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Effect of Protease Combination with Vitamin C Supplementation on Growth Performance and Sperm Quality in Noi Crossbred Cockerels at 24-36 Weeks Old

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Abstract: Semen quality is one of the key factors leading to the success of artificial insemination, however, it is rich in long-chain polyunsaturated fatty acids which are involved in the proliferation of reactive oxygen species and lipid peroxidation in sperm, which causes sperm destruction by lipid peroxidation and can lead to reduced fertility with reduced antioxidant capacity. Some suggested the use of dietary additives to enhance antioxidant capacity to improve sperm quality and fertility in roosters. Therefore, the study was aimed to evaluate the effect of vitamin C (vitC) supplementation in combination with protease on growth performance and sperm quality in Noi crossbred cockerels from 24 to 36 weeks of age. The experiment was conducted with a total of 36 Noi crossbred cockerels with initial bodyweight (BW) 1500 - 2150 ± 167.5g/bird, arranged in a completely randomized design with 4 treatments, respectively. The treatments were Control included a basal diet (KPCS); C75Pro included KPCS + (125mg Protease +75mg vitC)/kg of feed; C125Pro included KPCS + (125mg Protease + 125mg vitC)/kg of feed; and C250Pro included KPCS + (125mg Protease + 250mg vitC)/kg of feed. Each treatment was repeated 9 times. Each repetition was 1 cockerel. The results showed that the BW of 32 and 36 week old cockerels had a statistically significant difference among treatments ($P < 0.05$), the highest BW at 32 weeks was C125Pro (2278g) and at 36 weeks was C250Pro (2500g) compared to control; Feed conversion coefficient from 24 to 36 weeks of age had a statistically significant difference among treatments ($P < 0.05$), the most effective in C250Pro (1.46). Regarding the training time, there was no statistically significant difference among the treatments ($P > 0.05$), but in C250Pro, the training time and the non-response rate were better than the rest of the treatments. Regarding semen pH, sperm activity, survival rate, morphological ratio and concentration, there were statistically significant differences among treatments ($P < 0.05$), with the best sperm quality in C250Pro compared with other treatments.

Keywords: Noi Crossbred Cockerel, pH, Sperm Survival Rate, Sperm Concentration, Vitamin C, Protease

1. Introduction

Noi cock, also known as fighting cock, is a domestic breed of fighting cock in Vietnam that is raised for cockfights. They have a strong temperament, a majestic and majestic appearance, high fighting ability and dangerous and beautiful shots and is one of the typical chicken breeds of Mekong Delta, Vietnam. Besides advantages such as good resistance, few diseases, short raising time bringing high economic efficiency to farmers, this chicken breed has low growth rate and reproductive productivity, is one of the cause of difficulties in the enlarge of this breed. The conservation of genetic resources in particular poultry breeds is essential [1]. Currently, artificial insemination (AI) is one of the most optimal methods for genetic conservation because of its low cost and high profit. AI can overcome low fertility in chickens, due to large size cockerels, unable to complete the natural mating process. The key to success in using AI is the

quality of semen [2], since semen from a single breeding cock can be sown to many hens. However, the chicken semen contains high concentrations of polyunsaturated fatty acids (PUFAs) that have been implicated in increased reactive oxygen species (ROS) proliferation and lipid peroxidation in sperm [3], which is responsible for sperm destruction by lipid peroxidation and can lead to reduced fertility with reduced antioxidant capacity [4]. Normally, spermatozoa are protected from ROS and lipid peroxidation by antioxidants and enzymes present in the internal plasma [5].

Vitamin C plays an important role in reproductive performance, especially in improving semen quality in poultry species, but their bodies do not synthesize enough vitamin C to meet the body's needs. Nowaczewski and Kontecka [6] documented that vitamin C contributes 65% of the antioxidant capacity of the seminal plasma. McDaniel reported that supplementation of vitamin C improved semen quality and fertility in male broiler breeders [7]. Dobrescu [8] indicated that supplementation of up to 200 mg ascorbic acid per kg diet in the diets of breeder turkey toms improved semen volume and concentration.

Nowadays, many enzyme preparations added to animal feed have been widely used, including protease enzymes, which can help livestock improve protein digestibility in feed [9, 10]. As reported by Lopez-Otín & Bond [11], protease also known as peptidase or proteinase, an enzyme that catalyzes the hydrolysis of peptide bonds in proteins and converts them into amino acids, which are then uptake and use by cells. According to Nir [12], poultry has the ability to hydrolyze protein, but a significant amount of protein in the diet is not fully digested and excreted [13, 9] Therefore, the addition of enzymes to the diet is considered an effective measure to overcome the above problem. Indeed, incorporation of protease enzyme in poultry diets has been reported to increase nutrient utilization [14, 9]. The study results of Fru-Nji [15] showed that the addition of protease to the diet improved the protein and lipid digestibility in chickens. Saleh [16] reported that adding 200-300 mg of protease/kg feed increased growth performance in Egyptian chickens raised in open house at 0-9 weeks of age. However, Fang [17] reported that the addition of 500 mg protease/kg feed had an effect on improving feed efficiency of Cobb broilers reared in closed house. Similar results were found in Ri broiler chickens at 1-12 weeks of age [18]. However, our research's unpublished study on Noi crossbred chickens with open house condition showed that supplemention of 250 mg protease/kg feed improved their BW, FCR and ADG at 28-84 days of age. Little is known about the effect of protease and the combination of vitamin C and protease on semen quality of Noi cocks.

With such benefits, the combination of vitamin C and protease is expected to significantly improve growth performance and sperm quality for cockerels, so the topic "Effect of protease combination with vitamin C supplementation on growth performance and sperm quality in Noi crossbred cockerels at 24-36 weeks old" was performed.

2. Materials and Methods

2.1. Materials

Chicken sperm were collected from 36 crossbred cockerels from 24 to 36 weeks of age. The cockerels were fully vaccinated against diseases according to the local veterinary vaccination guidelines, dewormed before starting experiment.

The basal diet in pellet form had a ME value of 3050 kcal/kg and a CP of 19%, Ca and P were 1.15% and 0.85%, respectively. The care and experimental use of animals were approved by the Animal Ethics Committee of Can Tho University (CTU-AEC).

Vitamin C powder with milky white color, odorless, tasteless. Enzyme protease is fine powder, light brown in color, fragrant. Trade name is JEFO PROTEASE®, from JEFO Canada. The semen extender, crystalline solution, is a white, crystalline powder with a net weight of 6.9 g with the medium containing the components NaCl, $MnSO_4$, $NaHCO_3$, KCl, $CaCl_2$, Na_2HPO_4 and Glucose.

2.2. Methods

2.2.1. Experimental Design

The experiment was conducted with a total of 36 Noi crossbred cockerels at 24 - 36 weeks of age, arranged in a completely randomized design with 3 treatments and control, repeated 9 times, corresponding to 4 different diets was as follows:

Control: Basal diet (KPCS);

C75Pro: KPCS + (125mg Protease + 75mg vitamin C)/kg feed;

C125Pro: KPCS + (125mg Protease + 125mg vitamin C)/kg feed;

C250Pro: KPCS + (125mg Protease + 250mg vitamin C)/kg feed.

Each replicate was 1 cockerel with initial BW 1500 - 2150±167.5 g/head.

2.2.2. Housing, Animal Care and Data Collection

Experimental chickens were raised in an open, airy cage system with a corrugated iron roof, covered with tarpaulin and a ventilation system on the roof of the house. Each cockerel was kept in each individual cage with the size of each floor plot being 60 × 40 × 40cm. Automatic nipple drinker system, and was supplied with clean water continuously from the water supply factory. Feed was provided 2 times/day at 7 am and 14 pm, corresponding to a feed amount of 60% and 40%, respectively. Vitamin C and protease were mixed directly into KPCS. Feed intake and leftovers were recorded daily, and drinking water was freely provided. Feeders and drinkers were cleaned 2 times a week.

2.2.3. Collecting Semen from Cockerels and Recorded Parameters

Semen in cockerels were collected by abdominal massage method of Dao Duc Tha [19] and Peters [20]. Place the chicken breast on the right leg of the person holding the

chicken so that the body of the chicken is parallel to the body of the person taking the semen, with the other right hand holding the chicken's tail, shaking it slightly to stimulate the cock's excitement, avoiding the movement of the cockerel. Then, the second person used his left hand to hold the eppendorf tube to collect the chicken's semen, and at the same time, the right hand gently massages from the back to the tail of the chicken for about 30 seconds to 1 minute until mucus appears in the anus of the chicken, this is a sign of the beginning of ejaculation in chickens, using the eppendorf tube to collect the ejaculated sperm. Recorded training time for semen collection in cockerels.

Temperature (°C) and humidity (%) were measured daily at 7 am and 14 pm using a hygrometer (EXTECH® 45170, Taiwan). Feed consumption was determined by calculating the difference between feed intake and feed left over the next morning. Body weight (BW) of chickens was weighed at the start and end of the experiment.

The parameters of semen quantity and sperm quality were recorded.

Semen volume (ml) was measured measured using an eppendorf tube with volumetric divisions spaced 0.05 ml apart. Semen weight (g) was weighed before and after semen collection.

The color of semen was assessed sensory with 3 different colors such as clear white, milky white and creamy white.

The pH value was measured directly with a WINLAB pH/Ion meter (Japan), each sample was measured 3 times then the average value of 3 measurements was taken.

Sperm concentration was determined by the method of direct cell count using an improved Neubauer hemocytometer.

The viability of spermatozoa was evaluated by using an eosin-nigrosin staining technique [3]. Taking 5 µL drop of semen of each group was mixed with 20 µL of eosin/nigrosin solution. The prepared semen samples were smeared on microscope slides and fixed by air-drying at room temperature for 10 min before observation. Based on the color of the sperm after staining to distinguish, the surviving sperm remained unstained and the completely or partially dead cells were pink to red/brown in color. Sperm viability was assessed in the five micro-fields examined. Each slide was evaluated twice.

2.2.4. Statistical Analysis

Data were recorded and preliminarily processed using Microsoft Excel 2019 software, statistically analyzed by Minitab version 16 with GLM ANOVA model, then compared each pair when the difference was statistically significant by Tukey's test with 95% confidence intervals. Pearson correlation between parameters of sperm quality and BW of the experiment of Noi crossbred cockerels was analyzed. Temperature (°C) and humidity (%) of experimental farm were analyzed by descriptive statistics and training time to collect cockerel semen was analyzed by Chi-square.

3. Results and Discussion

3.1. Temperature (°C) and Humidity (%) of the Experimental House

The results of Table 1 showed that the lowest temperature and humidity in the experimental house were 25.2°C and 74.07% in the morning, and 26.6°C and 54.40% in the afternoon. Meanwhile, the highest temperature and humidity were 31.9°C and 88.37% in the morning and 35.9°C and 77.33% in the afternoon. High temperature and humidity cause heat stress in chickens, which in turn affects spermatogenesis [21]. The results of McDaniel [22] showed that males kept at 32°C had a 42% decrease in spermatogenesis compared with those kept at 21°C. From the above conclusions of the authors, it is found that the temperature recorded in this experiment was not the ideal condition for Noi crossbred cockerels to achieve the best sperm quality.

Table 1. *Temperature (°C) and humidity (%) of the experimental farm.*

Parameters	Maximum		Minimum		Average	
	am	pm	am	pm	am	pm
Humidity, %	88.37	77.33	74.07	54.40	83.60	65.52
Temperature, °C	31.87	35.87	25.23	26.57	28.37	32.01

3.2. Feed Intake (FI), Body Weight (BW) and Feed Conversion Ratio (FCR) of Noi Crossbred Cockerels at 24-36 Weeks of Age

The results of Table 2 showed that BW of chickens at 32 and 36 weeks of age had a statistically significant difference among treatments ($P < 0.05$), in which the highest BW was on C125Pro at 32 weeks of age and C250Pro at 36 weeks of age compared with the control. In contrast, FCR was lowest in C250Pro and C75Pro compared with the control ($P < 0.05$). No statistically significant differences were found among treatments in terms of ADG and FI ($P > 0.05$). The improvement in BW and FCR of the experimental chickens could be attributed to the effectiveness of protease enzymes in increasing nutrient utilization. This is consistent with the conclusion of Angel [23] and Fru-Nji [16] the addition of beneficial exogenous enzyme preparations such as protease enzymes in poultry diets reduces feed costs and improves intestinal physiology because it increases crude protein digestibility (CP) and metabolizable energy (ME) in diets with low CP, thereby improving the BW of chickens. The results of Freitas [24] showed that the addition of protease to the diet improved protein and lipid digestibility in chickens. Some other authors have suggested that the use of exogenous proteases can help animals improve the digestibility of protein in the feed [9, 10].

Table 2. *Noi crossbred cockerels body weight, AWG and FI at 24 - 36 weeks of age.*

Parameters	Treatments				SEM	P
	Control	C_{75}Pro	C_{125}Pro	C_{250}Pro		
BW24wks,g	1850	1839	1917	1906	57.16	0.70
BW32wks,g	2033^{b}	2189^{ab}	2278^{a}	2252^{ab}	60.06	0.03
BW36wks,g	2228^{b}	2361^{ab}	2456^{ab}	2500^{a}	62.68	0.02
AWG24-32, g	183.3	350.0	361.1	346.7	55.61	0.09
AWG33-36, g	194.4	172.2	177.8	247.8	28.12	0.23
AWG24-36, g	377.8	522.2	538.9	594.4	58.55	0.07
FI24- 22, g/b/d	71.60	70.10	73.79	66.19	3.066	0.36
FI33-36, g/b/d	69.31	63.44	63.96	64.64	4.242	0.75
FI24-36, g/b/d	71.19	67.88	70.75	65.67	3.109	0.56
FCR24-32	3.62	2.33	2.03	2.16	0.549	0.17
FCR33-36	1.56	1.85	1.64	1.19	0.273	0.39
FCR24-36	2.48^{a}	1.81^{b}	1.70^{ab}	1.46^{b}	0.253	0.04

BW: body weight; AWG: average weight gain; FI: feed intake; FCR: feed conversion ratio; Control: KPCS; C75Pro: KPCS + (75mg vitamin C + 125mg Protease)/1kg feed; C125Pro: KPCS + (125mg vitamin C + 125mg Protease)/1kg feed; C250Pro: KPCS + (250mg vitamin C + 125mg Protease)/1kg feed. Mean values with different letters in the same row were significantly different at the level P<0.05.

3.3. Training Time to Collect the Cockerel's Semen

The results in Table 3 showed that the percentage of cockerels with ejaculatory reflexes after 1 week of training was 38.89% with a massage time of 60 - 120 seconds, 22.22% after 2 weeks of training, and after 3 weeks of training reached 25% with massage time reduced to 30 - 120 seconds. Total number of cockerels with ejaculatory reflex reached 86.11% and up to 13.89% of the cockerels had no ejaculatory reflex, 28.78% of the roosters ejaculated erratically among the total number of cocks with ejaculatory reflex. The results showed that training time did not affect the ejaculatory reflex of cockerels among treatments (P > 0.05), although C250Pro and C75Pro still gave better ejaculatory reflex than other treatments. Total number of cocks with ejaculatory reflex reached 86.11%. Results in Dong Tao chickens when using their abdomental massage with a stimulation time of 60-120 seconds showed that the total reflexes of chickens were 85.48%, non-reflexes were 14.52% and erratic reflexes were 15.09% [25].

Bakst & Dymond [26] reported that up to 90-98% of cocks have ejaculatory reflexes when using their abdomental massage with a stimulation time of 60-120 seconds. These authors recommended that the duration of massage should not be prolonged, as it was easy to lose the ejaculatory reflex in cocks.

Table 3. *Reflexes of the experimental cockerels during training.*

Parameters	Cockerels (bird)	Percentage (%)	Treatments				P	Stimualation time (s)
			ĐC	C75Pro	C125Pro	C250Pro		
Total bird	36	100						
Reflexes								60 - 120
after 1week	14	38.89	2	5	2	5		60 - 120
after 2 week	8	22.22	3	1	2	2	0.198	60 -120
after 3 week	9	25.00	2	2	3	2		30 - 120
No reflex	5	13.89	2	1	2	0		
Abnormal reflexes	11	28.78	4	3	2	2		

Control: KPCS; C75Pro: KPCS + (75mg vitamin C + 125mg Protease)/1kg feed; C125Pro: KPCS + (125mg vitamin C + 125mg Protease)/1kg feed; C250Pro: KPCS + (250mg vitamin C + 125mg Protease)/1kg feed

3.4. Quantity and Quality Traits of Semen of Noi Crossbred Cockerels

The results in Table 4 showed that there was no statistically significant difference in semen volume and semen weight among treatments (P > 0.05), but could be clearly observed after protease addition, specifically on C125Pro and C250Pro, both had higher semen volume (0.3 ml) and weight (0.29 - 0.31g) than the control group. This result is still lower than that of Dong Tao chicken with 0.48 ml [27] and AA chicken with 0.44-0.57 ml [28]. The lower results may be due to the difference in breeds of chickens.

The results in Table 4 also showed that the semen pH, activity, survival rate, morphological rate and concentration of sperm had statistically significant differences among treatments (P<0.05), in which C250Pro gave the best results and the supplemented treatments were better than the control. However, the results of semen pH in this experimental cockerel were lower than that of domestic chickens at 7 - 7.5; sperm motility in control was 98.79% compared to 99.48% in C250Pro; sperm survival rate in control was 98.84% versus 99.56% in C250Pro; the highest morphological rate in control was 0.21% and the lowest in C250Pro was 0.09%; the lowest sperm concentration was found in control (1.13×10^9 sperm/ml) and the highest in C250Pro was 1.542×10^9 sperm/ml. The present result was that the concentration of 1.13×10^9 - 1.54×10^9 sperm/ml was higher than that of AA chickens with a concentration of 0.77 - 0.83 $\times 10^9$ sperm/ml [28]; of Ho chicken 0.95×10^9 sperm/ml [29]. However, it was lower than some other breeds such as Dong Tao chicken with 2.57×10^9 sperm/ml [29] and 2.09×10^9 sperm/ml in Ri chicken. The results of this experiment showed that the addition of protease combined with vitamin C in the diet of Noi cockerels increased semen quality. It concurred with McDaniel [7] that supplementation of vitamin C improved semen quality in male broiler breeders. This study was in agreement of Dobrescu [8] that supplementation of up to 200 mg ascorbic acid per kg diet in the diets of breeder turkey toms improved semen volume and concentration. Not many studies have addressed the role of protease or protease in combination

with vitamin C on sperm quality in roosters, but the results of this trial suggest a positive effect on sperm quality. The improvement in sperm quality characteristics of Noi chickens can be attributed to the fact that vitamin C acts as an antioxidant against peroxidation damage and is essential for maintaining sperm structure while protease helps increase the ability to absorb nutrients to nourish sperm, thus improving sperm quality.

Table 4. *Quantity and quality traits of semen of Noi crossbred cockerels.*

Parameters	Treatments ($\bar{X}$±SEM)				P
	Control	C_{75}Pro	C_{125}Pro	C_{250}Pro	
Semen weight, g	0.2088±0.03	0.2097±0.03	0.3000±0.03	0.2991±0.30	0.08
Semen volume, ml	0.1995±0.03	0.1942±0.03	0.3086±0.03	0.2878±0.03	0.09
pH	6.49^{b}±0.06	6.74^{a}±0.05	6.83^{a}±0.06	6.72^{a}±0.05	0.003
Motility, %	98.79^{b}±0.11	99.15^{ab}±0.10	99.36^{a}±0.11	99.48^{a}±0.10	0.001
Survival, %	98.84^{c}±0.09	99.21^{b}±0.09	99.41^{ab}±0.09	99.56^{a}±0.08	0.000
Abnormality, %	0.21^{a}±0.01	0.15^{ab}±0.01	0.11^{bc}±0.01	0.09^{c}±0.01	0.000
Concentration, × 10^{9}	1.132^{b}±0.10	1.400^{ab}±0.09	1.267^{ab}±0.10	1.542^{a}±0.09	0.038

Control: KPCS; C75Pro: KPCS + (75mg vitamin C + 125mg Protease)/1kg feed; C125Pro: KPCS + (125mg vitamin C + 125mg Protease)/1kg feed; C250Pro: KPCS + (250mg vitamin C + 125mg Protease)/1kg feed. Mean values with different letters in the same row were significantly different at the level P<0.05.

3.5. Pearson Correlation Between Parameters of Sperm Quality and BW of the Experiment of Noi Crossbred Cockerels

The results in Table 5 showed that there was a positive correlation between semen volume and cockerel weight at 32 weeks of age (0.362); between semen volume and BW at 32 weeks of age (0.358), with semen volume (0.981); between concentration and pH (0.509). The positive correlation between sperm motility and semen volume, semen weight and pH was 0.453, 0.449 and 0.560, respectively; between survival rate and pH and concentration was 0.618 and 0.422; between sperm survival rate and viability was 0.698. Finally, the negative correlation between sperm abnormality and pH, concentration, motility and survival was -0.493, -0.547, -0.612 and -0.600, respectively.

4. Conclusion

Supplementation of vitamin C combined with protease at C250Pro not only helped the Noi crossbred cockerels gain better weight, lower FCR, but also improved the time of semen collection training, the ability to adapt and limited the abnormal symptoms. Besides, C250Pro had the best motility rate, survival rate, morphological rate and concentration compared to other treatments.

It is recommended to use C250Pro in the cockerel's diet to improve growth, adaptability to the living environment and sperm quality.

Table 5. *Pearson correlation between chicken weight, semen quantity and sperm quality parameters in Noi crossbred cockerels.*

	BW24wks	BW32wks	Semen volume	Semen weight	pH	Concentration	Motility	Survival	Abnormality
BW24wks	1								
BW32wks	0,545**	1							
Semen volume	0,233	0,362*	1						
Semen weight	0,254	0,358*	0,981***	1					
pH	0,015	0,349	0,287	0,288	1				
Concentration	0,053	0,254	-0,146	-0,121	0,509*	1			
Motility	-0,135	0,218	0,453*	0.449*	0,560**	0,343	1		
Survival	-0,110	0,093	0,237	0,217	0,618***	0,422*	0,698***	1	
Abnormality	-0,074	-0,217	-0,218	-0,231	-0,493**	-0,547**	-0,612***	-0,600***	1

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