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Effects of Sex-sorted Spermatozoa on the Efficiency of *in vitro* Fertilization and Ultrastructure of *in vitro* Produced Bovine Blastocysts

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With 5 figures and 3 tables

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Summary

Frozen-thawed sexed semen from six bulls (Holstein) was used for studying their efficiency in an *in vitro* fertilization (IVF)-programme and to compare their ultrastructure with *in vitro* produced bovine blastocysts produced with non-sorted sperm. Progressive motility of sorted spermatozoa, their IVF rate, development of produced blastocysts and the ultrastructure of the blastocysts were analysed. The cleavage rates of sexed sperm of bulls (groups S1, S2 and S4) were significantly lower than that of unsorted control sperm ($P < 0.01$). Blastocyst development at day 7 of the sexed semen groups varied between 3.5% and 28.8% versus 33.6% for non-sexed semen. The individual blastocyst yield with sexed semen of group S5 (28.8%) was similar to the mean blastocyst production of the non-sexed control spermatozoa (C, 33.6%; $P > 0.05$). The remaining five sexed sperm groups resulted in significantly lower developmental rates of blastocysts on day 7 (S1, 4.9%; S2, 0%; S3, 0%, S4, 3.5%; S6, 25.8%, $P < 0.01$). Group S2 showed microbiological contamination in 50% (four of eight) and S3 in 100% of the experiments (eight of eight). Progressive motility of sexed sperm was significantly lower than that of unsorted sperm (S1, 48 ± 12.0%; S2, 41 ± 11.9%; S3, 39.0 ± 9.9%; S4, 42 ± 4.6%; $P < 0.01$; S5, 72 ± 7.1% and S6, 64 ± 9.3; $P < 0.05$ versus C 82 ± 4.6%). The percentage of progressive motile spermatozoa showed a good correlation with the developmental capacity of blastocysts (r^2: >0.70), the regression parameter was significant ($P < 0.01$). Furthermore, with a straw containing 10×10^6 sexed spermatozoa significantly lower number oocytes was fertilized than with the same concentration of non-sexed sperm ($P < 0.01$). Our results demonstrate that the suitability of sperm sorting for *in vitro* fertilization (IVF) is lower than no sexed sperm. Our ultrastructural studies showed that blastocysts produced with flow-cytometrically sex-sorted spermatozoa possessed deviations in the number and structure of organelles like mitochondria, rough endoplasmic reticulum (ER) and nuclear envelope. These morphological alterations may be responsible for compromised development that observed in embryos produced with sex-sorted spermatozoa. Thus, we conclude that sperm sex sorting can markedly affect the efficiency of an IVF-programme.

Introduction

The control of sex selection in cattle production has been an interesting goal, which has been followed intensively during the last years. Use of sexed spermatozoa in conjunction with *in vitro* embryo production is a potentially efficient means of obtaining offspring of predetermined sex (Wheeler et al., 2006).

In fact sex adequate pre-selection of implantation embryos, combined with embryo transfer and genetic improvement programmes, can increase profitability of dairy and beef cattle production (van Vleck et al., 1987; Pegoraro and Hossepian de Lima, 2001; Seidel, 2003). There are several approaches to select the sex of an embryo: determination of the sex of pre-implantation bovine embryos with the use of the polymerase chain reaction (PCR) by conventional (Shea, 1999) or non-electrophoretic protocols (Bredbacka, 1998) and transferring the sexed embryo to the uterus of a recipient, sperm sorting in conjunction with IVF (Johnson and Pinkel, 1986; Johnson, 1995, 2000; Seidel and Garner, 2002; Maxwell et al., 2004) and immunological sexing (Blecher et al., 1999; Ramalho et al., 2004). Sex sorting of spermatozoa using flow cytometry already has an impact on the commercial embryo production (Pinkel et al., 1982; Johnson and Pinkel, 1986; Johnson, 1995), whereas the other methods are still in a phase of experimentation and their results have only low impact on the commercial production of bovine embryos.

A high variability in the outcome of IVP of bovine embryos has been reported in several studies (Galli et al., 2003). Previous investigations have shown that at least a part of these variations may be due to different capacities of bulls to produce embryos and high male effect on the efficiency of an IVF-programme (Palma and Sinowatz, 2004). In addition, the sexed bull semen is certainly affected by the sorting procedure and by freezing. Fertilization rates were 10–20% lower compared to IVP using non-sorted sperm and traditional artificial insemination (AI; Seidel et al., 1999). No detailed information is available of the effects on the IVF efficiency and blastocyst development, when sexed and frozen bull sperm were used in a commercial IVP-programme.

Material and Methods

Unless otherwise stated, all chemical used in this study were Purchased from Sigma (St Louis, MO, USA).

Collection of bovine oocytes

Ovaries from Holstein cows were obtained from a local slaughterhouse and kept in phosphate-buffered saline (PBS,

pH 7.35) at 25°C during transport to the laboratory. Within 6 h cumulus–oocyte complexes (COCs) were aspirated from follicles with 2–8 mm in diameter using a 5 ml syringe as aspirator and a 20 g needle. Follicular fluid containing COCs was allocated in 130-mm Petri dish for morphological selection (de Loos et al., 1989). Under stereomicroscope control, only those oocytes with had compact multilayered cumulus were considered suitable for maturation. COCs were washed five times in 100-μl drops using TCM 199, supplemented with 0.1 mg/ml L-glutamine, 0.8 mg/ml $NaHCO_3$, 1.4 mg/ml HEPES, 2.5 mg/ml pyruvate, 0.6 L-lactate-calcium-salt and 50-μg/ml gentamycin.

Maturation, fertilization and culture of oocytes

In vitro maturation, fertilization and embryo culture was performed according to Palma et al. (1997, 2004) and Palma (2001). Briefly, for IVM all selected COCs ($n = 2182$) were cultured in groups of 35–40 in 400 μl TCM plus 0.05 IU/ml r-hFSH (Serono, Italy) for 20–22 h at 39°C in 5% CO_2. After maturation, 2502 COCs were fertilized with frozen/thawed-sexed semen of six Holstein bulls or non-sexed spermatozoa of Holstein bull of the same IA station. Sperm was sorted by Goyaike S.A.A.C.I. y F., in Argentina, using a MoFlo® SX sperm sorter (Dako Cytomation, Fort Collins, CO, USA). Each straw used contained 0.25 ml and a concentration of 10×10^6 sexed spermatozoa. Straws were thawed in a water bath at 39°C for 10 s and prepared for fertilization by Percoll gradient centrifugation (45%, 60% and 90%) in TL-Fert for 20 min. Motile spermatozoa were collected in Tyrode's lactate solution (TL-Fert), supplemented with 6 mg/ml BSA, 0.1 μl/ml sodium pyruvate and 75 μg/ml gentamycin. After Percoll separation, semen of all experimental groups was washed once in the same medium (3 ml) by centrifugation at 800 ***g*** for 10 min. The supernatant was discarded and the sperm pellet containing viable spermatozoa was resuspended in 100–200 μl of TL-Fert. Sperm concentration was determined and resuspended spermatozoa were added to a final concentration of 1.0×10^6/ml. Matured COCs were transferred to 250-μl drops Fert-Talp (Bavister and Yanigimachi, 1977) supplemented with 6 mg of BSA fraction V, 25 μg/ml streptomycin and 10 μg/ml heparin. IVF was performed at 38.5°C, 5% CO_2 in air and maximum relative humidity for 18–20 h.

After fertilization of oocytes (day 0), cumulus cells were removed from presumptive zygotes by vortexing at maximum power for 1 min. The denuded oocytes were transferred in groups of 35–40 in 400 μl droplets of CR-1aa (Rosenkrans and First, 1994), supplemented with 10% oestrus cow serum, covered with paraffin and stored for 7 days, after which a quantitative evaluation was made.

Sperm motility

After Percoll gradient centrifugation of all tested sperm groups and before IVF, progressive motility (percentage of spermatozoa moving in a linear way) were assessed by placing a drop of 3–5 μl well-mixed semen onto a pre-warmed (38.5°C) glass slide under a cover slip and examined using phase contrast microscopy at a magnification of 20× (Nikon, Kawasaki, Kanagawa, Japan).

Experimental design

After maturation all COCs ($n = 2182$) were divided into seven groups according to experimental conditions: 1852 oocytes were fertilized with sexed sperm of six Holstein bulls (S1, $n = 310$; S2, $n = 300$; S3, $n = 320$; S4, $n = 310$; S5, $n = 302$; S6, $n = 310$) and 330 oocytes with the non-sexed sperm of a control bull (C), which was suitable for IVP of blastocysts (30%, Palma et al., 1997; Palma, 2001). Evaluation was made at days 2 and 7 for cleavage and blastocyst rate, respectively, in eight replicates. Sperm motility after Percoll gradient centrifugation was assessed in all groups.

Statistical analysis

The percentages of cleaved embryos and blastocysts on days 2 and 7 after fertilization (day 0), respectively, were expressed as mean of all oocytes cultured for maturation. Each drop was considered as an experimental unit. The proportions of embryos that developed to various stages as well as the percentage of embryos on day 2 progressing to blastocyst stage on day 7 in each culture group of oocytes were compared by means of chi-squared analysis (χ^2, StatsDirect, UK, 1990). The number of fertilized oocytes groups *per* straw was expressed in mean ± SD. Differences were compared by ANOVA (StatsDirect, 1990). Sperm progressive motility was expressed as percentage of spermatozoa moving in a linear way. The results were compared by ANOVA. Linear regression analysis was used to asses associations between the individual sperm motility and blastocyst development. The goodness-of-fit of the regression models was determined by R^2 values. The number of contaminated droplets after IVF was expressed in mean ± SD. Morphometric results from our ultrastructural study were expressed in mean ± SD. Differences were compared by ANOVA. In all cases, *P*-value of < 0.05 was considered to be significant between results.

Transmission electron microscopy

Seven-day-old IVF embryos produced with sexed or non-sex sperm were removed from the medium and washed twice in cacodylate buffer (0.2 M sodium cacodylate, pH 7.2). Embryos obtained *ex vivo* were removed from the uterine flushing fluid and washed. After fixation in Karnovskys fluid (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer), embryos were post-fixed in 1% OsO_4 and 1.5% $KFe(CN)_6$. To optimize diffusion conditions for embedding, a small incision was cut into the zona pellucida (ZP) by using a small needle. Embryos were then transferred to a drop of 20% BSA in cacodylate buffer. By adding 25% glutaraldehyde, the BSA was polymerized to a pellet containing the embryo. The pellet was dehydrated in a graded series of ethanol and embedded in Epon (Polysciences, Eppelheim, Germany). In order to assess the cellular compartments of the embryos, semithin sections (1 m) were cut and stained with methylene blue. Ultrathin sections (50 nm) were mounted on grids, post-stained with OsO_4 and examined with a Zeiss electron microscope TEM 902 (Carl Zeiss AG, Oberkochen, Germany) at magnifications from 3000 to 25 000×.

Morphometry

The relative volume density of mitochondria in 7-day-old embryos was determined utilizing the point-count-method

according to Weibel (1979). Mature and immature mitochondria were discerned according to the following criteria: mature mitochondria are characterized by well developed, evenly stacked *cristae*, immature (embryonic) mitochondria show comparatively few peripheral *cristae* or a hooded appearance. For determining the volume density of mitochondria, a transparent grid consisting of 1024 fine and 64 coarse test-points was laid over each micrograph. The number of test points falling on the various types of mitochondria was recorded. The volume density of mitochondria was equivalent to the proportion of points falling on mitochondria divided by the total number of test points available on the test grid. Micrographs of 10 different embryos produced with sexed or non-sexed sperm, were taken using a Zeiss 903 EM electron microscope (Carl Zeiss AG). All micrographs were taken at a magnification of 6000×. In each embryo, an area of 351 m^2 was analysed. The volume density of mitochondria was regularly determined in the cells of the inner cell mass (ICM).

Results

The efficiency and variability of *in vitro* production of bovine embryos with sexed semen of six bulls are illustrated in the Figs 1 and 2 for cleavage and blastocyst rate, respectively. Out of a total of 56 replicates, S2 was contaminated in four (50%, four of eight) and S3 in all replicates (100%, eight of eight). The cleavage rates of sexed sperm groups showed a much greater variation between 0% and 89% (S1, 47.5–52.5%; S2, 15.0–52.50%; S4, 11.4–30.0%; S5, 65–89.4% and S6, 75.0–85.0%) compared to the control group (C, 82.9–90.2%). The proportion of cleaved embryos of sexed groups S5 and S6 did not differ from the control group ($P > 0.05$; Fig. 1). The proportion of 2-cell embryos of sexed sperm of bulls S1, S2 and S4 were significantly lower than non-sorted controls ($P < 0.01$). The production and variability of blastocysts after IVF with sexed semen are illustrated in the Fig. 2. The blastocyst rate at day 7 of the sexed semen varied between 3.5% and 28.8% (S1, 2.5–7.5%; S2 0.0–0.0%; S4, 0.0–7.5%; S5, 26.7–32.5%; S6, 22.5–30.0%) versus control (C, 30.0–38.1%). Blastocyst rate of the six tested groups was significantly lower when the microbiologically contaminated repetitions were included (10.4%, 193 of 1852) or excluded (15.7%, 193 of 1232) compared with the mean of control non-sexed (33.6%, 111 of 330; $P < 0.01$). The blastocyst yield with sexed semen of S5 was similar ($P > 0.05$) but S1, S2, S4 and S6 were different form the mean blastocyst production of the control group fertilized with non-sexed sperm (Fig. 2; $P < 0.05$). Taking into account only the oocytes, which were cleaved, the blastocyst formation on day 7 was similar to S5 (35.1%, 87 of 248) and S6 (32.0%, 80 of 250) but significantly lower when fertilized with sexed sperm of S1 (9.4%, 15 of 159) and S4 (20.4%, 11 of 54) than those of controls with non-sorted sperm (C, 39.1%, 111 of 284; $P < 0.01$). There was found a significantly high frequency of contaminated IVF-sessions when sexed semen of bulls S2 and S3 was used compared with other tested groups and the control group (Table 1, $P < 0.01$). When contamination effects were not included in the analysis, the mean of development rate was also lower than those of the control (57.7%, 711 of 1232 versus 86.1%; 330 of 269 and 15.7%, 193 of 1232 versus 31.5%; 111 of 330 for cleavage and blastocyst rates on day 2 and day 7, respectively; $P < 0.05$).

The fertilizing efficiency of each straw of sorted semen was tested by measuring the number of semen doses, which was necessary to inseminate 35–40 oocytes in 250 μl droplets by a final concentration of 1×10^6 spermatozoa/ml. The number of oocyte groups fertilized with one straw of sexed semen was significantly lower compared to controls ($P < 0.05$; Table 2). It was possible to fertilize only 35–120 oocytes with 10×10^6

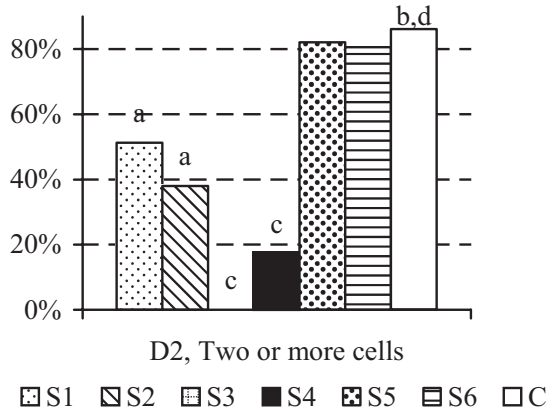

Fig. 1. Effect of x-sorted semen on the development of embryos produced *in vitro* on day 2 after fertilization. Total numbers of embryos were 310, 300, 320, 310, 302, 310 and 330 for groups 1–6 and control, respectively ($^{ab}P < 0.05$ and $^{cd}P < 0.01$; χ^2).

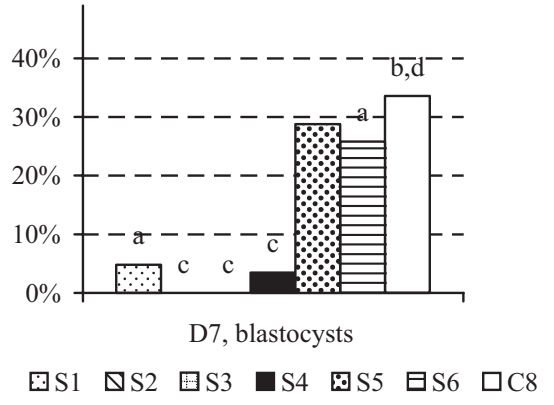

Fig. 2. Effect of x-sexed semen on the development of blastocysts produced *in vitro* on day 7 after fertilization. Total numbers of embryos were 310, 300, 320, 310, 302, 310 and 330 for groups 1–6 and control, respectively ($^{ab}P < 0.05$ and $^{cd}P < 0.01$; χ^2).

Table 1. Number and frequency of contaminations (+) after fertilization with sexed (S) semen

Trial	S1	S2	S3	S4	S5	S6	C
1	o	o	+	o	o	o	o
2	o	+	+	o	o	o	o
3	o	o	+	o	o	o	o
4	o	+	+	o	o	o	o
5	o	o	+	o	o	o	o
6	o	+	+	o	o	o	o
7	o	+	+	o	o	o	o
8	o	o	+	o	o	o	o
Total	0^d	4^c	8^c	0^d	0^d	0^d	0^d

o, no contaminated; C, control.
Values with different superscripts are significantly different (ANOVA, $^{cd}P < 0.01$).

Table 2. Number of *in vitro* inseminated droplets* containing 35–40 oocytes with one straw

	Semen						
Repetition	1	2	3	4	5	6	8†
1	1	2	2	1	2	2	7.5
2	1	1	1	1	3	2	7.0
3	4	1	3	2	1	2	6.5
4	2	2	3	1	3	2	7.5
5	3	2	1	2	2	1	6.5
6	2	1	2	1	2	3	8.0
7	2	2	1	2	3	3	7.5
8	3	3	1	1	2	3	8.5
Mean	2.0[a]	1.5[a]	1.4[a]	1.2[a]	2.0[a]	2.0[a]	7.3[b]
SD (±)	0.9	0.7	0.9	0.5	0.7	0.7	0.7

S, sexed sperm; C, control.
*250 μl volume.
†The efficiency of control group was adjusted to 10×10^6 sperms *per* straw.
[a,b]Values with different superscripts are significantly different ($P < 0.05$).

Table 3. Progressive motility (%) of sexed sperm (S) after Percoll gradient centrifugation

Repetition	S1	S2	S3	S4	S5	S6	C
1	40	60	40	40	70	60	90
2	60	40	30	50	80	50	90
3	50	30	30	40	80	70	80
4	40	30	60	40	70	80	80
5	70	40	40	50	60	70	80
6	40	40	40	40	80	70	80
7	40	60	50	40	70	60	80
8	60	50	40	40	70	60	80
Mean	46[c]	41[c]	39[c]	42[c]	72[a]	64[a]	82[b,d]
SD	8.9	11.9	9.9	4.6	7.1	9.3	4.6

C, control.
Mean values with different superscripts are different (ANOVA, [ab]$P < 0.05$ and [cd]$P < 0.01$).

sexed spermatozoa per straw compared to 210–320 with non-sexed control sperm.

The progressive motility of sperm after gradient density is presented in Table 3. The percentage of progressive motility with sexed sperm was significantly lower than that of the controls (S1, S2, S3 and S4, $P < 0.01$; S5 and S6 $P < 0.05$). Before IVF, the percentage of progressive motile spermatozoa showed no correlation with cleavage rate ($R^2 = 0.28$). However, the percentage of progressive motile spermatozoa showed a significantly good association with blastocyst developmental ability (S1, S4, S5, S6 and C; R^2: 0.78; $P < 0.01$).

Comparative electron microscopic studies of sexed blastocysts showed a clear differentiation of ICM and trophoectoderm cells in both groups. Whereas the polyhedral ICM cells had a spherical nucleus with large nucleoli, the flattened trophoblast cells were characterized by oval nuclei and occasional mitotic figures. The ICM of both groups revealed well-developed dictyosomes of the Golgi-apparatus, rough ER and a comparatively high amount of yolk material. The most abundant organelles were mitochondria, characterized by round to elongated shape and transverse *cristae*. Blastocysts of sexed groups showed significant higher proportion of immature mitochondria (33.7%; 120 of 356) than control

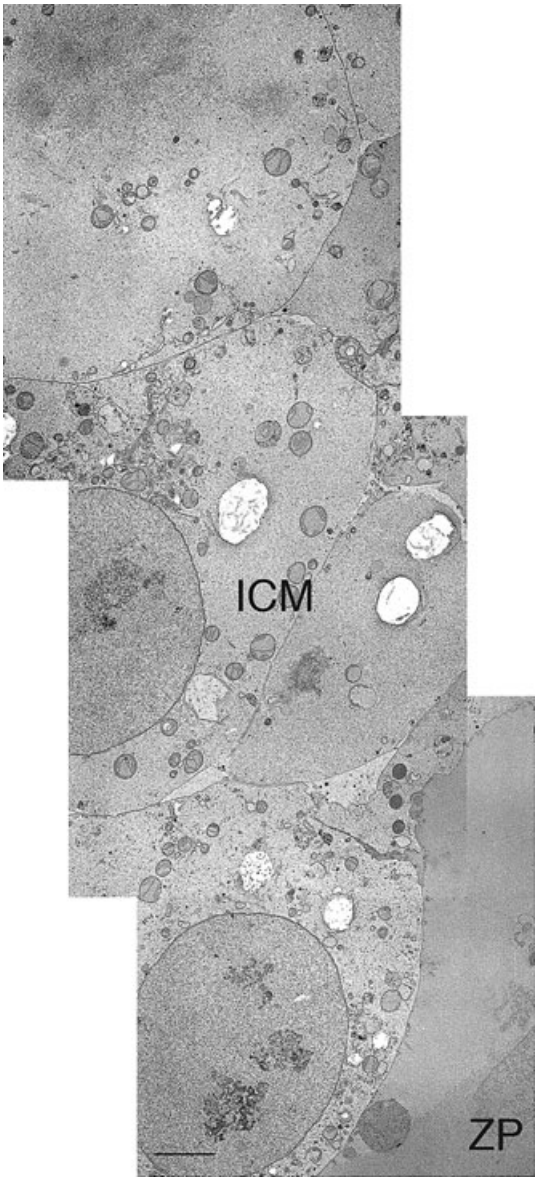

Fig. 3. Control embryo. The inner cell mass shows round euchromatin-rich nuclei with distinct nucleoli. The space between inner and outer nuclear membrane is narrow. The cytoplasm contains still a fair number of yolk granules and clear vesicles. The mitochodria appear mostly mature with transverse cristae. ZP = zona pellucida (bar = 2 μm).

(21.6%; 66 of 306; $P < 0.01$, Figs 3 and 4). The nuclei of sexed blastocysts showed significant lower percentage of intact nuclear envelopes membranes (33.3%; eight of 24) than the control (4.2%; one of 24; $P < 0.01$). Usually, small space between inner and outer nuclear membrane appeared quite frequently widened and in some cases protrusion of chromatin into the cytoplasm could be observed.

Discussion

Although a number of studies has been published on production of bovine embryos and using sexed sperm (Cran et al., 1993, 1994, 1995; Merton et al., 1997; Palma et al., 2004), this is the first study that investigated the potential of sexed sperm

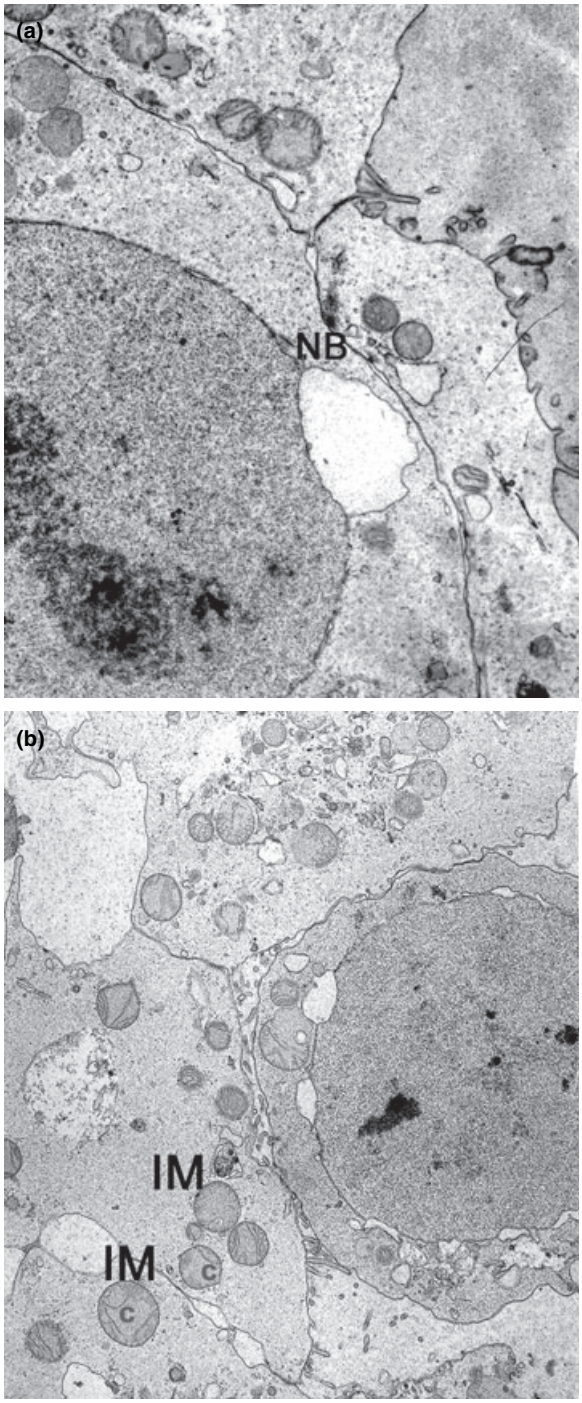

Fig. 4. In blastocysts produced with sexed semen, cell nuclei displayed frequently a localize protruding of the outer nuclear membrane ('nuclear bleb'; a and b). In many blastomeres, the cell organelles appear to be concentrated around the nuclei leaving a wide empty space in the cytoplasm towards the plasma membrane. Many mitochondria were still immature with only a few cristae (c). Bar = 1 μm.

for IVF in a commercial programme and the differential efficiency of the sexed sperm of a given bull for an IVF-programme. A high variation in development rates was observed among tested bulls. However, our results show that it was possible to produce blastocysts *in vitro* with sexed sperm and to obtain under special conditions a comparable yield in the IVF efficiency and developmental capacity, compared to controls with non-sexed sperm. Surprisingly, a high contamination was found in two cases (S2 and S3). Unfortunately, we could not carry out a detailed microbiological analysis to clarify the source of the contamination. The contaminations were only observed microscopically in the IVC droplet. The affected presumptive zygotes degenerated within 48 h and the culture media appeared turbid and replete with bacteria. Although the source of contamination we assume that ubiquitous microbes associated with the sorting or handling of sexed sperm was the cause of the contamination also between bulls.

Large differences in the capacity to produce embryos *in vivo* (Andersson et al., 2004) and *in vitro* (Hillery et al., 1990; Shi et al., 1990) have been observed between bulls. Our results showed that, under controlled conditions of adequate semen selection in AI-stations before or after freezing and also adequate non-return rates, it is possible to obtain a comparable yield in the IVP of bovine blastocysts. However, individual bulls differ in their ability to fertilize oocytes following IVF procedures (Hillery et al., 1990; Shi et al., 1991; Ward et al., 2001; Palma and Sinowatz, 2004). Thus, it is reasonable to consider that the capability of a bull to produce sperm for IVF does not predict its ability to produce embryos *in vitro* after flow sorting. While the combined effects of a bull and of sorting on fertility capacity remains to be clarified, it appears to be necessary to check bulls for *in vivo* but also *in vitro* fertility after sorting and freezing.

Besides the relatively high contamination frequency and low cleavage rates, there was also a significant decrease in the percentage of blastocyst of the remaining sexed sperm groups S1 and S4 on day 7, when only fertilized structures were analysed. These results are similar to those observed by Lu et al. (1999). However, in contrast to their results, we observed also a significantly low cleavage rate. One possible reason for the similar cleavage rate observed by Lu et al. (1999) could be their IVF conditions. The authors supplemented the fertilization medium with 20 μg/ml heparin, 5 mM caffeine and up to 2×10^6 spermatozoa; this could have promoted a higher fertilization rates than those obtained in our study (10 μg/ml heparin, without caffeine and 1×10^6 spermatozoa). On the other hand, we observed no correlation between progressive motility after Percoll separation and cleavage rate (R^2: 0.28). Our results were not consistent with those of Zhang et al. (2003). They reported that sorting sperm did not result in significantly lower blastocyst production than with non-sorted control sperm. The difference with our results may be due to an individual bull effect, which overrode the effect of sperm sorting.

The results obtained in this study are similar to those of Tanghe et al. (2002), who concluded that Percoll might decrease the variability between bulls with high and low fertility. Furthermore, in a previous study we observed that the use of spermatozoa from 63 different bulls from IA stations resulted in no differences of embryonic development on day 2 after fertilization (*F*-value: 0.8; Palma and Sinowatz, 2004).

We observed a significantly low progressive motility of sexed sperm before fertilization in all sexed sperm groups (S1–S6). As the blastocysts were frozen on day 7 in this study, we were not able to evaluate the potential of the blastocysts to develop on day 8 or 9 with sorted sperm, as underwent in the study by Lu et al. (1999). Thus, there is the possibility that lower

blastocyst development on day 7 in our study was due to a delay in embryonic development. Taking into account the development of blastocysts and considering only the number of cleaved embryos, a significant lower development rate was observed on day 7 in sexed groups with low cleavage rate. This suggests that the developmental capacity of embryos produced from sexed sperm with low fertility is significantly affected.

In agreement with our results, other field studies performed in Argentina and Brazil, showed that the applications of x-sorted sperms in IVF-programmes was associated with high variability of the results between bulls (Kaiser et al., 2003), with values varying between 6.2% and 70.6%; 0% and 52.9% and 0% and 52% for cleavage, blastocyst and pregnancy rates, respectively (Tonello et al., 2005). In accordance with our results, the authors showed a blastocyst rate from 13.8%, when evaluated the efficiency of sex-sorted spermatozoa in 168 OPU-donors, 3622 oocytes and 30 straws of sexed semen from nine different bulls.

Compared with embryos produced with non-sexed sperms, we observed that blastocysts produced after fertilization with flow-cytometrically sorted spermatozoa possess some ultrastructural differences that may be indicative of apoptosis (Palma et al., 2004; Fatehi et al., 2005) or aberrant transcription patterns (Rizos et al., 2002, 2003; Lonergan et al., 2003).

Our *in vitro* results confirm the low embryo viability *in vivo* observed by Bodmer et al. (2004) and Andersson et al. (2006) after IA of ovulated and Sartori et al. (2004) for superovulated heifers. The same authors reported that the sorting process might have caused damage of the sperm that compromised *in vivo* fertilization as well as subsequent embryonic development in superovulated heifers. Thus, the combination of factors for sperm sorting and superovulation may contribute to the increase in the percentage of degenerated embryos (Sartori et al., 2004). Recently, in a study published by Schenk et al. (2006) it was demonstrated that the number of recovered, transferable embryos was also lower when superovulated heifers and cows were inseminated with sexed sperm than with non-sexed controls. However, assuming a 90% desired sex ratio, the number of embryos of the selected sex resulting from the use of 20×10^6 sexed sperm was comparable to that theoretically expected for non-sex sperm (Schenk et al., 2006).

As discussed previously a source for the reduced fertilizing capacity of sorted spermatozoa could be the combination of the fluorescent dye and the high-energy laser light (Amann, 1999; Rath et al., 2003) during sorting. Beside these, reported stress factors, a possible stasis in the tubing of flow cytometer or suboptimal handling of sorted sperm could cause additional limitations (Seidel, 2003).

After Percoll differential gradient centrifugation, sperm progressive motility was significantly lower in sexed sperm. This caused a high variability of developmental rates. Progressive motility showed no correlation with cleavage (R^2: 0.28) but a strong correlation with blastocyst formation (R^2: 0.78). While Percoll sperm selection may increase progressive motility of bovine spermatozoa with low *in vitro* motility, this selection could significantly decrease the high variability in sperm motility between sexed sperm groups with low developmental rates, leading to a similar fertility rate (Tanghe et al., 2002). Furthermore, individual bull sperm do not respond with the same developmental potential to the same conditions (Palma and Sinowatz, 2004) and the proliferative potential of produced embryos may be affected differently by stress factors (Fleming et al., 2004).

The high blastocyst development rate observed in one of the sexed sperm group and the adequate embryonic progression of two other groups fertilized with sorted sperm showed that sexed and frozen bovine sperm can be used successfully in an IVF-programme. However, the high variability of the results, contamination problems, reduced fecundity and developmental capacity in the remaining groups indicates that additional work is still required to minimize damage of sperm during the cytometric sorting procedure for successful and economic adoption in commercial IVF-programmes.

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