—Mini Review—

Sperm Sexing in the Cattle Industry

Hiroyuki Hayakawa

Genetics Hokkaido Association Inc., Hokkaido 089-0103 Japan

Abstract: *Sperm sexing using flow cytometry and cell-sorting technology is now available for many mammalian species with approximately 90% accuracy. This technique has been applied only in the cattle livestock industry on a large-scale commercial basis. Frozen straw doses containing sexed bull sperm (usually marketed as 'sexed semen') have been sold in many countries for artificial insemination (AI) use. However, the cell-sorting process damages sperm physically/physiologically, consequently compromising fertility results compared to conventionally processed sperm when used for AI or in vitro fertilization (IVF). Also, the production of sexed sperm is still limited and costly. Despite these biological and economic restrictions, sexed semen is widely accepted and the industry is awaiting further refinements to it because of growing demand.*

Key words: *Cattle, Bull, Sperm sexing, Artificial insemination, Embryo transfer*

Introduction

In the last century, the cattle industry had grown up with the introduction of artificial insemination (AI), resulting in efficient breeding with vast genetic improvement [1, 2]. At the same time, manipulating of sex of calves has been of great interest to the industry [1, 3], because of sex related traits (milking, herd replacement, growth rate, etc.), and eliminating calves of unwanted sex at the time of AI offers various benefits in animal production management [3–5]. However, no practical and efficacious method of sexing sperm was established until the 1980s [6, 7].

In 1989, a major breakthrough was reported by Johnson *et al*. [8] at the United States Department of Agriculture (USDA). They were the first to successfully produce mammalian (rabbit) offspring with intended sex bias using sexed sperm [8]. Their novel technique is based on a system of a flow cytometer equipped with a cell sorter which can distinguish X-chromosome bearing sperm (X-sperm, female) from Y-chromosome bearing sperm (Y-sperm, male) in a given semen sample [6–7, 9].

Important trials were made in the cattle sector in the 1990s [10]. In 1993, Cran *et al.* [11] reported the world's first sex selected calves derived from transferred embryos that were fertilized *in vitro* using sexed sperm. Seidel *et al.* commenced AI trials and reported the first AI calves using unfrozen [12] and frozen [13] sexed sperm. A little later, a product termed 'sexed semen' (straw doses containing sexed frozen bull sperm) was released in the UK in 2000 [5]. After a decade, this technique still remains the only proven sperm sexing method and has been utilized for cattle and various other species [7, 10, 14]. Recently, several million doses of sexed semen have been produced yearly [10]. In most cases, sexed semen is marketed as 'female semen' because dairy operations constantly need replacement heifers.

Roughly 10 to 20 millions of sperm can be distinguished as 'male or female' and sorted per hour per sperm sorter currently in use [10, 14]. However, that number is equivalent to one (or less) dose of conventional semen so fewer sperm are deposited per insemination [10, 14], and the sexing process impairs sperm fertility [5, 7, 10, 14]. That is to say, sexed semen should be used for herds with excellent reproductive management, and is recommended for inseminating heifers that have inherently higher fertility than lactating dairy cows [7, 10, 14].

How are Sperm Sexed?

The principle and the procedure of sperm sexing have been described in previous review articles [7, 9, 14–15]. Briefly, sperm cells in fresh ejaculates are stained with Hoechst 33342, a vital dye which binds to DNA molecules stoichiometrically [16]. Since a bovine X-sperm contains approximately 4% more DNA than a Y-sperm [6, 7], X-sperm will bind 4% more dye. Hoechst 33342 dye only fluoresces when exposed to a particular wavelength of UV light. The basic instrument used is a flow cytom-

Received: May 16, 2012
Accepted: June 29, 2012
e-mail: hayakawa@gh-assoc.ne.jp

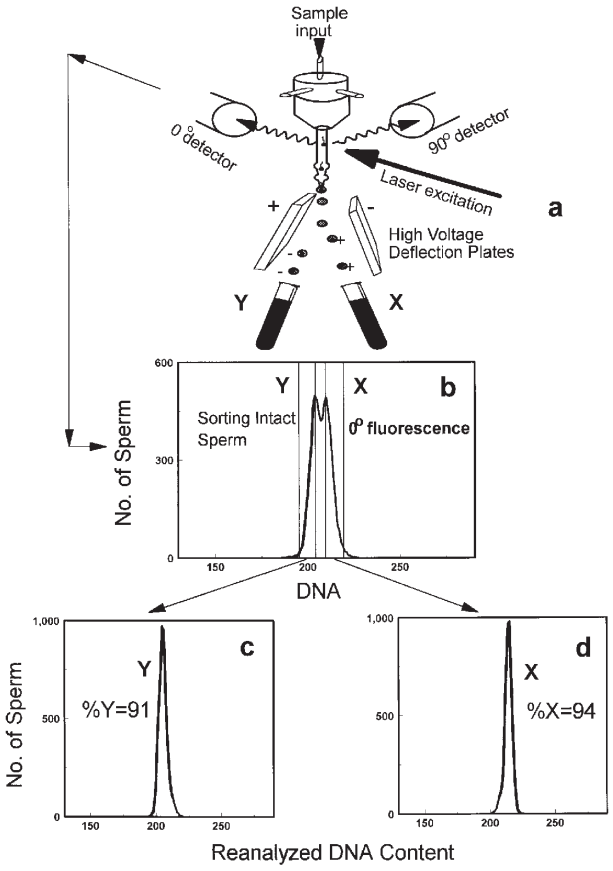

Fig. 1. Schematic diagram of flow cytometry and sex-sorting of sperm (a, b), and reanalyzed DNA content data from sorted fractions of Y-sperm (c) and X-sperm (d). Modified from Johnson, Welch and Rens. 1999 [9].

eter equipped with a cell sorter that consists of a pump to move the buffer (sheath fluid) containing sperm cells that receive excitation by a UV laser (Fig. 1a). Two detectors observe the fluorescence intensity of each sperm cell and a computer rapidly analyzes the digitized data allowing operators to recognize DNA content differences of sperm sub populations on a computer screen (Fig. 1b).

The cell-sorting unit of the system works as follows. When the stream of the sheath fluid exits the flow cytometer nozzle, a piezo vibrator breaks it forming 60,000 to 70,000 serial droplets per second. About one third of the droplets contain a sperm and others are empty. If a droplet is recognized as containing X-sperm, the system gives a positive charge to the droplet; and if the droplet contains a Y-sperm, multiple sperm, sperm with damaged membrane, undistinguishable sperm or no sperm, no charge is given. As the droplets fall after exiting the nozzle, they pass through a high voltage (2,000 to 3,000 V) field that is positive on one side and negative on the other. The droplets with positive charge (X-sperm) move toward the negative side of the field, and those with no charge continue falling straight down to the waste tube. The side stream of charged droplets is collected in a test tube, which fills with the X-sperm rich fraction. Conversely, Y-sperm can also be enriched in a similar way. This sorting process works quite well with a powerful computer combined with exquisitely designed hardware. Since the DNA content difference is small, the resulting fractions are not 100% pure populations of either X- or Y-sperm [7, 9].

Accumulated instrumental innovation including a custom designed nozzle [7, 9] led a manufacturer to produce a high speed sperm sorter 'MoFlo® SX' (Cytomation Inc., Fort Collins, CO, USA) [10]. The above procedures were originally patented by the USDA, and are exclusively controlled by XY LLC (Fort Collins, CO, USA), who develops and promotes the technology for commercial use with non-human species [10]. Currently MoFlo® sorters are manufactured by Beckman Coulter Inc. (Fullerton, CA, USA) and the sperm sorting model is distributed through XY LLC (currently located in Navasota, TX, USA) [10].

Artificial Insemination Trials and Fertility Results

While a conventional AI dose contains 10 to 30 million sperm per straw [1, 2], one dose of sexed semen currently contains about two to three million sperm [10, 14]. In the initial phase of trials, usable sexed sperm cell numbers were limited because of the complicated logistics [10, 12]. Thus, Seidel *et al.* proposed 'low dose AI' [12–15, 17] as a method for using sexed sperm efficiently with fewer sperm numbers per dose (0.1 million) as liquid (non frozen) semen [12]. After reports of successful cryopreservation of sexed bull sperm [13, 18], numerous field trials were done using frozen sperm [10]. Seidel and Schenk [17] compiled their AI trials using heifers and cows, and concluded that pregnancy rates of sexed, low dose, frozen-thawed sperm averaged about 75% of control sperm with a normal dose. Also, they observed no difference in pregnancy rates in the range between 1.5 and 6 million sexed sperm per dose in heifers.

Frijiters *et al.* [19] used seven Holstein sires to evaluate how a lowered dosage and the sexing process affected the AI fertility of sexed sperm. They showed that 2/3 of the decrease in the pregnancy rates was the result of lowered sperm numbers being depoisted and 1/3 was caused by the sorting process itself. DeJarnette *et al.* [20] found no difference in pregnancy rates between two million (commonly used) and 3.5 million doses of sexed sperm from six Holstein bulls in commercial herds (heifers and cows). DeJarnette *et al.* [21] further performed a

large AI trial using eight bulls and 350 straws per treatment per sire and compared sexed and unsexed sperm at dosages of 2 and 10 million sperm per straw. In the results for 51 dairy herds, sperm dosages and herds (management and technicians' skill) had an interaction affecting the pregnancy rates. Overall, inseminating doses of 10 million sexed sperm resulted in higher pregnancy rates than two million, but did not compensate to the level of the two million dose of unsexed control sperm [21].

These results suggest that the fertility of sexed frozen sperm is only slightly compensated by increasing the sperm number per insemination dose. Based on earlier trials [12, 13], one AI dose containing two million sexed frozen sperm is recognized as the minimum threshold to achieve acceptable pregnancy rates in heifers [10, 17]. Production of sexed sperm requires huge investments; instruments, laboratory facilities, skilled technicians and a license. Consequently, semen suppliers (AI organizations) are still adopting the 'low dose' concept to match the costs to the demand [10].

Why is Fertility of Sexed Sperm Reduced?

While Guthrie *et al.* [22] showed the laser power used for sperm sexing was not detrimental to embryonic development in swine, Suh *et al.* [23] reported that lowering sheath fluid pressure (50 to 40 psi) in a sperm sorter improved motility and cellular integrity of sorted bull sperm without compromising sorting efficiency. AI fertility [24] and *in vitro* produced (IVP) embryo yields [25] were improved using sexed bull sperm sorted at lowered pressure. This kind of mechanical stress is thought to be a major cause of the decreased fertility associated with the current sperm sexing process [14]. Virtually all the sperm sorters are now operated at 40 psi [10].

Palma *et al*. [26] observed that IVP bovine embryos from sexed sperm had more ultrastructural deviations than their non-sexed counterparts. This indicates sperm sorting might influence the subsequent embryonic development and cause the lowere pregnancy rates. Gosálvez *et al*. [27] investigated DNA fragmentation status in sexed frozen bull sperm. They suggested that DNA fragmentation increased faster in sorted, frozen-thawed sperm than in control sperm. It might also be linked to the reduced fertility of sexed sperm.

Besides the sexing procedure and lowered sperm number, individual bulls show vast variations of fertility *in vivo* [17, 28] and *in vitro* [25, 26, 29, 30]. It is important to select bulls in commercial settings for their field fertility and seminal tolerance for the sexing process [10, 14–15, 19, 21, 28].

Sex Bias and Normalcy of Calves

Purity (% of X- or Y-sperm) in a batch of sexed semen can be evaluated by 'reanalyzing' sperm DNA content using a flow cytometer [9] (Fig. 1c and 1d). With this technique, semen suppliers have set 90% as a minimum threshold for selling sexed semen [10]. With this virtual industry standard, sex bias of calves derived from sexed semen has been as expected (around 90%) in commercial settings [10, 19–21, 28].

On the other hand, people are concerned that the calves produced using a new biotechnology, such as sexing, might have abnormalities, because the current sperm sexing procedure uses a DNA-binding dye and exposure to UV laser light [7, 14, 16]. Tubman *et al*. [31] studied 1,169 calves from field trials with sexed sperm. They found no evidence of increased abnormalities in the fetal, perinatal and neonatal stages compared to the counterparts from control sperm. It is impossible to prove 'no effect'; however, millions of calves have been phenotypically normal, and resulted females have been producing progenies since this technique was introduced into the industry [10]. It is widely accepted that genetic damage resulting from the sperm sexing process is extremely low and not yet detectable [7, 14, 31].

Other Applications and Future Developments

Recently, researchers have been trying to inseminate sexed semen at time points closer to ovulation (s) [32, 33], since it is hypothesized that sexed sperm have shorter half-life (including accelerated capacitation) in the female reproductive tract than conventionally processed sperm [34]. These trials have shown promising results for timed insemination protocols and the approaches need further investigation to achieve general agreement for complete AI protocols.

Since the supply of sexed semen is still limited, there is great interest in using it in embryo transfer (ET) programs [11, 28–30, 35–36]. ET programs using multiple ovulation and embryo transfer (MOET) [28, 35–36] or IVP using *in vitro* fertilization (IVF) [29, 30] can increase the number of offspring produced from a unit of sexed semen used. Fertility of a transferred embryo in MOET is not compromised regardless if it is derived from sexed sperm [28]. However, inseminating sexed sperm in an ovarian-superstimulated female is challenging because of the higher costs of sexed semen and the program itself. Although increased sperm number per inseminate may improve the rate of usable embryos, the *in vivo* embryo yield is highly dependent upon each situation [28,

35–36]. The IVF-IVP program is an option for the production of embryos from slaughtered ovaries at relatively low cost using sexed sperm [29, 30].

The accuracy and production efficiency of sexed sperm have been dramatically improved by development of the sorting hardware and software [9, 10, 14, 37]. In contrast, the accompanying procedure of sperm processing has not been modified greatly since its initial establishment [12, 14, 18]. Each of sperm processing steps (handling ejaculates, Hoechst 33342 staining, high-dilution and centrifugation, cryopreservation, etc.) is indispensable for the current method and has ample room for further optimization. Recent work suggests the possibility of improving the quality and fertility of sexed sperm [38–40].

For the aforementioned reasons, techniques without cell-sorting have been anticipated for simpler, cheaper, faster and less invasive sperm sexing [10, 14]. For example, immunological approaches are being considered for mass sexing [41, 42]. However, it is not likely that any method will emerge soon as a commercially viable alternative [10, 14].

Conclusions

Many contributors have developed the current sperm sexing technology, mainly supported by the cattle industry. This review covered only a small part of their important accomplishments. Sexed semen products are successfully reproduced at multiple sites around the world. This means a paradigm shift in cattle reproduction, just as AI had an immeasurable impact on the industry. However, the current technology is not yet fully mature and requires further refinements in both procedures and applications.

References

1) Foote, R.H. (1996): Review: Dairy cattle reproductive physiology research and management-past progress and future prospects. J. Dairy Sci., 79, 980–990.
2) Vishwanath, R. (2003): Artificial Insemination: the state of the art. Theriogenology, 59, 571–584.
3) Seidel, G.E. Jr. and Johnson, L.A. (1999): Sexing mammalian sperm - Overview. Theriogenology, 52, 1267–1272.
4) Seidel, G.E. Jr. (2003): Economics of selecting for sex: the most important genetic trait. Theriogenology, 59, 585–598.
5) Seidel, G.E. Jr. (2003): Sexing mammalian sperm–intertwining of commerce, technology, and biology. Anim. Reprod. Sci., 79, 145–156.
6) Garner, D.L., Gledhill, B.L., Pinkel, D., Lake, S., Stephenson, D., Van Dilla, M.A. and Johnson, L.A. (1983): Quantification of the X- and Y-chromosome bearing spermatozoa of domestic animals by flow cytometry. Biol. Reprod., 28, 312–321.
7) Garner, D.L. (2006): Flow cytometric sexing of mammalian sperm. Theriogenology, 65, 943–957.
8) Johnson, L.A., Flook, J.P. and Hawk, H.W. (1989): Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol. Reprod., 41, 199–203.
9) Johnson, L.A., Welch, G.R. and Rens, W. (1999): The Beltsville sperm sexing technology: High-speed sperm sorting gives improved sperm output for *in vitro* fertilization and AI. J. Anim. Sci. 77 (Suppl 2), 213–220.
10) Garner, D.L. and Seidel, G.E. Jr. (2008): History of commercializing sexed semen for cattle. Theriogenology, 69, 886–895.
11) Cran, D.G., Johnson, L.A., Miller, N.G.A., Cochrane, D. and Polge, C. (1993): Production of bovine calves following separation of X- and Y- chromosome bearing sperm and *in vitro* fertilization. Vet. Rec., 132, 40–41.
12) Seidel, G.E. Jr., Allen, C.H., Johnson, L.A., Holland, M.D., Brink, Z., Welch, G.R., Graham, J.K. and Cattell, M.B. (1997): Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. Theriogenology, 48, 1255–1264.
13) Seidel, G.E. Jr., Schenk, J.L., Herickhoff, L.A., Doyle, S.P., Brink, Z., Green, R.D. and Cran, D.G. (1999): Insemination of heifers with sexed sperm. Theriogenology, 52, 1407–1420.
14) Seidel, G.E. Jr. and Garner, D.L. (2002): Current status of sexing mammalian spermatozoa. Reproduction, 124, 733–743.
15) Seidel, G.E. Jr. (2007): Overview of sexing sperm. Theriogenology, 68, 443–446.
16) Garner, D.L. (2009): Hoechst 33342: The dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology, 71, 11–21.
17) Seidel, G.E. Jr. and Schenk, J.L. (2008): Pregnancy rates in cattle with cryopreserved sexed sperm: Effects of sperm numbers per inseminate and site of sperm deposition. Anim. Reprod. Sci., 105, 129–138.
18) Schenk, J.L., Suh, T.K., Cran, D.G. and Seidel, G.E. Jr. (1999): Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology, 52, 1375–1391.
19) Frijters, A.C.J., Mulsaart, E., Roelofs, R.M.G., van Hoorne, R.P., Moreno, J.F., Moreno, O. and Merton, J.S. (2009): What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology, 71, 64–67.
20) Dejarnette, J.M., McCleary, C.R., Leach, M.A., Moreno, J.F., Nebel, R.L. and Marshall, C.E. (2010): Effects of 2.1 and 3.5 million sex-sorted sperm dosage on conception rates of Holstein cows and heifers. J. Dairy Sci., 93, 4079–4085.
21) DeJarnette, J.M., Leach, M.A., Nebel, R.L., Marshall, C.E., McCleary, C.R. and Moreno, J.F. (2011): Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen plausible? J. Dairy Sci., 94, 3477–3483.
22) Guthrie, H.D., Johnson, L.A., Garrett, W.M., Welch, G.R. and Dobrinsky, J.R. (2002): Flow cytometric sperm sorting: Effects of varying laser power on embryo development in swine. Mol. Reprod. Dev., 61, 87–92.

23) Suh, T.K., Schenk, J.L. and Seidel, G.E. Jr. (2005): High pressure flow cytometric sorting damages sperm. Theriogenology, 64, 1035–1048.
24) Schenk, J.L., Cran, D.G., Everett, R.W. and Seidel, G.E. Jr. (2009): Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm number per inseminate, sorting pressure and sperm storage before sorting. Theriogenology, 71, 717–728.
25) Barceló-Fimbres, M., Campos-Chillón, L.F. and Seidel, G.E. Jr. (2011): *In vitro* fertilization using non-sexed and sexed bovine sperm: sperm concentration, sorter pressure, and bull effects. Reprod. Domest. Anim., 46, 495–502.
26) Palma, G.A., Olivier, N.S., Neumüller, C.H. and Sinowatz, F. (2008): Effects of sex-sorted spermatozoa on the efficiency of *in vitro* fertilization and ultrastructure of *in vitro* produced bovine blastocysts. Anat. Histol. Embryol., 37, 67–73.
27) Gosálvez, J., Ramirez, M.A., López-Fernández, C., Crespo, F., Evans, K.M., Kjelland, M.E. and Moreno, J.F. (2011): Sex-sorted bovine spermatozoa and DNA damage: II Dynamic features. Theriogenology, 75, 206–211.
28) Hayakawa, H., Hirai, T., Takimoto, Y., Ideta, A. and Aoyagi, Y. (2009): Superovulation and embryo transfer in Holstein cattle using sexing sperm. Theriogenology, 71, 68–73.
29) Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P. and King, W.A. (2009): Analysis of bovine sexed sperm for IVF from sorting to the embryo. Theriogenology, 71, 30–38.
30) Xu, J., Chaubal, S.A. and Du, F. (2009): Optimizing IVF with sexed sperm in cattle. Theriogenology, 71, 39–47.
31) Tubman, L.M., Brink, Z., Suh, T.K. and Seidel, G.E. Jr. (2004): Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. J. Anim. Sci., 82, 1029–1036.
32) Sales, J.N.S., Neves, K.A.L., Souza, A.H., Crepaldi, G.A., Sala, R.V., Fosado, M., Campos Filho, E.P., de Faria, M., Sá Filho, M.F. and Baruselli, P.S. (2011): Timing of insemination in dairy and beef cattle receiving timed artificial insemination using sex-sorted sperm. Theriogenology, 76, 427–435.
33) Soares, J.G., Martins, C.M., Carvalho, N.A.T., Nicacio, A.C., Abreu-Silva, A.L., Campos Filho, E.P., Torres Júnior, J.R.S., Sá Filho, M.F. and Baruselli, P.S. (2011): Timing of insemination using sex-sorted sperm in embryo production with Bos indicus and Bos taurus superovulated donors. Anim. Reprod. Sci., 127, 148–153.
34) Mocé, E., Graham, J.K. and Schenk, J.L. (2006): Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. Theriogenology, 66, 929–936.
35) Schenk, J.L., Suh, T.K. and Seidel, G.E. Jr. (2006): Embryo production from superovulated cattle following insemination of sexed sperm. Theriogenology, 65, 299–307.
36) Peippo, J., Vartia, K., Kananen-Anttila, K., Räty, M., Korhonen, K., Hurme, T., Myllymäki, H., Sairanen, A. and Mäki-Tanilaa, A. (2009): Embryo production from superovulated Holstein-Friesian dairy heifers and cows after insemination with frozen-thawed sex-sorted X spermatozoa or unsorted semen. Anim. Reprod. Sci., 111, 80–92.
37) Sharpe, J.C. and Evans, K.M. (2009): Advances in flow cytometry for sperm sexing. Theriogenology, 71, 4–10.
38) Rath, D., Moench-Tegeder, G., Taylor, U. and Johnson, L.A. (2009): Improved quality of sex-sorted sperm: A prerequisite for wider application. Theriogenology, 71, 22–29.
39) Burroughs, C.A., Evans, K.M., Lenz, R.W. and Seidel, G.E. Jr. (2012): Effects of removing seminal plasma during 8-h storage before sex-sorting bovine sperm. Reprod. Fertil. Dev., 24, 213 [abstr].
40) Leahy, T. and Gadella, B.M. (2011): Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction, 142, 759–778.
41) Sang, L., Yang, W.C., Han, L., Liang, A.X., Hua, G.H., Xiong, J.J., Huo, L.J. and Yang, L.G. (2011): An immunological method to screen sex-specific proteins of bovine sperm. J. Dairy Sci., 94, 2060–2070.
42) Ellis, P.J., Yu, Y. and Zhang, S. (2011): Transcriptional dynamics of the sex chromosomes and the search for offspring sex-specific antigens in sperm. Reproduction, 142, 609–619.