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SPERM SEXING BY FLOW CYTOMETRY –PAST IMPROVEMENTS AND FUTURE PROSPECTS: A REVIEW

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Sex preselection is a subject that has held man's attention for generations. The most effective way to achieve sex predetermination is to resolve X and Y chromosome bearing sperm populations. In the past, various techniques have been evolved with paucity of scientific validation. Among them, Flow cytometry, which is based on the principle of difference in the DNA content, is the best and efficient method .This technique has been suitably modified and is being used commercially in several countries with about 90% accuracy in cattle. Recently, the process of commercialization of sexed sperm hastened. However, this technology has some impediment with respect to cost of production, implementation and pregnancy rate than control sperm. Despite these limitations, production of sexed semen usually followed by cryopreservation is being used commercially for cattle and horse production. Development of the instrument for increasing the sorting rate and also purity of sorting without affecting the viability and fertility is still an active area of research.

Key words: Flow cytometry, sex preselection, sperm sexing.

Being able to select the sex of offspring is something that animal producers have desired for many years. The desire is often greatest when farms have a high incidence of calves opposite sex, just by random chance. After years of research and testing, a way of preselection that has been tested thoroughly is now commercially available. Production of the offspring of predetermined sex is one technology which will have long lasting effects on the economics of the animal owners. Majority of dairy farmers desire the female offspring whereas, stud bull farmers are anxious to obtain male calf crop for selection of potential future bulls for use in progeny testing programmes. In natural breeding, a cow gives birth to either a male or female and the sex ratio of offspring is around 50:50 whereas, the insemination with sexed semen would allow a large calf crop of desired sex in minimal time and therefore, help in getting the increased rate of genetic improvement.

Historical perspective

Greeks during 470-402 BC, suggested that the right testis produce males, whereas the left testis produce females. Sometimes there are distortions of sex ratios without separating the male and female producing sperm. It is observed in cattle that normal AI or embryo transfer (ET) results in 51% males, whereas *in vitro* fertilization (IVF) results in about 54% males (Hasler *et al*.,1995), very old cows produce about 53% male calves (Skjervold and James, 1979), herds with poor management had 49% males as compared to 53% males in the herds having very good management (Skjervold and James, 1979), Others suggested that the timing of AI can also alter the sex ratio (Rorie,1999) however, these results were not repeatable. Based on several differences between the male and female producing sperm such as size, weight and density (Bhattacharya, 1962), swimming speed (Erricsson *et al*., 1973), electrical surface charges (Shirai *et al*.,1974;Shishito *et al*., 1974), surface macromolecular proteins, differential effects of pH, and differing effects of atmospheric pressure, methods such as sedimentation, electrophoresis,

sephadex filtration, centrifugation, albumin/percoll gradients, convection-counterstreaming galvanization, forced convection galvanization etc. has been used to separate X and Y chromosome bearing sperm with varying success. However, none of the method was able to significantly separate the viable sperms capable to achieve successful fertilization or not repeatable. Subsequent studies on this aspect revealed that there exists a difference in the amount of DNA among sex chromosomes (Moruzzi, 1979), Sex selection in domestic animals became a major objective once the ability to determine the success of X and Y sperm separation was achieved with flow cytometric analysis.

Principles of flow cytometric sorting of spermatozoa

The difference in DNA content is the basis for sperm separation by flow cytometry. Therefore, as the difference in the DNA content between X and Y spermatozoa increases, the efficiency and accuracy of the sorting process increases. The differences in the DNA contents differ among species (Moruzzi, 1979) and it varies from 3.6-4.2% (Johnson, 1992; Johnson and Welch, 1999). Ram, Rabbit, Bull and Boar spermatozoa have a difference of 4.2%, 3.9%, 3.8% and 3.7% respectively. In *Bos indicus*, the average X-Y sperm difference is 3.73%. Whereas, differences in DNA content for Murrah and Nili-Ravi buffalo were 3.59% and 3,55% respectively (Lu *et al.,* 2006. This means an optimum sorting accuracy and sorting rate would be more difficult for buffalo sperm compared to that of bovine sperm, whose difference in DNA content between X and Y sperm is slightly higher. Lu *et al*. (2007) obtained 94% and 895 accuracy of sorted X and Y sperm of Murrah and Nili-Ravi buffalo respectively by a modified BD FACSVantage SE flow cytometer (Becton, Dickinson and Company).

Besides, the differences in the DNA content, morphology and orientation of the cells to the excitation sources also affects the efficiency and accuracy of the DNA analysis and the sort rate of cells. For sorting of the spermatozoa by flow cytometry, the spermatozoa are first stained with a vital, fluorescent, bisbenzimide dye (Hoechst 33342; Johnson *et al.*,1987) and a non-toxic food dye. The fluorescent dye binds to the DNA whereas, the food dye penetrates into the non-viable spermatozoa due to damaged membrane and reduces the binding intensity of the fluorescent dye in the dead or membrane damaged spermatozoa. The food dye, thus help to eliminate the non-viable spermatozoa from the sorted population and increases the viable count in the gender selected spermatozoa. The stained spermatozoa after incubation at 34^0C are passed through a miniature nozzle in thin stream under pressure (40-50 psi). At the same time undulations of a piezo-electric crystal breaks this stream into approximately 90,000 droplets/second. Each droplet carrying a small number of spermatozoa is then passed through a laser beam (blue light). The laser beam fluoresce the stain bound to the DNA of the spermatozoa. X-chromosome-bearing sperm fluoresce with 4% more intensity than Y-chromosome bearing sperm in view of higher amount of stained DNA in the X chromosome bearing spermatozoa. The intensity of the fluorescence is collected by the two optical lenses located at 90^0 and 0^0 to the laser beam and transmitted to the photomultiplier tube for the analysis by a computer. Droplets carrying the spermatozoa are charged (+/- or no charge) depending on the amount of DNA before passing between the appositively charged plates. The droplets containing more than one sperm, dead sperm (stained by vital dye), or those where DNA content could not be measured accurately are not charged and therefore, these cells are not sorted and goes as waste. During their passage between the charged plates the charged droplets are deflected to either side and the uncharged droplets carrying debris or droplets without any cell passes un-deflected, Samples are then collected in three containers, X and Y- chromosome bearing and unsorted. This process allows sexing and collection of about 40% of the sperm going through the sorter at a speed of approximately 100 km/h. Thus, at an event rate of 20,000 total sperms, nearly 4,000 live sperms of each sex can be sorted

simultaneously (Schenk, 1990). The current system can produce approximately 10 to 13 $\times 10^6$ live sperm/h of each sex with 85-95% accuracy (Seidal et al., 1999).

Improvements in flow cytometric sorting of sperm

After development of flow cytometry (Sprenger et al.,1971), the first report of flow cytometrical sperm analysis was published by Gledhill et al, (1976), however, their experiments failed due to problems of the proper orientation of flat head of spermatozoa. It was Fulwyler in 1977 that solved the problem of orientation of flat chicken erythrocytes analysis by employing two sheath liquid streams. Dean *et al*. (1978) and Stove *et al.* (1978) further improved the resolution of analysis of flat cells by utilizing wedge shaped injection tube. Later, Pinkel *et al.* (1982) modified their system to orient spermatozoa in front of the laser beam. They first developed the technology of sperm sorting at Lawrence Livermore National Laboratory, California by separating the X and O sperm of vole, Microtusoregoni, having 9% DNA content difference of its sex determining chromosomes (Pinkel et al., 1982). Johnson and Pinkel (1986) modified a coulter EPICS V flow cytometer, adding a second fluorescence detector at 0^0 and developed a beveled tip for the sample injection tube. The bevelled tip favours a large proportion of sperm to pass the laser beam in correct orientation because it transforms the cylindrical sample stream into a thin ribbon. The sorting process with the modified standard flowcytometer was relatively slow and allowed separation of about 55 sperm heads/second. In late 1980's, a major breakthrough in sexing of sperm was reported by Johnson *et al.* (1989) at USDA Beltsville Research Centre. The research group here reported production of live offspring from sex-sorted, living rabbit sperm. This was the first verified report where the sex of offspring had been predetermined at conception by sorting live sperm into the respective X and Y chromosome bearing sperm populations.

Continuous efforts for refinement of this technology are being made since its invention. The efforts during this time mainly centered around two aspects:-

1. The development of new refined flow nozzle that could more effectively orient the sperm head to the laser beam, for increasing the purity of the sorted spermatozoa,
2. The development of high speed sorter that could sort a large number of spermatozoa for adoption of this technology for commercial production of sexed semen.

The success of the sorting process is dependent on the accuracy and efficiency of analyzing sperm. This is hampered by the flat shape of the sperm head, compactness of the chromatin and a high index of refraction, brighter fluorescence from the edge compared to flat surface and random orientation of sperm resulting in broad fluorescence distribution within the sample. The beveled needle was useful for orienting the sperm during passage through laser beam. This was however, true for sperms without tails. Also, the ribbon shaped sample stream exists only when the sample flow rate is slow and it moves under low pressure. These limitations restrict the use of this system for large scale sperm sorting. Since beveled needle primarily orient the tail less sperm, only 20-40% of intact viable sperm were correctly oriented by this system (Johnson, 1995) and 60-80% were not analyzed for DNA contents. This problem was solved by the discovery of a novel nozzle by Rens *et al.* (1998) for high efficiency flow sorting of asymmetrical or flattened cells. The nozzle is uniquely designed to have two interior ellipsoidal zones and an elliptical exit orifice capable to form stable droplets to orient sperm independently of sperm motility and sample flow rate. The new nozzle is capable to orient in excess of 60% of sperm for sorting. This elliptical nozzle achieves three times increase in orienting the sperm and a two fold increase in the efficiency of sorting compared to the standard conical nozzle in combination with beveled injection needle. A cell sorter equipped with this nozzle may sort the X and Y bearing sperm with 90% purities.

The high speed cell sorter was developed in 1985 at the Lawrence Livermore National Laboratories, Livermore, CA which was capable of processing and sorting cells at the rate of 20,000 per second compared to approximately 8,000 per second by the conventional sorters (Peters *et al.*, 1985). This sorter however, worked at a higher sorting pressure (200 Psi or 14078 g/cm^2) in comparision to conventional sorters (844 g/cm^2) .However sorting pressure exposes the spermatozoa to stress during sorting process resulting into death of all sorted sperm. Van den Engh and Stokdijk (1989) of the same laboratory improved this sorter to suit specially to sperm sorting by reducing the operational pressure from 14,078 g/cm^2 of the original high speed sorter to 1,408 to 7,038 g/cm^2 and a sorting rate of about 200,000 cells/second. This design licensed by Cytomation and developed for commercial production of sexed semen came to the market in 1996 with the trade name MoFlo (Johnson et al., 1999). This system equipped with the modified new nozzle had increased the overall production rate of sorted X and Y sperm from about 0.35 million sperm per hour (each population). In view of damage to the sperm by sort pressures continuous efforts have been made to reduce the sort pressures continuous efforts have been made to reduce the sort pressure till it was achieved to be around 50 Psi. Suha *et al.* (2005) conducted a series of experiments utilizing MoFlo® SX flow cytometer (MoFlo; Cytomation, Inc.Fort Collins, CO) to optimize the sort pressure that could return a high survivability of the sorted semen without compromising the sort speed. The routine operating pressure of the MoFlo® SX flow cytometer for sperm sorting for commercial production has been 50 Pounds per square inch (Psi), with a standard 70 µm standard nozzle tip. Sorting at 50 Psi with the 70 µm nozzle yielded post-thaw sperm motility of 40.5% and 30% at 30 minutes and two hour interval. Reducing the sort pressure to 30 psi increased the corresponding motility to 48.0% and 40.2% respectively. Similarly in another experiment, sorting at 50, 40, and 30 Psi, returned mean sperm motilities of 44.8, 48.6, and 49.6% at 30 minutes, and percentage of live spermatozoa were 51.7, 55.7, and 57.8% respectively. It was thus, evident that lowering pressure of the MoFlo® SX flow cytometer for sperm sorting from 50 Psi (standard pressure) to 40 Psi clearly improved sperm quality without a significant decrease in sorter performance.

Cryopreservation and Insemination dose of sexed semen

Flow sorting had improved to produce enough living sexed sperm so that oviductal insemination, *invitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) was possible. After sorting, sperm were concentrated by centrifugation to about 80x 10^6/ml so that they could be reconstituted to $20x10^6$/ml and cryopreserve sorted sperms at $1x10^6$ 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 sperm result in slightly lower post-thaw motilities and acrosomal integrities compared with control sperm. This damage is minor compared with that caused by routine cryopreservation (Amann, 2000). The numbers of sex-sorted, cryopreserved sperm used for insemination have ranged from 1 to 6×10^6.

Applications

Sperm sexing has significant implications with regard to maximizing the efficiency mainly in dairy and beef animal production. Following are the application of sexed semen in general:

1. Sperm sexing permit selection of a desired sex based on the producers need.
2. Use of sexed semen with in-vitro fertilization and embryo transfer in Marker Assisted Selection 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 maneuver for the repopulation of endangered species and as a breeding scheme in zoos, sperm

sexing can be applied into wildlife management.

5. Allows producers to curtail the economic loss that results from the culling of animals of un-desired sex.

It can be seen, sperm sexing has important implications in the livestock as well as other fields of research. Thus further advancement of sperm 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 sperm 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 sperm 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 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 the sorting instrumentation and biological handling, sorting efficiency should reach a point where commercially acceptable pregnancy rates may be achieved in a number of species after a 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 cytometric 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. 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. Most computer models suggest judiciously using sexed semen in virgin heifers, depending, of course on the above three factors.

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