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Factors influencing boar sperm cryosurvival[1]

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ABSTRACT: Optimal sperm cryopreservation is a prerequisite for the sustainable commercial application of frozen-thawed boar semen for AI. Three experiments were performed to identify factors influencing variability of postthaw sperm survival among 464 boar ejaculates. Sperm-rich ejaculate fractions were cryopreserved using a standard freezing-thawing procedure for 0.5-mL plastic straws and computer-controlled freezing equipment. Postthaw sperm motility (assessed with a computer-assisted semen analysis system) and viability (simultaneously probed by flow cytometry analysis after triple-fluorescent stain), evaluated 30 and 150 min postthaw, were used to estimate the success of cryopreservation. In the first experiment, 168 unselected ejaculates (1 ejaculate/boar), from boars of 6 breeds with a wide age range (8 to 48 mo), were cryopreserved over a 12-mo period to evaluate the predictive value of boar (breed and age), semen collection, transport variables (season of ejaculate collection, interval between collections, and ejaculate temperature exposure), initial semen traits, and sperm quality before freezing on sperm survival after freezing-thawing. In Exp. 2, 4 ejaculates from each of 29 boars, preselected according to their initial semen traits and sperm quality before freezing, were collected and frozen over a 6-mo period to evaluate the influence of interboar and intraboar ejaculate variability in the survival of sperm after cryopreservation. In Exp. 3, 12 ejaculates preselected as for Exp. 2, from each of 15 boars with known good sperm cryosurvival, were collected and frozen over a 12-mo period to estimate the sustainability of sperm cryosurvival between ejaculates over time. Boar and semen collection and transport variables were not predictive of sperm cryosurvival among ejaculates. Initial semen traits and sperm quality variables observed before freezing explained 23.2 and 10.9%, respectively, of the variation in postthaw sperm motility and viability. However, more that 70% of total variance observed in postthaw sperm quality variables among ejaculates was explained by boar. This indicates that boar is the most important ($P < 0.001$) factor explaining the variability among ejaculates in sperm cryosurvival, with most (14 of the 15 boars in Exp. 3) showing consistent ($P > 0.05$) sperm cryosurvival over time.

Key words: cryopreservation, pig, semen, variability

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INTRODUCTION

Efficient application of frozen-thawed (**FT**) boar semen in commercial AI programs is now feasible due to improvements in cryopreservation protocols and the development of new insemination procedures (reviewed by Roca et al., 2006). The promising fertility results achieved by Eriksson et al. (2002) and Roca et al. (2003) have revived the interest of commercial pig companies in establishing sperm cryobanks. Obviously, these companies require cryobanks that are based on frozen semen with high and consistent postthaw sperm quality. Unfortunately, it is not easy to achieve this goal because ejaculated boar sperm show great variability in their survival of the cooling and thawing processes.

To minimize this variability, an accurate knowledge of the factors influencing sperm cryosurvival is necessary, including boar variability, ejaculate quality, and semen handling conditions. Very few studies have evaluated the factors influencing sperm cryosurvival, apart from the evident difference between males (Larsson and Einarsson, 1976, among others).

Therefore, the objective of the current study was to identify factors responsible for variability among ejacu-

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lates in their ability to survive cryopreservation. Three experiments were carried out to evaluate the predictive value of boar breed and age, semen collection and transport variables, conventional semen measurements of fresh ejaculates and sperm quality before freezing on sperm survival after freezing and thawing, and to estimate the importance of inter- and intraboar variability on the sustainability of sperm quality after cryopreservation.

MATERIALS AND METHODS

Reagents and Media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO) and were made up under sterile conditions in a laminar-flow hood (HH48, Holten LaminAir, Denmark) with purified water (18 MΩ -cm; Elgastat U0HQPS, Elga Ltd., Buckinghamshire, UK).

The basic medium used for sperm extension was Beltsville Thawing Solution (**BTS**, composed of 205 m*M* glucose, 20.39 m*M* NaCl, 5.4 m*M* KCl, 15.01 m*M* $NaHCO_3$, and 3.35 m*M* EDTA, pH 7.2; Pursel and Johnson, 1975), containing kanamycin sulfate (50 μg/mL). The basic medium used for sperm cryopreservation was a lactose-egg yolk (**LEY**) extender composed of 80% (vol/vol) β-lactose solution (310 m*M* in water), 20% (vol/vol) egg yolk, and 100 μg/mL of kanamicyn sulfate.

Animals, and Source, Handling, Evaluation, and Processing of Ejaculates

Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of Murcia University.

Ejaculates from healthy boars housed at 3 commercial insemination stations were used in this study. Boars were housed in individual pens in environmentally controlled buildings. They were given ad libitum access to water and were fed commercial diets according to the nutritional requirements for adult boars.

Sperm-rich ejaculate fractions were collected using the gloved-hand method, extended (1:1, vol/vol) in BTS, and evaluated for conventional semen characteristics. Volume was assessed from a graduated collection test tube. Sperm concentration was evaluated by hemocytometer after extending (1:10 vol/vol) an aliquot of semen with a 0.3% solution of formaldehyde in PBS. The percentage of motile sperm was evaluated from 3 samples of the extended semen (1:1 vol/vol in BTS) placed under a coverslip in the center of a prewarmed (37°C) microscope slide and transferred to a heated microscope stage set at 37°C. Sperm morphology was assessed according to the proportions of sperm with normal morphology by viewing wet mounts of extended semen fixed in buffered 2% glutaraldehyde solution (Pursel and Johnson, 1974) under a phase contrast microscope at a magnification of 400×. Two hundred sperm were counted per preparation.

After evaluation, the extended semen was transferred to 50-mL tubes, cooled to 17°C, and sent by mail, packaged in insulated containers under conditions of monitored temperature (miniature data logger, Gemini Data Loggers, Ltd., Chichester, UK), to the sperm cryopreservation laboratory of the Faculty of Veterinary Medicine, University of Murcia. The extended semen arrived at the laboratory 14 to 15 h after collection of the ejaculate. At the laboratory, the semen was reevaluated (see Sperm Quality Assessment) and centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) for 3 min at $2{,}400 \times g$ (Carvajal et al., 2004). After centrifugation, the supernatant, containing mostly seminal plasma, was removed by aspiration.

Sperm Cryopreservation

Sperm pellets obtained after centrifugation of extended sperm-rich fractions were cryopreserved using the straw freezing procedure described by Westendorf et al. (1975), as modified by Thurston et al. (2001). Briefly, sperm pellets were reextended in LEY (pH 6.2 and 330 ± 5 mOsm/kg) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5°C in 90 min, the sperm were resuspended with LEY-Glycerol-Orvus ES Paste extender [92.5% LEY + 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA) and 6% glycerol, vol/vol; pH 6.2 and 1,650 ± 15 mOsm/kg] to a final concentration of 1×10^9 sperm/mL.

The resuspended and cooled sperm were packed into 0.5-mL, PVC, French straws (Minitüb, Tiefenbach, Germany) and frozen using a controlled-rate freezing instrument (IceCube 1810, Minitüb, Tiefenbach, Germany) as follows: cooled to –5°C at 6°C/min, cooled from –5°C to –80°C at 40°C/min, held for 30 s at –80°C, then cooled at 70°C/min to –150°C, and finally plunged into liquid N. The straws remained in the liquid N tank for at least 2 wk before thawing. Thawing of straws was done in circulating water at 37°C for 20 s. Thawed sperm from 2 straws per ejaculate were resuspended in BTS (1:2, vol/vol; 37°C) and incubated in a waterbath at 37°C for 150 min.

Sperm Quality Assessment

Each ejaculate was assessed for sperm motility and viability before freezing (immediately after arrival at the laboratory) and after thawing (at 30 and 150 min postthaw).

Sperm motility (proportion of total motile sperm) was objectively evaluated using a computer-aided sperm analysis system (Sperm Class Analyzer, SCA, Microptic, Barcelona, Spain). The extended and incubated FT sperm were reextended in BTS to a concentration of 20×10^6 sperm/mL. For each evaluation, a 4-μL sperm sample was placed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were analyzed at 39°C, assessing a minimum of 100 sperm/sample.

Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity. These characteristics were analyzed simultaneously using a modification of a triple-fluorescent procedure, described by Graham et al. (1990) and adapted for boar sperm by Carvajal et al. (2004), which includes the DNA-specific fluorochrome propidium iodide, the mitochondria-specific fluorochrome rhodamine-123 (R123), and the acrosome-specific fluorochrome fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin. Three hundred sperm were counted under 1,000× magnification (Eclipse E800, Nikon, Tokyo, Japan), using a BV-2A filter (excitation filter 400–440 nm, barrier filter 470 nm, dichroic 455 nm combination). Sperm showing only green fluorescence over their midpiece (R123-positive) were considered viable with an intact acrosome (viable sperm), and the values were expressed as a percentage of viable sperm.

Experimental Design

Experiment 1: Predictive Value of Boar, Semen Collection, and Transport Variables, and Initial Semen Traits, and Sperm Quality before Freezing on Sperm Survival after Cryopreservation. Ejaculates (n = 168) were collected over a 12-mo period from 168 boars (1 ejaculate per boar). The boars varied in breed and age (8 to 48 mo) and ejaculate collection frequency (interval between ejaculations), and initial semen traits also differed. All ejaculates collected were sent to the laboratory and cryopreserved, regardless of the initial semen traits recorded, or sperm quality assessed, before freezing.

Experiment 2: Inter- and Intraboar Variability of Sperm Survival after Cryopreservation. Four ejaculates were cryopreserved from each of 29 boars over a 6-mo period (1 ejaculate/boar every 6 to 10 wk). All boars were 1 to 3 yr of age, known to be fertile, and were undergoing regular semen collection (1 or 2 times per wk) for commercial artificial insemination. The ejaculates to be cryopreserved were preselected according to their initial semen traits and sperm quality before freezing. Therefore, only ejaculates with ≥ 200 × 10^6 sperm/mL, ≥ 85% sperm with normal morphology, and ≥ 75% and ≥ 80% of motile and viable sperm, respectively, before freezing, were retained for cryopreservation.

Experiment 3: Sustainability of Sperm Survival after Cryopreservation between Ejaculates over Time. Twelve ejaculates were cryopreserved from each of 15 boars over a 12-mo period (1 ejaculate·boar^{-1}·mo^{-1}). All boars were 2 to 3 yr of age, known to be fertile, and undergoing regular semen collection for AI. The boars were selected, on the basis of their consistent good sperm cryosurvival, from those used for Exp. 2. As for Exp. 2, the ejaculates to be cryopreserved were preselected according to their initial semen traits and sperm quality before freezing. Therefore, only ejaculates with ≥ 200 × 10^6 sperm/mL, ≥ 85% sperm with normal morphology, and ≥ 75% and ≥ 80% of motile and viable sperm, respectively, before freezing, were retained for cryopreservation.

Statistical Analysis

For data analysis, SPSS (version 13.0, SPSS Inc., Chicago, IL) and PATN (CSIRO, Canberra, Australia) software packages were used. Descriptive analyses of boar, semen collection, and transport variables, and initial semen traits and sperm quality before freezing and after thawing were made in Exp. 1. In Exp. 1 and 2, multiple regression analyses were performed using boar, semen collection, and transport variables, and initial semen traits and sperm quality before freezing to predict postthaw sperm quality variables. For these analyses, breed (in Exp. 1) and age (in Exp. 2) of individual boars, ejaculate collection frequency, temperature of semen exposure, initial semen traits (ejaculate volume, sperm concentration, total sperm output, sperm morphology, and total sperm motility), and sperm quality before freezing (sperm motility and viability) were treated as the independent variables. Breed of boar and individual boars were included in the models as dummy variables. The percentages of total sperm motility and viability at 30 and 150 min after thawing, analyzed at first separately and then combined for the 2 times, were treated as the dependent variables.

In Exp. 1, analysis was performed using the PATN pattern analysis package to identify naturally occurring subgroups within the ejaculate data set (Abaigar et al., 1999). The PATN analysis classifies the ejaculates, using postthaw sperm variables, into a small number of groups. Three groups were finally obtained from the nonhierarchical classification of the 168 ejaculates. The data for boar and semen collection and transport variables, and initial semen traits and sperm quality before freezing and after thawing of the 3 groups of ejaculates were calculated and compared using 1-way ANOVA.

In Exp. 2 and 3, 2 separate ANOVA were carried out to investigate interboar (among boars) and intraboar (among ejaculates within the same boar) variability on postthaw sperm quality. Initially, the fixed effects of boar and ejaculate within boar were included in the statistical model. When the latter was significant, a second ANOVA was carried out to assess the effect of ejaculate within boar (intraboar variability) for each of boars tested. In Exp. 3, intraboar variability was also estimated by calculating the CV. When means for the 2 postthaw evaluation times (30 and 150 min) did not differ, they were averaged across the 2 times. Statistical significance was defined as $P < 0.05$.

Table 1. Distribution of ejaculates among breeds of boars, and initial semen traits and sperm quality variables observed before freezing and after thawing (Exp. 1)[1]

Item	Mean ± SEM	Range
Initial semen trait[2]		
Volume, mL	116.15 ± 2.41	46 to 185
Sperm concentration, millions/mL	371.76 ± 8.87	147 to 680
Total sperm output, billions	43.26 ± 1.32	10 to 93
Sperm morphology, normal morphology, %	90.72 ± 0.72	54 to 100
Total sperm motility, %	82.13 ± 1.17	65 to 95
Sperm quality before freezing, 17°C		
Total sperm motility	76.31 ± 0.88	42 to 96
Sperm viability	83.94 ± 0.58	49 to 96
Sperm quality after thawing, 37°C		
Total sperm motility at 30 min	46.85 ± 1.14	11 to 77
Sperm viability at 30 min	51.86 ± 1.04	11 to 78
Total sperm motility at 150 min	37.88 ± 1.09	6 to 68
Sperm viability at 150 min	44.41 ± 1.06	9 to 69
Mean sperm motility[3]	42.37 ± 1.07	9 to 70
Mean sperm viability[3]	48.14 ± 1.02	10 to 71

[1]Breeds of boars represented included Landrace (n = 20; 11.9%), Large White (n = 42; 24.7%), Duroc (n = 20 (11.9%), Pietrain (n = 35; 20.6%), Yorkshire (n = 10; 5.9%), and Crossbred (n = 43; 25.3%). n = 168 ejaculates.

[2]Sperm-rich fraction.

[3]Mean values of 30 and 150 min assessment.

RESULTS

Experiment 1: Predictive Value of Boar, Semen Collection, and Transport Variables, and Initial Semen Traits, and Sperm Quality before Freezing on Sperm Survival after Cryopreservation

Age of the boars ranged from 8 to 48 mo (mean ± SEM: 19.03 ± 0.64), and frequencies of ejaculate collection ranged from 4 to 15 d (7.86 ± 0.14). Ejaculates (n = 168; 1 per boar) were collected during 1 yr with the following distribution among the 4 seasons: 31, 49, 22, and 66 for winter, spring, summer, and autumn, respectively. The temperature of diluted semen during transport from the AI center to the cryobiology laboratory ranged from 12 to 21°C (mean ± SEM: 16.69 ± 0.14). The distribution of ejaculates among breeds, the initial semen traits of ejaculates, the sperm quality variables assessed before freezing, and those assessed after thawing are summarized in Table 1.

To determine the predictive value of boar and semen collection and transport variables, initial semen traits and sperm quality before freezing on sperm quality assessed at 30 and 150 min postthawing, multiple regression models were generated for all possible combinations. The statistical models generated were similar for the 2 postthaw times. For simplicity of presentation, only statistical models generated from the combination of the 2 times were presented in detail. When all independent variables were included in the model, only 25 and 16.5% (adjusted R^2), respectively, of the variation in postthaw sperm motility and viability was accounted for. Three models derived by forward stepwise regression showed statistical results. There was a weak predictive value for both postthaw sperm variables. The best predictive value (23.2%) for postthaw sperm motility was achieved with a model including motility before freezing, morphology and concentration. For postthaw sperm viability, only one variable (sperm morphology) was significant and had a very low predictive value (10.9%).

After the PATN analysis of FT sperm quality variables, 3 groups of ejaculates (boars) were clearly identified. The ejaculates with best postthaw sperm quality were classified as good, whereas those that showed moderate and reduced sperm cryosurvival were classified as moderate or poor, respectively. A summary of the data for these 3 groups of ejaculates is shown in Table 2. Most ejaculates (66.7%) were classified as good. Differences between groups ($P < 0.05$ to 0.001) were achieved for 2 initial semen traits (sperm concentration and proportion of morphologically normal sperm) and the 2 sperm quality variables (motility and viability) assessed before freezing. Ejaculates classified as good showed the greatest percentages of normal morphology and sperm motility before freezing. Likewise, they showed a high sperm concentration and viability before freezing.

Experiment 2: Inter- and Intraboar Variability of Sperm Survival after Cryopreservation

Multiple regression analyses revealed one significant ($P < 0.01$) model, which included individual boars as the only variable. This model had a high predictive value for postthaw sperm quality, with postthaw sperm motility and viability accounting for 70.8 and 80.4% of total variance, respectively.

Variability among boars (interboar variability) was significant ($P < 0.001$) for postthaw sperm motility and viability. Variation between ejaculates within each boar (intraboar variability) was also significant ($P < 0.001$). However, interboar variability (F-values = 71.85 and 102.21, df = 28) was considerably greater than intraboar variability (F-values = 4.74 and 2.52, df = 87, for motility and viability, respectively). Moreover, intraboar variability was only significant ($P < 0.05$) in 4 of the 29 boars. Fifteen of the 25 boars with no statistical intraboar variability had consistently high values for motility and viability of sperm after thawing (over to 50% in all ejaculates at 30 min after thawing). Another 4 boars had very low postthaw sperm quality (below 35% in all ejaculates at 30 min after thawing).

Experiment 3: Sustainability of Sperm Survival after Cryopreservation between Ejaculates over Time

Figure 1 summarizes the data for the 15 boars in a series of box-whisker plots. Although high overall postthaw sperm motility and viability was obtained for all boars, interboar (boar differences) and intraboar

Table 2. Differences on boar, semen collection and handling conditions, and semen traits before cryopreservation among the 3 groups of ejaculates classified according their freezability (Exp. 1)[1]

Item	Sperm freezability group[2]			
	Good	Moderate	Poor	*P*-value
Number of boars, %	112 (66.7)	49 (29.2)	7 (4.2)	
Boar age, mo	18.47 ± 0.73 (10.1 to 34.3)	19.83 ± 1.39 (9 to 37)	23.13 ± 2.71 (10.9 to 32)	0.270
Ejaculate collection frequency, d	7.78 ± 0.17 (6 to 12)	7.88 ± 0.25 (5.5 to 15)	9.14 ± 0.71 (7 to 12)	0.146
Semen transport temperature, °C	16.74 ± 0.16 (13 to 19.5)	16.68 ± 0.31 (11.6 to 19.5)	15.93 ± 0.76 (11.5 to 17)	0.528
Initial semen trait				
Volume, mL	114.94 ± 2.82 (62.9 to 170.7)	118.10 ± 5.08 (48 to 167.5)	121.14 ± 13.11 (76 to 168)	0.772
Sperm concentration, millions/mL	374.28 ± 11.06[a] (203 to 640)	378.71 ± 16.11[a] (192.5 to 600)	279.99 ± 28.78[b] (147.5 to 375)	0.047
Total sperm output, billions	42.93 ± 1.50 (17.2 to 70.8)	45.17 ± 2.84 (14.2 to 84.4)	35.12 ± 5.64 (11.2 to 52.1)	0.331
Normal sperm morphology, %	93.10 ± 0.66[a] (79 to 100)	86.00 ± 1.63[b] (60.5 to 99)	84.57 ± 4.82[b] (68 to 100)	0.001
Total sperm motility, %	83.33 ± 0.95 (75 to 95)	81.66 ± 1.77 (70 to 90)	79.66 ± 4.48 (70 to 90)	0.156
Sperm quality before freezing, %				
Total sperm motility, %	78.62 ± 0.92[a] (59.1 to 90.79)	72.66 ± 1.82[b] (50.9 to 91.6)	64.79 ± 5.18[b] (44.5 to 79.5)	0.001
Sperm viability, %	85.11 ± 0.61[a] (73.3 to 93.4)	82.24 ± 1.19[ab] (69 to 93.5)	77.14 ± 4.47[b] (60 to 95)	0.004
Sperm quality postthawing, %				
Total sperm motility at 30 min	54.86 ± 0.92[a] (40.9 to 71.7)	32.81 ± 1.09[b] (20.8 to 44.8)	17.01 ± 2.36[c] (11.4 to 26.1)	0.001
Sperm viability at 30 min	59.37 ± 0.62[a] (48.3 to 70.4)	39.80 ± 1.18[b] (25.5 to 54)	18.29 ± 1.95[c] (11 to 25)	0.001
Total sperm motility at 150 min	45.27 ± 0.98[a] (27.5 to 63.5)	25.02 ± 0.83[b] (15.2 to 35.4)	9.81 ± 1.12[c] (5.1 to 12.8)	0.001
Sperm viability at 150 min	51.89 ± 0.76[a] (37 to 65)	31.90 ± 1.13[b] (20 to 46.5)	12.29 ± 1.77[c] (6 to 17)	0.001

[a–c]Different letters within rows denote significant differences ($P < 0.05$).
[1]Values are means (± SEM), with ranges in parentheses.
[2]The ejaculates with best, moderate, and reduced sperm cryosurvival were classified as good, moderate, or poor, respectively, using PATN analysis (Abaigar et al., 1999).

(among ejaculate of a same boar) variability was found for both postthaw sperm characteristics evaluated ($P < 0.001$). However, intraboar variability was less (F-values = 3.77 and 2.08, df = 165) than interboar variability (F-values = 17.48 and 5.50, df = 14, for motility and viability, respectively), with only 1 boar (the number 14 in Figure 1) having ejaculate differences in postthaw sperm motility ($P = 0.001$) and viability ($P = 0.003$). Therefore, in 14 of the 15 boars tested, a consistently high postthaw sperm motility and viability was achieved in the 12 ejaculates cryopreserved during the year. Indeed, the low coefficients of variation (below 15%) observed in 13 of the 15 boars in both postthaw sperm characteristics confirmed the consistent sperm cryosurvival achieved.

DISCUSSION

Optimum sperm cryopreservation is of primary importance if improvements are to be made in the efficiency of utilization of frozen-thawed semen for pig AI, particularly for commercial use. Obviously, there is a need to accurately determine which factors influence sperm survival after cryopreservation of ejaculated boar sperm. The results from Exp. 1, designed to evaluate the influence of boar, semen collection and transport variables, and conventional seminal measurements, demonstrated that semen collection and transport variables were not useful predictors of sperm quality postthaw. Especially noticeable was the lack of predictive value for boar breed, previously reported as important (Almlid and Hofmo, 1996; Thurston et al., 2001; Park and Yi, 2002). Although a significant influence of breed on postthaw sperm quality was observed in the current study, with ejaculates collected from Landrace and Pietrain boars having the greatest percentage of postthaw sperm motility and viability (results not shown), the variance in both postthaw sperm quality measurements was not explained by breed or any other boar and management variables. This suggests that the best mean

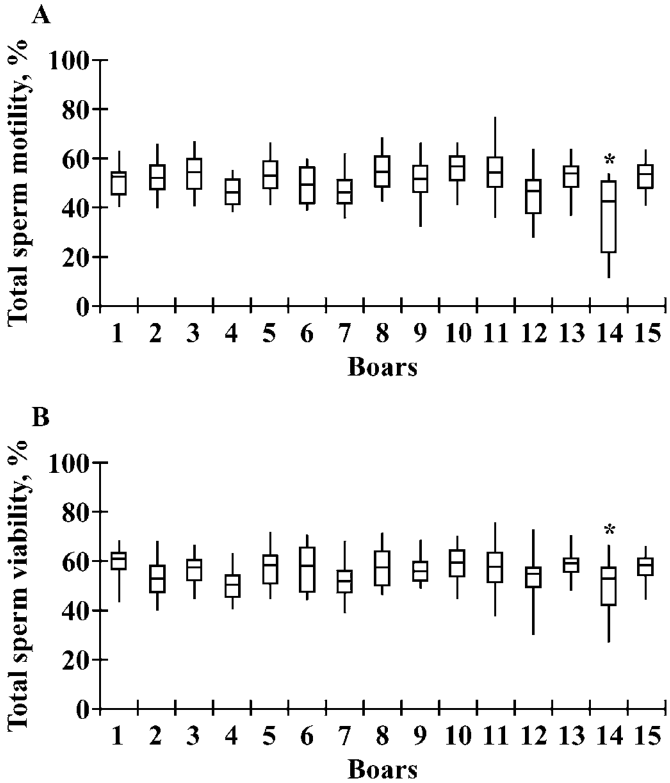

Figure 1. Box-whisker plots showing variation in total sperm motility (A) and viability (B) postthawing of cryopreserved semen of 15 boars (12 ejaculates per boar; Exp. 3). Boxes enclose the 25th and 75th percentiles, the line is the median, and whiskers extend to the 5th and 95th percentiles. Data are means of 2 postthaw evaluation times (30 and 150 min). *Boars with differences ($P < 0.05$) between ejaculates (intraboar variability).

postthaw sperm quality values could be expected from certain breeds, but there is likely to be considerable variability among ejaculates within those breeds. The same circumstance could occur with the other factors, such as age of the boar. For example, significant differences in postthaw sperm motility have been reported between ejaculates collected from boars grouped in different age ranges (Joyal et al., 1986). However, according to the multiple regression analysis in Exp. 1, age of the boar does not explain the variance observed in postthaw sperm quality.

Traditionally, great importance has been given to conventional measurements of semen quality for the selection of ejaculates suitable for cryopreservation. Commonly, only ejaculates exceeding certain quality limits are selected. However, to the best of our knowledge, there have been no specific studies correlating conventional semen traits or sperm quality assessments before freezing with sperm quality postthaw. In Exp. 1, multiple linear regression analysis indicated that sperm concentration together with sperm morphology and sperm motility before freezing were the only variables that should be considered for the selection of boar ejaculates for cryopreservation. However, only 23.2 and 10.9% of total variance in postthaw sperm motility and viability, respectively, could be explained by these variables. This weak predictive value of conventional semen measurements and sperm quality assessments before freezing was not surprising because we and others have observed previously, using individual ejaculates from the same boars, that sperm quality and the in vitro fertilizing ability of cooled semen are not statistically related to the values obtained postthaw (Rath and Niemann, 1997; Roca et al., 2000).

Despite having a weak predictive value, a positive correlation was found between ejaculate measurements and postthaw sperm quality. This suggested that those ejaculates with increased sperm concentration, percentage of normal morphology and motility before freezing should have the best sperm recovery after cryopreservation. In an attempt to establish possible limiting values for sperm measurements and determine which ejaculates should be selected for cryopreservation, a PATN analysis was performed to classify the ejaculates into groups according to postthaw sperm quality (Thurston et al., 2001; Gil et al., 2005). Focusing on the ejaculates classified as good, the PATN analysis confirmed the results of the multiple regression analysis, in that the good ejaculates were those that showed the greatest mean values for sperm concentration, normal morphology, and motility before freezing. However, with a careful evaluation of the data, it was clear that ejaculates showing good sperm cryosurvival had a wide range of sperm concentration, normal morphology, and motility before freezing. Moreover, a similar range was observed also in the ejaculates classified as moderate and poor according to sperm cryosurvival. Therefore, increased sperm concentration, together with the greatest proportions of sperm with normal morphology and motility before freezing, did not necessarily guarantee good sperm survival after freezing and thawing. Moreover, ejaculates with low sperm concentration and moderate sperm quality immediately after collection or before freezing could have good sperm quality postthaw. Overall, these data demonstrated that conventional semen measurements and sperm quality assessments before freezing were quantitative rather than qualitative with regard to their relationship with sperm survival after cryopreservation. Thus, they could be used to reduce the variability among ejaculates in postthaw sperm quality, but they did not provide an accurate prediction of the survival of sperm in an ejaculate after cryopreservation.

In Exp. 2, we attempted to reduce the variability in sperm survival postthaw attributed to ejaculate and sperm variables by preselection of ejaculates before cryopreservation. Hence, only ejaculates with more than 200×10^6 sperm per mL and with percentages of normal morphology, sperm motility, and viability before freezing above 85, 75, and 80%, respectively, were cryopreserved (4 ejaculates from each of 29 boars). Under these conditions, the ejaculate and sperm variables did not have any predictive value for sperm cryosurvival. Moreover, despite the rigorous ejaculate selection criteria, considerable variability of sperm cryosurvival among ejaculates was still observed, especially among boars. The interboar variability in postthaw sperm quality found in the current study was not surprising because it has been extensively demonstrated by others (Larsson and Einarsson, 1976; Thurston et al., 2001, 2002; Medrano et al., 2002; Saravia et al., 2005). Moreover, individual male differences in sperm cryosurvival are not exclusive to pigs because they have also been observed in horses (Janett et al., 2003) and sheep (D'Alessandro and Martemucci, 2003). However, this is the first study, evaluating several factors, that has demonstrated the importance of boar in explaining variability between ejaculates in sperm cryosurvival. More that 70% of total variance among ejaculates in postthaw sperm quality was explained by boar. This suggests that boar is the primary factor influencing ejaculate variability in sperm cryosurvival and that it should be the most important criterion for selecting ejaculates for cryopreservation.

The reason for boar variability in cryosurvival of sperm is unknown at present, although it may have a genetic origin. Differences in specific DNA sequences have been identified between boars in which postthaw sperm quality was classified as poor or good (Thurston et al., 2002). However, such individual variations may be minimized when epididymal sperm are cryopreserved. For example, in a limited study involving 3 boars from which epididymal and ejaculated sperm were cryopreserved, Rath and Niemann (1997) observed that boar differences in postthaw motility were only significant for ejaculated sperm, with epididymal sperm having a consistently high postthaw motility. Although no clear explanation was given by these researchers because it was not the aim of their study, 2 of the 3 boars had differences in postthaw survival between epididymal and ejaculated sperm. This could be related to variation in exposure of sperm to the complex secretions of the accessory sex glands during ejaculation, which could modify the cryosurvival of the sperm. In this case, differences in seminal plasma composition between good and poor boars could explain the variation in sperm cryosurvival. This hypothesis remains to be investigated.

As boar was found to be the primary factor explaining variability in sperm cryosurvival between ejaculates, it seemed logical to select boars with good postthaw sperm survival as cryobank founders. However, there was no evidence that boars could maintain good sperm cryosurvival in ejaculates over time. In Exp. 2, 25 of 29 boars were found to maintain consistent postthaw sperm quality from 4 ejaculates collected and cryopreserved at intervals over a period of 6 mo. This finding was confirmed in Exp. 3, where postthaw sperm quality was consistently high in all ejaculates cryopreserved from 14 of the 15 boars, indicating that boars with semen classified as having good freezability could maintain this condition over time. This suggests that the assessment of a single ejaculate after cryopreservation may be sufficient to identify those with good freezability scores for selection as cryobank founders. Further investigation is required to confirm this finding.

Intraboar variability in sperm cryosurvival was also observed in the current study, although it was less important than interboar variation. However, from a practical and commercial viewpoint, intraboar variability should be taken into account, as it was observed in 4 of the 29 boars in Exp. 2 and in 1 of the 15 boars in Exp. 3. The reasons provided by various authors for this variation are not consistent, but it may be related to poor sustainability of the cryopreservation process (Thurston et al., 2001). This was not the case in the current study because the ejaculates from all boars, with or without significant intraboar variability, were processed at the same time. Transitory changes in boar health and inappropriate ejaculate manipulation before freezing have been also suggested as possible causes of this variation (Roca et al., 2006). Therefore, good knowledge of boar health and careful manipulation of ejaculates before freezing are of practical importance to minimize intraboar variability in sperm cryosurvival between ejaculates.

In conclusion, the results of the current study demonstrate that the boar is the most important factor explaining the variability in sperm cryosurvival among ejaculates. Although a certain degree of intraboar variability was observed, most boars showed consistent sperm cryosurvival over time. Standard semen measurements in fresh ejaculates and sperm quality assessments before freezing were shown to have limited value in explaining variation in postthaw sperm quality between ejaculates. Nevertheless, those ejaculates containing a high sperm concentration and a high proportion of motile sperm with normal morphology before freezing are the best to select for cryopreservation.

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