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In vitro sperm quality and DNA integrity of SexedULTRA™ sex-sorted sperm compared to non-sorted bovine sperm

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ABSTRACT

SexedULTRA™ is an improved method of sex-sorting sperm creating a less damaging environment to retain sperm integrity through the sorting process. The aim of this study was to evaluate the *in vitro* characteristics of fresh and frozen bovine sperm using the SexedULTRA™ method, and compare it to conventional (non-sorted) sperm. For both methods, percent total sperm motility was estimated visually and also classified into total and progressively motile using a computer assisted sperm analyzer (CASA). Percent sperm with intact plasma membranes (VIA) and acrosomes (PIA) were assessed using flow cytometry and sperm DNA fragmentation index (DFI) was estimated using the Bull sperm Halomax® Kit. Two contemporaneous ejaculates from 10 bulls were processed and cryopreserved using one of the two procedures (SexedULTRA™ and conventional). Sperm motility, VIA and PIA were assessed post-thaw (0 h) and post-incubation (3 h at 37 °C, 8 h and 24 h at 18 °C). DFI was analyzed post-thaw (0 h) and after 6, 24, 48 and 72 h of incubation at 37 °C. In a second experiment, ejaculates from 7 bulls were split sampled into the two types of processing (SexedULTRA™ and conventional) and diluted using a fresh semen extender developed for sex-sorted bovine sperm. Sperm quality was assessed after dilution (0 h) and after incubation for 12, 24, 48, 72 h at 18°, and the same time points of incubation at 37 °C for DFI. Frozen-thawed SexedULTRA™ sperm was significantly ($P < 0.05$) better than conventional semen after a 3 h incubation at 37 °C for PIA, and after a 24 h incubation at 18 °C for percent visual motility and PIA. DFI was significantly lower for SexedULTRA™ compared to conventional at all time points of incubation (37 °C). Fresh SexedULTRA™ sperm showed improved quality compared to conventional at all time points of incubation at 18 °C for percent visual and total motile sperm, VIA, PIA, and DFI. Significant differences were also found in progressive motile sperm immediately after dilution (0 h), but not at any time point after incubation. The results show that the SexedULTRA™ process maintains the quality of sex-sorted sperm and, in many cases, has better *in vitro* longevity than conventional semen.

1. Introduction

Since the first publications showing that flow cytometry was a feasible and reliable method to separate X and Y chromosome bearing sperm, the process has been subject to continual refinement and is now a commercial product available worldwide [1–4]. Sex-sorted sperm was first introduced commercially by XY Inc. and Cogent in late 2000 in the United Kingdom, using the XY method [5]. Bovine sperm sorting laboratories are now operating in more than 25 locations, in 15 countries, with an estimated annual production of 10 million straws [6]. Most reputable cattle artificial breeding companies offer a sex-sorted sperm option as part of their portfolio and the use of this product is increasing. Earlier, the limitations to a more widespread use of sex-sorted sperm in cattle had been the slow sorting process, which reduced production efficiency and increased costs. The introduction of application specific Genesis™ Digital sperm sorting systems and the use of multi-headed sperm sorters have now reduced this limitation considerably. Increased sorter speeds and associated technology allow operators to produce over 300 sex-sorted straws per sorter head per day [7,8]. Also, it is common to see many Sexing Technologies and associated licensed production laboratories operating more than 10 sorter heads, with some laboratories operating over 70 sorter heads in one location (Jared Templeton, Global Production Manager at Sexing Technologies, personal communication).

The other limitation to sex-sorted sperm is the reduced fertility

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compared with conventional semen [4,5,9]. Successful sperm sorting must consider the susceptibility of gametes to each of the many stages of the sorting process, including high dilutions, staining at 34–36 °C, laser exposure, sorting in contact with a biocompatible sheath fluid, holding sperm after sorting, centrifugation followed by cryopreservation at low cell concentrations. Recognizing that an improvement in the quality of sex-sorted sperm would allow wider commercial application, a concerted effort in the last few years to understand the biochemistry of extenders used for sperm sex-sorting and the processing methodology, has resulted in substantial changes in media composition and alterations to the various stages of the process. This new method branded SexedULTRA™ was designed to provide a supportive environment that accommodates changes in pH, temperature and tonicity, and retains sperm integrity through the entire process.

The objective of these experiments was to evaluate the effects of the SexedULTRA™ sex-sorting method on *in vitro* sperm quality. We hypothesized that SexedULTRA™ would improve *in vitro* sperm quality resulting in equal or better longevity to that of conventional semen.

2. Materials and methods

2.1. Semen samples

All the animals used in this research were treated in accordance with the Federation of Animal Science Societies (2010) guide for the use of farm animals in research and teaching. Ejaculates from a total of 17 bulls owned by Sexing Technologies were collected via artificial vagina in Navasota (TX, USA) and Fond du Lac (WI, USA). Only ejaculates with a sperm motility ≥65%, and abnormal head ≤15% and tail morphology ≤15%, were included in the analysis.

2.2. Conventional and SexedUltra™ procedures

Conventional semen was processed using standard industry methods. For conventional cryopreserved semen, ejaculates were diluted with a Tris-citrate egg yolk medium at 19 °C, equilibrated to 4 °C for a minimum of 90 min, and then re-diluted to a final concentration of 90 million cells per mL with a Tris-citrate glycerol egg yolk medium. For conventional fresh semen, fresh extender (FSRD4+, Sexing Technologies, TX, USA) was used to adjust sperm concentration to 45 million cells per mL.

Sex-sorted sperm was processed using the basic method of semen dilution and staining as described in Seidel and Garner, 2002 [5] with modifications that are collectively termed SexedULTRA™. This method is a revision of the media and conditions under which semen is processed and sorted, including the use of Genesis™ Digital sperm sorting systems designed in collaboration with Cytonome ST LLC. The media formulations in the SexedULTRA™ method are trade secret and proprietary and protected as intellectual property of Inguran LLC through patent US 9,781,919. SexedULTRA™ sperm was sorted at approximately 90% X chromosome purity. Final concentration of sex-sorted sperm before cryopreservation was 18 million cells per mL. Fresh sex-sorted sperm was diluted with fresh extender (FSRD4+, Sexing Technologies, TX, USA) to 10 million cells per mL.

2.3. Sperm quality determination

Sperm concentration was determined using the SP1-Cassette, Reagent S100, and NucleoCounter SP-100 system (ChemoMetec A/S, Denmark). Visual motility was estimated at 37 °C on 100 sperm cells under bright field microscopy with a Nikon Eclipse 80i microscope (Melville, NY, USA). Motility on a minimum of 500 cells at 37 °C was classified into total and progressively motile using a computer assisted sperm motility analyzer (CASA-IVOS II system, Hamilton Thorne, MA, USA). Percent sperm with intact plasma membranes (VIA) and acrosomes (PIA) were assessed after staining with Hoechst 33342 (Sexing Technologies, TX, USA. Final concentration: 10.0 μg/mL), Propidium Iodide (PI; Life technologies, IL, USA. Final concentration: 2.0 μg/mL) and *Arachis hypogaea* conjugated with fluorescein isothiocyanate (FITC-PNA; Thomas Scientific, NJ, USA. Final concentration: 4.0 μg/mL). Sperm were then incubated at 34 °C for 15 min. A minimum of 10,000 events were analyzed using a modified MoFlo SX sperm sorter with Summit v4.0 software (Beckman Coulter, Miami FL). The sorter was fitted with a Vanguard HMD 350mW/355 nm laser (Spectra Physics, Santa Clara CA) that excited Hoechst 33342 to identify Forward and Side Angle Fluorescence This laser was used to improve accuracy of the analysis, allowing to gate on sperm cells and exclude debris or other material. The sorter was also modified with a Coherent Sapphire OPSL laser (Coherent Inc, San Jose, CA) operating at 488 nm to excite PI emitting at 620 nm and FITC-PNA emitting at 530 nm. The emission from the Coherent laser was split using a DCLP 555 dichroic mirror to divide the light in two separate photomultiplier tubes, one detector path utilized a 620/60 (PI) and the other path used a 530/40 (FITC-PNA) bandpass filter. VIA and PIA were calculated as the percentage of PI and FITC-PNA negative sperm populations, respectively. DNA fragmentation index (DFI) was assessed on 300 sperm cells using the Bull sperm Halomax® commercial Kit (Halotech DNA, Madrid, Spain).

2.4. Experiment 1 – frozen semen

Ejaculates from 10 Holstein bulls were processed at the Sexing Technologies production laboratory in Fond du Lac (WI, USA) and sent for quality analysis to the Research and Development Laboratory in Navasota (TX, USA). Two contemporaneous ejaculates were processed per bull for one of the two procedures (SexedULTRA™ and conventional). 20 million conventional or 4 million SexedULTRA™ sperm cells were cryopreserved per 0.25-mL straw using the automated freezing device IMV Digitcool (IMV, France), and stored under liquid nitrogen. For quality analysis, one conventional and one sex-sorted straw were thawed at 38 °C for 45 s. Contents of each straw were split in two aliquots and placed into pre-labeled 1.5-mL Eppendorf microcentrifuge tubes (Eppendorf North America, NY, USA) at 37 °C and at 18 °C. Post-thaw (0 h) and post-incubation (3 h at 37 °C, 8 h and 24 h at 18 °C) percent visual and CASA sperm motility, VIA and PIA were assessed. DFI was also assessed post-thaw (0 h) and post-incubation (6, 24, 48 and 72 h, at 37 °C) for both semen processing procedures.

2.5. Experiment 2 – fresh semen

Ejaculates from 7 bulls (4 Holstein, 2 Jersey, 1 Brown Swiss) were processed and analyzed at the Sexing Technologies R&D laboratory in Navasota Texas. One ejaculate per bull was split sampled into the two types of processing (SexedULTRA™ and conventional). Fresh extended samples were split in two aliquots and placed into pre-labeled 1.5-mL Eppendorf microcentrifuge tubes at 37 °C and at 18 °C. Percent visual, total and progressively motile sperm, and VIA and PIA were assessed after dilution in fresh extender (0 h) and after incubation at 18 °C (12, 24, 48, 72 h). DFI was analyzed at the same time points but incubated at 37 °C.

2.6. Statistical analysis

The *in vitro* sperm quality data were analyzed by analysis of variance with the fixed effect of treatment and random effect of bull