Assessment of bovine spermatozoa viability using different cooling protocols prior to cryopreservation

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Abstract

The aim of our study was to evaluate the effect of different cooling rates on the post-thawing quality of bovine spermatozoa. Ejaculated semen from a 24-month-old Jersey bull was collected using an artificial vagina and diluted in a commercial extender to evaluate spermatozoan concentration and motility subjectively before cooling and freezing and after thawing. Straws were allocated to four cooling curves: rapid (RD), semi-rapid (SRD), semi-slow (SSILW) and slow (SLW). The temperature was decreased from 25°C to 4°C in 10, 50, 110 and 135 min, which represents a cooling rate of 2.06, 0.40, 0.18 and 0.15°C/min, respectively. Then straws were frozen and stored at -196°C. After thawing, one aliquot of each straw was used for evaluation. Spermatozoan integrity and mitochondrial function were evaluated using a combination of fluorescent probes containing 100 μg/mL FITC-PSA, 0.5 μg/mL PI and 153 μM JC-1. At the end of cooling, spermatozoan motility did not differ among RD (63.3%), SRD (66.7%), SSILW (66.7%) and SLW (80.0%). However, normal spermatozoan morphology was lower in SRD (84.8%) compared to RD (91.7%), SSILW (91.7%) and SLW (90.3%) (P<0.05). In thawed semen, spermatozoan motility and normal morphology did not differ among RD (40.0%; 88.8%), SRD (43.3%; 82.5%), SSILW (40.0%; 87.2%) and SLW (36.7%; 88.0%). The percentage of damaged spermatozoa, including plasma and acrosome membrane damage and low mitochondrial potential, was higher in RD compared to the others (P<0.05). In conclusion, a rapid cooling curve is detrimental to the spermatozoa and affects the post-thaw spermatozoan integrity of bovine frozen semen.

Introduction

In cattle, artificial insemination (AI) has been used for many decades. In the 1940s and early 1950s, the basic principles of semen preservation were established, which resulted in the first calf born after AI with frozen-thawed semen (Phillips and Lardy, 1940; Stewart, 1951). Despite advances in cryopreservation techniques, freezing and thawing procedures applied to bovine semen result in roughly 50% reduction in spermatozoan viability and sublethal damages induced by cryopreservation (Salamon and Maxwell, 2000). Semen quality and spermatozoan viability have been assessed by subjective progressive motility, morphology, membrane integrity and mitochondrial potential (Ombelet et al., 1995; Celeghini et al., 2007). The structural and functional labelling of bovine spermatozoa with fluorescent probes is an important approach to evaluate sperm viability. Indeed, a combination of fluorescent dyes has been used to evaluate plasma membrane and acrosomal integrity together with mitochondrial potential (Nagy et al., 2003; Celeghini et al., 2007).

The cooling rate prior to spermatozoan cryopreservation seems to be an important step to minimise the deleterious effects of injury during the freezing process (Kumar et al., 2003). The cooling period is necessary to decrease the effect of temperature changes and to allow equilibration of the spermatozoa with the diluents before freezing (Foote et al., 2002). However, cooling is a highly stressful process, which leads to irreversible damages to the spermatozoan membrane that result in either cell death or premature capacitation-like changes (Watson, 1995; Garner et al., 2001). While the freezing rate must be between 80°C/min and 120°C/min to result in high survival rate (Watson, 1995; Woelders et al., 1997), the cooling rate is not well established.

Different approaches for cooling semen are commonly used in the field (e.g. in different containers with ice in water or liquid nitrogen, and in different types of refrigerators). These procedures result in different cooling curve rates and diverse cell death rates. Rapid cooling, from 20°C to 4°C, is known to induce cellular stress, affecting cellular viability and spermatozoan integrity (Ball and Vo, 2001; Batellier et al., 2001). For instance, initial cooling to 4°C was shown to induce fragmentation of spermatozoan DNA, while low temperature storage lead to premature capacitation, production of free radicals and DNA damage of equine semen (Baumber et al., 2000, 2003). Therefore, the cooling curve and the protocol used to decrease semen temperature seem to have a strong effect on cellular integrity during cryopreservation with potentially negative consequences on the pregnancy rate. Hence, the aim of our study was to evaluate different cooling curves on cellular motility, spermatozoan morphology and cellular function during the first step of the cryopreservation process of bovine semen.

Materials and methods

Semen from a 24-month-old Jersey bull was collected twice a week with a bovine artificial vagina. A total of four ejaculates were used in this experiment, and the bull was collected at different seasons of the year to avoid environmental factors (Table 1). Each ejaculate was checked for volume and spermatozoan concentration, motility and morphology prior to exposure to different cooling curves. The animal used in this study was free of any disease and fed ad libitum during the experiment. All media components were purchased from Sigma Chemical Company, St. Louis, MA, USA, unless otherwise indicated in the text.

Semen evaluation

Immediately after collection, semen was transported to the laboratory, maintained in a
water bath at 30°C, and spermatozoan motility and concentration were evaluated. To evaluate spermatozoan concentration, semen was diluted (1:200) in saline-formaldehyde and measured using a Neubauer haemocytometer chamber (American Optical Corp., Buffalo, NY, USA), in duplicates under a magnification of 400x. To evaluate spermatozoan motility before and after cooling treatments (Table 1), spermatozoa were examined under a bright-field microscope (Olympus BX40, Olympus Optical Co. Ltd., Tokyo, Japan) at a magnification of 1000x, in one aliquot of 5 µL of semen placed on a warmed (37°C) slide and covered with a 22×22 mm coverslip. Only ejaculates with spermatozoan progressive motility ≥70% (scale from 0 to 100%) and concentration ≥107 spermatozoa/mL were used for subsequent manipulations.

Aliquots of semen were fixed in a formaldehyde-citrate-buffered solution and air-dried to evaluate spermatozoan morphology prior and after cooling treatments. Three samples from different ejaculated semen specimens (Table 1) were used for spermatozoan morphology analyses. A total of 200 spermatozoa were assessed under a phase-contrast microscope (Olympus CHS) at a magnification of 1000x. Spermatozoan abnormalities (e.g. nuclear pochues, defective acrosomes, loose heads, proximal and distal cytoplasmic droplets, mid-pieces, and abnormal tails) were classified according to De Andrade et al. (2007) and Celeghini et al. (2007). The number of spermatozoa showing each class of abnormality was expressed as a percentage of the counted spermatozoa. The percentage of morphologically normal spermatozoa was calculated by dividing the number of normal spermatozoa by the number of total spermatozoa counted.

Semen was diluted with a commercial extender (Bovimix, Nutricell, São Paulo, SP, Brazil) to a final concentration of approximately 25×10⁶ spermatozoa/mL and packaged into identified 0.25 mL plastic straws (Minutube, Tiefenbach, Germany), and then submitted to different cooling protocols prior to cryopreservation. The temperature curve of each cooling-treated semen specimen was assessed using a digital thermometer (Topterm, Columbus, OH, USA). The thermometer sensor was placed inside a straw in the refrigerator and the digital reader was visualised from outside. A total of 20 straws per ejaculated semen specimen was subjected to one of the four different cooling protocols described in the following section.

**Experimental design**

**Rapid cooling (RD, conventional cooling curve) - Protocol 1**

Straws were cooled inside a refrigerator at 4°C for 4 h. The initial cooling curve was set at 25°C and reached 4°C after 10 min. This temperature remained at 4°C during the 4 h until freezing onset.

**Semi-rapid (SRD) cooling - Protocol 2**

Straws were placed inside a glass recipient containing 500 mL of water at 25°C. Then, the recipient was placed in a plastic container with 3.5 L of cold water at 4°C. After initiating the cooling process, one litre of ice cubes was immediately added in the plastic container. The temperature reached 4°C after 50 min of cooling.

**Semi-slow cooling (SSLW) - Protocol 3**

Straws were placed inside a glass recipient containing 500 mL of water at 25°C. Then, the recipient was placed in a plastic container with 3.5 L of cold water at 4°C. At the beginning of the cooling curve, ice cubes (100 mL) were added every 10 min up to a total of 1 L. The temperature reached 4°C after 110 min of cooling.

**Slow cooling (SLW) - Protocol 4**

Straws were placed inside a glass recipient containing 500 mL of water at 25°C. Then, the recipient was placed in a plastic container with 3.5 L of cold water at 4°C and set inside the refrigerator at 4°C. After 90 min, 1 L of ice cubes was added. The straws remained in this container inside the refrigerator for a total of 4 h. The temperature reached 4°C after 135 min of cooling.

**Temperature average from the cooling curves**

The different cooling protocols used in our study resulted in four different cooling curve rates. The RD, SRD, SSLW and SLW cooling curves decreased the temperature from 25°C to 4°C in 10, 50, 110 and 135 min, at a rate of 2.06, 0.40, 0.18 and 0.15°C/min, respectively (Figure 1). The semen remained in the refrigerator for 4 h.

**Table 1. Number of straws (samples) and spermatozoa used in the experimental design.**

| Semen evaluation | Samples / treatment | Total (4 treat.) counted cells / treatment | Total (4 treat.) | Counted cells/ treatment | Total (4 treat.) n | n |
|------------------|---------------------|------------------------------------------|-----------------|--------------------------|------------------|--|
| Motility §        | 24 (12+12)          | 96                                       | -               | -                        | -                | -|
| Viability         | 24 (0+24)           | 96                                       | 4800            | 19,200                   | 19,200            | -|

*An ejaculate provided semen for the four treatments and three replications. Thus, four ejaculates and 36 samples distributed in four treatments were used. Before and after cooling processes. §Cell number is not being shown because spermatozoan motility was subjectively assessed.

**Figure 1. Cooling curve temperature (°C) over time (minutes) of bovine semen exposed to four cooling rate treatments. RD, rapid; SRD, semi-rapid; SSLW, semi-slow; SLW, slow.**
Assessment of spermatozoan integrity using fluorescent probes

After each cooling protocol, straws were frozen in nitrogen vapour for 12 min and stored in liquid nitrogen (-196°C) in cryogensics tanks. The straws were held inside tanks for a minimum of 24 h before post-thaw evaluation. To thaw, the straws were transferred to a 37°C water bath for 1 min and spermatozoan motility and morphology were immediately assessed as described previously.

After cryopreservation, samples were subjected to spermatozoan integrity assessment using fluorescent probes. Spermatozoan viability was evaluated by the plasma membrane integrity, acrosomal membrane integrity and mitochondrial potential, using fluorescent probes. Propidium iodide (PI) and isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA) were prepared in Dulbecco’s phosphate-buffered saline solution (DPBS) at the concentration of 0.5 mg/mL and 100 µg/mL, respectively. The 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was diluted in dimethyl sulphoxide (DMSO) at the concentration of 153 µM. A volume of 3 µL of PI, 50 µL of FITC-PSA and 3 µL of JC-1 was added to a 150 µL aliquot of 25×10^6 spermatozoa/mL diluted in Modified Tyrode’s Medium (TALP). Spermatozoan integrity evaluations were pooled from six straws per each cooling treatment. A total of 24 samples (Table 1) were mixed and maintained in a dark chamber at room temperature for 8 min. Each aliquot of 7 µL was evaluated under an epifluorescence microscope (Carl Zeiss HBO 50, Inc., New York, NY, USA) at a magnification of 1000x and a total of 200 spermatozoa were counted in each group.

Spermatozoa were classified according to De Andrade et al. (2007). Briefly, the viable spermatozoa presented an intact plasma membrane, intact acrosomal membrane and high mitochondrial function (IPIAH), while the non-viable spermatozoa had injury of the membrane and/or abnormal mitochondrial function (e.g. intact plasma membrane, intact acrosomal membrane and low mitochondrial function, IPIAL; intact plasma membrane, damaged acrosomal membrane and high mitochondrial function, IPDAH; intact plasma membrane, damaged acrosomal membrane and low mitochondrial function, IPDAL; damaged plasma membrane, intact acrosomal membrane and high mitochondrial function, DPIAH; damaged plasma membrane, intact acrosomal membrane and low mitochondrial function, DPIAL; damaged plasma membrane, damaged acrosomal membrane and high mitochondrial function, DPDAH; damaged plasma membrane, damaged acrosomal membrane and low mitochondrial function, DPDAL). Representative images of fluorescence microscopy for viable and non-viable spermatozoa are shown in Figure 2.

Statistical analysis

Repeated measures analysis was performed to determine the effect of different treatments on temperature of the sample during the cooling protocol. The MIXED procedure with a repeated measure statement was run and the main effects of treatment group, time and their

Figure 2. Epifluorescence photomicrography of spermatozoa stained with a combination of fluorescent probes: propidium iodide, fluorescein isothiocyanate-conjugated Pisum sativum agglutinin and JC-1. A, IPIAH; B, IPIAL; C, DPDAL; D, DPDAH; E, IPDAH; F, IPDAL; G, DPIAH; H, DPIAH (×1000 magnification).
interaction were determined. Differences at a specific time point were compared between groups using the least square means (Neter et al., 1996). Spermatozoan motility, abnormal morphology and viability at the end of each treatment period were submitted to one-way ANOVA using the General Linear Models (GLM), and multi-comparison between groups was performed by least square means using the t-test. All percentage data were transformed to arcsine. Data were tested for normal distribution using the Shapiro-Wilk test and when P<0.05, raw data were transformed according to their distribution. All analyses were performed using the SAS software package (SAS Institute Inc., Cary, NC). The results are presented as means ± standard error of the mean (S.E.M.) and P<0.05 was considered statistically significant.

Results and discussion

In our study, we tested the hypothesis that the cooling rate affects the spermatozoan viability during the semen freezing process. Our most significant finding was that a rapid cooling curve (conventional cooling curve) impairs the spermatozoan plasma and acrosomal membranes and mitochondrial potential when the semen is submitted to freezing and thawing. We also observed that slower cooling curves cause less damage to spermatozoa prior to cryopreservation. These findings have important implications for the practical application of AI using frozen semen. Generally, the conventional cooling method used to decrease the temperature and to reach the equilibration step prior to the freezing step consists of placing the semen straws in a temperature of 4ºC inside a refrigerator. However, this simple method was demonstrated in our study to decrease the temperature very rapidly, damage the spermatozoan membranes and alter mitochondrial function.

There was no effect of cooling treatment on the percentage of motile spermatozoa before and after semen cooling and freezing/thawing (P>0.05). Before cryopreservation, spermatozoan motility averages, following different cooling protocols, were 63.3% (RD), 66.7% (SRD), 66.7% (SSLW) and 70.0% (SLW). After freezing, the percentages of progressive motility were 40.0% (RD), 43.3% (SRD), 40.0% (SSLW) and 36.7% (SLW).

Spermatozoan motility and abnormal morphology are highly associated with the rate of fertilisation (Tartaglione and Ritta, 2004). The percentage of spermatozoan motility has been reported to range from 40% to 60% after cryopreservation; however, only 10% to 30% of the spermatozoa showed normal plasma and acrosomal membranes (Salamon and Maxwell, 2000). Thus, the evaluation of the integrity of spermatozoan morphology, are highly associated with the rate of fertilisation capacity of bull spermatozoa.

After cooling, the percentage of normal spermatozoan morphology observed in the SRD group (84.8±1.42) was lower than that observed in the RD (91.67±0.44), SSLW (90.33±0.83) and SLW (91.17±2.48) groups (Figure 3; P<0.05). The spermatozoan tail defects were the most frequent, ranging from 8.0% to 18.5%. There was no difference among treatments: RD (88.83±0.92), SRD (82.50±1.75), SSLW (87.16±0.60) and SLW (88.0±3.81), on the rate of normal spermatozoan cells in thawed semen (P>0.05).

Interestingly, the semi-rapid but not the rapid cooling rate used in our study affected spermatozoan morphology, increasing mainly the spermatozoan tail defects. This seems not to be an important finding because the spermatozoan tail defects were found to be within the normal range (8.0%-18.5%) for bovine semen (Cupps, 1991). In addition, it is well known that cooling and freezing conditions may induce damages to spermatozoan tails (Graham and Moc, 2005). However, the spermatozoan morphology analysis has been an important parameter for semen evaluation and has been used to estimate fertility potential in many species (Ombelet et al., 1995; Verstegen et al., 2002; Zahalsky et al., 2003). The incapacity of spermatozoa to fertilise oocytes has been associated with morphologically abnormal spermatozoa (Amann et al., 1987; Hammerstedt et al., 1990).

Fluorochromes are commonly used to evaluate spermatozoan membrane integrity. The combination of different fluorescent probes, as used in our work for simultaneous evaluation of changes in the spermatozoan plasma and acrosomal membranes and mitochondrial potential, has been reported previously (Henley et al., 1994; Huo et al., 2002; Nagy et al., 2003). Percentages of spermatozoan plasma membrane, acrosomal membrane and mitochondrial potential integrity were lower in the RD cooling curve (19.0±2.0, 36.0±3.0, 14.0±3.0) compared with the SRD (30.0±3.0, 49.0±4.0, 24.0±7.0), the SSLW (27.0±1.0, 47.0±4.0, 20.0±5.0) and the SLW (27.0±2.0, 47.0±4.0, 24.0±4.0) curves (Figure 4; P<0.05).

The effect of cooling rate on spermatozoan morphology, are highly associated with the rate of fertilisation capacity of bull spermatozoa.

Figure 3. Percentage of morphologically normal bovine spermatozoa following the four cooling-rate treatments. Data were pooled from three replicates from each ejaculate. RD, rapid; SRD, semi-rapid; SSLW, semi-slow; SLW, slow. Different letters indicate differences between cooling curve protocols after cooling semen (P<0.05).
viability may be explained by significant changes in membrane potential during the cell cooling process. Spermatozoan plasma membrane integrity is an important parameter used to evaluate cell viability. When spermatozoa are subjected to low temperatures during cryopreservation, there is a destabilisation and rupture of the plasma membrane owing to the transition of the fluid phase to the gel-like phase (Holt and North, 1984; De Leeuw et al., 1990). These changes may affect cellular water removal and ion concentration, impairing the spermatozoan acrosome and tail (Toner et al., 1990; Parks and Graham, 1992). The rate of water efflux during spermatozoan freezing depends on the membrane permeability, which may be affected by rapid cooling where the spermatozoa may not be able to lose enough intracellular water, resulting in intracellular ice formation and cellular injury (Muldw and McGann, 1994). In addition, thermal shock induces membrane damage with consequent changes in metabolite input, mainly reducing the level of glucose and fructose, and consequently decreasing cellular respiration, leading to DNA degradation (White, 1993). A negative correlation was observed between fertility and positive PI staining using fresh (Anzar et al., 2002) and cryopreserved (Januskauskas et al., 2003) bovine semen in AI programmes. Therefore, the evaluation of spermatozoan viability using fluorescent probes is crucial to evaluate the spermatozoan fertilisation potential in cattle.

In the current study, we observed that the cooling rate affected spermatozoan viability, mainly detected by membrane and mitochondrial status. The rapid cooling rate resulted in 10 percentage points less viable spermatozoa than the slower cooling rate. Fluorescence evaluation using mitochondrial stains are commonly used to measure mitochondrial membrane potential (Garner et al., 1997). The mitochondria-specific stain (JC-1) used in this work is efficient in identifying spermatozoa with high and low membrane potential, revealing the spermatozoan functional status (Garner et al., 1997; Thomas et al., 1998). In cattle, spermatozoan plasma membrane and mitochondrial integrity are similarly affected by the freezing and thawing process (Bollwein et al., 2008). In agreement, we found that the spermatozoan plasma membrane and mitochondrial potential integrity are approximately 20% to 30%, on average, after spermatozoan thawing.

**Conclusions**

The conventional method using the rapid cooling curve for cooling and equilibrating semen before freezing consists of placing the straws inside a refrigerator at 4°C. Besides this being a very simple process, the rapid cooling rate seems to have a negative effect on spermatozoan viability. In our study, we concluded that a rapid cooling rate is detrimental to the spermatozoa and has important effects on the post-thaw quality of bovine frozen semen.

**References**

Amann, R.P., Cristanelli, M.J., Squires, E.L., 1987. Proteins in stallion seminal plasma. J. Reprod. Fertil. S. 35:113-120.

Anzar, M., He, L., Buhr, M.M., Kroetsch, T.G., Pauls, K.P., 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol. Reprod. 66:354-360.

Ball, B.A., Vo, A., 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22:1061-1069.

Batellier, F., Vidament, M., Faququant, J., Duchamp, G., Arnaud, G., Yvon, J.M., Magistrini, M., 2001. Advances in cooled semen technology. Anim. Reprod. Sci. 68:181-190.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J. Androl. 21:895-902.

Baumber, J., Ball, B.A., Linfor, J.J., Meyers, S.A., 2003. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24:621-628.

Bollwein, H., Fuchs, I., Koes, C., 2008. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. Reprod. Domest. Anim. 43:189-195.

Braundmeier, A.G., Miller, D.J., 2001. The search is on: finding accurate molecular markers of male fertility. J. Dairy Sci. 84:1915–1925.

Celeghini, E.C., de Arruda, R.P., de Andrade, A.F., Nascimento, J., Raphael, C.F., 2007. Practical techniques for bovine sperm simultaneous fluorometric assessment of plasma, acrosomal and mitochondrial membranes. Reprod. Domest. Anim. 42: 479-488.

Cupps, P., 1991. Reproduction in Domestic Animals. Academic Press, San Diego, CA, USA.

De Andrade, A.F., De Arruda, R.P., Celeghini, E.C., Nascimento, J., Martins, S.M., Raphael, C.F., Moretti, A.S., 2007. Fluorescent stain method for the simultaneous determination of mitochondrial potential and integrity of plasma and acrosomal membranes in boar sperm. Reprod. Domest.

![Figure 4. Spermatozoan plasma membrane and acrosome membrane integrity and mitochondrial potential function (mean ± SEM) measured by PI/FITC-PSA/JC-1 staining in frozen/thawed semen subjected to cooling rate treatments. RD, rapid; SRD, semi-rapid; SSLW, semi-slow; SLW, slow. Different letters indicate statistical differences among treatments (P<0.05).](image-url)
Anim. 42:190-194.

De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology 27:171-183.

Foote, R.H., Brockett, C.C., Kaproth, M.T., 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim. Reprod. Sci. 71:13-23.

Garner, D.L., Thomas, C.A., Gravance, C.G., Marshall, C.E., DeJarnette, J.M., Allen, C.H., 2001. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology 56:31-40.

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M., Marshall, C.E., 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 57:1401-1406.

Hammerstedt, R.H., Graham, J.K., Nolan, J.P., 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl. 11:73-88.

Henley, N., Baron, C., Roberts, K.D., 1994. Flow cytometric evaluation of the acrosome reaction of human spermatozoa: a new method using a photoactivated supravital stain. Int. J. Androl. 17:8-16.

White, I.G., 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod. Fertil. Dev. 5:639-658.

Zahalsky, M.P., Zoltan, E., Medley, N., Nagler, H.M., 2003. Morphology and the sperm penetration assay. Fertil. Steril. 79:39-41.