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# Antioxidant effects of clove bud (*Syzygium aromaticum*) extract used with different extenders on ram spermatozoa during cryopreservation ☆

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ABSTRACT

Clove bud (*Syzygium aromaticum*) extract was added at concentrations of 0, 35, 75, and 115 μg/ml to ovine semen extenders in order to investigate the antioxidant activities of clove bud extract and its effects on semen quality parameters after cryopreservation of ram spermatozoa. The basic extender was composed of Tris, egg yolk, and glycerol. Two other extenders were prepared by substitution of egg yolk with either LDL or egg yolk + SDS. The DPPH inhibition test was employed to assess the antioxidant activity of clove bud extract. Results showed that, compared to vitamin E, clove bud extract had a higher antioxidant activity. Better sperm motility and movement characteristics ($P < 0.05$) were observed in the semen diluted with medium containing egg yolk + SDS than in that containing egg yolk and LDL. Progressive motility and movement characteristics of the sperm were significantly improved ($P < 0.05$) by adding 35 and/or 75 μg/ml of clove bud extract to semen extenders. Sperm viability and plasma membrane integrity were also higher ($P < 0.05$) in the semen exposed to medium containing egg yolk + SDS and 75 μg of clove buds extract after cryopreservation processes. Higher levels of clove bud extract, however, had adverse effects on all the sperm quality parameters and significantly reduced ($P < 0.05$) the motility, movement parameters, viability, and plasma membrane integrity of ovine sperm. It was concluded that the clove bud extract had an antioxidant potential that makes it useful for addition to semen extenders and that the best results are obtained with a maximum clove bud extract of 75 μg/ml. Moreover, the combination of egg yolk and a detergent was found to improve sperm quality after the cooling and freeze–thawing processes.



## Introduction

Artificial insemination (AI) is a reproductive technique used in both the developed and developing countries for livestock farming. The technique is based on cryopreservation that involves preserving animal semen in frozen conditions. These conditions are always associated with oxidative stresses on spermatozoa, which induce biochemical and functional damages to the sperm. The detrimental effects result in reduced sperm motility, viability, acrosome integrity, and fertility potential [8,28,30]. A number of studies have implicated membrane lipid peroxidation as a cause of defective sperm function, especially in ram spermatozoa which contain higher amounts of long-chain polyunsaturated fatty acids [27,30].

During the last decades, egg yolk has become a common component of not only most semen cryopreservation extenders but liquid semen extenders as well [3,21]. However, it has been reported that diluents containing egg yolk may have detrimental effects on the viability and acrosome integrity of spermatozoa in some species [1]. Evidence indicates that low-density lipoproteins (LDL) like lecithin are the egg yolk fraction showing the highest protective ability and maintaining the membrane phospholipid integrity of sperm during cryopreservation [23]. In addition, some studies have reported that sperm motility after freezing is better by purified LDL than by whole egg yolk [3,23]. On the other hand, egg yolk could be solubilized by the addition of sodium dodecyl sulphate (SDS) in semen extenders to reduce its adverse effects. Adding SDS to the egg yolk extender mimics the hydrophobic environment existing in biological membranes without changing protein conformation [4]. El-Kon et al. [14] reported that Tris–egg yolk glycerol extender containing 0.05% SDS improved the viability, acrosome integrity, and the fertility of spermatozoa in goats.

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Reactive oxidative substances (ROS) are produced during the cryopreservation of spermatozoa that exert some physical and chemical changes in the sperm membrane [30]. Therefore, numerous research efforts have evaluated the effect of a various synthetic and natural antioxidants on sperm. Antioxidants are believed to neutralize the free radicals in lipid chains by contributing a hydrogen atom usually from a phenolic hydroxyl group which, in turn, converts phenolic groups into stable free radicals that do not initiate or propagate further lipid oxidation. Malo et al. [19], showed that supplementation of exogenous rosemary or cysteine to the freezing extender positively affected post-thawed viability and acrosome integrity of boar sperm. Clove bud (*Syzygium aromaticum*) is known to be an effective herbal antioxidant containing phenolic compounds (mainly eugenol, >50%) [15]. In an *in vitro* assay, Kabuto et al. [17] showed that eugenol reduced peroxidation and increased glutathione and L-ascorbate in mouse cells.

The aim of the present study was to evaluate the antioxidant properties of clove bud extract and to identify the effects of different concentrations of this natural antioxidant on cooled and frozen-thawed ram sperms. An additional objective of the study was to compare the profitability of extenders containing whole egg yolk, LDL, and egg yolk + SDS in the presence of a natural antioxidant.

## Materials and methods

All chemical reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

### *Clove buds extraction and DPPH inhibition method*

Fifty grams of dried clove buds were ground and extracted using a modified version of the method proposed by Khalaf et al. [18]. Briefly, the powder was soaked in 1 L of 80% ethanol at 4 °C in the dark. The soaked material was stirred every 12 h using a sterilized glass rod. After 72 h, the mixture was filtered on a filter paper (Whatman No. 1, England) and the residue was subjected to three further extractions under the same conditions. The extracts were combined and condensed at 35 °C under vacuum on a rotary evaporator. Finally, the condensed extract was dried using a freeze dryer (OSK 2139, Japan).

The antioxidant activities of both the clove bud extract thus obtained and vitamin E as the standard were assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity. The stock solution of clove bud extracts was prepared by dissolving a known amount of the dry extract in 98% methanol. The working solutions of clove bud extract were 0.1, 0.5, 1, 5, and 12.5 ppm. Vitamin E (50% pure) was used in 25, 50, and 100 ppm solutions. Five milliliters of the DPPH methanolic solution (0.1 mm) was mixed each time with 0.1 ml of the different solutions, separately. These solution mixtures were kept in the dark for 30 min before the optical density was measured at 517 nm (6505 UV/VIS Spectrophotometer, Jenway). Methanol with the DPPH solution was used as the blank. The percentage free radical scavenging activities of the samples were calculated using the following formula [10]:

$$I\% = [\text{A blank} - \text{A sample}/\text{A blank}] \times 100.$$

### *LDL extraction*

Fresh eggs were obtained from a flock of Lohmann Brown Classic laying hens. After disinfection, the eggs were broken and yolks were separated out from the albumen. The yolks were washed with saline solution three times to remove all the attached albumen. The vitellin membrane was then disrupted with a scalpel blade and the yolks were collected in a beaker maintained at 4 °C to prevent bacterial growth. LDL was extracted from the egg yolk according to the method described in Moussa et al. [23]. In brief, the purified yolk was diluted using isotonic saline solution (0.17 M NaCl) (w/w), stirred for 1 h at 4 °C, and centrifuged at 10,000*g* for 45 min at 4 °C. The supernatant was subsequently centrifuged again to remove all the trace granules. Ammonium sulphate (20.5 g/100 ml of plasma) was used to precipitate livetins. After 1 h of stirring at 4 °C, the mixture was centrifuged at 10,000*g* for 45 min before the supernatant was dialyzed for about 12 h with distilled water to eliminate ammonium sulfate. The solution was then centrifuged at 10,000*g* for 45 min at 4 °C and the floating residue, rich in LDL, was collected. Finally, the purified LDL was subjected to the lyophilization process [23].

### *Preparation of the extenders*

The basic extender in the present study was composed of 2.7 g Tris, 2.44 g citric acid, 0.5 g glucose, 7% glycerol (v/v), 100 IU penicillin for 100 ml of sterile milli-Q water. The osmolarity and pH were set at 335 mOsm and 6.5, respectively. Different extenders were prepared: 1) basic extender +15% egg yolk (control), 2) basic extender +15% egg yolk + SDS (0.5 mg per ml of egg yolk), and 3) basic extender +8% LDL (w/v). The final treatments were prepared using different levels of clove bud extract (0, 35, 75, and 115 μ/ml) added to the three extenders prepared.

### *Semen collection and processing*

Semen samples were collected from three mature Lori-Bakhtiyari rams (3.5 years of age and 105 kg of BW) using an artificial vagina twice a week during the breeding season. This breed is raised in a large area in the central and southwestern Iran and is known as a heavy-meat breed [29]. The rams were kept under a uniform and constant dietary regime in the Research Center of Lori-Bakhtiyari, Shahrekord, Iran. The semen thus collected was immediately assessed for volume, sperm concentration, and percentage of motile spermatozoa. Only ejaculates of 1–2 ml in volume, greater than 80% sperm progressive motility, and at least $2 \times 10^9$ sperm/ml were used. The semen samples ejaculated were pooled and diluted with the 12 different extenders prepared at 25 °C to a final concentration of $4 \times 10^8$ sperm/ml. The diluted semen was divided to two samples. One sample was cooled in 20 ml Falcon plastic tubes at 4 °C for 120 min in a refrigerator and analyzed as cooled sperm. The second sample was drawn into 0.25 ml French straws (IMV, Laigle, F-61300, France) which were maintained at 4 °C for 2 h. The straws were then placed 3 cm above the surface of liquid nitrogen where the temperature was approximately −120 °C. After 15 min, the straws were immersed directly into liquid nitrogen (−196 °C) for a long time storage. After 1 month, three straws from the same extenders were thawed in a 37 °C water bath for 30 s, pooled, and immediately subjected to analysis for evaluation.

### *Computer-aided sperm analysis (CASA)*

Sperm motility was assessed using a computer-aided sperm analysis system (WLJY-9000, WeiLi Software Co., Ltd., Beijing, China). The motility was evaluated in fresh, cooled, and frozen-thawed semen samples. For each extender, the semen samples were transferred into 1 ml plastic tubes and incubated at 37 °C for 5 min. Six microliters of the sample from each straw was examined and five fields were randomly chosen. The following motility patterns were studied: total motility (%), progressive motility (%), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s),

amplitude of the lateral head displacement (ALH, μm), linearity index [LIN = (VSL/VCL) × 100], average path velocity (VAP, μm/s), and straightness coefficient [STR = (VSL/VAP) × 100].

### *Assessment of sperm viability*

Viability of sperm was evaluated at pre-freezing and post-thaw stages using the eosin-nigrosin stain [9]. One drop of semen was placed with two drops of the stain on a warm slide to prepare the sperm smear. Viability was then assessed by counting 200 sperm cells using bright-field microscopy (400×) (Olympus CX21FS1, Olympos Optical Co. Ltd., Japan). Sperms with white or light pink heads were considered to be alive, while those with red or dark pink heads were regarded dead.

### *Assessment of plasma membrane integrity*

The hypoosmotic swelling test (HOS) was used to assess plasma membrane integrity at the post-thaw stage. For this purpose, 0.735 g of sodium citrate dehydrate and 1.351 g of D-fructose were dissolved in 100 ml of distilled water. The solution was stored at 4 °C until use. Three straws were thawed in a water bath at 37 °C for 30 s. Ten microliters of the semen was then added to 20 μl of the HOS solution previously warmed to 37 °C; the mixture was then placed in a water bath at 37 °C for 30 min. Sperm swelling was assessed by placing 10 μl of the well-mixed sample on a warm slide (37 °C). After spreading, the slide was read using a phase-contrast microscope. At least 200 spermatozoa were observed and classified as positive if characterized by swollen and/or curled tails (intact plasma membrane), or negative if characterized by non-curled tails (damaged plasma membrane).

### *Statistical analysis*

In this study, we had three different extenders and four levels of clove bud extract for each extender. All the data were analyzed using the Statistical Analysis System software version 9.1 (SAS, 2003). The factorial ANOVA models were used to determine the statistical differences among the main effects of the extenders, antioxidant levels and their interactions (extender × antioxidant). In the case of a significant interaction, the model was changed into an individual one-way ANOVA model for each antioxidant level. If a non-significant interaction was detected, the main effects (extenders and antioxidant levels) were interpreted. The comparison of means was performed using Tukey's test and the values $P < 0.05$ were regarded as statistically significant.

## Results

### *2,2-Diphenyl-1-picryl hydroxyl radical scavenging activity*

The data on the scavenging activities of clove dud extract and vitamin E are presented in Table 1. Vitamin E showed 14.8%, 55.0%, and 73.7% radical scavenging activities in 25, 50, and 100 μg/ml concentrations, respectively. Clove bud extract at concentrations of 0.1, 0.5, 1, 5, and 12.5 μg/ml showed 5.9%, 13.1%, 25.2%, 72.5%, and 97.9% inhibition of the DPPH radical, respectively. These results indicate that the antioxidant activity of the clove bud extract was more than 10-fold higher than vitamin E. Because the concentration of 5 μg/ml of clove bud extract had the same antioxidant activity with the level of 100 μg/ml of 50% vitamin E.

**Table 1**
Radical scavenging activity of different concentrations of clove buds extract and vitamin E by 2,2-diphenyl-1-picryl hydroxyl (DPPH) method.

| Antioxidant | Concentration (μg/ml) | Scavenging activity (%) |
|---|---|---|
| Clove bud | 0.1 | 5.9 |
| | 0.5 | 13.1 |
| | 1 | 25.2 |
| | 5 | 72. 5 |
| | 12.5 | 97.9 |
| Vitamin E | 25 | 14.8 |
| | 50 | 55.0 |
| | 100 | 73.7 |

### *Sperm motility and movement parameters*

Tables 2 and 3 present the motility characteristics of fresh as well as cooled and frozen-thawed spermatozoa. Clearly, the cooling and freeze–thawing processes decreased all the motility characteristics of spermatozoa as compared with fresh semen.

The extender and clove bud extract had significant effects ($P < 0.05$) on the motility characteristics of ram spermatozoa after the cooling and freeze–thawing processes. However, the interaction of extender and clove bud extract had no effects ($P > 0.05$) on certain parameters (Tables 2 and 3). Sperms in diluted semen containing egg yolk + SDS had the greatest ($P < 0.05$) total and progressive motility, VCL, VSL, VAP, ALH, LIN, and STR after cooling. The same was observed after freezing–thawing as well; however, no significant difference ($P > 0.05$) was observed in this stage between 15% egg yolk and 8% LDL with respect to total motility, ALH, and STR. The results of this study showed that addition of 75 μg/ml clove bud extract to the cryoprotective medium enhanced ($P < 0.05$) sperm motility characteristics. Nevertheless, there was no difference between the clove bud extract concentrations of 35 and 75 μg/ml with respect to total and progressive motility, ALH, LIN, and STR in cooled semen. Higher levels of clove bud extract (i.e., 115 g/ml) decreased ($P < 0.05$) the values already recorded for most parameters of sperm motility characteristics after the cooling and freezing–thawing processes.

### *Viability of Spermatozoa*

Results showed that both the extenders and antioxidant concentrations affected ($P < 0.05$) sperm viability after either cooling or freezing–thawing processes (Tables 2 and 3). However, the interaction of main effects was not significant in this respect ($P > 0.05$).

Compared to those containing 8% LDL and 15% egg yolk, the extender containing egg yolk + SDS had beneficial effects on the viability of spermatozoa in cooled and frozen-thawed semen. On the other hand, the use of 8% LDL, rather than 15% egg yolk, as a component of the extender decreased ($P < 0.05$) the viability of spermatozoa. Clove bud extracts of 35 and 75 μg/ml significantly improved ($P < 0.05$) sperm viability rate after cooling and that of 75 μg/ml of clove bud extract did so after the freezing–thawing process.

### *Plasma membrane integrity*

Table 3 presents the effects of different extenders and different concentrations of clove bud extract on plasma membrane integrity of frozen-thawed spermatozoa. The main effects of extender and antioxidant was significant ($P < 0.05$), but the interaction of the main factors was not ($P > 0.05$).

Including egg yolk + SDS in the semen extender increased ($P < 0.05$) sperm plasma membrane integrity as compared to 15%

**Table 2**
Effect of the egg yolk, lyophilized LDL and egg yolk + SDS extenders in combination with different levels of clove buds extracts on motility parameters of fresh and cooled ram spermatozoa.

| Extenders | Clove buds (μg/ml) | Total motility (%) | Progressive motility (%) | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) | ALH (μm) | LIN (%) | STR (%) | Viability (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Fresh semen | – | 89.07 | 85.83 | 117.64 | 102.02 | 106.82 | 2.84 | 77.14 | 86.40 | – |
| Egg yolk | 0 | 53.63 | 49.93 | $55.63^{bc}$ | $45.23^{bc}$ | $48.16^{bc}$ | 1.79 | 51.93 | 65.11 | 57.46 |
| | 35 | 60.84 | 56.53 | $60.81^{bc}$ | $48.69^{bc}$ | $52.26^{bc}$ | 1.93 | 56.11 | 67.70 | 65.20 |
| | 75 | 65.18 | 60.50 | $66.67^{ab}$ | $54.86^{ab}$ | $58.59^{ab}$ | 2.01 | 58.70 | 70.67 | 68.53 |
| | 115 | 43.45 | 37.49 | $37.31^{d}$ | $26.01^{d}$ | $29.69^{d}$ | 1.56 | 39.22 | 53.80 | 48.71 |
| LDL | 0 | 43.09 | 37.34 | $38.49^{d}$ | $25.93^{d}$ | $29.74^{d}$ | 1.61 | 38.05 | 52.21 | 45.81 |
| | 35 | 52.13 | 47.07 | $46.01^{cd}$ | $32.27^{cd}$ | $36.05^{cd}$ | 1.84 | 44.77 | 62.04 | 55.88 |
| | 75 | 51.84 | 47.26 | $45.30^{cd}$ | $31.79^{cd}$ | $35.72^{cd}$ | 1.92 | 46.75 | 60.92 | 56.23 |
| | 115 | 38.74 | 33.84 | $37.40^{d}$ | $24.76^{d}$ | $28.18^{d}$ | 1.61 | 38.26 | 54.84 | 42.21 |
| Egg yolk + SDS | 0 | 63.64 | 60.13 | $63.54^{b}$ | $53.67^{b}$ | $56.95^{b}$ | 1.88 | 58.80 | 69.13 | 68.13 |
| | 35 | 68.46 | 65.95 | $64.19^{b}$ | $51.18^{b}$ | $56.95^{b}$ | 2.14 | 62.00 | 74.00 | 75.92 |
| | 75 | 71.13 | 68.07 | $83.31^{a}$ | $71.18^{a}$ | $74.88^{a}$ | 2.26 | 64.26 | 74.97 | 77.09 |
| | 115 | 62.94 | 58.06 | $63.47^{b}$ | $51.94^{b}$ | $55.68^{b}$ | 1.89 | 56.84 | 68.18 | 64.93 |
| SEM | | 2.40 | 2.76 | 3.33 | 3.38 | 3.40 | 0.06 | 2.70 | 2.42 | 3.12 |
| Extenders | Egg yolk | $55.78^{b}$ | $51.11^{b}$ | $55.11^{b}$ | $43.07^{b}$ | $47.18^{b}$ | $1.82^{b}$ | $51.49^{b}$ | $64.32^{b}$ | $59.98^{b}$ |
| | LDL | $46.45^{c}$ | $41.38^{c}$ | $41.80^{c}$ | $28.69^{c}$ | $32.42^{c}$ | $1.75^{b}$ | $41.96^{c}$ | $57.50^{c}$ | $50.03^{c}$ |
| | Egg yolk + SDS | $66.54^{a}$ | $63.05^{a}$ | $68.63^{a}$ | $56.99^{a}$ | $60.61^{a}$ | $2.04^{a}$ | $60.48^{a}$ | $71.57^{a}$ | $71.52^{a}$ |
| Clove buds | 0 | $53.45^{b}$ | $49.13^{b}$ | $52.55^{bc}$ | $41.61^{b}$ | $44.95^{b}$ | $1.76^{b}$ | $49.59^{b}$ | $62.15^{ab}$ | $57.13^{b}$ |
| | 35 | $60.48^{a}$ | $56.52^{a}$ | $57.00^{b}$ | $44.05^{b}$ | $47.74^{b}$ | $1.97^{a}$ | $54.29^{a}$ | $67.91^{a}$ | $65.67^{a}$ |
| | 75 | $62.72^{a}$ | $58.61^{a}$ | $65.09^{a}$ | $52.61^{a}$ | $56.40^{a}$ | $2.06^{a}$ | $56.57^{a}$ | $68.85^{a}$ | $76.28^{a}$ |
| | 115 | $48.38^{b}$ | $43.13^{c}$ | $46.06^{c}$ | $34.24^{c}$ | $37.85^{c}$ | $1.69^{b}$ | $44.77^{b}$ | $58.94^{b}$ | $51.95^{c}$ |
| | | *P*-value | | | | | | | | |
| Extender | | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Clove buds | | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Extender × clove buds | | 0.1553 | 0.3014 | 0.0078 | 0.0077 | 0.0095 | 0.5227 | 0.1909 | 0.1348 | 0.9155 |

VCL: curvilinear velocity, VSL: straight line velocity, VAP: average path velocity, ALH: amplitude of lateral head displacement, LIN: linearity index (LIN = VSL/VCL × 100), and STR: straightness index (STR = VSL/VAP × 100).
a–d, within a column, the means without a common superscript are differ ($P < 0.05$).

**Table 3**
Effect of the egg yolk, lyophilized LDL and egg yolk + SDS extenders in combination with different levels of clove buds extracts on motility parameters of frozen-thawed ram spermatozoa.

| Extenders | Clove buds (μg/ml) | Total motility (%) | Progressive motility (%) | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) | ALH (μm) | LIN (%) | STR (%) | Viability (%) | Plasma membrane integrity (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Egg yolk | 0 | 27.70 | 23.58 | 22.93 | $11.72^{bc}$ | $14.98^{bc}$ | $1.36^{cdef}$ | $29.80^{bcde}$ | 47.03 | 29.35 | 24.50 |
| | 35 | 27.96 | 23.22 | 23.44 | $12.08^{bc}$ | $15.41^{bc}$ | $1.41^{cdef}$ | $33.74^{bcde}$ | 50.62 | 30.91 | 27.12 |
| | 75 | 38.97 | 30.51 | 32.49 | $15.04^{bc}$ | $19.84^{b}$ | $1.82^{a}$ | $29.88^{bcde}$ | 50.57 | 40.94 | 34.78 |
| | 115 | 21.56 | 14.63 | 19.87 | $7.28^{bc}$ | $10.72^{bc}$ | $1.32^{def}$ | $25.20^{de}$ | 43.92 | 22.79 | 19.45 |
| LDL | 0 | 23.66 | 18.14 | 21.62 | $8.58^{bc}$ | $12.26^{bc}$ | $1.40^{cdef}$ | $25.69^{cde}$ | 44.77 | 25.47 | 22.46 |
| | 35 | 24.47 | 18.58 | 21.40 | $8.20^{bc}$ | $11.66^{bc}$ | $1.42^{cde}$ | $24.84^{e}$ | 44.18 | 26.49 | 23.06 |
| | 75 | 37.66 | 33.21 | 28.95 | $16.84^{b}$ | $20.56^{b}$ | $1.53^{bc}$ | $35.39^{ab}$ | 51.38 | 40.48 | 38.40 |
| | 115 | 22.98 | 18.94 | 16.72 | $6.09^{c}$ | $9.02^{c}$ | $1.24^{f}$ | $27.64^{bcde}$ | 43.46 | 23.69 | 19.85 |
| Egg yolk + SDS | 0 | 32.38 | 26.81 | 23.66 | $11.76^{bc}$ | $15.16^{bc}$ | $1.48^{bcd}$ | $34.82^{abc}$ | 51.84 | 34.95 | 29.73 |
| | 35 | 35.12 | 29.70 | 29.22 | $16.86^{b}$ | $20.53^{b}$ | $1.49^{bcd}$ | $34.34^{bcd}$ | 51.76 | 38.43 | 32.89 |
| | 75 | 43.37 | 39.47 | 41.92 | $31.30^{a}$ | $34.70^{a}$ | $1.62^{b}$ | $43.75^{a}$ | 57.30 | 47.68 | 43.83 |
| | 115 | 22.04 | 18.53 | 18.90 | $8.54^{bc}$ | $11.66^{bc}$ | $1.26^{ef}$ | $27.84^{bcde}$ | 42.28 | 25.72 | 21.24 |
| SEM | | 2.12 | 2.05 | 1.93 | 2.02 | 2.00 | 0.04 | 1.83 | 1.80 | 2.23 | 2.02 |
| Extenders | Egg yolk | $29.05^{ab}$ | $22.99^{b}$ | $24.68^{b}$ | $11.53^{b}$ | $15.24^{b}$ | $1.48^{a}$ | $29.66^{b}$ | $48.04^{ab}$ | $31.00^{b}$ | $26.46^{b}$ |
| | LDL | $27.19^{b}$ | $22.22^{b}$ | $22.17^{b}$ | $9.93^{b}$ | $13.38^{b}$ | $1.40^{b}$ | $28.39^{b}$ | $45.95^{b}$ | $29.03^{b}$ | $25.94^{b}$ |
| | Egg yolk + SDS | $33.23^{a}$ | $28.63^{a}$ | $28.43^{a}$ | $17.12^{a}$ | $20.51^{a}$ | $1.46^{a}$ | $35.19^{a}$ | $50.80^{a}$ | $36.70^{a}$ | $31.92^{a}$ |
| Clove buds | 0 | $27.91^{b}$ | $22.84^{bc}$ | $22.74^{b}$ | $10.69^{bc}$ | $14.13^{bc}$ | $1.41^{bc}$ | $30.10^{b}$ | $47.88^{bc}$ | $29.92^{bc}$ | $25.56^{bc}$ |
| | 35 | $29.18^{b}$ | $23.83^{b}$ | $24.69^{b}$ | $12.38^{b}$ | $15.87^{b}$ | $1.44^{b}$ | $30.97^{b}$ | $48.85^{ab}$ | $31.94^{b}$ | $27.69^{b}$ |
| | 75 | $40.00^{a}$ | $34.40^{a}$ | $34.45^{a}$ | $21.06^{a}$ | $25.03^{a}$ | $1.66^{a}$ | $36.34^{a}$ | $53.08^{a}$ | $43.03^{a}$ | $39.00^{a}$ |
| | 115 | $22.19^{c}$ | $17.37^{c}$ | $18.50^{c}$ | $7.30^{c}$ | $10.47^{c}$ | $1.27^{c}$ | $26.89^{b}$ | $43.22^{c}$ | $24.07^{c}$ | $20.18^{c}$ |
| | | *P*-value | | | | | | | | | |
| Extender | | 0.0016 | 0.0003 | 0.0005 | 0.0001 | 0.0001 | 0.0092 | 0.0001 | 0.0034 | 0.0002 | 0.0005 |
| Clove buds | | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Extender × clove buds | | 0.2309 | 0.0746 | 0.0697 | 0.0088 | 0.0166 | 0.0009 | 0.0025 | 0.0931 | 0.4728 | 0.2826 |

VCL: curvilinear velocity, VSL: straight line velocity, VAP: average path velocity, ALH: amplitude of lateral head displacement, LIN: linearity index (LIN = VSL/VCL × 100), and STR: straightness index (STR = VSL/VAP × 100).
a–f, within a column, the means without a common superscript are differ ($P < 0.05$).

egg yolk and 8% LDL. There was no difference between LDL and egg yolk in this regard. The level of 75 μg/ml of clove bud extract led to the best integrity ($P < 0.05$) in sperm plasma membrane. On the other hand, 115 μg/ml of clove bud extract had an adverse effect on plasma membrane integrity of spermatozoa after the freezing–thawing process.

## Discussion

*Free radical scavenging activity of clove bud extract*

An increasing interest is being currently shown in the antioxidant activity of natural and synthetic substances and a variety of methods have been investigated for estimating the efficiency of such substances used as antioxidants. One currently popular method that has been widely used for free radical-scavenging assessment is the DPPH inhibition method [22,31]. The results of the present study revealed the powerful antioxidant activity of clove bud extract, which is consistent with the findings of Bamdad et al. [7] who compared the antioxidant activity of clove bud extract and BHT. This property of clove buds extract can be attributed to its phenolic compounds and its hydrogen donating ability [31].

*Sperm motility characteristics, viability, and membrane integrity*

Our results showed that the combination of egg yolk + SDS had more beneficial effects on sperm motility, movement characteristics, viability, and plasma membrane integrity after cooling and freezing–thawing processes. This finding is consistent with that of El-Kon et al. [14]. They concluded that the viability and fertility of frozen-thawed goat spermatozoa significantly improved as a result of adding 0.05% SDS to the Tris–egg yolk glycerol extender. Our results indeed confirm previous reports with bull [5], ram [13], goat [14], rabbit [6] and mice [12] to the effect that different detergents increase sperm quality following cryopreservation. Detergents may act as a lipid solubilizer of egg yolk in semen extenders and, thereby, be beneficial [12]. Moreover, it is noted that SDS is an anionic detergent that probably exerts its action by reducing lipid phase transitions and/or protecting the function of membranes by controlling the calcium influx [16,26]. Contrary to our expectation, substitution of 8% LDL with 15% complete egg yolk reduced sperm motility and most of the related characteristics. The discrepancy could be due to the methods used for preparing LDL from egg yolk. Moustacas et al. [24] maintained that the quality of lyophilized LDL was significantly lower than that of natural LDL as a cryopreservative in ram semen. They concluded that lyophilization was apparently unable to preserve the protective function of LDL.

It is known that cryopreservation produces physical and chemical stresses on the sperm membrane which affect its motility, viability, and membrane integrity [2,20]. The present study demonstrated that treatment with clove bud extract during cooling and freezing–thawing processes efficiently protects ram spermatozoa and that the best level of the extract used is 75 μg/ml. Malo et al. [19,20] evaluated the capability of rosemary for protecting boar spermatozoa against freezing–thawing damages and reported that rosemary-enriched freezing extenders improved the overall quality parameters of post-thaw epididymal and ejaculated boar semen. They concluded that 100 mg/ml of rosemary extract had a better protective effect compared to 25 and 50 mg/ml concentrations. There are limited reports on the effect of clove bud extract on cell protection so that the best concentration for this extract is unknown. Nassar et al. [25] reported that the ethanol extract of clove bud exhibited a remarkable hepatoprotective activity against paracetamol-induced liver injury in female rats. Depending on the concentration of clove bud extract, positive as well as negative effects were identified in our study and higher levels of the extract (e.g., 115 μg/ml) were observed to have adverse effect on all the parameters of ram spermatozoa. This result might be attributed to the spermicidal effect of high doses of eugenol [11].

Further studies are needed to identify the effect of clove bud extract on lipoproxidation of sperm plasma membrane, fertility capacity of sperm and activity of antioxidant enzymes in cooled and frozen-thawed semen.

## Conclusion

In our study condition, the presence of clove buds extract in semen extenders at the concentrations of 35 and 75 μg/ml had a beneficial effect on motility parameters of ram sperm after cooling and freezing–thawing process. The results showed that substitution of 15% complete egg yolk with 8% lyophilized LDL in extender had adverse effect on motility, movement parameters, viability, and plasma membrane integrity of cryopreserved spermatozoa. Though, combination of egg yolk with SDS increased the sperm quality after cryopreservation.

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