Cryobiology 80 (2018) 119–125

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Post-thaw boar sperm motility is affected by prolonged storage of sperm in liquid nitrogen. A retrospective study

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ARTICLE INFO

Keywords:
Porcine
Sperm
Cryopreservation
Liquid nitrogen storage

ABSTRACT

Owing to the quick genetic turnover of the pig industry, most AI-boar sires live 2-3 yr, a period during which for 1-2 yr their semen is extended and used in liquid form for AI. Despite showing low cryosurvival, affecting fertility after AI, boar semen is frozen for easiness of transport overseas and reposition of valuable genetics. For the latter, semen is stored in liquid nitrogen (LN_2, cryostorage) for many years, a controversial practice. Here we studied how length of cryostorage could affect sperm quality. Straws (0.5 mL) frozen using the same cryopreservation protocol at one specific location from AI- sires of proven fertility were stored in LN_2 for up to 8 yr. Post-thaw sperm quality was evaluated after 2, 4 or 8 yr of cryostorage, always compared to early thawing (15 d after freezing). Sperm motility and kinematics were evaluated post-thaw using CASA and sperm viability was cytometrically evaluated using specific fluorophores. Sperm viability was not affected by length of cryostorage, but total and progressive sperm motility were lower ($p < 0.01$) in sperm samples cryostored for 4 or 8 yr compared to those thawed 15 d after freezing. Cryostorage time affected sperm kinetics, but with greater intensity in the samples cryostored for 4 yr ($p < 0.001$) than in those for 2 yr ($p < 0.01$). The fact that the major phenotypic characteristic of boar spermatozoa, motility, is constrained by time of cryostorage should be considered when building cryobanks of pig semen. Attention should be placed on the finding that > 2 yr of cryostorage time can be particularly detrimental for the post-thaw motility of some sires, which might require increasing sperm numbers for AI.

1. Introduction

Freezing is the currently most efficient method for long-term preservation of mammalian spermatozoa for future use in artificial reproductive technologies [1]. Consequently, it is widely used in both humans and farm animals [2–4]. Once frozen, the spermatozoa are usually stored immersed in liquid nitrogen (LN_2). Despite early reassurances that sperm quality would not be affected [5,6], concerns have been lately arisen over whether storage in LN_2 (cryostorage) is totally harmless to quality and functionality of frozen-thawed (FT) spermatozoa [1,7].

Sperm cryosensitivity differs among species [1] and porcine spermatozoa are particularly sensitive to freezing because their plasma membrane has a high proportion of polyunsaturated fatty acids but low cholesterol [8]. Accordingly, FT-boar sperm shown variable results in both functionality and fertility after thawing, leading them to be scarcely used in swine artificial insemination (AI) programs [9,10]. This reality contrasts with the extensive worldwide use of liquid semen [11]. Despite, building cryobanks with semen from valuable boars and genetic lines for future use in AI-programs is usual practice in swine industry [10]. The putative modifications in quality or/and functionality experienced by frozen boar spermatozoa during cryostorage have received little attention. Only two studies have addressed this issue so far, whose results and conclusions might not be useful for swine Al because they used 6 mL straws [12] or 10 mL cryotubes [13] for sperm cryopackaging, systems that are far from the one package most currently used: 0.5 mL plastic straws [14]. In this context, it is well known that packaging is critical for sperm cryotolerance [15].

The present retrospective study aimed to evaluate whether cryostorage time, up to 8 yr, could affect post-thawing attributes of boar sperm. For this purpose, cryostored sperm samples from boars with good sperm freezability, packaged in 0.5 mL straws and frozen and thawed using the same cryopreservation protocol at one specific location were used. Special attention was focused on kinetics, as they are

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https://doi.org/10.1016/j.cryobiol.2017.11.004
Received 26 September 2017; Received in revised form 6 November 2017; Accepted 10 November 2017
Available online 13 November 2017

sperm attributes especially sensitive to cryopreservation [16] and related with fertility post-AI [17,18].

2. Material and methods

2.1. Reagents

Unless otherwise stated, all the chemicals and fluorochromes used in the experiments were of analytical grade, purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Media were prepared under sterile conditions in a laminar flow hood (MicroH; Telstar, Terrasa, Spain) using purified water (MilliQ Advantage A10, Merck, Darmstadt, Germany).

2.2. Boars, ejaculates and cryopreservation procedure

Over a period of eight years, the sperm-rich fraction of the ejaculate was collected using the gloved-hand method from healthy, mature (2-3 years old) and fertile boars of differed breeds (Landrace, Large White and Pietrain) housed in several Spanish AI-centres and regularly used as semen donors for commercial AI-programs. Immediately after collection, the ejaculates were extended (1:1, v/v) in Beltsville Thawing Solution (BTS: 205 mM glucose, 20.39 mM Na_3-$C_6H_5O_7$, 10.0 mM KCl, 15.01 mM $NaHCO_3$, 3.36 mM EDTA, supplemented with 50 mg/mL kanamycin sulphate; pH 7.2 and 290–300 mOsmol/kg) and cooled to 17 °C. Thereafter, they were transported overnight into insulated thermal boxes (17 °C) to the laboratory of Andrology of the Clinical Veterinary Teaching Hospital of the University of Murcia, where they were frozen at the morning. Only ejaculates with more than 200 × 10^6 sperm/mL, 75% of sperm motility and 85% sperm with normal morphology were used.

The ejaculates were frozen by the same working team following the standard 0.5 mL-straw protocol routinely used since 2004 [19]. Briefly, the liquid-stored semen samples were centrifuged (2400 × g for 3 min) and the recovered sperm pellets were re-extended to 1.5 × 10^9 sperm/mL in a lactose-egg yolk (LEY) extender (80% [v/v] β-lactose [310 mM in water] and 20% [v/v] egg yolk, supplemented with kanamycin sulphate [100 μg/mL], pH 7.2 and 295–300 mOsmol/kg). The extended samples were slowly cooled to 5 °C (during 120 min) and then re-extended to 1 × 10^9 sperm/mL in LEY (92.5%, v/v) plus Equex STM (1.5%, v/v; Nova Chemical Sales Inc. Scituate, MA, USA) and glycerol (6%, v/v) (pH 6.2 and 1650 ± 15 mOsmol/kg). Immediately after, the samples were packed into 0.5 mL PVC French straws (Minitüb, Tiefenbach, Germany) and frozen in a controlled-rate freezing machine (IceCube 1810, Minitüb) using the following freezing curve: to -5 °C at a rate of 6 °C/min, from -5 °C to -80 °C at 40 °C/min rate and to -150 °C at 70 °C/min speed. The straws were plunged into LN_2 and thereafter stored in large capacity LN_2 tank (GT40, Air Liquide, Paris, France) until thawing. The LN_2 tank remained in a cold room (5 °C) and it was always operating, with regular input and output of straws. The level of LN_2 within the tank continuously monitored, to ensure the straws remained always 20 cm below the surface of LN_2.

2.3. Cryostored sperm samples

A total of 58 cryostored sperm samples from 58 founding boars of a sperm cryobank were evaluated (one sample per boar). The cryostored sperm samples were grouped in three time-intervals of cryostorage: 2 yr (short), 4 yr (medium) and 8 yr (long), using a cryostorage time of 15 d as baseline control. Straws were thawed by the same working team using a standard procedure consisting in a vigorous agitation of the straws during 20 s inside of a circulating water bath at 37 °C. Two randomly chosen straws per cryostored sperm sample and storage time were thawed as technical replicate. Immediately after, the content of each straw was extended 1:1 (v/v) in BTS and stored in a dark chamber at 37 °C until the completion of *in vitro* analyses of quality and sperm functionality.

2.4. Post-thaw sperm assessment

Sperm quality was assessed at 30 and 150 min post-thawing in terms of motility and sperm viability. Sperm motility was evaluated objectively using a computer-assisted sperm analysis system (ISAS; Proiser RtD, Paterna, Spain). The assessment procedure was that described by Cremades et al. [16]. For each evaluation, 5 μL aliquots of thawed sperm samples (at 20-30 × 10^6 sperm/mL in BTS) were placed in a pre-warmed (38 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and 3-4 fields, with a minimum of 300 sperm per sample, were analysed. The trajectory of each identified and recorded spermatozoa was visually assessed to eliminate possible debris and to minimize the risk of including unclear tracks in the analysis. Sperm motility was recorded as the percentage of total motile spermatozoa (average path velocity ≥20 μm/s) and the percentage of sperm showing rapid and progressive movement (straightness of the average path ≥40%). Kinetic parameters were also recorded in FT-sperm samples cryostored during short- and medium time. Specifically, the curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of sperm movement (LIN, %), straightness of the average path (STR, %), wobble coefficient (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

Sperm viability was evaluated in terms of plasma- and acrosome membrane integrity using a triple-fluorescence procedure with later measurement by flow cytometry (BD FACSCanto II cytometer; Becton Dickinson Co, Franklin Lakes, NJ, USA). One hundred μl of each sperm sample (30 × 10^6 cells/mL) was extended (1:9, v/v) in EDTA-free phosphate-buffered saline (PBS; 139 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}7H_2O$; with 0.058 g/L penicillin G and 0.05 g/L streptomycin sulphate; pH 6.8 and 280–300 mOsmol/kg). Then, the sperm samples were stained with 3 μL Hoechst 33342 (H-42; 0.05 mg/mL in PBS), 2 μL propidium iodide (PI; 0.5 mg/mL in PBS) and 2 μL fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; 200 μg/mL in PBS). Thereafter, they were incubated at 38 °C in dark for 10 min, extended again in PBS (400 μl) and then cytometrically analysed. The viable sperm population (PI negative) showing non-reacted (PNA-FITC negative) or reacted (PNA-FITC positive) acrosome were recorded.

2.5. Statistical analysis

The IBM SPSS statistics package (version 19, IBM SPSS, Madrid, Spain) was used for statistical analyses. The Shapiro-Wilk test proved that the data population of each one of the sperm parameters evaluated were non-normally distributed. Therefore, the non-parametric Wilcoxon signed-rank and Kruskal-Wallis one-way ANOVA tests were used to evaluate the influence of cryostorage time (15 d, as control, vs 2; 4 or 8 yr later) and the rate of decrease among the different cryostorage times, respectively, at each of the two post-thawing evaluation times (30 and 150 min). $P < 0.05$ was considered statistically significant.

3. Results

Short cryostorage time (15 d, control, vs 2 yr) did not influence the percentages of total and progressive motile sperm recovered at 30 and 150 min post-thawing. However, the medium- (15 d vs 4 yr) and long (15 d vs 8 yr) cryostorage times caused a similar and significant ($p < 0.01$) decrease in both variables. The decrease was similar in sperm samples evaluated at both post-thaw incubation times, and it ranged between 5 and 10% for total motility and between 3 and 9% for progressive motility (Figs. 1 and 2).

The percentage of viable spermatozoa showing unreacted