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Research Article

Effect of Varying Levels of Protein and Energy in Pre-stater Feeds on Pectoralis Muscle Development of Kampung Super Chicks (*Gallus gallus gallus*)

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Abstract

Objectives: This study was performed to examine effect of varying level of protein and energy in pre-starter feed for 7 days old chicks on pectoralis muscle development of kampung super chicks. **Materials and Methods:** Fourthy kampung super day old chicks were fed pre-starter diet during the experimental period and were divided into four groups. The control group chicks were not fed for the first 3 days post-hatch and continued with a standard pre-starter diet to 7 days old. The second group was chicks with pre-starter type A diet with 20.19% of protein and 3300 kcal kg^{-1} of Metabolism Energy (ME) for the first day post-hatch to 7 days old. The 3rd group was treated with pre-starter diet type B for the first day post-hatch to 7 days old with protein level 21.84% and 3100 kcal kg^{-1} of ME and the last group was given pre-starter diet type C with with protein level 21.13% and 2800 kcal kg^{-1} of ME for the first day post-hatch to 7 days post-hatch. The parameters measured were body weight, pectoralis thoracicus weight, muscle area, cross-sectional area of myofibers and the number of Proliferating Cell Nuclear Antigen (PCNA)-positive nuclei. The data were analyzed using one way ANOVA, followed by Tukey tests. **Results:** The results showed that groups 4 had lower body weight, pectoralis thoracicus weight, muscle area, cross-sectional of myofibers area and number of PCNA-positive nuclei compared to group 2 and 3 ($p \leq 0.05$) which proved that early post-hatch feed in kampung super chicks was important to body weight and pectoralis muscle development. **Conclusion:** It was concluded from the study that diet with 21.84% of protein and 3100 kcal kg^{-1} of ME in 7 days old chicks promised optimum performance and pectoralis muscle development for kampung super chicks.

Key words: Kampung super chicks, muscle development, PCNA-positive nuclei, prestarter diet, protein and energy level, weight of pectoralis thoracicus muscle

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Early post-hatch feeding until the 14 days old which is called pre-starter feeding on broiler chicks is known to have significant effect on chicken development especially on body mass gain, pectoralis muscle size and small intestine development[1-3]. Protein and energy from the diet are essential for chickens, including the pre-starter phase. Energy is needed to maintain the body function while protein is needed for tissue maintenance. Protein is needed for energy production but the protein source on the other hand is relatively more expensive because in some countries it has to be imported from other countries[4]. As known on broiler, protein and energy necessity between temperate zone and tropical zone have different values[5]. High energy formulated in broiler pre-starter feed is leading to waste because the excessed energy will be converted to extra deposition of fat. The proper nutrient portion in the feed is important to ensure the maximum needs of each nutrient[6]. The ratio between energy and protein for broilers pre-starters is between 138-140. Furthermore, the optimum feed formulation in Iran contained 2900 kcal kg^{-1} energy and 21% Crude Protein (CP) for broiler in pre-starter period[7].

Chicken muscle development is used as an indicator of chicken meat quality. Pectoralis muscle is one of skeletal muscle which is located on thorax. It is used as chicken development parameter because it is reported contained low fat and has lower rate of post-mortem acidification[8]. Embryogenesis and post-hatch period are very crucial phase in chicken development, including pectoralis muscle development[9,10]. Muscle development in avian species occurs in 2 distinctive periods. Firstly, during the embryonic phase, the myofiber number (MFN) is established when a large number of precursor cells are committed to the expression of muscle-specific genes. Secondly, during the post-hatch period, the hypertrophy of the muscle occurs, mainly by accretion of protein and nuclei resulted from the proliferation and fusion of satellite cells[11]. New born chick skeletal muscle growth did not show an increase in myofiber number but did in myofiber size, which happened because DNA duplication was not followed by cell division. The increase in DNA coincident with myofiber growth occurs through the donation of nuclei from the mitotically active satellite cells population[1,12], that lies between the myofiber basal lamina and the sarcolemma[13]. Development of pectoralis muscle in post-hatch phase needs appropriate nutrient portion in its feed[9,10], including an appropriate ratio between protein and energy[14].

Developing chicken using cross-breeding is now a trend in Indonesia, between broiler or layer hen with local roaster for instance, for the taste of Indonesian local chicken is still the most favorite one. The advantage from this breeding is the production of fast growing chicken with Indonesian local chicken meat taste. The study of body weight of chicken needs the informations about both the skeleton and muscle development. Retnoaji *et al.*[15] reported the osteogenesis of hybrid chicken derived from crossbreeding between Indonesian local chicken (Pelung) and broilers. But until recently, no study on histological muscle development was conducted.

Kampung super chicken or commonly called as "Kamper" is one of the most desirable strain in chicken breeding. Kampung super chicken is resulted from cross-breeding of Lohman brown-type hen and Indonesian Pelung chicken. This strain is started to be developed due to the high consumer interest of local chicken meat but in opposite, it's production number decrease every year. This phenomenon is caused by the low development rate of local chicken and the reproduction rate of the hens is also low. Kampung super chicken's appearence is similar to Indonesian chicken but it has higher gain mass rate. Kampung super chicken can reach 1 kg in weight on the 3rd month of nursing[16].

The aim of this study was to investigate the effect of different level of protein and energy in pre-starter feed on pectoralis thoracicus muscle performance of kampung super chicks.

MATERIALS AND METHODS

Birds and housing: All procedures involving animals were approved by the Universitas Gadjah Mada, Indonesia Institutional Animal Care and Use Committee. In conducting the research, fourthy day old chicks were divided into four groups. All chickens were housed with semi-intensive system and kept under standard management conditions. Water was provided *ad libitum* during the experimental period for 7 days.

Every group was fed by different type of feed. Type A feed consisted of 21.84% protein and 3200 kcal kg^{-1} ME, type B feed consisted of 20.19% protein and 3300 kcal kg^{-1} ME and type C feed consisted of 21.13% protein and 2800 kcal kg^{-1}. The feed types were chosen by the ratio between ME and protein which were higher, equal and lower than the reference[7].

Experimental design used, which were the offspring of kampung super chicken were divided into 4 groups.

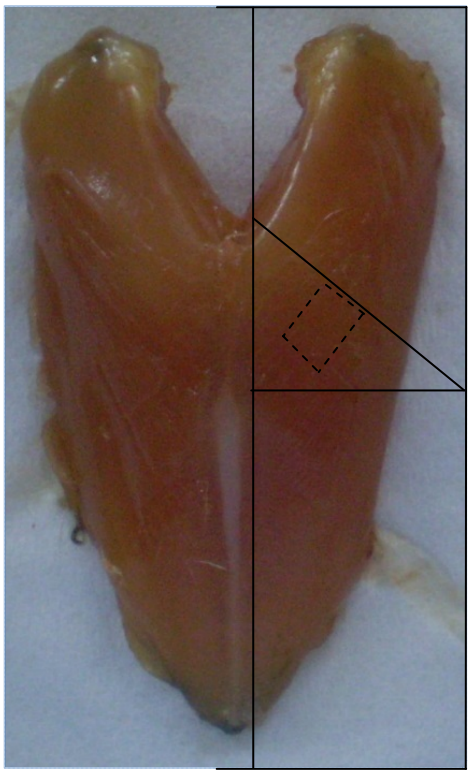

Fig. 1: Schematic of pectoralis thoracicus muscle which was still attached to the bone. Samples of muscle were taken by lateral cutting with dash orientation (----) then histological process to count area of myofiber. Sign solid orientation (-) was used to measure size of muscle area and tissues cutting for counting area of myofiber[17]

Each group consisted of 10 DOCs. The first group was the control group with no feed for the first 3 days post-hatch as the control group to convinve that the pre-starter feed is crucial for the weight gain and then it was continued with a standard pre-starter diet to 7 days post-hatch, which was the same feed as the type B feed. The type B feed was chosen for the frist group for it had the middle ratio of protein and ME as reported in the previous study[7]. Group 2 members were given type A pre-starter feed for 7 days post-hatch. Group 3 members were given type B feed for 7 days post-hatch. Group 4 members were given type C pre-starter feed for 7 days post-hatch. The feeds were provided *ad libitum* during the experiment period. The DOCs weight were measured on the 0, 3rd and 7th days old and then the left side of Pectoralis Thoracicus (PT) was measured to observe the muscles weight of PT and then the right side of PT was used to count the area width of PT with ott-planimeter (Fig. 1).

Myofiber measurement: The myofiber diameter was measured using micrometer software after histological preparation done. The measurement was conducted by counting 5 view area of fasciculus myofiber of every sample of each group. The histological preparation process was done by cutting the PT muscles into smaller pieces about 3×3 mm wide and then fixated in neutral buffer formaline. After fixation step, the next step was dehydration using alcohol ranging from 70% to absolute alcohol. Clearing process or dealcoholization was done using toluol. Afterward, infiltration was conducted by using paraffin and the muscle samples are embedded in paraffin blocks. Paraffin blocks then were cut using rotary microtome with 5 mm of coupes thickness and then affixed on microscope glass. The next step is deparaffination and rehydration with xylene and alcohol[18].

For immunohistochemical analysis pectoral muscle from 5 individuals was studied. The tissues were fixed in formalin buffer saline solution embedded in Paraplast (Sigma Aldrich). They were cut into transversal slices of 5 µm thickness using a microtome (Leica RM 2265, Leica Microsystems). Tissue sections were dewaxed with xylene and rehydrated. Endogenous peroxidase was blocked with a 3% solution of H_2O_2. Immunohistochemical analyses were performed using EnVision+System-HRP (DAB) used for mouse primary antibodies (Abcam) according to the procedures described by the manufacturer. Proliferating nuclei cells were identified via immunohistochemistry using monoclonal mouse antichicken antibodies at a 1:100 dilution (Abcam) directed against PCNA. The sections were incubated with the primary antibodies for 1 h at room temperature and then incubated with 3.3'-diaminobenzidine (DAB)+substrate-chromogen (DAKO kit), resulting in a brown-coloured precipitate at the antigen site. The number of PCNA-positive nuclei was estimated as PCNA-positive cells per 100 nuclei of myofiber. Furthermore, the number of PCNA-positive nuclei per 100 nuclei of myofiber, within one fasciculus of muscle fibres was counted, using cross sectional image of pectoral muscle[19].

Data analysis: Data of chicken body weight, muscle weight of PT, area of PT, myofiber area and PCNA-positive nuclei were analyzed using oneway ANOVA and Tukey-test at significance level of 5% in SPSS 13.0 software[20].

RESULTS

Proximate test: The feed used in this study was pre-starter feed which was given from post-hatch days until 7th days old. The treatment was given with varying level of protein and energy which was designed to accelerate DOCs growth in 7 days post-hatch. At the preliminary study conducted before, proximate test and bomb calorimetry were done in the certified laboratory in Animal Husbandry Faculty of Universitas

Table 1: Proximate test between type A, B and C feed

Code	Dry matter	Ash	Crude protein	Crude fat	Crude fiber	Nitrogen free extract	ME (kcal kg^{-1})	Ratio energy: Crude protein
Type A	89.41	6.68	20.19	7.66	2.51	62.95	3300	163.44
Type B	94.42	5.60	21.84	5.48	3.81	63.26	3100	141.94
Type C	88.10	16.95	21.13	4.08	3.25	54.59	2800	132.51

ME: Metabolism energy

Table 2: Average body weight (gram) of kampung super chicks from post-hatch, 3 and 7 days post-hatch

Days	N	Starved	Type A	Type B	Type C
0	8	32.6±0.6	33.6±0.67	32.0±0.95	32.8±0.73
3	8	28.0±0.44[a]	34.8±0.86[b]	35.2±0.8[b]	31.8±0.73[a]
7	8	40.0±0.7[a]	53.2±2[b]	54.0±0.54[b]	40.2±1.01[a]

[a,b]Values within rows without a common superscript are significantly different ($p \leq 0.05$). Values are Mean±SE, N: Number of chicks, Type A: Kampung super chicks fed 20.19% protein diet and 3300 kcal kg^{-1} ME, Type B: Kampung super chicks fed 21.84% protein diet and 3100 kcal kg^{-1} ME, Type C: Kampung super chicks fed 21.13% protein diet and 2800 kcal kg^{-1} ME

Table 3: Average of PT muscle weight (g), PT muscle area (cm^2), fasciculus of myofiber area (μm^2), area of myofiber (μm^2) and PCNA-positive cells kampung super chicks at 7 days post-hatch

Variables	N	Starved	Type A	Type B	Type C
Muscle weight	5	0.49±0.06[a]	1.02±0.1[b]	1.15±0.02[b]	0.36±0.02[a]
Muscle area	5	4.80±0.28[a]	6.12±0.27[b]	5.63±0.20[b]	3.92±0.36[a]
Fasciculus of myofiber area	5	3036.00±91.96[a]	9545.60±1.43[b]	9737.30±86.9[b]	2401.70±58.8[a]
Myofiber area	5	32.01±0.48[a]	57.64±0.68[b]	67.64±0.25[b]	41.53±1.09[a]
PCNA-positive cells	5	0.63±0.02[a]	0.78±0.02[b]	0.79±0.02[b]	0.68±0.01[a]

[a,b]Values within rows without a common superscript are significantly different ($p \leq 0.05$). Values are Mean±SE, N: Number of chicks, Type A: Kampung super chicks fed 20.19% protein diet and 3300 kcal kg^{-1} ME, Type B: Kampung super chicks fed 21.84% protein diet and 3100 kcal kg^{-1} ME, Type C: Kampung super chicks fed 21.13% protein diet and 2800 kcal kg^{-1} ME

Gadjah Mada (UGM) and Laboratory of Chemistry University Center of UGM. The analysis (Table 1) showed the level of protein and energy for type A feed was 20.19% and 3300 kcal kg^{-1}, type B feed was 21.84% and 3100 kcal kg^{-1} and type C feed was 21.13% and 2800 kcal kg^{-1}. The level of protein and energy from each group was used to calculate the ME-protein ratio. From the calculation it was shown that type A had the highest ratio of ME-protein, type B was at the middle and type C had the lowest ratio. From this calculation result, use type B feed for the first group for it had the sam ratio with optimal ratio of ME-protein reported in the previous study[7].

Body weight: Data on body weight performance during different of treatment are presented in Table 2. The body weight performance of kampung super chicks fed with type A feed containing 20.19% CP and 3300 kcal kg^{-1} and type B feed containing 21.84% CP and 3100 kcal kg^{-1} on the 3rd and 7th days olds was higher compared to type C feed containing 21.13% CP and 2800 kcal kg^{-1} and starved group (Table 2). The result showed that pre-starter feed which was given from day 0 had positive effect compared to the first group which was delayed to access feed for 3 days.

Pectoralis muscle performance: Pectoralis muscle performance data was obtained by measuring muscle weight, muscle area, myofiber area and PCNA-positive cells (PCNA). The data of pectoralis muscle performance on kampung super DOCs (Table 3) and area of myofiber (Fig. 2) showed the result of group 2 which was given with 21.84% protein and 3200 kcal kg^{-1} ME feed and group 3 containing 20.19% CP and 3300 kcal kg^{-1} ME feed were significantly different than group 4 which was given with feed containing 21.13% CP and 2800 kcal kg^{-1} and the group 1 which was delayed to access the fee for 3 days ($p<0.05$). It is shown that the feed which was given from day 0 gave better effect on muscle performance than the delayed feed.

DISCUSSION

The body weight in group 2 and 3 showed significant difference compared to groups 1 and 4 since the proportion of nutrients included the good ratios of protein and energy in type A and type B feed. The results were in line with the data that reported that in Asia, which is tropical zone, the proportion value of protein and energy needed by broiler chicken in starter phase[7] is 138. Another study also stated that the ideal ratio of energy and protein for 1-10 days old broilers[21] is 137. In addition, according to the body weight gain data in type A and B feed groups, it could be explained that chickens in early development have very high trypsin and other protease enzymes. Therefore, the intestinal absorption

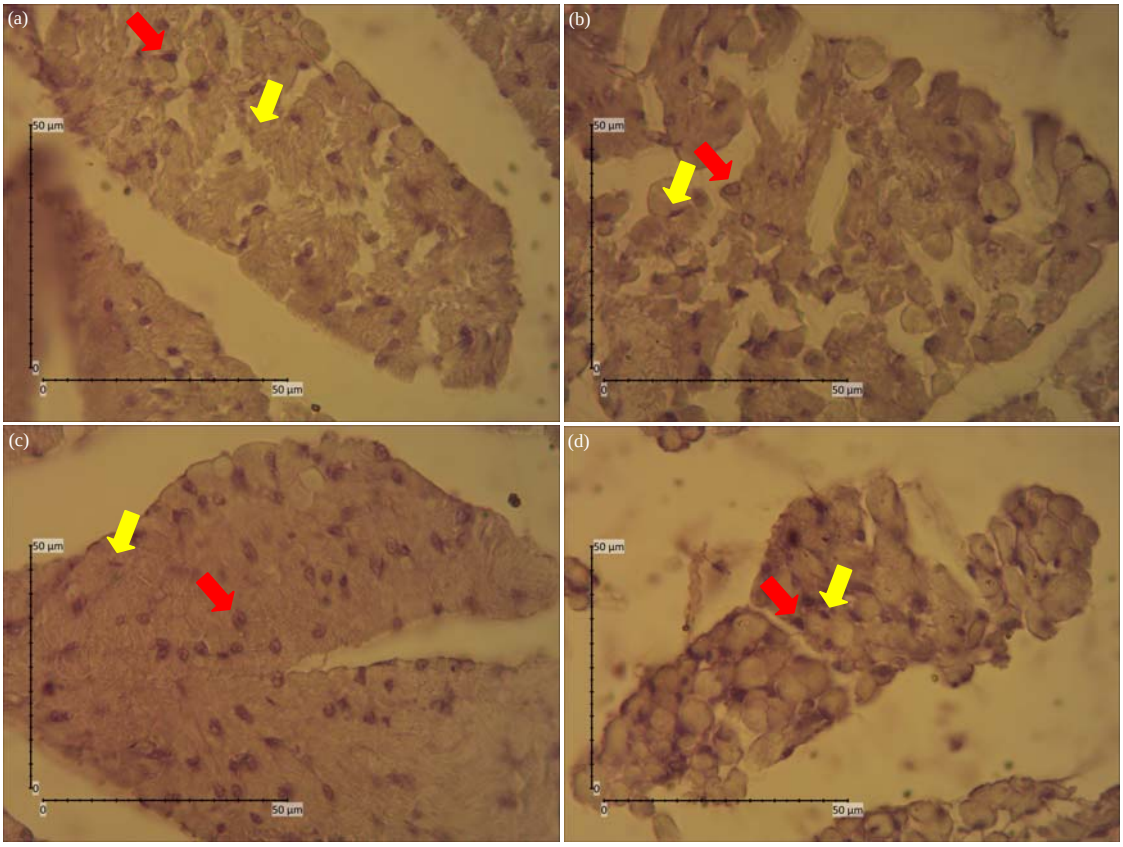

Fig. 2(a-d): Histological microscopy of myofiber area and PCNA-positive nuclei pectoralis muscle kampung super chicks, (a) Control group, (b) Type A feed group, (c) Type B feed group and (d) Type C feed group. It is clearly showed that the myofiber area, myonuclei (yellow arrow) and PCNA-positive nuclei (red arrow) of group B and C are larger and increase than the control and type D groups. Magnification 40x100

of amino acids, in this case lysine is better in younger chicks than the older ones. After they reach 3 weeks of age, the digestive enzymes activities will decrease[22,23]. However, the results of the present study did not agree with the previous study which reported that the optimum performance for broiler chickens at pre-starter phase with low ME and high CP diets[24].

It showed that type C, which has protein and energy of 21.13% and 2800 kcal kg^{-1} could not increase the body weight of kampung super chicks. It might be caused by the level of protein and energy was not balance so that the ratio between protein level and energy was too high, which became uneffective for body mass gain in chicks. High ratio between protein and ME in chicken feed would cause low mass gain because of the limited chicken ability to fulfill their protein-energy needs[25]. The results were also in line with the study which stated that feeding broilers with low CP feed failed to support growth performance[25].

Another study conducted by Van Emous *et al.*[26] using broilers chicks reported that the chicks which were fed with the medium and low crude protein diet showed a higher feed intake between days 18 and 27 and during the total growth period, compared to the chicks which were fed with high crude protein. Male broilers which were fed with low crude protein had higher breast meat yield than male broilers which were fed with high crude protein, while breast meat yield of female broilers was not affected by dietary protein levels. This experiment showed that a higher growth pattern during the rearing period influenced the fertility, decreased embryonic mortality and improved offspring performance in young breeders, on the other hand decreasing the dietary protein level did not affect those traits. The study of varying dietary levels of energy and protein on Vanaraja chicken revealed that feed containing 19 and 21% CP with 3000 kcal kg^{-1} ME showed significantly higher body weight and FCR and it also indicated better immune response against new castle disease[27].

Body weight for starved group was lower than the group 1 and 2. It showed that pre-starter feeding is important to DOC development in the future[1,2]. After the growth response test, a surgery was conducted to measure PT muscle performance, including muscle weight, muscle area width and area of myofiber. The surgery was conducted after growth response observation has been finished to confirm nutrient absorption in the formation of muscle. Pectoralis muscle was chosen because of it's bigger mass compared to other muscles. Pectoralis muscle development is commonly used as an indicator of chicken husbandry success.

Performance of PT muscle was related to body weight and it is indicated that formulation used in groups 2 and 3 induced muscle development rather than lipid deposition. It was because the nutrient components in type A and type B feeds were sufficient which could improve muscle development during early age. The results were also in line with the report which stated that the low protein and high energy feed for broilers might influence the breast muscle characteristic[28]. Post-hatch muscle development was conducted by the increase of new nuclei number from progenitor satellite cells of muscle in muscle fibers[29]. Good proportion feed on this stage is very essential because the stimulation of the proliferation of satellite cells, the incorporation satellite into myofibers and muscle growth highly occur on this stage[30]. Pectoralis muscle development with type C feed treatment did not show positive result on chickens growth. This was the result of incapability of nutrient compositions in type C to improve the muscle development. Unbalancing ratio between protein and energy in chicken feed can disturb its muscle development[14]. The starved group showed delayed pectoralis muscle development compared to group 1 and 2. It proved that pre-starter feeding had important effect to pectoralis muscle development[19].

To understand molecular mechanism of increasing number of muscle's nuclei, proliferating cells were identified by immunohistochemistry methods, using antibodies directed against proliferating cell nuclear antigen. Levels of PCNA are excellent markers of cell proliferation state for analysis the regulatory proteins cell cycle[24]. The PCNA-a protein molecule, which is found in nucleus is involved in regulation of polymerase delta (δ) and polymerase DNA. The peak concentration of PCNA protein is observed in nucleus during the interphase of cell cycle (G1, S, G2) and it is declining during G0 phase and division of cells[31].

The results showed increase of percentage of PCNA-positive cells in chicken pectoralis muscle treated with type A and B feed, indicating that bigger amount of muscle cells were present within interphase. Myocytes are multinuclear fibres, which result from fusion of myoblasts being in the post-mitotic G1 phase[31]. The expression of PCNA is a good marker of satellite cells, which are also precursors of myofiber, into S phase of cell cycle[30]. Assuming that an increase of PCNA-positive cells in the muscles reflects state when more cells are involved in non-dividing structure of myotubes, might be suggest improved balance between hypertrophy to hyperplasia in chicken post-hatch[30]. The result from this study revealed that the pre-starter feed which is given from day 0 showed positive effect on pectoralis muscle growth and performance and this is in line with the previous study reported by Modziak *et al.*[19].

CONCLUSION

Pre-starter feeding which contained 21.84% protein and 3100 kcal kg^{-1} metabolism energy fed from the 0 day of post-hatch is recommended for the kampung super chicken to support higher growth of pectoralis muscle.

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