, there is no significant difference in both treatments (P.kg1 and P.kg2), although if it is reviewed based on the results of the analysis of the feed, it is found that the treatment group with the combination of 10% casein and 10% blood cockle flour (P.kg1) has lower zinc level amounted to 19.65 mg/L, compared to P.kg2 with 34.19 mg/L. This result indicates that the status of zinc is affected by zinc intake from food. Components of blood cockle such as iron (Fe), protein, dietary fiber and phytic also affect zinc absorption. On the other hand, zinc absorption varies greatly depending on the content of zinc in the diet and bio-availability. Zinc from animal products is easily absorbed. Coppen and Davies[3] and Sandstrom and Cederblad[10] report that higher zinc intake will reduce the amount of zinc absorbed. The decrease in zinc absorption as a result of higher zinc intake is due to the saturation in transport mechanism. Zinc absorption is affected by the carrier components and diffusion processes. The amount of zinc absorbed increases linearly up to higher level, but after reaching the diffusion process, the amount of zinc absorbed will be consistent.

As a metalloenzyme, zinc is required in almost all aspects of cellular metabolism. The research results on experimental animals indicate that zinc is essential for DNA synthesis by mammalian cells. Thymidine kinase, RNA polymerase, DNA polymerase, ribonuclease and reserve transcriptase are dependent zinc enzymes serve as important catalysts in DNA replication and transcription during cell division[11] . Zinc finger is a nucleic acid that binds in the form of zinc cation that binds cysteine and histidine in the form of hydrophobic between the alpha bond and two anti-parallel beta strand of a self-folding structure. The structure of protein is essential to bind DNA. Zinc finger bond can describe the pattern of amino acids bond around the zinc observed on certain transcription factors that result in loop formation or finger in the protein and allow the parts that are bound to bind DNA (Deoxyribonucleic acid) in promoter gene. Therefore, without zinc, transcription factors can not bind DNA and stimulate gene transcription. Zinc-finger requires four residual amino acids as ligand namely two cysteines and two histidiles of each zinc molecule[2]. It is suggested that the biological role of zinc is always in the form of bivalent cation, does not undergo reduction and oxidation under physiological condition, so that zinc is a stable component of the protein complex[2,11]. The reason can explain that blood cockle that contains high protein and zinc may play a role in improving the immune system, in addition to its function as a cofactor enzyme in cell metabolism. According to Pond, *et al.*[12] zinc has effect on immunity and can increase the activity of lymphoid cells.

Thymus Tissue Repair

Thymus is essential in cellular specific immune system, as differentiation and proliferation of T cells or T lymphocytes occur in the thymus. The involution (developmental disorder) of the thymus gland leads to a decrease in T cells, such as $CD4^+$ [T helper 1 cells (Th1) and T helper 2 cells (Th2)][13].

Atrophy is shrinkage of an organ or tissue, making it smaller than its normal size. Another theory suggests that the atrophy of the thymus is characterized by the loss of lymphocyte populations and limit the disappearance of the distinction between medulla and cortex[14]. Atrophy can also be caused by malnourished in its function as the growth substance and thymus cell growth. The lack of complete nutrition makes the organ undergoes degenerative change. The change in thymus is atrophy in cortex and medulla. Table 3 shows that the size of cortex is smaller in treatment groups of malnourished rats fed with non protein feed. Moreover, the medulla is bigger than the cortex. This indicates that thymus undergoes atrophy due to the declining in the nutrient consumption which causes all cell components in the body grow improperly.

The size of thymus is varied, the biggest size can be found in newborn animal and the biggest absolute size is during puberty. As mature, thymus undergoes atrophy of parenchyma and cortex is replaced by fat tissue. Thymus which undergoes rapid atrophy is a reaction to stress due to malnourished. In this study, it is suspected that the experimental rats are stressed causing them experience physiological disorder as a result of low appetite which leads to barriers in the growth and development of cells or body tissues. That thymus will experience rapid atrophy because of stress, therefore animal that is dead due to a long illness has a very small thymus. It is also described that the main component of the cortex of thymus consists of epithelial reticulum and lymphocytes. As the consequence, the smaller cortex size, the fewer lymphocytes produced in this tissue.

Involution process is characterized by gradual decrease of lymphocytes primarily those in cortex area, the enlargement of reticular epithelial cells and the replacement of parenchyma by fat cells. Thymus also secures the area in which stem cells migrating from postnatal spinal cord proliferate and differentiate to be T lymphocytes [14]. This process is supported by some chemical materials such as polypeptide resulted from reticular epithelial cells[15].

Zinc serves as a strong antioxidant which is able to prevent cells damage, stabilize cell wall structure and improve immune system. Zinc also serves to prevent the occurence of free-radicals, therefore apoptosis process or a planned death cell can be suppressed[16]. Because zinc is influential on immunity and can enhance the activity of lymphoid cells including thymus.

Thymulin is a hormone which is secreted by thymus gland. The hormone functions as the controller of immune system in general such as T lymphocytes maturation. The increasing thymulin activity shows that immune system disorder such as malnourished due to thymus gland atrophy can be fixed through food intake with complete proteins such as blood cockle.

Nutrient in blood cockle and thymulin activity (thymus gland hormone) are presumed to be able to repair and protect thymus cells (cortex and medulla) from antigen attack. Therefore, cell intactness is maintained; and the maturation process, as well as differentiation and cell activation increase. The synergy of protein and zinc in blood cockle can spur thymus cells to work properly and eventually T lymphocytes production increased.

Conclusion

The supplementation of blood cockle (*Anadara granosa*) as much as 10% and 20% to malnourished rats (a) has an influence on the increasing number of T lymphocytes ($CD4^+$), (b) has an influence on the increasing serum zinc level, (c) has an influence on the histology of thymus through fixing the structure of cortical thickness and the diameter of medulla in thymus which experiences atrophy. The supplementation of 20% blood cockle flour has a better effect compared to the supplementation of 10% blood cockle flour.

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