Effect of cooling rate on the survival of cryopreserved rooster sperm: comparison of different distances in the vapor above the surface of the liquid nitrogen

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ABSTRACT

The aim of the present trial was to study the effect of different freezing rates on the survival of cryopreserved rooster semen packaged in straws. Slow and fast freezing rates were obtained keeping straws at different distances in the vapor above the surface of the nitrogen during freezing. Adult Lohmann roosters (n = 27) were used. Two experiments were conducted. In Experiment 1, semen was packaged in straws and frozen comparing the distances of 1, 3 and 5 cm in nitrogen vapor above the surface of the liquid nitrogen. In Experiment 2, the distances of 3, 7 and 10 cm above the surfaces of the liquid nitrogen were compared. Sperm viability, motility and progressive motility and the kinetic variables were assessed in fresh and cryopreserved semen samples. The recovery rates after freezing/thawing were also calculated. In Experiment 1, there were no significant differences among treatments for all semen quality variables. In Experiment 2, the percentage of viable (46%) and motile (22%) sperm in cryopreserved semen was greater when semen was placed 3 cm compared with 7 and 10 cm in the vapor above the surface of the liquid nitrogen. The recovery rate of progressive motile sperm after thawing was also greater when semen was stored 3 cm in the vapor above the surface of the liquid nitrogen. More rapid freezing rates are required to improve the survival of rooster sperm after cryopreservation and a range of distances from 1 to 5 cm in nitrogen vapor above the surface of the liquid nitrogen is recommended for optimal sperm viability.

Keywords: Rooster semen, Liquid nitrogen vapor, Straw, Cooling rate

1. Introduction

Genetic conservation and biodiversity are important and although mainly focused on endangered species, have a very important role in domestic animals, especially in intensive poultry breeding which has caused a rapid decrease of genetic diversity in local breeds (Váradi et al., 2013).

According to the DAD-IS (Domestic Animal Diversity Information System), more than 50% of poultry species are in the endangered category (Hoffmann, 2005). Consequently, there is an urgent need to create gene banks for these chicken breeds. In addition to *in vivo* management, *in vitro* conservation is a strategic technique to secure genetic diversity, taking into account the risk of epidemic diseases (Blesbois et al., 2007). Currently, local poultry genetic materials are stored *in vitro* in only four national gene banks (France, Netherlands, North-America and Japan; Blackburn, 2006; Blesbois et al., 2007; Blesbois, 2011; Woelders et al., 2006). Although cryopreservation is a valuable technique for the poultry industry, the freezing of rooster semen is not yet a commonly used commercial procedure (Bellagamba et al., 1993; Fulton, 2006; Long, 2006; Blesbois, 2007).

Many freezing methods with different cryoprotectants (glycerol, DMA- dimethyl acetamide, DMSO-Dimethyl sulfoxide, N-methylacetamide), different types of sperm packaging and with slow and rapid freezing procedures (Blanco et al., 2012; Blesbois et al., 2007; Sasaki et al., 2010; Seigneurin and Blesbois., 1995; Tselutin et al., 1999) have been studied. Even with these several studies, the average fertility in chickens with frozen semen is 60% (Blesbois, 2011), ranging from 0% to 90%. Greater study is, therefore, needed to gain knowledge as to how to increase the success of freezing methods for rooster semen. The most desirable fertility rates are realized with use of DMA as a cryoprotectent when the sperm are frozen in pellets (Blesbois et al., 2007) but this method does not permit the proper identification of semen samples and also can lead to cross-contamination (Wishart, 2009). All these problems can be avoided by the use of straws for semen packaging as required by the FAO guidelines (FAO, 2011).

In previous research assessing different freezing rates, a programmable freezing machine was used (Blanco et al., 2012; Blesbois et al., 2007; Santiago-Moreno, 2011) which is not always available, particularly in field conditions. The straws can be frozen on styrofoam floating on liquid nitrogen (e.g. Dong et al., 2009). It is difficult to compare different non-programmable freezing systems because these are influenced by many factors such as temperature inside and outside the straw, volume surface ratio of the straw and ventilation (FAO, 2011). Therefore, experimentation is needed to determine which conditions are optimal for freezing rooster semen.

The aim of the present study was to improve the success of cryopreservation of rooster semen packaged in straws and frozen in nitrogen vapor. The effect on sperm quality of different cooling rates in the vapor by placing samples at different distances from the surface of liquid nitrogen has been studied. Another objective was to identify a desired procedure to implement in a semen cryobank for conservation of Italian chicken breeds.

2. Materials and methods

Adult Lohmann roosters (*Gallus domesticus*; n = 27) were housed at 22 weeks of age in individual cages and kept at 20° C and 14L:10D photoperiod, at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were given *ad libitum* access to a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% crude protein, 3% ether extract, 10.5% ash, 3.10% calcium) and drinking water. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

After a 2-week semen collection training period, all males were routinely collected twice a week from March to July. Semen was collected according to the technique initially described by Burrows and Quinn (1935). Each day of collection, ejaculates were randomly pooled (nine ejaculates/pool) into three semen samples and pools obtained on different days were always formed with different ejaculates to reduce the effect of the bird. The ejaculates were pooled into graduated tubes and semen volume was recorded and sperm concentration was measured using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm. Pooled semen samples were subsequently diluted in modified Lake pre-freezing extender (8 g D-fructose, 5 g potassium acetate, 19.2 g sodium glutamate, 3 g polyvinylpyrrolidone, 0.7 g magnesium acetate, 3.75 g glycine, adjusted to 1 L with distilled water; pH 7.0, and osmolality 340 mOsmol/kg) to a concentration of 1.5×10^9 sperm/mL, cooled at 4° C for 20 to 30 minutes and transferred to the laboratory for further quality assessment, including sperm viability and motility, and freezing processing.

Sperm viability was measured using the SYBR14/PI (propidium iodide) dual staining procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano (2011) with minor modifications. In brief, the incubations were conducted at room temperature and the Lake's diluent (6 g glucose, 1.28 g potassium citrate, 15.2 g sodium glutamate, 0.8 g magnesium acetate, 30.5 g BES, 58 ml NaOH adjusted to 1 L with distilled water; pH 7.05, and osmolality 411 mOsmol/kg) was used. Assessment of 200 sperm cells was conducted in duplicate aliquots for every sample and evaluated microscopically at 100X total magnification using a Zeiss (Axioskop 40- AxioCamICc 1) microscope and FITC filter fluorescence. Green staining of only live sperm occurs with use of SYBR-14 and green staining occurs for dead sperm with use of the PI stain.

Sperm motility was assayed using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh pooled semen samples were further diluted in refrigerated 0.9 % NaCl to a concentration of 1.0×10^8 sperm/mL and incubated for 20 minutes at room temperature; then, 10 μL semen were placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature. The motion variables recorded were: motile sperm (%), progressively motile sperm (%), curvilinear velocity [VCL, (μm/s)], straight-line velocity [VSL, (μm/s)], average path velocity [VAP, (μm/s)], amplitude of lateral head displacement [ALH, (μm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)]. A minimum of three fields and 500 sperm tracks were analyzed at 10X magnification for each sample.

After the assessment of sperm viability and motility, semen samples were further diluted to 1 x 10^9 sperm/mL with Lake pre-freezing extender containing 18% DMA, leaving to 6% final DMA concentration (Zaniboni et al., 2014), equilibrated at 5 °C for 1 min and loaded into 0.25-mL French straws (IMV Technologies, France). Each pooled semen sample was divided into three aliquots and loaded into differently colored straws corresponding to different treatments during freezing in nitrogen vapor. The treatments were different distances between the straws and the liquid nitrogen bath, providing for different freezing rates.

Two consecutive experimental protocols were conducted with the first being performed in May and the second in July. In Experiment 1 the distances in the vapor when placement of samples occurred at 1, 3 and 5 cm from the surface of the liquid nitrogen were compared. Considering the results of Experiment 1, in Experiment 2 distances from the surface of the liquid nitrogen in the vapor were compared when placement of samples was at 3, 7 and 10 cm above the surface. Freezing of samples was performed as described by Nöthling and Shuttleworth (2005) with minor modifications. Floating racks consisted of a wire netting sustained by a styrofoam frame. These racks were placed at different distances above the surface of the liquid nitrogen and supported the straws at the experimental treatment distances of 1, 3, 5, 7, 10 cm above the surface. Three styrofoam boxes were loaded to a depth of about 4 cm with liquid nitrogen and the cover closed to allow the vapor to stabilize. The lid of the boxes was then removed and the racks with straws were placed so as to float on the liquid nitrogen. After 10 min, straws were plunged into liquid nitrogen and stored in a cryotank. A total of 9 (n. of replicates) pooled semen samples were processed per experiment and a total of 18 straws were stored per treatment. The temperatures inside straws (n = 3) with each of the treatments were measured simultaneously with a frequency of one per 20 seconds during the freezing process for both experiments.

The cooling velocity of the frozen semen was measured using a thermocouple thermometer (80PK-1 K, Fluke-51/RS, Fluke Corporation, USA). The straws were thawed in a water bath at 38

°C for 30 sec and sperm quality was assessed in thawed semen. Sperm viability and motility were measured as previously described in fresh semen samples, with the exception of using 0.9% NaCl at room temperature for sample dilution before sperm motility analysis.

Analysis of variance for sperm quality variables using fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS (SAS, 1999). Treatments (different distances in the vapor above the surface of the liquid nitrogen), time (fresh and thawed semen), and the relative interactions were considered as fixed effects and the pooled semen sample was considered as random effect. The *t* test was used to compare LSMeans. The recovery rates (%) of sperm viability, motility and progressive motility after cryopreservation were calculated as follows: [(mean on thawed semen*100)/mean on fresh semen]. Analysis of variance on the recovery variables was performed using the GLM procedure of SAS (SAS, 1999), and the treatment was the only source of variation included in the model. The *t* test was used to compare LSMeans. Data measured as proportions were transformed into arsin values before statistical analysis.

3. Results

The mean volume and sperm concentration recorded in fresh ejaculates were 0.2 ± 0.08 mL and 3.55 ± 0.84 x 10^9 sperm/mL respectively. As expected, the distance of the straws in the vapor above the surface of the liquid nitrogen had an effect on cooling rate as depicted in Figure 1.

3.1. Experiment 1: Freezing semen packaged in straws stored in the vapor at 1, 3 and 5 cm above the surface of the liquid nitrogen

Sperm quality variables were affected by the freezing–thawing process (Time Effect $P<0.001$) and not affected by the freezing rate (Treatments at 1, 3, 5 cm in nitrogen vapor $P>0.05$) and the interaction of time*treatment ($P > 0.05$). There were large numbers of motile sperm in all treatment groups (Table 1) and the recovery rate of motile sperm after freezing was highly acceptable, being between 36% and 41% (Table 2). There were lesser proportions of progressive sperm motility after processing, particularly after freezing/thawing (Table 1); therefore, there were lesser recovery rates and only 20% to 24% of progressively motile sperm survived cryopreservation (Table 2). This result indicates there is an increased sensitivity of the cells with during the freezing process as indicted by the decreased progressive motility after thawing. All kinetic variables decreased after freezing/thawing also and the same trend for the multiple variables was recorded for all treatments (Table 1). The mean values of sperm viability before and after cryopreservation were 74% and 41% respectively (Table 1). Large proportions of viable sperm were recovered after freezing/thawing and there were no differences among treatment groups (Table 2).

3.2. Experiment 2: Freezing semen packaged in straws in the vapor at 3, 7 and 10 cm above the surface of the liquid nitrogen

In Experiment 2, two distances not used in Experiment 1, 7 and 10 cm, were compared to the 3 cm distance above the surface of the liquid nitrogen. Sperm quality variables were affected by the freezing–thawing process (Time Effect $P < 0.001$). The freezing rate (3, 7, 10 cm above the surface of the liquid nitrogen) and the interaction time*treatment affected sperm motility ($P < 0.001$) and viability ($P < 0.001$), and three kinetic variables (STR, ALH and BCF; $P < 0.05$). The greatest percentage of viable and motile sperm in cryopreserved semen was observed when the storage occurred at 3 cm (46% and 22%, respectively) above the surface of the liquid nitrogen (Table 3). The recovery rate of viable sperm was greater when the sperm were placed at 3 cm above the surface of the liquid nitrogen with rate being 58% as compared to when storage occurred at 7 cm (47%) and 10 cm (44%) above the surface of the liquid nitrogen (Table 4).

This result indicates sperm integrity was preserved during freezing of the semen in nitrogen vapor at distances of 3 cm above the surface of the liquid nitrogen. The recovery rate of motile cells was also greater with the 3 cm treatment compared with the other treatments (26% compared with 16.6%). Progressive motility was greatly reduced after freezing/thawing and there were less than

1% of progressively motile sperm after thawing in all treatment groups (Table 3). However, the recovery rate of progressive motile sperm was greater when sperm were frozen at 3 cm above the surface of the liquid nitrogen compared with when storage occurred at 7 and 10 cm (Table 4) above the surface of the liquid nitrogen. The values for kinetic variables were all reduced as a result of the freezing/thawing process in all treatment groups (Table 3), with the only exception being the ALH value that did not differ between fresh and thawed semen for the 3 cm treatment. After freezing/thawing, mean values for STR, ALH and BCF for the 3 cm treatment group were greater compared with the mean values for the 7 and 10 cm treatment groups (Table 3).

4. Discussion

Freezing semen packaged in straws over nitrogen vapor is a simple, quick and inexpensive method, widely used for cryopreservation of mammalian semen, even in commercial AI (Artificial Insemination) Centers. This method has the great advantage to allow the adoption of temperature gradients suitable for freezing semen in liquid nitrogen without the need of expensive dedicated equipment. In fact, the distance between the straw and the liquid nitrogen bath indirectly determines the thermal gradient during the freezing phase when the change from the liquid to the solid state occurs. The present results provide the range of distances from the surface of the liquid nitrogen that is suitable to freeze rooster semen packaged in straws.

The optimal range is identified from 1 to 5 cm above the surface of the liquid nitrogen bath and freezing at this location allows for recovery of the greatest numbers of viable and motile sperm after freezing/thawing. The range of 1 to 5 cm above the surface of the liquid nitrogen provided for rapid cooling rates associated with the recovery of 56% viable and 36% motile sperm in Experiment 1, and of 59% viable and 26% motile sperm in Experiment 2. The distances of 7 and 10 cm above the surface of the liquid nitrogen provided for slower cooling rates associated with greater cell damage and loss in sperm viability and mobility, therefore, these treatments are not suitable for freezing rooster semen. Santiago-Moreno et al. (2011) obtained the most desirable percentage of sperm motility after using a moderate freezing rate, compared with slow or rapid freezing. The most desirable values (15%) from this pervious study were less than that from the present study (ranging from 22% to 31%). Furthermore, results from the present study were more desirable (40%-46%) than for the previous study (13%-24%) for sperm viability after thawing of samples. The sperm of some avian species, in particular imperial eagles and chickens, may remain viable after rapid cooling by maintaining acceptable percentages of sperm cell viability after thawing (Blanco et al. 2000).

Small proportions of progressively motile sperm (<1.4%) exist in rooster semen after freezing/thawing even when the rapid freezing rates are applied. Purdy et al. (2009) reported 15% motility and 1.8% progressive sperm cell motility in rooster semen after freezing in nitrogen vapor at the distance of 1 cm above the liquid surface for 7 minutes and subsequent thawing. Santiago-Moreno et al. (2012) found that there were similar values of percentage sperm cell progressive motility (<5%) and motility (25%), whereas, viability values (10%-30%) were less than in the present study.

Each distance above the surface of the liquid nitrogen corresponds to a different freezing curve. The temperature inside the straws placed at 1 and 3 cm above the surface of the liquid nitrogen decreased to below -40 °C within the first minute of placement at the 3 cm location. The same temperature in straws placed at 5 cm above the surface of the liquid nitrogen resulted after about 3 minutes. The temperature never decreased to below -24 and -39 °C even after 10 minutes of the time of placement in straws placed at 7 and 10 cm, respectively, above the surface of the liquid nitrogen. In a study of Morris et al. (1999), the most desirable human sperm survival was obtained with a freezing curve in which the temperature of -40 °C was attained earlier compared to the more delayed freezing curves. During the process of freezing, sperm cells undergo several changes and the cooling rate is important for cell survival, in particular avoiding intracellular ice formation (Mazur, 1977; Viveiros et al., 2001; Woelders and Chaveiro, 2004). The present results confirm that more rapid cooling rates are required to reduce damage to rooster sperm cells during the freezing process. The critical temperature of -25 °C must be attained within 30 s and -40 °C within 3 minutes for desirable viability to result after thawing.

Sperm motility is compromised as a result of poultry semen cryopreservation and 30% to 60% reductions occur after the freeze/thaw cycle (Long 2006). Also values for all the kinetic variables decrease after freezing and thawing. Froman and Feltmann (2000) reported the VSL to be the most accurate estimate of sperm cell velocity. Froman (2007) reported that the VSL must be >30 µm/s for sperm from an overlaid sperm suspension to penetrate an Accudenz solution. In the present study, VSL values were less than 30 µm/s and were consistent with the values obtained in a study of Santiago-Moreno et al (2012). With regard to other kinetic variables, values from the present study are similar or slightly less than those reported for the study of Santiago-Moreno et al (2012). This could be because the chickens in the previous study were of a native Spanish breed unlike the Lohmann birds used in the present study. Values for ALH (amplitude of lateral head displacement) in the present study, when sperm were placed 3 cm above the liquid nitrogen surface, were not different from the values obtained with fresh semen, which may be an important finding because it has been documented that the ALH value is a reliable predictor for successful *in vitro* fertilization in humans (Chan et al., 1990).

5. Conclusions

The present study provided evidence that the freezing of rooster semen can be conducted in nitrogen vapor using a wide range of distances (from 1 to 5 cm) above the surface of the liquid nitrogen which can be associated with a gradient of very rapid freezing, avoiding the use of a fiscally expensive programmable freezer. The freezing method employed in the present study preserved rooster semen by maintaining an acceptable percentage of viable and motile sperm and values for most kinetic variables related sperm cell progressive motility. The techniques employed in the present study may be able to enhance outcomes from inseminations using rooster semen and future research will be conducted to determine whether in vivo results are consistent with the *in vitro* results of the present study.

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Authors' contributions

M. Madeddu drafted paper, performed the statistical analysis, participated in design of the study, handled the animals and collected data. F. Mosca, L. Zaniboni, A. Abdel Sayed, M.G. Mangiagalli and E. Colombo participated in design of the study, contributed analyzing data, handled the animals and collected data. S. Cerolini conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All co-authors provided inputs during final manuscript preparation. All authors read and approved the final manuscript.

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Table 1

Sperm motility variables (mean ± SE) measured in fresh semen and in semen frozen in the vapor at three different distances above the surface of liquid nitrogen

Sperm variables	Fresh	Heights over nitrogen vapor		
		1 cm	3 cm	5 cm
Viability (%)	74.4 ± 1.9^{a}	42.1 ± 2.2^{b}	41.6 ± 2.2^{b}	40.4 ± 2.2^{b}
Motility (%)	78.1 ± 1.6^{a}	29.7 ± 2.3^{b}	31.1 ± 2.3^{b}	27.3 ± 2.3^{b}
Progressive motility (%)	10.8 ± 0.8^{a}	1.4 ± 1^{b}	1.3 ± 1^{b}	1.2 ± 1^{b}
VCL (µm/s)	47.2 ± 1.3^{a}	27.8 ± 1.7^{b}	27.6 ± 1.7^{b}	27.2 ± 1.7^{b}
VSL (µm/s)	15.2 ± 0.5^{a}	7.3 ± 0.7^{b}	7.2 ± 0.7^{b}	7.1 ± 0.7^{b}
VAP (µm/s)	26.9 ± 0.8^{a}	13.8 ± 1^{b}	13.7 ± 1^{b}	13.6 ± 1^{b}
LIN (%)	32 ± 0.4^{a}	26 ± 0.6^{b}	25.8 ± 0.6^{b}	26 ± 0.6^{b}
STR (%)	56.2 ± 0.5^{a}	52.4 ± 0.8^{b}	52.3 ± 0.8^{b}	52.3 ± 0.8^{b}
WOB (%)	56.9 ± 0.4^{a}	49.4 ± 0.5^{b}	49.3 ± 0.5^{b}	49.6 ± 0.5^{b}
ALH (µm)	3.2 ± 0.1^{a}	2.9 ± 0.1^{b}	2.8 ± 0.1^{b}	2.5 ± 0.1^{b}
BCF (Hz)	6.9 ± 0.2^{a}	5.2 ± 0.4^{b}	5.9 ± 0.4^{b}	5.1 ± 0.4^{b}

Motility, percentage of motile sperm; progressive motility, sperm swimming forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble

^{a,b} Values within each row with different superscript letters differ ($P < 0.001$)

Table 2

Recovery rates of sperm quality (mean ± SE) recorded in semen frozen in the vapor at three different distances above the surface of liquid nitrogen

Sperm variables	Recovery (%)		
	Distance from liquid surface into nitrogen vapor		
	1 cm	3 cm	5 cm
Viability (%)	56.6 ± 2.3	55.8 ± 2.3	54.7 ± 2.3
Motility (%)	39 ± 3.8	40.8 ± 3.8	35.8 ± 3.8
Progressive motility (%)	24.2 ± 6.6	20.5 ± 6.6	20.4 ± 6.6

Table 3

Sperm motility variables (mean ± SE) measured in fresh semen and in semen frozen in the vapor at three different distances above the surface of liquid nitrogen

Sperm variables	Fresh	Distance from liquid surface into nitrogen vapor		
		3 cm	7 cm	10 cm
Viability (%)	78.3 ± 2.1^{a}	46.1 ± 2.3^{b}	36.4 ± 2.3^{c}	34.8 ± 2.3^{c}
Motility (%)	86 ± 1^{a}	22 ± 1.3^{b}	14.1 ± 1.3^{c}	14.3 ± 1.3^{c}
Progressive motility (%)	16.2 ± 0.4^{a}	0.6 ± 0.4^{b}	0.3 ± 0.4^{b}	0.3 ± 0.4^{b}
VCL (µm/s)	53.3 ± 1.5^{a}	28.5 ± 1.7^{b}	27.3 ± 1.7^{b}	27.5 ± 1.7^{b}
VSL (µm/s)	18.1 ± 0.5^{a}	7 ± 0.6^{b}	6.2 ± 0.6^{b}	6.4 ± 0.6^{b}
VAP (µm/s)	30.9 ± 1^{a}	13.8 ± 1^{b}	12.9 ± 1^{b}	13.2 ± 1^{b}
LIN (%)	34.1 ± 0.5^{a}	24.4 ± 0.6^{b}	22.7 ± 0.6^{b}	23.2 ± 0.6^{b}
STR (%)	58.9 ± 0.5^{A}	50.4 ± 0.6^{B}	48.3 ± 0.6^{C}	48.3 ± 0.6^{C}
WOB (%)	57.9 ± 0.6^{a}	48.3 ± 0.7^{b}	46.9 ± 0.7^{b}	47.9 ± 0.7^{b}
ALH (µm)	$3.2 \pm 0.1^{\alpha}$	$2.8 \pm 0.2^{\alpha}$	$1.9 \pm 0.2^{\beta}$	$2 \pm 0.2^{\beta}$
BCF (Hz)	7.8 ± 0.3^{a}	4.4 ± 0.4^{b}	2.7 ± 0.4^{c}	2.1 ± 0.4^{c}

Motility, percentage of motile sperm; progressive motility, sperm swimming forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble

$^{a-c}$ Values within each row with different superscript letters differ ($P<0.001$).

$^{A-C}$ Values within each row with different superscript letters differ ($P<0.05$).

$^{\alpha-\beta}$ Values within each row with different superscript letters differ ($P<0.01$).

Table 4

Recovery rates for sperm quality variables (mean ± SE) recorded in semen frozen in the vapor at three different distances above the surface of liquid nitrogen

Sperm variables	Recovery (%) Distance from liquid surface into nitrogen vapor		
	3 cm	7 cm	10 cm
Viability (%)	58.7 ± 1.16[a]	46.6 ± 1.16[b]	44.1 ± 1.16[b]
Motility (%)	25.7 ± 2.3[a]	16.5 ± 2.3[b]	16.6 ± 2.3[b]
Progressive motility (%)	3.9 ± 0.5[a]	1.9 ± 0.5[b]	1.6 ± 0.5[b]

[a, b] Values within each row with different superscript letters differ (*P*<0.001).

Captions of illustrations

Fig. 1. Change in temperature of semen in straws during cryopreservation while being suspended in the vapor at 1, 3, 5, 7 and 10 cm above the surface of liquid nitrogen