**Improvement of post-thawed sperm quality and fertility of Arian rooster by oral
administration of D-aspartic acid**

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Abstract

This study was conducted to investigate the effect of D-Aspartic acid (D-Asp) on post-thawed sperm quality, fertility and hatchability outcomes in male broiler breeders. Twenty 55-week-old roosters were selected and equally split into four groups (n=5 rooster/group). Different daily D-Asp doses including 0 (D-0), 100 (D-100), 200 (D-200) or 300 (D-300) mg/kg BW were encapsulated and individually administered for 12 weeks to roosters in each group. Semen samples were weekly collected from 7th to 12th week of experiment. Sperm quality from 7th to 11th week was evaluated in both fresh (total and forward motility and plasma membrane functionality) and post-thawed (total and forward motility, plasma membrane functionality, apoptosis status and mitochondrial activity) conditions. Also, collected semen samples on the 12th week were frozen and artificially inseminated to evaluate fertility and hatchability. The results from fresh condition showed that total and forward motility and plasma membrane functionality were significantly higher in D-200 compared to other groups. Also, interaction effect of time and treatment was not significant for all assessed parameters in fresh condition. In post-thawed condition, D-200 showed significantly higher total and forward motility, fertility and hatchability compared to other groups. The higher value for plasma membrane functionality and mitochondrial activity was observed in D-200 compared to D-0 and D300 groups. However, the percentage of live, early apoptotic and dead spermatozoa were not significantly affected by applied treatment in the current study. No significant difference for time and treat interaction effect was observed for all assessed parameters except forward motility. In conclusion, it seems that D-Asp administration could improve fresh and post-thawed sperm quality and post-thawed sperm fertility in male broiler breeders.

Key words: D-Aspartic acid, Rooster, Sperm, Aging, Cryopreservation.

1. Introduction

Poultry sperm cryopreservation and fertility outcomes are highly variable and not reliable as much as other species in ex situ conservation of genetic resources [1]. Sperm cryopreservation of native and non commercial birds is very important method to protect their genetic resources. Although many researches are doing to optimize methods in the field of sperm cryobiology in commercial animals, a few studies have focused on sperm cryopreservation of native poultry species [1, 2]. In some countries (France, United State, Netherlands and Spain) cryopreservation of native and commercial poultry semen has been preformed as program for genetic resources conservation [2]. However, it seems that understanding biochemical and molecular-biological events during cryopreservation of native poultry possibly help to introduce new strategies to improve post-thawed sperm quality to save the gene pool for the future.

Ameliorating cryo-injury effect of freezing process has been considered by numerous studies in the last decades. However, most of them have focused on in vitro strategies to modify the effectiveness of some extenders including Minnesota Avian extender [3, 4] or Beltsville poultry semen extender (BPSE) [5]. Using antioxidants such as catalase [6, 7], superoxide dismutase [8], Vitamin E [7, 8] and different cryoprotectants such as glycerol and dimethylacetamide [9] or even different cooling rates [10] are among those strategies to improve poultry sperm recovery post cryopreservation.

Nutriceutical treatments by incorporating into the sperm plasma membrane or modifying its microenvironment have also been successfully improved fresh or frozen thawed sperm quality and its fertilizing ability in several species [11-17]. In the current study a new oral treatment with both endocrine and direct effect on sperm production pathways was assessed. Free D-Asp was

first introduced by D' Aniello and Giudatta in the brain of cephalopoda [18]. Three decades later, it was highly detected in Leydig, Sertoli and germ cells, particularly in spermatogonia, elongate spermatids and spermatozoa in several species including human [19], duck [20] and rat [21, 22]. Interestingly D-Asp levels in the gonad of frog and lizard fluctuated during reproductive cycle and the highest levels reported in sexually active animals [23]. These pieces of evidence directed scientists to consider the possible role of D-Asp in male reproduction. D-Aspartic acid can directly act on Hypothalamus-Hypophysis-Testes (HPG) or indirectly through Leydig cells to increase testosterone levels [24]. Testosterone as a main androgen possess a vital role during male reproduction. It plays a fundamental role in at least four critical processes during spermatogenesis: maintenance of Blood-Testis-Barrier, meiosis, Sertoli-spermatid adhesion, and spermiation [25]. Furthermore, D-Asp has a direct impact on sperm production. Including D-Asp to culture medium of spermatogonia GC-1 cells increased the protein expression of proliferating cell nuclear antigen (PCNA) and aurora kinase B (AURKB), both known as proliferation markers, and phosphorylation of MAPK3/1 and AKT proteins, participate in cell survival and proliferation [24, 26]. The impact of feeding D-Asp on both semen concentration, motility and forward motility have been well documented in human and rabbit bucks [27, 28]. Since existing a relationship between fresh and post-thawed sperm quality is rational and have been confirmed by flow cytometry analysis [29], the aim of the present study was to determine whether oral administration of D-Asp affect roosters sperm freezability and fertility.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from sigma chemical Co. (St. Louis, Mo, USA) and Merck (Darmstadt, Germany) unless otherwise indicated.

2.2. Animal ethics

All procedures during this study were approved by the Animal Care Committee of Department of Animal Science, University of Tehran.

2.3. Birds and Treatments

Twenty 55-week-old of Arian (Arian Line Breeding Center, Babol-kenar, Iran) broiler breeder roosters (kept in the research farm of the university of Tehran, Karaj, Iran) were individually housed in pens (1.2×1.2×1 m) under controlled environment (21-23°C and a 14L: 10D lighting program), fed a standard diet (Table 1) and allotted randomly to four treatment groups (n=5 rooster/group) consisting of oral administration of 0 (D-0), 100 (D-100), 200 (D-200) or 300 (D300) mg/kg BW of D-Asp per bird per day for 12 weeks. Being sure to receive the allotted doses, D-Asp was packed in capsules in advance and administered once daily at the time of feeding. Semen samples were weekly collected from 7th to 11th week to evaluate fresh and post-thawed sperm quality. Total and forward motility and plasma membrane functionality were evaluated in both fresh and post-thawed sperm, but apoptosis status and mitochondrial activity were tested only in post-thawed sperm. Also, frozen semen samples on the 12th week were artificially inseminated to evaluate fertility and hatchability.

2.4. Semen sampling, extending and cryopreserving

Semen samples were weekly collected from roosters by massage technique [30]. Following collection, sample from each rooster was evaluated to have standard criteria (80% motility and 10% > abnormality). Semen sample from each rooster was individually diluted with a modified BPSE composed of Potassium phosphate dibasic trihydrate (7.59g/L), Sodium l-glutamate (8.67 g/L), D-Fructose (5 g/L), Sodium acetate trihydrate (3.2 g/L), N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic (3.2 g/L), Potassium citrate tribasic monohydrate (0.6 g/L), Potassium Phosphate monobasic (0.7 g/L), Magnesium chloride anhydrous (0.3 g/L), Soybean lecithin (0.5% w/v) [31] supplemented by Dimethyl Sulfoxide (DMSO) (4%, v/v). Before DMSO and Soybean lecithin inclusion, osmotic pressure (Osmomat 030-D Gonotec Germany) and pH (3520 Jenway, United Kingdom) of the extender were adjusted to 310 mOsmol/kg and 7.4, respectively. Afterwards, diluted semen samples were loaded into 0.25 mL French straws with the final concentration of 400×10^6 /mL, sealed with polyvinyl alcohol powder and cooled for one hour in 5°C as an equilibration period. Using a cryobox, straws were placed horizontally 6 cm above liquid nitrogen for approximately 10 min. Finally, they were plunge in Liquid nitrogen for storage at -196°C [31, 32].

2.5. Sperm thawing and gross evaluation of fresh and post-thawed sperm

Sperm samples were individually thawed by immersion of the straws in a water bath at 4°C for 5 min [4]. Ten microliters of post-thawed semen was diluted with 20 µL of BPSE (without DMSO and lecithin). Then, 10 µL of extended sample was deposited on a clean glass slide preheated to 37°C, covered with a coverslip. Total and forward sperm motility were

subjectively evaluated using a phase contrast microscope (Labomed, Lx 400, USA; magnification: $\times 400$). To determine plasma membrane functionality via the hypo-osmotic swelling (HOS) test, a 10 μ L semen sample was diluted in a 200 μ L medium containing sodium citrate 1% (100 mOsmol/kg), incubated at 37°C for 20 min. Spermatozoa with functional plasma membrane were owning a curled or swollen flagellum, 200 cells per slide were considered (Labomed, Lx 400, USA; magnification: $\times 1000$) [33].

2.6. Flow cytometry analysis

Flow cytometry analyses were carried out with a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). An argon ion laser (488 nm) was used to excite fluorescent probes in the current study. Green fluorescence from Annexin V/ Fluorescein Isothiocyanate (FITC) and Rhodamine 123 was detected by FL1 photodetector and red fluorescence from propidium iodide (PI) was detected by FL3 photodetector. Ten thousands event were collected for each sample. Acquired data were analyzed by FlowJo software (version 9.5.3).

2.6.1. Apoptosis status

Apoptosis status was detected by a Commercial Kit (IQP, Groningen, the Netherlands) according to the manufacturers guide. Briefly, sperm samples were washed in calcium buffer and readjusted to the 1×10^6 spermatozoa/mL. Ten microliters Annexin V-FITC (0.01 mg/mL) was added to 100 μ L of sperm suspension followed by incubation at room temperature for 20 min. finally 10 μ L of PI (1 μ g/mL) was added to sperm suspension and incubated for 10 min prior to evaluation with flow cytometer. The analysis resulted in classifying spermatozoa in four distinct

groups: 1) A⁻/PI⁻ and 2) A⁺/PI⁻ were considered alive and early apoptotic cells, respectively. Both 3) A⁺/PI⁺ and 4) A⁻/PI⁺ referred to dead cells [34].

2.6.2. Mitochondrial status

Rhodamine 123 (R123; Invitrogen, Eugene, OR, USA) and PI were used to assess the mitochondrial activity of spermatozoa. Briefly, 5 µL of R123 (0.01 mg/mL) were added to the diluted semen sample (50×10⁶ spermatozoa/mL) and incubated in the dark at room temperature. Samples were washed (500 g for 5min) and pellet were resuspended in 250 µL PBS buffer. Then, 10 µL of PI solution (1 mg/mL) was added to the sample and assessed by flowcytometry to determine the number of live spermatozoa with active mitochondria (positive for R123 and negative for PI) [35].

2.7. Artificial insemination, fertility and hatchability

The fertilizing ability of post-thawed spermatozoa was estimated using intravaginal artificial insemination [1]. A total of 120 Arian broiler breeder hens (34 week-old) were individually caged and 6 hens were allotted to each rooster (a total of 30 hens per treatment) and fed a standard diet (15% Crude protein, 2800 kcal/kg diet Metabolizable energy, 3% Calcium and 0.35% available phosphorus). Straws from each rooster (6 straws/rooster) were thawed, pooled (to delete straw effect) and inseminated once with a dose of 200 µL (80 × 10⁶ spermatozoa/hen).

In this trial, eggs were collected from the second day following insemination for five consecutive days (A total of 412 settable eggs for 5 days, 110 settable eggs/D-0 group, 95 settable eggs/D-100 group, 101 settable eggs/D-200 group and 106 settable eggs/D-300 group)

and set daily with appropriate settings for setter (T= 37.7°C and RH= 60%) and hatcher (T= 37.2°C and RH=70%). Fertility (i.e. percentage of fertile per incubated eggs) and hatchability (i.e. percentage of hatched eggs per fertile eggs) were calculated for each rooster.

2.8. Statistical analysis

Using univariate procedure and Shapiro-Wilk test, data were checked for normal distribution. Arc sine (for percentage data) transformation of data were used when appropriate. Single and repeated measurements data were analyzed by PROC GLM and PROC Mixed SAS 9.1 (version 9.1, 2002; SAS institute Cary, NC, USA), respectively. Results are presented as the Lsmean $\pm$ SE. The Turkey's test was applied to compare Lsmeans the level of significance adjusted to $p < 0.05$.

3. Results

3.1. Fresh sperm parameters

Although, the effect of treatment was significant for sperm total and forward motility and plasma membrane functionality, but the effects of time and treatment $\times$ time were not significant for above mentioned parameters. Moderate level of D-Asp (D-200) significantly improved sperm total and forward motility and plasma membrane functionality in comparison with other levels (Table 2).

3.2. Post-thawed sperm parameters

Sperm total ($p=0.01$), forward motility ($p<0.01$), plasma membrane functionality ($p=0.02$) and mitochondrial activity ($p<0.01$) were among evaluated traits affected by different levels of D-Asp in a relatively similar dose response manner. However, the percentage of live ($p=0.83$), early apoptotic ($p=0.55$), and dead spermatozoa ($p=0.77$) were not influenced by the treatments (Table 3).

Moderate level of D-Asp (D-200) resulted in the highest values of sperm motility (59.4 ± 1.16) which was significantly different compared to D-0, D-100 and D-300 levels (53 ± 1.29 , 55.8 ± 0.68 and 54.8 ± 0.97 , respectively). No significant effect of time and treatment interaction was observed except for forward motility ($p=0.03$, Table 3).

Sperm from roosters in D-200 group exhibited the highest forward motility percentage among all treatments ($p<0.05$) (50.8 ± 1.17 vs 41.4 ± 0.89 , 43.8 ± 1.16 and 43.2 ± 1.03 for D-200, D-0, D-100 and D-300, respectively). The interactive effect of D-Asp and week of experiment on sperm forward motility percentage is illustrated in figure 1. A steady trend was observed for moderated level of D-Asp through the experiment which was started with 52% in the 7th week and ended with 51% on the 11th week. However, remaining levels (D-0, D-100 and D-300) had a relatively fluctuated tendency during the trial (Fig. 1).

Results of plasma membrane functionality are presented in Table 3. We observed an increasing trend in plasma membrane functionality with increasing D-Asp supplementation in the diet until 200 mg/kg BW above which there was a decrease with 300 mg/kg BW. Time and treatment interaction for plasma membrane functionality was not significantly influenced in this trial. The same trend was observed for mitochondrial activity.

Despite insignificant, early apoptic status and viability percentage were improved in D-200 level compared with other groups. The percentage of dead spermatozoa, however, was comparable among treatments (Table 3).

Fertility and hatchability of fertile egg were significantly affected by feeding different doses of D-Asp in the current study (Table 4). Among treated groups, D-200 resulted in significantly improved fertility (51 ± 1.87) and hatchability (84.06 ± 1.11) percentages compared to other groups.

4. Discussion

Structural, cellular and molecular changes during cryopreservation, known as physiological challenges, compromise sperm cell function which lead to lower fertilization capacity. Poultry sperm compared to other species are more sensitive to this phenomena because of unique structural and biochemical characteristics [36]. Thus far, feeding macro- and micronutrients have been successfully improved sperm quality and freezability through modification of sperm structure and its production process including initiation, meiosis, and maturation or even Leydig and Sertoli cells activity [37, 38]. Because of well documented direct and indirect impact of D-Asp on spermatogenesis, it was nominated to feed roosters in the current study. To the best of our knowledge, this is the first study evaluating possible effect of orally administered D-Asp on fresh sperm quality and freezability. Thus, we inevitably relied on mechanisms introduced by studies that evaluated the effectiveness of D-Asp acid on androgens synthesis pathways [39], testis microenvironment and fresh sperm parameters [27, 28, 40]. In the current trial, a mild dose of D-Asp effectively protected rooster spermatozoa against cryo-injuries

that occur during freeze-thawing process. Rooster's fresh (total and forward motility and plasma membrane functionality) and post-thawed sperm (total and forward motility, plasma membrane functionality, mitochondrial activity as well as fertility and hatchability rates) were improved by oral administration of 200 mg D-Asp/Kg Bw/day. The beneficial effects of D-Asp on male reproduction has previously been reported in several species [19, 21-24, 26-28, 39-45]. Administration of D-Asp led to 1.49 fold increase in sperm forward motility of sub-fertile patients with oligoasthenozoospermia and asthenozoospermia [27] who exhibit decreased D-Asp content of seminal plasma [19]. In another study, buck rabbits fed a concentrate containing DL-Asp showed higher concentration of D-Asp in blood and seminal plasma and improved sperm concentration and kinematic parameters. Furthermore, D-Asp inclusion in the medium of human sperm maintained its total motility and progressive motility during six hours of incubation while significant reductions observed in the control group [46]. Ours is the first report in which post-thawed sperm mitochondrial activity was improved following D-Asp administration. However, the exact mechanism behind this effect is not clear. Possessing more active mitochondria results in more adenosine triphosphate (ATP) production that is the major energy currency of the cell, providing energy for the most energy-consuming activities in the cell. According to the mentioned studies, improvement of sperm total and forward motility as well as plasma membrane functionality can be explained by the following mechanisms: 1) D-Asp acid increases testosterone levels which has a pivotal role in spermatogenesis especially in aged rooster with a lower testosterone concentration (our unpublished data). In the absence of testosterone or its active receptor, spermatogenesis rarely progresses beyond meiosis [47], 2) Elective storage of D-Asp in male genital tract and seminal plasma following administration may trigger survival cascade in germ cells or regulate sperm maturation and activity after emission [21, 26, 28]. 3) Improving mitochondrial activity, like other factors, play a key role in sperm kinematic

parameters and its membrane functionality through supplying cell energy currency. Long [36] reported a dramatic decrease in ATP content of fresh rooster spermatozoa after freeze-thawing. Therefore, more active mitochondria preserve during cryopreservation in the current study may correlate with higher ATP production (not measured in the current study), motility and forward motility. Plasma membrane functionality as well as total and forward motility were affected by supplementation of D-Asp in the diet. It has been accepted that ionic and osmotic equilibrium across the cell membrane depends on Na^+/K^+ pump (an electrogenic transmembrane ATPase). The Na^+/K^+ -ATPase enzyme pumps sodium out of cells while pumping potassium into cells, both against their concentration gradients. In this trial, higher mitochondrial activity may have resulted in more ATP production required to maintain Na^+/K^+ -ATPase activity and thus membrane functionality.

A decreasing trend was seen in most evaluated traits with doses above 200 mg/kg Bw/day in the current study. The effects of excessive D-Asp intake on testis physiology have been investigated by Chandrashekar et al [48]. Their finding suggests that excessive exogenous D-Asp elevates oxidative stress primarily due to its metabolism by D-Asp oxidase (D-AspO). Under normal physiological conditions, D-Asp is metabolized by D-AspO into oxaloacetate, ammonia and hydrogen peroxide. Hydrogen peroxide is immediately hydrolyzed either by catalase or glutathione peroxidase thereby minimizing the peroxidative injuries. Mitochondrial dysfunction is inevitable when the antioxidant capacity of cell is exceeded. Marked reduction in membrane potential and increased membrane transition pore in the presence of Ca^{+2} are the potential mechanisms perturb mitochondrial membrane integrity. Therefore, the pro-oxidant effect of D-Asp in the highest dose of the current experiment (D-Asp 300) might have contributed to compromised mitochondrial function and lower sperm quality.

Post-thawed sperm viability and apoptotic status were not affected by D-Asp acid in this trial. In the only related published study, incubation of mouse spermatogonia cell line [GC-1spg] with D-Asp activated cell survival pathway through activating ERK, PCNA and AKT [26]. The exact reason of this discrepancy is not clear, but the followings might be considered: 1) different effects of D-Asp in the *in vivo* and *in vitro* environments; 2) different doses of D-Asp used in the current study; 3) survival or mitotic pathways are active in germ cell but not in spermatozoa and 4) freezing/thawing cycle may attenuate survival pathways.

In the current study, the maximum fertility rate was observed in D-200 group (51 ± 1.87) which is comparable with the finding of Shahverdi et al. [32] who reported 49% fertility by using enriched BPSE extender with 4% Low-density lipoprotein (LDL). Freezing and thawing process dramatically decreases rooster sperm fertilizing ability. Furthermore, a relatively high number of alive sperm is needed within sperm storage tubules (SST) to ascertain fertilization between inseminations [36]. Semen traits associated with fertility such as sperm motility, viability and sperm forward progression may affect the penetration of the cervical mucus and are thus important for sperm preservation and fertilizing capacity [49]. Comparing post-thaw semen samples among four pedigreed commercial layer lines revealed a direct relationship between sperm ATP concentration ($\text{pm}/10^9$ spermatozoa) and membrane integrity with fertile eggs and hatched chicks' percentages [36]. The higher forward motility and plasma membrane functionality of spermatozoa may be reasons for increasing fertility in D-200 group. Furthermore, it was found that D-Asp administration for oligo-asthenozoospermia and asthenozoospermia men significantly improves the pregnancy rate of their partners in human model [27]. Direct relationship between sperm storage time in oviduct and embryonic death was reported previously [50]. Thus, it seems that higher sperm quality in D-200 group resulted in higher hatchability

among treated groups by maintaining more alive sperm in SST and affecting fertility in the current trial.

4.1. Conclusions

Oral administration of rooster with D-Asp positively affected sperm quality parameters, fertility and hatchability rates in a dose dependent manner. The optimum level of D-Asp in the diet was 200 mg/kg BW/day and higher amounts adversely affected sperm quality and fertility outcomes.

Acknowledgement

We would like to thank Iran National Science Foundation (INSF) for providing the financial support for this work (Grant No. 93041373).

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507 Fig. 1. Weekly variation of sperm forward motility in roosters (n=5/group) administered different
508 doses of D-Asp (0 (D-0), 100 (D-100), 200 (D-200) or 300 (D-300) mg/kg BW/day). Within each
509 week, LSmeans with different letters (a-c) significantly differ ($p<0.05$).

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Figure

Table 1. Ingredient and chemical composition of standard diet fed by roosters.

Item	Value (%)	Digestible amino acids	Value (%)
Corn	69.5	Lysine	0.46
Soybean meal	9	Methionine	0.39
Wheat bran	19.5	Methionine & Cysteine	0.49
Dicalcium phosphate	0.18	Tryptophan	0.12
Calcium carbonate	0.85	Arginine	0.67
Sodium chloride	0.35	Valine	0.5
DL-Met	0.12	Leucine	0.53
Vitamin premix*	0.25	Isoleucine	0.4
Trace mineral premix**	0.25	Threonine	0.37
Composition			
	ME (kcal/kg)	2754.5	
	CP (%)	12	
	Ca (%)	0.7	
	Available P (%)	0.35	
	Na (%)	0.15	
	Cl (%)	0.15	
	K (%)	0.6	

*Supplied per kilogram of diet: vitamin A, 15,000 IU; vitamin E, 100 IU; vitamin K3, 4 mg; vitamin B12, 25 µg; vitamin D, 3,000 IU; riboflavin, 7.5 mg; niacin, 50 µg; pantothenic acid, 18 mg; pyridoxine, 5.5 mg; biotin, 50 mg and folic acid, 1.5 mg.

**Supplied per kilogram of diet: Fe, 90 mg; Mn, 120 mg; Zn, 110 mg; I, 2 mg and Se, 0.3 mg.

Table 2. Fresh sperm quality of roosters administered D-Asp.

Feature	Treatments* (LSmean ±SE)				P Value		
	D-0	D-100	D-200	D-300	Treat	Time	Treat×Time
Total motility (%)	81.6 ^c ±0.38	83.18 ^c ±0.52	91.42 ^a ±0.32	87.11 ^b ±0.49	<0.01	0.18	0.54
Forward motility (%)	70.74 ^d ±0.52	73 ^c ±0.53	83.5 ^a ±0.54	78.22 ^b ±0.63	<0.01	0.05	0.6
Hos (%)	63.68 ^d ±0.38	66.45 ^c ±0.39	72.44 ^a ±0.35	69.89 ^b ±0.45	<0.01	0.6	0.59

^{a-d} values within a raw with different superscripts differ at p<0.05.

*The Rooster (n=25 evaluations/treatment) received different levels of D-Asp including: 0 (D-0), 100 (D-100), 200 (D-200) or 300 (D-300) mg/kg BW/day.

Table 3. Post-thawed sperm quality of roosters administered D-Asp.

Feature	Treatment*(Lsmean ±SE)				P Value		
	D-0	D-100	D-200	D-300	Treat	Time	Treat×Time
Total motility (%)	53 ^b ±1.29	55.8 ^b ±0.7	59.4 ^a ±1.16	54.8 ^b ±0.97	0.01	<0.01	0.67
Forward motility (%)	41.4 ^b ±0.89	43.8 ^b ±1.16	51.8 ^a ±0.95	43.2 ^b ±1.03	<0.01	0.25	0.03
Hos (%)	51.4 ^c ±1.45	57 ^{ab} ±1.08	58.8 ^a ±0.96	53 ^{bc} ±0.86	0.02	0.96	0.82
Live	48.57±1.72	48.22±1.91	49.12±2.17	47.01±1.37	0.83	0.04	0.23
Early apoptosis	17.08±1.43	15.96±1.64	14.93±1.59	17.1±1.37	0.55	0.01	0.08
Dead	34.32±0.9	35.05±1.08	35.97±1.15	35.74±1.34	0.77	0.14	0.92
Active mitochondria	51.74 ^{bc} ±1.24	53.01 ^{ab} ±1.59	56.23 ^a ±1.79	49.02 ^c ±1.37	<0.01	0.01	0.18

^{a-c} values within a raw with different superscripts differ at p<0.05.

*The Rooster (n=25 evaluations/treatment) received different levels of D-Asp including: 0 (D-0), 100 (D-100), 200 (D-200) or 300 (D-300) mg/kg BW/day.

Table 4. Fertility and hatchability in hatching eggs sired by roosters administered D-Asp.

Trait (%)	Treatment*(Lsmean±SE)				P value
	D-0	D-100	D-200	D-300	
Number of eggs	110	95	101	106	-
Fertility [#]	38.2 ^b ±2.59	43 ^b ±1.14	51 ^a ±1.87	41.6 ^b ±1.32	<0.01
Hatchability of fertile eggs [‡]	73.54 ^b ±1.39	76.77 ^b ±2.57	84.06 ^a ±1.11	76.67 ^b ±2.42	0.01

^{a-b} Values within a raw with different superscripts differ at p<0.05.

*The rooster (n=5/treatment) received different levels of D-Asp including: 0 (D-0), 100 (D-100), 200 (D-200) or 300 (D-300) mg/kg BW/day.

[#] Thirty Arian female broiler breeders were artificially inseminated per treatment.

[‡] Eggs were collected from second to seventh day after AI.