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SHORT COMMUNICATION

Cryopreservation of horse semen with a liposome and trehalose added extender

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ABSTRACT. The aim of the study was to evaluate the effect of cryopreserved equine semen in the presence of trehalose-loaded liposomes on the integrity and function of sperm cells. Six ejaculations of five stallions collected with an artificial vagina were used. The ejaculates were diluted with INRA 96® 2:1 v/v and transported at 22 °C to the laboratory. Before cryopreservation, the semen was diluted with INRA Freeze® to obtain the following treatments: T1) INRA Freeze® (control), T2) INRA Freeze® + liposomes, T3) INRA Freeze® + liposomes+trehalose. Data were analysed using the Kruskal Wallis test. The percentages of sperm with intact DNA were 54.5, 57.9, and 64.8% for T1, T2 and T3, respectively (P>0.05). When evaluating the acrosomal and capacitation state after filtering with Percoll®, the percentages of spermatozoa without acrosome reaction and without capacitation were 67.8, 79.2 and 68.1% in T1, T2, and T3, respectively (P>0.05), while the capacitated sperm without acrosome reaction and without capacitation was similar in T1 (47%) and T3 (32%) (P>0.05), and lower in T2 (16%) before filtering with Percoll®. The use of liposomes and liposome-trehalose did not affect on the functional status and nuclear chromatin of the equine sperm after freezing, but it did affect the percentage of capacitated sperm without acrosome reaction after selecting the thawed semen using the Percoll® gradient.

Key words: equine, semen, liposomes, trehalose.

INTRODUCTION

Variability in sperm viability after the freezing and thawing process is a factor that limits the frequent use of frozen semen in equine breeding programs. Differences are observed among individuals and ejaculates, and these changes can result in reduced fertility (Amann *et al* 1987). One of the causes of reduced sperm fertility is the freezing/thawing process which damages cell components. The plasma membrane is the first affected site (Quinn *et al* 1980). Studies in this field have shown that the addition of liposomes to diluents before freezing can optimise membrane composition (Pillet *et al* 2012). It has also been shown that this damage can be reduced by adding lipoproteins to cryoprotectants (Wu *et al* 2013). It was found that the combination of external and internal cryoprotectants (Corcuera *et al* 2007) stabilises cell osmotic pressure and reduces stress. Trehalose is a polysaccharide with extracellular protective action which diminishes lesions caused by ice crystals. Its hypertonic effect causes cell dehydration, leading to a greater resistance of sperm against damage during freezing and thawing, improving its viability and motility (Molinia *et al* 1994, Bucak *et al* 2007).

The effect of adding trehalose and liposomes during cryopreservation on equine sperm quality after thawing has not been reported yet. Therefore, it is important to determine the efficacy of the use of trehalose and liposomes on the integrity and functionality of cryopreserved equine sperm.

MATERIAL AND METHODS

EJACULATES

Six ejaculations from five stallions were used. All the ejaculations were collected during the reproductive season (spring-summer), twice on two consecutive days with an interval of one hour between collections. The stallions rested 48 h and ejaculations were collected again the day after resting. The last ejaculate was used for treatment application. To collect the semen, the penis was washed with warm water to remove smegma and avoid contamination of the sample. The semen was collected with an artificial vagina (Colorado model), using a mare in heat to stimulate the stallions. Only the fraction rich in spermatozoa was recovered and filtered. Ejaculations with 60% progressive motility were selected and evaluated by one technician, following recommendations of Maxwell *et al* (1998).

The ejaculates diluted with INRA 96® (IMV Technologies®, France) at a ratio of 2:1 v/v at 22 °C were transported at a temperature of 37 °C to the Reproduction Biology Laboratory of the Diagnostic Unit of the "Torreón del Molino" Zootechnical Farm, FMVZ-UV, where they were processed.

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CONCENTRATION AND SEMINAL DILUTION

The semen was assessed to determine sperm concentration, as described by Brito *et al* (2016). Once sperm concentration was obtained, the semen was centrifuged to form a pellet, which was later re-suspended with the commercial freezer diluent INRA Freeze® (IMV Technologies®, France), following the manufacturer's instructions, and the sample was homogenised. Once the semen was diluted, three aliquots were separated from each ejaculate to be used in the treatments.

TREATMENTS

After diluting the semen, a 5 ml aliquot was assigned to one of the following treatments:

1) T1 (control): Semen diluted in INRA Freeze® without egg yolk.
2) T2: INRA Freeze® and liposomes 50 µl.
3) T3: INRA Freeze® and liposomes 50 µl and 200 mM trehalose.

PREPARATION OF LIPOSOMES

To prepare the liposomes, we used a commercial kit (SIGMA-ALDRICH®) consisting of a mixture of lipids such as cholesterol (9 µmol), L-α-phosphatidylcholine (egg yolk, 63 µmol), and stearylamine (18 µmol). The preparation was made following the instructions of the manufacturer: 1000 µl of deionized water was added, the mixture was then homogenised by vortex for 30 seconds and stored under refrigeration until use.

PREPARATION OF DILUENT WITH TREHALOSE

To prepare the trehalose solution, 1 ml of deionized water was mixed with 200 mM of trehalose (Fluka Analytical, UK) and vortexed to homogenise the solution.

FREEZING

To determine the sperm concentration of each ejaculate, sperm count was performed in a hematocytometer, using the technique described by Brito (2016). Once subjected to the different treatments, the diluted semen was deposited into 0.5 ml straws at a concentration of 200 million sperm per ml. They were refrigerated at 4 °C for 75 min and exposed to liquid nitrogen for 20 minutes (Ramires *et al* 2014). The freezing curve was performed in two steps: the semen straws are first cooled at a rate of 3 °C to 5 °C per minute until they reach 5 °C. They are then frozen at a rate of 20 ° to 50 °C per minute until reaching –196 °C. Isothermal boxes were used for freezing: 45 L polystyrene foam boxes in which the nitrogen and straws were separated by 3 to 6 cm (Alvarenga *et al* 2015). After 30 minutes, the straws were immersed in liquid nitrogen and deposited inside a tank with liquid nitrogen.

THAWING

To assess the effect of the treatments on the motility, acrosome state, and integrity of the nuclear chromatin, straws of each treatment were thawed. Thawing was done by placing the straws in a water bath at 37 °C for 30 seconds (Ramires *et al* 2014).

SPERM SEPARATION

To obtain a more appropriate sample of viable sperm, the sperm was separated through Percoll® gradients. Sperm separation was done in the following steps: 3 conical tubes with a capacity of 15 ml were used. In each tube, 0.5 ml of Percoll® was placed with a concentration gradient of 60%. Then, to each tube, 0.5 ml of Percoll® was added at a concentration of 40%, and finally, 0.5 ml of semen samples from each treatment was added in each tube. The samples were centrifuged at 3000 rpm for 3 min, the pellet was separated, and each pellet was resuspended in PBS and incubated for 15 min at 37 °C. Then, mass motility was observed and viability was estimated.

EVALUATION OF THE INTEGRITY OF THE ACROSOME

To determine the acrosomal state, the chlortetracycline fluorescence technique was used (Das Gupta *et al* 1994).

The following solutions were used:

- Buffered solution: 130 µmol NaCl, 5 µmol cysteine, 20 µmol Tris-HCl (pH 7.8).
- Chlortetracycline solution (CTC): 750 µmol of CTC in buffered solution.
- 12% Paraformaldehyde in 0.5 moles Tris-HCl buffer (pH 7.4).
- DABCO: 0.22 mol DABCO® in glycerol solution and PBS (9:1).

The contents of a previously thawed semen straw were placed in a 2 ml vial and centrifuged for 3 min at 323 *g*. The supernatant was removed and reconstituted with 80 µl of PBS and 100 µl of the CTC solution and shaken; 10 µl of the paraformaldehyde solution was added to the mixture and stirred. The slides were prepared by placing 10 µl of the sample on a clean slide and adding a drop of DABCO® solution to prolong the fluorescence. A slide cover was placed on the sample and observed under a fluorescence microscope.

The following fluorescence patterns were observed:

- Uniform fluorescence throughout the head and the middle part: not trained, no acrosomal reaction.

- Non-fluorescent band in the post-acrosomal region: trained, no acrosomal reaction.
- No fluorescence in the head, only in the intermediate piece: trained, with acrosomal reaction.

EVALUATION OF THE INTEGRITY OF NUCLEAR CHROMATIN

DNA integrity was evaluated using the fluorescence technique with acridine orange (NA), as described by (Tejeda *et al* 1984). The contents of a previously thawed semen straw were deposited into a 2 ml tube and centrifuged at 300 *g* for 3 min. The supernatant was removed, 100 µl of PBS were added and a smear was made with 20 µl of the sample. When the smear was dry, it was placed in Carnoy's solution for 24 h, after which it was removed from the solution and dried, and 50 µl of citric acid was added. After 5 min, it was rinsed with deionized water, dried, stained with the orange acridine solution for 5 min, rinsed with deionized water, drained and covered with a slide cover. It was immediately observed in the fluorescence microscope (Leica DM 020-518500/LS) with blue excitation filter from 405 to 455 nm, with a 40x objective. Several fields were observed to count 200 cells. The following fluorescence patterns were observed: Green sperm = intact DNA; Orange or red sperm = denatured DNA.

STATISTICAL ANALYSIS

The results were analysed statistically by one-way analysis of variance with the statistical package of the program Statistics v. 10 and Sigmaplot v. 13.

RESULTS AND DISCUSSION

POST-THAWING MOTILITY

The use of liposomes and trehalose as cryoprotectants has been studied with different animal species. They protect sperm by limiting the damage to the cell membrane during cooling, freezing and thawing. Figure 1 shows the average individual motility obtained after the thawing process for each treatment. An increase in motility is observed in cryopreserved samples with liposomes (33.3%) relative to the control samples (25.8%), 7.5% higher. In contrast, samples with liposomes loaded with trehalose (15.8%) were 10% lower (P>0.05) in motility than the control samples. It has already been shown that in horses, the addition of liposomes, relative to other components (egg yolk, skimmed milk, and glycerol), are more effective in preserving post-thaw motility (Denniston *et al* 1997). In this project, greater post-thaw progressive motility was observed in the samples frozen with the freezing diluent INRA Freeze® and liposomes (33.33%) than in the control treatment and in that of liposomes with trehalose (P<0.05). These results are similar to the 31.4% spermatozoa with progressive motility reported by Belala *et al* (2016). On the other hand, the result of the treatment using only the INRA Freeze® diluent (25.8%) is similar to the 26.5% obtained by Najjar *et al* (2016). The difference observed between these two treatments (T1 and T2) can be attributed to the stimulating effect of liposomes (Pillet *et al* 2012) on the freezing diluent, compared with the use of egg yolk. Although trehalose helps to reduce damage to the plasma membrane, it could increase the density of the medium, causing a decrease in motility.

FUNCTIONAL STATUS OF SPERM AFTER THAWING, BEFORE, AND AFTER FILTRATION WITH PERCOLL®

The assessment of the integrity of the acrosome of the six thawed ejaculates and their comparison before and after filtering showed a statistical difference in the percentage of untrained sperm without acrosome reaction in the liposome and liposome treatments with trehalose (P<0.05). There were no differences between treatments after post-thaw filtration (P>0.05) (figure 2). After thawing, it is important to use sperm selection procedures to recover a highly functional sperm population and achieve optimal conception rates (Stoll *et al* 2013, Cabera *et al* 2014). This selection is made through centrifugation in density gradients, in this case Percoll®, which has been catalogued as a method to select significantly higher percentages of spermatozoa with intact acrosome (Brandies *et al* 1993, Somfai *et al* 2002). The results of this study showed that the proportion of untrained sperm with no acrosomal reaction was not statistically different (P>0.05) after filtration with Percoll®. However, it was observed that the process is effective for the selection of a cell population with greater viability since sperm quality is low (<45%) before filtration (figure 2). It has been shown that

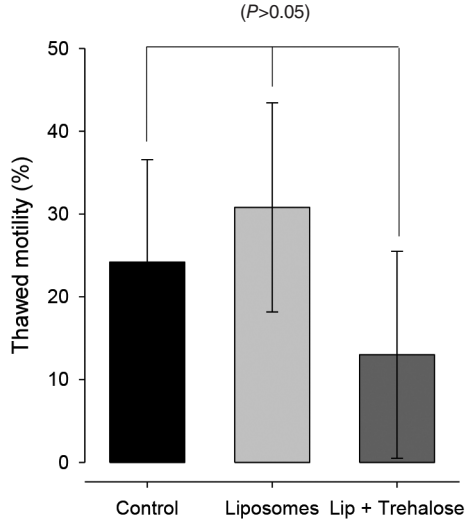

Figure 1. Post-thaw evaluation of motility of equine spermatozoa subjected to different cryopreservation treatments.

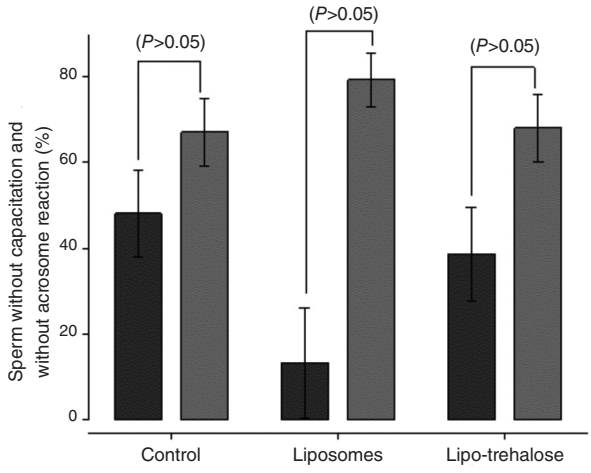

Figure 2. Post-thaw evaluation of the functional status of equine spermatozoa subjected to different cryopreservation treatments and filtering with Percoll®.

the addition of exogenous phospholipids and liposomes protect the sperm cell from damage during the freezing/thawing process since they maintain stability in the plasma membrane by fusing it (Köse *et al* 1998). Likewise, it has been demonstrated that the addition of polysaccharides such as trehalose contributes to the protection of the plasma membrane in the freezing/thawing process by dehydrating the cell and decreasing the transition temperature of the phospholipids (De Leeuw *et al* 1993).

INTEGRITY OF NUCLEAR CHROMATIN

The integrity of the nuclear chromatin in the thawed semen included in the three treatments was evaluated. The control treatment (54.5%) was statistically different ($P<0.05$) from that of the liposomes loaded with trehalose (64.8%). DNA integrity is a very important factor when evaluating sperm fertility as it is reflected in the ability to maintain embryonic development (Hamamah *et al* 1990). In this study, it was evaluated with acridine orange. The results showed no statistical differences between the proposed treatments ($P>0.05$) (table 1). However, our results are markedly different from those reported by other authors (Moreno *et al*, Belala *et al* 2016). This may be due to the cryopreservation technique used (Röpke *et al* 2011) or to the tolerance variability to the freezing/thawing process among stallions (Amann *et al* 1987).

Table 1. Post-thaw evaluation of the integrity of the nuclear chromatin of equine spermatozoa subjected to different cryopreservation treatments.

Treatments	Intact DNA %	Denatured DNA %
Control	54.5 ± 0.047	45.5 ± 0.052
Liposomes	57.9 ± 0.045	42.1 ± 0.053
Liposomes/trehalose	64.8 ± 0.042	35.2 ± 0.056

Without statistical difference between columns ($P>0.05$).

In conclusion, this study indicates that the enrichment of the freezing diluent INRA Freeze® with liposomes and liposomes plus trehalose in equine sperm does not provide better results in terms of progressive motility or preservation of DNA integrity compared with the use of INRA Freeze® diluent alone. However, filtering using density gradients with Percoll® can raise sperm quality after thawing by recovering the cells with greater viability.

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