Cryobiology 69 (2014) 203–210

Contents lists available at ScienceDirect

Cryobiology

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

Boar sperm cryosurvival is better after exposure to seminal plasma from selected fractions than to those from entire ejaculate ☆

Diego V. Alkmin [a], Cristina Perez-Patiño [a], Isabel Barranco [a], Inmaculada Parrilla [a], Juan M. Vazquez [a], Emilio A. Martinez [a], Heriberto Rodriguez-Martinez [b], Jordi Roca [a,*]

[a] Department of Medicine and Animal Surgery, University of Murcia, Murcia, Spain
[b] Department of Clinical & Experimental Medicine (IKE), University of Linköping, Linköping SE-581 85, Sweden

ARTICLE INFO

Article history:
Received 4 June 2014
Accepted 9 July 2014
Available online 15 July 2014

Keywords:
Ejaculate
Epididymis
Sperm
Seminal plasma
Porcine

ABSTRACT

Boar bulk ejaculates are now being collected instead of usual sperm-rich fractions (SRF) for artificial insemination purpose. The present study evaluated the influence of holding boar sperm samples before freezing surrounded in their own seminal plasma (SP), from either fractions/portions or the entire ejaculate, on post-thawing sperm quality and functionality. Ejaculates collected as bulk (BE) or as separate (first 10 mL of SRF [P1] and rest of SRF [P2]) from 10 boars were held 24 h at 15–17 °C and then frozen. Some bulk ejaculate samples were frozen immediately after collections as Control. In addition, epididymal sperm samples from the same 10 boars were collected post-mortem and extended in SP from P1 (EP1), P2 (EP2) and post SRF (EP3), and also held 24 h before freezing for a better understanding of the influence of SP on boar sperm cryopreservation. The sperm quality (motility, evaluated by CASA, and viability, evaluated by flow cytometry) and functionality (flow cytometry assessment of plasma membrane fluidity, mitochondrial membrane potential and intracellular generation of reactive oxygen species [ROS] in viable sperm) were evaluated at 30, 150 and 300 min post-thaw. Post-thawing sperm quality and functionality of P1 and P2 were similar but higher ($p < 0.01$) than BE samples. Control samples showed higher ($p < 0.01$) post-thaw sperm quality and functionality than BE samples. Post-thawing sperm quality and functionality of EP1 and EP2 were similar but higher ($p < 0.05$) than EP3. These results showed that boar sperm from BE are more cryosensitive than those from the SRF, particularly when held 24 h before freezing, which would be attributable to the cryonegative effects exerted by the SP from post SRF.

Introduction

Frozen-thawed (FT) boar sperm, other than the routinely used liquid-stored (LS), should be used in artificial insemination (AI) programs, given their additional benefits regarding biosecurity, international exchange and genetic improvement [29]. Despite these primary compelling benefits, FT-sperm is not yet widely used owing to its still notorious low cryosurvival and the short lifespan depicted by the sperm cryosurviving [26]. Therefore, research is still pursuing alternative ways to increase sperm cryosurvival.

Customary removal of the seminal plasma (SP) before freezing, in order to concentrate sperm for further extension in cooling and freezing extenders, has motivated alternative studies aiming to elucidate the role of native SP on boar sperm freezability. Despite many studies has attempted to tackle its role over the recent years, the topic is still pending, since results had been inconclusive [8]. It is, however, widely accepted that holding sperm suspended in their own native SP prior to cooling improves sperm resistance to cold shock [18,24]. Consequently, such procedure is praxis in conventional cryopreservation protocols, albeit there are substantial differences regarding either the duration of the SP-exposure [11,15,31, 35,36] or the evidence of its effectiveness on sperm freezability. Some authors reported that SP-exposure improves it [37], while others found that its influence is inconsequent or even detrimental [12,21]. One explanation for these divergent results

☆ *Statement of funding*: This experimental study was supported by Ministry of Economy and Competitiveness of Spain and European Regional Development Fund (AGL2012-39903) and Swedish Research Council Formas (2011–512). D.V. Alkmin, C. Perez-Patiño and I. Barranco were financially supported by the Science Without Borders program (Brazilian Scientific Mobility Program at CNPq/MCT), Seneca Foundation (Murcia, Spain) and Ministry of Education, Culture and Sport of Spain, respectively.

* Corresponding author. Address: Department of Medicine and Animal Surgery, Faculty of Veterinary Science, University of Murcia, E-30100 Murcia, Spain. Fax: +34 868 887069.
E-mail address: roca@um.es (J. Roca).

http://dx.doi.org/10.1016/j.cryobiol.2014.07.004
0011-2240/

would be the lack of agreement among studies for the origin and characteristics of the SP surrounding the sperm used for the testing. The boar ejaculate is expelled in rather easily identifiable fractions, each one of these containing varying sperm numbers (or even their absence) but, as importantly, different composition of the SP. The SP composition and volume follows a differential secretion of accessory glands, and the differential presence of epididymal fluid [29]. Thus, unless the ejaculate is collected in one single container, marking its classical large volume and low sperm numbers per mL; when fractions are collected they show clear differences among a sperm-rich fraction (SRF) and the following sperm-poor fraction, the so-called post-sperm-rich fraction (PSRF). With practice during sperm collection, portions of these can also be collected as for instance the sperm-peak portion [29]. While sperm numbers present defines these fractions, differences are also clear between fractions for the SP, particularly regarding its protein contents [30].

The traditional glove hand method used for manual ejaculate collection in pigs allows for easy sampling of different fractions or even portions within fractions. The SRF is usually the only fraction collected for freezing [9,17,18], although some cryopreservation protocols contemplate the entire ejaculation [4,14,27]. In contrast, other studies suggest only freezing the first 10 mL of the SRF, the sperm-peak portion because the SP of this portion seems to have a greater cryoprotective effect [29,32]. Although these results would prove to the SP surrounding sperm at ejaculation influences boar sperm freezability, it remains unclear the magnitude of this influence, which could depend of fractions/portions of the ejaculate. The main purpose of this study is clarifying this issue. Clarifying this issue is particularly relevant today because, for productivity and hygienic reasons, semi-automatic systems, such as Collectis® [2], are successfully replacing the usual method of the gloved hand for collection of boar ejaculates. These semi-automatic systems require collecting the bulk ejaculate, increasing the proportion of SP in the collected semen samples, and therefore, the putative influence of SP on the capability of boar spermatozoa to withstand the cryopreservation process may be more critical. In addition, it is likely that boar sperm will be held some time surrounded in SP before beginning the freezing process because AI centers are typically located far from the freezing facilities and the time required for ejaculate transport varies. The purpose of this experimental study was to evaluate the influence of native SP, from fractions/portions or the entire ejaculate, on boar sperm freezability. Additionally, epididymal sperm were held in SP before freezing for a better understanding of the influence of SP.

Materials and methods

Reagents and media

Unless otherwise stated, all of the chemicals used in the experiments were analytical grade and purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The basic medium used for semen extension was Beltsville Thawing Solution (BTS: composed of 205 mM glucose, 20.4 mM sodium citrate, 10.0 mM KCl, 15.0 mM $NaHCO_3$, and 3.6 mM EDTA, pH 7.2, and 290–300 mOsmol/kg) supplemented with kanamycin sulfate (0.05 mM). EDTA-free phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 6.8, and 280–300 mOsmol/kg) was used to dilute fluorochromes and extend sperm samples for flow-cytometric analysis. Sperm were frozen using a basic freezing medium (FE) containing 80% (v/v) Tris-citric acid-glucose extender (111 mM Trizma Base, 31.4 mM monohydrate citric acid, 185 mM glucose) and 20% (v/v) egg yolk, supplemented with 100 μg/mL kanamycin sulfate (pH 7.2; 295–300 mOsmol/kg).

Experimental design

The experimental design is shown in Fig. 1. All of the procedures that involved animals were performed according to international guidelines and were approved by the Bioethics Committee of Murcia University (research code: 639/2012). The boars (Large-White) were three years old, healthy and with a proven history of fertility after conventional AI with liquid semen. At the moment when experiments were drawn, semen was regularly collected (twice week) for commercial production of AI-semen doses in a commercial insemination center (AIM Iberica, Calasparra, Murcia, Spain). The boars were, 3–5 days after the month-long experimentation with ejaculates ended, slaughtered at a local slaughterhouse (Mercamurcia, Murcia, Spain). The testes and epididymides from each boar were removed immediately after slaughter and transported in insulated containers at 20–23 °C to the Andrology Laboratory of Veterinary Teaching Hospital of University of Murcia (VTH), arriving within 30 min after collection.

Ejaculates, bulk or in portions, were collected during the month prior to slaughter using the gloved-hand method and following the standard operating procedure. The portions of ejaculate collected separately were the first 10 mL of SRF (so-called P1), the remaining SRF (so-called P2) and the PSRF (only as a source of SP as it contained too few sperm). All semen samples from the bulk ejaculate or ejaculate portions met the following criteria: 70% total motile sperm (subjectively evaluated using light microscopy) and 80% sperm with normal morphology and intact acrosome ridges (evaluated using phase contrast microscopy of sperm samples fixed in buffered 2% glutaraldehyde solution). Semen samples were split in two aliquots immediately after collection; one was used for harvesting SP (see below) and the other was extended in pre-warmed (35 °C) BTS (2:1, v/v). Thereafter, semen and SP-samples were transported at 20–22 °C to the Andrology Laboratory of VTH, arriving within 2 h of collection. Once in the laboratory, the semen samples from bulk ejaculates were split into two aliquots and one was frozen immediately (as Control) whereas the other was stored at 17 °C during 24 h before freezing (BE sample). P1- and P2-semen samples were also stored at 17 °C during 24 h before freezing. A holding time as long as 24 h was chosen because it allows any AI center, even those located in remote locations, to ship semen samples overnight to freezing facilities. Also, to hold the semen samples 24 h before freezing has resulted in high sperm cryosurvival rates and excellent fertility outcomes when SRF were frozen using the cryopreservation protocol used in this experiment [16,28].

The SP from each one of SP-sources (first 10 mL of SRF [SP_1], rest of SRF [SP_2] and PSRF [SP_3]) was harvested via double centrifugation (Rotofix 32 A, Hettich Zentrifugen®, Germany) at 1500 × g for 10 min at rt. The SP was collected by aspirating fluid above the sperm pellet, preventing disturbance of the pellet. The SP samples were stored at −80 °C until used.

Epididymal samples were collected following the procedure described by Martinez-Pastor et al. [20] with slight modifications. Briefly, the epididymides were removed and dissected avoiding blood contamination. Cauda epididymal luminal fluid was collected by retrograde washing from the vas deferens using a syringe loaded with 1 mL BTS, injecting air afterwards until all the fluid was flushed out. The harvested caudal epididymal fluids, from both epididymides of the same boar, were collected and mixed in a sterile Petri dish, and the sperm concentration (using an SP-100 NucleoCounter; ChemoMetec A/S, Allerød, Denmark) and motility (see above) was then evaluated. All epididymal samples showed a total sperm motility above 70%. Epididymal samples were split into three aliquots, extended (to 300 × 10^6 sperm/mL) with each