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Original Research

Evaluation of Sperm Kinetics and Plasma Membrane Integrity of Frozen Equine Semen in Different Storage Volumes and Freezing Conditions

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ABSTRACT

In the present study, different freezing systems (Styrofoam box and Mini Digitcool ZH 400) and storage volumes (0.5- and 0.25-mL straws) were compared with regard to sperm kinetics and plasma membrane integrity of frozen and thawed semen. For that, three ejaculates from four animals were frozen in Styrofoam box and Mini Digitcool ZH 400 machine. The 0.5-mL straws were thawed at 46°C for 20 seconds, and the 0.25-mL straws were thawed at 46°C for 12 seconds. Statistical analysis was performed using program R of descriptive analysis box plot, followed by analysis of variance using PROC MIXED of SAS 9.1 package. Variances of 5% were considered as different. There was no interaction between the straw sizes and volumes; however, statistical differences were observed between the semen storage volumes. The 0.5-mL straws had higher total motility (%), progressive motility (%), average path velocity (μm/s), straight-line velocity (μm/s), curvilinear velocity (μm/s), and rapid sperm percentage (%) than the 0.25-mL straws. However, plasma membrane integrity analysis did not differ between the two straws. Thus, it is possible to conclude that equine sperm cryopreserved in 0.5-mL straws has better sperm kinetics than when stored in 0.25-mL straws. Additionally, it is possible to conclude that automated systems that enable faster freezing rates result in a seminal quality that is similar to the one obtained by the conventional system using Styrofoam boxes.

1. Introduction

The biological techniques involving cryopreservation of equine semen have been receiving greater importance to better use genetically superior animals, allow long-distance transportation of semen, and control sexually transmitted diseases [1].

However, irreversible damages to the structural integrity, biochemistry, and biophysics of the sperm cell are caused by cryopreservation, resulting in lower fertility rates [2]. Thus, methods that reduce damages to the sperm cell have been studied and improved, including the discovery of the cryoprotective effect of glycerol by Polge et al. [3].

Cryopreservation requires exposing spermatozoa to extreme variations in temperature and osmolarity. In this process, there is ice crystal formation, resulting in greater extracellular solute concentrations, and, consequently, hyperosmolarity. The cell responds to this insult by losing water and shrinking in volume to equilibrate the solute concentrations between intra- and extracellular compartments. Conversely, the cell volume increases by passive diffusion of water when cells are exposed to a hypotonic extracellular environment (as is the case during thawing). It is not known whether this osmotic stress results in sublethal irreversible damage to the cell membrane, thus decreasing fertilizing capacity [4,5].

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The injuries of cold shock occur during the transition phase, which is the process characterized by the plasma membrane passage from a liquid/crystalline state to a gel phase [1,6]. These alterations prevent the membrane phospholipids from moving laterally, resulting in the formation of small lipid regions in liquid state where proteins adhere. This protein aggregation results in both the increase of membrane permeability and decrease of metabolic activity [7].

The optimal freezing rate is extremely important to maintain cellular integrity. The cooling rate was −0.3°C/min from room temperature to 5°C, and the freezing rate was −15°C/min from 5°C to −80°C, and −10°C/min until −120°C. Subsequently, straws were plunged into liquid nitrogen (−196°C) for storage. An ideal rate must be slow enough to allow the sufficient dehydration of the cell (to avoid the formation of intracellular ice crystals) but fast enough to avoid prolonged exposure of spermatozoa to hypersaturated solutions of the extracellular environment [4,8-11].

Although automated systems for temperature control during the cryopreservation process have been developed, they are still costly. In this regard, several protocols combining different storage volumes and freezing rates have been developed and tested to improve the success of frozen equine semen and decrease the damages caused by cryopreservation [12].

This study aimed to compare the sperm kinetics and the plasma membrane integrity (PMI) of equine semen frozen using a passive vapor-phase technique in a Styrofoam box and a controlled-rate freezing technique in a Mini Digitcool ZH 400 machine (IMV Technologies, L'Aigle, France), with sperm packaged in either 0.5- or 0.25-mL polypropylene straws.

2. Materials and Methods

2.1. Animals

Four stallions of Arabian, Westfallen, Hannoverian, and Mangalarga Marchador breed, respectively, were used in the study. These animals were maintained under a regular semen collection regimen. Three ejaculates from each stallion (n = 12) were collected using a mannequin and Botucatu (Botupharma Ltda, Botucatu, São Paulo, Brazil) artificial vagina.

2.2. Semen Processing and Cryopreservation

After collection, the semen was filtered, and the sperm concentration was evaluated using Neubauer chamber (LO - Laboroptik Ltd, Marlborough Road Lancing, Business Park Lancing, UK). The sperm kinetics parameters were evaluated in a computerized sperm movement analyzer—CASA (HTM-IVOS 12; Hamilton-Thorne Research, Danvers, MA). The semen samples were diluted in Botu-Sêmen (Botupharma Ltda) at 37°C for 10 minutes at a concentration of 50×10^6 spermatozoa/mL. A 10-μL aliquot of diluted semen was deposited in a Makler chamber (Makler Counting Chamber, Sefi-Medical Instruments Ltd., Haifa, Israel) preheated to 37°C. The minimum of 500 cells was analyzed per sample, and only the ejaculates with total motility ≥60% were used for cryopreservation. Values for the Integrated Visual Optical System (IVOS) system consisted of the following: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 60 pixels; minimum cell size, 3 pixels; minimum static contrast, 30 pixels; straightness threshold for progressive motility (PM), 50; average-path velocity (VAP) threshold for PM, 70; static head size, 0.62 to 2.98; static head intensity, 0.24 to 1.19; static elongation, 100 to 0; illumination intensity, 2200. Experimental end points included percentage of motile sperm, percentage of progressively motile sperm, mean curvilinear velocity (VCL, μm/s), mean VAP (μm/s), and mean straight-line velocity (μm/s).

For the cryopreservation of the ejaculates, the technique described by Papa et al. [13] was used, in which each ejaculate was extended in a media containing skimmed milk and glucose (Botu-Sêmen) in 1:1 proportion (medium:semen) and centrifuged at 600 × *g* for 10 minutes. After the centrifugation, the supernatant was removed, and the pellets were resuspended to a concentration of 100×10^6 spermatozoa/straw in Botu-Crio extender (Botupharma Ltda). After the addition of the extender and adequate homogenization, the samples were packaged in 0.5- and 0.25-mL straws and sealed with polyvinyl alcohol.

The straws were then distributed on racks and cooled to 5°C for 20 minutes in Minitube refrigerator (Minitube Ldta). The rack containing the straws was then transferred to the suitable freezing system.

Freezing in Styrofoam box was performed using a 42-L isothermal box with 3.5 cm of liquid nitrogen (N_2), and the straws were placed horizontally at 6 cm above the level of N_2, with a freezing rate of −10°C/min between 5°C and −60°C and a velocity of −8°C/min between −60°C and −100°C (measured by thermometer inside the straw to determine the correct freezing rate).

Mini Digitcool ZH 400 equipment was programmed with the following rate: −15°C/min from 5°C to −10°C and velocity of −40°C/min from −10°C to −140°C. After freezing, the straws from both freezing systems were immersed into liquid nitrogen for storage.

Table 1
Means (± standard errors) of postthaw variables of TM, PM, and PMI in different freezing systems and in 0.5- and 0.25-mL straws

Volume	TM (%)		PM (%)		PMI (%)	
	SB	MD	SB	MD	SB	MD
0.5	67.3 (4.5)[a]	71.1 (4.6)[a]	27.8 (3.3)[a]	30.9 (3.6)[a]	38.9 (2.6)[a]	37.8 (2.8)[a]
0.25	60.5 (4.6)[b]	62.0 (5.1)[b]	24.3 (3.4)[b]	24.5 (3.7)[b]	33.4 (2.4)[a]	33.1 (2.4)[a]

SB, Styrofoam box; MD, Mini Digitcool ZH 400; TM, total motility; PM, progressive motility; PMI,plasma membrane integrity.
[a,b]Different letters in a column indicate differences ($P < .05$).

2.3. Evaluation of Semen after Thawing

Semen samples were thawed in water bath at 46°C, 20 seconds for 0.5-mL straws and 12 seconds for 0.25-mL straws as previously described (14), and evaluated using the computerized analyzer—CASA. These samples were diluted with the same extender used for cryopreservation (Botu-crio) at 37°C for 10 minutes, achieving a final concentration of 50×10^6 spermatozoa/mL. This final concentration is adequate for CASA evaluation. The following variables were considered: total motility (%), PM (%), percentage of rapid sperm (%), average-path velocity (VAP) (μm/s), straight-line velocity (μm/s), and VCL (μm/s). Additionally, PMI was analyzed (%) by associating fluorescein diacetate of 6-carboxyfluorescein and propidium iodide (as previously described) [14].

2.4. Statistical Analysis

For statistical analysis, the program R of descriptive analysis box plot was initially used. Then, the study variables of freezing/thawed semen were submitted to analysis of variance using PROC MIXED of SAS 9.1 package (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina, USA). The different freezing rates and straws were considered as fixed effects. The animals were considered as random effect. Variances of 5% were considered as different.

3. Results

Tables 1 and 2 show the mean values and standard errors of the seminal characteristics (evaluated using CASA) and PMI (evaluated using epifluorescence microscopy).

No differences were observed in CASA values between freezing systems ($P > .05$). However, when the two different straw volumes (0.5 and 0.25 mL) were compared, differences were found in all kinetics parameters ($P < .05$).

4. Discussion

Sperm stored in 0.5-mL straws presented increased kinetics values (according to CASA analysis) than sperm stored in 0.25-mL straws, but no significant differences were found regarding membrane integrity. Importantly, similar results were reported for ram and canine sperm by Maxwell et al. [15] and Nothling and Shuttleworth [16], respectively. According to Maxwell et al. [15], the reduced surface area to volume ratio in 0.5-mL straws results in decelerated sperm freezing, thus reducing cryopreservation-related injuries. According to Senger et al. [17], storing semen in 0.25-mL straws requires a milder freezing rate than storing in 0.5-mL straws. They also reported that both freezing rates and straw volumes have significant effects on sperm motility and acrosomal membrane integrity [15]. In our study, an equal freezing curve was used for both 0.5- and 0.25-mL straws to identify the best storing volume for the system already in use in our laboratory. The inferior results observed for 0.25-mL straws can be dependent (at least partially) on the freezing systems used in this experiment. Of note, such interference was already reported by Stevenson et al. [18].

Nascimento et al. [12] analyzed sperm freezing in 0.25- and 0.5-mL straws using an automated system and reported higher VCL in frozen–thawed semen stored in 0.25-mL than in 0.5-mL straws. However, further sperm parameters analyzed in this study did not significantly differ between the two straw volumes. These results differ from the observations of improved kinetics when cryopreserving sperm in 0.5-mL straws, compared with 0.25-mL straws, observed in our study.

Dell'Aqua Jr. et al. [19] compared the cryopreservation of equine semen in 0.5- and 0.25-mL straws and observed improved sperm kinetics when using 0.25-mL straws. This finding differs from our study, probably owing to the different freezing protocol, including the use of a different cryoprotectant substance. In the current study, a cryopreservation extender containing dimethylformamide (a substance of lower molecular weight and higher permeability to sperm) was used, possibly minimizing the injuries to the sperm, thus being more suitable for semen storage in 0.5-mL straws. Furthermore, in stallions with poor freezability, the use of amides in the cryopreservation extender confers better results when compared with glycerol [20].

Regarding the freezing protocol used in our experiment, the results of sperm parameter assessment using the automated system had similar results compared with the conventional method using the Styrofoam box. This finding demonstrates that the simple and inexpensive method of the Styrofoam box is indicated for significantly reducing the economical costs of equine spermatozoa cryopreservation without resulting in significant losses of semen quality after thawing [21].

The PMI is another important parameter regarding sperm quality [21]. It has been shown that the percentage of sperm with intact PMI is positively correlated with sperm motility [10] ($r = 0.78$). In contrast with the results reported by Salazar Jr. et al. [11], the results of our study showed no significant influence of semen-freezing method on PMI. Possibly, the differences among experiments occur owing to different protocols for processing, cryopreserving, thawing, or evaluating the semen, as each laboratory adopts an elected technique [22].

Table 2
Means (±standard errors) of postthaw variables to VAP, VSL, VCL, and RAP in different freezing systems and in 0.5- and 0.25-mL straws

Volume	VAP (μm/s)		VSL (μm/s)		VCL (μm/s)		RAP (%)	
	SB	MD	SB	MD	SB	MD	SB	MD
0.5	91.9 (4.3)[a]	96.5 (4.7)[a]	71.3(3.1)[a]	75.1 (3.3)[a]	172.8 (6.1)[a]	178.0 (6.3)[a]	50.4(6.8)[a]	55.4 (7.1)[a]
0.25	90.1 (4.3)[b]	90.3 (4.8)[b]	69.7(3.1)[b]	69.5 (3.4)[b]	168.0 (6.1)[b]	169.5 (6.9)[b]	43.9(6.9)[b]	45.4 (7.5)[b]

VAP, angular velocity; VSL, straight-line velocity; VCL, curvilinear velocity; RAP, rapid cells.
[a,b]Different letters in a column indicate differences ($P < .05$); regarding VAP, $P = .05$.

5. Conclusion

In conclusion, equine sperm cryopreserved in 0.5-mL straws presented higher sperm kinetics values evaluated using CASA compared with the ones preserved in 0.25-mL straws. Additionally, the freezing method did not have influence on the sperm characteristics of the stallions, showing that a simple and cheap protocol using a Styrofoam box could achieve the same results observed in a computerized and complex system.

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