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Evaluation of cooling and freezing systems of bovine semen

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ABSTRACT

Semen cryopreservation comprises different steps, among them are the cooling and freezing rates which significantly influence the quality of thawed sperm. Different systems with variable freezing rates are used for freezing bull semen in the field, with a consequence of variable success rates. The objective of this study was to compare different systems for freezing bull semen in the field. Five cooling methods of semen and two methods for the subsequent freezing phase (5 × 2 factorial scheme) were used. Two to four ejaculates were collected from 12 bulls with an electroejaculator. The ejaculates were diluted in BotuBov® to a concentration of 50 × 10^6 spermatozoa/mL in 0.5-mL straws. After dilution, the straws were cooled to 5 °C in five cooling systems: TK 4000® at a cooling rate of −0.25 °C/min (R1); TK 4000® at a rate of −0.5 °C/min (R2); Minitube® refrigerator at a rate of −2.8 °C/min (R3); Botutainer® at a rate of −0.65 °C (R4), and domestic refrigerator at a rate of −2.0 °C/min (R5). After stabilization at 5 °C for 4 h, these straws were then submitted to two freezing systems: TK 4000® at a freezing rate of −15 °C/min (C1) and Styrofoam box with liquid nitrogen at a rate of −19 °C/min (C2). Sperm kinetics were evaluated by computer-assisted sperm analysis at four time points: in fresh semen, after cooling, post-thawing, and after the rapid thermal resistance test (TRT). In addition, plasma and acrosomal membrane integrity, mitochondrial potential and intracellular H_2O_2 were analyzed after thawing by flow cytometry. The R1, R2 and R4 cooling systems were the most efficient in preserving sperm viability, membrane integrity and intracellular H_2O_2. Samples frozen in the C1 system exhibited better post-thaw and post-TRT kinetics than C2 samples. In conclusion, slower cooling curves in conjunction with a constant freezing rate obtained with the programmable unit were more efficient for freezing bull semen in the field.

1. Introduction

For years, gamete freezing has been a valuable procedure for the application of reproductive technologies such as artificial

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insemination, *in vitro* embryo production and embryo transfer since it eliminates the limitations of time and space (Parks, 1997). However, the cryopreservation process exposes the cells to stress induced by the low temperature and osmotic imbalances (Sieme et al., 2016). Approximately 40–50% of the cell population does not survive the freezing process and the subpopulation that remains viable after cryopreservation may have its function compromised (Curry, 2000; Watson, 2000).

Several factors are involved in the success of semen freezing and different strategies have been evaluated to optimize cryopreservation protocols (Mocé et al., 2010). In this respect, knowledge of the impact of cooling and freezing may help improve these protocols (Sieme et al., 2016). The cooling curve is a major step in the cryopreservation process, which is important to reduce the metabolism of sperm cells but can significantly affect post-thaw sperm survival (Mazur, 1984; Salamon and Maxwell, 2000).

The method of semen cryopreservation has changed little in recent years. Field techniques generally use simple materials for generation of the cooling curve, such as thermal boxes with ice or domestic refrigerators, and Styrofoam boxes with liquid nitrogen are used for construction of the freezing curve (De Lima et al., 2010). Despite their low cost, the problem of these methods is the lack of standardization of the cooling curve due to the wide variation in the size and thickness, time of use, brand, model and conditions of use of the Styrofoam box and refrigerator, which results in variations in the cryopreservation results (Abud et al., 2014). Another practical system for the transport of diluted semen that is also used in the field is Botutainer® (Botupharma, Botucatu, Brazil). According to the manufacturer, this system maintains the semen temperature at 5 °C for up to 24 h. This transport box for cooled semen have been reported to be efficient in maintaining semen viable after cooling (Monteiro et al., 2013; Crespilho et al., 2014; Moscardini et al., 2014; Borges-Silva et al., 2016). Recently, programmable units for semen cryopreservation have been developed, which permit the selection of the desired cooling and freezing curve. Although these freezing units are easily purchased, their cost is still very high when compared to simpler and less expensive cryopreservation techniques that are used in the field (Abud et al., 2014).

In an effort to improve post-thaw sperm survival, the present study simultaneously compared for the first time the *in vitro* quality of bovine semen cryopreserved in five cooling systems (programmable freezing unit at a cooling rate of −0.25 °C/min and −0.5 °C/min, Minitube® refrigerator, portable Botutainer® cooling system, and domestic refrigerator) and two freezing systems (programmable freezing units at a freezing rate of −15 °C/min and Styrofoam box with liquid nitrogen).

2. Material and methods

The study was approved by the Ethics Committee on Animal Use of the Institute of Zootechny, Nova Odessa, on 1st April 2015 (Protocol 205/15).

2.1. Collection, analysis and packaging of fresh semen

Twelve Nellore bulls (*Bos taurus indicus*), 3.1 ± 0.5 years of age, which belonged to Centro APTA Bovinos de Corte, the research unit of the Institute of Zootechny, Sertãozinho (latitude 21°10′ south and longitude 48°5′ west), SP, Brazil, were used. The animals were maintained on pasture with water and mineral salt available *ad libitum* and normality of andrological parameters was evaluated prior to semen collection according to the recommendations of the Brazilian College of Animal Reproduction (CBRA in the Portuguese acronym): mass motility, ≥ 3; sperm motility, ≥ 60%; sperm concentration, 350 million spermatozoa/mL; total number of spermatozoa in the ejaculate, 3–5 billion; morphologically normal sperm, ≥ 70%; major defects, ≤ 10%; minor defects, ≤ 20%; major individual defects, ≤ 5%; minor individual defects, ≤ 10% (Colégio Brasileiro de Reprodução Animal, 2013).

Two to four ejaculates were obtained per bull with an Autojac® electroejaculator (Neovet®, Uberaba, Brazil) in the manual mode, totaling 38 ejaculates. First, routine physical and morphological analysis was performed (sperm volume, motility, vigor, concentration, and morphology). After initial assessment, the ejaculates obtained were diluted conventionally in BotuBov® single-fraction diluent (BotuFarma®, Botucatu, Brazil), which contains 7% glycerol, to a final concentration of 50×10^6 spermatozoa/mL. The diluted fresh semen was assessed by Computer-Assisted Sperm Analysis (CASA; IVOS, version 14, Hamilton-Thorne Bioscience®, Beverly, MA, USA) and this analysis was defined as control (R0).

Before cooling, the semen was kept in a water bath for 10 min at 34 °C for stabilization and was packaged at room temperature (25 °C) in properly identified 0.5-mL straws (25×10^6 spermatozoa/dose).

2.2. Semen cooling

Fifty straws of each ejaculate were equally and randomly allocated to the five cooling systems (10 straws per treatment): programmable freezing unit (TK 4000®, Uberaba, Brazil), cooling rate of −0.25 °C/min (R1); programmable freezing unit (TK 4000®), cooling rate of −0.5 °C/min (R2); Minitube® refrigerator (Minitube®, Tiefenbach, Germany), cooling rate of −2.8 °C/min (R3); portable semen cooling device (Botutainer®, Botucatu, Brazil) in which the procedures were conducted as indicated by the manufacturer (maintain the recyclable ice in the freezer for at least 24 h before use and transfer it to the Botutainer® one hour before inserting the semen straws), cooling rate of −0.65 °C/min (R4); domestic refrigerator (Electrolux®, DC 45, 436 liters) regulated at a temperature of 5 °C, with the straws being placed in the proximal third of the refrigerator, cooling rate of −2.0 °C/min (R5). After reaching 5 °C, the straws were kept at this temperature for 4 h in all systems (equilibrium time). Two straws from each system (R1, R2, R3, R4, and R5) were subjected to CASA at the end of the cooling period described above before cryopreservation.

2.3. Semen freezing

After cooling, the remaining eight straws of each cooling system were frozen using two different methods (four straws each). In the first system (C1), a portable semen freezing unit (TK 4000®, Uberaba, Brazil) with a programmed freezing curve of -15 °C/min was used, starting at 5 °C until −80 °C. Once this temperature was reached, the rate was set to -20 °C/min until −140 °C were reached when the straws were immersed in liquid nitrogen.

In the second system (C2), a 45-l Styrofoam box (internal dimensions [height x length x width]: 36 × 40 x 31 cm and 4.5-cm thick wall) was used where the straws were placed 3 cm from the liquid nitrogen (2-cm column). The freezing rate was -19 °C/min. After 20 min, the straws were immersed in liquid nitrogen.

2.4. Monitoring of the cooling and freezing curves

2.4.1. Cooling curve

The cooling curves of the systems were monitored with a Digi Sense® Type K probe (Vernon Hills, IL, USA) inside one 0.5-mL straw containing semen and diluent whose temperature was recorded every minute. In the R1 and R2 systems, the temperature of the probe of the unit and that of the thermometer inside the straw were compared in quadruplicate. For monitoring of the R3, R4 and R5 systems, in addition to the thermometer inside the straw, another digital thermometer (Inconterm®, Porto Alegre, Brazil) was fixed outside the equipment to determine the temperature outside the straw in quadruplicate.

2.4.2. Freezing curve

The same sensitive thermometer with a Type K probe inserted into a 0.5-mL straw was used for both systems. Eleven replicates were performed per system.

2.5. Sperm thawing and post-thaw assessment

For all systems, the straws were thawed in a water bath at 37 °C for 30 s. For post-thaw assessment, two straws of each ejaculate were used per system to remove the effect of the straw. The following analyses were performed after thawing: CASA, analysis of sperm kinetics after the rapid thermal resistance test (TRT), and evaluation of plasma and acrosomal membrane integrity, mitochondrial potential and intracellular H_2O_2 by flow cytometry.

2.5.1. Computer-Assisted sperm analysis - CASA

Sperm kinetics were analyzed by CASA at four time points: fresh semen, after equilibrium time, after thawing, and after TRT. A previously heated (38 °C) semen aliquot (10 µL) was placed in the chamber of a Makler® reader (SEFI Medical Instruments Ltd®, Haifa, Israel) and inserted into the IVOS analyzer (version 14, Hamilton-Thorne Bioscience®, Beverly, MA, USA). The setup was adjusted for the analysis of bovine semen (number of frames: 30; minimum contrast: 60 pixels; minimum cell size: 6 pixels; straightness: 70%; average path velocity cutoff: 30 µm/s; minimum average path velocity: 40 µm/s; straight-line velocity cutoff: 20 µm/s; non-motile head intensity: 90; non-motile head size: 5 pixels; magnification: X 1.95; temperature: 38 °C). Five analysis fields were randomly chosen. The following sperm kinetic parameters were analyzed: total motility (MT, %), progressive motility (MP, %), percentage of rapid sperm (RAP, %), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %).

2.5.2. Rapid thermal resistance test

The semen of two thawed straws per system was transferred to a 1.5-mL microtube and kept in a water bath at 46 °C for 30 min. After this period, the samples were analyzed by CASA (Bacinoglu et al., 2008).

2.5.3. Flow cytometry

A BD LSR II (Becton Dickinson®, Mountain View, CA, USA) equipped with a blue (488 nm, 100 mW) and red laser (640 nm, 40 mW) was used for flow cytometry analysis. The data were analyzed with the program of the same manufacturer (BD FACSDiva™ software v6) regarding plasma and acrosomal membrane integrity, mitochondrial potential, and intracellular H_2O_2.

2.5.3.1. Evaluation of plasma and acrosomal membrane integrity. The sample was homogenized and incubated for 15 min at 37 °C. Propidium iodide (PI; P4170, Sigma®, St. Louis, MO, USA), FITC-PSA (L0770, Sigma®) and Hoechst 3342 (H342; 14533, Sigma®) were used as probes. For analysis, 5 µL H342 (100 µg/mL), 5 µL PI (50 µg/mL) and 0.5 mL FITC-PSA (2 mg/mL) were added to 200 µL semen diluted in TALP to a concentration of 10×10^6 spermatozoa/mL.

2.5.3.2. Evaluation of mitochondrial potential. The sample was homogenized and incubated for 15 min at 37 °C. JC-1 (T3168, Molecular Probes®, Eugene, OR, USA) and H342 were used as probes. For analysis, 5 µL H342 (100 µg/mL) and 10 µL JC-1 (10 µg/mL) were added to 200 µL semen diluted in TALP to a concentration of 10×10^6 spermatozoa/mL.

2.5.3.3. Determination of intracellular H_2O_2. Intracellular H_2O_2 was measured using 5-(-6)-carboxy-2,7-dichlorodihydrofluorescein

diacetate (D6883, DCFDA; Molecular Probes®, Eugene, USA) as the probe. For analysis, 0.5 µL of the DCFDA probe (final concentration of 20 µM), 5 µL PI (50 µg/mL) and 5 µL H342 (100 µg/mL) were added to 500 µL semen diluted in TALP to a concentration of 1×10^6 spermatozoa/mL and the mixture was incubated for 60 min at room temperature protected from light.

2.6. Statistical analysis

The experimental design was completely randomized in a 5 × 2 factorial scheme. The results were analyzed using least square method for performing variance analyses (F test) using the PROC GLM procedure (SAS Institute, Inc., Cary, USA). The model included the fixed effects of bull (i = 1, …., 12), ejaculate (i = 1, …, 38), cooling system (i = 1, …, 5), freezing system (i = 1, 2), and the cooling x freezing interaction. The least square means were estimated by the least square method. For significant fixed effects ($P < 0.05$), the means were compared by the Tukey test. Significant difference was declared at $P < 0.05$.

3. Results

The results obtained after cooling (R1, R2, R3, R4 and R5) and freezing (C1 and C2) were first analyzed together (5 cooling systems x 2 freezing systems = 10 treatments). However, since no effect of the interaction between the cooling and freezing systems was observed ($P > 0.05$) for any of the CASA or flow cytometry variables analyzed, the mean results obtained with the five cooling systems and two freezing systems were compared separately.

3.1. Monitoring of the cooling and freezing curves

3.1.1. Cooling curve

Fig. 1 shows the monitoring of the cooling curves. In the case of R1, the temperatures of the unit and of the thermometer inside the straw were similar and reached 5 °C about 80 min after the beginning of the cooling process. The temperatures measured inside the straw and the temperature of the system did not oscillate and did not differ throughout evaluation. The cooling rate in the R1 system was -0.25 °C/min. The temperatures of the R2 system and of the thermometer inside the straw were also similar and reached 5 °C about 40 min after the beginning of the cooling process. As observed for R1, the temperature of the R2 system also did not oscillate and was similar to that measured inside the straw. The cooling rate in the R2 system was −0.5 °C/min. In the R3 system, the temperature stabilized at 5 °C about 7 min after introduction of the straws. The temperature inside the straw and the outside temperature oscillated little and were very similar. The cooling rate in the R3 system was −2.8 °C/min. The R4 system stabilized at 6.5 °C within 30 min, with a cooling rate of −0.65 °C/min. The R5 system reached 5 °C about 10 min after the beginning of the measurement, with a cooling rate of −2.0 °C/min.

3.1.2. Freezing curve

Fig. 2 shows the freezing curves. In C1, a large difference was observed between the temperature measured with the thermometer and that indicated by the equipment. The freezing rate in the C1 system was −15 °C/min, starting from 5 °C until −80 °C. The freezing rate in the C2 system was −19 °C/min. and was stabilized at about −105 °C.

3.2. Evaluation of sperm kinetics

3.2.1. Fresh and cooled semen

As expected, better results were obtained for most of the variables assessed by CASA for fresh semen (R0) compared to post-cooling semen ($P < 0.05$). When the systems were compared post-cooling, VAP, VCL and ALH indicated poorer quality of semen post-cooled in Minitube® refrigerator with cooling rate of −2.8 °C/min (R3) compared to semen cooling in programmable freezing unit with cooling rate of −0.25 °C/min (R1). R3 semen only exhibited higher values for straightness and linearity when compared to R1 semen (Table 1).

3.2.2. Post-thaw and post-rapid thermal resistance test semen

After thawing, higher values ($P < 0.001$) of MT, MP, RAP and VCL were observed for semen cooling in programmable freezing unit with cooling rate of −0.25 °C/min compared to semen cooled in Minitube® refrigerator with rate of −2.8 °C/min and in domestic refrigerator with a rate of −2.0 °C/min (Table 2), while straightness and linearity were lower.

Regarding the freezing system, higher values were obtained for most variables when the semen was frozen in the portable semen freezing unit with a programmed freezing curve of -15 °C/min, except for beat cross frequency and linearity whose values were higher for semen frozen in the Styrofoam box with freezing rate of -19 °C/min.

The results of MT, MP and RAP obtained after the rapid thermal resistance test were similar to those observed in frozen semen. Semen cooling in a programmable freezing unit with a cooling rate of -0.25 °C/min exhibited higher MT, MP and RAP values than semen cooled in a Minitube® refrigerator (rate of −2.8 °C/min) and in domestic refrigerator (rate of -2.0 °C/min) and similar results compared to the semen of the other cooling systems (Table 3). VAP, VSL, VCL, ALH, BCF, STR or LIN obtained after the rapid thermal resistance did not differ between semen cooled in the different systems. Higher MT, MP, RAP, VAP, VSL, VCL and ALH values were observed for semen of the portable freezing system with freezing rate of −15 °C/min compared to semen freezing in Styrofoam box with freezing rate of −19 °C/min. No difference between portable semen freezing unit and Styrofoam box was found for the other

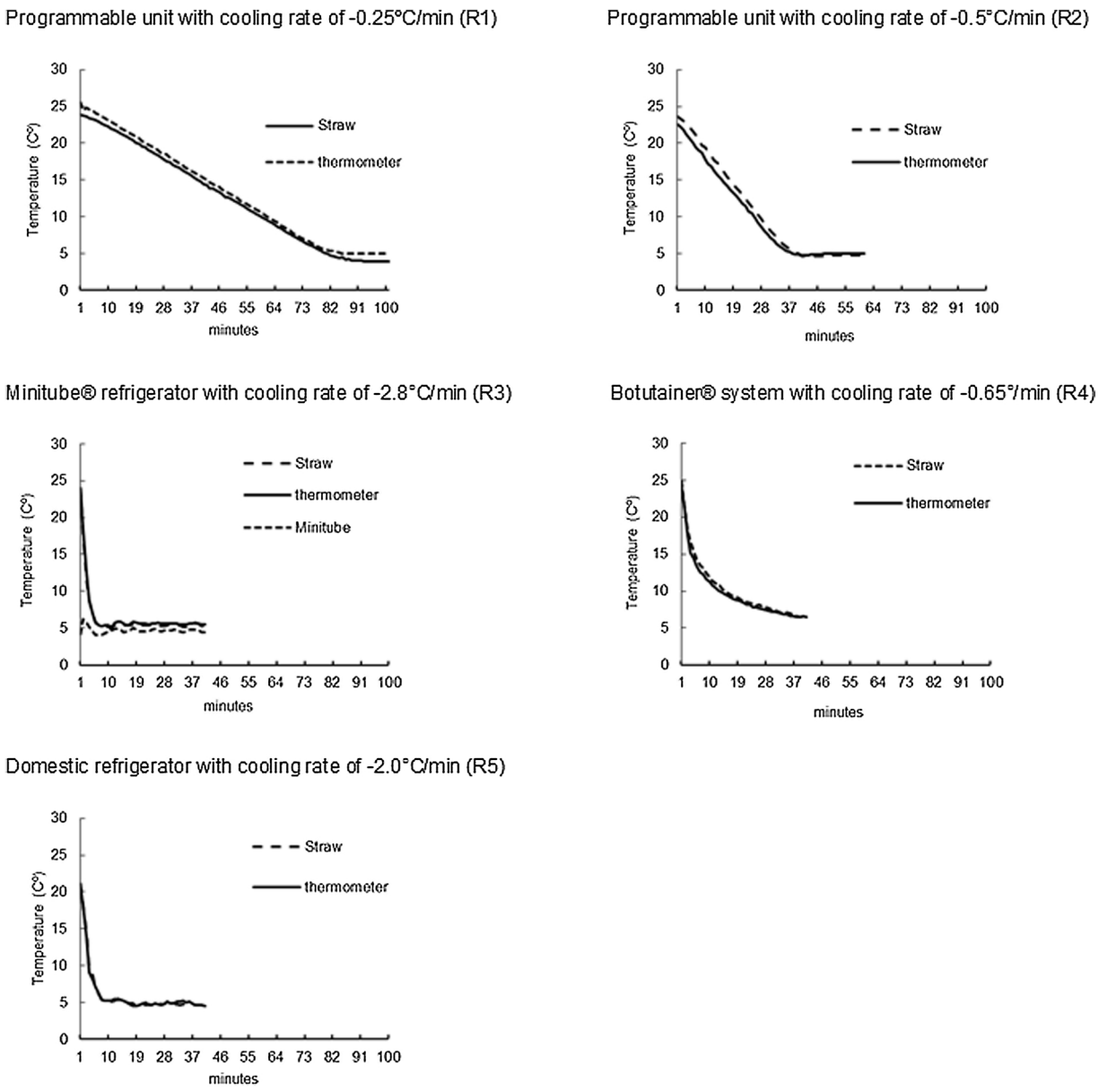

Fig. 1. The cooling curves for bovine semen.

variables assessed by CASA (Table 3).

3.3. Flow cytometry analysis

Table 4 shows the results of flow cytometry analysis. The R4 cooling system provided higher plasma and acrosomal membrane integrity (IPAM) of spermatozoa than semen cooled in Minitube® refrigerator (rate of −2.8 °C/min) and in domestic refrigerator (rate of −2.0 °C/min) and similar results compared to semen cooled in programmable freezing units (cooling rate of −0.25 °C/min and −0.5 °C/min). Comparison of membrane integrity between the two freezing systems revealed no difference. There were also no differences in mitochondrial potential between the five cooling systems or the two freezing systems. The percentage of intracellular H_2O_2 was lower for semen cooled in programmable freezing units with cooling rate of -0.25 °C/min and −0.5 °C/min compared to semen cooled in Minitube® refrigerator with rate of −2.8 °C/min and in domestic refrigerator with rate of −2.0 °C/min. Comparison of the two freezing systems showed no difference in the percentage of H_2O_2-positive intact cells (ICH_2O_2).

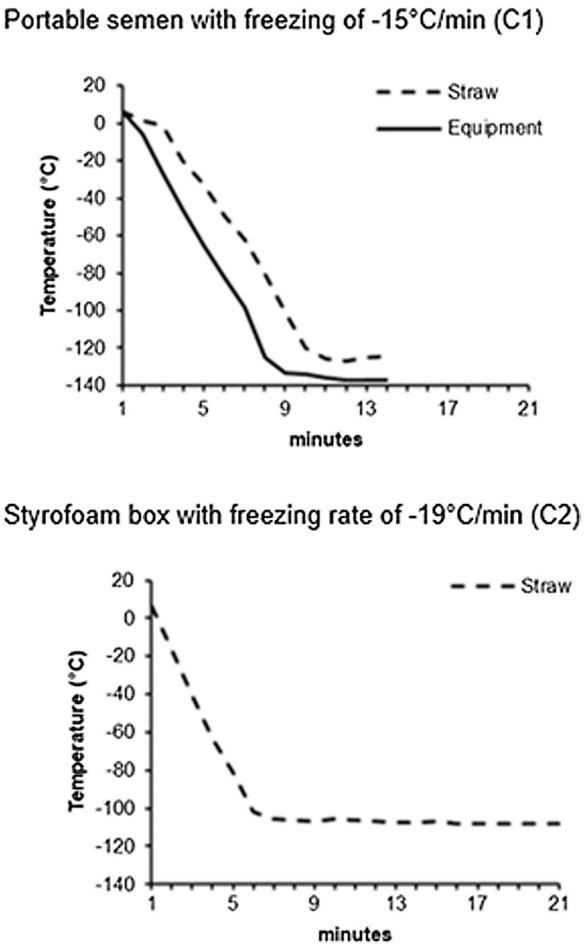

Fig. 2. Monitoring freezing curves in bovine semen.

Table 1
Mean sperm kinetic variables evaluated in fresh semen (R0) and after cooling in the five systems (R1-R5).

Variable	R0	R1	R2	R3	R4	R5	SEM
MT (%)	88.2^{a}	82.0^{b}	81.1^{b}	78.6^{b}	79.8^{b}	78.7^{b}	1.1
MP (%)	71.0^{a}	55.2^{b}	57.3^{b}	60.3^{b}	57.4^{b}	57.5^{b}	1.4
RAP (%)	84.6^{a}	79.3^{b}	78.8^{b}	75.7^{b}	77.1^{b}	76.0^{b}	1.2
VAP (μm/s)	91.3^{b}	97.3^{a}	95.2^{ab}	89.4^{b}	93.1^{ab}	93.3^{ab}	2.0
VSL (μm/s)	77.8^{a}	73.2^{b}	73.4^{b}	70.1^{b}	72.7^{b}	73.4^{b}	1.4
VCL (μm/s)	140.2^{b}	166.7^{a}	158.2^{ab}	150.2^{b}	156.9^{ab}	158.6^{ab}	4.0
ALH (μm)	5.4^{c}	6.8^{a}	6.7^{a}	6.1^{b}	6.4^{ab}	6.4^{ab}	0.1
BCF (Hz)	32.6^{a}	22.6^{b}	23.4^{b}	25.0^{b}	24.3^{b}	24.9^{b}	0.6
STR (%)	85.3^{a}	76.5^{c}	78.4^{cd}	81.8^{b}	79.6^{bc}	80.2^{bd}	0.8
LIN (%)	59.2^{a}	47.1^{c}	48.2^{bc}	50.8^{b}	50.1^{bc}	50.2^{bc}	0.9

R0: fresh semen; R1: programmed cooling curve at −0.25 °C/min; R2: programmed cooling curve at −0.5 °C/min; R3: Minitube; R4: Botutainer; R5: domestic refrigerator. MT: total motility; MP: progressive motility; RAP: percentage of rapid sperm; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity; SEM: standard error of the mean.
[a, b, c, d]Means in the same row followed by different superscript letters differ significantly from one another ($P < 0.05$).

4. Discussion

The search for the best cooling/freezing and thawing rates of sperm cells has encouraged studies in different animal species (Fiser and Fairfull, 1990; Blanco et al., 2000; Yu et al., 2002; Kumar et al., 2003; Thurston et al., 2003; De Vita et al., 2011; Abud et al., 2014). The most critical temperature range for cooling sperm is between 15 °C and 5 °C, which determines cold shock, the most sensitive temperature especially for bovine sperm (De Leeuw et al., 1990; Watson, 2000; Agca and Critser, 2002; Stornelli et al.,

Table 2
Mean sperm kinetic variables of post-thaw semen cooled in the five systems (R1-R5) and frozen in the two systems (C1 and C2).

Variable	R1	R2	R3	R4	R5	SEM	C1	C2	SEM
MT (%)	39.7[a]	34.9[ab]	30.9[b]	35.8[ab]	30.1[b]	1.6	37.6[a]	30.9[b]	1.1
MP (%)	30.7[a]	28.0[ab]	24.6[b]	28.4[ab]	24.0[b]	1.2	29.5[a]	24.8[b]	0.8
RAP (%)	37.2[a]	32.2[ab]	28.3[b]	33.3[ab]	27.8[b]	1.5	35.1[a]	28.4[b]	1.0
VAP (μm/s)	78.5[a]	77.0[ab]	75.6[ab]	77.5[ab]	75.0[b]	0.8	78.8[a]	74.6[b]	0.5
VSL (μm/s)	65.4	66.2	65.2	65.9	64.2	0.7	66.3[a]	64.5[b]	0.4
VCL (μm/s)	131.2[a]	126.3[ab]	122.1[b]	127.3[ab]	123.0[b]	1.6	131.2[a]	121.2[b]	1.1
ALH (μm)	5.4[a]	5.2[ab]	5.0[b]	5.2[ab]	5.1[ab]	0.1	5.4[a]	4.9[b]	0.05
BCF (Hz)	29.4	30.7	30.6	30.8	29.9	0.4	29.4[b]	31.2[a]	0.3
STR (%)	82.8[b]	85.8[a]	86.1[a]	85.2[a]	85.6[a]	0.6	86.2[a]	84.0[b]	0.4
LIN (%)	52.6[b]	55.8[a]	56.7[a]	54.8[a]	55.7[a]	0.6	53.7[b]	56.5[a]	0.4

R1: programmed cooling curve at −0.25 °C/min; R2: programmed cooling curve at -0.5 °C/min; R3: Minitube; R4: Botutainer; R5: domestic refrigerator; C1: programmed freezing; C2: freezing in a Styrofoam box. MT: total motility; MP: progressive motility; RAP: percentage of rapid sperm; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity; SEM: standard error of the mean.
[a, b]Means in the same row followed by different superscript letters differ significantly from one another ($P < 0.05$).

Table 3
Mean sperm kinetic variables of semen after rapid thermal resistance testing cooled in the five systems (R1-R5) and frozen in the two systems (C1 and C2).

Variable	R1	R2	R3	R4	R5	SEM	C1	C2	SEM
MT (%)	31.3[a]	25.5[ab]	23.4[b]	25.9[ab]	21.2[b]	1.6	29.5[a]	21.4[b]	1.1
MP (%)	26.4[a]	21.5[ab]	19.8[b]	21.8[ab]	17.9[b]	1.4	25.1[a]	17.9[b]	0.9
RAP (%)	29.5[a]	23.6[ab]	21.5[b]	24.1[ab]	19.3[b]	1.6	27.7[a]	19.5[b]	1.1
VAP (μm/s)	59.9	60.2	56.7	58.1	56.1	1.5	60.1[a]	56.3[b]	1.0
VSL (μm/s)	53.1	54.1	51.1	51.8	50.5	1.4	53.6[a]	50.6[b]	0.9
VCL (μm/s)	94.3	94.5	89.2	90.8	88.0	2.5	95.1[a]	87.6[b]	1.7
ALH (μm)	3.8	4.1	3.8	3.7	3.6	0.1	4.0[a]	3.7[b]	0.1
BCF (Hz)	26.8	28.3	27.5	27.5	27.3	0.8	27.1	27.8	0.6
STR (%)	77.2	81.5	78.3	78.9	79.4	2.0	78.5	79.6	1.3
LIN (%)	51.1	54.3	52.0	52.0	53.8	1.4	51.7	53.2	0.9

R1: programmed cooling curve at −0.25 °C/min; R2: programmed cooling curve at -0.5 °C/min; R3: Minitube; R4: Botutainer; R5: domestic refrigerator; C1: programmed freezing; C2: freezing in a Styrofoam box. MT: total motility; MP: progressive motility; RAP: percentage of rapid sperm; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity; SEM: standard error of the mean.
[a, b]Means in the same row followed by different superscript letters differ significantly from one another ($P < 0.05$).

Table 4
Mean flow cytometry variables of post-thaw semen cooled in the five systems (R1-R5) and frozen in the two systems (C1 and C2).

Variable	R1	R2	R3	R4	R5	SEM	C1	C2	SEM
IPAM (%)	37.2[ab]	37.0[ab]	33.2[b]	38.1[a]	33.4[b]	1.2	36.2	35.4	0.8
HMP (%)	48.7	48.0	45.9	50.9	45.5	1.5	48.7	47.0	1.0
LMP (%)	51.3	52.0	54.1	49.1	54.4	1.5	51.3	53.0	1.0
ICH_2O_2 (%)	25.5[b]	28.6[b]	39.8[a]	33.2[ab]	40.4[a]	2.7	31.5	35.5	1.8

R1: programmed cooling curve at −0.25 °C/min; R2: programmed cooling curve at −0.5 °C/min; R3: Minitube; R4: Botutainer; R5: domestic refrigerator; C1: programmed freezing; C2: freezing in a Styrofoam box. IPAM: intact plasma and acrosomal membranes; HMP: high mitochondrial potential; LMP: low mitochondrial potential; ICH_2O_2: H_2O_2-positive intact cells; SEM: standard error of the mean.
[a, b]Means in the same row followed by different superscript letters differ significantly from one another ($P < 0.05$).

2005). A considerable number of studies have been conducted in an attempt to determine the ideal cooling rates and to permit individual adaptation of the cooling curves for bulls, but little practical progress has been made (Vishwanath and Shannon, 2000).

Analysis of the results obtained for the five cooling systems revealed no difference in most of the variables analyzed (except for post-thaw STR and LIN) between the two programmable systems (R1 and R2). In addition, R4 semen cooled with the Botutainer® exhibited results very similar to those obtained with the programmable systems (R1 and R2) at all time points of sperm kinetic analysis (post-cooling, post-thaw and post-TRT) and by flow cytometry. These three cooling systems provided the slowest cooling rates (R1: -0.25 °C/min; R2: -0.5 °C/min and R4: -0.65 °C/min). Most cryopreservation protocols use a slow cooling rate situated between the collection temperature and 5 °C (Gao et al., 1997). Slower cooling curves have been associated with better preservation of bull sperm viability and fertility (Dhami et al., 1992; Dhami e Sahni, 1993; Januskauskas et al., 1999). Vishwanath and Shannon (2000) reported that slow cooling and equilibrium time before freezing are necessary for the processing of diluted semen to obtain

optimal post-thaw sperm recovery.

A disadvantage of passive cooling systems are possible variations in the cooling curve due to the influence of the ambient temperature and of the initial temperature and mass of the sample, factors producing exponential and negative cooling rates (Valle et al., 1999). The similar result obtained with the three systems can be explained by the fact that the study was conducted in the laboratory at a controlled temperature (25 °C). The advantage of passive cooling systems is their low cost (Pickett, 1993). Furthermore, De Vita et al. (2011) reported that the use of the Botutainer® for seminal stabilization before freezing can facilitate the freezing procedure in the field when compared to the Minitube® system because of greater practicality of the former.

With respect to the R3 and R5 cooling systems, the time to reach 5 °C was shorter than those obtained for the other systems, with faster cooling rates (R3: −2.8 °C/min and R5: −2.0 °C/min). This influenced the parameters of seminal quality. One factor that possibly contributed to the lower sperm quality of R3 and R5 semen was the fast cooling rate. The reduction in temperature up to 5 °C during cooling causes the transition of plasma membrane lipids from the liquid or fluid phase to the crystalline or gel form, resulting in a more rigid organization of the membrane (Graham, 1996; Watson 2000; Sieme et al., 2015). During this phase (15 °C to 5 °C), some irreversible damage to sperm cells may occur due to changes in the orientation of plasma membrane lipids, such as rapid loss of motility, reduced energy production, abnormal sperm movement pattern, acrosomal and plasma membrane damage, reduction in metabolism, loss of ions and intracellular components and release of intracellular material (Kayser et al., 1992; Graham, 1996; Watson, 2000; Medeiros et al., 2002; Silva and Guerra, 2011). If the spermatozoon is not cooled properly during this critical phase, it may suffer damage that results in the loss of fertility (Watson, 1995). In this respect, faster cooling rates have been associated with irreversible damage (Drobnis et al., 1993).

Under physiological conditions, sperm cells produce a large amount of reactive oxygen species (ROS), including superoxide (O2-), hydroxyl (OH-) and hydrogen peroxide (H_2O_2) (Aitken and Barker, 2004). Adequate amounts of ROS are required to stimulate tyrosine phosphorylation cascades which are associated with sperm capacitation. However, when ROS production are excessively produced, oxidative stress may leads to DNA fragmentation and peroxidative damage of sperm plasma membrane (Aitken and Barker, 2004). Gibb et al. (2014) observed a positive correlation between ROS levels and stallion fertility. On the other hand, Johannisson et al. (2014) reported a negative association between H_2O_2 concentrations and progressive motility, chromatin integrity and fertility in cooled equine semen. Gürler et al. (2016) observed that sperm DNA damage is related to H2O2 production, but not with other ROS. In the present work, the system techniques producing the highest H_2O_2 rates in sperm cells with plasma membrana integrity (R3 and R5) demonstrated significantly lower values of total motility, both in thawed semen and after thermal resistance challenge. Oxidative phosphorylation is responsible for a greater ATP production by sperm cells, increasing H_2O_2 generation. However, if sperm mitochondria is damaged during freezing process, the ATP production cycle may be affected, generating high levels of H_2O_2 and low final conversion of ATP, contributing to reduced sperm motility.

A sudden drop in temperature during cooling was observed for both the Minitube® refrigerator (R3) and the domestic refrigerator (R5), probably because the straws were in direct contact with the environment of the refrigerator. A sudden temperature drop has also been reported by De Lima et al. (2010) in sheep, with a decrease of −18.0 °C in the first minute of cooling in a refrigerator. These authors showed that the use of plastic bags containing water during cooling in a refrigerator controls the decrease in temperature (mean of −0.4 °C/min). This change in the protocol is interesting to improve the results obtained with the technique since it is an inexpensive procedure that provides a slower and more homogenous cooling curve. It should be noted that variations in the quality of the refrigerator can also interfere negatively with the results obtained (Abud et al., 2014). A homogenous and constant sperm cooling rhythm is important not only to prevent thermal shock, but also to standardize the techniques and to obtain more homogenous semen batches (De Lima et al., 2010). During the cooling and equilibrium time the sperm cells acquire resistance to the effects of freezing (England, 1993).

The freezing rate is extremely important for the maintenance of sperm integrity. A good freezing rate allows water to flow out of the sperm cell, but not in excess, and the formation of some crystals without causing damage to the cell (Graham, 1996; Watson, 2000). In general, the protocols for semen cryopreservation use freezing curves ranging from -10 to -100 °C/min, which provide good post-thaw survival rates (Forero-Gonzalez et al., 2012). In cattle, Chen et al. (1993) reported that freezing rates of −15, −25 and −35 °C/min resulted in similar sperm survival rates and were superior to freezing at −5 °C/min. Forero-Gonzalez et al. (2012) found no difference between the conventional protocol that uses a cooling rate of −0.55 °C/min, followed by a freezing rate of −19.1 °C/min, and an automated cryopreservation protocol using a cooling rate of −0.23 °C/min and a freezing rate of -15 °C/min.

Regarding the freezing systems, semen frozen in the programmable unit exhibited better post-thaw and post-TRT sperm kinetics than semen frozen in the Styrofoam box. These results possibly reflect the difference between the freezing curves of the two systems. The initial freezing curve of the C1 system was slower than that of the C2 system. A very fast freezing curve leads to inadequate dehydration of all cells and the formation of intracellular ice crystals that cause damage to spermatozoa, reducing their post-thaw viability (Watson, 2000; Sieme et al., 2015). An adequate freezing rate is sufficiently slow to permit appropriate dehydration of the cell, preventing the formation of intracellular ice crystals, but is sufficiently fast to prevent prolonged exposure of spermatozoa to the hypertonic solutions of the extracellular environment or mechanical tension caused by rapid shrinking (Mazur, 1984; Chaveiro et al., 2006).

Many universities, research centers and especially field technicians continue to perform the freezing step in Styrofoam boxes where the semen straws are subjected to uncontrolled freezing conditions that depend on the distance of the straw from the nitrogen layer, with freezing rates ranging from −15 °C/min to −30 °C/min (Vishwanath and Shannon, 2000). The advantage of conventional systems for semen cooling and freezing is their low cost; however, it is necessary to control all variables that can influence the cryopreservation process to obtain post-thaw semen of good quality. Variations in the size of the Styrofoam box and in the liquid nitrogen level can negatively affect the results obtained (Abud et al., 2014).

The cooling and freezing rates of automated systems are rigorously controlled and there is no influence of the ambient temperature (Abud et al., 2014). According to Purdy (2006), the programmable cryopreservation unit is suitable to freeze large numbers of semen straws since the system controls the velocity of cooling and freezing. These systems permit better control of the decrease in temperature, minimizing variations in sperm quality after cooling and freezing. In addition, these freezing units can be used in places with no electricity because they are powered from a battery. A disadvantage of programmable cryopreservation units is their high cost (Abud et al., 2014), while they have the advantage of being flexible and permitting to alter the curve until the best results are achieved, with adaptation to the diluent, cryoprotectant or any other factor that influences the result (Forero-Gonzalez et al., 2012). Several studies have used programmable units for the cryopreservation of semen (Carvalho et al., 2010; Leite et al., 2010; De Andrade et al., 2011; Franco et al., 2014; Campanholi et al., 2017).

Finally, it should be noted that different factors, alone or in combination, can alter the freezing rate and post-thaw sperm viability, including the number of solutes (cryoprotectants in the freezing medium), type of packaging, time and height of the straw in relation to the liquid nitrogen level in the Styrofoam box, and freezing rate in programmable units (Clulow et al., 2008).

5. Conclusion

Programmable systems are the best option for cooling semen in terms of post-thaw *in vitro* sperm quality. The passive Botutainer® is a good alternative for cooling semen as a less expensive method that can be easily applied in the field, exhibiting results similar to those of programmable systems. Better sperm kinetics are obtained when bovine semen is frozen in a programmable system compared to freezing in a Styrofoam box. Further studies are needed to evaluate the effect of different cooling and freezing systems on *in vivo* fertility in cattle.

Declarations of interest

None.

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