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Ejaculated compared with epididymal stallion sperm vitrification

C. Álvarez[a,*], N. González[b], V. Luño[b], L. Gil[b]

[a] Military Horse Breeding Center in Zaragoza, Spain
[b] Department of Animal Pathology, Faculty of Veterinary Medicine, Instituto Agroalimentario de Aragón IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

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ABSTRACT

The aim of this study was to evaluate the effect of trehalose and lactose extenders on ejaculated and epididymal stallion sperm vitrification. Ejaculated semen samples were collected from seven fertile stallions, and *cauda* epididymis samples were collected from ten stallion carcasses after slaughter. Both the ejaculated and the epididymis samples were diluted and vitrified using INRA 96® and bovine serum albumin as well as trehalose or lactose. As a control, ejaculated and epididymal samples were collected and frozen using the conventional method. Vitrification was performed by immersing sperm suspensions directly in LN2. After thawing or devitrification, there was assessment of samples for sperm motility using computer-assisted analysis. Viability was assessed using SYBR-14 and propidium iodide (PI) and acrosome integrity by fluorescein using isothiocyanate combined with peanut agglutinin (FITC-PNA) and PI. Epididymal sperm vitrification with trehalose (EPT) or lactose (EPL) resulted in greater progressive sperm motility than sperm of the control sample (EPC). After post-thaw/devitrification of sperm in the EPT group, sperm motility was greater ($P < 0.001$) compared to that using EPL (50.72 ± 5.09% compared with 34.21 ± 3.02%). The results from assessment of ejaculated sperm samples after undergoing the vitrification process indicated cells were less viable ($P < 0.001$) than the control (EJC) sample. In conclusion, vitrification of epididymal stallion sperm using trehalose might be a beneficial alternative for the long-term storage of sperm samples with great economic value. Spermatozoa from vitrified ejaculates of stallions, however, had lesser motility and viability rates than samples subjected to conventional freezing.

1. Introduction

Stallions generally have a marked decrease in spermatozoa fertilization capacity after storage than other sperm of other species (Salazar et al., 2011; Restrepo et al., 2012). There is a large amount of inter-individual variation of values for sperm quality variables after the freezing/thawing processes in which only few stallions produce semen that can be cryopreserved with retention of desirable viability after thawing ("Good freezers"; Hoffmann et al., 2011; Alvarenga et al., 2016). To improve semen quality after thawing, there have been recent studies conducted of new procedures with varying results. One of these procedures is sperm vitrification, which is still a relatively unexplored methodology. The vitrification method has some advantages in practice, such as the reduction of processing time, cost, and equipment needed. It can also be conducted in any andrology laboratory (Rahiminia et al., 2017; Le et al., 2019).

Vitrification consists of the ultra-rapid freezing of a sperm drop immersed directly in liquid nitrogen (LN2), thus avoiding the

* Corresponding author.
E-mail addresses: calvsan@gmail.com (C. Álvarez), noegorti@unizar.es (N. González), viluno@gmail.com (V. Luño), lydiagil@unizar.es (L. Gil).

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detrimental effects of intracellular ice crystallization (Jiménez-Rabadán et al., 2015; Slabbert et al., 2015) and DNA damage that can be caused with use of more prolonged freezing methods (Rahiminia et al., 2017).

In humans, results of different studies indicate vitrification processing of semen led to improvement of values for some sperm variables such as motility, viability, acrosome integrity and DNA integrity compared to use of conventional freezing methods. Inconsistencies in outcomes, however, remain in regard to the most desirable method of cryopreserving human sperm (Le et al., 2019). In animals, there are only few studies on vitrification protocols (Pérez-Marín et al., 2018) and in recent reports there has been little information regarding the vitrification process for stallion semen (Hidalgo et al., 2018; Pérez-Marín et al., 2018).

In stallions and in other animal species, cryoprotectants are essential for these processes to avoid adverse reactions (Pérez-Marín et al., 2018). Disaccharides are used as non-permeable cryoprotectants and the effects have been investigated (Isachenko et al., 2008; Wu et al., 2015; Caturla-Sánchez et al., 2018; Golshahi et al., 2018; Pérez-Marín et al., 2018; Isachenko et al., 2019). With use of disaccharides, there is a recovery rate of motility and protection of the plasma membrane DNA integrity of spermatozoa to the extent sperm viability rates are acceptable for use of these samples for AI (Isachenko et al., 2008).

Hidalgo et al. (2018) analysed the effect of sucrose supplementation, while Pérez-Marín et al. (2018) tested both trehalose and sucrose as cryoprotectants for stallion sperm vitrification. Furthermore, the use of extenders containing trehalose or sucrose as compared to samples without these inclusions, at a range of concentration of between 0.15 and 0.2 M, are associated with a greater protective effect on the quality of sperm. Cryoprotectant-free vitrification approaches to cryopreservation of human sperm compared with conventional freezing results in greater sperm viability after devitrification of the samples (Isachenko et al., 2019). Few studies on vitrification have been published regarding the use of trehalose and lactose as cryoprotectants for stallion semen, and to the best of our knowledge, there are no studies evaluating stallion epididymal sperm vitrification processes. Epididymal collection may be a useful technique that could be used when there is impaired fertility in stallions (e.g., serious traumas or injuries, sudden death, castration or any other incident that makes semen collection impossible).

The purpose of this study was to determine the effect of vitrification on stallion epididymal sperm and to compare the results with ejaculate vitrification after the incorporation of non-permeable cryoprotectant (trehalose or lactose).

2. Materials and methods

The care and treatment of the animals used in this study was conducted in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experimentation.

2.1. Reagents and media

All chemicals used in this study were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. The medium used for washing the semen and its centrifugation was INRA 96® (IMV Technologies; L'Aigle, France).

Lactose–egg-yolk extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste (Gil et al., 2013) and 5%(v/v) of glycerol, was used as the control freezing extender. Vitrification medium contained INRA 96®, 1% bovine serum albumin (BSA) and 0.15 M trehalose or 0.15 M lactose.

2.2. Experimental design

In Fig. 1 there is a depiction of the schematic of the experimental design.

Sperm samples (n = 7) from the ejaculate and epididymis (n = 10) were treated with identical conservation media.

There were six experimental groups with differences in extenders and sperm origin: EJC (frozen-thawed ejaculate sperm), EJT (trehalose vitrified ejaculate sperm), EJL (lactose vitrified ejaculate sperm), EPC (frozen-thawed epididymal sperm), EPT (trehalose vitrified epididymal sperm) and EPL (lactose vitrified epididymal sperm).

2.3. Semen handling methods

2.3.1. Ejaculate collection, handling and dilution

Ejaculates were collected from seven healthy stallions (age 7–12 years old) for which the semen had adequate viability characteristics after cryopreservation and thawing. Semen collections occurred during the breeding season at the Horse Breeding Military Centre (HBMC) in Zaragoza (Spain) using an artificial vagina (Missouri-model; Nasco, WI, USA).

Macroscopic and microscopic assessments were performed immediately after collection. The initial concentration of spermatozoa was determined using a Spermacue® 12300/0500 device (Minitube Ibérica S.L., Tarragona, Spain). Sperm motility was analysed using the CASA System (ISAS®, Projectes I Serveis R + D S.L., Valencia, Spain). Samples with a minimum sperm concentration of 200×10^6/ml and sperm motility of greater than 50% were selected for the study.

Semen samples were diluted 1:1 in INRA 96® medium and centrifuged at 1000 *g* for 5 min (Alvarenga et al., 2016) to remove seminal plasma. Samples were divided into three aliquots and re-suspended into different media based on the experimental design (EJC, EJT and EJL). The final semen concentration after dilution was 100 x 10^6 sperm/mL (Samper, 2009).

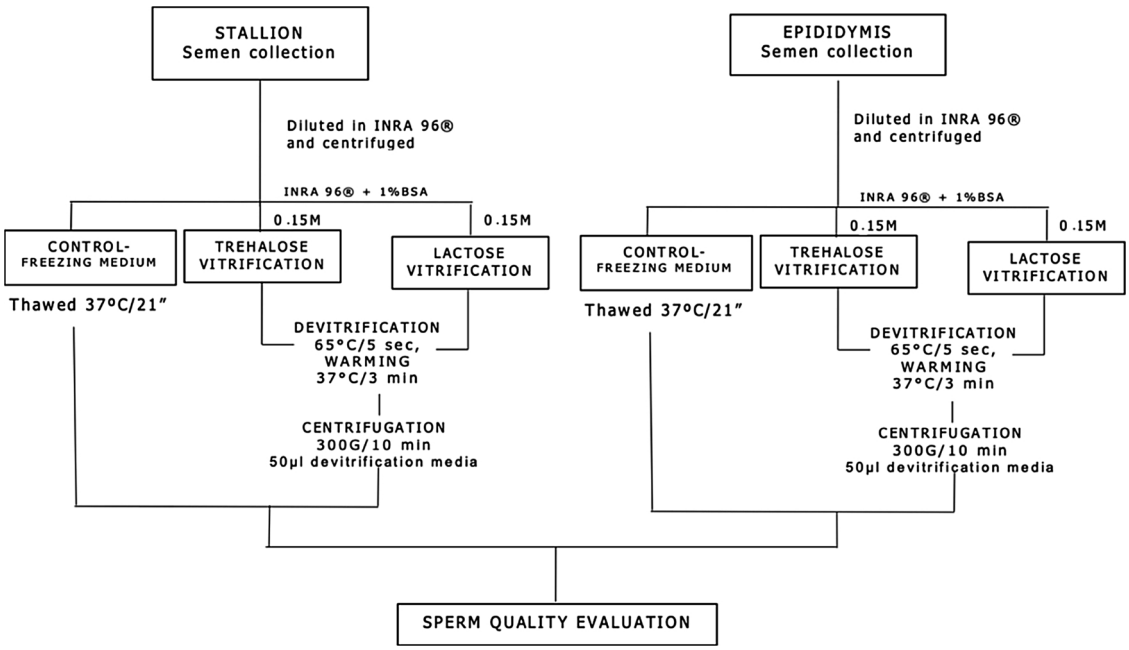

Fig. 1. Schematic of the experimental design – same preservation media was used for sperm samples ($n = 7$) from ejaculates and epididymides ($n = 10$); Samples were then placed in two vitrification media, INRA 96® + 1% bovine serum albumin + 0.15 M Trehalose or 0.15 M Lactose. A conventional freezing group was used as a control for both ejaculate and epididymides samples; After thawing/devitrifying, the quality of the sperm samples was evaluated.

2.3.2. Epididymal sperm collection, handling and dilution

Ten epididymides were collected from the carcasses of five stallions at the slaughterhouse (Mercazaragoza, Zaragoza, Spain). Sperm from the epididymis were collected by retrograde flushing of the *cauda* epididymis (Álvarez et al., 2014; Olaciregui et al., 2014).

The *cauda* epididymis was dissected and cannulated with a 25 G needle connected to a 10 ml of INRA 96® syringe at 20 °C. Manual pressure was then applied to the syringe, and spermatozoa were collected in a beaker. Sperm samples were centrifuged at 1,000 *g* for 5 min at room temperature and the supernatant was discarded (Olaciregui et al., 2014). The remaining sperm was extended to achieve a concentration of 100×10^6 sperm/mL with the medium (EPC, EPT or EPL).

2.3.3. Cryopreservation

2.3.3.1. Freezing and thawing. The samples were frozen using a stabilization step process at a gradual temperature-cooling rate of approximately 0.5 °C per minute. The samples were cooled to 4 °C in a conventional freezer for 2 h and packaged at 4 °C into 0.5 ml polyvinylchloride straws, (IVM technologies; L´Aigle, France). The samples were then frozen in LN2 vapour, 4 cm above the surface for 20 min. The straws were subsequently plunged directly into LN2 (-196 °C) in a styrofoam freezer box with a neopor insulation block (Minitub Ibérica S.L., Tarragona, Spain). Frozen sperm samples were thawed in circulating water at 37 °C for 21 s.

2.3.3.2. Vitrification and devitrification. Initially, 50 µl of each group were immersed directly in a cryotube containing 300 µl of LN2, which was subsequently sealed. The cryotubes, each containing three spheres, were then stored in LN2 tanks.

The devitrification process consisted of the vitrified sperm spheres being immersed in 500 µl of warming medium (INRA 96® + 1% BSA) at 65 °C for 5 s and maintained at 37 °C for 3 min. The samples were then centrifuged at 300 *g* for 10 min and the final pellet was re-suspended with 50 µl of warming medium for semen quality evaluation.

2.4. Semen quality evaluation

Total motility (TM) and progressive motility (PM) combined with kinematic variables were analysed using a computer-assisted analysis ISAS® (Projectes I Serveis R + D S.L., Spain; Holt et al., 2007). A total of 25 consecutive digital images were obtained with a time lapse of 1 s and the particle area was between 4 and 75 μ^2. With regard to the variables evaluated using the program, spermatozoa with an average path velocity (VAP) < 10 µ/s were considered slow, > 45 µ/s moderate, and > 90 µ/s fast. Spermatozoa with 75% of the straightness (STR) were designated as progressively motile. Spermatozoa were considered hyperactive if curvilinear velocity (VLC) was ≥180 and the amplitude of lateral head displacement (ALH; i.e., twice the maximum displacement of a sperm head from its fitted moving axis in a track segment) was ≥12 (Neuhauser et al., 2018; Rathi et al., 2001).

Sperm viability was evaluated using the LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands). A

volume of 100 µl of diluted sperm was mixed with 150 µl buffer (Becton Dickinson Immunochemistry, San Jose, CA, USA) with a final concentration of 20×10^6 sperm/ml. The SYBR-14 (20 nM) and propidium iodide (PI; 10 nM) were mixed with the different sperm samples. After incubation in the darkness for 15 min at room temperature, the proportion live/dead sperm cells was determined. Samples were analysed using a phase-contrast fluorescence microscope (Leika Microscope, DM2500 LED, l´Hospitalet de Llobregat, Spain). Two hundred spermatozoa were evaluated.

Acrosome status was assessed using fluorescein isothiocyanate combined with peanut agglutinin (FITC-PNA) and a propidium iodide (PI) stain. An aliquot of sperm suspension from each treatment group was supplemented with FITC-PNA solution (1 mg/ml in double distilled water) and PI solution (500 µg/ml), maintained at 38 °C for 5 min, and then fixed in paraformaldehyde (4% [v/v]) in a saline solution. At least 200 spermatozoa were examined using a fluorescence phase-contrast microscope.

2.5. Statistical analysis

All data obtained were analysed using computer software SPSS version 22.0 for Windows (SPSS. PC software, Chicago, Il, USA). The statistical procedure used for the analysis sample began with a visual exploration of data (boxplots) and an outline of centrality (mean and median) and variability (standard deviation) estimates. Kolmogórov-Smirnov tests were used to verify the normality of the data. Comparisons between the values of total motility, progressive motility, viability, acrosome integrity, VCL; VSL, VAP, SRT, BCF from different groups were analysed using a one-way analysis of variance (ANOVA). The LIN, WOB and ALH values were compared using the Kruskal–Wallis test.

In those cases where significant differences were observed, values were compared using the least significant difference pairwise multiple comparisons *post hoc* test (Tukey HSD test). The quantitative variables were expressed as mean ± standard error. All tests were assessed at the conventional significant level of 0.05.

3. Results

The primary variables for assessing sperm quality were affected by the stallion, sperm origin (ejaculate or epididymis) and process (conventional or vitrification) of sperm preservation ($P < 0.01$).

Results for ejaculate control samples (EJC; conventional freezing) indicated these samples had greater sperm quality ($P < 0.05$) than vitrified samples for the ejaculate when stored with diluents containing trehalose (EJT) or lactose (EJL) for all variables evaluated except progressive motility (Fig. 2). For epididymal sperm samples, the results were different. The EPC group had greater percentages for sperm TM as compared to the EPL group ($P < 0.01$), although there were no differences when there was comparison to the results with these two groups to those of the EPT group. For PM data, with the EPC group there was a greater ($P < 0.05$) PM than with the other experimental groups, except for the EPL group. The vitrification of ejaculate sperm samples resulted in reduced ($P < 0.05$) viability and acrosome integrity percentages as compared to those of epididymal sperm samples and the EJC group.

In addition, when results from sperm origin (ejaculate and epididymis) were compared, the vitrification process with supplementation of diluent with trehalose or lactose resulted in greater ($P < 0.05$) values for epididymal sperm than ejaculate sperm samples in terms of TM, PM, viability and acrosome integrity. There, however, were no differences in ejaculated sperm compared with epididymal sperm when conventional freezing procedures were used.

With regard to velocity variables (Fig. 3), all values were greater ($P < 0.05$) when vitrification methods were used. The ALH data were similar between frozen and vitrified samples regardless of the combination of the disaccharides used (Fig. 4). The BCF after vitrification when there was both trehalose and lactose supplementation, was greater ($P < 0.05$) than with use of conventional freezing procedures (EJC or EPC).

4. Discussion

In the present study, there were similar results to those of previous studies with use of the vitrification technique for ejaculated stallion spermatozoa (Hidalgo et al., 2018; Pérez-Marín et al., 2018). Sperm quality results were different when lactose and/or trehalose were used to supplement the diluents used for sample preservation. Nevertheless, to the best of the authors knowledge, there are no results from previous studies that have addressed vitrification as a method of preserving stallion epididymal spermatozoa, and the results from the present study for the cryopreservation of epididymal sperm varied from that when there was ejaculated sperm vitrification. The results of the present study provide evidence that vitrification of epididymal sperm using trehalose as a diluent supplement results in an enhancement of sperm quality.

Trehalose and sucrose are usually combined with BSA for sperm vitrification (Isachenko et al., 2008; Sánchez et al., 2011; Merino et al., 2012; Slabbert et al., 2015; Diaz-Jimenez et al., 2017; Consuegra et al., 2018). The BSA, among its other properties, reduces oxidative stress (Uysal et al., 2005) and maintains membrane integrity. The BSA used was concentrated to 1% (Hidalgo et al., 2018) because, at a greater concentration, it has negative effects on sperm quality after cryopreservation (Naijian et al., 2013; Nang et al., 2012). The 1% limit in concentration is also consistent with findings from previous studies where there was sperm vitrification in other species (Sánchez et al., 2011; Merino et al., 2012; Pradieé et al., 2018). In the present study, results obtained at a concentration of 1% were also consistent with results from these studies.

In contrast to recent studies, where the use of cryoprotectant free vitrification for humans and fish sperm cryopreservation yielded acceptable results (Merino et al., 2012; Isachenko et al., 2019), the use of non-permeable cryoprotectants was more effective for maintenance of sperm viability of cryopreserved semen of most animals including horses. Trehalose and sucrose are the most studied

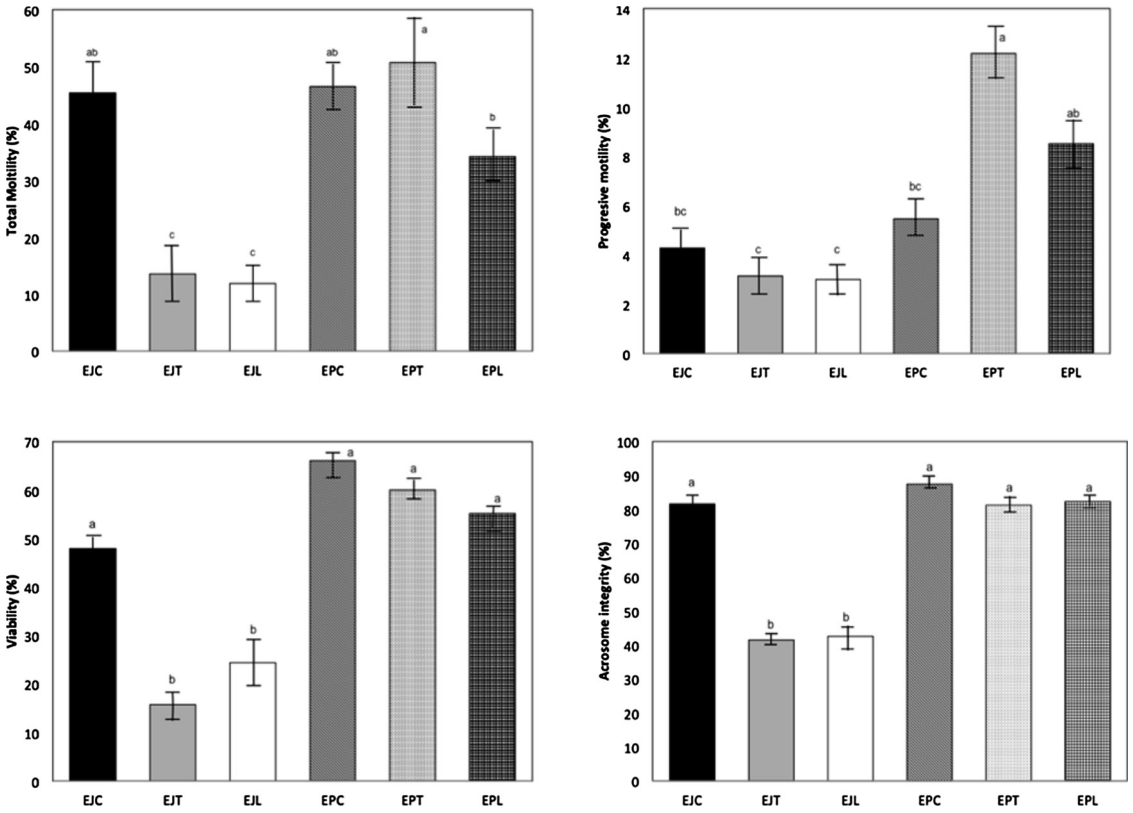

Fig. 2. Effect of sperm origin (ejaculated or epididymal) and extender on total motility, progressive motility, viability and acrosome integrity after preservation; EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$); Superscripts (^{a,b,c}) indicate differences ($P < 0.05$).

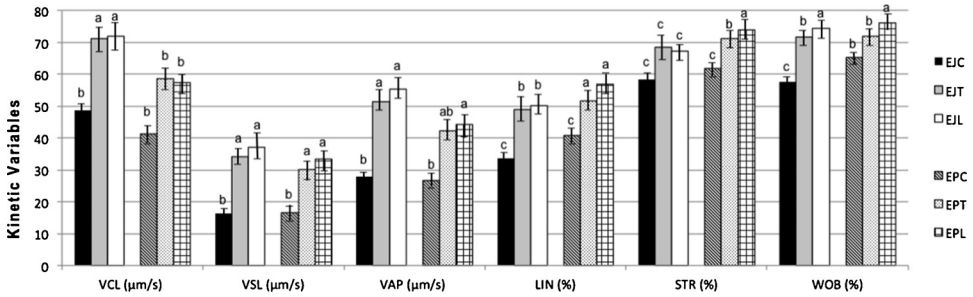

Fig. 3. Effect of sperm origin and extender on kinetic variables (VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness and WOB: wobble) after the conservation process.
EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$) Superscripts (^{a,b,c}) indicate differences ($P < 0.05$).

diluent supplemental components for spermatozoa preservation with the vitrification process (Caturla-Sánchez et al., 2018; Consuegra et al., 2018; Diaz-Jimenez et al., 2018; Hidalgo et al., 2018; Pérez-Marín et al., 2018). Results of only a few studies indicate trehalose is more effective than sucrose as a non-permeable cryoprotectant for sperm storage (Schulz et al., 2017). Results from the present research indicate that with supplementation of semen diluent with either trehalose or lactose there is no difference in sperm quality outcomes when there is use of these two supplements for cryopreservation of ejaculated vitrified samples. These results were consistent with those from recent studies (Pérez-Marín et al., 2018) in horses, or Caturla-Sánchez et al. (2018) in dogs. Nevertheless, with sperm epididymal samples, the addition of trehalose in the vitrification media resulted in a greater post-thaw motility than with use of lactose.

In results from recent studies (Pérez-Marín et al., 2018; Restrepo et al., 2019), there was a negative effect of the vitrification process on sperm quality as compared with using the conventional freezing process for the preservation of sperm. In addition, in these

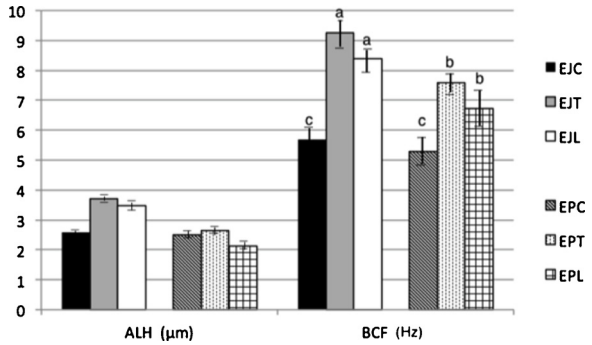

Fig. 4. Effect of sperm origin (ejaculated or epididymal) and extender on ALH (amplitude of lateral head) and BCF (beat cross frequency) variables after the preservation process; EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$); Superscripts (^{a,b,c}) indicate differences ($P < 0.05$).

previous studies sperm quality was markedly decreased when the vitrification process was conducted, both with trehalose and lactose in the vitrification medium, with the most desirable results at lesser concentrations (0.15 M). The present study, therefore, was conducted with a 0.15 M concentration and there were similar values for sperm quality when ejaculated sperm samples were evaluated whereas there was a marked decrease in sperm quality when there was use of the vitrification process compared with use of the conventional freezing method for sperm cryopreservation. Nevertheless, post-thaw sperm quality after the imposing of the vitrification process on epididymal samples was similar to that of the control samples. Hidalgo et al. (2018), reported that there were greater sperm quality values for ejaculated samples with the process of vitrification with sucrose plus BSA, but at different concentrations (sucrose at 20, 50, and 100 mM and BSA at 1%, 5%, and 10%).

In the present study, devitrification was performed at 65 °C for 5 s. Caturla-Sánchez et al. (2018) evaluated the optimal temperature during the devitrification process, while Pérez-Marín et al. (2018) reported that in stallions, devitrification at relatively greater temperatures for a short period of time (65 °C for 5 s) resulted in a greater sperm quality as compared with use of other devitrification conditions. Based on the findings from this previous research, in the current study, similar temperatures were applied for both ejaculated semen and epididymal samples. Results with vitrification of the epididymal samples indicated the sperm of these samples were of greater quality than those of ejaculated semen.

From results of the present study, it is concluded that stallion sperm vitrification is affected by sperm origin (i.e., ejaculated compared with epididymal sperm). Thus, vitrification with stallion epididymal spermatozoa resulted in greater sperm quality than that of sperm from stallion ejaculates. There is not exposure of epididymal sperm to seminal plasma which is different from what occurs with ejaculated sperm. Seminal plasma has a greater pH and greater Na^{++} and Cl^{-} content, which have been reported to be detrimental to sperm survival during the conservation process (Kareskoski et al., 2006; Monteiro et al., 2013). In addition, results of different studies indicate there is a deleterious effect of seminal plasma on sperm quality due to biochemical changes that increase membrane permeability (Aurich et al., 1996; Lozano et al., 2011) which cause greater susceptibility to freezing damage on the sperm plasma membrane.

The incorporation of 0.15 M of trehalose in the sperm vitrification medium could be a useful alternative for a long-term storage. Ejaculate-vitrified sperm, however, were of lesser quality for cryopreservation than sperm that are cryopreserved using conventional practices.

The findings of the current study indicate the importance of continued development of reliable protocols regarding the vitrification process for stallion sperm. These protocols could also be extended to other animal species.

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Declaration of Competing Interest

None.

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