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The effect of coenzyme Q10 on rooster semen preservation in cooling condition

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ABSTRACT

Oxidative stress has been known as a significant cause of the lower fertility rates correlated with liquid stored rooster semen. The effect of coenzyme Q10 (CoQ10), as a powerful antioxidant, seems be beneficial on semen storage of broiler breeder roosters at the cooled condition. Therefore, two experiments were performed to assess the effect of CoQ10 supplemented semen extender on sperm quality parameters, fertility, hatchability and sperm penetration (SP) rates of rooster semen stored at 5 °C. In the first experiment, semen samples of 12 roosters were weekly pooled for four weeks (47–50 weeks of age). The pooled semen was diluted by modified Beltsville poultry semen extender and divided into three equivalent parts containing different levels of CoQ10 [0 (Q-0), 100 (Q-100) and 200 (Q-200) μM/mL) and then stored for 24 h at 5 °C. Sperm quality including progressive motility, plasma membrane integrity and functionality were evaluated after 0 and 24 h storage. The results showed that progressive motility, plasma membrane integrity and functionality were improved in Q-200 compared to Q-0 after 24 h storage at 5 °C ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively). According to the results of the first experiment, Q-200 group was selected to be used to evaluate the fertility, hatchability and SP rate in the second experiment during next four weeks (51–54 weeks of age). The results of the second experiment showed that fertility rate was significantly increased in Q-200 compared to control group by approximately 10%, although no significant difference was observed in hatchability and SP rates between Q-200 and control groups. In conclusion, the results of the present research confirm that supplementation of rooster semen extender with CoQ10 might be potentially used to improve semen quality and fertility rates.

1. Introduction

The genetic selection of broiler breeder lines (Gallus gallus domesticus) is mainly concentrated on producing speedy growth chicks with reduced feed conversion ratio and high slaughter weights [1]. However, these production successes of the end output seldom are contrasted with the reproductive traits of the breeding flocks such as decreased fertility rate at the last weeks of production life [2–4]. The decreasing fertility rate from 45 weeks of age has been correlated with multiple factors such as a declining in testicular mass, sperm generation and the testosterone levels of broiler breeder roosters, and also because of the birds are grossly overweight, the lack of complete copulations may be possible to the low fertility [2]. In the poultry industry such as turkey, because toms unable to consistently transmit semen to the hen at the mating process, artificial insemination (AI) is widely applied to overcome depressed fertility [5]. It has not been necessary to implement AI programs before 45 weeks of age in commercial broiler breeder farms because of natural mating results in adequate fertility levels [2,6]. The young roosters at end production steps of broiler breeder herds, is identified as spiking, were introduced to overcome the deterioration of reproductive traits due to roosters aging. However, it seems that the biosecurity concerns associated with spiking would be eliminated by AI of aging broiler breeder farms. So, the use of AI in commercial poultry operations is becoming more common. Before AI of hens with undiluted or diluted semen, the avian semen begins to lose fertilizing ability when stored more than 1 h (h) [7,8]. Lipid peroxidation as a result of oxidative stress has been identified as a significant cause of the lower fertility rates associated with liquid-stored fowl semen [5]. Addition of antioxidant compounds such as vitamin E [9], oleic acid [10] and lycopene [11] to the semen extender has a preventing effect on lipid peroxidation of fowl sperm during semen storage.

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Coenzyme Q10 (CoQ10) is an antioxidant that is ubiquitous to almost all body tissues, with its tissue concentration being 5–10-fold higher than the other chief lipid-soluble antioxidant such as vitamin E [12]. Also, CoQ10 can regenerate α-Tocopherol from the radical α-Tocopherol [13]. Coenzyme Q10 is an isoprenylated benzoquinone that transfers electrons in the mitochondrial respiratory chain of the cell such as spermatozoa or each other cell type [14,15]. Several studies performed on *in vitro* cooled semen of different species such as bull [16], ram [17] and horse [13,18] they were proved that including CoQ10 to semen extender improved sperm quality. However, it seems that effects of adding CoQ10 to modified Beltsville poultry semen extender (modified BPSE) may be beneficial for long time semen storage of broiler breeder roosters at the cooled condition. Therefore, the aims this study were to determine (1) if adding CoQ10 to semen extender improves the rooster sperm quality stored at the cooled condition, (2) whether the optimum level of CoQ10 for supplementing rooster semen extender can improve *in vivo* fertility rate.

2. Materials and methods

The maintenance, housing, and utilization of roosters and hens for this study were allowed by the Animal Welfare Committee of the Department of Animal Science, University of Tehran.

2.1. Chemicals

All chemicals except for CoQ10 (Hangzhou Dingyan Chem Co., Ltd, Hangzhou, China) were purchased from Merck (Darmstadt, Germany).

2.2. Experiment 1

2.2.1. Birds and semen collection

Twelve mature Arbor Acres Plus broiler breeder roosters were selected at 45 weeks of age from a commercial flock. The roosters with at least 75% sperm motility and 80% sperm plasma membrane integrity were selected for the present study. The roosters were randomly assigned to 4- floor pens (1.25 m × 1 m) equipped with pan feeder and an automatic bell drinker at 22 ± 2 °C (60% humidity) under a 14L:08D photoperiod. Roosters were fed a usual breeder diet with 2700 kcal of MEn/kg and 11.5% crude protein and 0.7% calcium. During the experiment (45–54 wk of age), the standard commercial breeder male diet restricted to a mean of 146 g of feed/d was fed and water was supplied ad libitum. During the first two weeks (45 and 46 wk of age) of the experiment, roosters were adopted to semen collection by the method of Burrows and Quinn [19] and routinely ejaculated one weekly (47–54 wk of age) thereafter.

2.2.2. Extender preparation and sperm dilution

The modified Beltsville poultry semen extender (mBPSE; the pH and osmotic pressure were set at 7.2 and 310 mOsm/kg and, respectively) has been used in this study [20]. Because of CoQ10 is a fat-soluble substance, first 0.1 M of CoQ10 was dissolved in 99% methanol (according to our unpublished data, the dose of the methanol (0.002% in modified BPSE), as a positive control, has not adverse effect on sperm quality compared to negative control, so the positive control in experiment 1 was not used). Then, to obtain 100 and 200 μM/mL of CoQ10 (at pre-test, the effects of different levels of CoQ10 on sperm motility were assessed and the best results were chosen as levels of CoQ10; Fig. 1), the solution of 0.1 M of CoQ10 was diluted in modified BPSE. Immediately after semen collection, the semen samples were pooled and diluted (final concentration: 400×10^6 spermatozoa/mL) in the extender supplemented with different concentrations of CoQ10 containing 0 (Q-0), 100 (Q-100) and 200 (Q-200) μM/mL. After 0 and 24 h storage of semen at 5 °C, sperm quality parameters were evaluated. In the second part of this study (51–54 weeks of age), each week semen samples were collected for AI to test fertility, hatchability and sperm penetration (SP) rate.

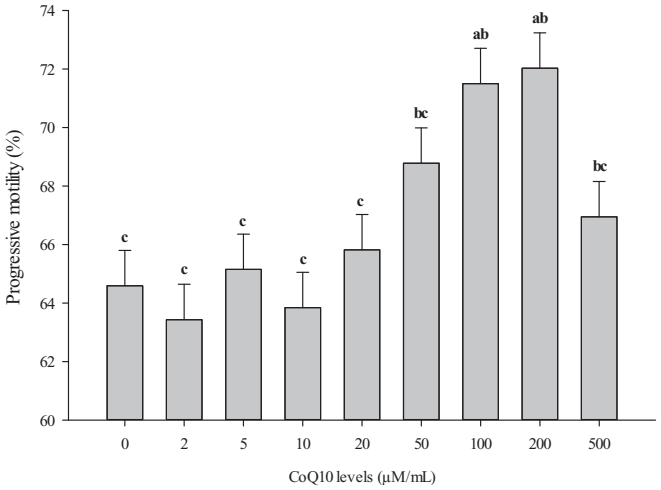

Fig. 1. Least squares means of sperm progressive motility of pooled semen following semen extender supplemented with different concentrations of coenzyme Q10 stored under 5 °C for 24 h in pre-test. Different letters (a, b, c) represent significant differences ($P < 0.05$).

2.2.3. Experiment I: sperm quality analyses

Sperm forward motility was evaluated by placing a diluted semen (1:20 in modified BPSE) on a pre-warmed microscope slide (37 °C) covered with a coverslip, using a light microscope (D-22976; Kruss, Hamburg, Germany) at 5 microscopic fields (400 × magnification). The percentage of forwarding motility was measured as spermatozoa exhibiting moderate to rapid progressive movement [21].

Sperm plasma membrane integrity was evaluated by eosin-nigrosin staining method [22]. Ten μL of stain was placed on a pre-warmed microscope slide (37 °C) and well mixed for 30 s with 10 μL of diluted semen (1:20), then spreading the stain with another slide. After drying, by counting 200 spermatozoa, Plasma membrane integrity was examined under oil immersion objective by light microscopy assessed (D-22976; Kruss, Hamburg, Germany) at 1000 × magnification. Sperm exhibiting stainless sperm heads were counted as sperm with integrated plasma membrane or live sperm, and sperm with stained or partially stained heads was numerated as sperm with unintegrated plasma membrane or dead sperm.

Sperm plasma membrane functionality was assessed by a hypo-osmotic swelling test [21]. A hypo-osmotic solution (100 mOsm/kg) was made by adding sodium citrate (1 g) to 100 mL of distilled water. 25 μL of diluted semen was mixed with 500 μL of the hypo-osmotic solution and incubated at 37 °C for 30 min. The percentage of spermatozoa showing coiled mid-pieces and tail segments were determined after stained by the eosin-nigrosine stain on a microscope slide and counting 200 spermatozoa under oil immersion objective by light microscopy assessed (D-22976; Kruss, Hamburg, Germany) at 1000 × magnification.

2.3. Experiment II: evaluation of fertility, hatchability rates and sperm penetration

2.3.1. Artificial insemination

According to the results of the experiment I, Q-200 group was selected to be used to evaluate sperm fertility potential (fertility,

hatchability and SP rate) in experiment II. For this purpose, 64 Arbor Acres Plus broiler breeder hens (32 hens/treatment) were used. The hens (51 wk of age) were weekly inseminated with Q-0 and Q-200 diluted semen (200×10^6 spermatozoa/hen). Eggs were collected from the second day after the first AI, for a period of 28 days. The eggs were weekly incubated in a commercial incubator (specific dry-bulb temperature of 37.7 °C and the wet-bulb temperature of 29 °C). Fertilizing capacity (fertilized eggs divided by total eggs set × 100) and hatchability of fertile eggs (hatched eggs divided by fertilized eggs × 100) were determined at the completion of each incubation period [23].

2.3.2. Sperm penetration assay

The number of SP sites in the inner perivitelline layer (IPL) can be applied to approximate the number of spermatozoa remaining in the employment sperm storage tubules at the time of ovulation [6]. The SP was weekly measured, on day 3 following each insemination, by using the method described by Bramwell et al. (1995) and Sharideh et al. (2016) [24,25]. Briefly, the eggs (12 eggs/treatment/week) were broken and the yolk was separated from the albumen. The yolks were immersed in a 1% NaCl solution for at least 5 min. The IPL overlying the germinal disc was removed with scissors and rinsed in 1% NaCl solution to remove excess yolk. The IPL was then transferred on a slide and several drops of neutral phosphate-buffered formalin (10%) were added on the IPL and decanted immediately. The IPL was stained with Schiff's reagent and air-dried. The holes were counted using light microscopy (D-22976; Kruss, Hamburg, Germany; 40 × magnification). The number of SP sites in one visual field (15.89 mm^2) was measured.

2.4. Statistical analysis

All data were tested for normal distribution by UNIVARIATE procedure and Shapiro–Wilk test, and all percentage data were normalized through $\sqrt{x}$ Arcsin transformation. All data (except fertility and hatchability rates) were analyzed using Proc Mixed of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC, USA). Also, SP data was analyzed by the GLM procedure of SAS 9.1. The results were represented as LSmean ± SEM, where they were compared with the Tukey's test at $P \leq 0.05$. Fertility and hatchability rates data were analyzed by the GENMOD procedure of SAS 9.1, and the results were compared by the chi-squared test at $P \leq 0.05$.

3. Results

3.1. Experiment I: sperm quality analyses

The percent of progressive motility was higher in Q-100 and Q-200 (69.3 ± 1.1% and 71.8 ± 1.1%, respectively) compared to Q-0 (63.9 ± 1.1%) at 24 h (Fig. 2). The percentage of progressive motility was higher ($P < 0.01$) at 0 h (82.89 ± 1.14%) compared to 24 h (67.97 ± 1.14%, Fig. 2). On the other hands, CoQ10 improved sperm motility during the time of semen storage. So, the interactive effects of CoQ10 and time on the progressive motility were significant ($P < 0.01$).

The percent of sperm plasma membrane integrity and functionality were higher ($P < 0.05$ and $P < 0.01$, respectively) in Q-200 (75.98 ± 1.93% and 71.79 ± 1.49%, respectively) compared to the Q-0 (65.54 ± 2.4% and 61.90 ± 1.49%, respectively) at 24 h (Figs. 3 and 4). The sperm plasma membrane integrity and functionality were higher ($P < 0.01$) at 0 h (86.29 ± 1.25% and 79.16 ± 0.90%, respectively) compared to 24 h (71.29 ± 1.25% and 67.70 ± 0.90%, respectively) (Figs. 3 and 4). On the other hands, CoQ10 improved sperm plasma membrane integrity and functionality during the time of sperm storage. So, the interactive effects of CoQ10 and time on them were significant ($P < 0.05$ *and* $P < 0.01$, respectively).

3.2. Experiment II: evaluation of fertility, hatchability rates and sperm penetration

Although CoQ10 treatment numerically increased the number SP hydrolysed in the IPL (Table 1), a significantly higher ($P < 0.05$) percentage of fertility rates was observed in 200 μM/mL CoQ10 compared to the control group (68.68% vs. 62.18%, Fig. 5). The effect of CoQ10 on hatchability of fertile eggs was not significant (Fig. 5).

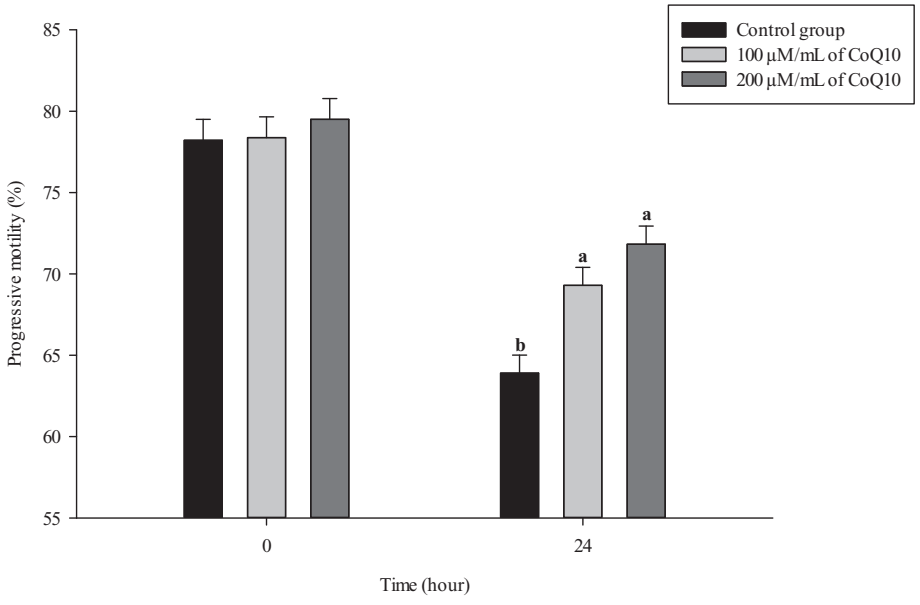

Fig. 2. Least squares means of sperm progressive motility of pooled semen (12 ejaculates/week during 47–50 weeks of age) following semen extender supplemented with different concentrations of coenzyme Q10 (CoQ10; 0, 100 and 200 μM CoQ10/mL) stored under 5 °C for 24 h in experiment 1. Different letters (a, b) within time represent significant differences ($P < 0.05$).

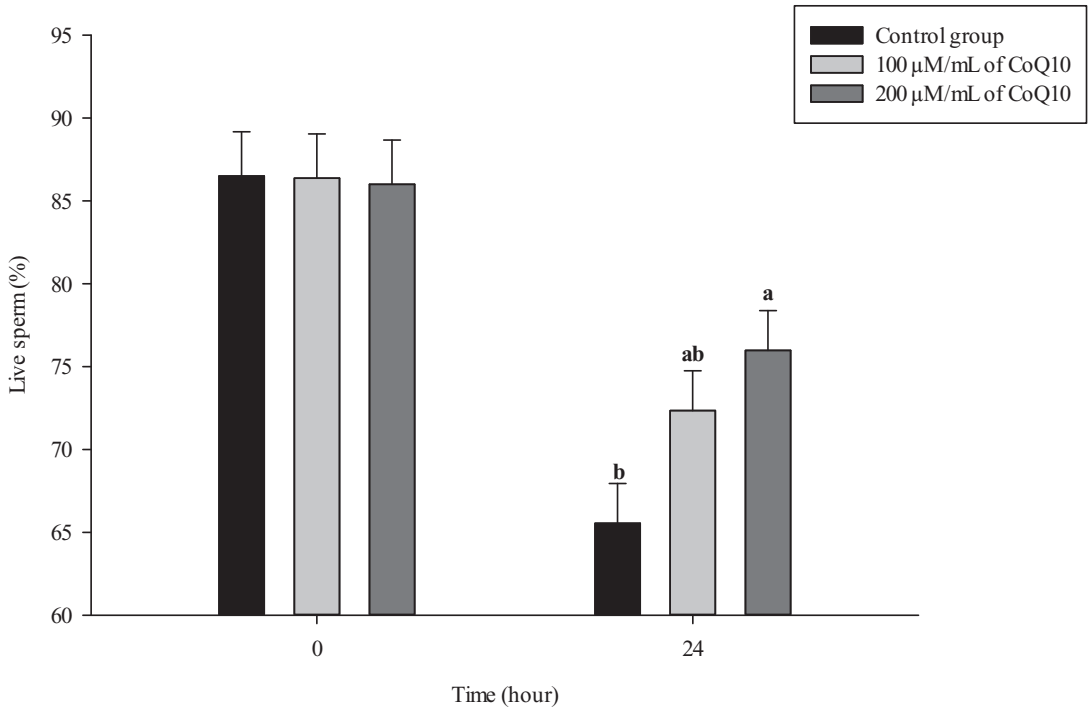

Fig. 3. Least squares means of sperm plasma membrane integrity (viability) of pooled semen (12 ejaculates/week during 47–50 weeks of age) following semen extender supplemented with different concentrations of coenzyme Q10 (CoQ10; 0, 100 and 200 µM CoQ10/mL) stored under 5 °C for 24 h in experiment 1. Different letters (a, b) within time represent significant differences ($P < 0.05$).

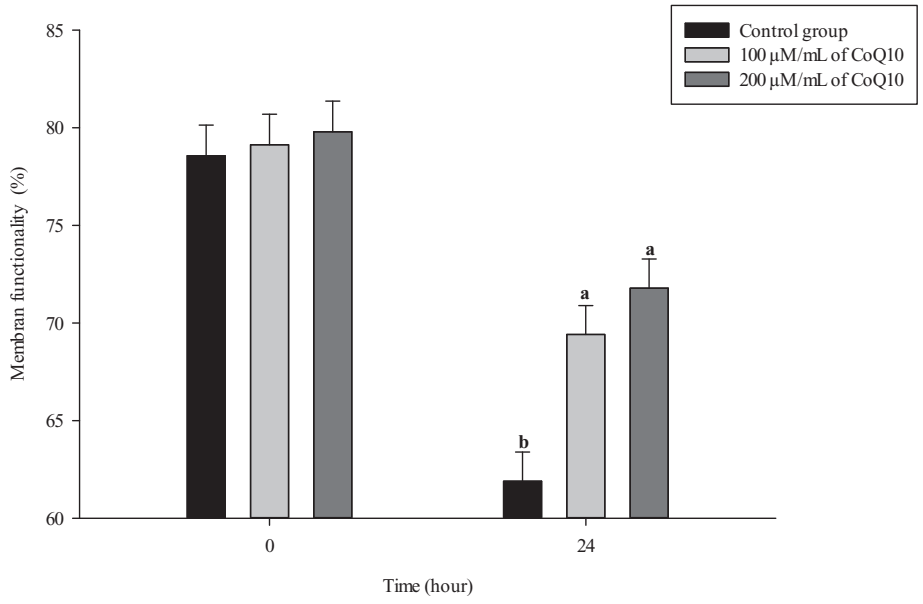

Fig. 4. Least squares means of sperm plasma membrane functionality of pooled semen (12 ejaculates/week during 47–50 weeks of age) following semen extender supplemented with different concentrations of coenzyme Q10 (CoQ10; 0, 100 and 200 µM CoQ10/mL) stored under 5 °C for 24 h in experiment 1. Different letters (a, b) within time represent significant differences ($P < 0.05$).

4. Discussion

Although the high proportions of polyunsaturated fatty acids (PUFA) in bird spermatozoa membrane keep the stableness and membrane flexibility during the fertilization process but increasing susceptible chicken semen to lipid peroxidation by ROS during *in vivo* and *in vitro* storage semen [5,26]. Lipid peroxidation has negative effects on morphology, motility, viability, and fertilization capacity of avian sperm [5]. In this study, sperm storage at 5 °C for 24 has damaging effects on plasma membrane functionality and integrity, and also motility of sperm. These results agreed with several studies performed on avian semen storage at 5 °C for a different time in which reported semen storage increased ROS and lipid peroxidation of spermatozoa, which in turn, contribute to the lower sperm viability, motility and fertility rates [5,10,27]. Oxidative stress can damage sperm by membrane and DNA fragmentation at

Table 1
The sperm penetration (SP) holes hydrolysed (Lsmean ± SEM; 48 eggs/treatment) in the inner perivitelline layer following artificial insemination of broiler breeder hens, by adding CoQ10 (0 or 200 μM CoQ10/mL) to rooster semen extender.

CoQ10 level (μM/mL)	Number SP
0	22.72
200	28.76
SEM	4.80
P-Value	0.3963

both nuclear and mitochondrial levels, which in turn, can have a negative effect on sperm motility and viability [15]. It occurs when ROS overcome the natural antioxidant defenses [16]. Mitochondrial DNA, unlike nuclear DNA, does not contain histones or introns, under oxidative stress, making the mitochondrial DNA more vulnerable to mutations and deletions, which in turn, leading to apoptosis of the cell [14]. In studies performed on bovine [28] and human [29] showed that supplementation CoQ10 to semen extender prevented chromatin fragmentation at ambient temperature for 5–6 h of incubation.

In avian semen, spermatozoa were protected against oxidative stress by natural antioxidants (such as vitamin E and vitamin C). However, these antioxidants are usually present in insufficient amounts to neutralize the oxidative stress that occurs during *in vitro* semen storage [11]. It is well demonstrated that suppression the oxidative stress can be provided through enrichment of different antioxidants such as vitamin E [9], oleic acid [10] and lycopene [11] to fowl semen extender. Coenzyme Q10 is concentrated in the mitochondria of a different cell such as spermatozoa and plays a vital role in energy metabolism [30]. Coenzyme Q10 moreover functions as a lipo-soluble chain-breaking antioxidant, is involved in the regeneration of the endogenous antioxidants such as superoxide dismutase, which in turn, restrained of oxidative stress [31]. In vivo studies on human and rat confirmed that including CoQ10 to diet improved TAC and semen quality [32,33]. It has been shown that the levels of CoQ10 have a significant correlation with sperm motility [34]. Moreover, in a study performed by our groups showed that adding CoQ10 to the diet of aged broiler breeder roosters improved seminal plasma TAC and sperm quality (unpublished data). In the present study, comparing to the fresh semen, chilled semen storage in extender supplemented with CoQ10 decreased spermatozoa motility, plasma membrane functionality and integrity by 8.16%, 12.16% and 8.62%, respectively, but lack of CoQ10 caused much bigger adverse changes in motility, plasma membrane integrity and functionality by 18.30%, 24.23 and 21.21% respectively. The results of previous studies showed that CoQ10 maintained the sperm quality, as in asthenozoospermic [35] and normospermic [29] human as well as in semen of cocks [36] and in horses [13]. Nath et al. (2015) showed an *in vitro* study on pooled semen of Rhode Island Red cocks incubated with 0.3 mg/mL of CoQ10 at 4 °C for 36 h which significantly improved the sperm motility, viability, and membrane integrity. Coenzyme Q10 is a more powerful antioxidant than lipid-soluble antioxidants such as vitamin E and is capable to oxidize vitamin E into a strong antioxidant [37]. It seems that CoQ10 can protect rooster spermatozoa from adverse effects oxidative stress on plasma membrane integrity and functionality.

Motility and viability of spermatozoa have a positive correlation with mitochondrial function and also shows a high correlation between the ATP concentrations, as an energy indicator, and motility [13]. In the avian, the production of energy for the motility of the spermatozoa provided by oxidative phosphorylation in the mitochondria rather than glycolysis [38]. So, a supply of molecular oxygen during *in vitro* storage semen, to more ATP production, is necessary. In addition, molecular oxygen is involved in the degradation of lipids through peroxidation [27]. Phospholipids, with a high peroxidizability index, are major components of avian sperm [38]. It has been shown that membrane-bound phospholipids are lost during *in vitro* storage of avian sperm, which in turn, decreased viability and plasma membrane integrity [27]. In studies performed on bovine [16], human [29] and cocks [36] as the current study

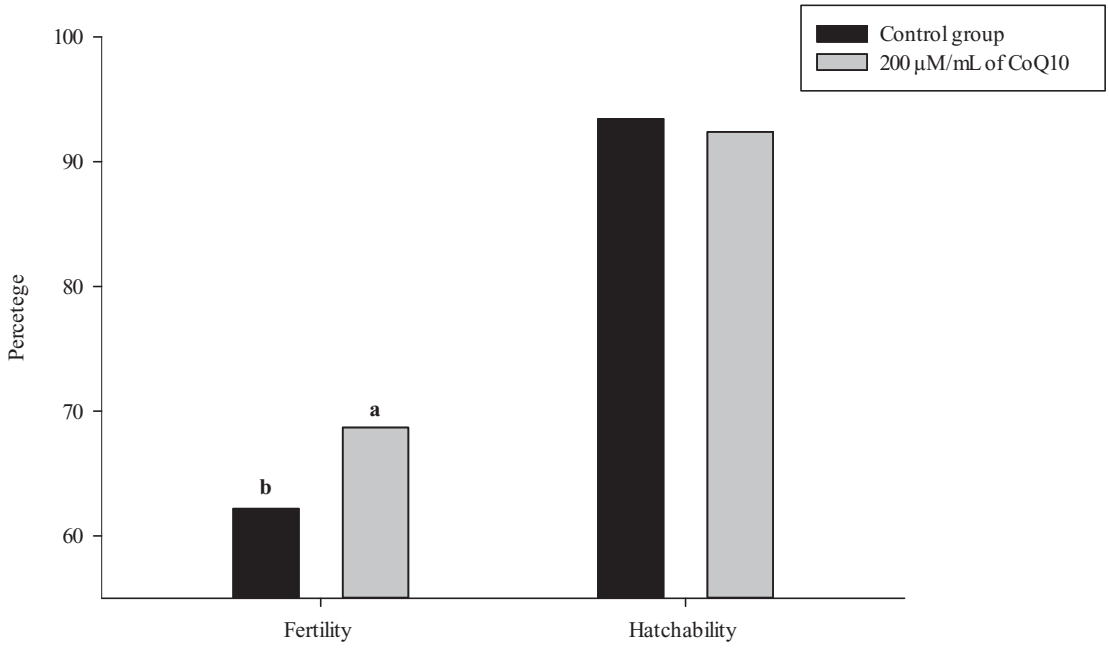

Fig. 5. Fertility (465 eggs/treatment) and hatchability (hatched eggs/fertilized eggs) rates following artificial insemination (200×10^6 sperm/hen) of hens (32 hens/treatment), by adding coenzyme Q10 (CoQ10; 0 or 200 μM CoQ10/mL) to the diluted rooster semen in experiment 2 (during 51–54 weeks of age). Fertility and hatchability rates were analyzed using chi-squared test. Different letters (a, b) represent significant differences in each parameter ($P < 0.05$).

showed that supplementation CoQ10 to semen extender improved plasma membrane integrity. So, it appears that since CoQ10 is the liposoluble antioxidant, it can diffuse directly in the poly-unsaturated lipid chains present in the plasma membrane and thus protect the sperm plasma membrane.

The storage avian semen for long periods at cooling conditions can induce ROS, a trigger of apoptosis through the oxidative stress, DNA fragmentation and diminution of fertility rate [20]. Coenzyme Q10 is able to clean free radicals that can induce damage to DNA proteins and lipids [39]. In the second experiment, although CoQ10 improved fertility by approximately 10%, hatchability and SP rates were not significantly altered by adding CoQ10 to the semen extender. In a study performed on the semen of Lohmann light breeder males showed that the fertility rate of semen diluted with the avian diluent significantly decreased 1 h after the ejaculate collection [8]. The effect of heat stress on spermatozoa may be one reason of decreased fertility rate. In the natural mating of avian, the semen as temperature as female vagina was ejaculated into the vagina by the rooster. But at AI, the diluted semen temperature was different with the vagina temperature. So, the semen may expose to heat stress. One of the defense systems of cells in response to injury during various stress conditions is the expression of heat shock proteins. Heat shock protein 70 is known to be induced in several stress conditions such as heat [40]. Xu et al. (2017) indicated that CoQ10 protected chicken heart cells from heat stress via the induction of Hsp70 expression. Moreover, Erata et al. (2008) proved that increased expression of Hsp70 of spermatozoa protected the cells of infertile men against apoptosis [41]. In this study, it seems that including CoQ10 to semen extender maybe has a protective role against heat stress during AI.

In conclusion, the outcomes of this study confirm that supplementation of CoQ10 (200 μM/mL) to semen extender rooster improved motility, sperm plasma membrane functionality and integrity, which in turn enhanced the fertility rate. So, enrichment of semen extender with CoQ10 might be potentially used to improve semen quality and fertilizing capacity of rooster sperm. However, we did not analyze some indicators such as the mitochondrial activity, antioxidant enzyme activities and lipid peroxidation, but basing on spermatozoa morphology and motility, CoQ10 addition to the rooster semen stored had a positive effect on improving the sperm quality, so further studies are necessary to determine the effects of CoQ10 on these indicators of the rooster semen stored.

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