

Sex-sorting of Spermatozoa in Mammals: A Review

Asit Jain^{1*}, Tripti Jain², Yathish H. M.³, B. Prakash⁴ and Ashwani Sharma¹
Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal-132001, Haryana

ABSTRACT

Sex-preselection by separating the male- and female-bearing spermatozoa is vital for efficient reproduction. In the past, various techniques have been evolved for sexing of semen. They were based on differences in the weight, density, size, motility, surface charges and surface antigen structures etc. There are many reports which have documented the favourable results but they have paucity of scientific validation. A flow cytometric sperm sorting is the best method for separation of X- and Y-chromosome bearing sperm, which is commonly used and relies on the difference of their DNA content. Efforts are in place to exploit this technique in animals. To date, the flow cytometrically sorted sperm are used in the production of sex preselected offspring by surgical intratubal insemination, in vitro fertilization and embryo transfer and intracytoplasmic sperm injection (ICSI). In last decade, the high-speed sperm sorting with the orienting nozzle resulted in production of X- and Y-sperm with 90% accuracy at the rate of 2-6 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. The process of commercialization of sexed sperm has accelerated recently. However, this technology has some impediments with respect to cost, implementation and pregnancy rate in comparison with insemination with fresh semen. Despite these limitations, production of sexed semen usually followed by cryopreservation is being used commercially for cattle and horse production in different parts of the world.

Key words: Sperm sexing; sexed semen; flow Cytometer; sex preselection

Present addresses: ¹NDRI, Karnal-132001, Haryana; ²Indian Veterinary Research Institute, Izatnagar-243122, UP; ³National Bureau of Animal Genetic Resources, Karnal-132001, Haryana. ;

***Corresponding author:** Dr. Asit Jain (Ph D Scholar), Dairy Cattle Breeding Division, NDRI, Karnal-132001, Haryana, INDIA. E-mail address: vetasit@gmail.com

INTRODUCTION

Modern livestock management requires efficient methods to optimize herd size avoiding none or sub-optimal producing animals (Seidel, 2003), especially, when products are sex related, the opposite sex diminishes farm efficiency. Advancements in biotechnology are pivotal in improving the efficiency of livestock reproduction and production. One current example that illustrates the importance of biotechnological development is a process that allows production of offspring whose sex was predetermined. This is based on the separation of X- and Y-chromosome bearing spermatozoa. It is known that sex is determined by presence or absence of Y-chromosome in mammals. During spermatogenesis in the testis, the sex chromosomes segregate into individual spermatocytes, and haploid spermatozoa therefore carry either the X- or Y- chromosome. Fertilization of an X- bearing haploid oocyte by either X- or Y- bearing spermatozoon determines the sex of the resulting embryo. Therefore, the most effective and convenient method for sex control is based on the use of

sexed semen. Several investigators have separated the X- and Y- spermatozoa by various techniques which are based on principles of differing mass & motility (Ericsson et al., 1973; Rohde et al., 1975; Ross et al., 1975; Schilling and Thormaehlen 1977; Dmowski et al., 1979; Quinlivan et al., 1982; Kaneko et al., 1983; Beal et al., 1984; Zavos, 1985; Iizuka et al., 1987; Lopez et al., 1993; Pyrzak, 1994; Wang et al., 1994a; Wang et al., 1994b; Flaherty et al., 1997; Kobayashi et al., 2004), differing swimming pattern (Check and Katsoff 1993), differences in surface charge (Sevinc, 1968; Shirai et al., 1974; Umland and Willems 1975; Engelmann et al., 1988; Blottner et al., 1994; Manger et al., 1997), sperm sorting based on volumetric differences (van Munster et al., 1999a; van Munster et al., 1999b; van Munster et al., 2002), centrifugal countercurrent distribution (Ollero et al., 2000) and immunologically relevant structures (Bennet and Boyse 1973; Hancock 1978; Erickson et al., 1981; Pinkel et al., 1985; Ali et al., 1990; Hemdriksen et al., 1993; Sills et al., 1998; Blecher et al., 1999). However, neither of these methods was able to produce statistically significant separation of fertile sperm

populations, nor was reproducible. The only method known so far is flow cytometry which uses the relative difference in DNA content of X- and Y- chromosome bearing spermatozoa (Johnson et al., 1995; Weigel, 2004; Seidel, 2007).

Spermatozoa bearing X and Y chromosomes are separated on the basis of DNA using flowcytometric sperm sorter. This is effective in many species (Johnson et al., 1989; Johnson, 1995). Sex predetermined offspring's have been produced using surgical artificial insemination in rabbits (Johnson et al., 1989), horses (Buchanan et al., 2000; Lindsey et al., 2002), pigs (Johnson et al., 2000a; Rath et al., 2003; Vazquez et al., 2003) and cattle (Doyle et al., 1999; Seidel et al., 1999), by intra cytoplasmic sperm injection in sheep (Catt et al., 1996) and using in vitro fertilization techniques in cattle (Cran et al., 1994,) and pig (Rath et al., 1993; Rath et al., 1997). Sex ratios have also been skewed in humans (Johnson et al., 1993; Fugger et al., 1998; Fugger et al., 1999).

One of the riddle associated with the research attempts of producing the sexed semen is the difficulty of warranting the proportion of X- and Y- bearing spermatozoa within putative sperms population (Windsor et al., 1993). Moreover, there are numerous confronting reports and many investigators were unable to corroborate enrichment of X or Y-bearing spermatozoa using some of these techniques. Quinacrine staining that evaluates the efficiency of sperm separation has been shown to be unreliable and not specific to Y body at Y chromosome (van Kooij and van Oost 1992).

In this review, we have discussed various techniques employed in separating X and Y- chromosome bearing spermatozoa to produce the sex predetermined offsprings. However, the information on flow cytometry will be furnished in depth when compared to other methods.

Techniques Employed in Sexed Semen Production: Virtually in every sector of commercial animal breeding there is an obvious preference for one sex over the other. Sperm Sexing Technology (SST) enables the producers of dairy, swine and beef animals to predetermine the sex of offspring prior to conception, thereby maximizing the genetic potential, productivity, and profitability. Numerous techniques of sexed semen production based on various principles are as follows.

1. *Difference in Mass and Motility: Albumin Gradient:* Successful separation of X- and Y-chromosome-bearing human spermatozoa using an albumin gradient was first reported by Ericsson et al., (1973). This method is based on the assumption that the smaller Y- bearing spermatozoa swims faster in fluid of high density and viscosity, and have a greater ability to penetrate the interface between fluids than X-bearing spermatozoa. In this technique, human serum albumin (Ericsson et al., 1973) or bovine serum albumin (Beal et al., 1984), a sticky protein solution, is layered into a column of increasing thickness. Centrifuged, washed, and diluted semen is layered on top of this albumin column. The column is allowed to stand for 1 hour, allowing sperm to penetrate the albumin. The top layer is discarded, and the column is allowed to stand for another 30 minutes. The next layer (originally the middle layer) is discarded. The remaining bottom layer is centrifuged, and of this only the pellet (sediment at the bottom) is retained and prepared for artificial insemination (Ericsson and Ericsson 1999). This procedure enhances motility and eliminates abnormal sperms along with partial separation of X- and Y- bearing spermatozoa. It has been reported that the percentage of male children born is increased to 70–80% on employing the latest version of this technique (Ericsson and Ericsson 1999). However, the legitimacy of sex pre-selection by this approach has remained controversial (Evans et al., 1975; Ross et al., 1975; Dmowski et al., 1979; Quinlivan et al., 1982; Brandriff et al., 1986; Ueda and Yanagimachi 1987). In domestic animals this technique does not effectively separate X and Y bearing spermatozoa (Beal et al., 1984; White et al., 1984). Reliable and repeatable alteration in the sex ratio is yet to be achieved by using this technique.

Percoll Gradients: Percoll method is a standard procedure for preparing sexed sperm for intra uterine insemination (IUI) or in vitro fertilization (IVF). Percoll consists of colloidal silica particles coated with polyvinyl pyrrolidone. Percoll was often used as the density medium. Other density media used were Ficoll (Hedge et al., 1977) and Sephadex microbead solution (Quinlivan et al., 1982; Lopez et al., 1993). In the early 1980s, researchers reported that density medium might also cause separation of X and Y-sperm. Later investigations testified that this was not the case, but the method has been promoted in some clinics for gender

selection. In this procedure, sperm is centrifuged through increasingly dense layers of a solution, on the theory that heavier X sperm will sediment to the bottom, and lighter Y sperm will migrate to top. A density medium, a liquid solution available in various thicknesses, is layered in a conical centrifuge tube, with the densest layer at the bottom and the least dense layer at the top. In the standard procedure of preparing sperm for assisted reproduction, two layers are used. For gender selection, 3 to 12 layers are used. Diluted semen is layered on top, and centrifuged for 30 minutes. For female sex pre-selection only the bottom layer, or "pellet", is retained, in which the heavier X-sperm have sedimented and that for male top layer of the liquid "supernatant" is retained, containing the lighter Y-sperm. The remainder is washed and centrifuged again to remove the density medium. The Percoll product was withdrawn from the market in 1996 because of its risk of contamination with endotoxins.

Iizuka et al., (1987) used 12-step discontinuous Percoll gradients (25-80%). Evaluation of this method by Wang et al., (1994b) using double label fluorescent in-situ hybridization (FISH) revealed 94% difference in the proportion of X-bearing spermatozoa as against 55% normal. This apparent discrepancy was elucidated earlier by van Kooji and van Oost (1992).

2. Difference in Surface Charge: Free Flow Electrophoresis: Electrophoretic separation of mammalian sex determining spermatozoa has been attempted by many investigators without substantial success (Kiddy and Hafs 1971; Mohri et al., 1987). Free-flow electrophoresis is a fast and promising method for separation of X- and Y-bearing spermatozoa on the basis of difference in the electrical charge on cell membrane, or differing amount of net charge (Engelmann et al., 1988). In this technique, pretreated seminal plasma-free spermatozoa were injected continuously as a fine stream into the buffer medium in the separation chamber flowing perpendicular to the forces of an electrical field, which separates the spermatozoa according to their differences in electrophoretic mobility into the two major classes (Kaneko et al., 1984). To identify these two classes of spermatozoa before and after separation, some stains (like quinacrine mustard stain used to identify the Y-chromosome-bearing spermatozoa) are used. Spermatozoa moved toward the anode and were separated into two main peaks. The fast moving fraction consists of nearly 80-90% Y-bearing spermatozoa. X-

bearing spermatozoa, because of greater concentration of sialic acid containing glycoproteins on the cell membrane were found in slower peak (Kaneko et al., 1983; Kaneko et al., 1984). In free flow electrophoresis, the sperm viability is nearly unchanged as determined by eosin staining with the reduction in sperm motility. It has been shown that experimental temperature and initial sperm motility had a significant effect on sperm distribution in the electric field (Manger et al., 1997). However, this technique is ineffective in separating spermatozoa as high variations have been observed in different research experiments.

3. Difference in Swimming Pattern: Swim-up Procedures: The story behind sperm swim-up for gender selection is almost exactly same as with the Percoll method. This methods based on the fact that Y-bearing sperm swim differently and more quickly than X-bearing sperm. In this procedure, semen is placed in a test tube (in some cases, it is washed and centrifuged). A culture medium is carefully placed on top of the semen. This medium is cordial to the sperms, and healthy sperms will swim up across the media. Slow and immotile sperms are left behind, along with most debris in the semen. The test tube is allowed to stand for an hour or so; in some cases it is placed at an angle, and/or in an incubator. In the standard procedure, the top layer is collected for use. In the modified swim-up technique for attempted male gender selection, a small fraction of the top layer is first discarded. This practice is based on the theory that a small fraction of X-sperm are the fastest of all, next fastest are the Y-sperm, and the slowest are the majority of X-sperm (Check et al., 1989). Finally, the portion retained for use may be washed and centrifuged again.

Check et al., (1989) reported 81% male offspring after insemination of women with spermatozoa prepared by a modified swim up procedure. They followed this initial report with a prospective trial in which the incidence of male birth was 88.5% when compared to 50% after preparation on standard Percoll gradients (Check and Katsoff 1993). Using quinacrine staining, the incidence of putative Y-bearing spermatozoa was found to be 49.2% in the Percoll samples and 83.6% in the swim-up procedure fractions. These data suggested that swim-up procedures can alter the ratio of X- to Y-bearing spermatozoa in the isolated motile sperm fraction and therefore could be used for male sex pre-selection.

In contrast, Han et al., (1993) used double label FISH, both before and after isolation of motile spermatozoa using a routine swim-up procedure, and found no enrichment of X- or Y-bearing spermatozoa in the motile sperm fraction. Lobel et al., (1993) also found no significant change in the X: Y ratio in the motile sperm fraction. These conflicting results might be explained by slight differences in the procedure used, such as the time of incubation (Claassens et al., 1989) or the centrifugation steps, but more likely emphasize the inadequacies of the quinacrine staining technique used by Check and Katsoff (1993).

4. Difference in Surface Antigen: Immunoassay: Cell surface antigens specific to either X- or Y-chromosome-bearing spermatozoa offer a potential means of separating two sperm populations. Histocompatibility-Y (H-Y) male specific antigen is an acidic hydrophobic glycoprotein with a monomeric molecular weight of 19,000 (Bradley and Heslop 1989) and it is secreted by sertoli cells lineage. Gonadal and non gonadal tissues express this H-Y antigen, however, the same antigen is absent in female tissues (Bradley, 1989). Immunohistochemical studies with male-specific antiserum revealed that serologically detectable male-specific antigen is present on approximately 50% of spermatozoa and is located on both the postacrosomal region of the head and the midpiece of the flagellum (Bradley et al., 1985 and Bradley et al., 1987).

Several attempts have been made to demonstrate that H-Y antigen expression is impounded to Y-chromosome bearing spermatozoa. Unpublished reports provided further evidence for the haploid expression of the H-Y antigen in Y-chromosome bearing spermatozoa (Bradley, 1989). However, some reports have shown the presence of H-Y antigen on both X- and Y-chromosome-bearing spermatozoa (Hoppe and Koo 1984). Detailed study is imperative before exploiting this technique in separating the sex chromosome bearing sperms.

Affinity Chromatography: A report suggests that a viable immunological sperm sexing procedure can be developed using a more meticulous method to isolate sex-specific proteins (SSPs). In this approach, non-SSPs were removed immunologically before the attempted isolation of SSPs because they are likely to be more highly conserved than non-SSPs. Antibodies to SSPs were raised and used to identify SSPs by affinity

chromatography. Antibodies to purified female fetal SSPs caused agglutination of approximately half of the bovine spermatozoa. Unagglutinated spermatozoa were isolated and used in bovine IVF, which produced more than 90% male embryos (Blecher et al., 1999). This immunological approach, which implies post-meiotic transcription or translation of SSPs that do not equilibrate through inter-spermatid cytoplasmic bridges, appeared promising.

5. Volumetric Differences: X-bearing spermatozoa are theoretically larger than Y- bearing spermatozoa. Van Munster et al., (1999a) demonstrated a difference between X- and Y-bearing bovine spermatozoa head volume that matched differences in DNA content. A method based on this principle has been developed for sorting live spermatozoa by using interference microscopy optics with a flow cytometer (van Munster, 2002). This method eliminates the use of DNA-specific dyes for sexing of sperms in mammals. The potential purity of spermatozoa separated using volumetric measurements cannot exceed 80% purity of either sex based on theoretical considerations (van Munster et al., 1999b), and recent efforts to make this pragmatic is disheartening (van Munster, 2002).

6. Centrifugal Counter current Distribution: In this method, X- and Y- bearing spermatozoa are separated by centrifugal counter current distribution using an aqueous two-phase system (Ollero et al., 2000). This is a chromatographic process that partitions cells, several times, into a stationary (lower phase) and a mobile (upper phase). Centrifugation was used to speed the partitioning process, so a set of 59 partitions was done in about 1h. The separation result was up to 75% Y-chromosome-bearing spermatozoa with reasonable viability using this procedure at certain salt concentrations. However, data on repeatability of the process or fertility of the spermatozoa was not presented (Ollero et al., 2000). Also, it may not be successful in species with less difference in DNA content than sheep between X- and Y-chromosome bearing spermatozoa (Johnson, 1995).

7. Differing DNA Content: Flow Cytometry : Detailed procedures for flow-sorting of mammalian spermatozoa are beyond the scope of this manuscript, and depend on the species and applications. However, a short overview on the flow cytometry is provided.

In late 1980's, this technology for sexing sperm was

developed (Johnson et al., 1989) and it has been in use till today. This technology was developed for use on conventional-speed cell sorters in the early 1970s and improved dramatically in 1980. There has been accretionary improvement in these standard sorting systems over the last three decades, mostly related to advancement in computer technology. Even with these improvements, only miniature advances were made in the sorting speed of commercial cell sorters. This has now changed with the development of commercial high speed flow sorting systems. Improvement in the Beltsville technology in the past two years has involved nearly two simultaneous approaches. First is the development of a new flow nozzle to more effectively orient the sperm head to the laser beam, and second is the development of a commercial high speed cell sorter, which could be modified to sort sperm, that we adapted with the improved nozzle to ameliorate sperm orientation.

Technology for sex ratio skewing based on separation of spermatozoa bearing either the X- or Y-chromosome, first developed in rabbits (Johnson et al., 1989). Now, it is well demonstrated in domestic animals like cattle (Cran et al., 1995; Seidel et al., 1997), sheep (Johnson, 1995; Catt et al., 1996; Cran et al., 1997) and swine (Johnson, 1991; Rath et al., 1997). It was developed in human (Johnson et al., 1993; Fugger et al., 1998) and non-human primates like gorilla, chimpanzee, baboon and marmoset (O'Brien et al., 2003; O'Brien et al., 2005a; O'Brien et al., 2005b) and farmed elk (Schenk and De Grofft 2003). Most notably, the first pre-sexed non-domestic species, the bottlenose dolphin, was born after insemination of sorted frozen-thawed spermatozoa derived from liquid-stored semen at Sea-World California (O'Brien and Robeck 2006). Most domestic animals have an X-Y DNA content difference between 3.6 and 4.2% (Johnson and Welch 1999) but this range widens when wild species such as gorilla (2.7%) and giraffe (4.4%) are included (O'Brien et al., 2003). The DNA content differences in the bimodal populations of sperm from bulls, boars, rams, and rabbits were 3.9%, 3.7%, 4.0%, and 3.9%, respectively (Garner et al., 1983). The development of high-speed flow cytometers and improved cell orientation systems (Johnson and Welch 1999; Seidel and Garner 2002) has led to substantial increases in the efficiency of sorting and sorted sperm quality. This

technique is sensitive to detect a minute difference in DNA content between X- and Y- bearing sperms and sort them with 90% purity with an efficiency of 10 million live sperm of each sex (X and Y) per hour (Siedel, 2003; Garner and Seidel 2003; DeJarnette et al. 2008).

The first step in flow cytometric separation of spermatozoa is to dilute the semen to a very low concentration. Then the sperm are dyed with Hoechst 33342, which is a DNA-binding fluorescent dye (Johnson, 1987; Garner, 2006). This dye binds to DNA molecules within the sperm. When the sperm are subsequently exposed to ultraviolet light, the DNA containing the dye will fluoresce. X- Chromosome bearing sperm contain 3.5% more DNA than Y- chromosome bearing sperm (Siedel, 2003), thus the X- chromosome bearing sperm will absorb more of the DNA-binding dye. Using this method the sperms are identified and separated based on their DNA content. The dyed sperms are then placed in the flow cytometer. The sperm enter the flow chamber one at a time and then each sperm is evaluated. The dyed sperm are then subjected to a laser; here the X- chromosome bearing sperm emit more intense light due to the high absorption of fluorescent dye (Senger, 2003). The computer recognizes this light intensity and can assign the sperm as either X or Y. The sperm then drop sequentially through the droplet charging collar of the apparatus, where they are assigned their charge (positive or negative). The sperm sequentially pass through a magnetic field where they are drawn to either the positive or negative side based on their assigned charge (Senger, 2003). On each side of the magnetic field are collection chambers. X and Y chromosome bearing sperms are collected separately on each side. Initially the sperms are also treated with another dye that suppresses the light signal from dead sperms. These dead sperms thus are not detected and are passed through the magnetic field without a charge and fall into the waste chamber.

Sperm Sorting Speed: Sorting speed is dependent on orientation of the sperm through the instrument. An improved system for orienting sperm during the sorting process was developed at USDA (Rens et al., 1998; Johnson et al., 1999; Rens et al., 1999; Welch and Johnson 1999; Johnson, 2000). Results of adapting the orienting nozzle (Rens et al., 1998) to the high-speed

sorter were remarkable. This nozzle design increased the proportion of sperms that could be correctly measured from 25% to more than 60% (Rens et al., 1998; Rens et al., 1999). The greater capability of the high-speed system to sort and acquire data resulted in a 10 to 15 fold amelioration in throughput attained than with the old epics systems using the bevelled needle (Johnson and Pinkel 1986). The new nozzle was adapted for a high-speed sorter and increased sorting rates from 2 to 6 $\times 10^6$ sperm/h for each sex (Rens et al., 1998; Johnson et al., 1999). Modification to the Cytomations SX MoFLo (Cytomations, Fort Collins, Colo) by XY Inc have significantly augmented the efficiency of sorting X and Y sperm and it is capable of sorting 15 $\times 10^6$ bovine sperm/h for each sex (Seidel, 2000; Schenk, 2001).

Cryopreservation and Insemination Dose of Sexed Semen: Flow sorting had improved to produce enough living sexed sperm so that oviductal insemination, in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was possible. After sorting, sperms were concentrated by centrifugation to about 80×10^6 /ml so that they could be reconstituted to 20×10^6 /ml and cryopreserve sorted sperms at 1×10^6 sperm per dose in 0.25ml straws (Schenk et al., 1999). This was almost twice the concentration that had been used successfully for unfrozen, sexed sperm to compensate for cell death due to cryopreservation and thawing. The cryopreservation of sexed sperms result in slightly lower post-thaw motilities and acrosomal integrities compared with control sperm. This damage is minor when compared with that caused by routine cryopreservation (Amann, 2000). The numbers of sex-sorted, cryopreserved sperm used for insemination have ranged from 1 to 6 $\times 10^6$ sperm/ straw (Seidel et al., 1999c). Normal insemination doses usually are 10–20 $\times 10^6$ /ml sperm.

Pregnancy, Calving Rates and Normalcy of Offspring: Optimal use of sexing technology requires an excellent management (feeding, disease control, oestrus detection, semen handling and insemination technique) because application of the current technology requires careful timing of inseminations (Seidel et al., 1999a; Seidel et al., 1999b; Seidel et al., 1999c). In heifers, reasonable pregnancy or fertility rates have been achieved with low-dose sexed and cryopreserved sperm, but there is a dearth of work done in lactating

cows (Seidel et al., 1999a). Repeatedly, it has been demonstrated that with good management, pregnancy rates in breeding heifers are only slightly lower than normal using low doses of sexed sperm (Tubman et al., 2004; Schenk et al., 2005). In heifers, the pregnancy rate for 2 $\times 10^6$ sexed sperm per inseminate was 56%, whereas the control pregnancy rate with 10 $\times 10^6$ unsexed sperm was 61%. However, pregnancy rates with sexed sperm are very low with animal under marginal management (Garner and Seidel 2003; Schenk et al., 2005; Schenk et al., 2007; DeJarnette et al., 2008). Pregnancy rates can be similar in lactating dairy cows to controls with sexed sperm when selecting only those cows with completely normal reproductive characteristics using different examination and records (Seidel, 2007). Such a pre-screening of cows is usually impractical. However, it is not recommended to use sexed semen in cows due to reduced conception rate (Andersson et al., 2006; Linderroth, 2008).

Seidel et al., (1999a) observed that in more than 1000 heifers there is very little difference in pregnancy rate after inseminations of 1.0 to 1.5 $\times 10^6$ /ml versus 3.0 $\times 10^6$ /ml cryopreserved sexed semen. Higher pregnancy rates have been observed in heifers for sexed, cryopreserved sperm if inseminated with 7 to 20 times more sperm/dose and when sperms are placed into the uterine body (Seidel et al., 1999).

More than 1000 live births using sexed sperm have been produced in different mammalian species with no gross perceivable abnormalities (Seidel et al., 1999a; Seidel et al., 1999b; Cran 2000). Although offspring born from pregnancies generated with sexed sperm appear normal from a general phenotypic standpoint, meticulous epidemiological studies need to be conducted to verify and strengthen these observations (Garner, 2001). The concern is that the process of sexing the sperm could damage sperm DNA and, thus, could increase the incidence of genetic abnormalities (Evenson, 1989; Garner, 2001). No increase in embryonic death has been detected during early pregnancy but with very few abortions (Seidel et al., 1999).

Limitations of Sexed Semen: There are several major limitations that have throttled the implementation of sex-sorted semen. Because sex sorting of sperm is a highly invasive procedure that negatively affects sperm viability and longevity compared to normally

cryopreserved semen, sexed semen reduces the conception rate is the primary impediment (Doyle et al., 1999; Seidel et al., 1999; Chebel et al., 2006; Schenk et al., 2007). The other most important hindrance is the loss of pregnancy. In cattle, the pregnancy loss is 1–2% higher during early gestation with low insemination doses of sexed spermatozoa than with normal insemination doses of unsexed spermatozoa (Seidel et al., 1999). In addition, the procedure is extremely torpid and inefficacious (Hasler, 2003). As the sperms pass through the laser and fluorescence detectors in the flow cytometer, sperms must be precisely oriented to sort properly. In practice, about 30–40% sperms are sexable. Thus, only out of these 50% of the sperm going into the machine are recovered as a marketable, sexed product (Seidel, 2007). Although the 3,000 to 5,000 sperm of each sex sorted per second sounds like a lot, this translates into ~1.3 hours of sorting to process enough semen for a standard dosage of 20 million sperm per straw. Thus, due to the slow sorting speed, commercialization is only possible with very low sperm numbers per dose (~2 million). Along with the above mentioned limitations, the high cost of flow cytometry equipment and intensive amounts of highly skilled labour to sort sperm makes the sexed semen production expensive (Amann, 1999).

Without sexed semen, little selection is possible on the dam side because all female calves to be raised as replacements. Sexed semen has been estimated to increase rate of genetic progress by 15% (Van Vleck, 1981). However, a later study suggested use of sexed semen in elite dams and sires would have a minor impact on rate of genetic progress (Baker et al., 1990)

Because of the low sperm numbers per dose and compromised sperm viability, sexed semen of the proven sires is recommended to be used in well-managed, highly-fertile, virgin heifers. While many research herds have realized very acceptable conception rates, it has been demonstrated repeatedly that with good management, pregnancy rates with breeding heifers are only slightly lower than normal using low doses of sexed sperm (Tubman et al., 2004; Schenk et al., 2005).

Improvement in Sexed Semen Potential: Potential fertility in each straw of sexed semen is maximized with extreme caution during semen handling. Conception rates will most likely be increased by accurate

identification of heifers in estrus, following the recommendations for thawing semen, maintaining the thermal protection of straws during AI gun assembly and transport to the heifer, using appropriate hygienic procedures, depositing the semen in uterus of the heifer as soon as possible (within 5 to 10 minutes after thawing). Frozen sexed semen must be stored, thawed and handled properly to maintain fertility and offer the greatest opportunity to obtain optimal conception rate.

Commercialization: Johnson et al. (1989) reported the first reiterable success using flow-sorting for sexing sperm. This sexing procedure was patented by USDA, which was originally developed at the USDA Beltsville Agricultural Research Center by Larry Johnson (Johnson et al., 1989). The first commercial license was issued to Master calf Ltd, and this group produced sex calves by using this sperm sexing procedure with IVF and transferring embryos to recipients (Cran et al., 1993; Cran et al., 1995). Sperm sexing as currently practiced is expensive due to high cost of equipment and its maintenance, personnel cost for many steps, and the inefficiency of the procedure adds to the high cost of producing the sexed semen. However, the procedure continues to improve. Despite these complexities, the procedure works and already appears to be commercially viable for niche applications in several species. Commercialization of sperm sexing has begun for bovine and is coming up for equine (Buchanan et al., 2000). In human, this methodology is being used on a limited scale, mainly to produce girls to avoid linked genetic disease (Johnson et al., 1993; Fugger et al., 1998).

Applications: Sex-sorting of mammalian spermatozoa has applications for genetic improvement, control of sex-linked disease, and for captive management and re-population of endangered species. Considerable research has been undertaken globally on the Beltsville sperm sexing technology, the only effective method for pre-selection of sex of offspring. The combination of this method with assisted reproductive technologies has resulted in the birth of offsprings in various mammalian species. This technology is exploited commercially in cattle, the only livestock species. Sperm sexing has significant implications with regard to maximizing the efficiency mainly in dairy and beef cattle production. Following are the application of sexed semen in general.

- 1) Semen sexing permit selection of a desired sex

based on the producers needs.

- 2) Use of sexed semen with in-vitro fertilization (IVF) and embryo transfer (ET) in Marker Assisted Selection (MAS) will enable to produce calves with more desirable traits or to select away from recessive traits.
- 3) Sperm sexing can be used in the biotechnological sphere for transgenic animal production and for cloning (Maxwell et al., 2004).
- 4) As a manoeuvre for the repopulation of endangered species and as a breeding scheme in zoos, semen sexing can be applied into wildlife management.
- 5) Allows producers to contain the economic loss that results from the culling of animals of the undesired sex.
- 6) In humans, sperm sexing can be applied for the genetic control of sex-linked diseases. This implicates the application of semen sexing well beyond the animal industry.

It can be seen, sperm sexing has important implications in the livestock as well as other fields of research. Thus further advancement of semen sexing techniques will prove to be useful if not critical to the future development of profuse industries.

Conclusion and Future Prospects: We have entered a new era in which molecular biology techniques can be used accurately to identify the gender of a pivotal mammalian species. Success of any semen sexing technique will only be determined by being able to demonstrate repeatedly effective separation of X- and Y- bearing spermatozoa with no reduction in normal fertility rate. There should be minimum loss during separation procedure. It should have a high recovery rate, low cost and easy application. Out of all the techniques of semen sexing, only flow cytometry based sperm sorting is the feasible method that has been shown to produce high purity (85%–95% accuracy) and clinical significance of sex chromosome selection. Several major improvements have been made in the past, especially by fabrication of high speed cell sorting and improved orientation of cells in front of the laser. Further research into sorting and preservation methods that incorporate strategies to prevent destabilization of sperm membranes may improve the fertilizing lifespan of flow cytometrically sorted spermatozoa. With continued improvement in sorting instrumentation and

biological handling, sorting efficiency should reach a point where commercially acceptable pregnancy rates may be achieved in many species after conventional or deep uterine insemination. More research needs to be carried out for achieving higher sorting rates and for avoiding mutagenic effects like chromosomal aberrations, low embryo-viability etc. in the flow cytometry method. In combination with other biotechnologies, bovine sexed spermatozoa are already commercially available. In other species intensive research is required to provide sufficient sexed spermatozoa. Semen cell sorting technology is continuously being improved and improvements in sorting capacity, fertility and reduction of sexed semen cost are expected. Ultimately, the decision to use sexed semen depends on three forces: the magnitude of conception rate drop, the additional cost of sexed semen production and the differential value of heifer calves versus bull calves. Most computer models suggest judiciously using sexed semen for first and sometimes for second service in virgin heifers, depending, of course, on the above three factors.

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REFERENCES

- Ali JI, Eldridge FE, Koo GC, Schanbacher BD, 1990: Enrichment of bovine X- and Y-chromosome-bearing sperm with monoclonal H-Y antibody-fluorescence-activated cell sorter. *Arch Androl* 24: 235-245.
- Amann RP, 1999: Issues affecting commercialization of sexed sperm. *Theriogenology* 52: 1441-1457.
- Amann RP, 2000: Issues impacting commercialization of sexed sperm. In: Proceedings of the British Society of Animal Science Annual Meeting. Scarborough, United Kingdom, pp. 37-38.
- Andersson MJ, Taponen J, Kommeri M and Dahlbom M, 2006: Pregnancy rates in lactating Holstein-Friesian cows after artificial insemination with sexed sperm. *Reproduction in Domestic Animals* 41: 95-97.
- Baker RL, Shannon P, Garrick DJ, Blair HT, and Wickham BW, 1990: The future impact of new opportunities in reproductive physiology and

- molecular biology on genetic improvement programmes. Proceedings of the New Zealand Society for Animal Production 50: 197-210.
- Beal WE, White LM, Garner DL, 1984: Sex ratio after insemination of bovine spermatozoa isolated using a bovine serum albumin gradient. *Journal of Animal Science* 58: 1432-1436.
- Bennett D, Boyse EA, 1973: Sex ratio in progeny of mice inseminated with sperm treated with H-Y antiserum. *Nature* 246: 308-309.
- Blecher SR, Howie R, Li S, Detmar J, Blahut LM, 1999: A new approach to immunological sexing of sperm. *Theriogenology* 52: 1309-1321.
- Blottner S, Bostedt H, Mewes K, Pitra C, 1994: Enrichment of bovine X and Y spermatozoa by free-flow electrophoresis. *Zentralbl Veterinarmed A* 41: 466-474.
- Bradley MP, Heslop BF, 1985: A biochemical and immunological approach to the identification of H-Y antigenic proteins secreted from Daudi cells. *Human Genetics* 71: 117.
- Bradley MP, Forrester IT, Heslop BF, 1987: Identification of a male-specific (H-Y) antigen on the flagellar plasma membrane of ram epididymal spermatozoa. *Human Genetics* 75: 362.
- Bradley MP, 1989: Immunological Sexing of Mammalian Semen: Current Status and Future Options. *Journal of Dairy Science* 72: 3372-3380.
- Bradley MP, Heslop BF, 1989: Recent developments in the serology and biochemistry of testicular sex specific (H-Y) antigens. In: Wachtel SS (ed), Evolutionary mechanisms in sex determination. eRe Press, Boca Raton, FL.
- Brandriff BF, Gordon LA, Haendel S, 1986: Sex chromosome ratios determined by karyotypic analysis in albumin-isolated human sperm. *Fertility and Sterility* 46: 678-685.
- Buchanan BR, Seidel GE Jr, McCue PM, Schenk JL, Herickhoff LA, Squires EL, 2000: Insemination of mares with low numbers of either unsexed or sexed spermatozoa. *Theriogenology* 53: 1333-1344.
- Catt SL, Catt JW, Gomez MC, Maxwell WMC and Evans G, 1996: Birth of a male lamb derived from an in vitro matured oocyte fertilized by intracytoplasmic injection of a single presumptive male sperm. *Veterinary Record* 139: 494-495.
- Chebel R, Braga F, Dalton J, 2006: Factors affecting reproductive performance of Holstein heifers. *Animal Reproduction Science* 101: 208-224.
- Check JH, Shanis BS, Cooper SO, Bollendorf A, 1989: Male sex preselection: swim-up technique and insemination of women after ovulation induction. *Arch Androl* 23: 165-166.
- Check JH, Katsoff D, 1993: A prospective study to evaluate the efficiency of modified swim-up preparation for male selection. *Human Reproduction* 8: 211-214.
- Claassens OE, Stander FSH, and Kruger TF, 1989: Does the wash-up and swim-up method of semen preparation play a role in sex selection? *Arch Androl* 23: 23-26.
- Cran DG, Johnson LA, Miller NGA, Cochrane D and Polge C. 1993: Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilization. *Veterinary Record* 132: 40-41.
- Cran DG, Cochrane DJ, Johnson DJ, Wei H, Lu KH, Polge C, 1994: Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF. *Theriogenology* 41: 183-183.
- Cran DG, Johnson LA, Polge C. 1995: Sex preselection in cattle: a field trial. *Veterinary Record* 136: 495-496.
- Cran DG, Mckelvey WAC, King ME, Dolman DF, Mcevoy TG, Broadbent PJ, and Robinson JJ, 1997: Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted sperm. *Theriogenology* 47: 267.
- Cran DG, 2000: Current status of semen sexing in cattle. In: Proceedings of the British Society of Animal Science Annual Meeting. Scarborough, United Kingdom, pp. 35.
- DeJarnette JM, Nebel RL, Marshall CE, Moreno JF, McCleary CR, Lenz RW, 2008: Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows. *Journal of Dairy Science* 91: 1778-1785.
- Dmowski WP, Gaynor L, Rao R, 1979: Use of albumin gradients for X and Y sperm selection and clinical experience with male sex preselection. *Fertility and*

Sterility 31: 52-57.

- Doyle SP, Seidel GE Jr, Schenk JL, Herickhoff LA, Cran D, and Green RD, 1999: Artificial insemination of lactating Angus cows with sexed semen. In: Proceedings Western Section, American Society of Animal Science 50, pp. 203-205.
- Engelmann U, Krassnigg F, Schatz H, Schill WB, 1988: Separation of human X and Y spermatozoa by free-flow electrophoresis. *Gamete Research* 19: 151-160.
- Erickson RP, Lewis SE, Butley M, 1981: Is Haploid gene expression possible for sperm antigens? *Journal of Reproductive Immunology* 3: 195-217.
- Ericsson RJ, Langevin CN, Nishino M, 1973: Isolation of fractions rich in Y-spermatozoa. *Nature* 246: 421-424.
- Ericsson RJ, Ericsson SA, 1999: Sex ratios. In: E Knobil and JD Neill (eds), *Encyclopedia of Reproduction*. Academic Press, London, pp. 431-437.
- Evans JM, Douglas TA, Renton JP, 1975: An attempt to separate fractions rich in human Y sperm. *Nature* 253: 352-354.
- Evenson DP, 1989: Flow cytometric analysis of toxic chemical induced alteration in testicular cell kinetics and sperm chromatin structure. In: Jolles G and Cordier A (eds), *New trends in Genetic risk assessment*. Academic press San Diego, CA, pp. 401-410.
- Flaherty SP, Michalowska J, Swann NJ, Dmowski WP, Matthews CD, and Aitken RJ, 1997: Albumin gradients do not enrich Y-bearing human spermatozoa. *Human Reproduction* 12: 938-942.
- Fugger ER, Black SH, Keyvanfar K, Schulman JD, 1998: Births of normal daughters after microsort sperm separation in intrauterine insemination, IVF or ICSI. *Human Reproduction* 13: 2367-2370.
- Fugger EF, 1999: Clinical experience with flow cytometric separation of human X- and Y-chromosome bearing sperm. *Theriogenology* 52: 1345-1440.
- Garner DG, Gledhi U BL, Pinkel D, Lake S, Stephenson D, van Dill MA, Johnson LA, 1983: Quantification of the X- & Y-chromosome bearing spermatozoa of domestic animals by flow cytometry. *Biology of Reproduction* 28: 312-321.
- Garner DL, 2001: Sex-Sorting Mammalian Sperm: Concept to Application in Animals. *Journal of Andrology* 22: 519-526.
- Garner DL, Seidel GE, 2003: Past, present and future perspectives on sexing sperm. *Canadian Journal of Animal Science* 83: 375-384.
- Garner DL, 2006: Flow cytometric sexing of mammalian sperm. *Theriogenology* 65: 943-957.
- Han TL, Flaherty SP, Ford JH, Matthews CD, 1993: Detection of X- and Y-bearing human sperm after motile sperm isolation by swim-up. *Fertility and Sterility* 60: 1046-1051.
- Hancock RJ, 1978: Comparison of effects of normal rabbit sera and anti-cock sperm sera on rabbit sperm, including comparison of effects on the sex ratio. *Biology of Reproduction* 18: 510-515.
- Hasler JF, 2003: The current statue and future of commercial embryo transfer in cattle. *Animal Reproduction Science* 79: 245-264.
- Hedge UC, Shastry PR, Rao SS, 1977: A simple and reproducible method for separating Y-bearing spermatozoa from human semen. *Indian Journal of blood Research* 65: 738.
- Hendriksen PJ, Tieman M, van Der LT, Johnson LA, 1993: Binding of anti-H-Y monoclonal antibodies to separated X and Y chromosome-bearing porcine and bovine sperm. *Molecular Reproductive Development* 35: 189-196.
- Hoppe PC, Koo GC, 1984: Reacting mouse sperm with monoclonal H-Y antibodies does not influence sex ratio of eggs fertilized in vitro. *Journal of Reproductive Immunology* 6: 1-9.
- Iizuka R, Kaneko S, Aoki R, Kobayashi T, 1987: Sexing of human sperm by discontinuous Percoll density gradient and its clinical application. *Human Reproduction* 2, 573-575.
- Johnson LA, Pinkel D, 1986: Modification of a laser-based flow cytometer for high-resolution DNA analysis of mammalian spermatozoa. *Cytometry* 7, 268-273.
- Johnson LA, Flook JR, Look MV, 1987: Flow cytometry of X and Y chromosome-bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. *Gamete Research* 17, 203-212.
- Johnson LA, Flook JP, Hawk HW, 1989: Sex

- preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol Reprod* 41, 199-203.
- Johnson LA, 1991: Sex preselection in swine: Altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. *Reprod Dom Anim* 26, 309-314.
- Johnson LA, Welch GR, Keyvanfar K, Dorfmann A, Fugger EF, Schulman JD, 1993: Gender preselection in humans? Flow cytometric separation of X and Y spermatozoa for the prevention of X-linked diseases. *Human Reproduction* 8, 1733-1739.
- Johnson LA, 1995: Separation of X and Y chromosome bearing sperm based on DNA differences. *Fertil Dev* 7, 893-903.
- Johnson LA, Seidel GE Jr, 1999: Current status of sexing mammalian sperm. *Theriogenology* 52, 1267-1484.
- Johnson LA, Welch GR, 1999: Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. *Theriogenology* 52, 1323-1341.
- Johnson LA, Welch GR, Rens W, 1999: The Beltsville sperm sexing technology: high-speed sorting gives improved sperm output for in vitro fertilization and AI. *J Dairy Sci* 82, 213-220.
- Johnson LA, 2000: Sexing mammalian sperm for production of offspring: the state-of-the-art. *Animal Reprod Sci* 60-61, 93-107.
- Johnson LA, Guthrie HD, Fisher P, Maxwell WMC, Welch GR, Gerrett WM, 2000a: Cryopreservation of flow cytometrically sorted boar sperm: effects on in vivo embryo development. *J Anim Sci* 78, 198.
- Kaneko S, Yamaguchi J, Kobayashi T, Iizuka R, 1983: Separation of human X- and Y-bearing sperm using percoll density gradient centrifugation. *Fertil Steril* 40, 661-665.
- Kaneko S, Oshiro S, Kobayashi T, Itzuka R, Mohri H, 1984: Human X- and Y-bearing sperm differ in cell surface sialic acid content. *Biochem and Biophys Res Commun* 124, 950-955.
- Kiddy CA, Hafs HD, 1971: Sex Ratio at Birth - Prospects for Control. American Society of Animal Science, Savoy, IL.
- Kobayashi J, Oguro H, Uchida H, Kohsaka T, Sasada H, Sato E, 2004: Assessment of bovine X- and Y-bearing spermatozoa infractions by discontinuous percoll gradients with rapid fluorescence in situ hybridization. *J Reprod Dev* 50, 463-469.
- van Kooij RJ, van Oost BA, 1992: Determination of sex ratio of spermatozoa with a deoxynucleic acid-probe and quinacrine staining: a comparison. *Fertil Steril* 58, 384-386.
- Linderoth S, 2008. Sexed semen primer. Dairy Herd management, 25-27.
- Lindsey AC, Schenk JL, Graham JK, Bruemmer JE, Squires EL, 2002: Hysteroscopic insemination of low numbers of nonsorted or flow-sorted spermatozoa. *Equine Vet J* 34, 128-132.
- Lobel SM, Pomponio RJ, Mutter GL, 1993: The sex ratio of normal and manipulated human sperm quantitated by polymerase chain reaction. *Fertil Steril* 59, 387-392.
- Lopez O, Mata A, Antich M, Bassas L, 1993: Sperm selection by PD-10 sephadex columns: comparison with SpermPrep filtration and Percoll centrifugation. *Hum Reprod* 8, 732-736.
- Manger M, Bostedt H, Schill WB, Mileham AJ, 1997: Effect of sperm motility on separation of bovine X- and Y-bearing spermatozoa by means of free-flow electrophoresis. *Andrologia* 29, 9-15.
- Maxwell WMC, Evans G, Hollinshead FK, Bathgate R, De Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK, 2004: Integration of sperm sexing technology into the ART toolbox. *Ani Reprod Sci* 82-83, 79-95.
- Mohri H, Oshio S, Kaneko S, Kobayashi T, Iizuka R, 1987: Separation and characterization of mammalian X- and Y-bearing sperm. In: Mohri H (ed), New Horizons in Cell Research. Japan Scientific Society Press, Tokyo/Gordon and Breach Scientific Publishers, New York, pp. 469-481.
- Van Munster EB, Stap J, Hoebe R, te Meerman GJ, Aten JA, 1999a: Difference in volume of X- and Y-chromosome bearing bovine sperm heads matches differences in DNA content. *Cytometry* 35, 125-128.
- Van Munster EB, Stap J, Hoebe RA, te Meerman GJ, Aten JA, 1999b: Difference in sperm head volume as a theoretical basis for sorting X- and Y-bearing spermatozoa: potential and limitations. *Theriogenology* 52, 1281-1293.
- Van Munster EB, 2002: Interferometry in flow to sort

- unstained X- and Y-chromosome bearing bull spermatozoa. *Cytometry* 47, 192-199.
- O'Brien JK, Hollinshead FK, Evans KM, Evans G, Maxwell WMC, 2003: Flow cytometric sorting of frozen-thawed spermatozoa in sheep and non-human primates. *Reprod Fertil Dev* 15, 367-375.
- O'Brien JK, Stojanov T, Heffernan SJ, Hollinshead FK, Vogelnest L, Maxwell WM, Evans G, 2005a: Flow cytometric sorting of non-human primate sperm nuclei. *Theriogenology* 63, 246-259.
- O'Brien JK, Stojanov T, Crichton EG, Evans KM, Leigh D, Maxwell WMC, Evans G, Loskutoff NM, 2005b: Flow cytometric sorting of fresh and frozen-thawed spermatozoa in the western lowland gorilla (*Gorilla gorilla gorilla*). *Am J Primatol* 66, 297-315.
- O'Brien JK, Robeck TR, 2006: Development of sperm sexing and associated assisted reproductive technology for sex preselection of aptive bottlenose dolphins (*Tursiops truncatus*). *Reprod Fertil Dev* 18, 319-329.
- Ollero M, Perez-Pe R, Gargallo I, Morlanes S, Osada J, Muino-Blanco T, Cebrian-Perez J, 2000: Separation of ram spermatozoa bearing X and Y chromosome by centrifugal countercurrent distribution in an aqueous two-phase system. *J Androl* 21, 921-928.
- Pinkel D, Garner DL, Gledhill BL, Lake S, Stephenson D, Johnson LA, 1985: High resolution DNA content measurements of mammalian sperm. *Cytometry* 3, 1-9.
- Pyrzak R, 1994: Separation of X- and Y-bearing human spermatozoa using albumin gradients. *Hum Reprod* 9, 1788-1790.
- Quinlivan WL, Preciado K, Long TL, Sullivan H, 1982: Separation of human X and Y spermatozoa by albumin gradients and Sephadex chromatography. *Fertil Steril* 37, 104-107.
- Rath D, Johnson LA, Welch GR, 1993: In vitro culture of porcine embryos: Development to blastocysts after in vitro fertilization (IVF) with flow cytometrically sorted and unsorted semen. *Theriogenology* 39, 293.
- Rath D, Johnson LA, Dobrinsky JR, Welch GR, Niemann H, 1997: Production of piglets preselected for sex following in vitro fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry. *Theriogenology* 47, 795-800.
- Rath D, Ruiz S, Sieg B, 2003: Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. *Vet Rec* 152, 400-401.
- Rens W, Welch GR, Johnson LA, 1998: A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X- and Y-chromosome bearing sperm. *Cytometry* 33, 476-481.
- Rens W, Welch GR, Johnson LA. 1999: Improved flow cytometric sorting of X- and Y-chromosome bearing sperm: substantial increase in yield of sexed sperm. *Mol Reprod Dev* 52, 50-56.
- Rohde W, Porstmann T, Prehn S, Dorner G, 1975: Gravitational pattern of the Y-bearing human spermatozoa in density gradient centrifugation. *J Reprod Fertil* 42, 587-591.
- Ross A, Robinson JA, Evans HJ, 1975: Failure to confirm separation of X- and Y-bearing human sperm using BSA gradients. *Nature* 253, 354-355.
- Schenk JL, Suh TK, Cran DG, Seidel GE Jr, 1999: Cryopreservation of flow sorted bovine sperm. *Theriogenology* 52, 1375-1391.
- Schenk JL, 2001: Applying sperm sexing technology to the AI industry. In: Proceedings of the 18th National Association of Animal Breeders Technical Conference. Milwaukee, Wis. pp. 73-78.
- Schenk JL, De Grofft DL, 2003: Insemination of cow elk with sexed frozen semen. *Theriogenology* 59, 514-572.
- Schenk JL, Brink Z, Suh TK, 2005: Use of competitive fertilization to evaluate a simple laser for flow cytometric sexing of bovine sperm. *Reprod Fertil Dev* 17, 306.
- Schenk JL, Everett RW, 2007: Insemination of Holstein cows with sexed sperm. *J of Dairy Sci* 90, 18.
- Schilling E, Thormaehle ND, 1977: Enrichment of human X- and Y-chromosome bearing spermatozoa by density gradient centrifugation. *Andrologia* 9, 106-110.
- Seidel GE Jr, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR, Graham JK, Cattell MB, 1997: Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. *Theriogenology* 48, 1255-1264.
- Seidel GE, Cran DG, Herickhoff LA, Schenk JL, Doyle

- SP, Green RD, 1999: Insemination of heifers with sexed frozen or sexed liquid semen. *Theriogenology* 51, 400.
- Seidel GE Jr, 1999a: Sexing mammalian spermatozoa and embryos-state of the art. *J Reprod Fertil* 54, 475-485.
- Seidel GE Jr, 1999b: Commercializing reproductive biotechnology-the approach used by XY, Inc. *Theriogenology* 51, 5.
- Seidel GE Jr, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD, Cran DG, 1999c: Insemination of heifers with sexed sperm. *Theriogenology* 52, 1407-1420.
- Seidel GE Jr, 2000: El sexado de semen bovino. In: Proceedings of the Vth Congreso Argentino de Reproduccion Animal. Rosario, Argentina, pp. 1-5.
- Seidel GE Jr, Garner DL, 2002: Current status of sexing mammalian spermatozoa. *Reproduction* 124, 733-743.
- Seidel GE Jr, 2003: Economics for selecting sex: The most important trait. *Theriogenology* 59, 585-598.
- Seidel GE Jr, 2003: Sexing mammalian sperm-intertwining of commerce, technology, and biology. *Anim Reprod Sci* 79, 145-156.
- Seidel GE Jr, 2007: Overview of sexing sperm. *Theriogenology* 68, 443-446.
- Senger PL, 2003: Pathways to Pregnancy and Parturition: 2ns Edition. Ephrata, PA: Cadmus Professional Communications.
- Sevinc A, 1968: Experiments on sex control by electrophoretic separation of spermatozoa in the rabbit. *J Reprod Fertil* 16, 7-14.
- Shirai M, Matsuda S, Mitsukawa S, 1974: Electrophoretic separation of X- and Y-chromosome-bearing sperm in human semen. *Tohoku J Exp Med* 113, 273-281.
- Sills ES, Kirman I, Colombero LT, Hariprashad J, Rosenwaks Z, Palermo GD, 1998: H-Y antigen expression patterns in human X- and Y-chromosome-bearing spermatozoa. *Am J Reprod Immunol* 40, 43-47.
- Tubman LM, Brink Z, Suh TK, Seidel GE Jr, 2004: Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. *J Anim Sci* 52, 1029-36.
- Ueda K, Yanagimachi R, 1987: Sperm chromosome analysis as a new system to test human X- and Y-sperm separation. *Gamete Res* 17, 221-228.
- Uwland J, Willems CM, 1975: Results of semen separation using a modified electromagneto-chemical method. *Tijdschr Diergeneeskde* 100, 911-914.
- van Vleck LD, 1981: Potential genetic impact of artificial insemination, sex selection, embryo transfer, cloning and selfing in dairy cattle. In: New Technologies in Animal Breeding. Academic Press. New York, pp. 222-242.
- Vazquez JM, Martinez EA, Parrilla I, Roca J, Gil MA, Vazquez JL, 2003: Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa. *Theriogenology* 59, 1605-1614.
- Wang HX, Flaherty SP, Swann NJ, Matthews CD, 1994a: Assessment of the separation of X- and Y-bearing sperm on albumin gradients using double label fluorescence in situ hybridization. *Fertil Steril* 61, 720-726.
- Wang HX, Flaherty SP, Swann NJ, Matthews CD, 1994b: Discontinuous Percoll gradients enrich X-bearing human spermatozoa: a study using double-label fluorescence in-situ hybridization. *Hum Reprod* 9, 1265-1270.
- Weigel KA, 2004: Exploring the role of sexed semen in dairy production systems. *J of Dairy Sci* 87, 120-130.
- Welch GR, Johnson LA, 1999: Sex preselection: laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA. *Theriogenology* 52, 1343-1352.
- White IG, Mendoza G, Maxwell WHC, 1984: Preselection of sex of lambs by layering spermatozoa on protein columns. In: Lindsay DR and Pearce DT (eds), Reproduction in Sheep. Cambridge University Press, Cambridge, pp. 299-300.
- Windsor DP, Evans G, and White IG. 1993: Sex predetermination by separation of X and Y chromosome-bearing sperms: a review. *Reproduction and Fertility Development* 5: 155-171.
- Zavos PM, 1985: Sperm separation attempts via the use of albumin gradients in rabbits. *Theriogenology* 23: 875-879.