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A Combination of *Anadara nodifera* Shell and Milkfish Thorns Powder Effectively Promote Springiness Index, Serum Testosterone, and Breast Muscle Testosterone in Bangkok Rooster

Akhmad Abror As Sidiqi[1], Claude Mona Airin[2], Sarmin Sarmin[2], Pudji Astuti[2*]

[1]Veterinary Sciences Master Program, Faculty of Veterinary Medicinie, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta 55281, Indonesia
[2]Department of Physiology,, Faculty of Veterinary Medicinie, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta 55281, Indonesia

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ABSTRACT

Aromatase blocker effectively promotes testosterone, which improves muscle performance and protein synthesis. Considerable muscle strength in Bangkok rooster is the outcome of testosterone activity. Clamshell contains aromatase blocker substances, while fishbone possesses a high amount of protein. This research aimed to evaluate the clamshell and fishbone combination potency towards testosterone levels and muscle performance in Bangkok roosters. This study employed 18 Bangkok roosters under 35 days of treatment. The rooster groups consisted of control (P0, n = 6); clamshell-fishbone (P1, n = 6): clamshell (6.6 gram/day) + fishbone (3.3 gram/day); fishbone (P2, n = 6): fishbone (3.3 gram/day). The blood was collected once every seven days. Enzyme immunoassay showed the highest serum and muscle testosterone levels in P1 ($p<0.05$). Immunohistochemistry presented the most and broadest myofiber and the highest proliferation activity in P1 ($p<0.05$). The texture analysis showed the topmost springiness index in P1 ($p<0.05$). Aromatase blockers in clamshell and protein in fishbone improve testosterone and muscle performance in Bangkok roosters.

1. Introduction

The general purpose of the Bangkok rooster (*Gallus gallus* domesticus) raising is the fighting cock, not only for the game but also for personal amusement. Bangkok rooster originates from fighting cock in Thailand, afterward is widely bred in Indonesia (Ulfah *et al.* 2017). Indonesian people raise the Bangkok chicken as a game, companion, and egg and meat production (Ulfah *et al.* 2015). The virtue of Bangkok chicken lies in strong posture, muscular strength, high libido, and high fertility rates. The fighting cocks have highly active and robust muscle characteristics (Endo *et al.* 2021). Muscle organ plays roles in body metabolism, including protein storage (Endo *et al.* 2021; Puspita *et al.* 2016). The characteristics of muscles are associated with testosterone due to its anabolic features. Anabolism is a part of metabolism that results in macromolecule synthesis, including protein synthesis. The counterpart is catabolism, which comprises macromolecule breakdown processes, such as fat or protein breakdown for energy usage. Testosterone is a steroid hormone that regulates male reproduction and protein metabolism in muscle (Hamdi and Mutungi 2011; Rosati *et al.* 2021). Anabolic steroids such as testosterone derivatives can trigger protein accumulation and increased muscle mass (Davidyan *et al.* 2021; DeChick *et al.* 2020; Gharahdaghi *et al.* 2021). However, the effect of testosterone activity on muscle in Bangkok roosters is still scarce of data.

Testosterone assists muscle protein storage by enhancing amino acid intake in muscle. High body protein availability promotes muscle growth, which involves myogenic cell proliferation. The activity of proliferating cell nuclear antigen (PCNA) is a hallmark of dividing cells because of its role in DNA replication. The muscle originates from myogenic stem cells or satellite cells that proliferate, differentiate, and fuse

* Corresponding Author
E-mail Address: pastuti2@ugm.ac.id

to become functional myofiber. The enlargement and multiplication of myofiber influence the physical characteristics of the muscle, such as hardness, tenderness, and springiness.

Testosterone has an anabolic effect that supports muscle recovery and improvement. Exogenous testosterone has effectively restored muscle wasting in spinal cord injury individuals (Gorgey *et al.* 2021; Holman and Gorgey 2019). Exogenous testosterone also solves muscle weakness in individuals with aging and heart failure (Huang and Wang 2021; Josiak *et al.* 2014). Exogenous testosterone therapy improves muscle performance but impairs the reproductive system (El Osta *et al.* 2016). Therefore, an alternative of exogenous testosterone is necessary to haste muscle improvement without reproductive system deterioration.

Aromatase facilitates the conversion of testosterone into estradiol. Aromatase inhibitors enhance testosterone activity by hindering the transformation of testosterone into estradiol. Furthermore, the inhibition increases testosterone availability and then promotes muscle performance. The body's zinc state can influence testosterone activity. Dietary zinc differences in Liaoning Cashmere goats affect blood testosterone levels (Liu *et al.* 2015). Zinc can induce testosterone production in animals by the action mechanism of zinc as a second messenger (Yamasaki *et al.* 2007). Clamshells and fish thorns are the waste output of the marine industry. Clamshell has potency as a natural aromatase blocker, while fishbone contains a high protein. The administration of clamshell increases testosterone in white rats (Astuti *et al.* 2019), testosterone and vocal frequency in canaries (Astuti *et al.* 2020), and testosterone and vocal frequency in *Pelung* chicken (Yuneldi *et al.* 2021a). The aromatase blocker property of clamshell arises from its high Zinc content (Astuti *et al.* 2019). Milkfish thorns contain 27.88% protein (Wulandari and Kusumasari 2019), calcium, phosphorus, and zinc, which inhibit aromatase activity (Astuti *et al.* 2019; Charlier *et al.* 2015).

Clamshell and fishbone may become natural materials to boost Bangkok roosters' testosterone and muscle performance. Therefore, this research aimed to evaluate the potential of clamshell and milkfish thorn combination towards testosterone level and muscle performance in Bangkok roosters. The testosterone activity in this research comprises serum and muscle testosterone levels. This study evaluates hardness, springiness, and tenderness to understand muscle performance. Moreover, PCNA activity, myofiber population, and area are also assessed to understand the underlying basis of muscle performance alterations. This present study revealed that 6.6 g/day clamshell and 3.3 g/day fishbone treatments enhanced testosterone levels and muscle performance in Bangkok roosters. These materials may be effective for recovering muscle wasting and improving livestock production. Bangkok rooster may have excellent muscle performance that originates from androgen activity.

2. Materials and Methods

2.1. Animal

This study used 18 three-month-old Bangkok roosters. The roosters originated from an ornamental chicken trader in Bantul Regency, Yogyakarta Special Region, Indonesia. Moreover, the maintenance and treatment took place in a Bangkok rooster private breeder in Bantul Regency, Yogyakarta Special Region, Indonesia, from February to March 2022. The animal housings were individual-stage wood cages with slit bases (50 × 50 × 50 cm). All procedures in this research had approval from the Ethics Committee of the Integrated Testing and Research Laboratory Universitas Gadjah Mada (LPPT UGM) with certificate number 00009/04/LPPT/III/2021.

The study design in this research was Randomized Control Trial (RCT) method. The Bangkok roosters (n = 18) underwent 35 days of treatment. Each treatment group consisted of 6 replications. The treatment groups were P0: control; P1: milkfish thorns 3.3 g/day + clamshell 6.6 g/day, and P2: milkfish thorns 3.3 g/day. The thorns originated from brackish water milkfish (*Chanos chanos*). Atomic absorption spectrometry (AAS) showed 35.75% protein content in the milkfish thorns. According to a previous study, the clamshell preserved 61.55 mg/kg of Zn (Astuti *et al.* 2019). Chicken feed and water supplies for roosters were ad libitum.

2.2. Texture Profile Analysis of Muscle

Muscle tenderness (kg/cm^2) was estimated using Warner-Bratzler (WB) device. Muscle hardness (N) and springiness index were measured using Texture Analyzer TA1 (AMETEK Lloyd instrument Ltd., Fareham, UK). The tenderness analysis proceeded by testing a 1.5 × 0.67 cm meat block taken parallel to the muscle fiber. The scale changes in the WB device indicated the tenderness score. The hardness

and springiness analysis required a meat block at a minimum size of 1 × 1 × 1 cm. The operation of the Texture Analyzer and obtained data analysis employing NexygenPlus software (AMETEK Lloyd Instruments Ltd.).

2.3. Serum Testosterone

The serum testosterone analysis applied an Enzyme-linked immunosorbent assay (ELISA) according to the manual (Calbiotech Testosterone ELISA TE373S). The sera were sampled every seven days on days 0, 7, 14, 21, 28, and 35, then stored at -20°C until the analysis.

2.4. Muscle Testosterone

The muscle testosterone analysis also applied ELISA (Calbiotech Testosterone ELISA TE373S). Before the assay, 100 mg squeezed breast muscle was homogenized in 1 ml PBS. After 24 hours of frozen incubation, the mixture was thawed into liquid at room temperature (RT) and refroze for about 4-5 hours until solid. Cold centrifugation at 5,000 rpm at 2-8°C proceeded following the second thawing. The supernatant was extracted and stored at -20°C until the assay.

2.5. Hematoxylin-Eosin Staining

Hematoxylin-eosin (HE) staining aimed to calculate myofiber area and population (Saragih *et al.* 2019). A 1 × 1 cm pectoralis muscle sample fixation with 10% neutral buffer saline (NBF) initiated HE staining. Specimen processing employed the paraffin method. Finally, the histological documentation used a light microscope (Leica, Germany) with a digital camera system. The myofiber number and area calculation employed ImageJ 1.53k (National Institute of Health, USA) software with the documented histological images.

2.6. Immunohistochemical Staining

Immunohistochemistry estimated cell proliferation using proliferating cell nuclear antigen (PCNA) reactivity (Yuneldi 2022). The tissue specimens were fixed with 10% NBF and then embedded with paraffin. Afterward, tissue sections were cut transversely at 5 micrometers width (Leica RM 265, Leica Microsystems, Germany). Xylene and rehydration were used in tissue dewaxing. Mouse monoclonal anti-chicken PCNA primary antibody (Abcam) was used for PCNA detection, based on the manual. The secondary antibody was polyclonal goat anti-mouse IgG (DAKO). The sections were incubated with primary antibody for 1 hour at RT and followed by 3,3'-diaminobenzidine (DAB) + substrate-chromogen (DAKO kit) incubation. The proliferative cells would present brown-colored PCNA. Subsequently, the PCNA positives in each fasciculus were counted. PCNA immunoreactivity was transformed into a percentage by calculating the number of PCNA positives divided by 100 and then multiplied by 100%. Immunohistochemistry documentation used a Leica microscope with a digital camera system. Calculations were performed on a cross-sectional image of the pectoralis muscle tissue.

2.7. Statistical Analysis

This study applied one-way ANOVA at a 95% confidence level ($\alpha = 0.05$) with SPSS software version 16.0. Then, the Duncan test was performed following ANOVA.

3. Results

3.1. Serum Testosterone Level

Clamshell (6.6 g/bird) and brackish milkfish thorns (3.3 g/bird) powder for 35 days caused the highest serum testosterone in the rooster (P1). ELISA showed a significant difference ($p<0.05$) in serum testosterone on day 28 and day 35 in P1 compared to P2 and P0 (Table 1). The testosterone levels of P2 remained the highest among other groups on days 0, 7, and 21 ($p<0.05$). The trend showed that the highest testosterone levels shifted to P1 at the end of the experiment.

Table. 1. Serum testosterone level of Bangkok rooster after treatment

P	Mean ± SD level Testosterone (ng/ml) day -					
	0	7	14	21	28	35
P0	0.06±0.04^{b}	0.10±0.03^{b}	0.02±0.01^{b}	0.10±0.02^{b}	0.11±0.05^{b}	0.13±0.04^{b}
P1	0.04±0.01^{b}	0.04±0.01^{b}	0.14±0.07^{a}	0.06±0.03^{b}	0.48±0.14^{a}	0.44±0.09^{a}
P2	0.12±0.03^{a}	0.28±0.16^{a}	0.12±0.08^{a}	0.21±0.03^{a}	0.14±0.11^{b}	0.23±0.15^{b}

^{a,b} Different letters in the same column show significant differences ($p<0.05$). P0: control group, P1: fishbone 3.3 g + clamshell 6.6 g treatment group, P2: fishbone 3.3 g treatment group. SD: deviation standard

3.2. Muscle Testosterone Level

The administration of clamshell (6.6 g/bird) and brackish milkfish thorns (3.3 g/bird) powder (P1) for 35 days produced higher muscle testosterone compared to controls. There was a significant difference ($p<0.05$) between P1 and P0 in muscle testosterone (Table 2). However, muscle testosterone levels in P2 were not significantly different from P1 and P0.

3.3. Texture Analysis (Springiness Index, Tenderness, and Hardness) and Myofiber (PCNA+, Number, and Area) of the Pectoralis Muscle

The study results showed that the group treated with clamshell (6.6 g/bird) and milkfish thorns (3.3 g/bird) powder (P1) gained the highest springiness index of pectoralis muscles (Table 3). The texture analysis revealed that P1 had the highest springiness index ($p<0.05$) among others. Although, hardness and tenderness profiles showed no significant difference among groups ($p>0.05$).

Table. 2. Testosterone level of *Bangkok* chicken pectoralis muscle after treatment

P	Mean ± SD
	Pectorales muscle testoterone
P0	1.46±0.10[b] ng/100 mg
P1	1.61±0.08[a] ng/100 mg
P2	1.55±0.10[ab] ng/100 mg

[a,b] Different letters in the same column show significant differences ($p<0.05$). P0: control group, P1: fishbone 3.3 g + clamshell 6.6 g treatment group, P2: fishbone 3.3 g treatment group. SD: deviation standard

Histological observations illustrated the microscopic alterations in the muscle. Table 4 exhibited the pectoralis muscle myofiber profile in the Bangkok rooster. Moreover, Figure 1 demonstrated the PCNA immunoreactivity in the myofiber. The calculation of myofiber number and area employed HE observation on muscle tissue. Pectoralis muscle histology showed that the P1 group had the greatest quantity and area of myofiber ($p<0.05$). Afterward, the calculation of PCNA activity in the myofiber applied the immunohistochemical method. Groups P1 and P2 showed an increase in the percentage of PCNA, with P1 showing the highest activity. ANOVA analysis proved significant differences between treatment groups ($p<0.05$).

4. Discussion

4.1. Aromatase Blocker in Clamshell Improves Testosterone Levels

Aromatase is an essential enzyme that regulates estrogen synthesis (Astuti *et al.* 2019). The role of Zn as an aromatase blocker can maintain high testosterone levels (Fallah *et al.* 2018; Sankako *et al.* 2012). Aromatase blockers can block aromatase action from converting testosterone into estradiol (Astuti *et al.* 2019; Santen *et al.* 2009; Yuneldi *et al.* 2021a). The absence of aromatase expression indicates inhibition of testosterone conversion in the seminal tubules (Astuti *et al.* 2019).

The Zn aromatase blocker in *Anadara granosa* shell powder (0.036 mg/40 g BW) can increase testicular

Table 3. Springiness index, hardness, and tenderness value of *Bangkok* chicken chest muscle after 35 days of treatment

P	Parameter, Mean ± SD		
	Springiness index	Hardness	Tenderness
P0	0.52±0.01[b]	177.44±3.68[a] N	3.37±0.04[a] kg/cm^2
P1	0.60±0.04[a]	163.54±35.78[a] N	3.30±0.09[a] kg/cm^2
P2	0.52±0.02[b]	130.60±42.76[a] N	3.36±0.07[a] kg/cm^2

[a,b] Different letters in the same column show significant differences ($p<0.05$). P0: control group, P1: fishbone 3.3 g + clamshell 6.6 g treatment group, P2: fishbone 3.3 g treatment group. SD: deviation standard

Table 4. The number and area of myofiber and PCNA immunoreactivity in Bangkok chicken pectoralis muscle after 35 days of treatment

P	Parameter, Mean ± SD		
	Myofiber area	Number of myofibers	PCNA
P0	4275.99±108.57[c] µm^2	100.67±7.09[c]	6.50±0.84[c]%
P1	6234.72±654.08[a] µm^2	166.33±11.27[a]	14.50±2.07[a]%
P2	5093.55±658.24[b] µm^2	125.83±10.42[b]	10.67±4.18[b]%

[a,b,c] Different letters in the same column show significant differences ($p<0.05$). P0: control group, P1: fishbone 3.3 g + clamshell 6.6 g treatment group, P2: fishbone 3.3 g treatment group. SD: deviation standard

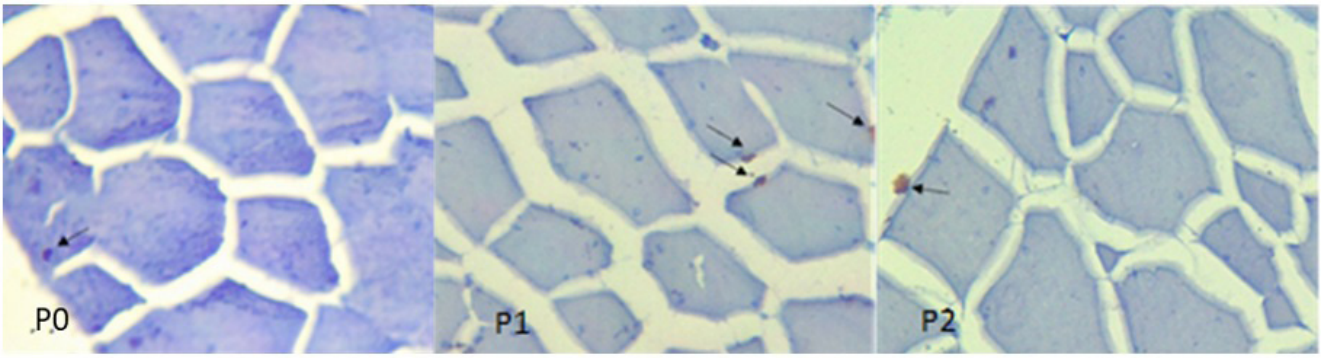

Figure 1. Immunohistochemistry of pectoralis muscle in Bangkok rooster from the different treatment groups. P1 groups showed considerable and clear positive PCNA, while the P2 group showed lesser and, lastly, faint and very low PCNA in the P0 group. P0: control group, P1: fishbone 3.3 g + clamshell 6.6 g treatment group, P2: fishbone 3.3 g treatment group. SD: deviation standard

weight at week 5th and testosterone levels in male layer chicken serum at week fourth (Yuneldi *et al.* 2021b). These reports agree with 28 and 35 days of fishbone 3.3 g/day + clamshell 6.6 g/day treatment that shows the topmost serum testosterone levels in P1. *Pelung* rooster shows blood testosterone levels at 0.13 ng/ml and an increase up to 1.42 ng/ml after 56 days of clamshell powder treatment (0.9 mg/kg BW) (Yuneldi *et al.* 2021a). Aging also influences the blood testosterone levels in roosters. Cockerels have serum testosterone less than 0.5 ng/ml before 12 weeks old and begin to rise more than 1.5 ng/ml after 16 weeks old (Zawacka *et al.* 2017). The drop of serum testosterone in roosters occurs at 34-37 and 48-51 weeks old (McGary Brougher *et al.* 2005). Rooster breeders may have serum testosterone lower than 0.5 ng/ml at 48-51 weeks old (McGary Brougher *et al.* 2005). Estradiol takes part in the negative feedback of testosterone in the brain. Therefore, aromatase elevates brain estradiol to assist the negative feedback (Charlier *et al.* 2015). The blockage of aromatase decreases brain estradiol activity, resulting in weak negative testosterone feedback. Low negative feedback of testosterone maintains high testicular testosterone secretion into the circulation system. This mechanism may underlie the higher serum testosterone in P1 but lower serum testosterone in P2 after 28 and 35 days. P2 could not retain the high testosterone level because of the absence of an aromatase blocker that reduced its negative feedback.

The body's zinc state is closely associated with animal reproduction. High dietary zinc (110 mg/kg BW) increases blood testosterone levels in the male broiler (Jafari *et al.* 2021). Zn ions in the P1 group would be absorbed through the intestine and circulated throughout the body. Zinc translocation into the testes passes the ZRT- and IRT-like protein (ZIP) transporters on the cell membrane (Kambe *et al.* 2015). The intracellular Zn^{2+} activates casein kinase (CK2), which increases phosphorylation. Zinc becomes a second messenger that elevates intracellular phosphorylation (Yamasaki *et al.* 2007). Besides, some species of Zn transporter induce the release of Zn from intracellular stores (Kambe *et al.* 2015). Zn^{2+} ions will subsequently enter the mitochondria, then bind to cholesterol and P450scc to stimulate the biosynthesis of testosterone (Santos and Teixeira 2020; Zhang *et al.* 2018). Zinc stimulates the steroidogenic acute regulatory (StAR) protein in the Leydig cells to escalate testosterone (Zhang *et al.* 2018). Intracellular CK2 inhibits aromatase by phosphorylation and increases testosterone levels (Astuti *et al.* 2019; Charlier *et al.* 2015; Yuneldi *et al.* 2021a; Zhang *et al.* 2018).

Higher testosterone levels in serum affect the increase of testosterone levels in muscles. The mean blood testosterone level in roosters is 1.55 ng/ml during spring and 0.88 ng/ml during winter (Purohit *et al.* 1978). The blood testosterone level in an untreated rooster is 1.58 ng/ml, but 45 weeks of organic zinc (110 mg/kg BW) treatment increases the testosterone to 3.68 ng/ml (Jafari *et al.* 2021). Approximately 2% or 0.5-3.0% of circulating testosterone can freely enter the cells to affect cellular metabolism (Gharahdaghi *et al.* 2021). Gambel's white-crowned sparrows demonstrate that a slight increase in muscle testosterone coexists with a significant androgen elevation during the pre-migration period (Pradhan *et al.* 2019). However, the

data about muscle testosterone in Bangkok roosters are still lacking. The richness of zinc in clamshells inhibits aromatase activity in organisms (Astuti *et al.* 2019). Table 2 shows that the aromatase blocker in clamshell powder inhibited the muscle testosterone conversion into estradiol. Treatment of fish thorns and clamshells provides additional protein and minerals for animals (Bechtel *et al.* 2019; Wulandari and Kusumasari 2019). The protein richness in fishbones can stimulate testosterone synthesis and muscle growth (Hanai and Esashi 2012; Haun *et al.* 2018). The abundant protein in the fishbone supplies amino acids and protein needed to maintain the enzyme availability for testosterone biosynthesis and signaling. The dietary protein scarcity depletes 17β-hydroxysteroid dehydrogenase (17β-HSD) (Hanai and Esashi 2012). Aromatase inhibition can increase testosterone levels, which promotes muscle growth. Previous studies have reported that aromatase converts testosterone into estradiol in fat, testes, brain, bone, liver, blood vessels, and muscle tissues (Balthazart *et al.* 2005; Charlier *et al.* 2015). The ability of aromatase inhibitors to elevate several reproductive hormones enables it as a therapy for testosterone deficiency in males (de Ronde and de Jong 2011).

Local biosynthesis of testosterone can maintain muscle testosterone levels. Testosterone synthesis proceeds with serial hydrogenations of dehydroepiandrosterone (DHEA). These hydrogenations involve the 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD (Aizawa *et al.* 2008; Sato and Iemitsu 2015). Androgen steroids consist of androstenedione, testosterone, and dihydrotestosterone (DHT). The 5-alpha reductase proceeds testosterone-DHT transformation to amplify its effect in muscles (Hamdi and Mutungi 2011). Muscle 17β-HSD also transforms DHT into androstenedione to produce intramuscular testosterone (Swerdloff *et al.* 2017). Muscle aromatase eliminates testosterone by converting testosterone into estradiol (Schiffer *et al.* 2018). The highest muscle testosterone in P1 demonstrated the aromatase blocker effect that was absent in the other group (Table 2).

4.2. Testosterone Elevation and High Protein Consumption Enhance Muscle Performance

Muscle mass and strength in poultry depend on testosterone (Haun *et al.* 2018; Li *et al.* 2020). Testosterone binds with androgen receptors (AR) in the myonucleus and satellite cells. The testosterone-AR interaction stimulates the proliferation of active satellite cells in skeletal muscle, and transcription processes occur in the myonucleus (Josiak *et al.* 2014; Seale *et al.* 2000; Sinha-Hikim *et al.* 2004). Testosterone also associates with high physical activity through dopaminergic activity (Jardí *et al.* 2018). Increased physical activity in individuals can improve the population of myofiber IIa which supports rapid contraction and fatigue resistance (DeChick *et al.* 2020; Ferry *et al.* 2014; Gharahdaghi *et al.* 2021).

The protein content in milkfish thorns acts as a protein source, while aromatase blocker in clamshell boosts testosterone activity. A previous study shows that testosterone is an anabolic hormone for protein synthesis and maintenance of muscle growth stimulation (Krause Neto *et al.* 2017; Schiaffino *et al.* 2013). Broiler chicken muscle comprises approximately 75% water, 20-25% protein, and 0.86–3.18% intramuscular fat (Weng *et al.* 2022). The 5-alpha reductase enzyme increases amino acid absorptions through testosterone-dihydrotestosterone (DHT) conversion in muscle cells (Hamdi and Mutungi 2011). The absence of an aromatase blocker allows testosterone elimination for estradiol production. Therefore, the administration of milkfish thorns alone (P2) resulted in lower testosterone levels than a mixture of milkfish thorns and clamshell (P1) (Table 1 and 2). Consistent with higher testosterone levels, P1 showed the highest springiness index after 35 days of treatment (Table 3). This study showed that a combination of aromatase blockers in clamshell and protein in milkfish thorns (P1) was necessary to increase the springiness index.

Hardness, tenderness, and springiness are muscle strength characteristics. Springiness is essential for fast, frequent, and energy-efficient muscle contractions (Kraemer and Looney 2012; Reich *et al.* 2000; Rodríguez-Rosell *et al.* 2018). The population, composition, and structure of myofiber affect muscle hardness and tenderness (Jaturasitha *et al.* 2008; U-chupaj *et al.* 2017; Weng *et al.* 2022). The abundance and sturdy construction of myofiber increase the strength capacity of muscles. Abundant myofibers produce powerful strength. However, muscle contraction only occurs after the innervation of motor neurons. An elastic myofiber can result in

powerful, faster, and more efficient contractions. Good hardness, tenderness, and springiness muscle character can boost muscle performance during physical activity.

Springiness or elasticity is one of the muscle characteristics that determine muscle strength. Myofiber can thicken and form intramuscular spaces that increase muscle springiness (U-chupaj *et al.* 2017). Myofiber is a skeletal muscle contraction unit whose thickness will support muscle strength. The elasticity of myofibers and tendons accelerates the formation and repetition of muscle contractions (Kraemer and Looney 2012). Muscle springiness accelerates the rate of force development (RFD), which is the ability of the neuromuscular system to increase the contraction force quicker (Rodríguez-Rosell *et al.* 2018). The RFD variable is significant in animals and athletes with high skeletal muscle activity. High muscle elasticity also results in highly efficient energy contractions and faster relaxation (Kraemer and Looney 2012; Rodríguez-Rosell *et al.* 2018). The contraction cycle will be faster in muscles which an elastic or high springiness index.

4.3. Myogenic Cell Proliferation Increases Myofiber Number and Size

Muscle hardness and tenderness are related to the myofiber population in the muscle. High levels of hardness and tenderness are associated with the density, abundance, and composition of muscle myofibers in chicken (U-chupaj *et al.* 2017; Weng *et al.* 2022). Larger myofiber sizes are found in indigenous chicken muscles in Thailand with higher tenderness values (Jaturasitha *et al.* 2008). The number of myofibers determines the force capacity of muscle contraction. High collagen is also related to high muscle tenderness in chickens (Jaturasitha *et al.* 2008). The higher myofiber activation produces more powerful muscle contractions (Kraemer and Looney 2012). High hardness and tenderness indicate strength and the number of myofibers in the muscle.

Immunohistochemistry showed the effect of clamshells and fish thorns on proliferating cell nuclear antigen (PCNA) activity in muscle. The high PCNA activity occurred in P1 and P2, with the highest results in P1. This result indicated that a clamshell and fishbone combination treatment is more potent for cell proliferation than a sole fishbone treatment. PCNA proteins regulate deoxyribonucleic acid (DNA) replication and repair processes in cells (Tang *et al.* 2020). DNA clamp proteins, including PCNA, will bind and prevent DNA polymerase from being separated from the DNA strand (Maga and Hubscher 2003). The presence of PCNA in the nucleus is essential for DNA replication. Nevertheless, PCNA is also found in the cytoplasm and performs various functions (Tang *et al.* 2020).

Elevation in PCNA of muscle in groups P1 and P2 indicates high DNA replication activity. Frequent DNA replication occurs in proliferating cells to produce identical cells. Since PCNA is an essential protein in DNA replication, PCNA activity becomes a cell proliferation marker (Johnson and Allen 1993; Piestun *et al.* 2017; Tang *et al.* 2020). In addition, elevation in PCNA immunoreactivity of muscle tissue showed efficacy after treatment on cell proliferation. High PCNA activity in broiler chicken muscles aged 13 days showed high muscle cell proliferation (Piestun *et al.* 2017). The higher PCNA immunoreactivity of the P1 group than P2 (Tabel 4) indicated that the combination of clamshells and fish thorns stimulated cell proliferation better than only fish thorns. PCNA also influences cell differentiation. Osteoclast cell differentiation is an example of cytoplasmic PCNA activity in cell differentiation (Tang *et al.* 2020). The translocation of nuclear PCNA into cytoplasm enables its binding to actin to support the process of osteoclast differentiation. Myogenic cells such as myoblasts and satellite cells must differentiate and fuse to become functional myofibers (Geiger *et al.* 2018; Seale *et al.* 2000). Actin also performs a fundamental function in muscle contraction and is one of the cytoskeletons of myofibers. This evidence suggested that aromatase blockers and high protein promoted muscle proliferation.

PCNA immunoreactivity also confirmed the multiplication and expansion of myofibers in the treatment groups. The increase of the myofiber population in P1 and P2 (Table 4) with high localization of myofiber PCNAs indicated the proliferation of myogenic cells to produce functional myofibers. High PCNA immunoreactivity coexisted with large and numerous myofibers in P1. Myofibers rise from the myoblasts and satellite cells proliferation, which subsequently underwent differentiation (Seale *et al.* 2000). The following fusion of differentiated myogenic cells forms a novel myofiber or thickens the existing myofiber. The increase in springiness in the P1 group showed functional myofiber improvement that supported muscle contraction. PCNA activity initiates the proliferation of myogenic cells, which then differentiate to form functional myofibers.

Aromatase blockers and additional protein from clamshells and fish thorns increase testosterone, which increases skeletal muscle performance. Several studies reveal that high protein intake excels muscle performance. Dietary protein and carbohydrate enhancement with Spirogyra jaoensis enlarge the cross-sectional area and the number of myofibers in broilers (Saragih *et al.* 2019). Feeding with high energy and protein levels increases PCNA activity in the pectoralis muscle of super Kampung chicken (Puspita *et al.* 2016). Protein can stimulate poultry muscle performance, but androgens mediate circulating amino acid absorption to muscle and trigger proliferation (Hamdi and Mutungi 2011; Schiaffino *et al.* 2013). Testosterone can cause androgenic effects through DNA-binding dependent pathways that affect DNA replication and transcription in the cells (Davey and Grossmann 2016). The non-DNA-binding dependent effect of testosterone can also occur through a signal transduction system that involves second messengers (Davey and Grossmann 2016). An increase in the number and area of myofibers in Bangkok roosters increased muscle springiness. Elastic muscles can accelerate and strengthen the resulting contractions.

This study concluded that clamshells and fish thorns boosted testosterone levels and muscle performance in Bangkok roosters. Aromatase blocker increases systemic and muscle testosterone levels. High testosterone and additional protein can subsequently improve muscle performance as the elevation of springiness, total number and area of myofibers, and PCNA immunoreactivity are present. These findings suggested that clamshells and fish thorns might be valuable materials for myopathic treatment and meat production.

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