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Comparison between the conventional and automated systems for frozen cooled equine semen

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Abstract

This study aims to compare the efficiency of the automated system (controlled-rate freezer) and the conventional system (manual system) for freezing the equine semen after cooling at 16ºC. The parameters evaluated were: motility, strength, plasmatic and acrosomal membrane integrity of spermatozoa from twelve stallions. Ejaculates from stallions were collected three times per week, during four weeks. Gel-free semen was diluted in skim milk extender and cooled at 16ºC for 24 h. After cooling, extended semen was centrifuged at 600 x *g* for 10 min. The supernatant was removed and sperm pellets were re-suspended using the freezing extender. Samples were then packed into 0.5 ml straws, which were divided into two parts: one for cryopreservation in a controlled-rate freezer (CR) and the other for a manual system (MS). In this study, CR showed higher values for motility (44.6%), viability (57.9%) and plasmatic membrane integrity (29.3%) when compared with MS (20, 35.7 and 5.1%), ($P < 0.05$), respectively, after 24 h of cooling at 16ºC. The automated system for cryopreservation of cooled semen at 16°C for 24 h was more efficient, with higher values of motility, viability and plasmatic membrane integrity when compared with the manual system.

Keywords: cryopreservation, frozen semen, genetic resource, spermatozoa, stallion.

Introduction

Important advances in the use of frozen semen have occurred in the 57 years since the birth of the first foal using this technology. However, pregnancy rates remain low due to many variables (Miller, 2008). The collection and shipment of cooled-semen to a specialized laboratory makes the cryopreservation process easier, without transporting the stallion to reproduction centers (Crockett *et al*., 2001). However, manipulation of equine semen during these processes can reduce sperm viability and fertility due to several factors (Cocchia *et al*., 2011).

The utilization of frozen semen has created a new dimension in the breeding of horses by allowing the indefinite preservation of this material and its worldwide distribution, which maximizes the use of stallions with higher genetic merit and reduces transportation costs and risk of disease dissemination; furthermore, geographical barriers are abolished and it is possible to use frozen semen from stallions which are in competition or recovering from pathologies that prevent them from breeding, even semen from dead stallions can be used (Miller, 2008).

Most of the semen cryopreserved in Brazil outside of a research laboratory is processed in a refrigerator/liquid nitrogen vapor system. This technique has been shown to be viable, however, it is difficult to standardize the cooling and freezing curves since several factors contribute to inducing variations, such as: brand and model of the refrigerator, type of Styrofoam box, number of doses to be frozen and the level of liquid nitrogen used.

Since the freezing procedure used in these cases may interfere in the final viability of semen, the aim of this study was to compare the efficiency of the automated system (controlled-rate freezer) versus the conventional system (manual system) for freezing equine semen after cooling it at 16°C.

Materials and Methods

Semen collection and cooling

The experiment lasted from March to May. Stallions' semen was collected once a day for 7 days to stabilize extragonadal reserve and daily sperm output. Afterwards, ejaculates were collected three times per week from 12 fertile light-horse stallions between the ages of 5 and 15 years, using the Colorado model (Equine Artificial Vagina; ARS, Chino-CA, USA), with a total of 36 ejaculates obtained. Semen samples were collected in a plastic bottle and filtered immediately after collection to create gel-free semen. Only ejaculates with more than 60% total motility were used for cooling (Colégio Brasileiro de Reprodução Animal - CBRA, 2013).

The sperm-rich fraction (gel-free) was diluted in a 2:1 ratio using a skim milk-glucose extender with penicillin G procaine (Kenney *et al*., 1975) and cooled

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(16ºC) for 24 h in a transport box (Max-Sêmen Express, Agrofarma, São Paulo, Brazil).

The average temperature of the experiment days was 24°C. After 24 h of cooling, the temperature of semen was measured immediately after opening the box. In all samples the temperature measured was 16°C.

Sample freezing

After the 24 h of storage, extended semen was centrifuged at 600 x *g* for 10 min, and sperm pellets were resuspended in a freezing extender (Botucrio; Botupharma, Botucatu, Brazil) to a concentration of 200 x 10^6 cells/ml and were packed into 0.5 ml straws and divided in two groups for the freezing process: one half was used in the controlled-rate freezer system (CR; TK 3000 SE, TK Tecnologia em Congelação Ltda, Uberaba, Brazil) and the other half in the manual freezing system (MS), to stabilize the cooling and freezing rates.

For stabilization in CR, straws were placed on a straw holder inside the cooling tube until it reached 5°C at a rate of -0.25°C/min, remaining 20 min in this temperature. After this time, the straw holder was moved to a thermal box containing liquid nitrogen, at a freezing rate of 15°C/min from 5°C until reaching -80°C and from 10°C until reaching -120°C. Once this temperature was reached, the straws were plunged into liquid nitrogen (-196°C) and stored in a liquid nitrogen holding tank.

For stabilization in MS, straws were placed in a stainless steel support and kept inside the refrigerator (5°C; Eletrolux R130, Eletrolux Brasil, Curitiba, PR, Brazil) for 20 min. Subsequently, these straws were kept at a height of 6 cm from the liquid nitrogen for 20 min in an isotherm box (20L model) and plunged into liquid nitrogen.

Post-thawing analysis

Three straws from each treatment, from the same stallion, were thawed in a water bath at 37°C for 30 s 24 h after storage in the cryogenic container. Variables analyzed were: total motility, strength, viability and acrosomal and plasmatic membrane integrity.

Assessment of sperm motion characteristics

The subjective evaluation of total motility (0-100%) and strength (0-5) was done with a drop of semen (10 µl) placed between a slide and coverslip previously heated at 37°C and visualized by a contrast phase optical microscope at 200X magnification. The strength expresses the speed with which the spermatozoa move into the field. The result was expressed on a scale of 0-5, 0 being no motion and 5 intense speed (CBRA, 2013).

Assessment of viability, plasma and acrosomal membrane integrity

For the evaluation of viability, the supravital eosin-nigrosin staining technique (live/dead) was used, where equal volumes (20 µl) of semen and stain were mixed and transferred to a preheated (37°C) labeled microscope slide and smeared by sliding a cover slip in front of it. The smears were air dried and examined directly. Samples were evaluated by microscopy (magnification 1,000X). Five hundred sperm cells were counted per sample, and unstained cells were classified as those that were viable (Dott and Foster, 1972).

To evaluate sperm plasmatic membrane integrity, the hypo-osmotic swelling test (HOST) was used by incubation of 100 µl of semen in 1.0 ml of a sucrose solution of 100 mOsm/l in a water bath at 37°C for 30 min. After this time, 20 µl of this solution was analyzed in a humidity chamber, using phase-contrast microscopy at 1,000X magnification. A total of 200 spermatozoa were counted, and those considered swollen (coiled) were determined to possess membrane integrity after the subtraction of the percentage of tail alterations found in the morphologic evaluation (Melo and Henry, 1999).

To evaluate acrosome membrane integrity, trypan blue/Giemsa staining was used. Equal volumes of semen and trypan blue, 0.2% (20 µl; Sigma Chemical Co.) stain were placed on a microscope slide, heated at 37°C, and mixed to prepare a smear, which was air dried. Dried smears were fixed in neutral red solution (Sigma Chemical Co.) for 5 min, washed in running water, air dried again, and then immersed in 7.5% Giemsa stain solution (Sigma Chemical Co.) for 4 h (Kútvölgyi *et al*., 2006).

Evaluation was performed at 1,000X magnification, and 200 spermatozoa were counted and classified as live if the acrosomes were stained pink or purple and postacrosomal regions were unstained; and as dead, if the postacrosomal region was stained blue and acrosomes stained purple or pink. True acrosome reaction was observed if acrosomes and postacrosomal regions were unstained; and false acrosome reaction if acrosomes were unstained and postacrosomal regions were stained blue (Didion *et al*., 1989).

Statistical analysis

Data analysis was performed using SAS UNIVARIATE procedure (Statistical Analysis System, version 9.2) to determine if the experimental error of variables had normal distribution for variance probability and homogeneity. Since the studied variables did not present normal distribution, a non-parametric test, Wilcox on test (WinStat module; Microsoft), was used.

Results

Table 1 shows the results of fresh semen, immediately post collection and after cooling at 16°C for 24 h. As expected, there was a decrease in motility (70 ± 5.9%) *vs*. (51.8 ± 4%), viability (76.5 ± 7.8%) *vs*. (69.8 ± 8.1%), plasmatic membrane integrity (60 ± 14%) *vs*. (49.3 ± 12.2%) and acrosomal membrane integrity (82.2 ± 8.3%) *vs*. (77.2 ± 10.2%) when comparing fresh semen with cooled semen respectively ($P < 0.05$).

Table 2 shows the results for total motility, strength and viability for the different freezing protocols. Total motility was higher ($P < 0.05$) in controlled rate freezer (CR; 44.6 ± 15.6)% than in manual system (MS; 20 ± 10.9)%. Strength results were similar for both protocols (CR/3.4 ± 0.6) x (MS/3.4 ± 1.1; $P > 0.05$). Viability was higher ($P < 0.05$) in CR (57.9 ± 14.7)% when compared to the MS (35.7 ± 8.7)%.

Table 2 shows that plasmatic membrane integrity was higher ($P < 0.05$) in the CR (29.3 ± 14.7%) when compared to MS (5.1 ± 2.8%). Acrosome membrane results were similar for both protocols (CR/71.1 ± 2.8%) x (MS/68.0 ± 4.3%; $P > 0.05$). To discuss the results, acrosomal integrity will be taken into account only as the percentage of live cells within tact acrosome.

Table 1.Results of analysis of fresh and cooled semen at 16ºC for 24 h for total motility, strength, viability, plasmatic membrane integrity (PMI) and acrosomal membrane integrity (AMI).

Semen	Motility (%)	Strength (%)	Viability (%)	PMI (%)	AMI (%)
Fresh	70.7 ± 5.9^a	4.0 ± 0.5^a	76.5 ± 7.8^a	60.0 ± 14^a	82.2 ± 8.3^a
Cooled	51.8 ± 4^b	3.0 ± 0.5^a	69.8 ± 8.1^b	49.3 ± 12.2^b	77.2 ± 10.2^b

Within a column, values without a common letter differed ($P < 0.05$).

Table 2.Results of post-thaw analysis for total motility, strength, viability, plasmatic membrane integrity and acrosomal membrane integrity of equine frozen semen using two different freezing systems (controlled rate freezer-CR; Manual-MS) after 24 h of cooling at 16ºC.

Systems	Motility (%)	Strength (%)	Viability (%)	Plasmatic membrane integrity (%)	Acrosomal membrane integrity (%)
CR	44.6 ± 15.6^a	3.4 ± 0.6^a	57.9 ± 14.7^a	29.3 ± 14.7^a	71.1 ± 2.8^a
MS	20.0 ± 10.9^b	3.4 ± 1.1^b	35.7 ± 8.7^b	5.1 ± 2.8^b	68.0 ± 4.3^a

Within a column, values without a common letter differed ($P < 0.05$).

Discussion

The cryopreservation of equine semen is limited by factors such as the high cost of equipment, the need for specialized technicians, and on farm locations to manipulate semen (Crockett *et al*., 2001). It is possible to ship cooled-semen to specialized laboratories.

Cooled equine semen is used to allow its storage for variable periods of time. During transportation the semen should maintain the fertility, and that depends on factors such as storage temperature, composition of the extender and spermatic concentration per insemination dose (Heckenbichler *et al*., 2011).

The cooled shipping system chosen for this study maintains semen at 16ºC for 24 h. It is a low cost procedure, widely used during the breeding season and allows the long distance shipment of semen (Melo *et al*., 2007). It was chosen becauseit simulates the real situation faced by the farms.

Melo *et al*. (2007), after storing the semen in two cooling systems (16 and 5ºC) for 24 h and freezing it with the conventional system, found no differences in motility between the equine spermatozoa immediately frozen after being collected and the spermatozoa frozen after being chilled passively for 24 h, although some variations regarding the plasmatic membrane integrity were observed.

The results in Table 1 show that there was a decrease in sperm motility from 70.7 to 51.8% after cooling for 24 h, but these variables remained within the limits considered appropriate for equine spermatozoa after cooling (CBRA, 2013). Reduction was also observed for viability and integrity of plasmatic and acrosomal membranes when semen has been cooled at 16°C for 24 h.

When the automated freezing system was used, motility, viability and plasmatic membrane integrity were higher when compared to the manual system. This can be explained by the standardization and non-oscillation of cooling and cryopreservation curves found in the machine.

The manual system method has a lower cost than the controlled-rate freezer, but it does not have a control setting for the cooling curve and may undergo variations in temperature during cooling and freezing curves because it is affected by ambient temperature, stabilization of the refrigerator, quality and dimensions of the Styrofoam box and nitrogen level. Thus, the cryopreserved semen may present poor quality if all these variables are not controlled, which was verified in Table 2.

The results of this experiment contradict the results found in cattle (Forero-Gonzalez *et al*., 2012; Abud *et al*., 2014) and horses (Maziero *et al*., 2013.), where no differences in motility and plasmatic membrane integrity were observed when comparing the

conventional system with the automated system, but corroborate the data found by Clulow *et al*. (2008) which demonstrated that the programmable freezer provided a more consistent and reliable freezing rate than liquid nitrogen vapor.

The superior post-thawing result found in the experiment related to motility, viability and plasmatic membrane integrity can be explained by what was found by Clulow *et al*., 2008 who observed an overall faster decline in temperature in the manual system when compared to the programmable freezer. Moreover, the manual system may have provided a more variable freezing rate than the programmable freezer, as the level of liquid nitrogen within the box was subjectively estimated and subjected to evaporation, and it was difficult to standardize it for each freezing run. The removal of the lid and the number of straws may also influence the temperature of the vapor in the box and therefore the cooling rate in subsequent freezing runs.

Although Vishwanath and Shannon (2000) claim that in the automated system, for freezing large numbers of straws, considerable variations in the individual freezing rate of each straw occurred due to the fact that they were arranged in different levels, this may not have occurred in this study since a small number of straws were frozen in the different systems, being distributed homogeneously.

Despite the high cost of the automated system used in the experiment, which would be the biggest drawback, it has as an advantage in the fact that it might be used in places without electricity, because it can work with an internal battery, thus replacing the refrigerator and thermal container used for positive and negative slope, respectively, which can vary according to the ambient temperature, size, maintenance, etc., which means that it has more homogeneous freezing curves within the machine. The main advantage of the manual freezing system method is that it is cheap, easy to use, has low liquid nitrogen requirements and is not made from fragile material, which makes it better for transportation.

In conclusion, the automated system for cryopreservation of cooled semen at 16°C for 24 h was more efficient with higher values of motility, viability and plasmatic membrane integrity when compared with the manual system.

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