New Technical and Biological Approaches to Increase the Fertilizing Capacity of Sex Sorted Bovine Sperm

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Introduction

Since the first reports of a successful and repeatable method to separate X- and Y-chromosome bearing sperm populations by flow cytometry (Johnson et al. 1989) the technology has been improved substantially (Sharpe and Evans 2009). High throughput techniques allowed the transfer from the research laboratory to the field and sexed bovine semen was introduced into commercial application a decade ago. Nowadays sexed semen is available in several countries, especially in the USA, where the demand for replacement heifers is higher than in other countries (Ettma et al. 2007; DeVries et al. 2008). The pre-selection for sex of offspring is a very valuable tool to optimize herd management, increase the replacement of young heifers, minimize hygiene risks introduced into closed herds by sales animals and to increase the export of female animals. According to Hutchinson and Norman (2009) since 2006 9.2% of all Holsein heifers were bred at least once with sexed sperm in the USA. This is 6.8% of all heifer breedings. As fertility in cows is lower than in heifers, only 2.4% of Holstein cows (0.9% of all cows) were inseminated with sexed sperm at least once. About 91% of calves were female. This is highly correlated to the prediction based on re-analyses of sorted samples.

However, the sorting technique still has limitations caused by the necessity to identify and sort individual sperm and by technical stressors that diminish the lifespan of sperm after freezing and thawing. Sex selected sperm are a different product compared to unsorted semen and require more attention during processing and handling to make the sorting process effective and efficient in order to gain fertility result approaching that of unsexed semen. The process has to be reasonably inexpensive as the cost/quality relationship is critical and the utilization of sexed sperm is strongly affected by the economiy of the milk market as seen currently.

Even in the bovine where more than 4 million doses were sold in 2008 (Seidel, 2009) and many offspring have been produced with sexed sperm, the fertility after sorting and freezing is highly variable, mainly due to the technical process itself. After semen collection and dilution sperm are labeled with a fluorescent dye (Hoechst 33342) in order to discriminate quantitatively between X- and Y-chromosome bearing spermatozoa in a high speed flow cytometer where they are exposed to UV Laser light produced with an Argon laser or

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preferably with a pulsed solid state laser avoiding damaging effects of low UV wave length that is absorbed by nucleic acids and proteins (Seidel et al. 2002). Those droplets containing a single oriented sperm cell are then electrically charged before they pass an electrostatic field for separation. The separated droplet streams are pushed into prefilled collection tubes at high speed. The samples are centrifuged and the remaining sperm pellet is extended with an appropriate extender for liquid or frozen preservation. This treatment may cause further cell damages. Due to the requirement of individual sperm identification, even with the latest high-speed flow cytometers less than 20 million spermatozoa can be sexed per hour, and this equals one normal AI dosage with unsorted frozen semen. To make the sorting method more efficient and economically attractive, only a tenth of the normal sperm number is used for AI with sexed sperm and independently but additional to the limited lifespan after sorting, the higher dilution further lowers the fertilizing potential of sexed sperm. Frijters et al. (2009) estimated that reduced pregnancy rates after AI with sexed sperm (approx -14%; NRR56) are 2/3 related to high dilution effects and to 1/3 related to the sorting technology. Consequently lower pregnancy rates have been reported in several cross species studies (Johnson et al. 1989; Johnson 1991,1995; Cran et al. 1993; Seidel 1999; Hollinshead et al. 2002; Seidel and Garner 2002; Maxwell et al. 2003). The portion of stillbirths is similar between pregnancies from unsorted sperm and those withX-chromosomal sperm, whereas the stillbirth rate is increased in male calves. The reasons are not clear. One explanation might be aneuploid sperm that the sorter includes in the male population (De Jarnette et al. 2009).

Technical and biological approaches to increase the fertilizing capacity of sex sorted bovine sperm

Since the first sperm sorters were developed more than 25 years ago, many technical improvements have been made in flow cytometry like noise reduction, data zoom, data tracking and rotation of the dot plots. Especially in the latest version of high-speed flow cytometers digitalization has increased the sort efficiency significantly (Sharpe and Evans 2009). Beside these general improvements to flow technology, specific modifications improved the sort efficiency for spermatozoa, including optical filters with high numerical aperture, noise reduction in photo multipliers (PMTs) and digital technology have increased throughput by approximately 35%. In parallel, refinement of the nozzle system improved sperm orientation and currently 60% of the cells are correctly positioned in front of the Laser beam. However, there is still room for improvements because still 50% of the introduced spermatozoa are sorted into the waste.

Labeling. So far only the different quantity of DNA in X- and Y- chromosome bearing sperm can be used to significantly separate both sperm populations by flow cytometry. The labeling with a fluorochrome is rather unspecific but as the chromatin is highly condensed until the sperm enters the oocyte, it is difficult to use a labeled DNA sequence to directly identify Y-chromosome bearing sperm. When using Hoechst 33342 for quantitative fluorescence signals, Seidel and Garner (2002) reported no difference in motility and DNA integrity in sperm that passed the sorter with and without staining. Sperm labeling itself did not affect embryo cleavage and blastocyst development rates (Merton et al. 1997; Zhang et al. 2003). Presumably, DNA damages are due to combined effects of dye and UV Laser. Higher laser intensity is more damaging than the lower laser intensity as shown for rabbit

sperm (Johnson et al. 1996) as well as for bovine semen (Schenk and Seidel 2007). As long as the Laser power is however not higher than 125 mW, no effects due to sperm exposure to UV light are recognizable on embryo development (Guthrie et al. 2002; Catt et al. 1997) and no changes were found in the frequency of endogenous DNA nicks. This is also agreement with a recent studies from Parrilla et al. (2004) and Garner (2009) indicating no genotoxic effects of Hoechst 33342 in porcine spermatozoa. Meanwhile alternative dyes have been developed and current experiments have shown that these dyes have similar DNA staining capacity to Hoechst 33342 but can be used at very low concentrations (Rath, unpublished).

The droplet stream. Laminar cell flow is required to permit individual analysis of single spermatozoa. Once sperm have passed the jet system in the core stream they are enclosed by sheath fluid, maintaining the shape of the core stream so that sperm heads are oriented properly in front of the laser and form droplets around the sperm that can be charged and electrically separated. A stable hydrodynamic pressure is important for the consistent positioning of sperm and a fixed time delay between recognition and charging. The percentage of live spermatozoa increased significantly when the fluid pressure was lowered from 50 to 40 psi, resulting in increased developmental rates with bovine IVF embryos (Campos-Chillon and de la Torre, 2003; Suh et al. 2005).

Separation. A significant factor during sorting seems to be electrical charging and electrostatic deviation. Membranes of the mid-piece of the sperm tail are sensitive to the electric field and may undergo depolarization. We aspect also that the mitochondrial activity is reduced due to the presence of reactive oxygen species (ROS) produced by electric forces (Klinc and Rath 2007). SEM images of the mid-piece clearly indicate severe damage to mitochondrial cristae and membranes. Reduction of the electric capacity requires further investigation. Typically more than 90% of the sperm are sorted correctly. Purity validation of sorted samples is performed either by reanalysis (Johnson et al. 1997; Welch and Johnson 1999), by fluorescence in situ hybridization (FISH) (Kawarasaki et al.1998; Rens et al. 2001), or by PCR (Welch et al. 1995). New software tools were currently developed to simplify the reanalysis and make it more accurate.

Sperm reduction in AI dosages. Utilization of sex-sorted semen requires a significant reduction of spermatozoa per AI to meet economic demands. In the bovine commercially 2.1 million sexed spermatozoa are filled per straw, which seems to be sufficient for certain bulls. Their usability however depends only partly on the ability of the spermatozoa to survive the sorting process. It is well known that the fertilizing capacity even with unsorted spermatozoa varies among bulls when low sperm concentrations are used for AI (Den Daas et al. 1998; Andersson et al. 2004) as compensable and non compensable effects of an ejaculate are bull specific. In a recent field trial using commercially sexed sperm, Andersson et al. (2004, 2006) found less than half of heifers became pregnant as compared to AI with unsorted semen from the same bulls and concluded that the average insemination dose for sexed sperm should be above 2 million sperm. Similar results were obtained by DeJarnette et al. (2008) using sorted sperm from three different bulls at different concentrations (2.1, 3.5, and 5.0×10^{6}sperm/AI). In opposite, Seidel et al. (1997) found no excessive embryonic loss between 1 and 2 months of gestation in heifers inseminated with sorted sperm. Recently, we reported an improved method to maintain the fertilizing capacity of sex sorted frozen sperm

(Rath and Johnson 2008; Klinc and Rath 2007) The new sperm handling protocol named Sexcess® comprises several modifications of sorting and preservation. Beside other components it includes a reversible inhibition of sperm motility during sorting as well as supplementation with different radical scavengers and a three step cooling and freezing protocol that minimizes the exposure of sperm to toxic substances including glycerol above freezing temperatures. Overall our field data indicate that conception rates and calving rates are no longer significantly different in heifers with sorted sperm vs. unsorted semen (Klinc et al. 2007). It should be mentioned that insemination does no longer require special handling (thawing at 37°C for 20 sec; AI into the uterine body or distal horn) because the life span of sperm after thawing has been at least doubled with the Sexcess® procedure.

Thawing. Several recommendations for the thawing process of sexed sperm are provided by the producers. As each preservation protocol requires different thawing time and temperature, a thawing trial was setup for Sexcess®. In parallel we wanted to elucidate effects on post thaw quality using CASA and flow cytometry to investigate sperm motility pattern and membrane integrity. Sorted dosages from a commercial producer and from Sexcess® were thawed at 40 sec/35°C, 20 sec/38°C and 5 sec/70°C. Within the semen preparation protocol, no significant differences were found for motility and morphological integrity. However, results were significantly reduced for all thawing protocols in commercially produced semen as compared to Sexcess®. It is recommended to thaw semen stored according to the Sexcess® protocol at 37°C for 20 sec. and inseminate into the uterine body or if accessible into the ispi-lateral uterine horn.

IVF. Effects of the sorting process not only have impact directly on sperm but also on the subsequent embryo development after AI or IVF with sexed sperm, resulting in reduced bovine blastocyst development [Blondin et al. 2009, Merton et al. 1997; Cran et al. 1994; Lu et al. 1999). In a recent study we demonstrated that bovine IVF-embryos derived from sex-sorted spermatozoa display a reduction in the relative abundance of developmentally important genes including Gluc-3 and G6PD (Morton et al. 2007) requiring further epigenetic studies. Similarly, Bermejo-Alvarez et al. (2008) reported reduced cleavage rates after IVF with sex sorted spermatozoa and diminished blastocyst formation on day 8, which is in accordance to fewer cell cycles (Beyhan et al. 1999) and disturbed timing (Cran et al. 1993; Lu et al. 1999; Cran and Johnson 1996; Morton et al. 2005). Ultrastructural studies show that blastocysts produced with flow-cytometrically sex-sorted spermatozoa possess deviations in the number and structure of organelles including mitochondria, rough ER and the nuclear envelope. These morphological alterations may be responsible for the compromised development observed in embryos produced in vitro with sex-sorted spermatozoa (Palma et al. 2008).

Conclusions

Pre-selection of offspring gender based on the Beltsville Sperm Sexing Technology has gained from several technical and biological developments and it has been implemented as a biotechnological tool into current breeding strategies. However, the technology still suffers

from reduced fertility in AI and IVF. Especially the life span after thawing is highly reduced and requires further technical and biological strategies to improve the efficiency.

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