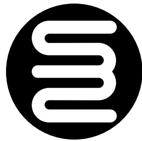

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Use of sex-sorted and unsorted frozen/thawed sperm and *in vitro* fertilization events in bovine oocytes derived from ultrasound-guided aspiration

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ABSTRACT - The objective was to investigate the effects of sex-sorting on early fertilization events in ovum pick up (OPU) derived oocytes fertilized *in vitro* with frozen-thawed sperm at different co-incubation lengths. Eighty-four OPU sessions were carried out in 18 cyclic, dry and non-stimulated Holstein Friesian and German black pied cows. Ovum pick up oocytes were matured *in vitro* for 24 hours and fertilized with frozen-thawed sex sorted or unsorted sperm from the same ejaculate. Fertilization was achieved by two experimental protocols: 1) short gamete co-incubation length: 4, 8 and 12 hours; and 2) long gamete co-incubation length: 18 and 24 hours. After *in vitro* fertilization, ova were fixed and stained to identify early fertilization events. Sperm penetration, monospermy, pronuclear formation and syngamy did not differ, whether sexed or unsexed sperm was used. Overall, the findings demonstrate similar fertilizing potential between sex-sorted and unsorted sperm.

Key Words: bovine IVF, co-incubation length, early fertilization, ovum pick up, sex-sorted sperm

Introduction

Separation of sperm into X- and Y-chromosome-bearing populations by flow cytometry is an emerging reproductive technology. However, a number of studies have reported deleterious effects on sperm due to the sorting process, which include reduced fertility *in vivo* (Schenk et al., 1999; Maxwell et al., 2003; Bodmer et al., 2005) and *in vitro* (Cran et al., 1993 and 1994; Merton et al., 1997; Lu et al., 1999; Xu et al., 2009) and abnormal embryo developmental patterns (Maxwell et al., 2004). Several causes have been proposed for the reduced fertility of sorted sperm, including lower spermatozoa concentrations (Bodmer et al., 2005), structural damages (Schenk & Seidel Jr., 2007) and differences between sires (Rath et al., 2009). In fact, *in vitro* assessment of flow cytometrically sorted spermatozoa has revealed altered patterns of motility when compared with unsorted spermatozoa (Suh & Schenk, 2003; Suh et al., 2005), higher proportion of capacitated spermatozoa (Maxwell et al., 1998), acceleration of the acrosome reaction (Mocé et al., 2006) and reduced lifespan (Hollinshead et al., 2003). Despite these issues, offspring of pre-determined sex have been produced using sex-sorted spermatozoa in both artificial insemination (AI) and *in vitro* fertilization (IVF) systems (Maxwell et al., 2004). Offspring derived from sex-sorted sperm were morphologically normal, in contrast to some reports (Seidel Jr. & Garner, 2002).

Prolonged gamete co-incubation during IVF results in the exposure of oocytes and embryos to high levels of reactive oxygen species (ROS) produced by spermatozoa (Baker & Aitken, 2004), which may have detrimental effects on embryonic development (Dirnfeld et al., 1999). Reducing the duration of gamete co-incubation has been reported to increase fertilization (Gianaroli et al., 1996a), blastocyst formation (Grupen & Nottle, 2000) and embryonic development (Gianaroli et al., 1996b). Events such as penetration (Hyttel et al., 1988; Johnson, 1991; Puglisi et al., 2004), monospermy (Xu & Greve, 1988; Morton et al., 2007; Alomar et al., 2008), pronuclei formation (Lin et al., 2000; Puglisi et al., 2004; Alomar et al., 2008) and syngamy (Maxwell et al., 2004; Machatkova et al., 2008; Rath et al., 2009) are key in evaluating fertilization.

The objective was to investigate the effects of sex-sorting on penetration, polyspermy, formation of pronuclei and syngamy of OPU-oocytes fertilized *in vitro* with sex-sorted sperm at various co-incubation lengths.

Material and Methods

All procedures involving animal experiments in this study were carried out in accordance with the German

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Animal Welfare Law. Fourteen cycling Holstein-Friesian cows and four European Black Pied cows (approximately 4 years-old) from the experimental herd of the Institute of Farm Animal Genetics (FLI, Mariensee, Germany) were used. Reproductive soundness was based on rectal palpation of uterus and ovaries. All cows had an average body condition score (BCS) of 6 (scale of 1-10).

The chemicals used in this study were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany), unless otherwise stated. Eighty-four OPU sessions were carried out at intervals of 3-4 days. Ovum pick up was performed according to procedures described by Zaraza et al. (2010). After aspiration of three to four follicles, follicular fluid was flushed with Dulbecco PBS medium containing 1% heat-inactivated fetal calf serum (Invitrogen, Karlsruhe, Germany), 50 IU/mL penicillin G, 50 µg/mL streptomycin sulphate (Serva, Heidelberg, Germany), 0.327 mM pyruvate, 5.55 mM D-glucose (Carl Roth GmbH, Karlsruhe, Germany), 0.9 mM $CaCl_2$ 2 H_2O (Merck, Darmstadt, Germany) and sodium heparin (2.2 IU/mL; Serva) to prevent clotting. The fluid aspirated from each donor was stored in a 50 mL conical tube in a 37 °C water bath; contents were filtered (50 µm; Jürgens, Hannover, Germany) and washed in fresh Dulbecco PBS to eliminate blood. Filter contents were then washed into a Petri dish (15036, Nunc, Denmark) and cumulus-oocyte complexes (COC) were retrieved under a stereomicroscope (Nikon, Düsseldorf, Germany).

From 84 OPU sessions, a total of 420 oocytes (5.11 per OPU session/animal) were obtained. The total number of viable oocytes (categories I, II and III) (Zaraza et al., 2010) was 356 (82.98% of the total recovered oocytes). The mean number of viable COC obtained by OPU session/animal was 4.24. The interval between puncture and isolation of individual COC was always under 30 min. Cumulus-oocyte complexes were collected with a micropipette (20 µL; Brand, Wertheim, Germany) and placed into individual droplets of TCM-air medium at 37 ºC for morphological assessment. TCM-air medium consisted of TCM 199 tissue culture medium, containing L-glutamine (2 mM) and Hepes (25 mM), supplemented with 0.2 mM pyruvate, 4.2 mM $NaHCO_3$ (Riedel-de Haën AG, Seelze, Germany), 50 µg/mL gentamycin and 0.1% fatty acid-free BSA.

Ejaculates were collected with an artificial vagina from one mature Holstein-Friesian bull of proven *in vitro* and *in vivo* fertility and only ejaculates with initial progressive motility of at least 75% were used. Semen was diluted to a concentration of 100×10^6 spermatozoa/mL with Sexcess® sample fluid (Masterrind, Verden, Germany) and were stained with 125 µg Hoechst 33342 and incubated for 1.25 h at 37 ºC. Stained samples were filtered through a 51 µm nylon mesh (Falcon 2235; Becton Dickinson, Franklin Lakes, NJ, USA) and 0.001% (w:v) food dye (Warner Jenkinson, Inc., St. Louis, MO, USA) was added.

Sperm were then separated into X- and Y-chromosome-bearing populations using a high-speed cell sorter (SX MoFlo®, Beckman-Coulter, FL, USA) modified for sperm sorting (Johnson & Pinkel, 1986; Rens et al., 1999), operating at 2.76 bar. Labelled spermatozoa were passed through an orienting nozzle (Johnson et al., 1999) and were illuminated with a 200 mW solid state UV-laser (Coherent Palladin, Coherent, USA). During flow cytometric sorting, gates were placed around viable and correctly oriented sperm to achieve purities greater than 92% in each of the enriched X- and Y-chromosome-bearing sperm populations. Sorted sperm were collected into 10 mL centrifuge tubes containing 500 µL of TEST catch medium (Johnson & Pinkel, 1986) supplemented with 20% (v:v) egg yolk.

Semen was cryopreserved using the Sexcess® treatment for sexed sperm. Briefly, sorted sperm in catch medium were centrifuged for 20 min at 800 x *g*. The supernatant was discarded and pellets were resuspended in Sexcess® cooling extender at 26.4×10^6 sperm/mL. Samples were then cooled in 2 steps to 5 ºC for 2 hours and diluted with Sexcess® freezing extender (Masterrind, Verden, Germany) to a concentration of 20.5×10^6 spermatozoa/mL (final glycerol concentration 6.4%). Resuspended sperm were then loaded into 0.25 mL straws (IMV, L'Aigle, France). Samples were then frozen in an automated freezer (IceCube, Minitub, Landshut, Germany). Sperm from the same ejaculate which had not been sorted were frozen as described above as controls. Reanalyses were performed by resorting of aliquots of sorted samples. Briefly, sperm were re-stained with ten-fold less concentrated DNA dye (Hoechst 33342) as for sperm sorting and were incubated at 37 °C for 30 min. Sperm were then sorted again at a sort rate of 60-80 events per second. Histogram data at a resolution of 256 channels were tested in a curve fitting program (Gaus 7, anonymous) to obtain the best fitting probability. Only samples with a purity of more than 92% were used for further experiments.

Immediately after filtering, COC were classified into five categories (Zaraza et al., 2010). Cumulus-oocyte complexes classified into categories I, II and III were considered suitable for in IVM/IVF. Before being placed into a maturation medium, COC were washed three times in TCM 199. The maturation medium consisted of TCM 199, containing L-glutamine (2 mM) and Hepes (25 mM);

supplemented with 0.2 mM pyruvate, 26.2 mM $NaHCO_3$, 50 µg/mL gentamycin, 0.1% fatty acid-free BSA, 10 IU/mL eCG and 5 IU/mL hCG (Suigonan, Intervet, Tönisvorst, Germany) and COC complexes were matured for 24 h at 39 ºC under 5% CO_2 in air.

For *in vitro* fertilization, COC were placed, after IVM, in 50 µL drops of Fert-TALP medium containing 10 µM hypotaurine, 0.1 IU/mL heparin (Serva), 1 µM epinephrine [HHE] and 6 mg/mL BSA (Fraction V). Straws containing frozen X-chromosome-bearing sperm and straws with unsorted sperm from the same ejaculates of the same bull were thawed at 30 ºC for 60 s, layered on 1 mL Bovipure® gradient (Bottom layer, Nidacom, Sweden) in an Eppendorf tube and centrifuged at 300 x *g* for 10 min and twice at 400 x *g* for 3 min. The final sperm concentration added per fertilization drop was adjusted to 1×10^6 spermatozoa/mL.

Gametes were co-incubated during 4, 8 and 12 hours (experiment 1) and 18 and 24 hours (experiment 2) at 37 °C, 5% CO_2 in humidified air under the same temperature and gas conditions described for maturation. After co-incubation, oocytes were fixed in ethanol:acetic acid (3:1, v/v), stained with 1% (w/v) lacmoid and examined at 400x magnification under a phase-contrast microscope (Olympus, Düsseldorf, Germany). The sequence of total sperm penetration into ooplasm, i.e., including the sperm tail, sperm head decondensation, completion of second meiotic division, and male and female pronuclear development, were recorded and classified microscopically in 6 developmental stages, according to Xu & Greve (1988). The experiment was performed in five replicates. Male and female pronuclei sizes were classified subjectively on a 1 (smallest) to 5 (largest) scoring scale by a single observer.

Two experiments were conducted in a completely randomized design, with the effect of sperm type (sex-sorted *vs*. unsorted) tested at 4, 8 and 12 hours of co-incubation in the first trial, and at 18 and 24 hours of co-incubation in the second trial. Data were analyzed by the GENMOD procedure (Statistical Analysis System, SAS®, version 10) with the binomial distribution option. Least square means were compared by orthogonal contrasts and, for statistical significance, the alpha level was set at $P<0.05$, unless otherwise specified. The proportions of penetrated oocytes were calculated in relation to the total matured oocytes, e.g., oocytes achieving metaphase-II only. Proportions of monospermic, female, male and syngamic pronuclei were calculated relative to the total oocytes penetrated. Yield is defined as the proportion of oocyte showing simultaneously monospermic penetration and female and male pronuclei (Yield 1) plus syngamy (Yield 2).

Results

Degenerate oocyte and germinal vesicle proportions ranged from 0.0 to 9.7% and from 0.0 to 3.2%, respectively, within 4 and 12 hours of co-incubation. The type of sperm did not influence ($P>0.05$) most of the variables studied (Tables 1, 2 and 3), except for metaphase-II, which, at 4 hours of co-incubation, was greater ($P = 0.03$). Some features had frequencies equal to zero, e.g., germinal vesicle at 4 and 12 hours, and therefore could not be analyzed.

Proportions of penetrated, polyspermic and female pronucleus bearing oocyte were not influenced by semen type at 12 hours of co-incubation (Table 4). On the other hand, the proportion of oocytes with a male pronucleus and Yield 1 were greater ($P = 0.02$ and $P = 0.04$, respectively) for the sex-sorted semen. At 4 and 8 hours of co-incubation there was no semen effect.

Table 1 - Effect of semen type on early fertilization events after 4 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
n	32	31	
Degenerate	6.3±3.3 (2/32)[1]	0.0±3.3 (0/31)	1.0
Germinal vesicle	0.0±0.2 (0/30)	0.0±0.2 (0/31)	-
Metaphase-I	26.7±7.3 (8/30)	48.4±7.2 (15/31)	0.08
Metaphase-II	66.7±8.8 (20/30)	38.7±8.6 (12/31)	0.03

Values are least square means ± mean squared error.
[1] Numbers within parentheses indicate total number of oocytes exhibiting the event in relation to the total oocytes examined. The degenerate oocytes were subtracted from the total oocytes examined for each event (32 - 2 = 30).

Table 2 - Effect of semen type on early fertilization events after 8 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
n	34	32	
Degenerate	0.0±3.2 (0/34)[1]	3.1±3.3 (1/32)	0.23
Germinal vesicle	2.9±1.8 (1/34)[2]	3.2±1.8 (1/31)	0.95
Metaphase-I	18.2±7.0 (6/33)	10.0±7.4 (3/30)	0.35
Metaphase-II	45.5±8.4 (15/33)	40.0±8.8 (12/30)	0.66

Values are least square means ± mean squared error.
[1] Numbers within parentheses indicate total number of oocytes exhibiting the event in relation to the total oocytes examined. The degenerate oocytes was subtracted from the total oocytes examined for each event (34 - 0 = 34).
[2] Numbers within parentheses indicate total number of oocytes exhibiting the event in relation to the total oocytes examined. The germinal vesicle was subtracted from the total oocytes examined for each event (34 - 1 = 33).

Table 3 - Effect of semen type on early fertilization events after 12 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
n	31	32	
Degenerate	9.7±3.4 (3/31)	3.1±3.3 (1/32)	0.28
Germinal vesicle	0.0±1.9 (0/28)	0.0±1.9 (0/31)	-
Metaphase-I	7.1±7.7 (2/28)	25.8±7.3 (8/31)	0.05
Metaphase-II	32.1±9.1 (9/28)	22.6±8.6 (7/31)	0.41

Values are least square means ± mean squared error.

After 18 hours of co-culture, proportions of oocytes classified as degenerate, or as exhibiting germinal vesicle and metaphase- I were similar among types of sperm (Table 5). There was a significant (P<0.05) effect of sex-sorting on the proportion of oocytes exhibiting polyspermy and male pronuclei. The proportion of polyspermic oocytes was greater (P = 0.005) for the sex-sorted semen. On the other hand, the proportion of male pronuclei oocytes was greater (P = 0.002) for the unsorted semen. None of the remaining events were influenced by semen type (P<0.05) (Table 5).

After 24 hours of co-culture, the proportion of metaphase-II was greater (P = 0.02) in sex-sorted semen and penetrated oocytes were in greater proportion (P = 0.02) for unsorted semen (Table 6). None of the remaining variables were affected by semen type.

The proportion of oocytes achieving Yield-1 at 18 hours was greater (P = 0.01) for unsorted (87.5% - 21/24) compared with sorted sperm (56% - 14/25), while at 24 hours, results were 72.7% (16/22) and 66.7% (18/27), respectively and did not differ (P = 0.64) between semen types. Yield-2 was similar between semen types at 18 hours (P = 0.097) (12.5% - 3/24 and 32.0% - 8/25 for unsorted and sorted semen, respectively) and at 24 hours (18.2% - 4/22 and 25.9% - 7/27 for unsorted and sorted semen, respectively; P = 0.52).

Table 4 - Effect of semen type on early fertilization events after 12 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
Penetrated	65.38±9.1 (17/26)[1]	69.57±9.7 (16/23)[1]	0.76
Polyspermic	17.65±11.0 (3/17)	25.00±11.4 (4/16)	0.61
Female pronuclei	82.35±11.1 (14/17)	75.00±11.4 (12/16)	0.61
Male pronuclei	64.71±8.9 (11/17)	25.00±9.2 (4/16)	0.02
Yield-1*	44.00±5.8 (11/26)	17.39±6.1 (4/23)	0.04

Values are least square means ± mean squared error.
[1] Degenerate and Metaphase-I oocytes were subtracted from the total oocytes which were considered to calculate the total for each event (32 - 3 - 2 = 26 and 31 - 1 - 8 = 23 for sorted and unsorted semen, respectively).
*Yield 1- Proportion of oocytes showing simultaneously monospermic penetration and female and male pronuclei in relation to the total.

Table 5 - Effect of semen type on early fertilization events after 18 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
Degenerate	12.50±5.3 (4/32)	6.67±5.5 (2/30)	0.43
Germinal vesicle	0.00±2.5 (0/28)	3.57±2.5 (1/28)	0.24
Metaphase-I	10.71±6.0 (3/28)	11.11±6.1 (3/27)	0.96
Metaphase-II	20.00±7.5 (5/25)	12.50±7.7 (3/24)	0.48
Penetrated	80.00±7.5 (20/25)	87.50±7.7 (21/24)	0.48
Polyspermic	25.00±6.9 (5/20)	0.00±6.8 (0/21)	0.005
Female pronuclei	5.00±3.5 (1/20)	0.00±3.4 (0/21)	0.23
Male pronuclei	70.00±7.3 (14/20)	100.00±7.2 (21/21)	0.002
Syngamy	40.00±9.7 (8/20)	14.29±9.5 (3/21)	0.60

Values are least square means ± mean squared error.

Table 6 - Effect of semen type on early fertilization events after 24 hours of co-incubation

Event	Type of semen		Probability (P)
	Sorted (%)	Unsorted (%)	
Degenerate	3.23±4.5 (1/31)	10.00±4.6 (3/30)	0.28
Germinal vesicle	0.00±2.4 (0/30)	3.70±2.6 (1/27)	0.22
Metaphase-I	10.00±6.1 (3/30)	15.38±6.6 (4/26)	0.54
Metaphase-II	14.81±5.2 (4/27)	0.00±5.7 (0/22)	0.02
Penetrated	85.19±5.2 (23/27)	100.00±5.7 (22/22)	0.02
Polyspermic	17.39±8.5 (4/23)	22.73±8.7 (5/22)	0.65
Female pronuclei	4.35±5.3 (1/23)	9.09±5.4 (2/22)	0.52
Male pronuclei	78.26±9.4 (18/23)	68.18±9.6 (15/22)	0.44
Syngamy	30.43±9.1 (7/23)	18.18±9.3 (4/22)	0.34

Values are least square means ± mean squared error.

Discussion

Early *in vitro* oocyte fertilization events were investigated, under the overall assumption that specific early developmental steps would signal negative effects of sperm sex-sorting (Lin et al., 2000; Morton et al., 2005 and 2007; Liang et al., 2008). Primarily, this trial aimed to compare between sex-sorted and non-sorted sperm, the proportion of oocytes showing metaphase-II, sperm penetration, polyspermy, formation of pronuclei and syngamy. Events related to *in vitro* oocyte maturation such as oocyte degeneration, germinal vesicle and metaphase-I were compared between semen types to demonstrate that the culture conditions were similar for sex-sorted and unsorted semen (Tables 1, 2 and 3). These proportions are similar to those observed in other experiments (Xu & Greve, 1988). Pronuclei size was analysed, but, since it is relatively subjective and in low numbers, it will only be discussed briefly.

The proportion of metaphase-II oocytes at 4 hours of co-incubation was greater (P = 0.03) for the sex-sorted semen, but similar between both semen types at 8 and 12 hours. Somehow, the sorted semen was associated with an earlier resumption of the second meiotic division, which could not be related to faster penetration in this study. According to Xu & Greve (1988), sperm penetration occurs around 5-6 hpi (hours post-insemination) or earlier (3-4 h) (Machatkova et al., 2008), which would suggest that metaphase-I at only 4 h of co-incubation could be, generally, disregarded as a semen-related effect. Perhaps, a larger number of oocytes could reveal no consistent differences in such an early fertilization event.

Oocytes with pronuclei were first observed in significant numbers at 12 h of co-culture, which is comparable to other reports (Morton et al., 2007; Alomar et al., 2008). In the present study, between 75.0 and 82.4% of oocytes showing female pronuclei were detected at 12 h, comparably higher

than the values between 22.0 and 70.0% overall (male and female pronuclei) reported by Alomar et al. (2008) with unsorted sperm. Similar proportions of oocytes bearing pronuclei to reported in the present study were observed in human oocytes (Lin et al., 2000) fertilized *in vitro* after one hour (79.4%) and 16-18 hours (86.7%) of co-incubation and in bovine oocytes by Barceló-Fimbres et al. (2011), who observed values between 42 and 72% with unsorted semen. Interestingly, those authors found that higher pronuclei rates were correlated to higher cleavage rates. This indicates that the pronuclear formation in the present work is within the range observed in the literature and may be quite variable and possibly more dependent on the semen source (bull) and insemination dose than on the sex-sorting process *per se*. The fertilization pattern in bovine oocytes between 4 and 12 hours of culture is not different whether sexed or unsexed sperm is used, except for a greater proportion of metaphase-II at 4 hours co-incubation for sorted semen.

The percentage of penetrated oocytes at 12 h ranged from 65.4 to 69.6% in this experiment. These values compare well to the literature (Morton et al., 2007; Sattar et al., 2011).

The percentages of polyspermic oocytes observed at 12 h were higher than those reported by Barceló-Fimbres et al. (2011), but comparable to those found by Sattar et al. (2011), which ranged from 16.0 to 19.0% at 4 and 12 hours of co-culture, respectively. Similarly to the present trial, Morton et al. (2007) found no difference between sex-sorted and unsorted semen, although their polyspermy percentages (1.1 – 1.9%) were much lower than the ones observed in this trial. Overall, the results from the present work and from the literature show high variability as far as polyspermy is concerned; however, the end results in terms of blastocyst production were not strongly affected by this variation. Unfortunately, this comparison was not under the scope of this work. However, we observed that the blastocyst rates were not significantly different when unsorted or sex-sorted sperm were used for IVF with bovine oocytes derived from OPU (31.58 vs. 29.63%, respectively) (Cebrian-Serrano et al., 2013).

This second experiment sought to verify the effects between two longer co-incubation lengths and sperm-sex sorting on events that should lead to normal zygote development. Data relative to degenerate, germinal vesicle and metaphases- I and II are shown on tables 5 and 6 in order to illustrate the origin of the total numbers of oocytes and will not be discussed. Penetration rate proportions were similar to those reported by Morton et al. (2005, 2007) and higher than those observed by Dode et al. (2002) and Machatkova et al. (2008) for *in vitro* cultured bovine oocytes. The penetration rates observed in the present work may be indicative of adequate blastocyst formation potential for sorted sperm, since Machatkova et al. (2008) have linked higher penetration rates with increased blastocyst formation in bovine oocytes produced *in vitro*. Interestingly, penetration was higher in unsorted semen at 24 hours but not at 18 hours, indicating an interaction between semen type and co-culture length. Despite this difference at 24 hours, the percentage of penetration for sex-sorted semen was above 85%, which still should be adequate for good blastocyst yields. Higher polyspermy could be expected (Sattar et al., 2011) for unsorted semen, since penetration was greater, but this link was not observed here.

Since polyspermy is an undesirable fertilization characteristic (Rath et al., 2009), it could be speculated that oocytes co-incubated with unsorted sperm exhibited higher fecundation potential at longer incubation periods compared with sex-sorted sperm, but the data found herein does not support this concept. This is in disagreement with the literature (Maxwell et al., 2004; Rath et al., 2009), as it implies no possible disadvantage for sorted sperm, dependent upon co-culture length under the present culture and sex-sorting conditions.

The proportion of fertilized oocytes with male pronuclei decreased at 18 hours of co-incubation with sex-sorted semen, but was similar at 24 hours. This pattern is similar to that observed for polyspermy, also indicating higher competence of unsorted sperm-derived oocytes dependent upon co-culture length, which is in agreement with the literature (Xu & Greve, 1988; Alomar et al., 2008). In this scenario, however, a longer incubation time was not associated with any harmful effects of sex-sorted semen on *in vitro* fertilization.

Syngamy and female pronuclei proportions were similar between both sperm types. This finding also would appear to contradict what has been reported (Maxwell et al., 2004; Rath et al., 2009), as it implies better *in vitro* performance for sorted sperm. In part, the present results may actually indicate that the sperm sorting protocol used did not cause any additional detrimental effect, which is different from some reports (Bodmer et al., 2005; Klinc & Rath, 2007; Morton et al., 2007), but also in agreement with similar studies with rams (Morton et al., 2005) and buffalos (Liang et al., 2008).

The proportion of oocytes achieving Yield-1 was higher at 18 hours of co-incubation for unsorted semen. Nearly 30% more oocytes fertilized with unsorted semen exhibited more development potential than those fertilized with sorted semen at 18 hours of co-culture. However, this finding was not repeated at 24 hours of culture, e.g., yield was similar, indicating that sex-sorting may lower embryo

yield at specific time-points of co-culture and that this is not a linear relationship. There are few references on the performance of early fertilization events; most have data only in terms of cleavage and blastocyst production rates (Morton et al., 2007; Barceló-Fimbres et al., 2011; Sattar et al., 2011).

Conclusions

The results presented herein are consistent with reports in the literature, but they also indicate novel differences in the development of the two sperm-dependent types of oocyte. There are differences between semen types with respect to fertilization events that depend on the co-culture length. Overall, the findings demonstrate similar fertilizing potential between sex-sorted and unsorted sperm. This may reflect improved semen handling procedures during sorting and may also reflect the fact that, at these specific co-culture time points, the differences observed may only impact later oocyte or embryo culture yields. These findings may be used for further experimentation which could strategically lead to improvements in bovine oocyte fertilization rates and embryo yields in *in vitro* culture systems using sex-sorted sperm.

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