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Cryopreservation of Boar Semen

II: Effect of Cooling Rate and Duration of Freezing Point Plateau on Boar Semen Frozen in Mini- and Maxi-Straws and Plastic Bags

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Bwanga, C. O., S. Einarsson and H. Rodriguez-Martinez: Cryopreservation of boar semen. II: Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags. Acta vet. scand. 1991, 32, 455–461. – The post-thaw motility and the acrosome integrity of semen from 4 boars frozen with a programmable freezing machine, in mini (0.25 ml) and maxi (5 ml) plastic straws and in 10 × 5 cm Teflon^R FEP-plastic bags (0.12 mm thick, 5 ml), were compared. The freezing of the semen was monitored by way of thermocouples placed in the straws and the bags. Three freezing programmes were used, namely **A:** from + 5° C, at a rate of 3° C/min, to –6° C, held for 1 min at –6° C, and followed by a cooling rate of 20° C/min to –100° C; **B:** a similar curve except that there was no holding time at –6° C and that the cooling rate was 30° C/min, and **C:** from +5° C to –100° C, with a cooling rate of 35° C/min, followed by storage in liquid N_2. Despite the treezing curve assayed, both the mini-straws and the bags depicted much shorter freezing point plateaus as compared to the maxi-straws. Post-thaw sperm motility as well as the amount of normal apical ridges were equally significantly higher when semen was frozen in mini-straws or in bags than in maxi-straws. Significant differences in these post-thawing parameters were obtained between the freezing curves used. The stepwise freezing procedure **A** appeared as the best alternative for boar semen, considering this in vitro evaluation.

deep freezing; cryobiology; swine.

Introduction

Successful cryopreservation of porcine sperm depends upon the understanding of many variables such as the cryoprotectant's concentration, the composition of the diluent, the cooling and warming rates, degree of supercooling, etc. Limited information is currently available concerning the effect of the cooling velocity, upon the packaging systems in general use (pellets, maxi-straws) which restrict the range of useable cooling rates. In previous studies (*Bwanga et al.* 1990, 1991), a satisfactory method for freezing large volumes of boar semen in plastic bags and straws has been reported from our laboratory. The freezing curve assayed produced a freezing point plateau (dissipation of latent heat of fusion) of 1 min or less for volumes of up to 5 ml of semen, and a rapid post-crystallization freezing rate. Nevertheless, since boar semen has been reported to require fast rates of freezing and thawing (*Polge* 1976), faster cooling rates aimed at inducing shorter freezing plateaus within the range of the programmable biological freezing machines available ought to be tested.

The aim of the present investigation was therefore to study the effect of 3 different cooling rates and their freezing point pla-

teaus onto the viability of boar semen frozen into mini-, maxi-straws or plastic bags.

Material and methods

Semen was collected twice weekly from 4 Swedish Yorkshire boars, housed at the Department of Obstetrics and Gynaecology. The age of the boars averaged 2 years.

The filtered sperm-rich fraction (*Einarsson* 1971) of the ejaculate was collected by the glovedhand method (*Hancock & Lovell* 1959) being filtered through 2 layers of gauze fixed to prewarmed (37° C) thermos flasks. All collections were done in the morning, and ejaculates from each boar were sampled with at least 2 days apart. The volume of the split-ejaculate was measured and the cell concentration determined with a photometer (Elextrolux Mecatronic AB, Sweden) and later confirmed with the aid of a Bürker chamber. Sperm motility was determined by direct observation at 37° C in a microscope equipped with phase contrast optics (250 ×). The evaluation of acrosomal integrity was done by examination with phase contrast microscopy (1000 ×) of formol-saline fixed samples.

Semen for freezing was required to have at least 70 % motile spermatozoa. The semen was processed and frozen by a modified procedure based on the straw freezing method originally described by *Westendorf et al.* (1975). Shortly after collection, the semen was diluted 1:3 with Beltsville thawing solution (BTS, extender I; *Pursel & Johnson* 1975) at pH 7.4, both at 30° C into 250 ml centrifuge flasks. The flasks with the diluted semen were placed into a general purpose refrigerated centrifuge (IEC Centra 4R, England), at 15° C for 3 h. Thereafter, the diluted semen was centrifuged (800 × g for 10 min), the supernatant skipped and the concentrated semen rediluted (1:1 at 15° C) with Extender II: (80 ml of 11 % Lactose (0,15 M) solution and 20 ml egg yolk). The diluted semen was gently mixed and the concentration of sperm cells counted. Depending on the cell count, the necessary amount of Extender II was added, until the concentration of semen to be frozen was 1×10^9 spermatozoa/ml, the flasks being then replaced into the centrifuge. The temperature of the centrifuge chamber was set to 5° C, and the diluted semen was allowed to cool down for 2 h. Extender III (89.5 ml of extender II, 1.5 ml Orvus Es Paste, 9.0 ml glycerol) was then added, 2 parts of semen: 1 part of extender at 5° C. The final diluted semen was manually put into 5 ml maxi-straws (Mini-tüB GmbH, Landshut, West Germany) closed with metallic sealing balls; into 10 × 5 cm plastic bags made up of Teflon^R FRP-film (Dupont, Switzerland, 0.12 mm thick), sealed with a thermo-sealer (Auto-Seal 102, Nitech AB, Sweden); and into 0.25 ml mini-straws (with an automatic fillersealer, Cassou^R, L'Aigle, France). The filled straws were put in carrier racks and locked. Half of the Teflon^R FRP-bags were placed horizontally on a metallic net, while the other half were compressed between 2 parallel thin aluminium nets clamped together and frozen vertically. The straws and the bags were placed into the chamber of the programmable biological freezing machine (Model PTC 200, Planer Products Ltd., England).

Three freezing programmes were run (**A:** 5° C to –6° C with 3° C/min, 1 minute at –6° C, and a further decrease from –6° C to –100° C with 20° C/min freezing rate; **B:** 5° C to –6° C with 3° C/min, and further decrease from –6° C to –199° C with 30° C/min, and **C:** 5° C to –100° C with a cooling rate of 35° C/min). The frozen straws and bags were stored in liquid N_2 until thawing. For representative groups of straws being frozen, one 0.2 mm in diameter type K copperconstan-

tan thermocouple was held in the center of a mini- and maxi-straw as well as into a plastic bag. The tips of the thermocouples were placed about 30–60 mm from the ends of the straws and the border of the bag, by way of plastic cushions, when they were filled prior to freezing. A 4th thermocouple was held within the freezing chamber. All thermocouples were connected to a temperature recorder (Chessel[R] Model 4001, Chessel Ltd., West Sussex, England) and the variations in temperature of the semen and the freezer chamber were recorded throughout the experiment. The freezing curves were consistently repeatable.

Thawing was done by immersion into circulating water at 50° C for either 12 sec (mini-straws), 40 sec (maxi-straws) or 25 sec (Teflon[R] FRP-film bags). In all cases, immediately after thawing, 0.5 ml of semen was added to 4.5 ml of BTS at room temperature. For motility evaluation, triplicates (sub-samples) of each sample were incubated at 37° C and the samples examined after 30 min (*time 0*) and after 2 h (*time 2 h*) of incubation. The test straws and bags holding the thermocouples were thawed as described above. The recorded temperatures reached at complete thawing were 44–47° C for mini- while the maxi-straws attained between 0–10° C in the core. The bags attained temperatures between 18–20° C.

Sperm viability (progressive motility and acrosome integrity) was estimated on fresh semen shortly after dilution with BTS, at various stages of the cooling procedure and post-thawing. The results, pooled from individual boars, are expressed as means ± s.e.m. and differences were compared for statistical significance with a Student's t-test and ANOVA (one-way classfication) (*Steel & Torrie* 1960).

Results

The temperature curves monitored during freezing by the thermocouples inside the chamber, bags and straws, using the 3 freezing curves assayed (**A-C**), are presented in Fig. 1. When employing the freezing programme **A** the temperatures within the

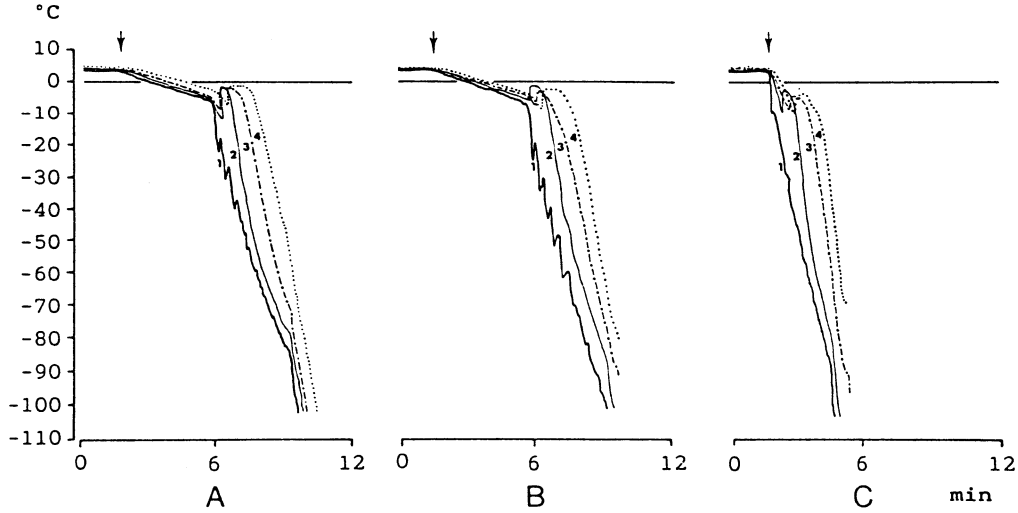

Figure 1. Tracings of the temperature changes monitored in the chamber (**1**) test mini-straw (**2**), plastic bag (**3**) and maxi-straw (**4**) during the assayed freezing procedures, initiated at arrows (**A:** +5° C to – 6° C with 3° C/min, 1 min at – 6° C, followed by 20° C/min till –100° C; **B:** +5° C to –6° C with 3° C/min followed by 30° C/min till –100° C; **C:** +5° C to –100° C with a cooling rate of 35° C/min).

straws and bags followed the cooling rate of the freezing chamber to temperatures beyond the crystallization point (–5 to –12° C). A rebouncing effect was subsequently observed both in straws and the bag with the inner temperature rising to –2–0° C, while the chamber temperature was already down to –20° C. The mini-straw then showed a curve of temperature decrease closely associated to the designed cooling rate. As expected – the mini-straw – on the other hand, depicted the longest freezing point plateau – longer than a minute – before showing a temperature fall in the rest of the procedure. The temperature curves of the plastic-material bags assayed (Teflon^R FEP), showed a much shorter freezing point plateau – of about 30 sec – with their freezing curves traced in between the 2 above mentioned volumes of straws (Fig. 1). There were no obvious differences in the temperature curves between the horizontally (flat) or vertically (standing) frozen Teflon^R FEP bags.

The use of a faster cooling rate than in the original (**A**) procedure, as assayed in (**B**); showed a faster rebouncing effect and a larger freezing point plateau for both straws and bags (Fig. 1). Furthermore, when the chamber and the mini-straw reached –100° C, the temperatures within the maxi-straws and the plastic bags still ranged between –80–90° C. A more dramatic temperature delay was seen when a direct and faster cooling was applied, as in programme **C** (Fig. 1), which neither eliminated supercooling.

The results of the thermoresistance test ran after the spermatozoa, frozen with any of the 3 freezing programmes above mentioned, were thawed, diluted in BTS and incubated at 37° C, are presented i Tables 1 and 2. Post-thaw motility (%) was, for freezing rate **A**, clearly comparable and significantly higher ($P < 0.01$) in the bags and the mini-straws than in the maxi-straws, 30 min after thawing and incubation at 37° C (Table 1).

Table 1. Post-thaw motility (%) of boar semen frozen in different containers and 3 freezing programmes (**A:** +5° C to –6° C with 3° C/min, 1 min at –6° C, and a further decrease from –6° C to –100° C with 20° C/min freezing rate; **B:** +5° C to –6° C with 3° C/min followed by 30° C/min till –100° C; **C:** +5° C to –100° C with a cooling rate of 35° C/min) assessed after 30 min incubation at 37° C (Values as mean ± s.e.m., n = 12).

	Freezing procedures		
Container	**A**	**B**	**C**
Mini-straw	51.8 ± 3.72^{1a}	36.3 ± 3.55^{1b}	25.4 ± 4.48^{1b}
Maxi-straw	28.8 ± 4.10^{2a}	19.6 ± 4.09^{2ac}	12.9 ± 3.14^{1bc}
FEP-bag^H	51.9 ± 3.33^{1a}	37.5 ± 4.91^{1b}	15.0 ± 5.44^{1c}
FEP-bag^V	55.0 ± 4.00^{1a}	36.5 ± 4.84^{1b}	22.0 ± 6.44^{1b}

^{H,V} horizontally and vertically frozen Teflon^R FEP bags, respectively.
^{1,2,} values between columns with different superscripts differ ($p \mp 0.05$).
^{a,b,c} values within columns with different superscripts differ ($p < 0.05$).

Table 2. Percentage of post-thaw acrosome integrity (as normal apical ridges = NARs) in boar semen frozen in different containers and 3 freezing programmes (**A:** +5° C to –6° C with 3° C/min, 1 min at –6° C, and a further decrease from –6° C to –100° C with 20° C/min freezing rate; **B:** +5° C to –6° C with 3° C/min followed by 30° C/min till –100° C; **C:** +5° C to –100° C with a cooling rate of 35° C/min) assessed, using phase contrast microscopy, after 30 min incubation at 37° C (Values as mean ± s.e.m., n = 12).

	Freezing procedures		
Container	**A**	**B**	**C**
Mini-straw	76.8 ± 4.55^{1a}	45.0 ± 2.82^{2a}	44.4 ± 1.84^{2a}
Maxi-straw	59.3 ± 6.02^{1b}	30.0 ± 1.17^{2b}	29.5 ± 1.51^{2b}
FEP-bag^{H}	77.3 ± 3.82^{1a}	58.6 ± 3.31^{2c}	47.7 ± 1.22^{3a}
FEP-bag^{V}	81.6 ± 3.43^{1a}	49.6 ± 4.35^{2ac}	48.1 ± 2.21^{2a}

^{H,V} horizontally and vertically frozen Teflon^{R} FEP bags, respectively.
^{1,2,} values between columns with different superscripts differ ($p \mp 0.05$).
^{a,b,c} values within columns with different superscripts differ ($p < 0.05$).

In all cases, motility decreased significantly ($P < 0.01$) during the incubation interval, which was extended up to 2 h at 37° C. A similar trend was seen for freezing programme **B**, but not for programme **C** (Table 1). In any case, a significantly lower post-thaw motility ($P < 0.05$–0.001) was recorded for programme **B** and **C**, both at 30 min (Table 1) and 2 h (data not shown), irrespective of the container in question.

When acrosome integrity after freezing/thawing was evaluated (Table 2), the percentage of morphologically intact acrosomes 30 min after thawing and incubation at 37° C was significantly higher ($P < 0.05$–0.01) for mini-straws and Teflon^{R} FEP film-bags than for maxi-straws (Table 2), independent of the freezing programme used. Acrosome integrity was anyhow, significantly higher ($P < 0.05$–0.01) when using programme **A** than with either **B** or **C**, the latter attaining unacceptable low NAR values.

Discussion

The effect of 3 different cooling rates, with subsequent different freezing point plateaus was assayed onto the post-thawing viability of boar spermatozoa frozen with a programmable freezing machine in mini-, maxi-straws and plastic bags. A stepwise freezing procedure, with a slow cooling to the initial extracellular freezing point, followed by a cooling rate of 20° C/min, appeared to be the best alternative in terms of highest post-thaw sperm motility and acrosome integrity for all types of container tested.

The cooling rate is the most important factor controlling the life or death of a cell during freezing (*Mazur* 1984). Each type of cell has an optimal cooling rate for surviving the freezing proces, the rate varying widely depending upon a number of cellular characteristics, the cryoprotectant and its concentration, the extender, and even the warming rate (*Mazur* 1984). The cooling velocity greatly affects sperm motility and acrosomal

integrity, interacting significantly with glycerol concentration (*Fiser* 1990). While irreversible injuries obtained with slow freezing rates are not influenced by the addition of low concentrations of glycerol (*Polge et al.* 1949, *Smith* 1961, *Mazur* 1985), a combination of low concentrations of glycerol and high velocities of freezing seems to be elective for boar spermatozoa (*Polge* 1976, *Mazur* 1977).

This seemed to be the case for various procedures tested (*Pursel & Johnson* 1975, *Westendorf et al.* 1975, *Paquignon & Courot* 1975, *Larsson et al.* 1977), and a quite optimal freezing rate for several containers has been described recently (*Bwanga et al.* 1990, 1991) for boar spermatozoa. Nevertheless, the duration of the freezing point plateau described was rather prolonged (approx. 1 min) and responsible for major membrane and acrosomal damages (*Park & Pursel* 1985, *Pursel & Park* 1985). The present results indicate that despite the faster cooling rates assayed, supercooling was not significantly reduced, and the low NAR figures obtained at thawing made the fast procedures unacceptable for semen preservation.

Freezing injury is likely to be caused by a sequential chain of events, such as the formation of extracellular ice, increase in osmotic pressure across the plasmalemma, membrane damage and formation of intracellular ice (*de Leeuw et al.* 1990). Apparently, the temperature rate used by our group (*Bwanga et al.* 1990, 1991) allows movement of water and cryoprotectant without either major intracellular ice formation or irreversible membrane change in the processed boar semen which makes this method a promising one. However, the accurate determination of the presence and distribution of intracellular ice (*Courtens & Paquignon* 1985) in these processed boar spermatozoa remains to be done and it is being tested at our laboratories.

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Sammanfattning

Djupfrysning af galtspermier. II: Inverkan av infrysningshastighet och fryspunktsplatåns längd på galtspermier infrysta i ministrån, maxistrån och plastpåsar.

Spermiemotilitet och akrosommorfologi efter djupfrysning-upptining undersöktes hos spermier från fyra galtar som frysts med hjälp av en programmerbar frysmaskin i ministrån (0,25 ml), maxistrån (5 ml) och plastpåsar (5ml, 10 × 5 cm) tilverkade av 0,12 mm tjock Teflon FEP-film. Frysningsförloppet studerades med hjälp av "termocouples", som placerats i strån och plastpåsar. Följande 3 frysprogram användes: **A**- från 5° C till –6° C med 3° C/min, uppehåll under en minut vid –6° C, från –6° C till –100° C med 20° C/min; **B**- likartad frysningsförlopp, men inget uppehåll vid –6° C och 30° C/min från –6° C till –100° C; **C**- från 5° C till –100° C med 35° C/min, åtföljt av nedsänkning i flytande kväve. Trots fryskurvans utseende, hade båda ministrån och påsar kortare fryspunktplatåer än maxistrån. Såväl upptiningsmotiliteten som akrosommorfologin var signifikant bättre hos spermier som frysts i ministrån och plastpåsar jämfört med maxistrån. Signifikanta skillnader påvisades också mellan de 3 frysprogrammen. Frysprogram **A** lämpade sig bäst för galtspermier att döma av resultaten från in vitro testerna efter djupfrysningupptining.

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