—Review—

Sex Preselection in Farm Animals by Flow Cytometric Separation of X- and Y-Chromosome Bearing Spermatozoa

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Abstract: *Sex preselection by use of X- and Y- chromosome bearing spermatozoa has been recognized to be more efficient. A flow cytometric sperm sorting based on the difference of their DNA content is the best method for separation of X- and Y-sperm. To date, the flow cytometrically sorted sperm has been involved in the production of sex preselected offspring by surgical intratubal insemination, in vitro fertilization and embryo transfer and intracytoplasmic sperm injection. At first, flow cytometer was modified for DNA confirmation and sorting of sperm with high resolution. Especially, the beveled insertion tube could regulate orientation of flat-shaped sperm head. The forward fluorescent detector was essential for measuring DNA contents of sperm. Recently, the high-speed sperm sorting with the orienting nozzle resulted in production of 90% purity of X- and Y-sperm at rate of 6 million sperm per hour. This application can enable to accomplish more conventional technology for both artificial insemination and cryopreservation of X- or Y-sperm in farm animals.*

Key words: *Sex preselection, Spermatozoa, DNA, Flow cytometer, Farm animals*

Preselecting the sex of progeny is the most efficient for animal production and improvement. Since the 1950s, it has been known that in mammals sex is determined by the presence or absence of the Y chromosome. Most of the initial attempts have been carried out to identify either the X- and Y-chromosome bearing spermatozoa [1] or the sex in embryos [2]. In the embryo, both sex can be produced by fertilization: XX (female) and XY (male) embryos, which sex is determined by the chromosomes of the sperm. In the process of spermatogenesis in the testis, two type of the sperm can be produced: X-chromosome bearing sperm (X-sperm) and Y-chromosome bearing sperm (Y- sperm). Therefore, most effective and convenient method for sex control is based on the use of either X-sperm or Y-sperm separated. To date, using X- or Y-sperm separated by flow cytometric cell sorter, sex predetermined offsprings have been born in the rabbit [3] and pig [4] by surgically artificial insemination, in sheep [5] and cattle [6] by intracytoplasmic sperm injection and in cattle [7] and pig [8] by *in vitro* fertilization techniques, although the number of actual birth was a few.

This paper will present briefly the historical aspects of X- and Y-sperm separation, confirmation and the use of flow cytometer for X- and Y-sperm separation in order to produce the offspring with desired sex in farm animals.

X- and Y-Sperm Separation: Historical Aspects

For the long period, the difference between X- and Y-sperm has been believed to exist in their size, density, motility, antigen, or surface charge, and numerous studies to separate X- and Y-sperm have been attempted by these assumptive differences. Table 1 illustrates some of the physical characteristics of sperm for use of potentially means to distinguish X- from Y-sperm. Burstein and Schenker [9] reported that a sex ratio of 73% males was obtained in human sperm population treated with a albumin gradient method, which was modified from Ericsson *et al.* [10]. Clinical data compiled from various laboratories of the world suggested that about 75% of the offsprings obtained from the this method were male [11]. Afterward, however, Brandriff *et al.* [12] reported 43% Y-sperm in the samples by

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Table 1. Summary of potential differences between X- and Y-spermatozoa

Parameter	Proposed differences	Species	References (No.)
DNA	X-sperm more	Mammals	Moruzzi, Pinkel *et al.* [5, 6]
Size	X-sperm larger	Human	Cui and Matthews [18]
Density	X-sperm greater	Human	Ericsson *et al.* [20]
Motility	Y-sperm faster	Human	Ericsson *et al.* [20]
Surface charge	X-sperm negative	Human	Kaneko *et al.* [29]
Surface antigen	Y-sperm with H-Y antigen	Swine	Hendriksen *et al.* [70]

treatment of albumin gradient and 50% Y-sperm in untreated samples. Ueda and Yanagimachi [13] found an average of 48% Y-sperm in the treated sample, which was not differed from the untreated control sample. Beal *et al.* [14] reported 45% males obtained from inseminations with semen treated with albumin gradient and 54% males from those with control semen, which confirmation was made by flow cytometer. They concluded that the treated sample did not enriched Y-sperm. Amann [15] suggested that the Ericsson's procedure might not produce a sample enriched for Y-sperm, but rather differentially alter some nature of either Y-sperm or X-sperm, so that there was a greater inclination of Y-sperm to fertilize human oocytes that complete gestation.

Several investigators attempted to use antibodies against the H-Y antigen [16], which might be localized on the plasma membrane over the head and middle piece of the sperm [17]. Zavos [18] also used monoclonal H-Y antibody in separation with an immunofiltlation column. Theoretically, however, it is considered that the amount of H-Y antigen in X- and Y-sperm is similar.

Successful data [19, 20] showed that human sperm could be separated into two distinct populations by free-flow electrophoresis, which is on the basis of surface charge difference. There, however, may be the combined effects between surface charge and swimming characteristics when applied to separation of motile sperm. Amann [15] claimed that difference on surface charge between X- and Y-sperm could not be explained by the modification after exposure with epididymal fluid and/or seminal plasma.

In mammals [21], it is known that there are a few differences in DNA content between X- and Y-sperm, because the X chromosome is larger than the Y chromosome: in the bull, there is a 3 to 4 percentages difference [22]. Thus, X-sperm has more DNA content than Y-sperm and may have greater sedimentation velocity than Y-sperm. Iizuka [23] reported that female babies were born after artificial insemination with semen collected from the bottom fraction of eight layers of Percoll gradients. Upreti *et al.* [24] were not able to isolate bovine X- and Y-sperm after centrifugation with a continuous Percoll gradient method. By the chromosome analysis of sperm penetrated into zona-free hamster oocytes, Hamano and Uchiyama [25] confirmed no difference in the X- and Y-sperm ratio of the bull semen collected from bottom fraction separated by 8 fractions of Percoll density gradient centrifugation.

As the described above, there has been little reliable evidence to support the claims made for various physical, morphological or non-DNA based separating procedures. The majority of attempts using these methods ended in failure [26].

Flow Cytometry and Its Application to Sperm

In most mammals, the X chromosome is larger and has more DNA content than the Y chromosome. The DNA difference between X- and Y-sperm varies among species from about 2.8% in the humans to 12.5% in the creeping vole [27, 28]. In farm animals, the DNA difference between both sperm ranges from 3.5% to 4.2% [29]. Thus, the only scientifically validated and measurable difference between X- and Y-sperm is the DNA content. This fact was initially reported by Gledhill *et al.* [30] who analyzed X- and Y-sperm by flow cytometry. Flow cytometry provides the measurement of cellular DNA and accumulates the distribution of DNA. Base on this principle, flow cytometer has been used for immunological analysis and separation of the somatic cell. Theoretically, flow cytometer can distinguish X- sperm from Y-sperm. Pinkel *et al.* [31] suggested that DNA analysis to differentiate X- from Y-sperm was possible using an epi-illumination flow cytometer. Flow cytometry was successfully applied to the differentiation of DNA in X- and Y-sperm [31–33]. After axial adjustment with flow cytometer of standard-speed cell sorter, Hoechst 33342 stained sperm was analyzed and sorted (Fig. 1). Hoechst 33342 can bind to the adenine-thymine region of the nucleic acids. Analysis of sperm DNA content was performed by setting the gate with a detector at 90 degree angle to identify oriented sperm, which was

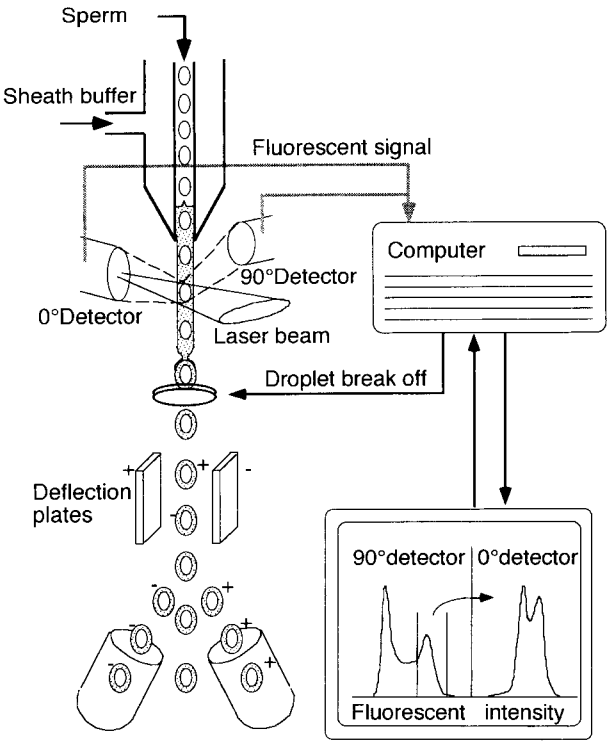

Fig. 1. Flow analysis and sorting of X- and Y-spermatozoa.

placed on a 0 degree histogram. The fluorescent signals represent both the orientation and amount of DNA content of single sperm. These signals that were gathered from both 90 and 0 degree fluorescent detectors mean as frequency distributions (fluorescent intensities). After flow analysis, sorting was carried out by setting a sort window on 0 degree fluorescent signals out of each peak. And then the droplets containing single sperm had a electrically positive or negative charge according to the DNA content. Finally, the deflected droplets of either X- or Y-sperm fell into a collection tube. Thus, sorted sperm can be fractionated (Fig. 1). Recently, Johnson *et al.* [34, 35] recommended that to stain dead sperm, 1 μl of food coloring, FD&C #40 was mixed into sperm suspension stained with Hoechst 33342. Hoechst 33342 is highly permeable to the living sperm membrane through incubation at 35°C [33], whereas the food coloring penetrates the membrane of the dead sperm and quenches the intensity of fluorescence of the dead sperm, resulting in eliminating dead sperm from the viable sperm population [34–36]. In general, propidium iodide also is used for the same purpose but has drawback of being on intercalating dye. McNutt and Johnson [37] demonstrated the direct damage of laser power on mammalian embryo development. Johnson *et al.* [35] examined laser output powers of 25 and 125 mW in case of sorting with high-speed and showed that 125 mW did not affect subsequent fertilization rate or embryo development in pigs.

Sorting of the sperm was accomplished only in sperm head [38] and later has been developed as a successful method for viable sperm by flow cytometry [3]. It, however, appears to be difficult to distinguish the small difference in fluorescent intensity between X- and Y-sperm population, because the fluorescent signal intensity could be affected more by morphology and orientation of the sperm to the laser excitation than by DNA content. Since the shape of the sperm head is flat, the sperm can not maintain their orientation in the ordinary fluid stream. The misorientation of sperm head causes high refraction of fluorescent emission. Johnson and Pinkel [32] modified commercially acquired flow cytometer to sort sperm heads: the ribbon-shaped fluid stream made by beveled insertion tube keeps the sperm head orientating easily [30, 31]. The emitted fluorescence from the edge of the sperm head was brighter than that from the flat side. Since the edge fluorescence is used to characterize the orientation of sperm, 90 degree fluorescent detector could gather the edge emission of the sperm head (Fig. 1). For high resolution of sperm DNA content, fluorescent signals should be collected from oriented sperm head. Forward additional fluorescent detector is essential for measuring the fluorescent emission from the flat side of the sperm head. Modified flow cytometer could collect the emitted fluorescent signals from both the edge and the flat side of the bull sperm head [22]: in each sample, about 65–95% of the sperm head were properly oriented, whereas 20–40% of them were properly oriented to the laser beam, fell into the orientation gate and seen by the 0 degree detector. These modifications contributed to establishment of the current sperm separation technology by commercial flow cytometer. For artificial insemination with sorted X- and Y-sperm in farm animals, however, efficiency of the sorting for viable sperm is required. For this purpose, improvement of the efficiency of sperm orientation is essential. Recently, Rens *et al.* [39] developed the orienting nozzle. This novel nozzle resulted in greater orienting percentage [34]. Rens *et al.* [39] and Johnson *et al.* [34] suggested that the hydrodynamic forces affected duration of the orientation force. The modification of the hydrodynamic forces using a orienting nozzle resulted in 2 to 3 times increase of the number of oriented sperm. More recently, Johnson *et al.* [34, 35] developed the commercially available high-speed sorter and stated that the high-speed

sorter can sort up to 2 million sperm per hour with the standard beveled needle and to 6 million sperm with the orienting nozzle. At present, the commercial available high-speed sorter with these 3 improvements is capable of producing up to 6-11 million of X- and Y-sperm per hour [34, 35].

X- and Y-Sperm Identification: Historical Aspects and Actual Methods

A convenient, precise and accurate procedure to identify X- and Y-sperm is essential for successful separation of X- and Y-sperm. About fifty percentages of human sperm had single brightly fluorescent spot, F-body, after stained with quinacrine mustard [40]. There, however, was not a consistency in evidences for location of F-body in the Y-sperm, detection of every Y-sperm and accuracy of F-body test [13]. In other mammalian sperm, 16–60% of the Y-sperm were not stained with quinacrine mustard [41]. Also, chromosome analysis is available for confirmation of X- and Y-sperm. After penetrated into zona-free hamster oocytes, sperm chromatin undergoes decondensation, so that a set of the chromosomes suitable for karyotyping is provided [13, 42, 43]. In bull sperm, only 37–54% of the hamster oocytes provided preparation with chromosomal spreads, in which the X- or Y-chromosome could be identified [43], suggesting that this method may require many oocytes per sample for accuracy.

By development of molecular biology and polymerase chain reaction (PCR) technology, identification of single bull Y-sperm with high accuracy has been enabled by amplifying the bovine Y specific DNA fragment. This method must be used for monitoring the separation of Y-sperm from X-sperm, especially when data on individual sperms can be obtained. Welch *et al.* [44] applied PCR to analyze single X- and Y-sperm previously sorted into 48 well plates, and found that each plate with single sperm could be determined within less than 2 min with 96% accuracy. PCR is also effective for identifying the sex of single embryo after in vitro fertilization with separated sperm. However, the labor required to conduct PCR analysis of single sperm or embryo likely prohibits its use as a routine monitoring of sex identification.

More recently, new technologies have been developed in molecular biology for DNA hybridization and fluorescent labeling. Of these, fluorescence in situ hybridization (FISH) is a more efficient and convenient method for detection of specific DNA and RNA in tissues and cells within a short period. This procedure with probes for identifying the Y- and X-chromosome gives us an opportunity to use it as an independent technique for validation of sperm population separated [46–50]. Considered that a large number of samples are monitored in separation of X- and Y-sperm by flow cytometer, FISH could be an efficient technique, because of possible potential to examine 200 or more sperm per sample. Rens *et al.* [48] developed a new protocol for sexing bovine X- and Y- sperm with the X- and Y-chromosome paints. They developed sex chromosome-specific paints obtained by degenerate oligonucleotide-primed-PCR (DOP-PCR) [51] amplification from a yak cell line. The application of these paints to a FISH protocol for bull sperm resulted in clear identification in more 90% of sperm. When the fluorescence-labeled X- and Y-specific paints were tested in samples of bull sperm sorted by Beltsville sperm sexing technology [34], the results indicated that the FISH procedure is robust, reproducible and simple with high efficiency to evaluate the sex of sorted sperm [48].

Fertilization by X- and Y-Sperm Sorted with Flow Cytometer and Production of Sex Preselected Offspring

Although sex confirmation of embryos is currently performed in cells collected by bisection or biopsy using X- or Y-specific DNA analysis with PCR, the production of embryos sex-preselected by methods with X- or Y-sperm, such as artificial insemination (AI), *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) has been recognized to be more efficient. To date, the fertility of X- and Y-sperm sorted by flow cytometer has been certificated in rabbits [3], pigs [4] and cattle [7, 52]. Table 2 shows data of the offsprings successfully produced by sperm sorted with flow cytometer followed by surgical insemination [3, 4], IVF [7, 8, 52] and ICSI [5, 6]. Before development of high-speed sorting technology [34, 35], the primary limiting factor to use sorted sperm by flow cytometer in artificial insemination is that only a small numbers of sperm were available in a given time period [53], which should be overcome, because of good motility and a large number of sorted sperm required for calf production. ICSI can be done with single sperm head without motility and so seems to be an efficient method for sorted sperm. In farm animals, the production rate of offsprings derived from ICSI oocytes with flow-sorted sperm is lower than that from IVF [5–7]. In sheep, Catt *et al.* [5] obtained cleaved embryos at rate of 46% at 6 days after ICSI with sorted ram sperm, and produced one male offspring from transfer of 28 embryos. Goto *et al.* [54]

Table 2. Sex preselected offspring production with flow sorted spermatozoa

Methods	Species	Sperm	No. offspring	Sex (X/Y) (%)	References
Surgical AI	Rabbit	whole	37	81/94	Johnson et al. (1989)
IVF	Bovine	whole	43	100/90	Cran et al. (1993, 1995)
ICSI	Sheep	whole	1	-/100	Catt et al. (1996)
AI	Bovine	whole	14	100/75	Seidel et al. (1997)
ICSI	Bovine	head	10	-/80	Hamano et al. (1999)

produced bovine blastocysts at rate of 0.4% using ICSI with unsorted bull sperm heads. Although the fertilization rate in ICSI oocytes was lower than that with IVF, their results were comparable to the previous reports using ICSI with sorted sperm [4, 37] and unsorted sperm heads [54]. In cattle, so far, there is only one report [6] of calf production by transfer of blastocysts derived from oocytes injected with flow cytometrically sorted Y-sperm heads; the production of 8 males from 10 calves using flow cytometrically sorted Y-sperm heads. They stated that calf production rate after transfer of sex preselected embryos was low but comparable to those in the previous studies using ICSI and IVF with sorted sperm.

For ordinary AI in cattle, at least 10 million sperm per trial are necessary for successful pregnancy. Ten years ago, it was difficult to separate 10 million X- or Y-motile sperm by the flow sorting method in a whole day. As the technique of sorting by flow cytometer has progressed as mentioned before, the number of sorted sperm has increased. Seidel *et al.* [55] demonstrated AI in cows using sorted bull sperm (0.1–0.2 million sperm/ 0.1 ml) by depositing the sperm deep into the uterine horn, and obtained sex-predetermined offspring or fetuses. Cran *et al.* [56] inseminated sheep with a low dose of sorted sperm by the laparoscopic intrauterine procedure, and showed offspring production with low rate. Recently, in cattle, insemination by a deep uterine procedure with low dose of X- or Y-sperm has been achieved by Seidel *et al.* [57]: a total of 0.2 million sperm per dose was sufficient to get pregnancies and to produce 17 calves. The average percentage of a female calf derived from sorted X sperm was 82%. This technique provided a useful semen-delivery avenue needed to bring sexed semen to producers. In IVF of porcine oocytes, the study using sexed sperm after high-speed sorting demonstrated effectiveness of use of the sexed sperm to produce sexed offspring [58]. High-speed sorting of bull sperm can provide sexed sperm for AI in the conventional manner. When some high-speed sorter pressures were tested, there was no differences in fertilization rate among pressures of 1.76, 2.81 and 4.22 kg/cm^2 [59]. In cattle, Johnson *et al.* [34] demonstrated AI directly into body of the uterus with 2 to 3 million motile sperm sex-sorted within one hour, resulting in pregnancies and calves with the predicted sex. Thus, high-speed sorting offers opportunity to perform conventional AI with sorted sperm as well as deep-uterine insemination [36].

Cryopreservation of sorted sperm extends the utility of them in many species. Johnson [35] suggested that the use of frozen sorted semen in addition to fresh one adds an important commercial dimension to the process, since frozen semen is highly important to widespread use of this sorting technology in farm animals' production. In cattle, Seidel *et al.* [60, 61] demonstrated the effectiveness of frozen, sorted sperm. Johnson *et al.* [52] applied standard freezing technology to bull sorted sperm: flow cytometrically sorted bull sperm was frozen and thawed. The resultant average sperm motility and acrosomal integrity was 30% and 40%, respectively. One and 2 million sperm per 0.5 ml straws could be available after sorting [52].

Feature and Aspects

At present, sex preselection using high-speed sorting technology enables, not completely but almost, us to perform artificial insemination with flow cytometric separated sperm. Morrell and Dresser [62] reported the subsequent development of the offspring derived from sorted sperm. No offspring showed any morphological abnormality in pigs and rabbits by the observation through two generations of reproduction. Seidel *et al.* [60] demonstrated that one thousand heifers have been inseminated with sexed sperm by high-speed sorter and that its accuracy of producing sex of offspring has approached to 90%. Thus, X- and Y-sperm separation using high-speed sorter could be the basic method for more efficient and commercial techniques of sex preselection in farm animals. So far, however, the cost of instruments for high-speed sperm sorting technology is too expensive to be widespread. One of alternative approaches should involve the use of antibodies to sorted sperm. To date, there has been experiments that at-

tempted to identify a marker related to sex specific surface membrane [63] and also other studies in progress [64]. If any antibody specific to either X- or Y-sperm be identified, these techniques would allow a much wider application of sexed semen in farm animal industry.

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