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Comparison of the developmental competence and quality of bovine embryos obtained by *in vitro* fertilization with sex-sorted and unsorted semen from seven bulls

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ABSTRACT

In artificial insemination, semen quality is considered to be closely related to the resultant pregnancy rate and the ratio of embryo development into fetus. The aim of this study was to evaluate the quality of sex-sorted versus unsorted semen used for *in vitro* fertilization (IVF). Semen was collected from seven bulls (A)–(G); each sample was divided in two, sex-sorted or not. Following IVF, the fertilization rate, embryonic cleavage rate, blastocyst formation rate, total cells number (TCN) of blastocysts, inner cell mass (ICM)/TCN ratio and apoptotic cell ratio (ACR) were determined. The results showed that except for bull C, the unsorted semen yielded a significantly higher IVF rate than sex-sorted semen ($p < 0.05$). A significantly higher percentage of embryos reached the two-cell stage using unsorted semen than the matched sex-sorted semen from the seven bulls (both $p < 0.05$), and the blastocyst stage from bulls A, C, D, E and G. However, there was no significant difference in the ICM/TCN ratio between the IVF blastocysts and the sexed blastocysts from bulls A, C, D, E, F and G ($p > 0.05$). Also, there was no significant difference in the expression of *IGF1* gene in bovine embryos produced with sorted or unsorted semen ($p > 0.05$). However, the *GDF9* gene expression in sexed embryos from bull D was significantly higher than in the others ($p < 0.05$). No significant difference was found in ACR among the seven bulls, while there were differences of *Bax* and *Bcl-2* expression in the sorted sperm of only one bull. The results obtained from the sex-sorted or unsorted semen from the seven different bulls varied, although semen from bull A yielded the highest embryonic cleavage rate, blastocyst rate and ICM/TCN ratio. In conclusion, the quality of unsorted semen was generally better than that of sex-sorted semen in the respect to embryo productivity *in vitro*. Additionally, individual differences in fertility were observed among different bulls.

1. Introduction

The possibility of sex pre-selection represents a significant advantage in livestock production. Sex sorting of sperm by flow cytometry is commonly used and advantageous because it allows collection of X-chromosome sperm for dairy herds or Y-chromosome sperm for meat production (Maxwell et al., 2004; Johnson et al., 1999). Therefore, sorting of X- and Y-bearing sperm has been used to impact selection schemes, and has enabled the animal husbandry industries to accelerate the efficiency using *in vitro* fertilization (IVF) (Presicce et al., 2010; Panarace et al., 2014).

Currently, the accuracy of sex pre-selection by sorting sperm with flow cytometry is more than 90% (Khamlor et al., 2014). Sexed semen had contributed to the development of available sexed embryos produced *in vitro* (Barcelo-Fimbres et al., 2011; Wilson et al., 2006). Indeed, a previous study has shown that the sperm concentration leading to optimal cleavage and blastocyst production was higher and more variable with sexed sperm compared with unsorted sperm (Barcelo-Fimbres et al., 2011). However, sexed sperm may be associated with impaired embryo development (Wilson et al., 2006).

In addition, disadvantages of using sex-sorted sperm are the increased expense of the sorting process and the lower fertility of sorted sperm (both fresh and cryopreserved) (Jo et al., 2014; del Olmo et al., 2013). Furthermore, sex-sorted sperm has been suggested to lead to a reduction in the proportion of fertilized oocytes developing to blastocysts (McNutt and Johnson, 1996). It is thought that the sorting process may cause damage to the sperm (Schenk et al., 1999), with some studies reporting DNA damage in semen

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sorted by flow cytometry, possibly related to the use of lasers in the excitation of fluorescent markers (Lu and Seidel, 2004; Suh et al., 2005). Similarly, sorted semen has altered patterns of motility and has a reduced lifespan compared with unsorted semen (Hollinshead et al., 2003). However, whether the sex-sorting process have effects on the quality of embryo after, especially the total cell number, ICM cell number, apoptosis and genes expression profile after fertilization, was reported rarely.

Previous studies have shown that individual bulls have significant influence on fertilization outcomes both *in vivo* (Andersson et al., 2006) and *in vitro* (Ward et al., 2001; Zhang et al., 2003). Also, there is evidence that spermatozoa from individual rams differ in their ability to fertilize oocytes when used for IVF (Fukui et al., 1988). Previous studies examined the effect of sorting sperm on production performance, but with variable results (Barcelo-Fimbres et al., 2011; Wilson et al., 2006; Jo et al., 2014; del Olmo et al., 2013; McNutt and Johnson, 1996). Therefore, selection of sex-sorted sperm with high fertilizing capacity for use in IVF is of great importance in maximizing the efficiency of embryo production, especially in cattle. Till now, the difference among bulls in ICM/TCN proportion, apoptosis rate, expression of developmental genes and apoptotic genes in bovine IVF embryos has not been reported.

Gowth differentiation factor 9 (GDF9) is a member of TGF-β family and its primary role is to act on granulosa cells, which in turn supply the oocyte with the support necessary for future healthy embryo or fetal development (Gilchrist et al., 2008). The insulin-like growth factor (IGF) system is related to quality of oocytes and embryos (Satrapa et al., 2013). IGF1 can improve the development of morulae or blastocysts, as well the reduction of apoptosis in bovine after added to the *in vitro* culture medium (Bonilla et al., 2011). Bcl-2 and Bax are members of Bcl-2 family and regulate apoptosis. Overexpression of Bcl-2 enhances cell survival by suppressing apoptosis, while overexpression of Bax accelerates cell death upon growth factor withdrawal (Sedlak et al.,1995). Detection of expressions of those four genes in bovine blastocysts, produced *in vitro* with unsorted and sex sorted sperm from different bulls was important to show the development competence and apoptosis.

Therefore, the aim of the present study was to compare the developmental competence and quality of bovine embryos fertilized *in vitro* with sex-sorted and unsorted semen from seven bulls and then to evaluate the difference between unsorted and sex-sorted semen in terms of ICM/TCN, apoptosis rate and expression of genes.

2. Materials and methods

2.1. In vitro maturation of bovine oocytes

A total of 567 bovine ovaries were obtained from a local slaughterhouse, washed three times with preheated 0.9% NaCl and transported to the laboratory at 35–37 °C within 2 h of collection. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2–8 mm in diameter by vacuum suction. COCs that were completely surrounded by three or more layers of cumulus cells were selected for maturation. These COCs were washed twice and matured in 4-well dishes (Nunc, Roskilde, Denmark) containing 50 COCs per well in 750 μL of maturation medium under oil at 38.5 °C in a humidified 5% CO_2 atmosphere. The maturation medium contained M199 medium with Earle's salts (GIBCO, Life Technology, Carlsbad, CA, USA), 0.01 IU/mL follicle-stimulating hormone (Sigma, St Louis, MO, USA), 1 IU/mL luteinizing hormone (Sigma, USA), 1 μg/mL estradiol 17-β (Sigma, USA), and 10% fetal bovine serum (FBS; GIBCO, Life Technology, USA).

2.2. In vitro fertilization with semen of seven bulls

The semen was sex-sorted by flow cytometry from the same batch of fresh unsorted semen from each of the seven bulls (A–G, Shandong Dairy Cattle Center, Jinan, China). The IVF procedure was carried out according to the method described by Nedambale et al. (2006), with minor modifications. One straw of unsorted frozen semen (1.2×10^8/mL) and three straws of sex-sorted semen (2.5×10^7/mL) from each bull were thawed at 38 °C for 1 min and washed twice in sperm wash medium by centrifugation at 500 g for 8 min. The sperm wash medium consisted of modified Brackett–Oliphant (m-BO) medium supplemented with 10 μg/mL heparin (Sigma, USA), 10 mM caffeine (Sigma, USA), and 4 mg/mL bovine serum albumin (fatty acid free BSA; Sigma, USA). Subsequently, sperm pellets were re-suspended with sperm fertilization medium (m-BO medium supplemented with 4 mg/mL BSA and 10 μg/mL heparin) and counted using a standard count 4-chamber slide (Leja, Nieuw-Vennep, The Netherlands) under a microscope at 200× (Nikon, Japan). Except for the dead and abnormal ones, the viable sperms were counted and the concentration was adjusted to 1×10^6/mL.

Following 22 h of maturation *in vitro*, bovine COCs were removed from the maturation medium and washed three times in fertilization medium. They were then allocated into groups of 30 and transferred into 100 μL droplets of fertilization medium with sperm. At 18–20 h after IVF, the presumptive zygotes were washed three times and cultured in modified-CR1aa medium supplemented with 6 mg/mL BSA for the first 48 h, and 10% FBS (GIBCO, Life Technology) thereafter. Presumptive zygotes were cultured under mineral oil (Sigma, USA) at 38.5 °C in a humidified 5% CO_2 atmosphere until day 7. The cleavage and blastocyst formation rate was recorded 48 h and 7 days after insemination, respectively. Cleavage formation rate was calculated as the cleaved embryo number divided by the total oocyte number. Blastocyst formation rate was calculated as the blastocyst number divided by the cleaved embryo number.

2.3. Staining of pronuclei of bovine zygotes

Ten hours after incubation of sperm and oocytes, 30 presumptive zygotes in each group were collected to stain the embryonic pronuclei. The experiment was repeated thrice. The cumulus cells and sperm attached to the zona were removed with 0.25% (w/v) hyaluronidase (Sigma, USA) in phosphate-buffered saline (PBS; GIBCO, Life Technology) for 2 min. Following three washes with 1% BSA in PBS (PBS–BSA), the zygotes were transferred into 5 μg/mL of Hoechst (H-33342) (Sigma, USA) in PBS for 15 min to stain the pronuclei. The samples were then mounted on slides and imaged using a fluorescence microscope (Nikon, Tokyo, Japan; EX 330-380, DM 400). Cells with two pronuclei were regarded as fertilized oocytes under the microscope. The fertilization rate was calculated as the fertilized oocytes number divided by the total number of oocytes.

2.4. Immunofluorescence staining of bovine blastocyst

To determine the total cell number (TCN), blastocysts were stained with H-33342 and the trophectoderm (TE) was labeled using antibodies against CDX2 (Biogenex, San Ramon, CA, USA) (Wydooghe et al., 2011). Briefly, ten blastocysts per group (performed in triplicate for a total of 30 blastocysts) at day 7 were fixed in 2% paraformaldehyde in PBS (GIBCO, Life Technology, USA) for 20 min at room temperature (RT). Next, the blastocysts were transferred into permeabilizing solution containing 0.5% Triton-X100 and 0.05% Tween 20 in PBS overnight at 4 °C. After three washes with PBS–BSA, the samples were incubated in blocking