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Flow cytometry sex sorting affects bull sperm longevity and compromises their capacity to bind to oviductal cells

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ABSTRACT

This study assessed the effect of flow cytometry sexing on sperm longevity and the capacity of sperm to bind to oviductal cells. Each ejaculate from four bulls was divided into two fractions: the first was immediately frozen as non sexed sperm (NS) and the second was sexed originating X- and Y-bearing sperm. The fourth treatment had sex-sorted X and Y sperm (XY) combined. Sperm from each group was assessed for sperm characteristics after thawing, after washing and at 2, 4, 8 and 12 h of incubation at 39 °C in 5% CO_2 in air. For the binding test, sperm were incubated in sp-TALP medium for 30 min or 24 h with oviductal explants. Percentages of motility (58.1 ± 4.3 and 35.2 ± 4.4), progressive motility (46.1 ± 6.1 and 25.7 ± 4.8), mitochondrial membrane potential (79.2 ± 8.3 and 69.0 ± 6.3), plasma membrane stability (77.4 ± 4.6 and 19.4 ± 4.2), and live sperm with intact acrosome (57.2 ± 8.5 and 31.3 ± 7.9) were higher in NS than in XY, respectively ($P < 0.05$). Those differences were maintained for up to 8 h. The sexing process did not affect the sperm binding to the explants after 30 min. However, after 24 h, XY had less (6.7 ± 2.0) sperm bound to explants than NS (23.6 ± 7.2). In conclusion, even though XY was of lower quality than NS, the decreases in quality in both NS and XY groups were similar between groups during incubation. Moreover, the sex-sorting process affected the ability of sperm to remain bound to oviductal explants.

1. Introduction

Ever since the flow cytometry sexing process was developed and sexed sperm became commercially available, several studies have evaluated its use for in vivo and in vitro embryo production. Although some studies have shown low rates of in vitro embryo production (Morton et al., 2005; Palma et al., 2008) improvements to the sexing process (Sharpe and Evans, 2009) and modifications of in vitro embryo production (IVP) protocols (Blondin et al., 2009; J.O. Carvalho et al., 2010; Rodriguez Villamil et al., 2012) have increased embryo production to rates similar to those obtained with non-sexed sperm (Blondin et al., 2009; J.O. Carvalho et al., 2010). Previous studies in our laboratory (J.O. Carvalho et al., 2010) identified that the sex sorting process affected some structural characteristics of bovine sperm but did not reduce their capacity to produce embryos in vitro. However, for in vivo embryo production via artificial insemination (AI) after ovarian superstimulation, the use of sexed sperm has been limited due to the high cost and reduced fertility rates (Seidel et al., 1999; Sartori et al., 2004; Bodmer et al., 2005; Andersson et al., 2006; Blondin et al., 2009; Peippo et al., 2009; Underwood et al., 2010a, 2010b). Therefore, it seems that the damage caused by sexing sperm is not as critical for in vitro fertilization as for in vivo fertilization. During in vitro embryo production, oocytes are co-incubated with a high sperm concentration in a single drop of fertilization medium. However, during in vivo fertilization, the sperm must reach the oviduct, bind to their cells, forming a sperm reservoir and finally reach the fertilization site. Therefore, sperm must be kept alive for a long period until ovulation has occurred in order to fertilize an oocyte

Considering these differences between in vitro and in vivo sperm conditions to fertilize the oocyte, we can hypothesize that the sexing process compromises sperm features that are required for in vivo fertilization, but not for in vitro fertilization. Features such as longevity in the female reproductive tract and their capacity to bind to oviductal cells during sperm reservoir formation are included in this category.

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There is evidence that the sexing process can induce capacitation (Bucci et al., 2012), plasma membrane damage and acrosome reaction (Moce et al., 2006). Therefore, it is expected that sexed sperm would remain viable for a shorter period after AI, compromising in vivo fertilization. Furthermore, we recently identified that the sexing process changes the sperm head shape, and some surface characteristics, such as the roughness of the plasma membrane (Carvalho et al., 2013). Therefore, the association between head shape modification and destabilization of plasma membrane caused by sexing, could induce the loss of plasma membrane proteins that are important for maintaining sperm bound to the oviduct reservoir. Therefore, the aim of this study was to evaluate the sexed sperm longevity and their capacity to bind to oviductal cells using an in vitro model.

2. Material and methods

2.1. Experimental design

Semen collected from four sexually mature Nelore bulls was used to obtain X and Y sperm cells sorted by flow cytometry. Each ejaculate was divided into three fractions: non-sexed (NS) sperm, sorted for X (X) or Y (Y) chromosome-bearing fractions. For each bull, a fourth group was formed, pooling X and Y samples (XY). In a first experiment, the samples were used to assess the sperm longevity during 12 h in culture media. Sperm motility and progressive motility, acrosome integrity, mitochondrial membrane potential and plasma membrane stability were evaluated at different time points up to 12 h of culture. In a second experiment, the capacity of sperm to bind to oviductal cell explants was tested. In both evaluations, one replicate per bull was performed, and each bull was considered a replicate.

The Animal Research Ethics Committee of Superior School of Agriculture “Luiz de Queiroz” (ESALQ)/University of São Paulo (São Paulo, Brazil) approved all procedures involving animals in this study (CEUA-ESALQ/2013-25).

2.2. Semen collection and sorting

One ejaculate from each of the four bulls was collected using an artificial vagina, and only ejaculates with > 60% motility and < 20% morphological abnormalities were used. The proportion of semen designated for immediate freezing (NS group) was diluted in a Tris-base freezing diluent with 4% egg yolk, cooled at 4 °C for 90 min and then diluted with Bioxcell (IMV, L’Aigle, France). Thirty millions of sperm were loaded into 0.5 mL straws (IMV, L’Aigle, France) and frozen in a programmable freezer TK 3000 (TK, Uberaba, Brazil). At the end of the program, the straws were submerged into liquid nitrogen for storage. All procedures for semen collection, sorting and freezing were performed by a commercial company as their routine techniques (ABS Pecplan, Uberaba, Brazil).

The remainder of the ejaculate was diluted to 200×10^6 sperm/mL with Tris medium, supplemented with 49–65 mM Hoechst 33342 (Invitrogen Molecular Probes, Eugene, USA) and incubated for 45 min at 35 °C. After staining, samples were diluted at 1:1 with Tris medium supplemented with 4% egg yolk and 0.0015% food dye (FD&C #40; Warner Jenkinson Company Inc., St. Louis, USA) and filtered through a 50 μm filter (GCAT, Fort Collins, USA) to remove debris or agglutinated cells prior to sorting.

A high-speed cell sorter (MoFlo SX, Beckman Coulter, USA) was operated at 40 psi with a diode pumped solid-state pulse laser (Vanguard 350 HMD-355; Spectra Physics, Mountain View, USA) at 125 mW with bovine sheath fluid (CHATA Biosystems Inc., Fort Collins, USA). Gates were set to attain 90% purity, and sexed sperm were sorted into Tris I medium. After being cooled at 4 °C for 90 min, the sexed sperm were centrifuged and diluted in Bioxcell (IMV, L’Aigle, France). Two million of sperm were packaged into 0.25 mL straws and frozen as described above for non-sexed sperm.

2.3. Sperm processing and incubation in culture medium

Sperm were thawed in a water bath at 37 °C for 30 s, and after thawing (before washing, BW), one aliquot per group was removed to assess different sperm characteristics. The remaining sample was washed for 5 min at 700 × g in 1 mL of synthetic oviduct fluid (SOF), which was supplemented with 5% fetal calf serum (FCS), 2.77 mM myo-inositol and essential and non-essential amino acids (Holm et al., 1998). After centrifugation (time 0), the supernatant was removed, and an aliquot was used for evaluation. The remaining sample was incubated in 200 μL of the SOF medium under silicone oil (Alphatec, Curitiba, Brazil) for 2, 4, 8 and 12 h (time 2, 4, 8 and 12, respectively) at 39 °C in 5% CO_2 in air. For incubation, final sperm concentration was adjusted to 2×10^6 sperm/mL. At each time point, total motility, and progressive motility were evaluated by CASA, and acrosome integrity, mitochondrial membrane potential, and plasma membrane stability were evaluated by flow cytometry.

2.4. Sperm quality assessment

The percentages of motile sperm and progressive motility were determined using a CASA system (Sperm Analysis System, Hamilton Thorn Motility Analyzer - HTMA – IVOS 12 – Hamilton Research. Beverly, USA). The software was set using the manufacturer's recommendation, with a slight adjustment on the light intensity, to allow clear identification of all sperm. For this assessment, 6 μL of sperm was placed on a pre-warmed Makler counting chamber (Sefi Medical Instrument, Haifa, Israel), under a coverslip. At least seven fields were selected for reading and analysis.

The acrosome status was assessed using fluorescent probe fluorescein isothiocyanate-conjugated with peanut agglutinin (FITC-PNA, Invitrogen, Eugene, USA) and propidium iodide (PI) as described by (Klinc and Rath, 2007). An aliquot (3 μL) of sperm sample was diluted in staining solution (30 μL) that consisted of 1 μL of buffered formol saline, 25 μL of sodium citrate (3%), 2 μL of PI (0.75 mM), and 2 μL FITC-PNA solution (1 mg/mL in PBS). In the PNA/PI dot plots, the regions were set to differentiate viability and integrity of acrosome. Sperm with PI negative were considered alive, and PI positive were considered dead. Alive or dead cells were classified as acrosome-reacted (PNA positive), or as acrosome-intact (PNA negative).

The mitochondrial membrane potential was determined using Mito Tracker Green (Molecular Probes, Eugene, USA), prepared according to Celeghini et al. (Celeghini et al., 2007), plus PI. An aliquot (3 μL) of sperm sample was diluted in 100 μL of sodium citrate (3%) with staining working solution, consisting of 2 μL of the Mito Tracker Green FM (1 mM) and 2 μL of PI (2 mg/mL). Both work solutions were prepared in dimethyl sulfoxide (DMSO). In the PI/mito tracker green dot plots, the regions were set to differentiate viable sperm and mitochondrial membrane potential. Viable sperm without mitochondrial membrane potential (PI negative and mito tracker negative); viable sperm with mitochondrial membrane potential (PI negative and mito tracker positive); nonviable sperm without mitochondrial membrane potential (PI positive and mito tracker negative); and nonviable sperm with mitochondrial membrane potential (PI positive and mito tracker positive).

Plasma membrane stability was assessed according to Hallap et al. (2006). Briefly, an aliquot of sperm sample (3 μL) was diluted in a 100 μL of sodium citrate (3%), with staining working solution, consisting of 2.6 μL of merocyanine 540 (M540; 1 mM; Molecular Probes) and 1 μL of YO-PRO-1 (25 μM), being both prepared in DMSO. In the Yo-PRO1/merocyanine-540 dot plots, the regions were set to differentiate viable and non-capacitated. Viable sperm without destabilized membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); nonviable sperm without destabilized membranes (YP positive and M540 negative); and nonviable sperm with destabilized membranes (YP positive

and M540 positive).

Fluorescent intensities of the samples were detected by flow cytometry (BD Accuri™ C6, BD Biosciences, San Jose, USA) to determine acrosome integrity, mitochondrial membrane potential, and plasma membrane stability. To differentiate sperm from non-sperm events, the protocol described by Petrunkina et al. (2010) was used based on sperm's osmotic intolerance. A minimum of 5000 events was included in each analysis. To specifically define the setting of the bovine sperm population, forward-scatter-cluster-height (FSC-H) was plotted against side-scatter-cluster-height (SSC-H) gated according to forward/side-scatter profile in scatter plot, based on size and complexity differences, according to Cheuqueman et al. (2012). Data were provided on a logarithmic scale and analyzed using the CFlow Plus software (BD Biosciences, San Jose, USA). In each analysis, sperm samples were transferred to micro tubes containing the fluorescent probes and then, were incubated for 8 min at 39 °C.

2.5. Collecting and processing oviductal epithelial cells

Bovine oviductal epithelium cells (BOECs) were collected and prepared according to Kadirvel et al. (2012), with modifications. Oviducts were collected immediately after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 μg/mL) at 35 °C. Upon arrival, oviducts ipsilateral to ovaries without CL were dissected from the surrounding tissues and rinsed in TCM-199 medium (TCM-199 Hank's salt base, Invitrogen, Carlsbad, USA) supplemented with 10% FCS and amicacyn, 0.075 mg/mL. To recover the BOECs, the oviduct was placed into a petri dish and covered with sp-TALP. Then, the isthmus was squeezed by pressure with a glass slide and the recovered BOECs were transferred to a 15 mL conical tube. After sedimentation, the supernatant was removed, and 5 mL of fresh TCM-199 medium was added to the pellet. The cells were de-aggregated by pipetting. The oviductal cells were cultured in 200 μL of TCM-199 medium supplemented with 10% FCS in 100 mm Petri dishes for 18–24 h at 39 °C, allowing the re-aggregation to form oviductal explant cells (Fig. 1). Spherical aggregates that were 150–450 μm in perimeter were selected for the experiments.

2.6. Assay of sperm binding to oviduct epithelial cells

For each sperm group, 90 spherical explants per group/bull/time (30 min or 24 h of co-incubation) were selected, washed twice and distributed similarly into three droplets of 50 μL of sp-TALP (30 explants/drop) under silicone oil. One straw from each sperm group/bull was thawed and washed in 1 mL of SOF medium by centrifugation at 700 × g for 5 min. After washing, sperm concentration was determined using a hemocytometer (Neubauer chamber) with 1:20 dilution, and then sperm was added into the drop with explants at a final concentration of 0.5×10^6 motile sperm/mL. Sperm and explants were co-incubated for 30 min or 24 h at 39 °C in 5% CO_2 in air. After 30 min and 24 h of co-incubation, free and loosely attached sperm were removed by washing the explants in 100 μL of sp-TALP. Explant-sperm complexes from each group were transferred to a microscope slide in a volume of 30 μL and covered with a coverslip. Two images from each explant-sperm complex (Fig. 1A) were captured using a digital Zeiss Axioskop and Axio Cam HRc (Carl Zeiss, Thornwood, USA). The perimeter of the explants and the number of sperm bound to the periphery of each explant were evaluated using AxioVision v4.5 software (Carl Zeiss, Thornwood, USA; Fig. 1B). The number of sperm bound per mm of explant was calculated for each aggregate.

2.7. Statistical analysis

For the statistical analysis of the variables related to sperm evaluation at different incubation times and the number of sperm bound to oviduct explants, repeated measurement analyses were used. The bull*treatment*incubation time was used as a subject. The data were analyzed utilizing the theory of generalized linear models (PROC GLIMMIX; SAS 9.1, SAS Inst. Inc., Cary, USA). Model adjustment was performed considering a binomial distribution of the results of ratio variables and Poisson distribution for count variables, with mean and variance having the logit and logarithm as link functions, respectively. Significant differences were determined with least squares means and were reported as least squares means ± standard error (SEM) in original scale, using the inverse link option for an easy interpretation. The least squares means were compared using a Tukey-Kramer test, at a significance level of $\alpha = 0.05$.

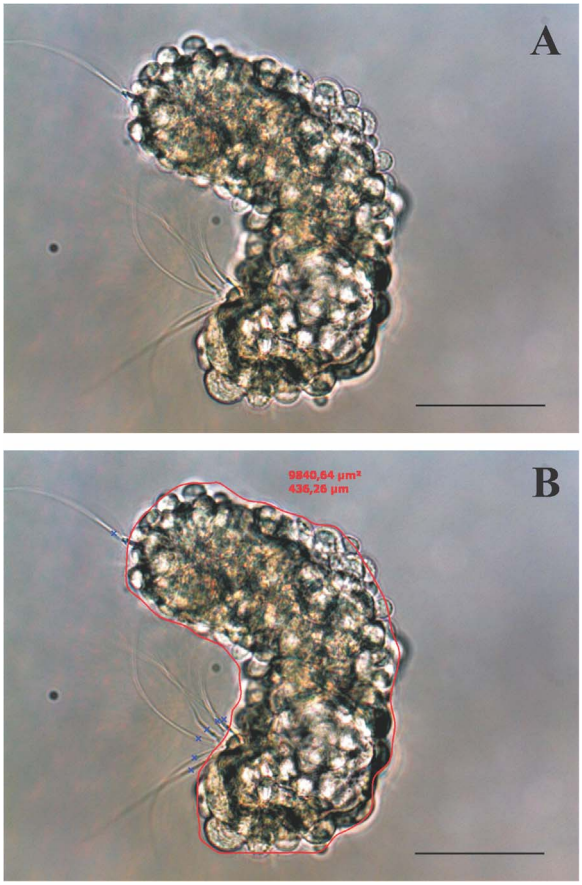

Fig. 1. Phase contrast images (magnification ×40) showing sperm cells bound to oviductal explant cells after 24 h of co-incubation (A); or after sperm counting (marked with the blue "x") and measuring the perimeter of the explants (red line) to calculate the number of sperm bound to mm of the explants (B). Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Assessment of sperm quality characteristics in culture medium

Total (Fig. 2A) and progressive (Fig. 2B) motilities were higher in the NS group than in XY group up to 8 h of incubation. However, after 12 h of incubation, the NS group was similar to the XY group for both variables. Although the XY group had fewer moving cells, a decrease in motility during all incubation times was similar for NS and XY groups. When the same comparison was performed for X and Y group, no differences in total motility were found (Fig. 3A). Regarding the progressive motility, the only difference found between X and Y groups was observed at time 0, with higher motility for the X group than for the Y group (Fig. 3B).

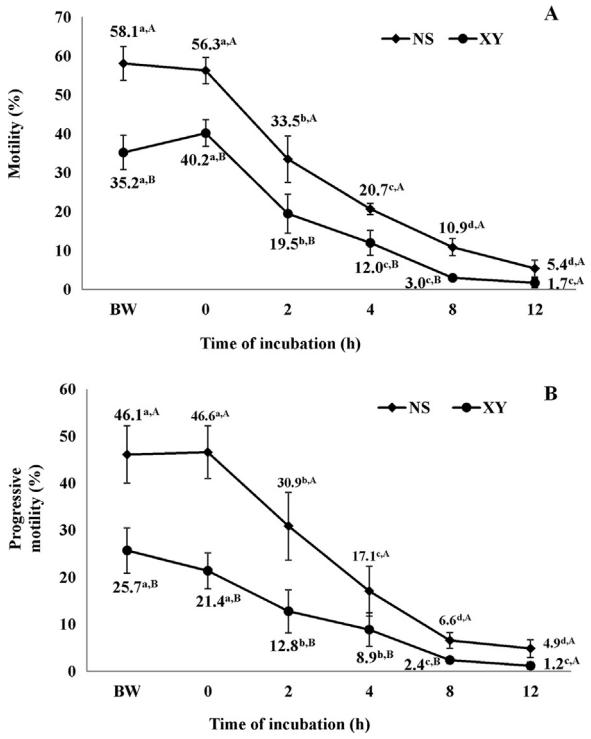

Fig. 2. Percentage of motility (A) and progressive motility (B) of non-sexed sperm (NS) and pool of sorted X and Y sperm (XY) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C in 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

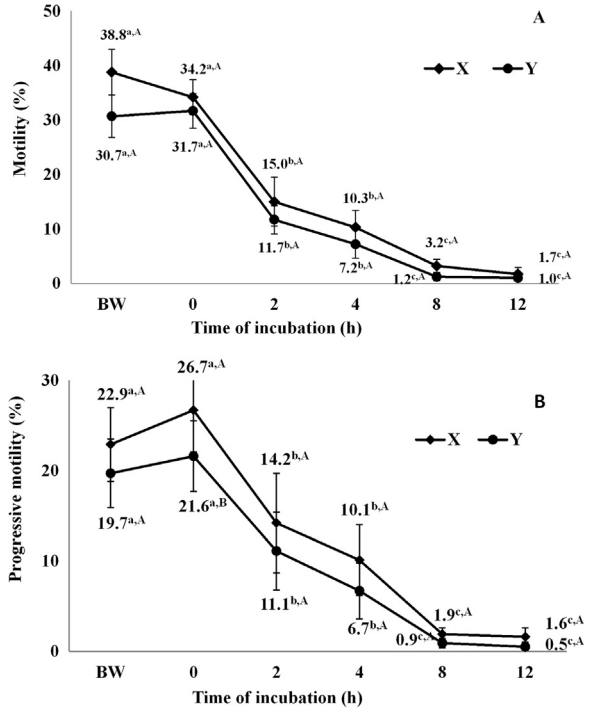

Fig. 3. Percentage of motility (A) and progressive motility (B) of X-bearing sperm (X) and Y-bearing sperm (Y) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C in 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

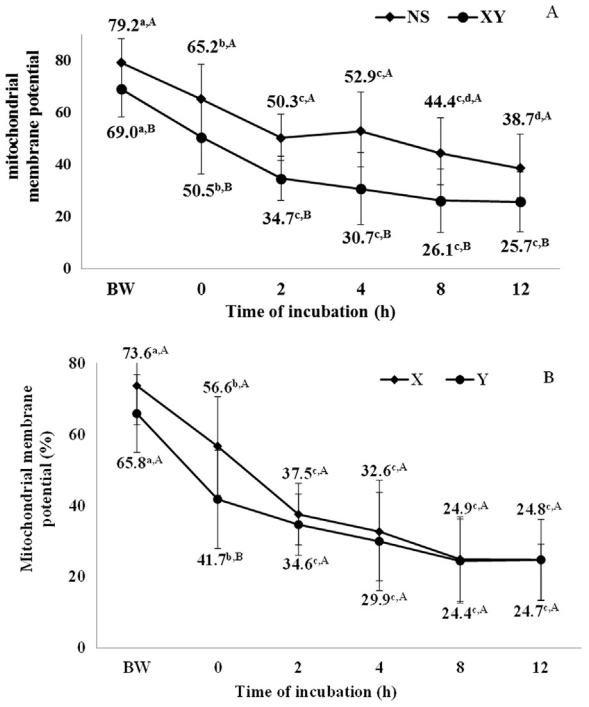

Fig. 4. Percentage of sperm with mitochondrial membrane potential of non-sexed sperm (NS) and pool of sorted X and Y sperm (XY; A) and in X-bearing sperm (X) and Y-bearing sperm (Y; B) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C with 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

The percentage of sperm with high mitochondrial membrane potential was higher in the NS group than in the XY group during all incubation periods (Fig. 4A). However, a negative effect of the washing process was observed for both groups. Between 2 and 12 h of incubation, the percentage of sperm with mitochondrial membrane potential remained stable in the XY group, whereas this percentage decreased in the NS group. A higher percentage of sperm with mitochondrial membrane potential was detected for the X group after washing (Fig. 4B); however no differences were observed between the X and Y groups during the incubation time.

When analyzing the plasma membrane stability, the sexing process was found to induce membrane destabilization, as indicated by the higher percentage of sperm with destabilized plasma membrane in the sexed sperm group than in the non-sexed group during all incubation times (Fig. 5A). However, after 2 h of incubation, the percentage of sperm with plasma membrane destabilization remained constant until 12 h in both groups. No differences in membrane stability were observed between the X and Y groups, with the percentage of sperm with stable and destabilized plasma membrane varying between 18.8 ± 4.3 and 56.0 ± 2.6 after washing and between 4.1 ± 2.3 and 42.5 ± 3.1after 12 h of incubation (Fig. 5B).

The percentage of live sperm with intact acrosome for NS compared with XY is shown in Fig. 6. After thawing, a higher percentage of living sperm with intact acrosomes were found in the NS group than in the XY group. This difference remained for up to 8 h of incubation. No differences were found between X and Y groups concerning the percentage of live sperm with intact acrosome (Fig. 7). However, a higher percentage of live sperm with reacted acrosome was observed for the X group than for the Y group at 0 h (39.1 ± 9.3 vs. 22.7 ± 2.8), 2 h (45.2 ± 6.1 vs. 27.3 ± 5.3) and 4 h (47.3 ± 5.8 vs. ± 32.0 ± 5.2) of incubation.

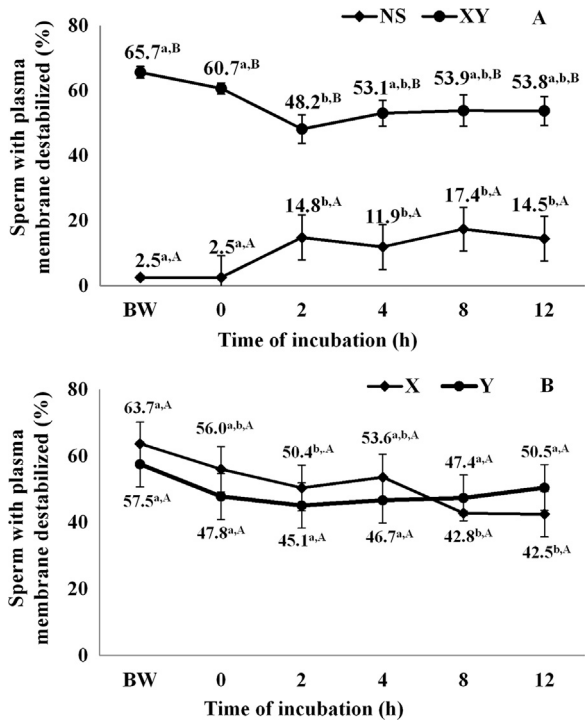

Fig. 5. Percentage of sperm with plasma membrane destabilized of non-sexed sperm (NS) and pool of sexed X and Y sperm (XY; A), and in X-bearing sperm (X) and Y-bearing sperm (Y; B) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C with 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

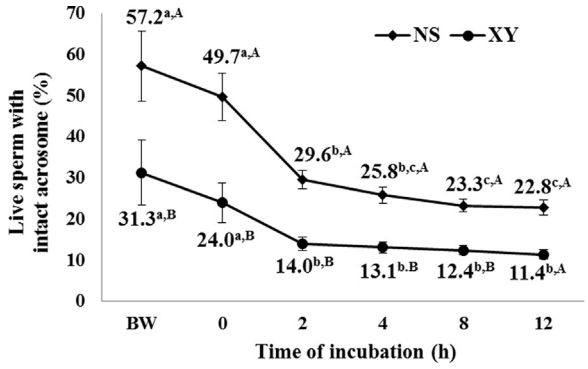

Fig. 6. Percentage of live sperm with intact acrosome of non-sexed sperm (NS) and pool of sexed X and Y sperm (XY) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C with 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

3.2. Capacity of sperm to bind to oviductal explant cells

The sexing process did not affect the ability of sperm to bind to oviductal explants after 30 min of co-incubation (Fig. 8A). However, after 24 h, the NS group had more sperm bound per mm of oviductal explants than the XY group (Fig. 8A). Conversely, the Y group had larger numbers of sperm bound per mm of oviductal explants than the X group at 30 min; however, no difference was found at 24 h between these two groups (Fig. 8B).

4. Discussion

The effect of the sexing process on different characteristics of frozen/thawed sperm viability has previously been reported (Blondin et al., 2009; J.O. Carvalho et al., 2010). However, in this study we also assessed the longevity and the ability of sexed sperm to bind to oviductal cells. Those characteristics are both needed for the sperm to fertilize the oocyte in vivo.

To determine the longevity of the sexed sperm, several characteristics related with sperm viability were assessed by flow cytometry, at different time points over 12 h of culture. The greatest decrease in sperm viability was noted for all groups after the first 2 h of incubation. This rapid loss was expected, since similar results of sperm viability after thawing have been reported in cattle (Alomar et al., 2006) and horses (Choi et al., 2003).

After 12 h of incubation, the majority of the sperm parameters evaluated was similar between treatments. However, the percentage of sperm with mitochondrial membrane potential, stable or destabilized plasma membrane remained dissimilar between the NS and XY groups. Among those parameters, the stability of plasma membrane, assessed by M540, had the greatest difference during all incubation times. It is interesting to note that at 2 h of incubation a decrease in plasma

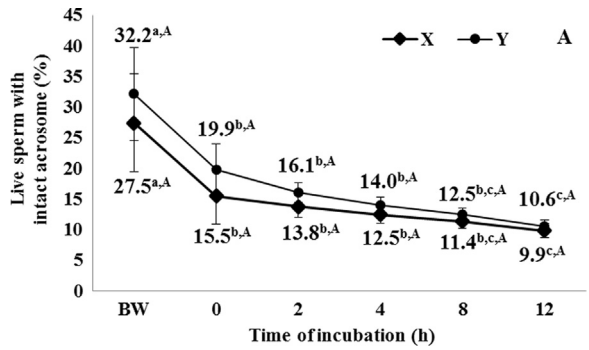

Fig. 7. Percentage of live sperm with reacted acrosome of X-bearing sperm (X) and Y-bearing sperm (Y) after thawing (before washing, BW), after washing (0) and 2, 4, 8 and 12 h of incubation in synthetic oviduct fluid at 39 °C with 5% CO_2 in air. Data are the average of four bulls. ^{a,b,c,d}Differences between each time of incubation for each group ($P < 0.05$). ^{A,B}Differences between groups for each incubation time ($P < 0.05$).

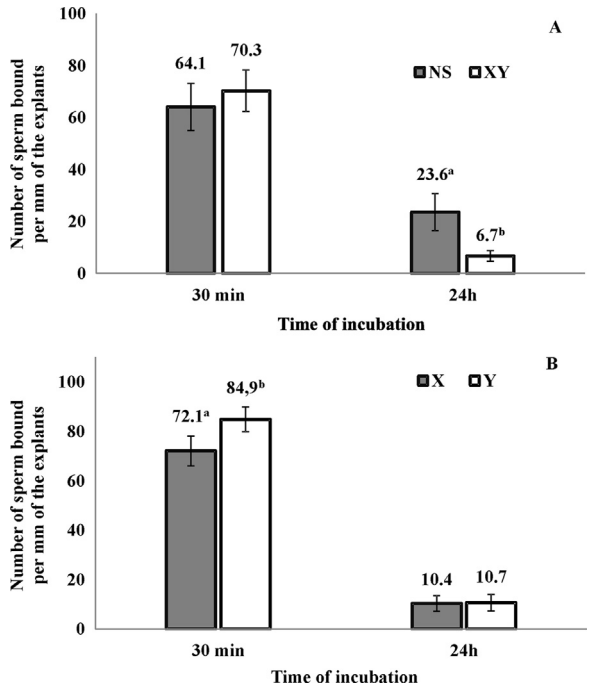

Fig. 8. Number of sperm bound per mm of oviductal explant cells after 30 min or 24 h of co-incubation of the explants with non-sexed sperm (NS) and pool of sexed X and Y sperm (XY; A); or X-bearing sperm (X) and Y-bearing sperm (Y; B). Data are the average of four bulls. ^{a,b}Differences between groups within each incubation time ($P < 0.05$).

membrane destabilization was observed for the XY group, while an increase in the NS group was observed. Conversely, the XY group had already begun the incubation period with a high population of cells with destabilized plasma membrane. Then, within the first 2 h of incubation, part of that population migrated to the population of non-viable sperm, reducing the percentage of cells with destabilized membrane. The M540 dye can be used to monitor the scrambling level of the phospholipids of the plasma membrane lipid bilayer. Plasma membrane which have more lipid disorders (Gadella and Harrison, 2000) have an increase in the intensity of fluorescence. Such disorders are indicative of membrane destabilization, similar to what occurrs during the first steps of capacitation (Flesch and Gadella, 2000). Although, there are other methods to assess changes in sperm plasma membrane, we chose M540 because it has been routinely used to evaluated status of capacitation and lipid membrane disorder (Bergqvist et al., 2006; Caballero et al., 2009; Schmid et al., 2013). Therefore, high percentage of sexed sperm with destabilized plasma membranes could be an indication that the sexing procedure induced the beginning of the capacitation process or induced changes in the plasma membrane, which resembles physiological changes for capacitation. However, to be sure if those alterations were, in fact, capacitation or capacitation-like changes, additional analysis should be performed. Nevertheless, the main significance of those findings is that when those changes occur, sperm lifespan is reduced, impairing fertilization due to the reduced number of viable sperm.

Taking these results together, we can assume that the survival time was similar for sexed and non-sexed sperm, since the decrease in most sperm variables was similar between NS and XY groups, even though the XY group had lower viability during most of the incubation time. It is important to point out that the sperm samples that we used had different volumes. The XY were cryopreserved in 0.25 mL and NS in 0.5 mL straws. Although one could raise the hypothesis that the volume of the samples could influence some sperm characteristics, we think that it would be irrelevant in face of the possible effects of the sexing process. Moreover, the volume of 0.25 or 0.5 mL used for bovine semen cryopreservation is very well characterized and widely used in several studies. Furthermore, all semen preparations were performed by a commercial company, similar to when it is prepared for sale.

Considering the differences found in the viability of sexed and non-sexed sperm, one could suppose that increasing sperm concentration in the straw for AI might compensate for the reduced quality of sexed sperm, improving fertility in vivo. However, Dejarnette et al. (2011) used 2.1 or 10×10^6 sperm per dose of sexed or non-sexed sperm in AI, and observed higher pregnancy rates per AI for non-sexed sperm than sexed sperm, regardless of the concentration used. According to these authors, factors other than concentration may be responsible for the lower pregnancy rates per AI obtained when sexed sperm is used. Therefore, we proposed that the sexing process could alter not only the lifespan but also the ability of sperm to bind to oviductal cells, which occurs during sperm reservoir formation. To test this hypothesis, we evaluated the number of sperm bound per mm of oviductal explants after 30 min or 24 h of co-incubation.

Similar analysis has been performed on sexed sperm of rams (Hollinshead et al., 2003; de Graaf et al., 2006) with conflicting results. While de Graaf et al. (2006) did not find differences between the number of sexed and non-sexed sperm bound to oviductal cell, Hollinshead et al. (2003) showed that sorted sperm were released more rapidly from oviductal cells than non-sorted sperm. However, those studies used oviductal epithelial cell monolayer, instead of oviduct explants. It is very well known that when oviductal cell are cultured in monolayer, they lose several structural and biochemical characteristics (Gualtieri et al., 2012), not being the type of culture to be used when functional tests are performed. A variety of studies (Lefebvre et al., 1997; Petrunkina et al., 2001; Gwathmey et al., 2003; Kadirvel et al., 2012) has tested the ability of sperm to bind to oviduct explants using co-incubation time of 15–30 min. However, in vivo, the bovine sperm must remain bound to the oviductal cells for several hours (Druart, 2012) before ovulation. In this regard, De Pauw et al. (2002) identified a positive correlation between the number of sperm bound to oviduct explants after 24 h of sperm co-incubation and non-return rate in cattle. Therefore, we chose to examine not only the ability to bind to explants after 30 min of co-incubation, but also the ability of sexed sperm to remain bound until 24 h of co-incubation. We have shown that although sexed sperm are able to bind to oviductal cells until 24 h of incubation with oviductal cells, fewer sexed sperm were observed bound per mm of oviduct explants compared with NS sperm. The difference in binding abilities between sexed and NS sperm could be due to greater sperm damage, a higher percentage of dead sperm and/or higher percentage of sperm with plasma membrane destabilized and reacted acrosome. Sperm reservoir formation is dependent on the presence of sugars and proteins in the sperm membrane (Green et al., 2001; Gwathmey et al., 2003; Foye-Jackson et al., 2011; Kadirvel et al., 2012), which are lost after capacitation and acrosome reaction (Gwathmey et al., 2003).

Based on the high number of sperm with plasma membrane destabilized, we could suggest that the sexing process induced the capacitation or capacitation-like changes, which would reduce sperm lifespan. However, we do not know the real cause of the lower number of sexed sperm bound to the explants after 24 h of co-incubation. Although this study was done on an in vitro model, we could assume that these results indicate that a smaller number of viable sperm are present in the oviduct reservoir at the time of ovulation. Moreover, there is evidence that the sperm interaction with oviductal cells leads to changes in the oviductal transcriptome (Fazeli et al., 2004; Kodithuwakku et al., 2007) and proteome (Georgiou et al., 2005, 2007; Artemenko et al., 2014) profiles, preparing the oviduct to receive the embryo. These findings could explain the higher number of unfertilized oocytes (Sartori et al., 2004; Schenk et al., 2006; Peippo et al., 2009) or lower quality embryos (Schenk et al., 2006; Peippo et al., 2009; Larson et al., 2010) reported when sexed sperm were used.

In addition to comparing sexed and NS sperm, we also verified whether differences existed between the X and Y chromosome-bearing sperm concerning their viability during culture and their ability to bind to oviductal cells. Many studies have not found differences between X and Y sperm regarding several sperm characteristics and fertilizing ability (Morton et al., 2007; Bermejo-Alvarez et al., 2008; J.d.O. Carvalho et al., 2009; J.O. Carvalho et al., 2010). However, in the present study, a lower longevity of up to 4 h of incubation was observed for the X sperm in relation to the Y sperm. This finding is consistent with the work of Van Dyk et al. (2001), that observed a lower number of X sperm bound to the hemizona pellucida after 48 h of incubation with X or Y sperm. These authors concluded that a different functional survival existed between X-bearing and Y-bearing spermatozoa, with the Y sperm exhibiting longer survival under in vitro conditions. Moreover, the difference in the percentage of living sperm with reacted acrosomes, could be responsible for the higher number of Y sperm bound to the oviduct explants after 30 min of co-incubation. In this regard, Alminana et al. (2014) found that the X or Y spermatozoa did indeed induce sex-specific transcriptomic responses in the oviduct. This could be due to the difference in the ability of the X or Y sperm to bind to the oviductal cells.

In conclusion, our study has shown that flow cytometry sex sorting affects the structural characteristics of sperm cells. Moreover, although XY presented lower quality than NS sperm at thawing, the decreases in quality in both NS and XY groups were similar between groups during incubation. It was also shown that the sexing process compromises the capacity of sperm to remain bound to the oviduct cells explants.

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Conflict of interest

None.

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