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# Evaluation of linoleic acid on lipid peroxidative/antioxidative parameters, motility and viability of rooster spermatozoa during cold storage

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**Abstract** Sustainable reduction in semen quality due to detrimental effects of primary and secondary peroxidative products was occurred during liquid storage. The objective of the current experiment was to explore the influence of bovine serum albumin conjugated linoleic acid (LA) on the rooster spermatozoa routine tests and lipid peroxidative/antioxidative levels stored at 4 °C over 48 h. For this purpose, collected ejaculates (≥ 80% progressive motile spermatozoa) pooled and extended with the phosphate buffer medium without (control) or enriched with different amounts of LA (0.125, 0.25 or 0.50 mM). Contents of total antioxidant status (TAS) and thiobarbituric reactive substances (TBARS) were measured separately in the medium and spermatozoa, as well as percent of viability and motility at 0, 24 and 48 h intervals. Viability was not affected by treatment during the study intervals ($P > 0.05$). While, higher motility was recorded in LA 0.50 mM group (77.98 ± 1.89 and 57.02 ± 2.45) compared to the control group (68.78 ± 1.29 and 45.09 ± 1.86) at 24 and 48 h, respectively ($P < 0.03$). Amounts of TBARS in medium and spermatozoa were lower in LA 0.25 and 0.50 groups compared to the control at 48 h ($P < 0.01$). Moreover, TAS levels of medium and spermatozoa were lower in control samples compared to LA treated groups at 48 h ($P < 0.03$). Because of the ability of the LA to lowering the quantities of lipid peroxidation index and improving motility especially at 0.5 mM levels, it can be proposed as an additive during liquid storage of rooster semen.

**Keywords** Lipid peroxidation · Linoleic acid · Rooster · Spermatozoa

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## Introduction

Polyunsaturated fatty acids (PUFAs) have 2–5 double bonds within their structures and divided into three classes: omega-3 (n-3), omega-6 (n-6) and omega-9 (n-9). The n-3 (such as α-linolenic acid) and n-6 (such as linoleic acid) cannot be synthesis de novo, because of the inability of specific desaturase enzymes in the animals, then, should be provided by diet (Paulenz et al. 1995). These mentioned PUFAs are necessary for development of central nervous system, eyesight,

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reproduction and body growth (Gurr et al. 2001). Moreover, the n-6 PUFAs of the spermatozoa membrane have a central role in flexibility and fluidity of spermatozoa, which are necessary in normal motility and final fusion of sperm-oocyte (Surai et al. 2001).

Previous report indicated the relationship between fertilizing ability of the rooster spermatozoa and lipid characters and amounts in the spermatozoa membrane (Cerolini et al. 1997). Moreover, avian spermatozoa are predispose to lipid peroxidation (LPO) induced by free radicals because of high amounts of PUFAs in the membrane (Surai et al. 2001). Because spermatozoa lose most of their cytoplasm contents during maturation phase, naturally encounter lack of antioxidant capacity and consequently be susceptible to deleterious effects of LPO (Zhao et al. 2011), especially during in vitro storage. High amounts of PUFAs in the spermatozoa cell membrane and possible high risk of LPO necessitates the presence of a sufficient amounts of antioxidant power to preserve the spermatozoa against injury due to intermediates and end products of the peroxidation process (Wishart 1984; Alvarez and Storey 1989). The mark sustainable decrease in quality of semen was reported when it kept at low temperature as liquid form (De Lamirande et al. 1997). Lewis et al. (1995) reported that amounts of total antioxidant status (TAS) in the semen of infertile men were less compared to the normal fertile men. Previous studies tried to fed different PUFAs and vitamin sources such as arachidonic acid (Surai et al. 2000), α-linolenic acid (Kelso et al. 1997), fish oil, docosahexaenoic acid and vitamin E (Cerolini et al. 1999, 2006) or enrich the medium (extender) by adding materials with antioxidant properties such as oleic acid (Eslami et al. 2016), palmitoleic acid (Rad et al. 2016), vitamin E (Blesbois et al. 1993), vitamin C (Amini et al. 2015), selenium nanoparticles (Safa et al. 2016) and sodium pentobarbital (Fiser et al. 1978) to reduce the risk of LPO and its detrimental consequences on avian semen. Little number of studies investigated the effects of in vitro administration of unsaturated fatty acids on avian semen quality (Eslami et al. 2016; Rad et al. 2016). Moreover, the effect of adding linoleic acid (LA) on rooster semen quality during cold storage was not investigated. Therefore, the current experiment was designed to evaluate rooster semen supplementation with different amounts of LA on thiobarbituric reactive substances (TBARS; the known end product of lipid peroxidation process) and total antioxidant capability of spermatozoa and medium, as well as viability and motility during storage at refrigerator temperature for up to 48 h.

## Materials and methods

### Roosters, diets and semen collection

The 40-week old Ross 308 broiler breeder roosters (n = 12) were used and kept at 22–24 °C during the experiment. Roosters were fed a standard commercial diet (110 g/day/head), had free access to water and were exposed to 14 h light per day during the experiment. They were trained to semen collection method by abdominal massage previously described by Lake (1957) in a 3-week period. After that, ejaculates collection carried out by the same person two times in a week. Samples contaminated with urine, blood or debris were excluded from the experiment.

### Ejaculate evaluation and treatment

At the ejaculate collection day, the same volume of ejaculates with ≥ 80% forward progressive motility were pooled and used for the experiment. Ten pooled ejaculates were used in the present experiment (each pooled sample contains at least ten individual ejaculates). The phosphate buffer extender (medium) was used to dilute the sample at a final concentration of $1 \times 10^9$ spermatozoa per mL (Wilcox et al. 1961). Neubauer hemocytometer was used to calculate the number of spermatozoa. Diluted semen sample supplemented with 0.125 (LA 0.125), 0.25 (LA 0.25), 0.50 (LA 0.50) mM bovine serum albumin (BSA)-conjugated LA (L 2376, Sigma-Aldrich) or served as control. Treated samples preserved at refrigerator for up to 48 h. Percentage of viability and forward progressive motile spermatozoa were evaluated in control and LA treated samples at 0, 24 and 48 h of storage. On the other hand, amounts of TBARS, total antioxidant status (TAS) and total protein were measured separately in the medium and spermatozoa at the mentioned intervals.

The viability of rooster spermatozoa in the treated samples was assessed by eosin-nigrosin staining previously described by Bakst and Cecil (1997). After preparation of smear, at least two hundred

spermatozoa were evaluated under light microscope (1000 ×, oil immersion). Unstained spermatozoa were considered as live cells and spermatozoa showing purple color were considered non-viable cells.

To evaluate forward progressive motility, sample was diluted ($25 \times 10^6$) with sodium citrate solution, then a portion of sample placed on a slide, covered with a coverslip. Samples were assessed by using a phase contrast microscope (Olympus, BX41, Japan; 400 × magnification) equipped with a warm stage. In the present experiment, spermatozoa showing moderate to rapid forward movement were considered as progressive forward motile class (Ommati et al. 2013). At least two hundred spermatozoa were evaluated for each sample.

After evaluation of viability and motility, spermatozoa separated from the medium by centrifugation (Blesbois et al. 1993). In order to re-suspend pellet of spermatozoa, 500 µL sodium chloride (0.9%) was mixed with the pellet. Finally, to break the spermatozoa membrane, trichloroacetic acid 50% was added and mixed well. Medium and spermatozoa suspension samples were preserved at freeze temperature (− 20 °C) until measurement of TBARS, TAS and total protein.

### Preparation of BSA conjugated linoleic acid

In order to optimize utilization of linoleic acid by rooster spermatozoa, the method of Van Harken et al. (1969) was used to conjugate the linoleic acid with BSA. Initially, linoleic acid was dissolved in ethanol to prepare linoleic acid solution. Twenty four percent's of BSA solution was provided by sodium chloride 0.9%. Then, a definite volume of linoleic acid solution was dissolved in sodium chloride 0.9% to provide sodium-linoleate. Finally, BSA was added to sodium-linoleate and mixed completely, then stored at − 20 °C until usage.

### Measurement of TBARS in the medium and spermatozoa

To measure TBARS quantities, reagent of trichloroacetic acid, thiobarbituric acid and hydrochloric acid was prepared according to the reference (Frederick 2010). Then, two milliliters of mentioned reagent were mixed with 200 µL sample in a test tube. Tubes were immersed in a boiling water for 15 min. In the next step, tubes centrifuged for 10 min at 1500 *g*. the optical density of the supernatant was recorded at wavelength of 532 nm. In the result section, TBARS levels were shown as µmol/g protein.

### Amounts of TAS in medium and spermatozoa

In order to determine TAS levels, PBS, sodium benzoate, acetic acid, Fe-EDTA and $H_2O_2$ were added, respectively to the 10 µL sample and mixed completely (Koracevic et al. 2001). After that, tubes were immersed in a water bath (37 °C) for 1 h. Then, thiobarbituric solution was added to the test tubes. After 10 min incubation at 100 °C, the optical density of the tube contents was read at wavelength of 532 nm. Amounts of TAS were reported as mmol/g protein in the medium and spermatozoa samples.

### Measurement of total protein in the medium and spermatozoa

To measure amounts of protein in samples, Bradford reagent was prepared according to the mentioned protocol (Bradford 1976). Bradford reagent was mixed with sample in a tube and incubated for 15 min at room temperature. Then, the optical density of the resultant mixture was recorded at 595 nm wavelength. Different concentrations of BSA were used as standard plot to estimate the protein concentrations in the samples.

### Statistical analysis

Analyses were carried out by using SigmaStat software (Version 3.5; Chicago, IL). Values of different parameters are presented as the mean ± standard error. Arcsine transformation carried out on percentage data. The data obtained were analyzed by one way ANOVA and Tukey's post hoc test to reveal differences among groups at any measurement time. Moreover, changes of variable among 0, 24 and 48 time points in a group were analyzed using a Repeated Measure ANOVA. The level of significance was set at $P < 0.05$.

## Results

### Viability

Analysis showed no significant differences among groups in spermatozoa viability during the study periods ($P > 0.05$; Table 1). Lower percent of viability was observed at 48 h compared to 0 h in all groups ($P < 0.001$; Table 1).

### Motility

Forward progressive motility was not significantly differed among groups at 0 h ($P > 0.05$; Table 1), while it was greater in LA 0.25 and 0.50 mM at 24 h and in LA 0.50 mM at 48 h compared to the control sample ($P < 0.03$; Table 1). As observed in viability, motility was less at 48 h compared to 0 h in all treated groups ($P < 0.001$; Table 1).

### TBARS at medium (μmol/g protein)

Analysis showed that the indictor of lipid peroxidation did not differ among groups at 0 h ($P > 0.05$; Fig. 1). Amounts of TBARS were significantly lower in LA treated groups compared to the control group at 24 h of storage ($P < 0.001$; Fig. 1). Moreover, higher amounts of TBARS were indicated in control group compared to the LA 0.25 and 0.50 groups at 48 h ($P = 0.001$; Fig. 1). Within group analysis revealed higher amounts of TBARS at 24 and 48 h compared to 0 h in control group ($P < 0.001$; Fig. 1), while no significant changes were recorded among different time points in LA treated groups ($P > 0.05$; Fig. 1).

### TBARS at spermatozoa (μmol/g protein)

Amounts of TBARS in spermatozoa did not show any significant differences among groups at 0 and 24 h of storage ($P > 0.05$; Fig. 2). While, lower levels of TBARS were detected in LA 0.25 and 0.50 groups compared to the control one at 48 h ($P = 0.01$; Fig. 2). No significant changes were detected in TBARS levels among different time points in LA treated groups ($P > 0.05$; Fig. 2), but, greater amounts of TBARS were detected at 48 h compared to 0 and 24 h in the control group ($P = 0.006$; Fig. 2).

### TAS at medium (mmol/g protein)

Amounts of TAS showed any noticeable differences among groups at 0 h ($P > 0.05$; Fig. 3), while TAS levels were significantly lower in control samples compared to the LA treated samples at 24 and 48 h of storage ($P < 0.002$; Fig. 3). Lower levels of TAS were recorded at 48 h compared to 0 h in control and LA 0.50 groups ($P < 0.03$; Fig. 3). No significant changes were observed in TAS levels of LA 0.125 and 0.25 groups among different time points ($P > 0.05$; Fig. 3).

**Table 1** Influence of medium enrichment with linoleic acid (LA) on percent of viability and forward progressive motility of rooster spermatozoa stored for different time points at refrigerator temperature

| Parameter | Treatment | Time of storage (h) | | |
|---|---|---|---|---|
| | | 0 | 24 | 48 |
| Viability | Control | $92.41 \pm 2.82^{a}$ | $79.67 \pm 3.40^{b}$ | $62.81 \pm 3.11^{c}$ |
| | LA 0.125 mM | $93.80 \pm 2.22^{a}$ | $83.83 \pm 1.70^{b}$ | $66.55 \pm 1.02^{c}$ |
| | LA 0.25 mM | $89.92 \pm 1.87^{a}$ | $84.75 \pm 3.23^{a}$ | $64.72 \pm 2.66^{b}$ |
| | LA 0.50 mM | $90.30 \pm 2.74^{a}$ | $80.69 \pm 3.06^{ab}$ | $68.87 \pm 3.54^{b}$ |
| Forward progressive motility | Control | $89.27 \pm 1.62^{Aa}$ | $68.78 \pm 1.29^{Ab}$ | $45.09 \pm 1.86^{Ac}$ |
| | LA 0.125 mM | $86.07 \pm 2.52^{Aa}$ | $76.10 \pm 2.90^{ABa}$ | $47.63 \pm 2.77^{ABb}$ |
| | LA 0.25 mM | $87.33 \pm 3.03^{Aa}$ | $82.78 \pm 2.86^{Ba}$ | $51.26 \pm 2.03^{ABb}$ |
| | LA 0.50 mM | $85.02 \pm 1.89^{Aa}$ | $77.98 \pm 1.89^{Ba}$ | $57.02 \pm 2.45^{Bb}$ |

$^{A,B}$Values with different superscripts indicate difference ($P < 0.05$) among groups at each time point

$^{a,b,c}$Values with different superscripts indicate significant difference ($P < 0.05$) between the data at the same raw

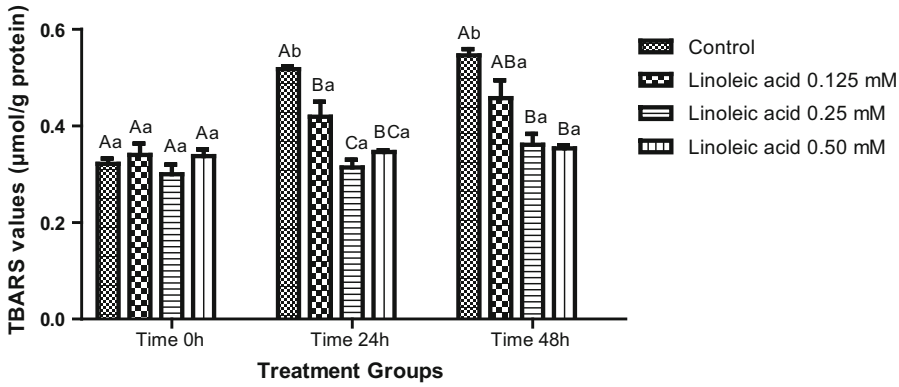


**Fig. 1** Quantities of thiobarbituric acid reactive substances (TBARS; µmol/g protein) in medium following treatment with different amounts of linoleic acid. [A,B,C]Values with different letters indicate difference ($P < 0.05$) among experimental groups at each time point. [a,b]Values with different letters indicate difference ($P < 0.05$) over time within the experimental groups

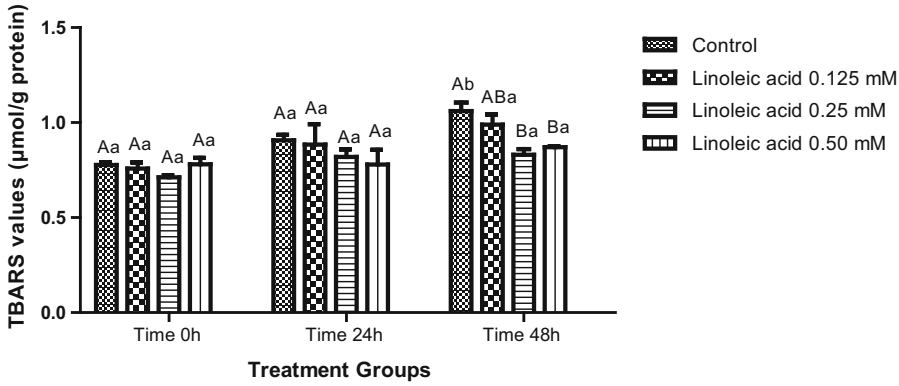


**Fig. 2** Quantities of thiobarbituric acid reactive substances (TBARS; µmol/g protein) in rooster spermatozoa following treatment with different amounts of linoleic acid. [A,B]Values with different letters indicate difference ($P < 0.05$) among experimental groups at each time point. [a,b]Values with different letters indicate difference ($P < 0.05$) over time within the experimental groups

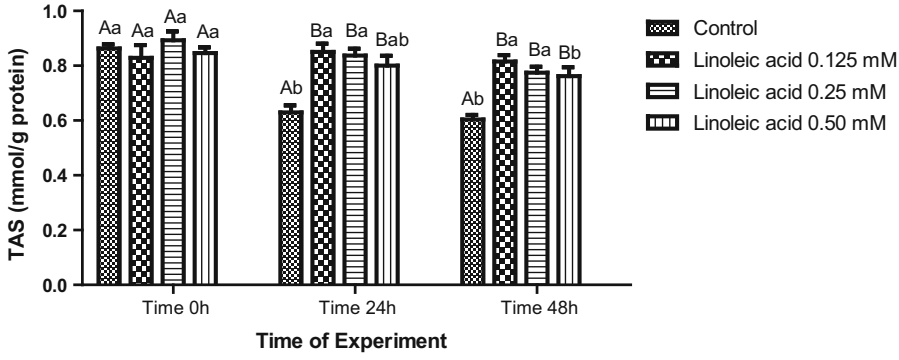


**Fig. 3** Amounts of total antioxidant status (TAS; mmol/g protein) in medium following treatment with different levels of linoleic acid. [A,B]Values with different letters indicate difference ($P < 0.05$) among experimental groups at each time point. [a,b]Values with different letters indicate difference ($P < 0.05$) over time within the experimental groups

TAS at spermatozoa

There were no differences among group in TAS levels at 0 h ($P > 0.05$; Fig. 4). Higher amounts of TAS values were recorded in LA 0.25 and 0.50 at 24 h and in all LA treated groups at 48 h compared to the control ($P < 0.001$; Fig. 4). Amounts of TAS were greater at 48 h compared to 0 h in LA treated groups ($P < 0.001$; Fig. 4).

## Discussion

The objective of the current experiment was to evaluate the effectiveness of BSA-conjugated LA on preservation of rooster spermatozoa during storage at refrigerator temperature. The important and undeniable role of lipid profiles of the cell membrane on spermatozoa function such as fluidity, flexibility, attachment to oocyte, signal transduction and fertility in fresh or cold semen have been well documented (Bearer and Friend 1982; Hammerstedt 1993). Due to presence of high amounts of PUFAs in avian and mammals spermatozoa cell membrane, it is very susceptible to lipid peroxidation event and the subsequent harmful effects (Howarth et al. 1977; Ravie and Lake 1985). In order to protect spermatozoa against peroxidative damage and associated spermatozoa dysfunction, especially during in vitro storage, it is necessary to provide an efficient antioxidant source (Alvarez and Storey 1989). It was previously reported that TBARS is more chemically stable and membrane permeable than the other primary and secondary lipid peroxidation products (Esterbauer et al. 1991). Thus, in the present work, TBARS was measured to evaluate the extent of lipid peroxidation and its association to spermatozoa motility. In the present study rooster semen enrichment with LA at 0.25 and 0.5 mM levels lowered the TBARS amounts of medium 39% and 33% at 24 h and 34% and 35% at 48 h compared to the control, respectively. Moreover, in comparison to control group, 22% and 18% reduction in intracellular TBARS levels were observed in 0.25 and 0.5 mM LA treated groups at 48 h of storage, respectively. Previous research indicated that supplementation of rooster spermatozoa medium with different doses of palmitoleic and oleic acid decreased the intracellular amounts of secondary end product of lipid peroxidation process at 24 and 48 h of storage (Eslami et al. 2016; Rad et al. 2016). In an experimental model, linoleic acid showed lower susceptibility to lipid peroxidation than the other isomers of conjugated linoleic acid (Fagali and Catalá 2008). In another experiment, ram semen enrichment with kinetin resulted in lower TBARS levels compared to the control samples (Zadeh Hashem and Eslami 2018). However, not all substances with antioxidative properties affect TBARS levels when added to the semen in comparison with their control (Bucak et al. 2008, 2010). On the other hand, our results are compatible with the previous studies about the negative association between extent of lipid peroxidation and motility of spermatozoa in rooster and bull (Surai et al. 2001; Akhlaghi et al. 2014). As shown in the current experiment, higher amount of TBARS in the control group is associated to lower spermatozoa motility.

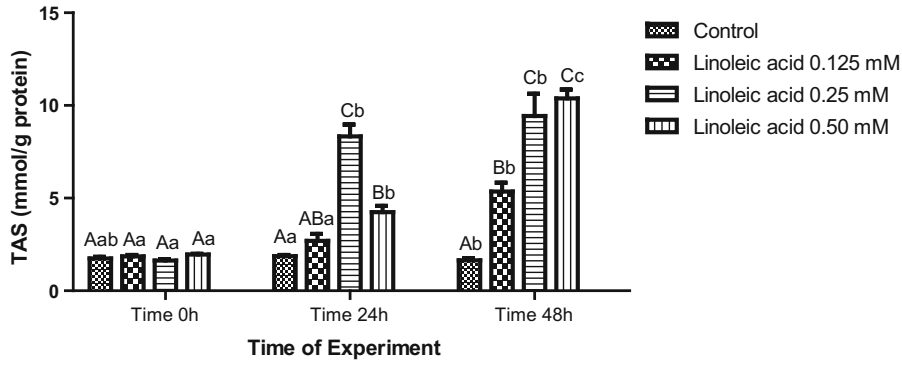


**Fig. 4** Amounts of total antioxidant status (TAS; mmol/g protein) in rooster spermatozoa following treatment with different levels of linoleic acid. $^{A,B,C}$Values with different letters indicate difference ($P < 0.05$) among experimental groups at each time point. $^{a,b,c}$Values with different letters indicate difference ($P < 0.05$) over time within the experimental groups

Total antioxidant status comprises enzymatic and non-enzymatic antioxidants in a system. Because spermatozoa lost most of their cytoplasm during maturation phase in the male reproductive system, their antioxidant capability is limited to counteract the damaging effects of peroxidation process (Zhao et al. 2011). Not only, the amounts of TBARS associated with motility and fertility of spermatozoa, but also infertile men showed lower amounts of enzymatic and non-enzymatic antioxidants in their semen compared to normal fertile men (Lewis et al. 1995). Moreover, the quantity of TAS is an important factor in maintaining viability and motility of animal spermatozoa (Bilodeau et al. 2001; Gadea et al. 2004). The association between amounts of TAS and spermatozoa motility in rooster, ram and bull has been evidenced (Eslami et al. 2016, 2017; Rad et al. 2016; Zadeh Hashem et al. 2017). The results of the current experiment showed that rooster semen enrichment with 0.25 and 0.5 mM LA, enhanced TAS levels in the medium and spermatozoa at 24 and 48 h of storage compared to control sample. In accordance with our results, oleate and palmitoleate improved amounts of TAS when added to the semen of roosters over in vitro storage (Eslami et al. 2016; Rad et al. 2016). Recent study indicated that linoleic acid significantly improved catalase activity and superoxide dismutase 1 gene expression in primary culture of hen hepatocytes (Qi et al. 2018). Moreover, the results of the present work verified the findings of previous studies about the ability of unsaturated fatty acids to improving antioxidant power, especially enzymatic one, in rat cardiomyocytes and ram spermatozoa (Narang et al. 2004; Eslami et al. 2017; Zadeh Hashem et al. 2017).

Predominant amounts of n-6 PUFA are present in the spermatozoa cell membrane of avian (Cerolini et al. 1997). On the other hand, linoleic acid is the essential precursor of all the other n-6 PUFAs which participate in a high amounts of avian spermatozoa cell membrane (Cerolini et al. 1997). Conflicting result have been published about the influence of n-6 PUFA rich diets on semen quality. Surai et al. (2000) reported that feeding arasco oil (rich in arachidonic acid, the n-6 fatty acid) from 26 to 60-week of age to roosters, increased the number of spermatozoa and weigh of the testes compared to its control (maize oil; rich in linoleic acid), while the fertility rate and concentrations of spermatozoa did not differ between groups. Moreover, motility and fertility of spermatozoa did not significantly differ at 54 and 72 weeks of age between the roosters received a feed rich in linoleic and linolenic acids (Kelso et al. 1997). An improvement in post-thaw motility of ram spermatozoa were reported after feeding of a diet rich in linoleic acid (Milovanov and Golubj 1973). The present experiment showed that enrichment of medium with various doses of BSA-conjugated LA did not affect percent of spermatozoa viability, but improved progressive motility especially at 0.5 mM levels at time points 24 and 48 h. Little information is present about enrichment of medium with linoleic acid on percent of motility and viability of avian and mammals spermatozoa. Our results are in consistent with the previous published study about the ineffectiveness of BSA-conjugated palmitoleic acid in improving viability compared to its control during low temperature liquid storage of fowl semen (Rad et al. 2016). However, another study indicated the positive effect of oleic acid supplementation on percent of rooster spermatozoa viability (at 0.25 mM level) and progressive motility (at 0.25, 0.5 and 1 mM levels) during chilled storage (Eslami et al. 2016). The antioxidant potency of LA and conjugated LA isomers has been indicated by previous studies (Bergamo et al. 2011; Qi et al. 2011, 2018; Chinnadurai et al. 2013; Basiricò et al. 2017). Higher motility achieved in 0.25 and 0.5 mM LA treated groups is related to LA potential in improving antioxidant capacity, especially enzymatic class and decreasing the production of secondary end products of the lipid peroxidation process and their detrimental consequences. It seems that, LA can improve enzymatic antioxidant activities and their expression at gene level via facilitating nuclear translocation of Nrf2, the key transcription factor that regulates the antioxidant response (Qi et al. 2018).

In conclusion, LA caused a significant enhancement in intracellular and extracellular antioxidant levels, probably due to influence on enzymatic antioxidants class, and provide a suitable antioxidative/oxidative ratio, especially at 0.5 mM level for preservation of rooster semen during cold storage over 48 h. Further studies are required to reveal the mechanism of action by LA on gene expression, protein levels and activity of enzymatic antioxidants in different species of avian and non-avian spermatozoa.

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**Compliance with ethical standards**

The experimental procedure carried out on the roosters for semen sampling was approved by Animal Care Committee of the Urmia University.

**Conflict of interest** The authors declare that they have no conflict of interest.

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