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Effect of sorting boar spermatozoa by sex chromosomes on oviduct cell binding

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ABSTRACT

This study examined the hypothesis that flow sorting sperm by sex chromosomes affects oviduct cell binding which would influence formation of the sperm reservoir in the oviduct. The sperm-rich fraction from boars (n = 5) was collected, sperm were stained with Hoechst 33342 and sorted. Sperm were sorted based on the presence of either an X or Y chromosome and placed into the following treatments: 1) sperm selected for the Y chromosome, 2) selected for the X, 3) an equal mixture of sorted X and Y, and 4) a control of non-sorted sperm from the same collection. Samples were tested for oviduct cell binding within 12 h of sorting. Additionally, sperm were analyzed for motility characteristics, acrosome status, and binding to the two oviduct glycan motifs that bind porcine sperm, biantennary 6-sialylated *N*-acetyllactosamine on a mannose core (bi-SiaLN) and sulfated Le^X trisaccharide ($suLe^X$). The disaccharide found within both glycan motifs, *N*-acetyllactosamine (LacNAc), was used as a control. Sperm binding to oviduct cells was reduced by more than half in the three sorted samples when compared to the control sperm that were not sorted. The percentage of sperm that were motile 24 h after sorting was also decreased significantly in each of the sorted sample groups when compared to the unsorted control. In contrast, sorting did not decrease the percentage of sperm that bound purified soluble glycans or the location on sperm to which they bound. There was also no difference in sperm acrosome status among the four groups. In summary, sorting reduced sperm binding to the complex matrix around oviductal cell aggregates but sperm binding to purified soluble oviduct glycans was not affected. The requirement for higher affinity and motility to bind glycans immobilized on oviduct cells may explain this difference. The reduction in sperm fertility observed following sex-sorting may be explained partially by a reduced or altered ability to bind to the oviduct epithelium.

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1. Introduction

Sorting sperm by sex chromosomes has been practiced successfully for decades and is used regularly in dairy cattle [1–4]. In contrast, sex sorting of porcine sperm is still undergoing development to make it practical for the swine industry. There is interest in using sex-sorted porcine sperm for several reasons. Increased legislation against physical castration requires alternatives such as sex biasing to reduce the percentage of males, which eliminates castration and the associated costs. It also allows for the improved production of seedstock replacement gilts. The strategic use of sorted sperm can also speed up genetic improvement and the incorporation of transgenic animals into swine industry genetics [5–7].

The sorting index, a value used to approximate the possible sorting efficiency, is defined as the product of the sperm head area (μm^2) and the DNA difference between Y- and X-bearing spermatozoa [8]. The sorting index of porcine sperm is similar to bovine sperm due to their large flat heads and a 3.6% difference in DNA, making them good candidates for sex sorting [8]; however, many factors reduce the feasibility of sorting porcine sperm. Similar to

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sperm from many species, porcine sperm are damaged by staining [9,10], the physical stress of sorting [9,11,12] and the high dilution rates sperm undergo during sorting [13,14]. In addition, there are two major challenges to overcome to use sex-sorted porcine sperm. First, it takes a long time to sort an adequate number of sperm for an insemination dose. Flow cytometers currently sort 20 $\times$ 10^6 sperm cells per hour [11]. Traditional practice for swine insemination is to inseminate approximately 3 $\times$ 10^9 sperm twice about 24 h apart [15]. A common AI dose in cattle is 10–20 $\times$ 10^6 sperm and they are inseminated once per estrus [16]. An AI dose of bovine sex-sorted sperm contains fewer sperm than a dose of non-sorted sperm, which makes sorting bovine sperm practical. Methods of reducing the number of sperm needed for fertility, such as deep uterine insemination (DUI) [17–21], in-vitro fertilization (IVF) [22–24], intracytoplasmic sperm injection (ICSI) [25], and surgical insemination [26,27] have allowed the production of offspring from sorted porcine sperm. Deep uterine insemination of as few as 50-70 $\times$ 10^6 sperm following ovulation induction, produced similar numbers of piglets as conventional AI using the same concentrations [19,20]. Furthermore, double deposition laparoscopic insemination (oviducts and uterine horns) of sows with a low dose of 3 $\times$ 10^6 per insemination resulted in an average litter size of 9.2 $\pm$ 0.6 piglets [27]. Greater improvements in AI technology could make the use of sex-sorting sperm feasible in swine.

In addition to the long sorting time, the second challenge in using sex-sorted boar sperm is a lack of post-sorting storage methods. Rather than freezing, liquid storage is most often used, due to poor fertility following cryopreservation, so it is critical to have a liquid extender adequate to prolong the lifespan of the sorted spermatozoa. Additives to extender such as egg yolk [28], spermadhesins such as PSP-I/PSP-II [14,29], and antioxidants [30,31] have improved viability as well as fertilizing ability. Still, IVF was necessary to produce offspring from sex-sorted frozen-thawed sperm [21].

We hypothesized that one reason for lower fertility of sex-sorted porcine semen is that sorting causes capacitation-like changes to occur. Sperm that are capacitated are less able to bind the oviduct epithelium and form the sperm reservoir that supplies fertile sperm to the site of fertilization [32–34]. Modifications of the sperm membrane induced by sorting promote capacitation [35] and could affect membrane function [36]. Membrane stability may also be affected by the removal of seminal plasma components from sperm during sorting, which otherwise inhibit capacitation and help maintain an intact acrosome [13].

The sperm reservoir in the porcine oviduct is formed by retention of sperm by oviduct epithelial glycans containing two motifs, either a biantennary 6-sialylated *N*-acetyllactosamine on a mannose core (bi-SiaLN) or a Lewis^X trisaccharide (Le^X) [37,38]. Both glycan motifs bind with high affinity to the head of sperm prior to capacitation and are necessary for sperm binding to the oviduct [37,38].

In this study, our aim was to assess the effect of sex-sorting on the ability of sperm to bind oviduct epithelial cells and soluble oviduct glycans. The results could be used to understand the changes that occur during the sex sorting process and perhaps devise techniques to improve the fertility of sex-sorted sperm.

2. Materials and methods

2.1. Sperm collection and sorting

Commercial boars (n = 5) of proven fertility, housed in Wisconsin, were collected in July–September of 2015 for this project. The sperm rich fraction was collected using the gloved-hand technique, diluted 1:1 in a pre-warmed (36 °C) proprietary media and transported to the laboratory. Semen samples were evaluated for concentration, total motility, and normal morphology characteristics. Samples were then diluted to 200 $\times$ 10^6/ml with the same dilution medium and stored at 17 °C overnight. Semen (1 ml) was stained with both 5 μl of Hoechst-33342 (from a stock of 5 mg/ml) and 3 μl of FD&C Yellow 6 (Sigma Aldrich, St. Louis, MO USA) from a 1:5 stock solution diluted in ddH_2O and incubated in the dark for 60 min at 34 °C. Following incubation semen was filtered through a 30 μM filter into a new 5 ml flow tube. The X and Y chromosome-bearing sperm were separated using a flow sorter (Genesis MoFlo®, Cytonome-ST, Boston, MA, USA) operated at 40 psi with a UV laser (Spectra Physics, Mountain View, CA USA). Sperm were sorted using a Tris based proprietary medium as the sheath fluid and collected in a 50 ml conical tube containing sheath fluid and 2% egg yolk. A total of 20 $\times$ 10^6 sperm/ml in 25 ml were collected, centrifuged at 2400X*g* for 4 min and the pellets resuspended in Tris based proprietary extender at a concentration of 30 $\times$ 10^6 sperm/ml. Immediately following sorting, the samples were analyzed for purity (more than 93% of sperm had the expected sex chromosome, Table 1). The motility of each population was over 85%, the viability over 90% and the percentage of morphologically normal cells was over 80% (Table 1). Sperm were cooled to 17 °C and transported immediately to the laboratory at the University of Illinois.

2.2. Motility and membrane analyses of spermatozoa

Post-sort motility and acrosome integrity were evaluated for each of the following treatments 1) sperm sorted for the Y chromosome (Y-bearing), 2) sorted for the X (X-bearing), 3) an equal mixture of sorted X and Y (XY-remixed), and 4) a control of non-sorted sperm from the same collection (C-not sorted). Motility analyses were conducted using Computer Assisted Sperm Analysis (Hamilton Thorne, Beverly, MA, USA). Sperm were warmed for 10 min at 38° C and 3 μl of each sample were loaded onto a pre-warmed 4-chamber Leja4 slide (IMV Technologies, Maple Grove, MN, USA). A minimum of 8 fields and 750 cells were observed at 37° C and 45 frames were acquired at a rate of 60 Hz with a minimum contrast of 55, minimum cell size of five pixels, and magnification of 1.73. Samples were measured at approximately 24 h after sorting. Parameters included total motility and progressive motility.

Coomassie Blue staining was done to determine acrosome integrity for each sample [39]. Around 24 h post-sort, 10 μl of spermatozoa from each group were fixed with 4% paraformaldehyde to fix cells. Cells were centrifuged at 2000X*g* for 1 min, supernatant was discarded and the pellet resuspended in 50 μl of 0.1 M ammonium acetate. The suspension was centrifuged again at 2000X*g* for 1 min, supernatant discarded and pellet resuspended in 25 μl of ammonium acetate. The sperm suspension (10 μl) was placed onto a slide and allowed to air dry. Dried slides were stained with Coomassie Blue G-250 solution for 4 min. Slides were rinsed with distilled water and allowed to air dry. A drop of 1X PBS was added and topped with a cover glass. A minimum of 300 sperm/sample was evaluated using a Zeiss Axioskop and AxioCamHRc (Zeiss Microscopy, LLC, Thornwood, NY, USA) at 400× magnification. Spermatozoa with a stained acrosome were considered to be acrosome intact.

2.3. Oviduct-sperm binding assay

One ejaculate from each of the 5 boars was tested on an experimental day. Oviduct-sperm binding assays were completed on the same day as sorting. For each experiment, female reproductive tracts were collected from pre- and post-pubertal females at an abattoir. Tracts were brought back to the lab and 20 to 30 oviducts were removed and held in 1X PBS on ice before being

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