Effect of the rooster semen enrichment with oleic acid on the quality of semen during chilled storage

M. Eslami,[*,1] A. Ghaniei,[†] and H. Mirzaei Rad[*]

[*]*Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; and*
[†]*Department of Poultry Diseases, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*

ABSTRACT Liquid storage of avian spermatozoa is currently being employed in programs utilizing the artificial insemination to optimize the management of genetically superior males. It is mandatory to use efficient semen storage techniques in order to prevent the reduction of the fertilizing ability of stored semen. The present study was designated to evaluate the effect of oleic acid on rooster semen quality stored at 4°C for 48 h. Semen was collected from 10 roosters twice a week. Good quality ejaculates were pooled and after dilution, the semen was enriched with 0 (control), 0.125 (O 0.125), 0.25 (O 0.25), 0.5 (O 0.5), and 1 (O1) millimolar oleate. Forward progressive motility and viability of spermatozoa were evaluated at 0, 24, and 48 h. Moreover, malondialdehyde (MDA) and total antioxidant activity (AOA) levels were measured in seminal plasma and spermatozoa at the mentioned time points. Motility was 80.33 ± 1.45, 80.00 ± 2.08, and 66.00 ± 2.30% at 24 h and 56.33 ± 1.45, 57.33 ± 2.18, and 41.33 ± 2.02% at 48 h in O 0.125, O 0.25, and control, respectively ($P < 0.001$). Total AOA concentrations of seminal plasma were significantly higher in oleate treated groups than the control at 24 and 48 h ($P < 0.03$). Moreover, concentrations of AOA in spermatozoa revealed that oleate treated group showed higher AOA values compared to the control group at 24 and 48 h ($P < 0.001$). MDA concentrations of seminal plasma and spermatozoa were lower in oleate treated groups in comparison with control group at 24 and 48 h ($P < 0.05$). In conclusion, rooster semen enrichment with low doses of oleate would exert beneficial effects on the quality of semen during cooled storage.

Key words: oleic acid, antioxidant activity, lipid peroxidation, semen, rooster

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INTRODUCTION

The practice of AI is now widely utilized in most domestic species including mammals and fowl; this requires a broad use of semen storage techniques, including liquid storage. Fertilizing ability of the undiluted fowl semen stored in vitro usually decreases a few h after collection (Blesbois et al., 1999). Therefore, it is important to develop an efficient system of semen preservation to maintain the fertilizing ability of stored semen (Gliozzi et al., 2003). It is necessary to dilute the spermatozoa in a buffered and osmotically equilibrated saline diluent (Clarke et al., 1982) to limit excessive energy expenditure in order to subsequently retain metabolism and fusion capability of the gametes.

An important factor in the fertilizing capability of sperm is the lipid content of their plasma membranes (Cerolini et al., 1997). Because avian sperm plasma membranes are high in polyunsaturated fatty acids (**PUFA**), spermatozoa are susceptible to lipoperoxidation by reactive oxygen species (**ROS**) during in vitro storage. An increased production of ROS is associated with male infertility (Surai and Sparks, 2001). Lipoperoxidation irreversibly abolishes the fructolytic and respiratory activity, which may thereby result in a considerable decline in their respiratory rate and motility (Surai and Sparks, 2001). Although the antioxidant activity (**AOA**) of sperm is low, enzymatic and non-enzymatic anti-oxidative compounds in seminal plasma are capable of protecting sperm against ROS (Zhao et al., 2011). Additionally, it was indicated that a lower level of total AOA occurs in the seminal plasma of infertile men when compared with fertile men (Lewis et al., 1995). In light of the result of the study, several approaches have been introduced to enhance semen quality and the anti-oxidative capacity of seminal plasma, including the use of caproic acid (MacPherson et al., 1977), pentobarbital (Fiser et al., 1978, 1980), vitamin E (Blesbois et al., 1993), carnitine (Neuman et al., 2002), inosine and adenine (Esashi et al., 1969), dried tomato pomace (Saemi et al., 2012), and dried ginger rhizome (Akhlaghi et al., 2014) in birds and ascorbic acid (Aurich et al., 1997), taurine (Alvarez and Storey, 1983), superoxide dismutase, catalase, trehalose, glutathione, (Maxwell and Stojanov, 1996; Bucak and Tekin, 2007) glutamine, and hyaluronan

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[1]Corresponding author: M.eslami@urmia.ac.ir

(Bucak et al., 2009) in other domestic animals. Nevertheless, improvement in semen quality still appears to be a challenge in poultry production.

The beneficial effects of oleic acid, the n-9 monounsaturated fatty acid with an 18-carbon chain, on AOA and signal transduction alteration during in vitro and in vivo experiments have been well documented (Parthasarathy et al., 1990; De Vries et al. 1997; Ruiz-Gutiérrez et al., 1999; Maedler et al. 2003; Menendez et al., 2006). The aim of the present study was to investigate the effect of oleic acid on the motility and survival of spermatozoa during chilled storage of rooster semen. To do this, the values of malondialdehyde (**MDA**), the indicator of lipid peroxidation, and total AOA were measured in seminal plasma and spermatozoa.

MATERIALS AND METHODS

Birds, Diets, and Semen Collection

Ten Ross 308 broiler breeder roosters were obtained from a local integrator, and maintained at 20 to 24°C during the experiment. The birds were 39 to 53 wk of age during the trial. The photoperiod 14L:10D was supplied and a standard commercial breeder male diet restricted to 110 g feed/d was fed and water was supplied ad libitum. During 3 wk of adoption, birds were trained to semen collection by the method of Lake [1957] and routinely ejaculated twice weekly thereafter. Collected semen samples (n = 58) were inspected to eliminate samples with debris, urine, or excessive particulate matter, and pooled for use. The care, housing, and use of roosters for this project were approved by the Animal Care of the Urmia University.

Treatment and Semen Evaluation

Each pooled semen sample (n = 9) was split into 5 equal aliquots to treat with different concentrations of the oleic acid. Semen was diluted by the phosphate buffer diluent (Wilcox et al., 1961) at a final concentration of 2×10^9 spermatozoa per mL. Then, diluted semen was supplemented with 0 (control), 0.125 (O 0.125), 0.25 (O 0.25), 0.5 (O 0.5), and 1 (O1) mmolar bovine serum albumin (**BSA**)-conjugated oleic acid. Following enrichment, the semen was stored for 48 h at 4°C. The percentage of forward progressive motility and viable spermatozoa was evaluated at 0, 24, and 48 h. Moreover, total AOA, MDA, and protein values were measured in the seminal plasma and the spermatozoa at the mentioned time points. Oleic acid was purchased from Sigma Company (O 1008, Sigma-Aldrich, USA) and other materials used in this project were bought from the Merck Company, Germany.

Forward progressive motility of spermatozoa was assessed by placing a portion of ejaculate diluted with 2.9% sodium citrate solution (1:200) on a slide covered with a coverslip, using an Olympus (BX41, Tokyo, Japan) compound light microscope (400× magnification), equipped with a warm stage.

The percentage of viable spermatozoa was evaluated, using a portion of ejaculate stained with eosin-nigrosin solution (Bakst and Cecil, 1997). The stained seminal smear was prepared in duplicate, and 200 spermatozoa per slide were evaluated. The slides were evaluated for viability, where unstained spermatozoa were considered as live. Concentration of spermatozoa was determined in duplicate, using a Neubauer hemocytometer.

Spermatozoa and seminal plasma were separated by centrifugation after evaluation of semen quality. The semen was centrifuged for 10 min at 550 g. The resulting pellet was then termed spermatozoa. The supernatant was centrifuged twice more, first for 10 min at 550 g, then for 30 min at 3,000 g. The resulting supernatant was called the seminal plasma (Blesbois et al., 1993). The pellet of spermatozoa re-suspended in 1 mL phosphate buffer saline (**PBS**), then 50 μl trichloroacetic acid 50% was added to the PBS in order to break down the spermatozoa membrane.

Preparation of BSA Conjugated Oleic Acid

Oleic acid was conjugated with BSA according to the method described by Van Harken et al. (1969) for better utilization of oleic acid by spermatozoa. In brief, BSA solution (24%) was prepared in NaCl 0.09%. Then, 0.12 g oleic acid was dissolved in 3 mL ethanol 95%. Sodium-oleate was prepared by dissolution of 0.13 g dissolved oleate in 15 mL NaCl 0.09%. Finally, sodium-oleate was mixed with BSA solution and stored in the freezer −200°C until usage.

Total AOA Values in the Spermatozoa and Seminal Plasma

Total AOA values were measured according to Koracevic et al., (2001) with a slight modification. In brief, 490 μl PBS solution was added to 10 μl sample. Additionally, sodium benzoate, acetic acid, Fe-EDTA, and H2O2 were added to the tubes, respectively. Tubes were incubated in 37°C for 60 min. Later, acetic acid and thiobarbituric solution were added to the tubes. Finally, the tubes were incubated in a 100°C water bath for 10 min. The absorbance of the samples was measured at 532 nm wavelength. Total AOA values were expressed as mmol/g protein in the spermatozoa and seminal plasma samples.

Concentrations of MDA in the Spermatozoa and Seminal Plasma

Concentrations of MDA were measured using the thiobarbituric acid reaction, in accordance with the method described by Frederick (2010). Briefly, spermatozoa suspension or seminal plasma was added to

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thiobarbituric solution, mixed, then double-distilled water was added and shaken. Tubes were heated in boiling water, then cooled and centrifuged for 10 min in 1,000 g. The absorbance of the upper layer was read at 532 nm wavelength. Concentrations of MDA were expressed as μmol/g protein in the spermatozoa and seminal plasma samples.

Total Protein Measurement in Spermatozoa and Seminal Plasma

Total protein was evaluated based on Bradford protocol (1976). Briefly, Bradford reagent and stock solution of BSA were prepared, then Bradford regent was added to the spermatozoa suspension and seminal plasma samples. The absorbance of the test tubes was measured at 595 nm wavelength after 15 min. Concentration of total protein was evaluated according to the standard plot.

Statistical Analysis

Mean values of motility, viability, MDA, and AOA were analyzed using a one-way analysis of variance, followed by the Tukey's post-hoc test to determine significant differences in all the parameters among groups using the SigmaStat computer program (Version 3.5; Chicago, IL). Changes in motility, viability, MDA, and AOA levels over time were analyzed through Repeated Measure ANOVA to reveal the differences among different time points in any treatment group. Results were presented as the mean ± standard error. Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Motility

Time of sampling and interaction between time of sampling and treatment group were significantly associated ($P < 0.05$) with forward progressive motility. Motility was higher at 0 h (92.00 ± 0.56%) than the 24 h (71.13 ± 2.15%) and 48 h (50.53 ± 1.71%) time points ($P < 0.001$); moreover, the percent of forward progressive motility was lower at 48 h compared to 24 h ($P < 0.001$). The interaction between time of sampling and treatment group indicated that the percent of forward progressive motility was higher in O 0.125 (80.33 ± 1.45%) and O 0.25 (80.00 ± 2.08%) groups than the control (66.00 ± 2.30%) group at 24 h ($P < 0.001$; Figure 1), while there were no differences among O 0.5 (65.00 ± 2.30%), O 1 (64.33 ± 2.96), and the control group at 24 h ($P > 0.05$; Figure 1). Moreover, forward progressive motility was higher in oleate treated groups than the control group at 48 h ($P < 0.05$; Figure 1). Within-group analysis showed that motility

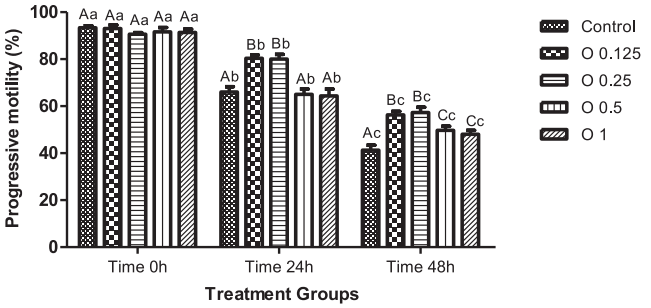

Figure 1. Forward progressive motility of rooster spermatozoa following treatment with different concentrations of oleic acid at 0, 24, and 48 h of experiment. [A,B,C]Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point. [a,b,c]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

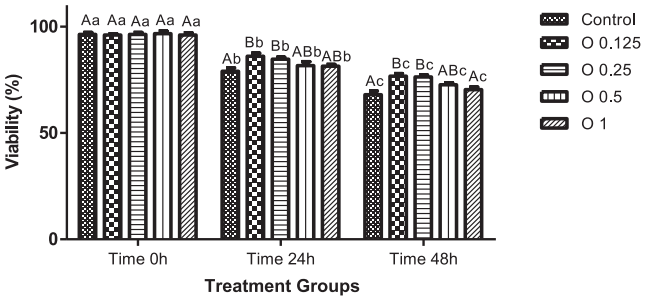

Figure 2. Live percentage of spermatozoa of rooster semen following treatment with different concentrations of oleic acid at 0, 24, and 48 h of experiment. [A,B] Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point. [a,b,c]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

decreased during the experiment in all treated groups ($P < 0.05$; Figure 1).

Viability

Time of sampling and interaction between time of sampling and treatment group were associated ($P < 0.05$) with the percent of viability. Viable spermatozoa was higher ($P < 0.001$) at 0 h (96.26 ± 0.35%) in comparison with 24 h (82.536 ± 0.83%) and 48 h (72.80 ± 1.01%); furthermore, significant differences were observed between 24 and 48 h ($P < 0.001$). The interaction between time of sampling and treatment group showed that the percent of the viable spermatozoa did not differ among treated groups at 0 h ($P > 0.05$; Figure 2), while the percent of viability was higher in O 0.125 (86.00 ± 1.52%) and O 0.25 (84.66 ± 0.88%) compared to the control (79.00 ± 1.48%) at 24 h ($P = 0.041$; Figure 2). Moreover, the percent of the viable spermatozoa was higher in O 0.125 (76.66 ± 1.20%) and O 0.25 (76.33 ± 0.88%) groups compared to the control (68 ± 1.73%) and O 1 (70.33 ± 1.20%) groups at 48 h ($P = 0.002$; Figure 2). Within-group analysis showed that the percent of viable spermatozoa was lower at 24 h compared to 0 h,

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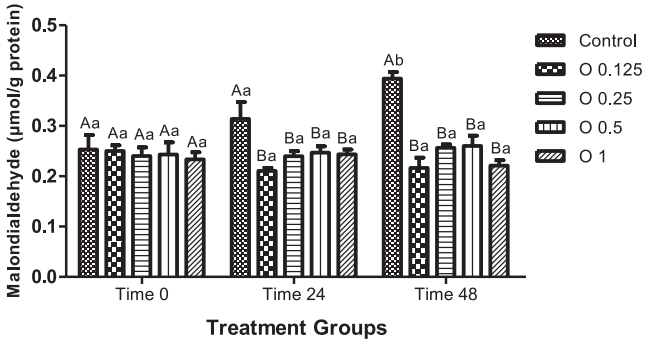

Figure 3. Malondialdehyde concentrations (μmol/g protein) in seminal plasma of rooster following treatment with different concentrations of oleic acid. [A,B]Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point. [a,b]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

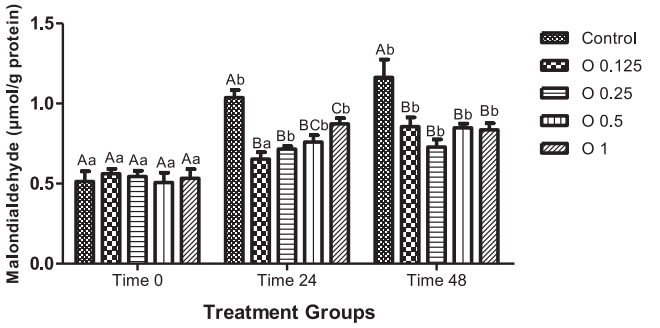

Figure 4. Malondialdehyde concentrations (μmol/g protein) of rooster spermatozoa following treatment with different concentrations of oleic acid. [A,B,C]Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point. [a,b]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

and 48 h compared to 24 h in all treated groups ($P < 0.001$; Figure 2).

MDA Values (μmol/ g protein) in Seminal Plasma

Treatment group and interaction between treatment group and time of sampling were significantly associated ($P < 0.05$) with MDA levels. Concentrations of MDA were significantly lower in oleate treated groups compared to the control group ($P < 0.001$). The interactions between time of sampling and treatment group showed that MDA levels did not differ among treated groups at 0 h, while MDA concentrations were lower in oleate treated groups in comparison with the control group at 24 and 48 h ($P < 0.019$; Figure 3). Within-group analysis revealed that MDA levels were higher at 48 h (0.39 ± 0.01) compared to the 0 (0.25 ± 0.02) and 24 h (0.31 ± 0.03) in the control group ($P = 0.023$; Figure 3), but there were no significant differences observed in oleate treated groups among different time points ($P > 0.05$; Figure 3).

MDA Values (μmol/ g protein) in Spermatozoa

Time of sampling and interaction between time of sampling and treatment groups were associated significantly ($P < 0.05$) with MDA levels. Treatment group was not associated with MDA levels ($P = 0.096$). MDA levels were higher ($P < 0.001$) at 24 h (0.80 ± 0.03) and 48 h (0.88 ± 0.04) compared to the 0 h (0.53 ± 0.02). The interactions between time of sampling and treatment group showed that MDA levels did not differ among the treated groups at 0 h ($P > 0.05$; Figure 4), whereas MDA levels were lower in oleate treated groups compared to the control group at 24 h ($P < 0.001$; Figure 4) and 48 h ($P = 0.007$; Figure 4). Within-group analysis showed that MDA levels were higher at 48 h than the 0 h in all treated groups ($P < 0.05$; Figure 4).

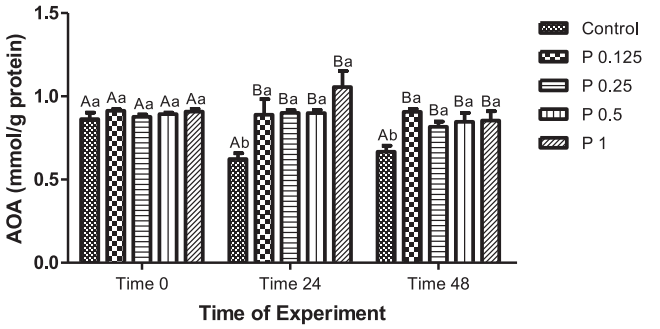

Figure 5. Total antioxidant activity (AOA) levels (mmol/g protein) in seminal plasma of rooster following treatment with different concentrations of oleic acid. [A,B]Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point.[a,b]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

Total AOA Levels (mmol/ g protein) in Seminal Plasma

Treatment group, and interaction between time of sampling and treatment group were associated ($P < 0.05$) with AOA values in the seminal plasma. Moreover, time of sampling tended to be associated with MDA levels ($P = 0.088$). Total AOA values were higher in oleate treated groups than the control group ($P < 0.001$). The interaction between time of sampling and treatment group showed that there were no significant differences of AOA values among groups at 0 h, whereas AOA levels were higher in oleate treated groups than the control group at 24 h ($P = 0.009$; Figure 5) and 48 h ($P = 0.022$; Figure 5). Within-group analysis indicated that total AOA values were lower ($P = 0.032$) at 24 h (0.62 ± 0.03) and 48 h (0.66 ± 0.03) in comparison with 0 h (0.086 ± 0.03) in the control group, while there were no significant differences observed in the other treated groups ($P > 0.05$; Figure 5).

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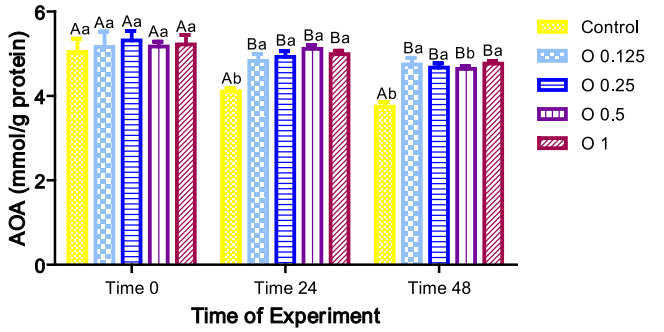

Figure 6. Total antioxidant activity (AOA) levels (mmol/g protein) in spermatozoa of rooster following treatment with different concentrations of oleic acid. [A,B]Values with different superscripts indicate significant ($P < 0.05$) differences among groups at each time point.[a,b]Values with different superscripts indicate the significant ($P < 0.05$) difference over time within experimental groups.

Total AOA Levels (mmol/ g protein) in Spermatozoa

Time of sampling, treatment group, and interaction between time of sampling and treatment group were significantly associated ($P < 0.05$) with the AOA values in the spermatozoa. Higher values of total AOA were observed at 0 h (5.18 ± 0.10) compared to 24 h (4.79 ± 0.10) and 48 h (4.51 ± 0.11) time points ($P < 0.001$). Additionally, the total AOA values were higher in oleate treated groups in comparison with the control group ($P = 0.004$). The interaction between time of sampling and treatment group revealed that there were no significant differences for AOA values among groups at 0 h, while the AOA values were higher in oleate treated groups compared to the control group at 24 h ($P < 0.001$; Figure 6) and 48 h ($P < 0.001$; Figure 6). Within-group analyses indicated that the AOA values were higher ($P = 0.012$; Figure 6) at 0 h (5.04 ± 0.31) compared to 24 h (4.10 ± 0.07) and 48 h (3.74 ± 0.11) in the control group, whereas there were no significant differences among different time points in the oleate treated groups except in the O 0.5 group between 0 and 24 h with 48 h (P = 0.041; Figure 6).

DISCUSSION

The purpose of the present study was to investigate the effect of oleic acid on the forward progressive motility of the spermatozoa, percent of viability, and concentrations of MDA and AOA in rooster semen. The present experiment showed that: 1) oleic acid would increase the percent of the forward progressive motility compared to the control group; 2) the total AOA values of the seminal plasma and the spermatozoa were significantly higher in the oleate treated groups in comparison with the control group; and 3) semen enrichment with oleate decreased the lipid peroxidation of seminal plasma and spermatozoa compared to the control group.

Chicken spermatozoa are characterized by comparatively high levels of 20:4 n-6 and 22:4 n-6 fatty acids within their phospholipids (Blesbois et al., 1997). As a result of this high proportion of PUFAs, chicken semen is susceptible to lipid peroxidation (Surai et al., 1997), which can lead to spermatozoa deterioration during storage (Surai et al., 1998). It has been reported that, as a likely result of high proportions of PUFAs, avian spermatozoa showed a significant susceptibility to lipid peroxidation, which was associated with the loss of viability, motility, and fertilizing ability of the spermatozoa in vitro (Wishart, 2004). Antioxidant nutrients are important for limiting damaging oxidative reactions in cells, which may predispose to the development of major clinical conditions such as oxidative stress disorders. It has been reported that the application of taurine to the semen of goat caused a decline in the MDA concentrations compared to the controls (Ateşşahin et al., 2008). Moreover, Neuman et al. (2002), stated that the MDA levels in spermatozoa were decreased by feeding carnitine to roosters. Additionally, administration of phosphatidylcholine to the semen of turkey reduced the harmful effects of lipid peroxidation during semen storage (Long and Conn, 2012). Also, semen enrichment with vitamin C and vitamin E (Amini et al., 2015a) and catalase (Amini et al., 2015b) reduced the concentrations of MDA in post-thawed rooster sperm. On the other hand, the addition of glutathione, oxidized glutathione, or cysteine to ram semen (Bucak et al., 2008) and curcumin, inositol, and carnitine to goat semen (Bucak et al, 2010) did not affect the levels of MDA compared to the control, while simultaneous administration of ethylene glycol and cysteine to bull semen caused an increase in the MDA levels (Büyükleblebici et al., 2014). In the present experiment oleic acid reduced the MDA levels in the seminal plasma and spermatozoa. It seems that the semen enrichment with the antioxidants may cause a decrease or an increase or have no effect on the MDA levels according to the animal species, types of usage (in vivo or in vitro), and the type of antioxidant.

The present experiment showed that semen enrichment with oleate caused an increase in total AOA levels of the spermatozoa and seminal plasma compared to control at 24 h and 48 h. The present study would be in line with the published literature about the improvement of AOA of semen following administration of dried ginger rhizome to the diet of aged roosters (Akhlaghi et al., 2014). Additionally, semen supplementation with antioxidants taurine and cysteine increased the AOA levels in ram (Bucak and Tekin, 2007; Bucak et al., 2008). Moreover, the result of the present experiment was similar to the studies performed on the antioxidant enzymatic activity of dog and sheep semen where a positive effect of superoxide dismutase activity on the semen quality was reported (Marti et al., 2003; Cassani et al., 2005). Our result was in contrast with the findings of Bucak et al. (2009) who revealed that supplementation of goat semen with the antioxidants glutamine and hyaluronan did not elevate the AOA. Previous studies showed that the increase in somatic cell

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antioxidant indices following the treatment with monounsaturated fatty acids could be a result of their potency to elevate essential enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase (Narang et al., 2004). Moreover, it was reported that monounsaturated fatty acids could strengthen the antioxidant defense and PI3 kinase levels, which are important factors in the viability of the cells (Oudit et al., 2004). Hence, it seems to be indicative to evaluate the activities of the antioxidant enzymes following administration of oleate to the semen (Brown and Borutattie, 2007).

The present experiment showed that oleate decreased the levels of MDA, increased the AOA levels, and finally improved the forward progressive motility of rooster spermatozoa. Cytoprotective effects of oleic acid have been reported in somatic cells (De Vries et al., 1997; Maedler et al., 2003; Yamasaki et al., 2008). One of the most important factors contributing to poor quality semen such as a low motility rate, has been associated to high MDA levels (Bucak et al., 2010). Supplementation with antioxidants has been proven to maintain the viability and motility of liquid or cryopreserved sperm cells of several species, e.g., the ram (Maxwell and Stojanov, 1996; Bucak et al., 2008), goat (Bucak et al., 2010), bull (Foote et al., 2002), dog (Cassani et al., 2005), red deer (Fernández-Santos et al., 2009), turkey (Donoghue and Donoghue, 1997), and rooster (Blesbois et al., 1993; Akhlaghi et al., 2014; Amini et al., 2015a,b). Recent studies showed that supplementation of broiler breeder diets with biotin (Daryabari et al., 2015) and canthaxanthin (Rosa et al., 2012) improved the fertility rate. Oleic acid might therefore provide protection in rooster spermatozoa via reducing damage to the spermatozoa and enhancing AOA levels.

In conclusion, enrichment of semen with oleic acid would decrease the harmful effects of the lipid peroxidation, as measured by MDA, in rooster seminal plasma and spermatozoa. Moreover, oleic acid would increase the antioxidant levels at both seminal plasma and spermatozoa levels; thus, it could be considered as an antioxidant additive during the storage of fowl semen.

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