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ScienceDirect
Theriogenology 74 (2010) 1521–1530
Theriogenology
www.theriojournal.com

Review

Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in *in vitro* embryo production

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Received 26 November 2009; received in revised form 5 June 2010; accepted 23 June 2010

Abstract

The objective was to evaluate the structural and functional quality of bull sperm after sexing by flow cytometry. Frozen non-sexed (NS), sexed for X (SX) and sexed for Y (SY) sperm from four bulls was used. Frozen-thawed sperm was analyzed for motility, sperm head agglutination, morphology, capacitation, and integrity of the plasma membrane, acrosome, and chromatin. After Percoll centrifugation (45:60% gradients), the pellet was used for sperm analysis or IVF. Data were analyzed using generalized linear models ($P < 0.05$) and were reported as least squares means ± standard error (SEM). Based on sperm evaluations, NS sperm had better ($P < 0.05$) quality than sexed sperm, including higher motility and greater percentages of cells with an intact membrane and acrosome (58.0 ± 3.0, 58.2 ± 3.0, and 60.9 ± 3.3) than SX (29.6 ± 1.3, 36.0 ± 2.9, and 37.1 ± 3.3), and SY (26.2 ± 2.1, 36.4 ± 2.9, and 37.5 ± 3.3). There were no differences ($P > 0.05$) among groups for fertilization and cleavage rates. Similarly, blastocyst rate on Day 8 (Day 0 = day of insemination) did not differ among groups (22.2 ± 3.2, 18.1 ± 3.3, and 14.8 ± 2.9 for NS, SX, and SY, respectively). Regarding embryo development kinetics, all groups had similar developmental stages from Days 6 to 9. Although the sex-sorting procedure affected sperm characteristics, it did not significantly affect fertilization or embryo development.

Keywords: Sexed semen; Semen sorting; Gender preselection; In vitro fertilization; Bull

Contents

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doi:10.1016/j.theriogenology.2010.06.030

1. Introduction

The only proven method for sex-sorting of sperm, which is effective for commercial use, is fluorescence-activated cell sorting using flow cytometry [1]. Although sexed sperm can have a great impact on breeding programs, the high cost and reduced pregnancy rates especially for artificial insemination (AI), limit application in cattle breeding. Indeed, lower pregnancy rates or embryo production in AI and embryo transfer (ET) programs using sexed sperm by flow cytometry have been well documented [2–8]. Moreover, there was higher pregnancy loss for sexed sperm compared to non-sexed sperm [4,8]. There is speculation that the sexing process damages sperm, perhaps due to exposure to the laser, the high velocity inside the collecting tube, electric charges, and room temperature before being processed [9].

Although the efficiency of *in vitro* embryo production (IVP) using sexed sperm has improved, with blastocyst rates as high as 45% [10], in the majority of the studies the mean rate was ~25% [11–14]. Nevertheless, IVP was considered a valuable and meaningful method to use sex sorted sperm.

Since sexed sperm are submitted to a variety of adverse conditions during sorting, an evaluation of the possible damages caused by the sexing process is needed. It is expected that this will help to develop procedures to improve results when using sexed sperm.

The majority of the studies with sexed sperm evaluated a specific feature such as their motility [15,16], and integrity of the DNA [17,18] and acrosome [19]. In some studies, only features of embryos produced with sexed sperm were assessed, e.g. gene expression [14,20] and embryo ultrastructure [21]. To our knowledge, there are only a few studies [13] that evaluated the various structural characteristics of the sperm and its ability to produce embryos *in vitro*. Furthermore, there are apparently no studies that compared sexed for X and Y with non-sexed sperm obtained from the same ejaculate from several bulls. It is noteworthy that this approach enables comparison of kinetics and developmental potential of male and female embryos produced *in vitro*, which is a controversial issue in *in vitro* embryo production systems [22–25].

The objectives of the present study were to investigate sperm structural and functional quality after sexing by flow cytometry and, additionally, the kinetics of development of male and female embryos generated from oocytes fertilized with sexed sperm.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated all chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Experimental design

The sperm used in this study were obtained from four Nellore bulls. One ejaculate from each bull was collected. Each ejaculate was divided into three fractions; one fraction was used as the non-sexed (NS) sperm group, whereas the other two were submitted to flow-cytometry and sorted for X (SX) and Y (SY) bearing fractions. Thereafter, each of the three fractions were placed in straws and frozen by the same procedure. Sexed and non-sexed sperm from each bull was used for quality assessment before and after Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) selection and/or for IVF, which were repeated three times in independent replicates. In each replicate, a straw from each group (NS, SX, and SY) was concurrently thawed and evaluated. The

number of sperm per straw was 35.0 ± 2.7 × 10^6, 2.4 ± 0.8 × 10^6, and 2.2 ± 0.4 × 10^6 for unsorted, X-bearing, and Y-bearing sperm samples, respectively.

2.3. Semen collection, preparation, and sorting

Semen was collected with an artificial vagina from four sexually mature Nelore bulls. Sperm was checked for motility and morphology; only ejaculates with >60% motility and <20% morphological abnormalities were used. The proportion of semen designated for immediate freezing was diluted in a Tris-base freezing diluent with 4% egg yolk, cooled at 4 °C for 90 min, and then diluted with the Bioxcell® (IMV, L'Aigle, France). Sperm were loaded into 0.5 mL straws (IMV, L'Aigle, France) and frozen in a programmable freezer (TK 3000®, TK, Uberaba, MG, Brazil). At the conclusion of the freezing program, straws were plunged into liquid nitrogen and stored there until retrieved for thawing.

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

A high-speed cell sorter (MoFlo SX, Beckman Counter, CA, USA) was operated at 40 psi with a diode pumped solid-state pulse laser (Vanguard 350 HMD-355; Spectra Physics, Mountain View, CA, USA) at 125 mW, with bovine sheath fluid (CHATA Biosystems Inc., Fort Collins, CO, USA). Gates were set to attain 90% purity, and sexed sperm were sorted into Tris I medium. After being cooled at 4 °C for 1 h and 30 min, the sexed sperm was centrifuged and diluted in Bioxcell® (IMV, L'Aigle, France). Semen was packaged in 0.25 mL straws and frozen as described above.

2.4. Sperm quality assessment

2.4.1. Assessment of motility, morphology, head aglutination and sperm concentration

The percentage of motile sperm and progressively motility was determined with a CASA system (Sperm Analysis System, Ivos-Ultimate 12's, Hamilton Thorne Biosciences, Beverly, MA, USA). For this assessment, 2 μL of sperm were placed in pre-warmed glass-slide (Leja® standard count, SC20.01.04.B, 20 μm, Leja Product B.V., the Netherlands). At least seven fields were selected for reading and analysis.

Sperm morphology was evaluated, according to Barth and Oko [26], using a phase contrast microscope (1 000× magnification). A total of 200 cells were counted and the results were expressed in percentages.

To evaluate head-to-head agglutination, 200 cells were counted at random under phase contrast microscopy at 400× magnification. For this analysis, head-to-head agglutination was considered when the head of two or more cells where grouped, as described by Maiti et al [27]. Results were expressed in percentage of head-to-head agglutinated cells.

Sperm concentration was determined with a hemocytometer (Neubauer chamber) after a 1:20 dilution. The recovery rate was calculated as described by Machado et al [28],using the following formula: (final concentration × final volume) × (initial concentration × initial volume)$^{-1}$ × 100.

2.4.2. Assessment of capacitation

Capacitation status was evaluated using chlortetracycline (CTC) staining, as described by Cormier et al [29], with slight modifications. The CTC stock solution contained 0.75 mM CTC, 20 mM tris and 5 mM DL-cysteine and was prepared daily; 300 mL of CTC stock solution was mixed with 10 μL of 12.5% paraformaldehyde in 20 mM tris and 60 μL of 0.22 M 1,4 diazabiciclo [2.2.2] octane (Invitrogen Molecular Probe®, Eugene, OR, USA). The final CTC stain solution (15 μL) was added to the sperm (10 μL) and the stained suspension (5 μL) was placed on a slide, covered with a coverslip, and observed under epifluorescence microscopy (400×, Axiophot Zeiss®; barrier filter 440/470 nm excitation/emission). A total of 200 sperm were counted and classified into three groups, as decribed by Fraser et al [30]: bright fluorescence over the whole head (uncapacitated cells, F pattern); fluorescence-free band in the postacrosomal region (capacitated cells, B pattern); and full fluorescence over the whole head except for a thin, bright band of fluorescence along the equatorial region (acrosome-reacted cells, AR pattern).

2.4.3. Assessment of plasma membrane and acrosome integrity

Sperm membrane integrity was assessed using 6-carboxy-fluorescein diacetate (C-FDA; propidium iodide [PI], Invitrogen Molecular Probe®, Eugene, OR, USA), as described by Harrison and Vickers [31]. An aliquot of sperm (10 μL) was added to the stain solution (40 μL) and incubated for 10 min. The stain solution was composed of buffered formal saline (96 mL of 0.9% saline solution and 4 mL of 40% formal), sodium citrate (3%), PI (0.75 mM), and C-FDA solution (0.46 mg/mL solution in dimethyl sulphoxide). An

aliquot (5 μL) of stained suspension was placed on a slide, covered with a coverslip, and observed under an epifluorescence microscope (1 000X; Axiophot Zeiss®; barrier filter 494/517 nm excitation/emission and 536/617 nm excitation/emission). For each slide, 200 sperm were counted and classified as having an intact or damaged plasma membrane. Cells stained green with C-FDA were considered to have an intact membrane, whereas those stained red with PI were deemed to have a damaged membrane.

Acrosome status was examined using fluorescent probe isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and PI, as described by Klinc and Rath [32]. An aliquot (10 μL) of a thawed sperm sample was diluted with staining solution (30 μL) and incubated for 10 min. The staining solution consisted of buffered formal saline, sodium citrate (3%), PI (0.75 mM), and FITC-PNA solution (1 mg/mL in PBS). An aliquot (5 μL) of stained suspension was placed on a slide and covered with a coverslip. At least 200 sperm were examined under a phase contrast and epifluorescence microscope (1 000 X; Axiophot Zeiss®; barrier filter 484/518 nm excitation/emission). Sperm labeled in red with PI were considered alive. Living cells were classified as acrosome reacted, if the acrosome had uniform FITC-PNA green fluorescence, or as acrosome intact, if no fluorescence was visible.

2.4.4. Assessment of chromatin integrity

Chromatin integrity was determined using the acridine orange staining procedure. Three smears were prepared for each sample, air-dried, and fixed overnight in freshly prepared fixation solution (methanol:acetic acid, 3:1). Then, the slides were again air-dried and incubated in a solution containing 80 mmol/L citric acid and 15 mmol/L Na2HPO4 (pH 2.5) at 75 °C for 5 min to test chromatin stability. Subsequently, slides were stained for 5 min with a solution containing acridine orange stain (0.2 mg/mL), citric acid (0.1 M) and disodium phosphate (0.3 M), and analyzed within a few hours, using an epifluorecense microcope (Axiophot Zeiss®: barrier filter 490/530 nm excitation/emission). For all samples, 500 cells were examined on each slide at 1,000 × magnification. Sperm with normal DNA had green fluorescence, whereas those with abnormal DNA emitted fluorescence in a spectrum varying from yellow-green to red.

2.5. Embryo production

2.5.1. Oocyte recovery and in vitro maturation (IVM)

Ovaries from crossbred cows (*Bos indicus* × *Bos taurus*) 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. Cumulus oocyte complexes (COC) were aspirated from 2 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 mL conical tube. After sedimentation, COC were recovered and selected using a stereomicroscope. Only COC with homogenous cytoplasm and at least three layers of cumulus cells were used. The selected COC were washed and transferred (groups of 25 to 30) to a 200 μL drop of maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% CO_2 in air. A total of 2,271 COC were matured *in vitro* throughout the experiment.

2.5.2. Sperm selection procedures

To have a concurrent control of the laboratory IVP system, a fifth group was added to each replicate. In this group, oocytes were fertilized with sperm from a control bull (CB) of known *in vitro* fertility, which has been used for several years as the reference bull for *in vitro* embryo production in our laboratory.

For the CB group, sperm selection was performed by centrifugation of the semen samples in a total volume of 800 μL, comprised of 400 μL of 45% Percoll gradient and 400 μL of 90% Percoll gradient, placed in a 2-mL microtube, and centrifuged for 5 min at 5 400 × g, as previously described [28]. For sexed (SX and SY) and non-sexed control sperm (NS), a modified Percoll selection method described by Blondin et al [13], was used. Briefly, thawed sperm was layered on a top of a gradient composed of 0.4 mL fraction each of 45% and 60% Percoll and centrifuged for 5 min at 700 × g. After centrifugation, the supernatant was discarded and the pellet was centrifuged for 2 min in fertilization media at 300 × g (NS, SX and SY) or 5 min in sp-TALP at 700 × g (CB). Then, the resultant pellet was re-suspended with fertilization medium and used for sperm analyses or IVF. In each replicate, one straw from each group was used.

2.5.3. In vitro fertilization (IVF) and embryo culture (IVC)

Following maturation, COC were transferred to a 50 μL (NS, SX, and SY) or 200 μL (CB) drop of fertilization medium. The sperm concentration after Percoll selection was determined for each group using a hemocytometer and then, added into the fertilization drop in a final concentration of 1×10^6 sperm/mL. Sperm and oocytes were co-incubated for 18 h at 39 °C with 5% CO_2 in air; the day of *in vitro* insemination was considered Day 0. After co-incubation, presumptive zygotes were washed and transferred to 200 μL drops of SOF medium supplemented with 5% fetal calf serum (FCS), and cultured at 39 °C and 5% of CO_2 in air for 9 d. Embryos were evaluated on Day 2, (48 h post-

Table 1
Percentage (least squares mean ± SEM) of various characteristics of non-sexed (NS), sorted for X (SX), and sorted for Y (SY) bull sperm before passage through a Percoll gradient.

Sperm	Motility	Progressive motility	Normal morphology	Agglutinated head	Intact membrane	Live sperm with intact acrosome	Intact chromatin
NS	58.0 ± 3.0[a]	8.3 ± 1.2	89.6 ± 0.8	2.6 ± 0.4	58.2 ± 3.0[a]	60.9 ± 3.3[a]	98.7 ± 0.1
SX	29.6 ± 1.3[b]	9.3 ± 0.9	91.2 ± 0.7	1.4 ± 0.3	36.0 ± 2.9[b]	37.1 ± 3.3[b]	98.6 ± 0.1
SY	26.2 ± 2.1[b]	6.8 ± 1.1	88.9 ± 0.8	0.8 ± 0.2	36.4 ± 2.9[b]	37.5 ± 3.3[b]	99.5 ± 0.1

Average of four bulls (three replicates per bull).
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

insemination) for cleavage, and on Days 6, 7, 8, and 9 for rates of blastocyst formation.

2.5.4. Assessment of fertilization rate and kinetics of blastocyst development

To evaluate fertilization rate, 294 embryos/ova were used. Presumptive zygotes were removed from culture 18 h post-insemination, fixed with acetic acid:alcohol (1:3), and stained with 1% solution of lacmoide in 45% glacial acetic acid. Then, they were examined under phase contrast microscopy (Nikon Eclipse E200, 1,000 X) and classified as: a) nonfertilized (presence of female and absence of male chromatin); b) fertilized (presence of female and sperm chromatin in the cytoplasm, decondensed sperm head, pronucleus or cleaved); or c) fertilized with polyspermia (presence of female chromatin and two or more decondensed sperm heads and/or pronuclei).

The kinetics of male and female embryo development was evaluated by the speed that they reached specific blastocyst stages. To measure these events, we used a system described by Souza et al [33], in which each embryo stage was graded as: 5 = initial blastocyst, 6 = blastocyst, 7 = expanded blastocyst, and 8 = hatched blastocyst (according to the International Embryo Transfer Society). Then, on each day of evaluation (Days 6, 7, 8, and 9), the average embryo stage was calculated.

2.6. Statistical analysis

Data were analyzed utilizing the theory of generalized linear models (PROC GLIMMIX; SAS 9.1, SAS Inst. Inc., Cary, NC, USA). Adjustment of the model 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.

The model considered the fixed effects of bull as block, treatment, time, and treatment*time, as well as the effect of repeated measures in the subject bull*treatment, with a first order autoregressive covariance structure beyond the random residual effect.

Signficant differences between treatments were determined with least square means and were reported as least squares means ± standard error (SEM) in original scale, using the inverse link option for ease of interpretation. The least square means were compared using a Student's t-test, at a significance level of $\alpha = 0.05$.

3. Results

3.1. Sperm quality assessment

Sperm characteristics of the different groups before and after Percoll selection are shown (Tables 1 and 2). Both before and after Percoll selection, SX and SY groups were not significantly different for all sperm characteris-

Table 2
Percentage (least squares mean ± SEM) of various characteristics of non-sexed (NS) sorted for X (SX), and sorted for Y (SY) bull sperm after passage through a Percoll gradient.

Sperm	Motility	Progressive motility	Normal morphology	Agglutinated head	Capacitated	Intact membrane	Live sperm with intact acrosome	Intact chromatin	Recovery rate
NS	70.4 ± 2.9[a]	38.5 ± 3.0[a]	92.8 ± 0.7	22.6 ± 2.9	30.5 ± 13.4	65.9 ± 2.9[a]	61.0 ± 3.3[a]	98.7 ± 0.1	47.9 ± 4.0
SX	37.1 ± 4.0[b]	10.3 ± 2.4[b]	94.9 ± 0.6	27.2 ± 2.5	34.7 ± 13.8	39.6 ± 3.0[b]	37.8 ± 3.3[b]	98.7 ± 0.1	52.7 ± 3.9
SY	43.4 ± 3.2[b]	13.2 ± 1.4[b]	93.5 ± 0.7	25.1 ± 2.5	32.4 ± 13.6	41.3 ± 3.0[b]	40.4 ± 3.3[b]	98.5 ± 0.1	48.7 ± 4.3

Average of four bulls (three replicates per bull).
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

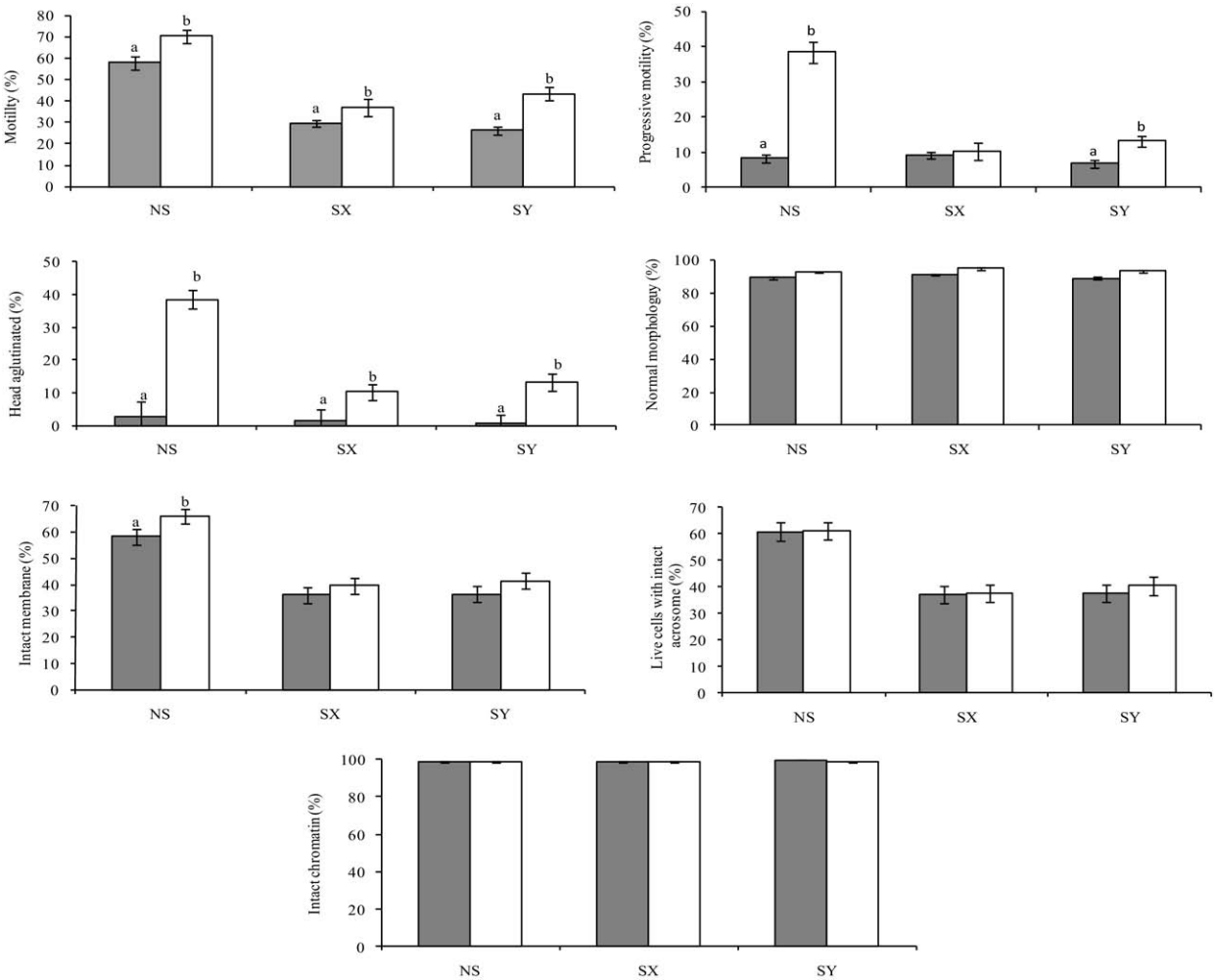

Fig. 1. Percentage (least squares mean ± SEM) of various aspects of sperm of non-sexed (NS), sorted for X (SX), and sorted for Y (SY) bull sperm, before and after passage through a Percoll gradient (shaded and non-shaded columns, respectively).

[a,b]Difference between pre- and post-Percoll gradients within each treatment group ($P < 0.05$).

tics evaluated. However, there was an effect of sperm sexing of various sperm quality end points after thawing (Table 1) and after Percoll selection (Table 2).

To evaluate the effect of Percoll on sperm characteristics in each group, an analysis comparing each variable before and after Percoll selection was performed (Fig. 1). There was no significant effect of Percoll for percentage of cells with normal morphology and with intact chromatin. However, for all groups, there were post-Percoll increases for motility and head agglutination.

There were no significant changes in percentage of sorted sperm (X and Y) with an intact membrane and an intact acrosome after Percoll separation (Fig. 1). However, passage through the Percoll gradient increased the percentage of non-sexed sperm with an intact membrane.

3.2. Fertilization rate

There was no significant differences in percentages of fertilized oocytes among NS (63.2 ± 5.7), SX (67.2 ± 5.7) and SY (55.8 ± 5.9) groups. Regarding fertilized oocytes, the stage of the fertilization process at 18 hpi was similar for all groups, with the majority of the oocytes having a female and a male pronucleus (Fig. 2).

3.3. Embryo production

There was no significant difference between sexed and non-sexed sperm in cleavage rate (Table 3). Furthermore,

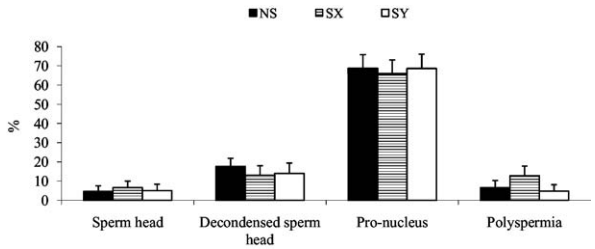

Fig. 2. Distribution (least squares mean ± SEM) of male chromatin stage in zygotes 18 h post-insemination, using non-sexed (NS), sorted for X (SX) and sorted for Y (SY) bull sperm after Percoll selection ($P > 0.05$).

blastocyst production on Days 6, 7, 8, and 9 of culture was not significantly different among groups. When the blastocyst rate was calculated in relation to the number of cleaved oocytes, the same pattern for embryo production was observed (Table 4).

For IVP using the control bull, the cleavage rate was 80.3 ± 4.1% and the blastocyst production on Days 6, 7, 8, and 9 of culture was 18.5 ± 3.2, 38.8 ± 3.9, 43.7 ± 4.0, and 44.5 ± 4.1%, respectively.

Regarding the kinetics of embryo development, there was no significant difference among groups in the speed that embryos reached the various stages of blastocyst development on Days 6, 7, 8, and 9 (Fig. 3).

4. Discussion

In the present study, structural and functional viability of sperm sexed by flow cytometry were evaluated using sperm cell characteristics and their ability to fertilize oocytes and to produce embryos *in vitro*. The use of non-sexed, X-sexed, and Y-sexed sperm from the same ejaculate of each bull facilitated comparisons of the kinetics of *in vitro* development of male and female embryos. The sexing process did not reduce *in vitro* embryo production, and male and female embryos had similar developmental kinetics.

As expected, sexed sperm had lower quality than the non-sexed after thawing, with lower motility and percentages of cells with an intact membrane and an intact acrosome. The reduction in sperm quality in the present study could have been caused, for example, by exposure to Hoechst 33342 stain, the laser light, or exposure in the droplets to electric charges, as previously discussed [16,34,35]. According to Smith [36], the effect of exposure to dye and then the laser may reduce mitochondrial activity in bovine oocytes. This type of damage could explain the decreased motility of sexed sperm, since mitochondria produce ATP as an energy source for sperm [37].

Another physical characteristic affected by the process of sexing was integrity of the membrane. This change may have been due to mechanical stress [35]; in that regard, decreased pressure during the process of sexing increased the survival of sexed sperm, and consequently rates of fertilization [16] and pregnancy [38].

Although there were some indications that exposure of sperm to UV radiation during sexing by flow cytometry affected chromatin integrity [17,18], there was no significant difference between non-sexed and sexed sperm for that end point. This result, based on a staining technique, was confirmed with IVF data, in which rates of sperm head decondensation, cleavage, and blastocyst formation were not affected by sexing. Perhaps bovine sperm are somewhat less sensitive to UV exposure than sperm from other mammals, due to their greater content of protamine in the DNA [39], which augments chromatin stability. In that regard, DNA which was more tightly packed could account for the apparent lack of damage in the sexed-sperm chromatin. However, the possibility that the deleterious effects of the exposure to UV would be manifested only at later stages of development (i.e. after blastocyst formation) cannot be excluded.

The lack of post-thaw differences between sex-sorted and unsorted sperm were maintained after Percoll selection. The most noticeable changes observed in all groups after Percoll selection was for motility and percentage of head-to-head agglutination. The effect of Percoll in selecting sperm with higher motility has been

Table 3
Least squares mean (± SEM) rates of cleavage and blastocyst production (%) on Days 6, 7, 8, and 9 of culture (after IVF) using non-sexed (NS), sorted for X (SX), and sorted for Y (SY) bull sperm after Percoll selection.

Sperm	No. oocytes	Cleavage	Blastocysts			
			Day 6	Day 7	Day 8	Day 9
NS	516	55.6 ± 3.6	5.9 ± 1.7	19.3 ± 3.0	22.2 ± 3.2	23.1 ± 3.2
SX	431	54.0 ± 4.2	5.5 ± 1.8	16.4 ± 3.1	18.1 ± 3.3	20.3 ± 3.3
SY	481	45.2 ± 4.0	3.4 ± 1.4	12.0 ± 2.6	14.8 ± 2.9	15.9 ± 3.4

Table 4
Least squares mean (± SEM) blastocyst production (%), in relation to the number of cleaved oocytes, on Days 6, 7, 8, and 9 of culture (after IVF), using non-sexed sperm (NS), sorted for X (SX), and sorted for Y (SY) bullsperm after Percoll selection.

Sperm	No. cleaved oocytes	Blastocysts			
		Day 6	Day 7	Day 8	Day 9
NS	281	10.7 ± 3.0	35.9 ± 5.1	41.7 ± 5.3	43.5 ± 5.3
SX	228	10.6 ± 3.5	31.9 ± 5.5	35.3 ± 5.6	39.7 ± 5.8
SY	215	7.5 ± 3.1	26.6 ± 5.4	32.9 ± 5.8	35.4 ± 5.9

well documented [28,40–44]. It is noteworthy that there were no significant differences between unsorted sperm treatments in the magnitude of motility improvement.

The increase in sperm head-to-head agglutination, which is related to sperm capacitation, could very easily have been induced by Percoll selection [42,44]. This assumption was supported by the great magnitude of post-Percoll increase in this end point for all groups. Passage through a Percoll gradient can alter the plasma membrane, causing membrane destabilization and capacitation [43]. However, that hypothesis was not confirmed in the present study since a CTC technique was not done before Percoll selection, due to the presence of cryoprotectant and diluents in the samples. Although the percentage of capacitated cells was similar for all groups, the percentage of cells with an intact acrosome was higher in non-sexed than in sexed sperm fractions. An acrosome reaction is the final step of the capacitation event [13]; therefore, we inferred that capacitation can also be induced by sorting, which agrees with results reported by Maxwell et al [45].

The use of sexed-sperm did not reduce embryo development relative to unsorted-sperm; therefore, changes in sperm quality did not affect rates of fertilization or blastocyst formation. Similarly, Underwood [10], Peippo [46] and Gutiérrez-Adán [47] did not detect differences in blastocyst formation rates between sorted and non sorted sperm. However, in other studies [12,20,21], embryo development was reduced when sexed sperm was used for IVF. Perhaps variations in the methods of sperm selection contributed to these apparent differences. In that regard each study used a different protocol with various types and volumes of gradient, as well as varations in the duration and force of centrifugation.

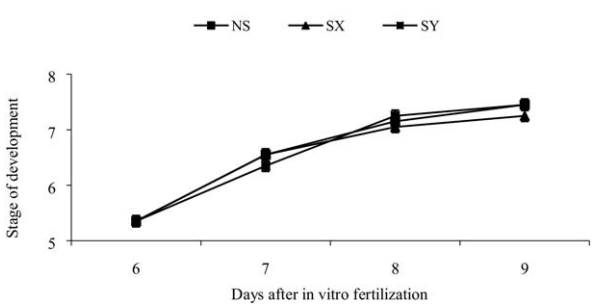

Fig. 3. Distribution (least squares mean ± SEM) of the stage of embryo development using non-sexed (NS), sorted for X (SX), and sorted for Y (SY) bull sperm after Percoll selection (no significant effect of group). Embryo development was coded as: 5 = initial blastocyst, 6 = blastocyst, 7 = expanded blastocyst, and 8 = hatched blastocyst.

Several studies have demonstrated that *in vitro* produced male bovine embryos developed faster than female embryos [22,25,47–50]. In the present study, there was no significant difference in blastocyst formation or the speed of blastocyst development between male and female embryos derived from sexed sperm. These results were supported by others [14,20,23,24]. Reported variation has been attributed to the effect of sire, IVF protocol, or culture system [50].

Overall, the present study clearly detected an effect of sperm sexing on sperm quality, but not on embryo development. Perhaps a decrease in motility and percentage of cells with intact membrane and acrosome caused by the sex-sorting procedure were not as critical to *in vitro* conditions as they are *in vivo*. Another possibility is that sex-sorting can cause sperm damage that was not detected by the evaluations performed in this study. In that regard, embryos derived from sex-sorted sperm had a higher proportion of immature mitochondria and damaged nuclear membranes [21], as well as reductions in expression of developmentally important genes [20], compared with their counterparts derived from unsorted sperm. These differences could explain the higher embryonic loss at 30–90 d of pregnancy, when sexed-sperm was used to inseminate cows and heifers [4,8]. Furthermore, some bulls are more adversely affected by the sexing process than others [13]. For commercial companies, the best way to improve fertility of sexed sperm is to monitor the results and preferentially sort semen from bulls with highest

fertility for sexed sperm. Considering that all samples used in the study were purchased from a commercial company, perhaps a previous selection had already been made and consequently, *in vitro* fertility was similar for sexed and non-sexed sperm. Finally, perhaps improvements in the sorting process achieved by the companies in the last years, e.g. changes in sorting speed, may have reduced sperm damage.

In conclusion, the sex-sorting procedure by flow cytometry affected some structural characteristics of bovine sperm, but did not reduce their capacity to produce embryos *in vitro*. Furthemore, male and female embryos produced by sorted sperm had similar development *in vitro*.

Acknowledgments

The authors thank Dr. Maurício Machain Franco for his thoughtful contribution. This research was supported by funding from Embrapa, Brazil.

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