Poultry Science Journal
ISSN: 2345-6604 (Print), 2345-6566 (Online) http://psj.gau.ac.ir
DOI: 10.22069/psj.2019.16840.1471

Effect of Guanidino Acetic Acid Consumption and Management Methods on the Reproductive Potential of Elderly Cockerel Broiler Breeders

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Poultry Science Journal 2019, 7(2): 195-204

Keywords
Spiking
Fertility
Creatine
Intra-spiking
Elderly rooster

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Article history
Received: 29 June, 2019
Revised: 1 November, 2019
Accepted: 20 November, 2019

Abstract

This study conducted to determine the effect of guanidino acetic acid consumption and management methods on the enhancement of aged rooster's reproduction. A total of 486 broiler breeders (Ross 308) were used in a 3 × 3 factorial experiment from week 41 to 62. Three levels as 0, 1200, and 1600 (mg/kg) of guanidino acetic acid, as a precursor of creatine (CreAmino) in combination with three management methods (no-management, spiking, and, intra-spiking methods) were evaluated. Quantitative and qualitative characteristics of sperm were determined every other week. At the end of the experiment, three roosters from each treatment were slaughtered and their testicles were evaluated for morphological and histological characteristics. The volume of sperm was a significant reduction in CreAmino (1200 mg/kg) + spiking. Sperm progressive motility reduced in CreAmino (1200 mg/kg) + no-management and CreAmino (1600 mg/kg) + intra-spiking. Also, CreAmino (1200 mg/kg) +no-management, increased the weight of the testicles. CreAmino (1600 mg/kg) + spiking showed the highest number of spermatozoa. The results showed that using 1600 mg CreAmino in combination with management methods, spiking and intra-spiking, can reduce and delay the occurrence of signs of aging in roosters. So, with using this level of CreAmino it is expected fertility in broiler breeder flock improved.

Introduction

In late years, genetic studies on broiler chickens for selection of traits such as high growth rate and larger size of the body shows there is a negative correlation between reproductive traits and increasing yield. So that's in the roosters, physiological disorders like decreased libido, decreased mating numbers and reduced sperm production have increased (Dawkins and Layton, 2012). In addition to the strong negative genetic correlation between productive and reproductive traits, the management of high-yielding birds is also more difficult. Often management operations are not produced consistent with genetic advancements. Hence, to reach the most productive capacity of birds, a steady and coordinated management program is needed (Arnould and Leterrier, 2007).

The effect of cock in herd fertility depends on the two chief factors: mating activity and sperm quality. As the rooster age increases, the semen volume and number of spermatozoa per ejaculation, and also the fertility ability of spermatozoa decreases (Mangiagalli *et al.,* 2010). The physical force of a fertile cock depends on the powerful muscle and the amount of available and sufficient energy in these muscles. As age increases, the rooster's physical capacity decreases to display mating behavior. The main reason for which is muscle weakness and lack of energy storage in these weak muscles. creatine is a constituent of animal tissue that plays an important role in the metabolism of energy in cells, especially muscle cells. Approximately 65-75% of the daily body needs of creatine for the body is obtained by re-synthesis of guanidino acetic acid, the rest of the body needs to be provided by the food (Ringel *et al.,* 2008). The efficiency of the synthesis process is not complete, and at each stage of conversion, some part of the creatine is eliminated and converted into creatinine and is excreted in the urine. This suggests

Please cite this article as Yaghobfar A, Javaheri Barfourooshi H & Hamedi S. 2019. Effect of Guanidino Acetic Acid Consumption and Management Methods on the Reproductive Potential of Elderly Cockerel Broiler Breeders. Poult. Sci. J. 7(2): 195-204.

that there is a constant demand for the replacement of lost creatine. The body must continuously build creatine from essential amino acids such as arginine and methionine. A part of creatine is supplied through food which, of course, cannot meet the needs of the body (Wyss and Kaddurah-Daouk, 2000). Lately, after a ban on the use of animal sources in poultry feeds, a decline in their performance has been observed, which is attributed to the lack of creatine in plant sources (Heger *et al.,* 2014). In general, the body's need for creatine is provided by animal proteins such as meat and fish powder or by *in vivo* production. For this reason, animals that are fed with rations that contain fewer amounts of animal protein, suffer from a shortage of creatine. By reducing the protein in the feed, due to their variable quality and the probability of contamination, it is possible to restore the amount of creatine in the body by adding creatine or guanidino acetic acid supplement to rations (Michiels *et al.,* 2012).

Thus, it can be anticipated that the use of guanidino acetic acid in the diet of roosters after 40 weeks of age will increase the likelihood of increased physical and powerful strength, as well as increased fertility and sperm quality.

Materials and Methods

The procedures of this research were approved by the Animal Care and Welfare Committee of the Animal Science Research Institute of Iran. This study was extended out in a factorial 3 × 3 experiments using 486 broiler breeders (Ross 308) in 9 treatments with 6 replicates and 9 birds (8 hens and 1 cock) in each replicate. To become accustomed to standard conditions, the test began at 38 weeks of age. Records of experimental treatments were performed from weeks 41 to 62 (after peak production). The status of the breeding farm in terms of moisture content, temperature, weight control, management of feed intake, and other parameters was carried out by following the guide for raising broiler breeder of Ross 308 (2016). The experimental treatments consisted of three management methods (no method, spiking method, and intra-spiking method) and three levels of CreAmino (zero, 1200 mg/kg and 1600 mg/kg). The basal diet compound was presented in Table 1.

Table 1. Ingredients and chemical composition of basal diet (g/Kg)

Components	Amounts (g/Kg)
Maze, Yellow	534.40
Wheat	208.50
Soybean oil	0.8
Soybean Meal (CP=44%)	159.30
Oysters shell	74.50
Di-calcium phosphate	12.60
Sodium chloride	3.1
Vitamin premix*	2.5
Mineral premix**	2.5
DL-Methionine	1.4
L-Lysine Hydrochloride	0.2
L-Threonine	0.2
Metabolism energy (Kcal/ Kg)	2800
Crude Protein (g/Kg)	144.40
Ether Extract (g/Kg)	21.70
Linolenic acid (g/Kg)	14.0
Crude Fiber (g/Kg)	38.40
Calcium (g/Kg)	33.0
Available Phosphorus (g/Kg)	3.40
Sodium chloride(g/Kg)	2.20
Digestible arginine (g/Kg)	6.40
Digestible threonine (g/Kg)	4.60
Digestible lysine (g/Kg)	6.40
Digestible methionine (g/Kg)	3.50
Digestible SAA (g/Kg)	5.4

*Vitamin premix (g/ Kg): Vitamin A (Retinoic acid), 4.4 g; vitamin D_3 (Cholecalciferol), 0.72 g; vitamin B_1 (Thiamin), 0.306 g; vitamin B_2 (Riboflavin), 1.5 g; vitamin B_6 (Pyridoxamine), 0.306 g; vitamin B_{12} (Cyanocobalamin), 1 g; vitamin E (alpha-tocopherol), 7.2 g; biotin, 1 g; vitamin K, 1 g; niacin, 2.48 g; folic acid, 0.306 g; pantothenic acid, 6.08 g; choline chloride, 220 g.
** Mineral premix (g/Kg): manganese, 2 g; iron, 10 g; zinc, 13 g; iodine, 0.2 g; cobalt, 0.02 g; selenium, 0.04 g.

Collection of semen samples
The roosters were trained for two weeks to enable them producing enough semen during the semen collection procedure. An abdominal rubbing method was used for collecting semen from the cocks (Hermiz *et al.* 2016). In summary, in this method, the rooster was placed on the technician's leg, and by the rubbing of the belly and back of the bird, an appropriate stimulation was made and the ejaculated semen was collected using 1.5 mL graduated microtube. During the experiment, semen was collected four times. To avoid the occurrence of a cold shock to the sperm, the microtubes are placed inside a nylon bag (to prevent the penetration of water into the microtube). Then the nylon bags inside a 37°C warm water flask transferred to the lab. Qualitative and quantitative sperm characteristics were evaluated in terms of semen volume, sperm density, sperm motility percentage, progressive sperm motility and percentage of dead and live sperm using an optical microscope.

Determination of semen volume
Immediately after taking samples from cockerels and transferring to the laboratory, the semen volume was determined by using the graduated body of the microtube.

Sperm Motility Survey
To investigate the sperm motility, sperm samples were incubated for 30 minutes at a temperature of 37 ° C and then diluted in a physiologic serum of 1 to 200 mL (Talebi *et al.* 2018). A drop of diluted sperm was placed under a microscope on a warming plate and analyzed by 400x magnification. Using a graded screen, several fields were randomly selected and progressive sperms, non-progressive sperms, and non-moving sperm were counted. The total counted sperm was between 200 and 400 sperm per specimen.

Sperm Concentration
To determine the concentration of the semen, the sample was first diluted 1 to 400 mL in distilled water. Then a drop of diluted sample was placed on a Neubauer chamber or Hemocytometer and placed under a microscope with a magnification of 400 fold. The spermatozoa were counted in 4 lateral squares and one square in the center. The concentration of the semen sample was obtained using the following formula:

$$C = \frac{(NOSC \times D)}{5(NOS) \times 4(SCS)}$$

Where: C = concentration (nL), NOSC = count of sperm count, D = dilution, NOS = number of sperm counted per square, and SCS = sperm concentration in each home (nL).

Determination of the percentage of live and dead sperm
To determine the percentage of live and dead sperm in
semen samples, 10 µL of diluted specimen in distilled water (1: 400 mL) was placed on a glass slide and one drop of Eosin-Nigrosin was mixed and one drop of the solution obtained on another glass slide was placed and extended to 45° with another glass slide. After drying, a 400-magnified microscope was used to count the spermatozoa that had been discolored (as dead sperm) and those without color (living sperm).

Determination of testicular characteristics
On day 156 of the experiment, a cock was randomly selected from each nest and slaughtered. Then, the testes were evaluated by weighting and ranked in terms of their size, color and extending the capillary network. Then transverse sections with a maximum thickness of 0.5 centimeters from each testicle were cautiously prepared. The tissue samples were placed in 10% formalin solution and transferred to the veterinary lab. The process for preparing tissue samples, coloring and preparing slides was performed according to established methods. After the preparation of the slides, the number of Spermatogonia cells, primary Spermatocyte cells, Spermatid cells, Spermatozoa cells, Sertoli cells, Leydig cells were counted by using a graticule 10×10 grid-scale, and the diameter of the seminiferous duct and the thickness of the germinal epithelium with Linear graticule were measured.

Statistical data analysis
The SAS program version 9.4 was used to analyze the data. Parametric data analyzed with Proc GLM and factorial method. To the traits of color, the span of the capillary network and the size of the testicles are not measured quantitatively, they were ranked and analyzed using nonparametric tests. The significance of the treatments was determined using the Kruskal-Wallis non-parametric test and two-way comparison with the Mann-Whitney test by using the SPSS program. The significance level was considered as 5%.

Results
The main and interaction effects of the use of spiking and intra-spiking management methods and different levels of 1200 and 1600 mg of CreAmino on the qualitative and quantitative characteristics of cockerel's sperm are shown in Table 2.

Semen volume
As shown in Table 2, the using of different management methods or the use of different levels of CreAmino did not have a statistically significant effect on the intensity of sperm obtained from the

cockerels. It should be noted that the volume of semen extracted was lower than the normal because the roosters were kept in nests with chickens and had regular mating. Nevertheless, it was observed that the interaction effect on the volume of sperm caused a significant reduction in CreAmino (1200 mg/kg) + spiking in comparison with control, CreAmino (1600 mg/kg) + spiking, CreAmino (1200 mg/kg) + Intra-spiking and CreAmino (1600 mg/kg) + Intra-spiking ($P < 0.05$).

Table 2. Main and interaction effects of management methods and CreAmino levels on qualitative and quantitative characteristics of rooster sperm

	Volume (mL)	Concentration ($\times 10^6$)	Motility (%)	Progressive motility (%)	Live sperm (%)
Main effects					
No management	0.07	5268.6	78.74	50.08	75.11
Spiking	0.07	5723.1	77.96	52.58	76.04
Intra-spiking	0.09	5504.5	79.16	50.47	74.63
Significant level	0.17	0.46	0.88	0.45	0.64
CreAmino* (zero)	0.08	5599.7^{a}	79.55	53.22	74.68
CreAmino (1200 mg/kg)	0.07	4940.7^{b}	78.18	50.58	75.67
CreAmino (1600 mg/kg)	0.09	5955.8^{a}	78.12	49.33	75.44
Significant level	0.37	0.03	0.78	0.17	0.77
Interaction effect					
Control	0.09^{a}	5293.2^{ab}	79.54	51.69^{ab}	75.44
No management + CreAmino (1200 mg/kg)	0.07^{ab}	4795.1^{b}	75.77	47.82^{ab}	73.71
No management + CreAmino (1600 mg/kg)	0.07^{ab}	5717.4^{ab}	80.90	50.74^{ab}	76.19
CreAmino (zero) + Spiking	0.07^{ab}	5906.0^{ab}	77.70	54.20^{a}	75.64
CreAmino (1200 mg/kg) + Spiking	0.05^{b}	4973.2^{ab}	77.96	52.26^{ab}	76.82
CreAmino (1600 mg/kg) + Spiking	0.09^{a}	6290.2^{a}	78.22	51.29^{ab}	75.68
CreAmino (zero) + Intra-spiking	0.07^{ab}	5600.0^{ab}	81.40	53.77^{a}	72.95
CreAmino (1200 mg/kg) + Intra-spiking	0.10^{a}	5052.8^{ab}	80.83	51.66^{ab}	76.48
CreAmino (1600 mg/kg) + Intra-spiking	0.10^{a}	5859.7^{ab}	75.25	45.97^{b}	74.46
Standard Error of Means	0.01	453.12	2.89	2.56	1.82

*CreAmino ®: as a precursor of creatine. Significant level is $P < 0.05$.

The concentration of sperm

The management methods used for elderly roosters did not have any significant effect on the concentration of sperm, but different levels of CreAmino significantly altered sperm concentration. So that CreAmino (1200 mg/kg) significantly reduced the sperm concentration. The interaction of these two factors also reduced the sperm concentration in CreAmino (1200 mg/kg) + no-management against the CreAmino (1600 mg/kg) + Spiking ($P < 0.05$). Indeed, the use of CreAmino (1600 mg/kg) + spiking improved sperm concentration, significantly ($P < 0.05$).

Sperm Motility and Progressive motility

The use of management methods and different levels of CreAmino alone or in combination with each other did not have any significant effect on sperm motility. Also, these treatments have not any significant effect on the percentage of progressively motile sperm alone. However, the interaction of these two factors reduced the percentage of progressively motile sperm in CreAmino (1600 mg/kg) + intra-spiking in comparison with the use of two management methods ($P < 0.05$).

Live sperm

The use of management methods and different levels of CreAmino alone or in combination with each other did not have a significant effect on the percentage of live sperm.

Body and testicle weight

Roosters' body weight, also total, right and left testicular weights are shown in Table 3. Management methods and different levels of CreAmino did not have a significant effect on all of these traits alone. But, in combination, there was a significant reduction for CreAmino (1600 mg/kg) + spiking compare with CreAmino (1600 mg/kg) + intra-spiking or no management + CreAmino (1200 mg/kg). Also, for total, right and left testicular weights there were significant reductions in no management + CreAmino (1600 mg/kg) against no management + CreAmino (1200 mg/kg) ($P < 0.05$).

Testicular color, capillary network, and size
There was no significant difference between different treatments for testicle color. The span of the capillary network on the surface of the testicles, in no management + CreAmino (1600 mg/kg), CreAmino (1200 mg/kg) + spiking, CreAmino (1600 mg/kg) + spiking, as well as CreAmino (1200 mg/kg) + intra-spiking- spiking were significantly reduced compare with CreAmino (1600 mg/kg) + intra-spiking ($P < 0.05$). For the size of the testicles, there were the same changes like the span of the capillary network, but there was an increase in size for control, also ($P < 0.05$).

Histological studies
The effect of different management methods and levels of CreAmino in spermatogonia cells, spermatocyte cell, spermatid cell, spermatozoid cell, Sertoli cell, Leydig cell, seminiferous duct diameter (micrometer), germinal epithelium thickness (micrometer) in rooster's testis tissue at the end of the period (age 64 weeks) is shown in Table 5.

Spermatogonia cells
The spermatogonia cells were significantly increased by spiking compare to control ($P < 0.05$). The zero levels of CreAmino increased the number of spermatogonia cells in comparison with two other levels, 1200 or 1600 mg/kg ($P < 0.05$). Roosters in CreAmino (1200 mg/kg) + no-management treatment had the lowest number of spermatogonia cells among other treatments ($P < 0.05$).

Primary spermatocyte cells
The number of primary spermatocyte cells significantly increased in spiking treatment compare with two other management methods ($P < 0.05$). Also, CreAmino (1600 mg/kg) had the highest number of primary spermatocyte cells among the other two levels of CreAmino ($P < 0.05$). The lowest number of these cells were for CreAmino (zero) + Intra-spiking among all treatments ($P < 0.05$).

Spermatid cells
The spermatid cell numbers increased by the spiking management method ($P < 0.05$). The lowest number of spermatid cells were observed in CreAmino (zero) + intra-spiking and CreAmino (1600 mg/kg) + intra-spiking treatments ($P < 0.05$).

Spermatozoid cells
The number of spermatozoa cells reduced by the intra-spiking management method, but in CreAmino (1200 mg/kg) treatment increased its number ($P < 0.05$). The lowest number of spermatozoa cells were observed in CreAmino (zero) + intra-spiking and CreAmino (1600 mg/kg) + intra-spiking ($P < 0.05$).

Table 3. Main and interaction effects of management methods and CreAmino levels on rooster's body weight, total, right and left testes weights.

	Body Weight (g)	Weight testes (g)	The weight of right testicle (g)	The weight of left testicle (g)
Main effects				
No management	5508.89	23.28	12.19	11.23
Spiking	5283.89	18.17	8.94	9.30
Intra-spiking	5375.56	21.63	11.32	9.95
Significant level	0.80	0.61	0.45	0.76
CreAmino* (zero)	5270.00	22.79	11.36	11.42
CreAmino (1200 mg/kg)	5432.78	22.14	11.94	10.01
CreAmino (1600 mg/kg)	5365.56	18.14	9.15	9.05
Significant level	0.98	0.66	0.57	0.69
Interaction effect				
Control	5326.67^{ab}	22.90^{ab}	11.50^{ab}	11.47^{ab}
No management + CreAmino (1200 mg/kg)	5940.00^{a}	34.83^{a}	18.83^{a}	16.33^{a}
No management + CreAmino (1600 mg/kg)	5260.00^{ab}	12.10^{b}	6.23^{b}	5.90^{b}
CreAmino (zero) + Spiking	5750.00^{ab}	19.47^{ab}	9.77^{ab}	9.77^{ab}
CreAmino (1200 mg/kg) + Spiking	5375.00^{ab}	14.07^{ab}	7.20^{ab}	6.93^{ab}
CreAmino (1600 mg/kg) + Spiking	4726.67^{b}	20.97^{ab}	9.87^{ab}	11.20^{ab}
CreAmino (zero) + Intra-spiking	5033.33^{ab}	26.00^{ab}	12.80^{ab}	13.03^{ab}
CreAmino (1200 mg/kg) + Intra-spiking	4983.33^{ab}	17.53^{ab}	9.80^{ab}	6.77^{ab}
CreAmino (1600 mg/kg) + Intra-spiking	6110.00^{a}	21.35^{ab}	11.35^{ab}	10.05^{ab}
Standard Error of Means	413.25	32.60	3.18	3.23

*CreAmino: CreAmino® as a precursor of creatine. Significant level is $P < 0.05$.

Sertoli cells

The management method had not any significant effect on the number of Sertoli cells, but using CreAmino in both levels (1200 and 1600 mg/kg) increased the number of these cells ($P < 0.05$). The lowest number of Sertoli cells was observed in CreAmino (zero) + spiking or intra-spiking ($P < 0.05$).

Table 4. Average testicular color, capillary network and testicular size for experimental treatments.

Testicular traits	Testicular color	Capillary network	Testicular size
Control (no management, no CreAmino*)	14.50	14.50^{ab}	22.00^{a}
No management + CreAmino (1200 mg/kg)	14.50	19.00^{ab}	17.50^{ab}
No management + CreAmino (1600 mg/kg)	14.50	10.00^{b}	8.50^{b}
CreAmino (zero) + Spiking	14.50	14.50^{ab}	17.50^{ab}
CreAmino (1200 mg/kg) + Spiking	14.50	10.00^{b}	8.50^{b}
CreAmino (1600 mg/kg) + Spiking	14.50	10.00^{b}	8.50^{b}
CreAmino (zero) + Intra-spiking	10.00	14.50^{ab}	13.00^{ab}
CreAmino (1200 mg/kg) + Intra-spiking	14.50	10.00^{b}	8.50^{b}
CreAmino (1600 mg/kg) + Intra-spiking	14.50	23.50^{a}	22.00^{a}
X^2	8.00	13.68	18.02

*CreAmino: Creamino® as a precursor of creatine.

Leydig cells

The number of Leydig cells decreased by intra-spiking management method ($P < 0.05$). The lowest number of Leydig cells was observed CreAmino (1600 mg/kg) +
intra-spiking ($P < 0.05$).

The diameter of the Seminiferous Ducts

The diameter of the seminiferous ducts was increased by the spiking management method compared to the intra-spiking management method ($P < 0.05$). The highest diameter of the seminiferous ducts was observed in CreAmino (1600 mg/kg) ($P < 0.05$). The lowest diameter of the seminiferous ducts was observed in CreAmino (zero) + intra-spiking treatment ($P < 0.05$).

The germinal epithelium thickness decreased by the intra-spiking management method ($P < 0.05$). The largest germinal epithelium thickness was observed in CreAmino (1600 mg/kg) group ($p < 0.05$), and the lowest thickness of germinal epithelium was observed in CreAmino (zero) + intra-spiking and no management + CreAmino (1200 mg/kg) groups ($P < 0.05$).

Discussion

The reproductive potential in rooster depends on the quantity and quality of the sperm produced in the testicles. Since in every broiler breeder's flock, each cock mates with some hens, so the characteristics of the sperm will have a significant effect on the fertility of the flock. In the determination of the rooster fertility, three parameters are usually used to evaluate the sperm: sperm concentration, survival, and mobility of sperm. In elderly rooster, semen volume and number of spermatozoids per ejaculation, and the spermatozoids fertilization's ability reduced (Mangiagalli *et al.,* 2010). But, Sonseeda *et al*. (2013) reported that age did not affect semen quality between the 10^{th} and 30^{th} month of age in Thai native cocks. In the present study, it was observed that using 1200 mg of CreAmino with the spiking management method reduced the sperm volume against other interactions of CreAmino levels or management methods. Also, sperm concentration was decreased by using 1200 mg of CreAmino compared with used 1600 mg of CreAmino and spiking. Although sperm motility was not affected by different treatments, progressive sperm motility was decreased by using 1600 mg of CreAmino and intra-spiking compared with when used just management methods. These results are consistent with the report of Namazizadegan *et al.* (2016). They reported that adding guanidine acetic acid to rooster diets did not observe any differences in the quantitative and qualitative parameters of sperm. Also, Murakami *et al.* (2014) did not observe differences in the number of spermatozoids that they were able to penetrate the vitelline membrane using different levels of guanidine acetic acid in the quail meat breeds. While, Sharideh *et al.* (2015) observed by using guanidine acid levels (0, 0.6, 1.2 and 1.8 g/kg) in broiler breeder males in 53 weeks of age, an increase of the number of spermatozoids penetrated to the perivitelline membrane and an improvement in flock fertility. Also, Shahabi Tapeh *et al.* (2017), using levels of 0, 600, 1200, and 1800 mg guanidine acetic acid per kg of diet for 24 weeks at the rooster in age of 29 weeks, observed that the level of 1200 mg guanidine acetic acid increased sperm concentration, total sperm count and progressive sperm motility. Ahangar *et al.* (2017) also observed that for all levels of L-arginine (0, 1.35, 3.33 and 3.22 g / kg of ration) in broiler breeder rooster at 37 weeks of age, all reproductive traits of rooster showed a dramatic improvement, especially at a level of 2.33 g L-arginine. They reported this is because of increasing glycolysis by the effect of L-arginine, which could lead to higher levels of adenosine triphosphate and lactate in the sperms.

Table 5. Main and interaction effects of management methods and CreAmino levels on the average number of spermatogonia, spermatocyte, spermatid, spermatozoid, Sertoli and Leydig cells, seminiferous duct diameter, and germinal epithelium thickness.

	Spermatogonia cell (number)	Spermatocyte cell (number)	Spermatid cell (number)	Spermatozoa cell (number)	Sertoli cell (number)	Leydig cell (number)	Seminiferous duct diameter (µm)	Germinal epithelium thickness (µm)
Main effects								
No management	79.76^{b}	76.82^{b}	82.23^{b}	83.30^{a}	4.38^{a}	3.13^{a}	149.51^{ab}	59.98^{a}
Spiking	83.31^{a}	91.09^{a}	92.48^{a}	86.53^{a}	3.95^{b}	3.23^{a}	156.56^{a}	57.13^{a}
Intra-spiking	81.28^{ab}	67.52^{c}	62.21^{c}	53.34^{b}	4.32^{ab}	2.90^{b}	142.31^{b}	51.84^{b}
Significant level	0.04	<0.01	<0.01	<0.01	0.06	0.03	<0.01	<0.01
CreAmino *(zero)	85.59^{a}	78.18^{b}	77.28	70.80^{b}	3.93^{b}	3.07	144.40^{b}	54.71^{b}
CreAmino (1200 mg/kg)	77.30^{c}	73.81^{c}	77.72	79.37^{a}	4.31^{a}	3.08	147.18^{b}	54.44^{b}
CreAmino (1600 mg/kg)	81.46^{b}	83.44^{a}	81.92	73.01^{b}	4.41^{a}	3.12	156.80^{a}	59.80^{a}
Significant level	<0.01	<0.01	0.12	0.01	0.04	0.90	0.01	<0.01
Interaction effect								
Control	84.07^{abc}	84.00^{b}	84.67^{b}	84.83^{abc}	4.57^{ab}	3.30^{ab}	168.47^{a}	71.60^{a}
No management + CreAmino (1200 mg/kg)	72.93^{d}	66.03^{d}	71.47^{c}	80.90^{bc}	4.50^{abc}	2.73^{cd}	131.00^{c}	43.00^{e}
No management + CreAmino (1600 mg/kg)	82.27^{bc}	80.43^{bc}	90.57^{ab}	84.17^{abc}	4.07^{bc}	3.37^{a}	149.07^{b}	65.33^{b}
CreAmino (zero)+ Spiking	87.63^{a}	94.40^{a}	93.60^{a}	90.23^{ab}	3.33^{d}	2.90^{bcd}	149.87^{b}	52.67^{d}
CreAmino (1200 mg/kg) + Spiking	79.40^{c}	81.67^{b}	87.87^{ab}	76.43^{c}	4.17^{abc}	3.40^{a}	149.47^{b}	59.60^{bc}
CreAmino (1600 mg/kg) + Spiking	82.90^{abc}	97.20^{a}	95.97^{a}	92.93^{a}	4.37^{abc}	3.40^{a}	170.33^{a}	59.13^{c}
CreAmino (zero) + Intra-spiking	85.07^{ab}	56.13^{e}	53.57^{d}	37.33^{d}	3.90^{cd}	3.00^{abcd}	114.87^{d}	39.87^{e}
CreAmino (1200 mg/kg) + Intra-spiking	79.57^{c}	73.73^{c}	73.83^{c}	80.77^{bc}	4.27^{abc}	3.10^{abc}	161.07^{ab}	60.73^{bc}
CreAmino (1600 mg/kg) + Intra-spiking	79.20^{c}	72.70^{cd}	59.23^{d}	41.93^{d}	4.80^{a}	2.60^{d}	151.00^{b}	54.93^{cd}
Standard Error of Means	1.71	2.56	3.02	3.71	0.24	0.16	5.41	2.24

*CreAmino: Creamino® as a precursor of creatine. Significant level is $P < 0.05$.

But with the advancement of studies in this field, it has been suggested that these amino acids and their metabolites play a more complex role in the regulation of sperm properties (Ahangar *et al.* 2017) Perhaps one of the reasons for the difference among the results of their study with the present study was the age of the roosters. Of course, sperm quality may not be as effective as the mating behavior on fertility (Gumułka and Kapkowska, 2005; Abudabos, 2010; Malik *et al.* 2013).

Indeed, as age increases, the abilities of roosters for showing the mating behaviors reduce. The main reasons for this phenomenon are muscle weakness and lack of energy storage in these weak muscles. Muscles' requirements to some amino acids (such as arginine, isoleucine, valine, methionine, and tryptophan) are important for proper function in breeder flocks. This is especially important due to the occurrence of mating behavior in roosters, which required intense and tedious muscular activity. Thus, in that aspect, there is a vital need for using energy producers for muscles and an appropriate amino acid profile, such as creatine, which acts as a significant part of the energy metabolism of cells, in particular, muscle cells. Likewise, the use of the spiking management method and the creatine additive for this determination is necessary for a rooster with a higher age (Wyss and Kaddurah-Daouk, 2000; Dilge *et al.,* 2013). It has been reported that the use of creatine in the diet of broiler breeders (aged 50 to 60 weeks) increases fertility and hatchability (Carpena *et al.*, 2015). Also, Abbaspour *et al.* (2019) reported that semen quality and spermatogenesis index were improved with dietary supplementation of L-arginine (0.68%) in aged roosters.

Male broiler chickens weighing less than 3,800 g were infertile or had subclinical infertility, as well as low levels of testosterone and high concentrations of corticosterone. Conversely, heavier roosters have larger and healthier testicles, higher testosterone levels, and fewer corticosterone concentrations are characteristic of good breeders with better fertility. However, these roosters did not have much success in mating because of their heavy weight. The want of homogeneity between roosters and the existence of pecking order in the flock, along with a reduction in the percentage of hatches that is considered a weakness of the rooster in mating or limitation of access to hens. Reducing fertility in roosters after 45 weeks of age was associated with a diminishing in testicular weight, sperm production, and testosterone levels (Fragoso *et al.,* 2013). Since most of the testicular tissue is assigned to spermatogenesis, it is possible to calculate the production of sperm from the size of the testicle. Larger testicles produce more sperm than the smaller testicles. This physiological characteristic affects their fertility (Talebi *et al.,* 2018). It has also been observed that in summing up to the relationship between body weight and testicular size, there is also a positive correlation between the age of the rooster and the size of the testicles so that older roosters tend to have larger testicles than young ones (Laskemoen *et al.,* 2008).

Along with the observations of CreAmino positive effects on fertility, the study also found that the use of 1200 mg CreAmino alone increased the weight of the testicles in comparison with using 1600 mg CreAmino. In 37-week-old roosters with four levels 0, 35.1, 33.3 and 22.3 g/kg of L-arginine diet for 8 weeks, it was observed that the level of 2.33 g/kg L-arginine increased testicular weight, sperm volume and forward sperm motility (Ahangar *et al.,* 2017). However, in terms of the span of the capillary network on the surface of the rooster testicles, the 1600 mg CreAmino along with intra-spiking management had the widest capillary network. The size of the testicle and the size of the blood vessel network, which provides sufficient blood for testicular tissue, leads to optimal spermatogenesis (Aire, 2014; Talebi *et al.,* 2018). As age rises, testicles have been retrograded and similar to the same trend in sperm production and testosterone concentration (Fragoso *et al.,* 2013). According to the results of this study, we can use the 1600 mg CreAmino level with the intra-spiking management method has postponed this retrogression to some extent in the testicular size.

The histological findings of the present study showed that using some management methods apart from the use of different levels of CreAmino can increase or decrease the number of different cells in the testes. CreAmino (1200 mg/kg) or intra-spiking, each one alone, had the lowest number for different cells. In 37-week-old roosters, which had four levels (zero, 1.35, 2.33 and 3.22 g / kg of L-arginine diet for 8 weeks), the level of 3.22 g / kg L-arginine increased the diameter of the seminiferous duct, Leydig, spermatids, and sperm cell numbers, but the birds consuming 2.33 g / kg of L-arginine had more diameter of the seminiferous, the number of Sertoli and spermatogonia cells than other groups (Ahangar *et al.,* 2017).

Conclusion

According to the results of this study, it seems that the use of 1600 mg of CreAmino in combination with spiking and intra-spiking management techniques can reduce and delay the occurrence of symptoms caused by increasing roosters' ages, like decreased libido, decreased mating numbers and reduced sperm production. This will improve the fertility of the breeders' broiler flocks.

Acknowledgments

The authors would like to express their gratitude to the Animal Science Research Institute for providing

the possibility of this project to be implemented by the Kowsar Agricultural Research and Development Company in terms of approval and funding of this research.

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