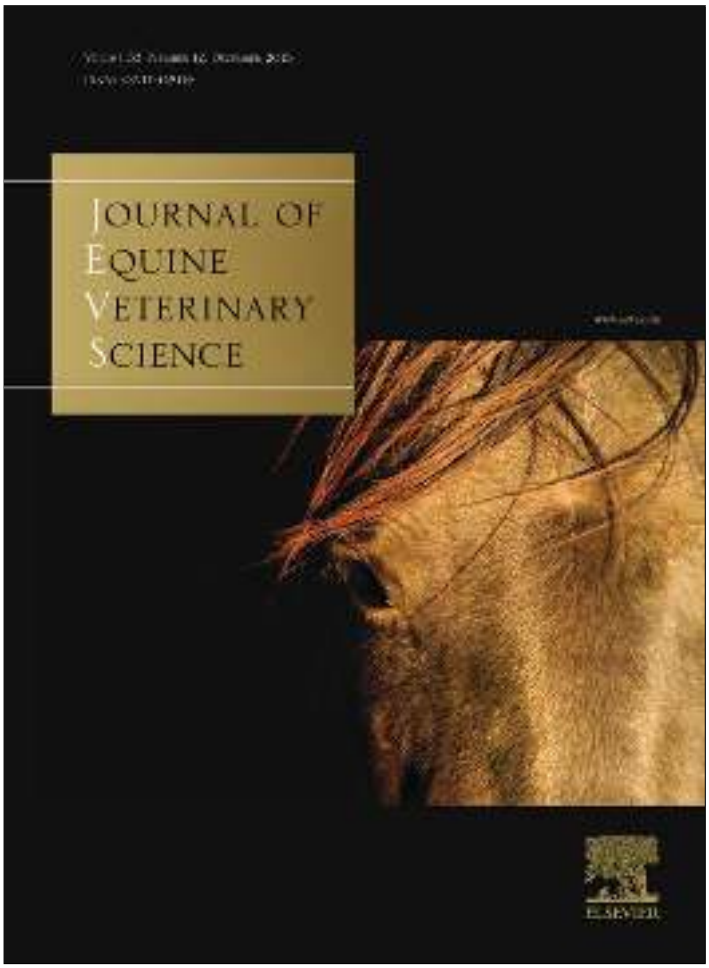

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Original Research

The Effects of Centrifuged Egg Yolk Used with INRA Plus Soybean Lecithin Extender on Semen Quality to Freeze Miniature Caspian Horse Semen

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ABSTRACT

The aim of this study was to determine the synergistic effects of centrifuged egg yolk (EY) and soybean lecithin on post-thaw Caspian horse sperm motility, morphological abnormalities, and assessment of membrane integrity. The centrifuged EY (CEY) was added at concentrations of 2% and 4% to a defined INRA plus 1.25% soybean lecithin extender used to freeze Caspian horse semen. In this experiment, ejaculates collected from each Caspian horse (n = 4) were divided into three equal aliquots and diluted in CEY 2% (INRA2), 4% (INRA4) supplemented, and without any CEY (INRA0) in INRA plus 1.25% soybean lecithin extender, respectively. Thereafter, samples were frozen and thawed following a standard protocol. Sperm cryosurvival was evaluated in vitro by microscopy assessments of post-thaw sperm motility (by means of computer-assisted semen motility analysis [CASA]), acrosomal and other abnormalities (head, mid-pieces, and tail) and plasma membrane integrity (evaluated by HOST). In Caspian stallion, semen extended with INRA2 had significantly higher CASA motility and CASA progressive motility than those extended with the rest of extenders after freezing and thawing ($P < .001$). There was no significant difference in path velocity (VAP), VCL, and ALH among three groups ($P > .05$). For straight line velocity ($P < .01$) and LIN ($P < .001$), the highest values were obtained from the INRA4 group. The highest percentages of acrosomal and other abnormalities were found in semen diluted in INRA4 ($P < .001$). In the group frozen INRA2, the percentage of membrane integrity was significantly higher than that of the other groups ($P < .001$). The use of CEY 2% in combination with soybean lecithin significantly improved Caspian horse semen freezability.

1. Introduction

Modern horse reproduction in most breeds is based on the use of artificial insemination (AI). The widespread use of AI has accelerated genetic progress by making selected stallions available to breeders outside the country or region where the stallion is located. Microscopic quality based on motility, functional and morphologic properties is very important after thawing. However, the nucleus, plasma, acrosome, and mitochondria membranes of sperm cells are susceptible to freezing/thawing processes [1-3]. Cold shock is the major reason for reduced motility after the freezing thawing. It generally leads to swelling and the protuberance of the acrosomal membrane and disruption or increased permeability of the plasma membrane [4]. Because of that, the use of frozen semen is limited by this unfavorable condition. The irreversible damages can be reduced by using proper extenders and cryoprotectant additives [5,6]. Egg yolk (EY) has been successfully replaced by soybean lecithin in extenders for cryopreservation of equine semen [7]. EY depends on containing cholesterol, phospholipids, and low-

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density lipoprotein prevents the formation ice crystal formation, thus protecting integrity of sperm plasma membranes against cold shock during the freeze-thaw process [8]. Although new extenders without animal ingredients are available, for example, soybean lecithin-based extender, egg yolk-containing extenders have still been widely used to freeze semen [2,7,9]. However, it was stated that EY, an animal originated product, carries microbial sanitary risk [10,11], and it also reduces respiration and motility of sperm cells [12] and consequently might affect AI success [2]. Besides, EY has been complicated to evaluate biochemical, metabolic and microscopic investigations, but this may be accomplished by removing some components in EY by centrifugation [13]. Lecithin of plant origin (soybean) has successfully replaced EY in laboratory and field trials for livestock semen [4,7,14-17]. The base influential compound of soybean lecithin is the low-density lipoprotein fraction, like EY, which protects the membrane phospholipid integrity during cryopreservation [12,18]. Recently, it has been declared that the soybean lecithin-based extender was used successfully in cryopreservation of both, for bull [17], ram [16,19], and stallion semen freezing. The improvement of semen cryopreservation technologies requires in-depth knowledge of the properties of the current extender. However, no studies have investigated the effects of centrifuged EY in combination with soybean lecithin on stallion semen freezability. We investigated the effects of adding centrifuged EY (CEY) at two different concentrations in INRA plus soybean lecithin extender on computer-assisted sperm motility assay (CASA) of motility, motility characteristics, acrosomal and other abnormalities (head, mid-pieces, tail), and membrane integrity during cryopreservation.

2. Material and Methods

2.1. Stallions and Semen Collection

This study was performed using four fertile miniature Caspian horses between 10 and 12 years of age from Animal Breeding Center of Karaj, Iran. The stallions were fed sufficient coastal Bermuda grass hay and grain mix to maintain good body condition. Semen was collected from these stallions on a regular basis (3 times a week) by using a Missouri artificial vagina. Seven ejaculates per stallion were processed. The four stallions' semen was collected on the same day (n = 7 days of collection). After collection, raw semen was filtered through gauze to exclude the gel fraction of the ejaculate and immediately processed.

2.2. Extender Preparation

Clarified EY was prepared as described by Holt et al. [20]. Briefly, fresh hen eggs were manually broken. Yolks were separated from the albumen and were carefully rolled on a filter paper to remove chalazas and traces of albumin adhering to the vitelline membrane. The latter was then disrupted with a scalpel blade, and yolk was diluted in distilled water (1:3) and centrifuged in sterile tubes at 4000 × *g* for 40 minutes at room temperature. After centrifugation, the lipid material at the top of each tube was removed, the water-soluble clear fraction was saved, and the pellet at the bottom of the tube was discarded.

A commercial extender, INRA96 (IR) (IMV, France) was used to dilute the semen before centrifugation. The samples were frozen using the commercial INRA96 (INRA) extender plus 1.25% soybean lecithin (Sigma, St. Louis, MO), to which the centrifuged EY (CEY) was added in different concentrations (2% and 4%), CEY 2% (INRA2), 4% (INRA4), and CEY %0 (INRA0) as a control.

2.3. Freezing Protocol

After collection, the ejaculates were diluted 1:1 with INRA96 extender, divided into aliquots according to the number of freezing extenders to be tested, and centrifuged at 1000 × *g* for 7 minutes. The supernatant was removed, and the sperm pellets were resuspended with a specific freezing extender (INRA plus 1.25% soybean lecithin + 2% CEY+ 2%G(INRA2), INRA plus 1.25% soybean lecithin + 4% CEY + 2% G [INRA4], INRA plus 1.25% soybean lecithin + 2%G [INRA0]) to obtain 200 × 10^6 sperm/ml. Each tube of diluted semen was cooled to 4°C over 75 minutes. Cooled semen was loaded into 0.5-ml polyvinyl chloride straws (IMV Technologies, France) that were then sealed with polyvinyl alcohol sealing powder. The samples were packaged into 0.5-ml straws, incubated at 5°C for 20 minutes, and frozen at 4 cm above the level of liquid nitrogen for 12 minutes before being plunged into liquid nitrogen for storage.

2.4. Semen Evaluation

Sperm motility, morphological abnormalities, and plasma membrane integrity were assessed for each sample to determine in vitro sperm quality. Three straws per ejaculate and per stallion were thawed at 37°C for 30 seconds and pooled in a test tube for each of the three extenders and then evaluated for total and progressive motility by using CASA version 12 (IVOS; Hamilton-Thorne Biosciences, Beverly, MA). The system parameters for CASA were 30 frames acquired at 60 frames per second; minimum contrast, 30; minimum cell size, 5 pixels; path velocity (VAP) cutoff, 30 μm/s; and VAP cutoff for progressive cells, 70 μm/s and straightness 80%; and straight line velocity (VSL) cutoff, 0 μm/s. The slow cells were considered static. A 10-μl drop of each sample was placed on a preheated (37°C) Makler counting chamber (10 μm depth). Analyses were performed in duplicate (2 × 3 Ml sampling/straw) with three fields analyzed (i.e., 6 observations/straw and 18 observations/stallion/ejaculate) and following motility values were recorded: motility (%), progressive motility (%), VAP (average path velocity, μm/sec), VSL (straight linear velocity, μm/sec), VCL (curvilinear velocity, μm/sec), ALH (amplitude of lateral head displacement, μm), LIN (linearity index; LIN = [VSL/VCL]× 100). Morphological abnormalities were assessed by phase-contrast microscopy examination (×1000 magnification, oil immersion). At least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and other abnormalities were determined by counting a total of 400 spermatozoa. Plasma membrane integrity was

assessed by means of the hypo-osmotic swelling test (HOST) as described by Buckett et al. [21]. Briefly, 5 μl of semen was diluted to 50 μl of a hypo-osmotic solution (100 mOsm) and incubated at 37°C for 60 minutes. After incubation, a smear was preparedm and 200 spermatozoa were evaluated (×1000 magnification) with brightfield microscopy. Sperm with swollen or coiled tails were recorded.

2.5. Statistical Analysis

Results were expressed as means ± SEM. Means were analyzed by analysis of variance (ANOVA), followed by the Duncan test to determine significant differences in all parameters between groups, using SPSS/PC version 12.0 software (SPSS, Chicago, IL). Differences with P values <.01 were considered to be statistically significant.

3. Results

The influence of two different CEY concentrations in a defined soy lecithin-based extender on semen parameters after the freeze-thawing is shown in Table 1. Regarding Caspian horses, semen extended with INRA2 had significantly higher post-thaw CASA motility and CASA progressive motility than those extended with the rest of extenders after freezing and thawing ($P < .001$). There were no significant differences in VAP, VCL, and ALH among the three groups ($P > .05$). For VSL ($P < .01$) the highest values were obtained from INRA4. The highest percentages of acrosomal and other abnormalities were found in semen diluted in INRA4 ($P < .001$). In the group with frozen INRA2, the percentage of membrane integrity was significantly higher than that of the other groups ($P < .001$).

4. Discussion

All the processes of cryopreservation, including the addition of cryoprotectants, cooling, freezing, and thawing that also create cold shock on the sperm membrane, resulting in the reduction of semen parameters are important factors [22]. As sperm cells contain high concentrations of polyunsaturated fatty acids, they are highly susceptible to freezing. In this sense, a source of lipoprotein and high-molecular weight compound such as EY or soybean lecithin has been routinely used in most cryopreservation protocols to protect sperm membrane phospholipids against cold shock [7,11,16,17,19]. However, previous studies have demonstrated that EY significantly diminishes the percentage of intact acrosomes and motility in stallion [7], ram [23], and bull [17] semen during cryopreservation. Results of the current study indicated that the effect of CEY concentration were statistically significant for sperm motility (CASA), membrane integrity (HOST), and functional integrity after thawing. Thus, based on our results, the post-thaw motility and membrane and functional integrity of Caspian horse semen frozen in diluents with INRA4 were significantly lower than that of the INRA2. The concentration of EY (4%) in 1.25% soybean lecithin-based extender increased the viscosity of the extender, which might also have complicated motility and cell membrane integrity. These data confirm the results of earlier studies [2,17,23], where a similar observation was found with frozen/thawed semen that was extended in EY containing extender. Besides, it has been also indicated that the concentrations of EY or EY constituents could be reduced by centrifugation without decreasing sperm cryosurvival [13] and centrifuged EY provided a higher protection than whole EY during the freeze-thaw process [24]. In this study, 2% CEY provided higher protection than INRA0 and INRA4 extender after freezing thawing. It can be inferred that EY contains some substances harmful to semen quality parameters. Therefore, additives may be preferred as external cryoprotectants in extenders include soybean lecithin, having less viscous fluid, in combination with centrifuged EY supplementation for cryopreserving miniature Caspian horse semen. Diluents containing soybean lecithin maintained the sperm motility and plasma membrane integrity similar to the diluent containing EY. These results are similar to those obtained by others using stallion [4,7,14] and bull sperm [2,5,25]. However, others have reported that bull sperm survive freezing more efficiently in EY-containing diluents than in diluents containing soybean lecithin [26,27]. Because of the elimination of some harmful components from EY by centrifugation, it is possible that CEY 2% provided the best cryoprotective effect on post-thaw motility, membrane, and morphologic integrity during the freeze-thaw process. In addition, it was also found that centrifuged CEY 2% was suitable for conducting CASA studies.

Table 1
Sperm parameters in frozen-thawed Caspian horse semen (mean ± SE)

Parameter	Extenders			P
	INRA2	INRA4	INRA0	
% of total motility	58.1 ± 5.19[c]	32.8 ± 2.67[a]	49.3 ± 3.46[b]	<.001
% of progressive motility	29.8 ± 2.9[c]	12.2 ± 0.80[a]	21.3 ± 1.64[b]	<.001
VAP (μm/s)	105.1 ± 3.88	94.4 ± 2.91	102.1 ± 2.39	>.05
VSL (μm/s)	83.7 ± 8.11[b]	89.0 ± 7.28[c]	71.1 ± 5.69[a]	<.01
VCL (μm/s)	204 ± 12.37	197 ± 15.43	192 ± 16.55	>.05
ALH (μm)	7.8 ± 0.46	7.0 ± 0.50	7.3 ± 0.23	>.05
% of LIN	43.2 ± 2.1[b]	49.9 ± 1.9[c]	41.6 ± 2.1[a]	<.001
% of acrosome abnormality	9.6 ± 0.95[a]	18.6 ± 1.2[b]	11.2 ± 0.75[a]	<.001
% of other abnormalities	13.4 ± 0.47[a]	21.3 ± 0.54[c]	16.7 ± 0.68[b]	<.001
% of membrane integrity	55.9 ± 2.16[c]	31.4 ± 1.19[a]	48.8 ± 1.46[b]	<.001

ALH, amplitude of lateral head displacement; LIN, linearity index; LIN = [VSL/VCL]×100); VAP, average path velocity; VCL, curvilinear velocity; VSL, straight linear velocity.

[a,b,c]Different superscript letters within the same row demonstrate significant differences among groups.

5. Conclusion

The results of this study indicate that the concentration of EY is important for Caspian horse semen freezing, because, when CEY at 2% concentration was added to the 1.25% soybean lecithin-based extender, a synergistic effect between EY and lecithin provided even higher post-thaw survival of sperm cells such as motility, morphology, functional integrity than that of INRA0 and INRA4 during freezing/thawing. Whereas the positive effect of INRA2 was demonstrated on semen quality, the CEY concentration 4% (INRA4) leads to decline of motility and membrane and functional integrity. Additionally, the defined soybean

lecithin-based extender with centrifuged CEY 2% facilitates motility evaluating in CASA because it has less viscous fluid. Furthermore, the results show that freezability of Caspian horse semen can be affected by EY concentration and type (centrifuged versus whole). Finally, the present results suggest that the use of a low concentration of CEY (2%) and soybean lecithin in combination significantly improved reproductive traits of Caspian horse semen, indicating its beneficial effect during the freeze-thaw process. Nevertheless, further studies should be carried out in order to confirm with AI trials of this presented results.

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