Techniques for semen sexing

The karyotype of buffalo has 25 pairs of chromosomes including the sex chromosome pair. The difference in size of the X and Y-chromosomes is the basis for most sperm sexing techniques. In buffalo, the size of the X-chromosome is approximately four-times larger than the Ychromosome, resulting in total DNA content of the X sperm being more than that of the Y sperm. In Bos indicus, the average X-Y sperm DNA content difference is 3.73%. Similarly, Nili–Ravi and Murrah buffalo's X and Y sperms differ by 3.55% and 3.59% in terms of total DNA content in sperms16. This size difference in X and Y chromosomes can also result in difference in sperm shape and density. The size difference can be utilized to separate the two types of sperms. The sperms can be rendered immotile by cooling to 1□C. On the egg yolk medium, the X sperms sediment faster than Y sperms under the influence of gravity due to difference in density17. Antigen H-Y is a male specific protein on the cell surface, coded by Y chromosome. This protein has a role in gender determination during embryo development. However, there are also claims that X and Y sperms can be separated using this antigen for immunolabelling18. It is claimed that monoclonal antibodies against antigen H-Y, binds preferentially to Y-chromosome bearing sperm which can then be separated using FACS. There have been various studies to find the difference between X and Y sperms in terms of gene expression during spermatogenesis, which can be used for an immunological method of separation of X and Y spermatozoa. But most of these gene products are shared between X and Y spermatids through inter-cellular bridges built during spermatogenesis. The X and Y sperms separated by FACS technique show no significant difference in terms of proteins on the surface, including antigen H-Y19. There have been several studies to determine X- and Y-sperm specific biomolecules on the surface of sperms. However low enrichment, low viability and high cost have been prohibitive in commercialization of immunological sperm sorting methods20. There have also been not so successful attempts to separate X and Y sperms through free flow electrophoresis, assuming that there is a difference in surface charge of X and Y sperms due to a difference in fatty acid composition of the membrane21. Rather, such differences between X and Y sperms are not well established. Percoll density gradient centrifugation was also tried, but the success in enriching X chromosome bearing sperm was insignificant22. Swim up method of semen sexing is based on the assumption that the Y sperm is relatively smaller and therefore swims faster. However, subsequent studies have found it to be false23. Other methods utilizing DNA staining dyes are prone to mutagenicity and reduce the viability of sperms24. There have also been attempts to use thin layer counter current distribution (TLCCD) chromatography to sort sex sperms25. Even transgenics has been used to produce specific X or Y sperms26. In this method, the promoter of testis specific promoter gene, protamine 1 on Y chromosome is used to express an anti sense mRNA which is toxic to gamete. This anti sense mRNA can stop the expression of genes such as fertilin B, sperm adhesion molecule (spam-1),glyceraldehyde phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase which are crucial for a functional sperm. Therefore, sperms of undesirable types can be selectively made non-

functional. The separation efficiency of various techniques is checked by PCR, using Y-chromosome specific DNA sequence. The separated sperm solutions can be tested for their percentage of Y sperms using real time PCR. Y-chromosome-linked SRY gene specific primers and X-chromosome-linked PLP gene specific primers were designed to amplify markers for X and Y sperm content estimation in sexed semen27. Evolutionarily conserved sequences on Y chromosome have been utilized for designing Y specific primers R-IV and U-IV. These primers have been successfully used to detect the Y chromosome even in embryonic tissue of Zebu and Taurine28. A highly repetitive sequence S4, localized on the Y chromosome can be amplified using PCR primer set which gives a 178 bp male-specific product and a 145 bp product which appear both in X and Y sperms29.

Challenges and upcoming developments in sperm sexing

The differences between X and Y sperms in terms of weight, size or density are very minute. Therefore a high precision technique is required to separate them on the basis of physical characteristics. Since several years, undoubtedly, various methods have been used to enrich the semen with X sperms, but all these methods are expensive and inefficient. Moreover, the X sperm enrichment procedure also makes it less viable for fertilization. The famous Ericsson's albumin layer based separation method for humans has been found ineffective30. The present technique of X sorting from bull semen involves the staining of DNA by Hoechst dye and further separation using FACS31. Hoechst 33342 is a DNA specific, nonintercalating dye. Therefore, it does not induce DNA damage and binds only in the minor groove of the A-T regions of the DNA32. The exposure of sperms to laser light and exposure of droplets to electric charge in FACS, reduces the motility of sperms as well as damages the acrosome and membrane33. Exposure to the dye in combination with the laser may reduce mitochondrial activity in bovine sperms. This results in reduced motility of sperms, because mitochondria produce ATP which is an energy source for sperm motility34. The shorted sperm in sheath fluid is then concentrated by centrifugation. Centrifugation also damages spermatozoa through lipid peroxidation. Due to high dilution in sheath fluid, the natural antioxidants present in seminal fluid are lost. The storage of shorted sperms in liquid nitrogen further increases peroxidation of membrane lipids. Various steps in FACS lead to reactive oxygen species (ROS) production causing damage to the membrane and mitochondria which can be reduced by a supplementing medium with antioxidants like sodium pyruvate and catalase35. Centrifugation and microfluidics may hold the key to the development of better techniques of sperm sorting with minimal tampering of cell structure. The miniature separation column has been used for continuous cell separation through density gradient centrifugation36, which may be adapted for sperm sorting based on the density difference between the X and Y sperms. The seminal fluid itself can be a better medium for centrifugation, and optimization of centrifugation speeds, time and volumes can result in a more viable sexed semen. Microfluidic channels can be used as cell sorters and separation of X and Y sperms on the basis of the negligible difference they have is a fitting challenge for this emerging high precision technology37. Microfluidics can be explored for separation of sperms on the basis of

their charge, density or shape. The UV-absorbance spectroscopy can be coupled with microfluidic channels to sort the X sperms from Y sperms38. The Y sperm has less DNA content than the X sperm. This difference can be picked up by UV absorbance of DNA at 260 nm. The sperms move in a spiral path in a stagnant fluid. However in a flow stream, the angular velocity of the sperm is reduced and the path of the sperm movement becomes almost a straight line. This effect on angular velocity is more in the case of X sperm, probably due to more weight. Therefore, in a flow stream the X sperms moves comparatively straight whereas Y sperms move in a spiral path. This difference can be utilized for shorting X and Y sperms through microfluidics. The gradient of velocity across the axis of flow has been used successfully to short the X and Y sperms39. The difference in swimming behaviour of sperms is expected to be more pronounced in case of cattle sperms. Advanced microfluidics may utilize this behaviour for X and Y sperm sorting in a setup similar to the one used for separating motile and nonmotile sperms (Figure 1)35,41. North Cyprus IVF Center has developed a microfluidic device to separate live human sperms from dead ones. Microfluidic channel may be designed to separate sperms on the basis that X sperms move in straight line in a flow whereas Y sperms move in an angular path. Microfluidic channels can also be coupled to various types of spectrophotometer42, which can be used to detect Y sperms on the basis of DNA content, and then use high power laser for inactivation of individual Y sperms, leaving only X sperms alive. The dead sperms can be removed using a micro scale sperm sorter developed by Chung et al.43 which is a point of care device driven by passive reservoir pumps. Size selective separation techniques used for nanoparticles in liquids may also be useful for picking up minute size differences between X and Y sperms44. Flow field-flow fractionation (FlFFF) has been used to separate carbon ink particles based on their size difference. Such high precision and delicate techniques also hold promise for the challenging task of semen sexing45. Nano size particles in natural samples have also been separated using symmetric as well as asymmetric FlFFF systems46). These techniques have a size resolution of □10 nm and are very gentle, and therefore adapted for semen sexing. Microfluidic systems are emerging as a cost effective and portable alternative to FACS for cell sorting. Microfluidic devices have the potential to develop into on-site devices which will also avoid freeze-thaw of semen and preserve the viability of sperms.

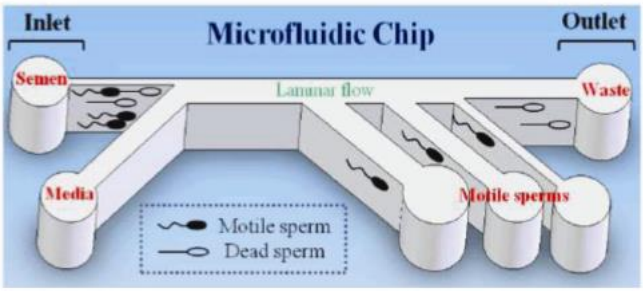

Figure 1. Multiple channel microfluidic chip. The dead sperms move straight with fluid whereas live sperms enter side channels due to their random motility.