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**—Original Article—**

# Sex-sorting of spermatozoa affects developmental competence of *in vitro* fertilized oocytes in a bull-dependent manner

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**Abstract.** The aim of the present study was to clarify if flow-cytometric sex-sorting of bovine sperm affected *in vitro* blastocyst production in different bulls, either in terms of its ability to fertilize the oocyte or by interfering with post-fertilization embryo development. We performed *in vitro* fertilization (IVF) using both commercially available frozen-thawed X-sorted and non-sorted sperm of 4 Holstein bulls at 3 concentrations ($1 \times 10^6$, $2 \times 10^6$, and $5 \times 10^6$ sperm/ml). When fertilization rates were compared, a variation in fertilization rates among different sperm concentrations was detected in 2 bulls, with similar results for X-sorted and non-sorted sperm. However, we found no evidence that the fertilization rates were affected by the sorting process. To investigate effects on embryo development, we determined the optimum sperm concentration for IVF in each bull, which resulted in similar fertilization rates among bulls. We next performed IVF using both X-sorted and non-sorted sperm of the 4 bulls at their optimum sperm concentration and compared *in vitro* embryo development. Cleavage rates with X-sorted sperm were similar to their non-sorted counterparts. However, significantly reduced blastocyst development was associated with the use of X-sorted sperm in one bull, whereas in the other three bulls, blastocyst development after IVF with X-sorted and non-sorted sperm was similar. In conclusion, in our system, X-sorting affects *in vitro* blastocyst production by reducing the developmental competence of fertilized oocytes rather than affecting the fertilization ability of the sperm. However, the occurrence of this phenomenon varies among bulls.

**Key words:** Bull, Embryo development, *In vitro* fertilization (IVF), Sex-sorted sperm

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Technical improvements in flow cytometry over recent decades have allowed large-scale production of sex-sorted bull sperm, leading to its use on the commercial level [1]. To date, numerous calves have been produced using sex-sorted sperm either by artificial insemination (AI) or *in vitro* fertilization (IVF). Despite the great success of this technology in achieving high accuracies (over 90%) to determine the desired sex of the resultant offspring, it still suffers from certain shortages [2]. It is evident that even with high-speed sorters, only limited amount of sperm can be prepared and the process of sorting reduces to some extent the viability in terms of motility, DNA integrity and fertilizing capacity which in many reports resulted in reduced pregnancy rates [2]. Nevertheless, AI and IVF protocols can be optimized by adjusting either sperm concentrations (AI, IVF) or heparin concentrations (IVF) allowing fertilization, embryo development and pregnancy rates similar to those with non-sorted sperm [3–5]. On the other hand, increasing sperm quantities used for each AI or IVF increases the costs of embryo production. According to a recent study from Japan, the most cost effective method of calf production with sex-sorted sperm is IVF (Ushijima, personal communication) which combined with ovum pick up (OPU) allows the production of high quality blastocysts with traceable genetic backgrounds [6]. However, the effect of sperm sorting procedure on post fertilization events (embryo and fetal development) is still a matter of debate. Some studies reported reduced blastocyst developmental rates [7, 8] whereas others reported similar blastocyst development rates after IVF with sex-sorted and non-sorted sperm [9, 10]. Previous papers described reduced fertilizing ability of sperm caused by sorting which is manifested in reduced blastocyst development or reduced pregnancies [4, 11] and there is evidence that this phenomenon occurs in a bull dependent manner [3, 12]. In addition, conflicting results have been published on the subject of embryo quality as well; some authors described ultrastructural alterations in blastocysts produced from sex-sorted sperm [13] whereas others reported similar embryo quality and pregnancy rates after IVF with sex-sorted and non-sorted

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sperm [14]. Nevertheless, to date we have no solid evidence whether sperm sorting affects embryo developmental competence of fertilized oocytes to the blastocyst stage, or possible contributions of bull effects.

The aim of the present study was to clarify if flow-cytometrical sex-sorting of bovine sperm affected *in vitro* blastocyst production by different bulls, either in terms of the sperm's ability to fertilize the oocyte or by interfering with post-fertilization embryo development. To investigate this point, we purchased X-sorted and non-sorted sperm of 4 commercially available Holstein bulls. With each sperm sample we performed IVF at 3 sperm concentrations and compared fertilization rates among different sperm concentrations within each bull. Based on these results, we determined the optimum sperm concentration for each bull that resulted in the highest frequency of normal fertilization by IVF. Using the optimum concentration for each bull, we compared fertilization rates after IVF groups with X-sorted and non-sorted sperm. Then, using the optimized IVF parameters for each sperm lot, we compared embryo development after IVF between X-sorted and non-sorted sperm in each bull.

## Materials and Methods

### *Oocyte collection and in vitro maturation IVM*

Ovaries from Holstein cows were collected at a local slaughterhouse, transported to the laboratory and then washed in Dulbecco's phosphate buffered saline (DPBS). The ovaries were then stored in DPBS supplemented with 100 IU/ml penicillin G potassium (Meiji Seika Pharma, Tokyo, Japan) and 100 μg/ml streptomycin sulfate (Meiji Seika Pharma) at 15°C for approximately 15 h until bovine spongiform encephalopathy (BSE) testing results of each ovary-donor animals was proven negative according to the Abattoirs Law of Japan. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (2–8 mm in diameter) using a 5-ml syringe with a 19-gauge needle and used for IVM. The medium used for IVM was TCM 199 (12340-030, Medium 199, GIBCO by Life Technologies, Grand Island, NY, USA) supplemented with 5% (v/v) newborn calf serum (NCS; GIBCO), 0.02 Armor Units (AU)/ml FSH (Antrin R10; Kyoritsu Seiyaku, Tokyo, Japan) and 100 IU/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. Oocytes with homogenous ooplasm surrounded by compact multiple layers of cumulus cells were submitted to IVM. After washing twice in pre-incubated IVM medium, groups of 5–20 COCs were cultured in 100 μl droplets of IVM medium covered by paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan) in 35-mm Petri dishes (Falcon 1008, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 20–22 h at 38.5°C in 5% $CO_2$ in a humidified incubator.

### *In vitro fertilization (IVF) and culture*

Sperm preparation for IVF was performed as previously described [6] with slight modifications. Frozen X-sorted (minimum 90% purity) and non-sorted sperm of different ejaculates from four proven Holstein bulls were purchased from Genetics Hokkaido (Hokkaido, Japan). Each frozen straw was thawed in a 37°C water bath for 30 sec, layered on Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient (45 and 60%), and centrifuged at 710 × *g* for 10 min at 37°C. After centrifugation, the pellet of sperm was resuspended in 5.5 ml of IVF-100 medium (Research Institute for Functional Peptides, Yamagata, Japan) and centrifuged again at 500 × *g* for 5 min at 37°C. The resulting supernatant was removed and the final concentration of sperm was adjusted according to experimental design in IVF-100. COCs were washed in IVF-100 and transferred to a 100 μl droplet of the sperm suspension covered with paraffin oil. Gametes were co-incubated for 6 h at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air. Presumptive zygotes were denuded by gentle pipetting with a fine glass pipette preincubated in Charles Rosenkrans 1 medium (CR1; [15]) with amino acids (CR1aa; [16]) supplemented with 5% NCS and 0.25 mg/ml of linoleic acid albumin (CR1aa-LAA; L-8384; Sigma-Aldrich, St Louis, MO, USA; [17]). Then, 15 to 20 embryos were placed separately in culture drops. IVC was performed in 100 μl drops of CR1aa-LAA medium covered with paraffin oil. Embryos were cultured at 38.5ºC in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ for 9 days. Cleavage and blastocyst formation were assessed on Day 2 (IVF = Day 0), and Day 7, 8 and 9, respectively.

### *Analysis of fertilization events*

Fertilization status of oocytes was assessed 12 h after IVF. In brief, denuded presumptive zygotes were mounted on glass slides and fixed with acetic alcohol (acetic acid 1: ethanol 3) for at least 3 days, then stained with 1% (w/v) orcein (Sigma) in acetic acid, rinsed in glycerol/acetic acid/water (1:1:3), and examined under a phase-contrast microscope at 400 × magnification. The presence and numbers of female and male pronuclei and/or a sperm head(s), and extrusion of the two polar bodies, were then investigated. Oocytes were considered to have been penetrated when a sperm head(s) or a male pronucleus (pronuclei) with the corresponding sperm tail(s) were detected in the cytoplasm. Oocytes with a female pronucleus but lacking a penetrating sperm were considered to have been activated parthenogenetically. Oocytes with one penetrating sperm in the cytoplasm were defined as monospermic. Normal fertilization was defined by the presence of one female pronucleus and one male pronucleus, and the extrusion of both the 1st and 2nd polar bodies.

### *Experimental design*

Experiment 1: The aim of this experiment was to determine the optimum concentration of X-sorted and non-sorted sperm for IVF in each bull and to compare X-sorted and non-sorted sperm lots in terms of their fertilizing ability. In brief, for each bull both X-sorted and non-sorted sperm were used simultaneously for IVF at concentrations of 1, 2 and 5 × $10^6$ sperm/ml (15–20 oocytes each). Six hours after insemination the oocytes were denuded and cultured as described above. Twelve hours after insemination, the fertilization status of oocytes was determined. Total and normal fertilization rates were compared among different sperm concentration groups within the same sperm lot. The lowest sperm concentration to achieve the highest frequency of normal fertilization for each ejaculate was considered the optimum concentration for IVF in further experiments. Furthermore, we have compared fertilization results between X-sorted and non-sorted sperm and among bulls using the optimum concentrations. The experiment was replicated 4 times with totals of 49–62 oocytes analyzed in each group.

Experiment 2: Comparison of embryo development after IVF between X-sorted and non-sorted sperm in each bull using optimized

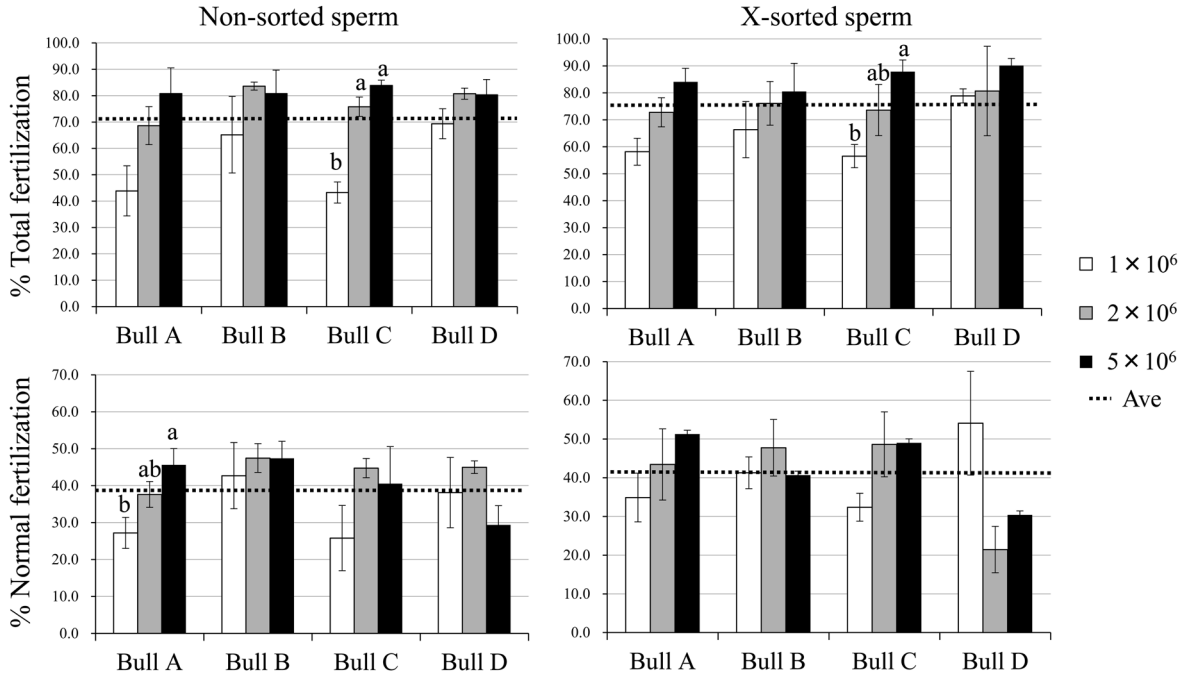


**Fig. 1.** The frequencies of total and normal fertilization after IVF with non-sorted and X-sorted sperm of 4 different bulls at different sperm concentrations. Data are presented as mean ± SEM. Within the same bull, a and b differ significantly at $P < 0.05$. Dotted line (Ave) denotes the mean level for the total average value for fertilization or normal fertilization rates from the four experimental replication calculated from all fertilization groups irrespective of bull and sperm concentration.

sperm concentrations. The aim of this experiment was to investigate if the sorting process affects the developmental competence to the blastocyst stage of fertilized oocytes. IVF was performed with X-sorted and non-sorted sperm in each bull using the optimum concentration determined in Experiment 1. Cleavage rates on Day 2 (IVF = Day 0) and blastocyst rates on Days 7, 8 and 9 were compared between X-sorted and non-sorted sperm in each bull and among different bulls using either sorted or non-sorted sperm. The experiment was replicated 4 times with totals of 150–214 oocytes analyzed in each group.

*Statistical analysis*

Each experiment was replicated at least 4 times. All data were analyzed by one-way ANOVA and Fisher's PLSD test using StatView software (Abacus Concepts, Berkeley, CA, USA). Differences with a probability value (P) of 0.05 or less were considered significant. Percentage data were arcsine transformed before analysis.

## Results

*Optimization of sperm concentration for IVF with X-sorted sperm*

Significant differences were observed in the total fertilization rates of Bull C between the IVF groups of $1 \times 10^6$ and $5 \times 10^6$ sperm/ml concentrations both using non-sorted and X-sorted sperm of these bulls, whereas IVF with $2 \times 10^6$ sperm/ml resulted in intermediate values with X-sorted sperm but was similar to $1 \times 10^6$ using non-sorted sperm (Fig. 1). In the other 3 bulls, there were no significant differences in total fertilization among different sperm concentrations irrespective of sorting (Fig. 1). When non-sorted sperm was used for IVF, there was a significant difference in the frequency of normal fertilization of $1 \times 10^6$ and $5 \times 10^6$ sperm/ml concentrations for Bull A whereas IVF with $2 \times 10^6$ sperm/ml resulted in an intermediate value (Fig. 1). On the other hand, there was no significant difference in normal fertilization rate among different sperm concentration groups in the other 3 bulls (Fig. 1) among non-sorted groups. When X-sorted sperm was used, there was no significant difference in normal fertilization rates among the three sperm concentrations in each bull.

For each bull, the optimum sperm concentration for IVF was defined as the lowest sperm concentration with the mean value for normal fertilization reaching or exceeding the total average value of the 3 sperm concentrations. These concentrations were as follows; Bull A = $5 \times 10^6$ sperm/ml, Bull B = $1 \times 10^6$ sperm/ml, Bull C = $2 \times 10^6$ sperm/ml and for Bull D = $1 \times 10^6$ sperm/ml (Fig. 1). For each sperm lot these optimum sperm concentrations were used for IVF in the subsequent experiment.

Comparison of the normal fertilization rates achieved with optimum sperm concentrations showed there were no differences among bulls within X-sorted and non-sorted groups (ranging between 38.1% and 54.1%), or between X-sorted and non-sorted sperm within each bull (Fig. 2).

*The effect of sperm sorting on embryo development after IVF*

When IVF was performed with optimized sperm concentrations for each bull, the cleavage rates were very similar between X-sorted and non-sorted sperm groups for Bulls A, B and D (Fig. 3). However,

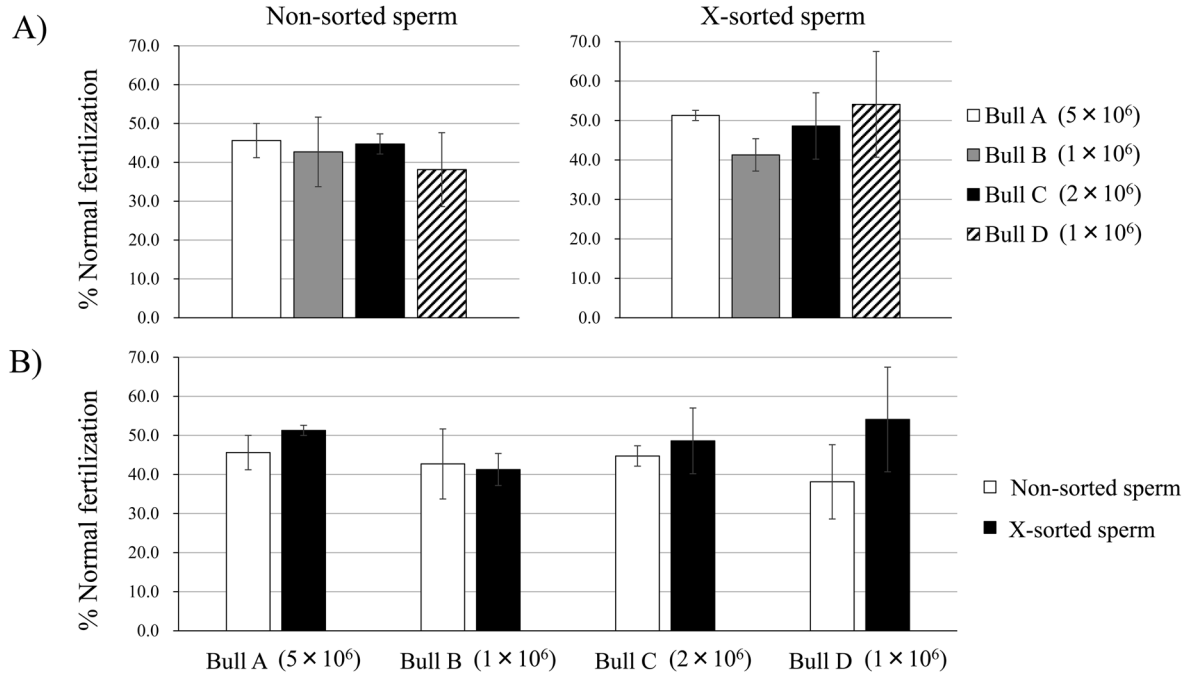


**Fig. 2.** Comparison of normal fertilization rates among bulls within X-sorted and non-sorted groups (A) and between X-sorted and non-sorted sperm within each bull (B) after IVF with sperm concentrations optimized for each bull. Data are presented as mean ± SEM. Significant differences were not detected ($P > 0.05$).

for Bull C, the cleavage rate appeared to be significantly lower in the non-sorted group compared with the X-sorted group (Fig. 3). When blastocyst rates were compared on Day 9, a significantly higher blastocyst development was achieved with non-sorted sperm than with X-sorted sperm in Bull A. On the other hand in Bulls B, C and D, the blastocyst development rates were not significantly different when using non-sorted and X-sorted sperm for IVF (Fig. 3).

There was no statistical difference in cleavage rates among bulls using either X-sorted or non-sorted sperm (Fig. 4). When using non-sorted sperm for IVF at optimized sperm concentrations, there was no statistical difference in blastocyst development rates amongst bulls. When X-sorted sperm was used for IVF at optimized concentrations, blastocyst development was significantly lower for Bull A than Bull D (Fig. 4).

## Discussion

The results of Experiment 1 have confirmed that there is variation amongst different bulls in terms of optimum sperm concentration for IVF. Furthermore, the same pattern of this variation amongst the bulls was observed when non-sorted and X-sorted sperm were used. This suggests that low fertilization abilities observed for Bulls A and C at $1 \times 10^6$ sperm/ml were not caused by the sperm sorting process and the variation amongst bulls in terms of fertilizing ability of their sperm existed prior to sorting. Such variation amongst bulls in fertilizing ability in IVF systems have been described previously using non-sorted semen in several reports [18–22].

As shown in Fig. 1, the highest fertilization rates were sometimes associated with reduced normal fertilization rates (such as in case of Bull D) because increased incidences of polyspermic fertilization (data not shown). Therefore we defined the optimum sperm concentration for each bull as the lowest concentration resulting in a mean value of normal fertilization rate that reaches or exceeds the average value of normal fertilization amongst all bulls. As demonstrated in Fig. 2, when optimized sperm concentrations were used for IVF, there was no difference amongst bulls in terms of normal fertilization irrespective of sorting, which to some extent verified the efficacy of sperm concentration optimization. Furthermore, there was no significant difference between X-sorted and non-sorted sperm within each bull in normal fertilization rates. Taken together with the above mentioned this demonstrates that in the 4 examined bulls, the sorting process for X chromosome bearing sperm did not affect their fertilizing ability at least in our IVF protocol.

In Experiment 2, we performed IVF with the optimized sperm concentration for each bull and compared their embryo development *in vitro*. On Day 2 of *in vitro* culture, cleavage rates were nearly identical between oocytes fertilized with X-sorted and non-sorted sperm in Bulls A, B and D, whereas in Bull C the cleavage rate was lower when non-sorted sperm was used for IVF, for reasons that remain unclear. Nevertheless, on Day 9 of culture, blastocyst development was significantly higher with non-sorted sperm than with X-sorted sperm in Bull A. On the other hand blastocyst development was similar between oocytes fertilized with X-sorted sperm and non-sorted sperm in Bulls B, C and D. Furthermore, the blastocysts obtained from non-sorted and X-sorted sperm did not differ in terms of the numbers of inner cell mass, trophectoderm and total cells in any of

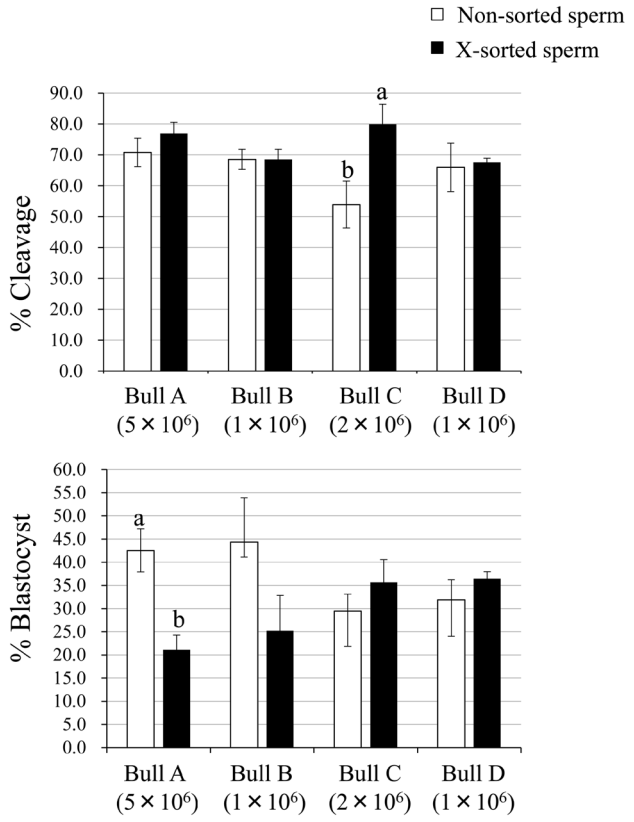


**Fig. 3.** Comparison of embryo development between X-sorted and non-sorted sperm within each bull after IVF with sperm concentrations optimized for each bull. Data are presented as mean ± SEM. a and b differ significantly at $P < 0.05$.

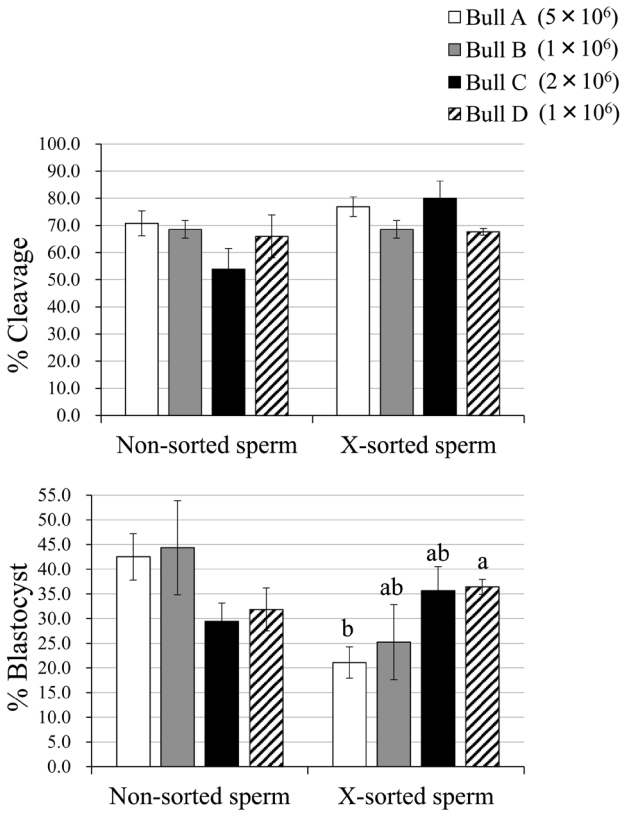


**Fig. 4.** Comparison of embryo development among bulls within X-sorted and non-sorted groups after IVF with sperm concentrations optimized for each bull. Data are presented as mean ± SEM. Within the same category, a and b differ significantly at $P < 0.05$.

the 4 bulls (data not shown). Since fertilization rates were similar between X-sorted and non-sorted sperm in all bulls, in the present case the differences in embryo development could only be attributed to anomalies in penetrating sperm caused by the sorting process to contribute to normal embryo development. Since in the present study, cleavage rates with X-sorted sperm were never reduced compared to their non-sorted counterparts, it seems evident that developmental arrest of embryos caused by sperm sorting occurred following the cleavage stage, presumably during the transition from maternal to embryonic genomic control that occurs at the 8-cell stage in bovine [23]. Furthermore, our results reveal that this phenomenon varied among bulls. A plausible explanation for this phenomenon may be the different extent of DNA fragmentation in sperm caused by the sorting process that is known to vary amongst bulls [24]. Supporting this suggestion, previous studies have shown that spermatozoa with damaged DNA can indeed fertilize the oocyte and the extent of DNA fragmentation greatly affects the ability of fertilized oocytes to reach the blastocyst stage and to implant in humans [25–27]. Another possible cause might be the side effect of Hoechst 33342 retained in spermatozoa that is used to label DNA to enable differentiation of X and Y chromosome bearing spermatozoa during sorting. There is evidence that Hoechst 33342 is transmitted into the oocytes by the fertilizing sex-sorted sperm and is detectable in the cytoplasm of the resultant embryos even until the 8-cell stage [28]. In fact, treatment of oocytes or zygotes with Hoechst 33342 has been found to impair subsequent embryo development in various mammalian species [29–31]. Although the dose of Hoechst 33342 transmitted by the penetrating sperm into the oocyte may be much lower than that transmitted during direct oocyte staining, Hoechst 33342 is still believed to affect post-fertilization events such as male pronucleus formation [2], consequently resulting in a delay in the timing of the first embryonic cleavage [8] and also altered gene expression in embryos [32] which have been observed in bovine embryos generated from sex-sorted sperm. Nevertheless at this point we have no solid evidence if altered epigenetic features contribute to the reduced developmental and pregnancy rates in embryos generated from sex-sorted sperm. In fact, another study found no difference between bovine blastocysts produced by IVF with sex-sorted or non-sorted sperm in the expression of 9 developmentally important genes [8]. Similarly, chromosome alterations could theoretically also contribute to reduced embryo development; however, such alterations caused by sperm sorting were not detected in the resultant embryos in a previous report [33]. Clarification of the exact mechanism by which sperm sorting impairs the developmental competence of fertilized oocytes in occasional cases remains an important task for the future.

When discussing the present results we must consider that in the

present study X-sorted and non-sorted sperm were obtained from different ejaculates of the same bulls. It is known that the number and percentage of motile sperm of bovine semen ejaculates can vary [34] which may affect fertilization results after AI or IVF. Experiment 1 solved this problem since the optimized sperm concentrations resulted in similar fertilization rates by the X-sorted and non-sorted sperm despite the different ejaculates from each bull. It is not clear if post-fertilization embryo development varies between ejaculates from the same bull. Nevertheless, DNA integrity in spermatozoa (which could theoretically affect post-fertilization embryo development) does not seem to vary greatly amongst different ejaculates from the same bull [35] which suggests that ejaculate differences might not affect embryo production as long as the health condition of the bull is maintained.

In conclusion, in our system, variation of the fertility of semen amongst bulls was observed; however, it was not associated with X-sorting. When sperm concentration for IVF was optimized for each bull in a way that resulted in similar fertilization rate amongst the 4 bulls, reduced blastocyst development was associated with X-sorted sperm in one bull. Further research will be needed to clarify if bull-dependent developmental anomalies caused by sperm sorting affect pregnancy and calving rates following embryo transfer.

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