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Intra- and interboar variability in flow cytometric sperm sex sorting

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ABSTRACT

To improve the efficiency of porcine sperm sex sorting using flow cytometry, the aims of the present study were to determine the relevance of inter- and intraboar variability in sperm sortability and to evaluate the significance of ejaculate semen characteristics in such variability. In addition, the variability among boars in the ability of sex-sorted spermatozoa to survive liquid storage at 15 °C to 17 °C was also evaluated. In total, 132 ejaculates collected from 67 boars of different breeds that were housed at an artificial insemination center were used in three experiments. X- and Y-chromosome–bearing sperm were simultaneously separated according to the Beltsville sperm-sorting technology using a high-speed flow cytometer. In the first experiment, interboar variability in the ability of the ejaculated spermatozoa to undergo the flow-based sex-sorting procedure was observed; the ejaculates of nearly 15% of the boars ($n = 67$) did not exhibit well-defined X- and Y-chromosome–bearing spermatozoa peaks in the histogram, and the ejaculate sperm concentration demonstrated good predictive value for explaining this variation, as indicated by the area under the receiver operating characteristics curve (0.88, $P < 0.001$). In the second experiment, a certain degree of intraboar variability was observed only in the boars that showed poor sperm sortability (measured according to the presence or not a well-defined split together with sperm sortability parameters) in the first ejaculate ($n = 3$). In contrast, boars classified as having good sperm sortability in the first ejaculate ($n = 5$) maintained this condition in five ejaculates collected over the subsequent 5 months. In the third experiment, sex-sorted spermatozoa from boars with good sperm sortability ($n = 5$) remained viable and motile (above 70% in all boars) after 48 hours of storage at 15 °C to 17 °C, which may facilitate the commercial application of sex-sorted spermatozoa in swine artificial insemination programs.

1. Introduction

The technology for sexing mammalian sperm for the production of offspring has progressed considerably in recent years, as is evident in cattle, with more than 10 million calves of predetermined sex born in the last 10 years following artificial insemination (AI) with spermatozoa that were sex sorted using flow cytometry [1]. For the swine industry, the commercial use of sex-sorted spermatozoa may have genetic, management, and financial benefits [2]. To make commercial use possible, several substantial advances were made in recent years that affect all steps in the flow cytometric sperm sex-sorting process [3–6], thus achieving very promising fertility outcomes [7]. Despite this encouraging progress, not all of the critical points in the flow-based process have been resolved. An unresolved critical issue is the variability among ejaculates in the labeling of the

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spermatozoa with Hoechst 33342 (H-42) [8]. Individual differences in the abilities of the spermatozoa to withstand different semen processing techniques, such as liquid storage [9] and cryopreservation [10], are intrinsic features of the boar ejaculates. Individual variability in flow-based sex-sorted sperm has been shown in bulls [11], horses [12], and dogs [13]. It has been suggested that this individual variability may also exist in boars [14]. Keeping in mind this possibility and with the ultimate purpose of improving the efficiency of the flow-based sperm sex-sorting procedure in boars, the first aim of the present study was to identify the relevance of inter- and intraboar variability in the sortability of boar spermatozoa.

The factors responsible for the individual variability in flow-based sperm sex sorting are still not completely known. It is hypothesized that the initial ejaculate semen parameters could be one factor affecting this variability [11–13]. The variability of the semen parameters among boar ejaculates is well known [10,15]. Therefore, the present study also aimed to determine how ejaculate semen parameters influence the ability of boar spermatozoa to withstand the flow-based sex-sorting process and, therefore, to determine their contribution to the presumed variability among boars or the variability among ejaculates from the same boar. This study paid particular attention to the labeling of the spermatozoa with H-42, with the purpose of optimizing the H-42 concentration to minimize the expected variability among boars or among ejaculates from the same boar, thus increasing the efficiency of flow-based sperm sex sorting. Additionally, the interboar variability in the ability of sex-sorted spermatozoa to survive liquid storage was evaluated.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, all media components were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All of the chemicals used in these experiments were of analytical grade, and the media were prepared under sterile conditions in a laminar-flow hood (Micro-R, Telstar; Barcelona, Spain) with purified water (Milli-Q, Millipore, Advantage, Elgastat, UK).

The basic medium used for semen extension was Beltsville thawing solution (BTS; 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 0.05 mM kanamycin sulfate. A PBS extender (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 6.8 and 280–300 mOsmol/kg) was used to dilute the fluorochromes and sperm samples for the flow cytometric evaluation of sperm viability. The sheath fluid used for sperm sorting was the same PBS supplemented with 0.1% (wt/vol) EDTA, 0.058 g/L penicillin G, and 0.05 g/L streptomycin sulfate [6].

2.2. Animals

All of the procedures involving animals were performed according to the international guidelines and were approved by the Bioethics Committee of Murcia University (research code: 639/2012).

Healthy and sexually mature 2- to 4-year-old boars (Pietrain, Duroc, Landrace, and crossbred) were used as semen donors. The boars had a proven history of fertility and were undergoing regular ejaculate collection (twice/week) for commercial AI semen doses (AIM Iberica, Calasparra, Murcia, Spain). The boars were housed in individual pens in an environmentally controlled (15 °C–25 °C) building with windows to expose the animals to natural daylight and supplementary light for a total of 16 hours per day. The boars had free access to water and were fed a commercial diet according to the nutritional requirements of adult boars.

2.3. Semen collection and sperm preparation for sex sorting

Sperm-rich fractions were collected using the gloved-hand method, according to the standard operating procedure; the samples were diluted (1:1, vol:vol) in prewarmed (35 °C) BTS and transported at 20 °C to 22 °C to the Andrology Laboratory of the Veterinary Teaching Hospital within 2 hours of collection. Once in the laboratory of the Veterinary Teaching Hospital, the semen samples were evaluated for sperm quantity and quality (see Section 2.5). Thereafter, the semen samples were diluted in BTS to a concentration of 200×10^6 sperm/mL and processed for flow-based sex sorting as originally described by Johnson et al. [16] and recently modified by Parrilla et al. [14]. Briefly, the semen samples (200×10^6 sperm/mL) were stained at a 0.09-mM or 0.135-mM final concentration (see Section 2.6) of H-42 (5 mg/mL (9 mM) stock solution). After incubation at 35 °C for 50 minutes in the dark, the semen samples were filtered through a 30-μm nylon mesh filter, and 2 μL of food dye (0.002% wt/vol; FD&C no. 40; Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to the semen samples immediately before flow cytometric sorting.

2.4. Sperm sorting by flow cytometry

X- and Y-chromosome–bearing sperm were simultaneously separated according to the Beltsville sperm-sorting technique [17] using a high-speed flow cytometer/sorter (SX MoFlo; Dako Cytomation Inc., Fort Collins, CO., USA) operating at 40 psi and equipped with a UV laser set to a 175-mW output (Spectra Physics 1330, Terra Bella Avenue, Mountain View, CA, USA). The samples were collected in 50 mL tubes that were precoated with 1% BSA (wt/vol in PBS) and contained the collection medium (2.5 mL TES–Tris–glucose supplemented with 2% egg yolk, vol/vol). A total of 20×10^6 sex-sorted spermatozoa were collected in each tube in an approximate volume of 25 mL. After collection, the samples were centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) for 4 minutes at $3000\times g$, and the sperm pellets were diluted in BTS to 20×10^6 sperm/mL. When appropriate (see Section 2.6), the BTS-diluted sex-sorted sperm samples were stored at 17 °C for 48 hours.

All sorted sperm samples were gated to achieve 90% purity. Reanalysis of sorted samples was used to check the accuracy of sorting. For sort reanalysis, immediately after