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Effect of sex-sorted sperm on development and quality of *in vitro*-produced bovine embryos derived from ovum pick up oocytes*

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A combination of sex-sorted sperm and ovum pick up (OPU) -*in vitro* fertilization (IVF) techniques could improve the overall efficiency of the process and develop the commercial opportunities for sex-sorted sperm. Despite intrinsic differences which have been found between bovine oocytes collected post-mortem from slaughterhouse ovaries and those collected by OPU, only a few studies about IVF with sex-sorted sperm have used oocytes collected by OPU. In addition, to our knowledge, the effect of sex-sorted sperm on embryo development and quality using OPU-oocytes have been studied in buffalo (*Bubalus bubalis*) and in *Bos indicus,* but not in *Bos taurus*, therefore we aim to address this issue in this study. Oocytes were retrieved by OPU from mature dry cows and *in vitro* matured. *In vitro* fertilization was performed with sex-sorted or unsorted sperm, and afterwards presumptive

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zygotes were *in vitro* cultured for 9 days. Oocytes fertilized with sex-sorted sperm showed a lower cleavage rate (52.43 *vs*. 69.09%, respectively; P<0.05) but similar blastocyst rate compared to oocytes fertilized by unsorted sperm (29.63 *vs*. 31.58%, respectively). Moreover, the percentages of excellent or good blastocyst (87.50 *vs.* 85.71%) as well as the timing of the blastocyst formation at day 7 (10.53 *vs.* 5.56%) and day 8 (30.26 *vs*. 24.07%) post IVF was not different between sex-sorted and unsorted groups. In conclusion, sex-sorted sperm and OPU-IVF are efficient and valuable tools to produce bovine embryos of predetermined sex and from individual donors.

KEY WORDS: cattle / embryo quality / embryo development / IVF / ovum pick up / sex-sorted sperm

The possibility of determining the sex in domestic animals is a scientific challenge that has great implications for the productivity of livestock systems. The advantages of controlling the sex ratio of progeny are numerous: higher productivity, faster genetic progress, improvement in animal welfare (by decreasing obstetric difficulties in cattle and avoiding castration in pigs) and reduction of environmental impact due to the elimination of the unwanted sex before they grow to adulthood [Rath *et al*. 2009]. For the dairy industry, females (together with a few breeding males) are the most profitable animals, but little benefit can be obtained from male calves. In contrast, male animals have better growth rates than females [Seidel 2003], so they are usually the desired sex in the beef industry. Nowadays, sperm gender preselection is a reality, as technical prerequisites such as adaptation of flow cytometers [Kamentsky and Melamed 1967], identification of the X- and Y- bearing sperm differences [Moruzzi 1979] and non destructive DNA labelling [Johnson *et al*. 1989] have been developed. In fact, sex-sorted sperm is one of the most important advances in biotechnology applied to agriculture over the past twenty years and offspring of predetermined sex have been born in several domestic mammals and humans using sex-sorted sperm [Maxwell *et al*. 2004].

Bovine sex-sorted sperm are commercially available in numerous countries. The method is efficient as the probability of the right sex is higher than 90% [Garner 2006]. However, despite the numerous optimistic predictions, the low fertilization efficiency of sex-sorted sperm still hampers its broader application because fertilization rates are lower and prices per AI are higher than non-sorted sperm doses [Seidel 2003, Cran 2007]. A combination with different biotechnologies has been suggested to improve the efficiency of the process and develop the commercial opportunities for sex-sorted sperm [Rath and Johnson 2008]. *In vitro* fertilization (IVF) is a preferred technique to be associated with sex-sorted sperm, as twenty oocytes could be fertilized with approximately 20,000 to 30,000 spermatozoa [Puglisi *et al*. 2006]. In fact, sex-sorted sperm has been used for IVF to produce *in vitro* sexed bovine embryos, either employing the steps of conventional IVF [Lu *et al*. 1999] or intracytoplasmic sperm injection [Hamano *et al*. 1999]. As for AI, the efficiency of *in vitro*-production of embryos with sex-sorted sperm is still lower than with unsorted sperm [Vazquez *et al*. 2008].

Ultrasound guided ovum pick up (OPU) [Pieterse *et al*. 1991] allows us to obtain oocytes from highly valuable live donors during their reproductive life [Galli *et*

al. 2001], which substantially increases the value of the *in vitro* produced embryos (IVPE) in breeding programmes. A combination of sex-sorted sperm and OPU-IVF techniques could optimise the use of genetic and economic resources to produce sexed embryos. In this regard, some trials have been reported for *Bos indicus* [Underwood *et al*. 2010, Pontes *et al*. 2010], *Bubalus bubalis* [Liang *et al*. 2008] and *Bos taurus* [Pontes *et al*. 2010, Presicce *et al*. 2010, Senatore *et al*. 2010]. However, as far as we know, the effects of sex-sorted sperm on embryo development and quality using oocytes collected by OPU, have been studied in buffalo [Liang *et al*. 2008] and in *Bos indicus* [Underwood *et al*. 2010], but not in *Bos taurus*.

The majority of IVF studies using sorted-sperm have made use of bovine oocytes derived from slaughterhouse ovaries to investigate embryo development and quality [Xu *et al*. 2009]. However, intrinsic differences were found between OPU oocytes and oocytes from slaughtered ovaries [Presicce *et al*. 2010, Merton *et al*. 2003, Lopes *et al*. 2006, Plourde *et al*. 2012]. On the other hand, the quality of the IVPE from sex-sorted sperm may be affected due to the stress inherent to the sex-sorting process [Rath and Johnson 2008]; nevertheless, the results in this regard are not conclusive yet. For instance, the mRNA expression patterns of some genes in IVPE with sex-sorted sperm were found to be different compared to those produced with unsorted sperm [Morton *et al*. 2007], but in other studies expression of studied genes were similar [Bermejo-Alvarez *et al*. 2010, Stinshoff *et al*. 2012].

The purpose of the present work was to study the effect of sex-sorted sperm on embryo development and quality, assessed as timing of blastocyst formation and blastocyst morphology, from bovine oocytes collected by OPU.

Material and methods

Animals

The experiment was performed at the Institute of Farm Animal Genetics (Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany), in the period from July to October. All procedures involving animal experiments in this study were carried out in accordance with the German Animal Welfare Law. Fifteen healthy and sexually mature dry Holstein-Friesian and Deutsche Schwarzbunte cows were used as oocyte donors.

Follicle aspiration and oocyte retrieval

Chemicals used in this study were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany), unless otherwise stated. OPU sessions were performed according to procedures described by Zaraza *et al*. [2010] with some modifications at 3 to 4 day intervals. Before each procedure, faeces were removed from the rectum and perineal area was cleaned with water and Octenisept® (0.1% octenidine dihydrochloride, 2% phenoxyethanol, Sulke and Mayr). Epidural anaesthesia was applied to each donor cow prior to OPU (5 mL of 2% procaine hydrochloride solution, Procasel®, Selectavet). Follicles bigger than 3 mm in diameter were aspirated using

an ultrasound system (CS 9000, Picker) with a 6.5 MHz ultrasound transducer (EUP-F331, Picker) placed in a PVC tube with a needle guide. A 20G x $2^{3/4}$" needle (Neolus®, Terumo) was inserted in a plastic rod for follicle aspiration and connected to a vacuum pump adjusted to 65 mm Hg pressure (Aspirator 3, Labotect). Dulbecco's PBS (DPBS) (D5773) medium containing 1 g/L BSA (11924, Serva GmbH), 50 UI/mL penicillin G (PENP), 50 µg/mL streptomycin sulphate (35500, Serva GmbH), and 2.2 IU/mL sodium heparin (24590, Serva GmbH) was used to retrieve the oocytes and flush the collection needle. A 50-mL conical tube was used to store the aspirated fluid from each animal; the contents were immediately passed through a 50 µm filter and collected in a Petri dish with DPBS. The cumulus-oocyte complexes (COCs) were found using a stereomicroscope and placed in TCM-air medium, consisting of TCM 199 (M2520) supplemented with 22 µg/mL pyruvate (P3662), 350 µg/mL $NaHCO_3$ (31437, Riedel–de Haen AG), 50 µg/mL gentamycin (G3632), and 0.1% BSA-fatty acid free (A7030). Oocytes were graded morphologically based on the cumulus investment according to Chaubal *et al.* [2006] with some modifications, as follows: category I, more than four layers of cumulus cells; category II, three or four layers of cumulus cells; Category III, one or two layers of cumulus cells; category IV, denuded oocytes; category V, oocytes with expanded cumulus; category VI, degenerated or lysed oocytes. Oocytes from categories V and VI were not used for IVF.

In vitro maturation (IVM)

Groups of up to 20 COCs were washed and *in vitro* matured in 100 µL drop of maturation medium consisting of TCM 199 containing 22 µg/mL pyruvate (P3662), 2.2 mg/mL $NaHCO_3$ (31437, Riedel–de Haen AG), 50 µg/mL gentamycin (G3632), 10 IU/mL eCG and 5 UI/mL hCG (Suigonan-Intervet), and 0.1% bovine serum albumin (BSA) (A7030). COCs were *in vitro* matured for 22 to 24 h in humidified atmosphere at 38.5°C and 5% CO_2 in air. All the culture media containing bicarbonate were covered with silicone oil (35135, Serva GmbH) and equilibrated for at least two hours in culture conditions before use (see below).

Collection, preparation and sex sorting of sperm

Briefly, sperm sorting was performed according to the BeltsvilleSperm Sorting Technology [Johnson *et al*. 1999]. 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 then stained with 125 µg bisbenzimide H 33342 trihydrochloride (bisbenzimide) (B2261) and incubated for 1.25 h at 37°C. 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 and Pinkel 1986, Rens *et al*. 1999], operating at 2.76 bar. Labelled spermatozoa were passed through an orienting nozzle [Johnson *et al*. 1999] and 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 and Pinkel 1986] supplemented with 20% (v:v) egg yolk.

Cryopreservation of sperm

Semen was cryopreserved using the Sexcess® treatment for sexed sperm [Rath *et al*. 2009]. Briefly, sorted sperm in catch medium were centrifuged for 20 min at 800×g. The supernatant was discarded and pellets resuspended in Sexcess® cooling extender to 26.4×10^6 sperm/mL. Samples were then cooled in 2 steps to 5°C over 2 h 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 aliquots of sorted samples. Briefly, sperm were restained with ten-fold less concentrated DNA dye (bisbenzimide) as for sperm sorting and then incubated at 37°C for 30 min. Sperm were then sorted again at a sort rate of 60 to 80 events per second. Histogram data at a resolution of 256 channels were tested in a curve fitting program (Gauss 7, anonymous) to obtain the best fitting probability. Only samples with a purity of more than 92% were used for further experiments.

***In vitro* fertilization (IVF)**

Two semen straws (Y sex-sorted and unsorted sperm) of 0.25 mL from a bull with proven fertility for IVF were thawed at 30°C in a water bath for 30 s and centrifuged for 10 min at 300 × g through a gradient of 1 mL of Bovipure® Bottom Layer (Nidacon). The sperm pellet was isolated and washed twice through 750 µL Fert-TALP medium [Parrish *et al*. 1988] by centrifugation at 400 × g for 3 min. In the first washing, heparin-hypotaurine-epinephrine (HHE) was omitted, but in the second wash HHE was included. Following IVM, COCs were washed thrice and co-cultured with spermatozoa in IVF medium in groups of up to 20 COCs per 35 µL drops, for 18 to 20 h at 38.5°C in an atmosphere of 5% CO_2 in humidified air.

In vitro culture (IVC)

Presumptive zygotes were denuded from surrounding cumulus cells in TCM-air medium, washed and transferred to 30 μL drop of culture medium in groups of 5 to 8 embryos. Modified synthetic oviductal fluid amino acids supplemented (mSOFaaci) following Holm's recommendations [Holm *et al*. 1999] and supplemented with 4 mg/mL BSA (A7030) was used as culture medium [Zaraza *et al*. 2010]. Culture drops were placed in a modular incubator chamber (Billups-Rothenberg- USA) with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 at 39°C for a total of 9 days. Cleavage rate was evaluated on day 2 (IVF = day 0). Blastocyst rate, morphological quality and timing formation of the blastocyst produced were assessed on day 7, day 8, and day 9. Blastocysts were classified according to their morphological quality as: excellent or good blastocysts (code 1 in the International Embryo Transfer Society [IETS] morphological classification [Robertson and Nelson 1998]) and fair blastocysts (code 2 in the IETS morphological classification [Robertson and Nelson 1998]).

Experimental design

To evaluate the effect of sex-sorted sperm following IVF on embryo development and quality, two hundred and seventy-two oocytes were retrieved from forty-one OPU sessions. Oocytes recovered in different OPU sessions on the same day were pooled and randomly allocated into two different groups. Following IVM, IVF with sex-sorted or unsorted sperm was performed as described above. For the experiment, ten IVF sessions were carried out.

Statistical analysis

OPU-derived oocyte results are expressed as means±SEM (standard error of the mean). Results of cleavage, blastocyst, morphological blastocyst quality, and timing of blastocyst formation rates were analysed using the Chi-Square test. A probability of $P<0.05$ was considered statistically significant.

Results and discussion

OPU-derived oocyte

The average collection rate was 6.8±0.56 oocytes per OPU session and cow, of which 6.1±0.52 were selected for maturation (see section "*Follicle aspiration and oocyte retrieval"*). The number of oocytes recovered and classified as category III was the largest (2.3±0.32), followed by category II (1.3±0.19), category I (1.3±0.20), category IV (1.2±0.25), category VI (0.5±0.15) and category V (0.2±0.07).The number of oocytes recovered per OPU session in our experiment was similar to the values reported by Bungartz *et al*. [1995] and higher than those reported by other authors previously [Lopes *et al*. 2006, Chaubal *et al*. 2006, Goodhand *et al*. 1999, Hasler *et al*. 1995, Rizos *et al*. 2005]. Regarding the oocyte morphology classification, the absence

of standardized classification makes it difficult to compare results between different scientific publications.

Effect of sex-sorted sperm on embryo development

The development rates of the bovine blastocysts *in vitro* fertilized with unsorted and sex-sorted sperm are shown in Table 1. We observed a higher cleavage rate in the oocytes fertilized with unsorted sperm in comparison with those fertilized with sex-sorted sperm (69.09 *vs*. 52.43%, respectively; $P<0.05$). Interestingly, Ruiz *et al*. [2009] found no differences in penetration rate and syngamy between sex-sorted or unsorted sperm using a similar OPU and IVF setup to ours. In other studies but with oocytes recovered post-mortem from slaughtered ovaries, zygotes from sex-sorted sperm showed a delayed first cell cycle [Bermejo-Alvarez *et al*. 2010, Beyhan *et al*. 1999]. This might not only be due to the *in vitro* conditions, since McNutt and Johnson [1996] observed *in vivo* a delay in embryo development when rabbits were inseminated with sex-sorted sperm. The effect only appeared 42 h after insemination, and was not evident at days 7, 14 or 21 post-insemination. Therefore, it would be interesting to look more intensively into the first cleavage cycle on a molecular basis.

Table 1. Development rates of bovine blastocyst derived from ovum pick up oocytes and *in vitro* fertilized with unsorted or sex-sorted sperm

Group	IVF sessions	Total oocytes	Cleavage (%)	Blastocyst/cleaved embryos (%)	Blastocyst/oocytes (%)
Unsorted	10	110	69.09[a]	31.58 (24)	21.82
Sex-Sorted	10	103	52.43[b]	29.63 (16)	15.53

[ab]Means bearing different superscripts differ significantly at $P<0.05$.

The blastocyst rate that we observed in our experiment was not significantly different when unsorted or sex-sorted sperm were used for the IVF (31.58 vs. 29.63%, respectively). Using OPU-derived oocytes, this finding has already been reported in buffalo [Liang *et al*. 2008] and in *Bos indicus* [Underwood *et al*. 2010]. Recently, Pontes *et al*. [2010], Senatore *et al*. [2010] and Presicce *et al*. [2010] showed the efficiency of *in vitro* embryo production in dairy *taurus* cattle utilizing sexed sperm and oocytes recovered by OPU, but the absence of control group (unsorted sperm) in all three experiments makes it difficult to establish connections between the results of these studies and our data.

Comparing the IVF efficiencies of both sperm types, the use of sex-sorted sperm did not affect significantly the percentage of oocytes forming blastocysts compared to unsorted sperm (15.53 *vs*. 21.82%, respectively). With the aim to simulate the conditions of commercial OPU-IVF programmes, in our experiment denuded oocytes were used for IVF and embryo culture was performed with small numbers of embryos (five to eight), even though the supposed poor quality of those oocytes and the reduced

number of embryos in culture would have been a handicap in this study [Hazeleger *et al*. 1995, O'Doherty *et al*. 1997, Nagao *et al*. 2008, Salvador *et al*. 2011]. Nevertheless, the efficiency of the IVF in our experiment was similar to other works [Zaraza *et al*. 2010, Chaubal *et al*. 2006].

In other studies where oocytes were derived *post mortem* from slaughterhouse ovaries, IVF data are variable. Zhang *et al*. [2003] and Puglisi *et al*. [2006] obtained analogous results to ours for cleavage and blastocyst rates, whereas Peippo *et al*. [2010] did not find differences between sex-sorted and unsorted sperm. This is in comparison to other studies reporting lower cleavage and blastocyst rates [Presicce *et al*. 2010, Bermejo-Alvarez *et al*. 2010, Beyhan *et al*. 1999] or lower blastocyst rate [Lu *et al*. 1999, Morton *et al*. 2007, Merton *et al*. 1997, Lu and Seidel 2004, Wilson *et al*. 2006]. The reported differences may be due to varying methods of oocyte and bull selection, quality of sorted sperm or type of culture medium.

Effect of sex-sorted sperm on the timing of the blastocyst formation and on the blastocyst morphology

Regarding the timing of the blastocyst formation (Tab. 2), no significant difference was found between unsorted or sex-sorted groups at day 7 (10.53 vs. 5.56%, respectively), day 8 (30.26 vs. 24.07%, respectively), or day 9 (data mentioned above). In addition, the percentages of excellent or good blastocyst yielded were similar between unsorted or sex-sorted sperm (87.50 vs. 85.71%, respectively). The kinetics of blastocyst formation was not influenced by the type of spermatozoa (unsorted vs. sex-sorted) in the work of Carvalho *et al*. [2010]. Morton *et al*. [2007] reported that bovine embryos derived from sex-sorted sperm had a similar timing of development, morphology, and cell number to those derived from unsorted sperm, but a lower mRNA abundance of some developmentally important genes. In contrast, two recent studies found similar mRNA abundance between embryos produced with sorted or unsorted spermatozoa [Bermejo-Alvarez *et al*. 2010, Stinshoff *et al*. 2012]. Lu *et al*. [1999] also observed delay of embryonic development from one-half- to one-day when bovine oocytes were inseminated with sorted sperm. Hayakawa *et al*. [2009] on

Table 2. Effect of *in vitro* fertilization with unsorted or sex-sorted sperm on morphological embryo quality and timing of blastocyst formation on bovine pre-implantation embryos derived from Ovum Pick Up oocytes

Group	Total blastocyst	Percentage of excellent or good blastocyst*(n)	Timing of blastocyst formation(n)		
			blastocyst rate at day 7	blastocyst rate at day 8	blastocyst rate at day 9
Unsorted	24	87.50 (21)	10.53 (8)	30.26 (23)	31.58 (24)
Sex-Sorted	16	85.71 (12)	5.56 (3)	24.07 (13)	29.63 (16)

*International Embryo Transfer Society (IETS) morphological classification [Robertson and Nelson 1998].
Blastocyst rate calculated from cleaved embryos.

the other hand used sex-sorted bull sperm in a multiple ovulation and embryo transfer programme and the quality of the *in vivo* produced embryos was no different to that in unsorted groups.

In conclusion, OPU derived-oocytes fertilized with sex-sorted sperm cleaved less than those fertilized with unsorted sperm. The embryo development to blastocyst stage was not further disturbed and the morphological quality and timing of blastocyst formation were similar to normal IVF. In conclusion, sex-sorted sperm and OPU-IVF are efficient and valuable tools to produce bovine embryos of predetermined sex and from individual donors.

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