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Semen Sexing in Bovine: Current Status and the Need to Develop Alternative Techniques

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ABSTRACT

Livestock industry is one of the important pillars for country economy. Sperm sexing has the potential of significantly improving the quality and quantity of production in the livestock industries. Over years several attempts, involving several different parameters of sperm cells, have been made to develop an efficient sperm sorting procedure. Till date, the only commercially available method for bovine sperm is flow cytometry. The disadvantages associated with this approach, however, points towards the need for developing a more non-invasive and cost efficient approach for separation of X and Y chromosome bearing spermatozoa. In this review paper, we attempt to highlight all the previously developed approaches, as well as other emerging immunological and proteomics based methods that might have the potential of being developed as a promising breakthrough approach for semen sexing. Furthermore, we discuss the advantages and applications of semen sexing in India and around the world.

Introduction

Semen sexing or sperm sexing deals with separation/sorting of X and Y chromosome bearing spermatozoa present in the semen before insemination. Sexed semen as its name and definition suggests that may create ethical issues if applied to human beings but sex predetermination of the offspring is of health and commercial importance if we talk about dairy and farm animals. A female calf has a higher demand in dairy industry due to their dependence on milk and milk products whereas the male calves are required in the meat industry. J. L. Lush (1925) conducted a study controlling prenatal sex. It was based on density difference of X- and Y- bearing spermatozoa in species of rabbit (Lush, 1925). A number of other physical methods for X- and Y-chromosome separation based on mass and motility differences, changes in swimming patterns, presence of surface charges and immunologically based differences have also been developed. However, Dr. Lush was not successful in demonstrating the practical sense of distortion in the sex ratios. Semen sexing was found to be dependent on the differences in the amount of DNA between X and Y sperm as well as others sperm parameters. X sperm has 3.8-7.5% more DNA than Y sperm (Rahman and Pang, 2020).

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Furthermore, Y encodes less than 700 genes, whereas the X chromosome encodes about 3,000 genes (Umehara et al., 2019). Several studies showed that there are few to no differences were observed between these sperm in terms of parameters like ratio, shape, motility etc. A more interesting technique, "Modified swim up technique using RT-PCR" has been used for separation of live and dead spermatozoa. Y-bearing spermatozoa are more motile in terms of swimming capability than X bearing spermatozoa due its smaller size (Sarkar et al., 1984). Success rate in this method was reported to be 81%. Efficacious technique like flow cytometry grew in the 90s with an accuracy of 80% production rate of the desired sex (Garner, 2006). In addition, Raman spectroscopy (RS) has also stands out to be an attractive approach in detecting vibrations of chemical bonds through the phenomenon of inelastic scattering of light as a non-invasive means for differentiating between X and Y sperm cells (Ferrara et al., 2015). The difference in DNA content between X and Y spermatozoa can be considered as one of the potential criteria and can be further studied. A set of proteins and genes that are differentially expressed between X and Y spermatozoa were identified by various proteomics and bioinformatics studies. The sorting of X and Y chromosome bearing sperm on the basis of DNA content differences can be considered as a reliable and repeatable technique for producing sex preselected animals. All chromosomes have active gene transcription, including the sex chromosomes and male germ cell that is essential for cell survival. During the process of spermatogenesis, the developing spermatozoa remain connected via cytoplasmic bridges. These intercellular cytoplasmic bridges allow free communication between the connected cells having different genotypes. This results in a mostly shared protein content between X and Y chromosome bearing sperm cells irrespective of the difference in their genotype. Mature sperm cells are transcriptionally and translationally inactive. However several recent studies have highlighted the possibility of difference in the protein content either due to certain level of RNA polymerase activity after closing of these bridges or the inability of some proteins to be shared. Several studies reported an extensive deep analysis of the possibility of differentially and/or uniquely expressed proteins that are not common in X and Y spermatozoa or are the reasons for phenotypic variations.

A brief overview of semen sexing known till date

Advances in semen sex sorting have enabled incorporation of many previously studied technology and belief that had led to advancement in sexing industry. This includes identification of H Y antigen, albumin gradient, free-flow electrophoresis, volumetric differences, Percoll density gradient, flow cytometry, etc.

H Y antigen employed to inactivate the Y sperm

Cell surface antigens specific for either X or Y chromosome bearing sperm cells can be used for their separation. One of first antigens identified was H Y antigen, a male specific minor histocompatibility antigen. It was suggested that insemination of female mice using anti-HY antibody treated spermatozoa reduced the percentage of male pups produced (Bennett and Boyse, 1973). Others reported that anti-HY antibody shows binding with half of the sperm cell population but showed no difference in sex ratio of off-springs (Ali et al., 1990). However, it reported that the HY antigen was present on the cell surface of X sperm cells as well as found in erythrocytes and premeiotic germ cells. Thus, it does not specifically bind with Y chromosome bearing spermatozoa and so cannot be used for separation of these cells (Yadav et al., 2017). Recently, Shen et al. (2021) reported that CLNR3 and SCAMP1 proteins are X and Y sperm specific antigens respectively. By screening these surface specific antigens, they attempted to study the molecular mechanisms leading to biological differences between X and Y spermatozoa. Inactivation of Y sperm through this method helped in identification and separation of X and Y bearing sperm.

A Label-free method induced semen sexing

Raman spectroscopy (RS) was reported by De Luca et al., in 2014. The X and Y chromosome bearing bovine sperm can be discriminated by using the label free and non-invasive sorting approach of RS. They suggested that the spectral component of the sperm such as DNA, lipids, protein and other cellular macromolecules can be used as the basis for differentiating between X and Y sperm. Although the nucleus having main biochemical differences between X and Y sperm, they observed that there was no difference in the peaks for three nuclear regions- the head, neck and acrosome. The variations in Raman peaks were observed due to the difference in DNA content as well as the difference within the sperm membrane of different sex (Ashok and Dholakia, 2012; De Luca et al., 2014). Hence, they suggested that RS can be used for the development of a sensitive, selective and a non-invasive technique that will be

highly efficient in sperm sexing (Fig 1). Recently, attempts are being made by Jiva Bioscience Pvt. Ltd, Bengaluru, India for commercializing this technique.

Percoll column based sexing method

Percoll gradient column is used in which semen is layered on the top and spermatozoa are allowed to penetrate the column. Separation takes place based on the difference in their densities and separate layers are forms based on the density of the solution. Viable sperms are separated into the lower layer containing high density solution. Abnormal or non- viable sperms are separated in the upper layer containing lower density solution. White blood cells and other debris are obtained in the topmost layer. Viable sperms can then be collected carefully from the lower layer (Kaneko et al., 1983). The ROS levels were higher in the spermatozoa that were separated using Percoll and no difference was observed in the integrity of sperm DNA. Percoll density gradient method was also efficient in increasing the progressive motility of the sample as compared to control. This technique, however, was not effective in separating X and Y chromosome bearing spermatozoa (Arias el al., 2017).

Based on difference in staining, motility and swim up methods

Several attempts were also made for the identification and separation of X and Y chromosome bearing sperms using different staining methods. One of these methods based on the use of Quinacrine staining. It was suggested that Y chromosome bearings sperms will possess a fluorescent spot due to the F body whereas X chromosome bearing sperms will be emitted no fluorescence and it remained unstained (Barlow and Vosa, 1970). However, later on it was noted that this method was not efficient and resulted in false positive results. The polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) based methods are used for assessing the purity of sexed semen. These methods are accurate and easy to use (Reinsalu et al., 2019). Their results concluded that a dual colour FISH protocol is a reliable method for validation of sexed bull semen. Low pH and stressful conditions retarded motility in Y spermatozoa, whereas motility of X spermatozoa rapidly declined when incubated in a high pH condition. Based on these observations it was noted that under certain conditions, separation of sperms is also possible based on difference in their motility. Several attempts have been made for the separation of sperm cells using swim-up techniques. In 1984, Sarkar et al. experimentally concluded a success rate of 81% in separation of X and Y sperm cells using swim-up protocol. Recently, Asma-Ul-Husna and colleagues (2017) developed a modified swim-up method and used it for separation of X and Y sperm cells from sample collected from Nilli Ravi buffalo. They used SYBR green based real time PCR to show X and Y chromosome specific enrichments in different fractions from the supernatant (Asma-Ul-Husna et al., 2017).

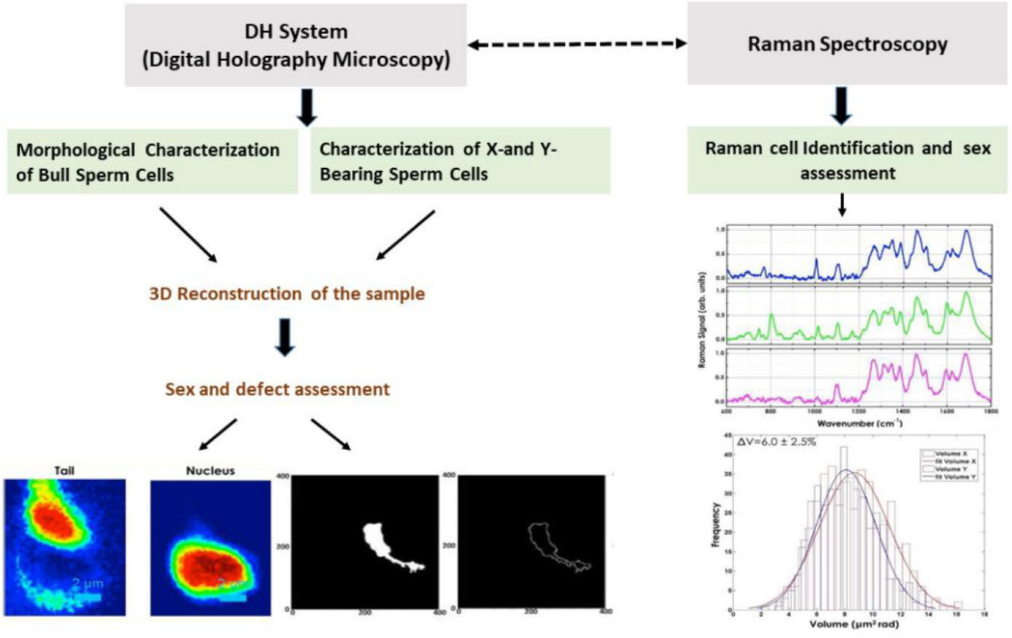

Fig. 1. Experimental flow of combined digital holographic microscopy and Raman spectroscopy for sex and defect assessment in spermatozoa (De Luca et al., 2014)

Immunological approaches for sperm sorting

In 1999, Hendriksen, et al. hypothesized the possibility of difference in gene expression which in turn can lead to difference in the protein content between X and Y sperms which, eventually, can be used for developing a feasible method for separation of these sperms. Immunological methods are developed by exploiting these proteomic differences between X and Y chromosome bearing sperms. These approaches may be based on the possibility of identification of different biomarker, surface antigens specific for either X or Y sperm cells or development of different antibodies specific for unique sperm proteins. These immunological methods can be considered as less aggressive and more efficient than other approaches (Yadav et al., 2017; Quelhas et al., 2021).

Identification of differentially expressed proteins between X and Y spermatozoa

Several attempts have been made for profiling the differentially expressed proteins between X and Y spermatozoa by using various proteomics based approaches. Howes and colleagues (1997) were the first ones to work on this approach; however, their work remained inconclusive (Howes et al., 1997). Chen et al. (2014) identified and characterized 31 differentially expressed genes present in sperm cell. Out of these 31 genes, 27 were up-regulated in X chromosome bearing sperm cells and only 4 genes were up-regulated in Y- chromosome bearing sperm cells. De Canio et al, (2014) used a label free shotgun nUPLC-MS/MS and identified 15 and 2 proteins up-regulated in X and Y spermatozoa respectively. Scott el al. (2018) identified 8 proteins that were differentially expressed between X and Y sperm cells using SWATH-MS analysis on flow cytometry sorted sperm sample. Recently, Shen et al, (2021) profiled differentially expressed proteins in X and Y sperm cells for Holstein bulls. They reported that 8 and 23 surface proteins were significantly up-regulated and down-regulated in X spermatozoa. They also reported that 81 and 151 proteins were specifically expressed in X and Y spermatozoa respectively. These differences in the proteomes of different sperm types can form the basis for development of various immunological approaches for semen sexing. These unique or differentially expressed proteins can be used a biomarker for identification and ultimately separation of X and Y chromosome bearing sperm cells.

Development of antibodies for specific sperm surface proteins

Sperm surface proteins or antigens that are specific for either X or Y sperm cells are considered as a good candidate for antibody based separation of sperm cells as indicated in Fig 2 (Goldberg et al., 1973; Quelhas et al., 2021). Sex determining region Y (SRY) is localized on Y spermatozoa specifically and attempts have been made for developing antibodies against recombinant SRY protein. Soleymani et al. (2019) developed polyclonal antibodies against SRY protein (anti-rbSRY) in goats. These antibodies specifically showed binding with Y chromosome bearing sperm cells and not with X chromosome bearing sperm cells. They also developed bovine antibodies against rbSRY (mAbSRY2). These antibodies were bound to a Sepharose column and only Y chromosome bearing sperm cells are bound to the column (Soleymani et al., 2021). A monoclonal antibody, WholeMom®, was developed against Y sperm surface epitopes by Chowdhury and colleagues (2019). They used these monoclonal antibodies for selection of Y sperm cells from cryopreserved sample (Chowdhury et al., 2019). A single chain fragment variable (scFv) antibody was developed against epitopes present on surface of sperm cells. These antibodies were composed of a single variable heavy chain (VH) and a variable light chain (VL). They were generated from the parent monoclonal antibody (mAB-1F9) but scFv antibodies showed higher specificity for Y chromosome bearing sperm cells and had lower cross reactivity with X chromosome bearing sperm cells (Thaworn et al., 2020).

Flow cytometry based semen sexing and its impact on fertility rate in bovines

Flow cytometry based sorting has emerged as one of the most efficient sorting technique that is now commercially available. It has been used for separation of sperm cells in many species like sheep, goat, pig but is commercially available and encouraged for only bovine. This method is primarily based on sperm DNA staining with Hoechst 33342 in order to differentiate sperm cells bearing the X and Y chromosome (Garner, 2009; Garner et al., 2013). Since X chromosome has 4% more DNA than Y chromosome and so it emits a stronger fluorescence. A high speed specialized cell sorter is used to separate subpopulations of fluorescently stained sperm cells and are collected into biologically supportive media (Fig 3).

This technique has been patented and commercialized for application in bovine sperm separation in USA,

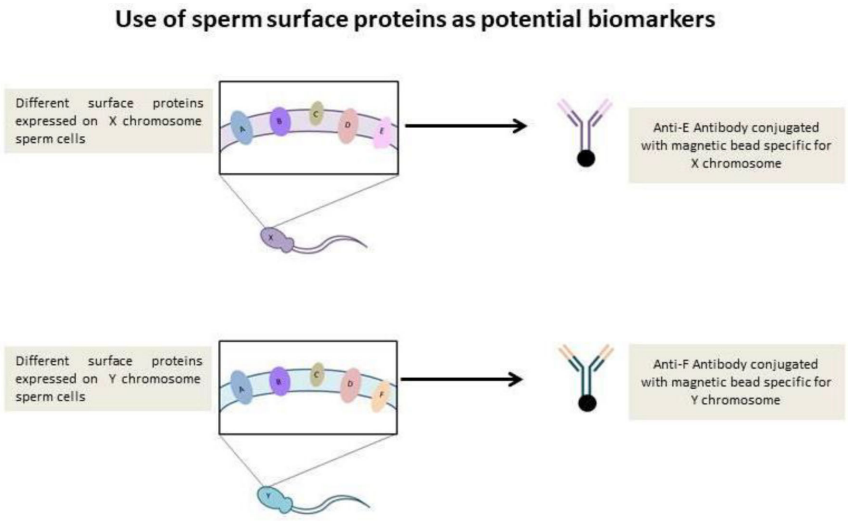

Fig. 2. A proposed method based on the use of antibodies for identification of differentially expressed plasma membrane proteins and separation of X and Y sperm cells.

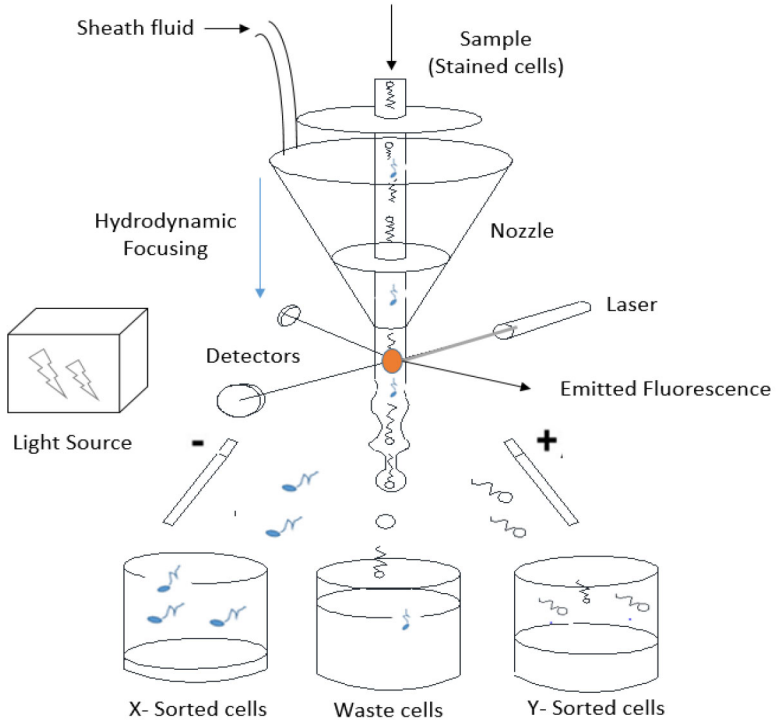

Fig. 3. Flow cytometry based semen sorting (Naniwa et al., 2018)

Europe and other countries. However, these sorting procedures exert stress on sperm cells which reduces their fertility, viability and lower conception rates than unsorted sperms, thereby making it a lesser preferred approach. This also makes it necessary to develop a new approach that will not cause harm to the quality of sample and will have comparatively higher conception rates.

Glycocalyx forms the outermost surface of the sperm cells and is essential for survival in the female reproductive tract and other capacitation processes. Flow cytometry procedure including high pressure sorting systems and lasers may have a detrimental effect on the glycocalyx layers. Analysis of the changes occurring in the gylcocalyx layer can be used to evaluate the potential reason for lower sperm viability. Umezu et al. (2017) attempted to study the changes that occur in the glycocalyx due the flow cytometry. A deeper study into the changes that occur in this layer after different treatments and procedures can help in improving the techniques.

Recent approaches to reduced constraints and produce cost effective semen

Protein profiling of sexed versus conventional semen in bovines differentially

Differential protein profiling of expressed proteins between X and Y chromosome bearing sperm cells, performed under pooled samples comparative study of the sexed sperm, shotgun nUPLC-MS/MS method was used for differential profiling. Followed by EMRTs and protein normalization, profiling was further performed and the results obtained were analysed using PLGS statistic filter. Modulated proteins were selected with a confidence level that was greater than 95 %. Application of these filtering criteria resulted in identification of 17 differentially expressed proteins, 15 proteins were found to be up-regulated in X sperm cells while 2 were up-regulated in Y sperm cells. Isolated proteins were found to be associated with structural function that includes five isoforms of tubulin, three of outer dense fibres and A-kinase anchor protein 3 while some of them were enzymes used in glycolysis i.e. two isoforms of glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase and L-lactate dehydrogenase A. Apart from these 4 of the proteins were found to be expressed in X cell: amino acid catabolism protein L-asparagine, regulation by calmodulin, seminal plasma protein PDC109 and a chaperon-like protein, strongly associated to spermatozoa membranes and sperm acrosome membrane-associated protein 1 (Soggiu et al., 2013). Validation of shotgun results was performed by applying a targeted label-free strategy in order to evaluate the expression of the proteins by studying and analysing their proteotypic peptides (Soggio, Alessio et al, 2013). Triplicate runs were given for digestion of X and Y cell proteins. Tryptic digestion of yeast enolase was taken as internal control and data acquisition performed using nUPLC-MS/MS in expression mode (Fig 4).

Sorting of conventional bovine semen using of sperm surface proteins as potential biomarkers

Germ cells comprise autosomes and an X or Y sex chromosomes. The sex of an offspring is determined based on a sex chromosome carried by the spermatozoon, which is the male germ cell that is capable of fertilizing an oocyte and also carry its genetic information. X and Y spermatozoa follows the same origin and maturation phenomenon, however, certain differences exist between them (Fig 5). Many genomics and proteomics studies have found

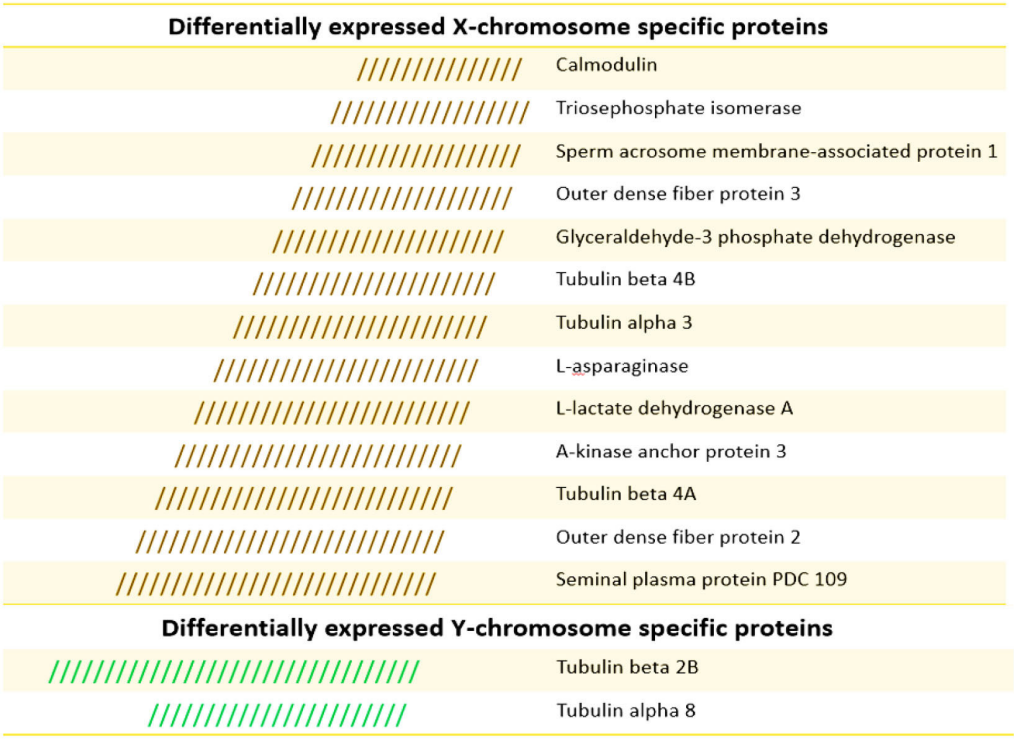

Fig. 4. Differentially expressed proteins with representation of fold change of modulated proteins in label-free comparative analysis (Soggiu et al., 2013)

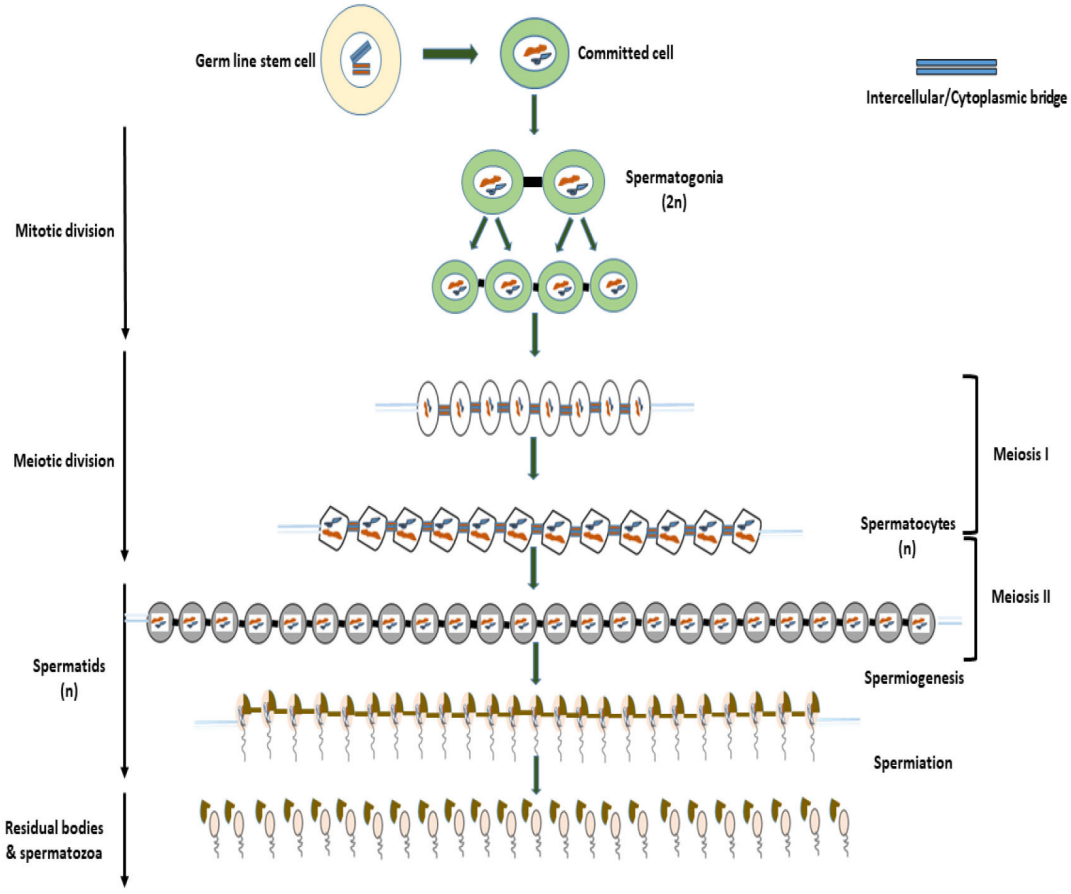

Fig. 5. Intercellular bridges connect developing male germ cells. These bridges are established after commitment to differentiation and ensure cytoplasmic continuity between the progeny of all subsequent mitotic and meiotic divisions. Sperm cell individualisation only occurs immediately prior to the release of the fully differentiated sperm from the gametogenic tissue (spermiation). The intercellular bridges are large enough to accommodate the flow of mRNAs, proteins, and even organelles between cells. Note that the degree of sharing of any given product is determined by a source–sink process, potentially resulting in increased levels near the site of production (as illustrated by the two cytoplasm hues in post-meiotic cells, matching the sex chromosome they contain) mRNA, messenger RNA (Paula et al., 2020)

out a set of proteins and genes that might be differentially expressed between X and Y bearing sperm. Difference in DNA content was thought to be responsible for the differential expression of certain genes and ultimately proteins between them (Rahman and Pang, 2020). Studies have also reported that an active gene transcription is selective in the chromosomes, including the sex chromosomes (Hu and Namekawa, 2015). Expected ratio was affected if the composition of sex chromosomes has been considered to change the gene expression due to the post-meiotic modifications and differential survival of spermatozoa during epididymal maturation (Bean, 1990). In a recent study, Umehara et al. (2019) had reported that Toll-like receptors 7 and 8 (TLR7/8) are selectively expressed by the X chromosome and whose activation by a suitable ligand, suppresses the motility of X sperm without any noted alteration in its fertilization ability. TLR7/8 procedure was successful in producing 90% of the male embryos which was followed by IVF (in vitro fertilization) using motile spermatozoa selected after treatment with the ligand. Other study carried by them used a knockout (KO) mice model, Rathje et al. (2019) showed that when Y chromosome is deleted partially (Yqdel) produces an equal number of X and Y spermatozoa in males. Although sperm of both kinds are equally responsible or capable of fertilizing oocytes at the fertilization site and exhibit a function difference in terms of motility or morphology from each other responsible for skewing offspring sex ratio.

Presently available sexing method is expensive from all dimensions thereby cost of sexed semen is high and it does not require any special equipment or professional skills, it can easily be applied in laboratories where IVF is performed.

Proteomics based approach can lead to: (1) Less damage to sexed sperm which increases fertility, (2) Bulk production of sexed semen with minimum cost involvement (Buragohain et al., 2017)

TLR7/8 as a potential biomarker for separation of X and Y sperm cells based on difference in progressive motility

Umehara et al. (2019) also reported that (TLR7/8) encoding X chromosome expressed by 50% in the round spermatids and the epididymal sperm. TLR7/8 ligand activation suppresses mobility of the X sperm selectively without change in Y sperm mobility. Separation of Y sperm from X sperm was possible due to difference in sperm motility. Using these ligand-selected high-mobility sperm, when used these sperm for IVF found 90% of the embryos having XY chromosome and further 83% of the pups obtained through embryo transfer were XY males. Functional differences between Y sperm and X sperm motility were found to be related to different gene expression patterns, specifically TLR7/8 on X sperm. The expressions of TLR7/8 protein was analysed using Western blot and immunofluorescence (IF). Expression of TLR7/8 was also found in spleen, used as a positive control. TLR7 and TLR8 that are expressed on the X chromosome have a ligand in common- Resiquimod (R848) binds both TLR7 and TLR8 while Imiquimod (R837) was specific for TLR7 only. Motility assay showed the percent of highly active sperm that swim up to an upper layer was decreased by the addition of R848/R837 in a dose-dependent manner (0.3 μM to 3 μM). Mobility was reduced and this was evident with sperm incubation with 0.3 μM for greater than 60 min. Computer-assisted sperm analysis (CASA) showed sperm tracks that were incubated with 0.3 μMR848 or R837 were shorter in compared to sperm incubated without TLR7/8 ligand i.e. control. VAP i.e. average path velocity of sperm was found decreased by R848 or R837 significantly. Treatment with the TLR7/8 ligand (R848) is responsible for separation of X sperm from Y sperm. Any special equipment or professional skills is not required, and can be applied in laboratories where IVF is performed. Semen sample incubated with 0.03 μM R848 in 1 mL of calcium-enhanced human tubal fluid (mHTF) medium for mouse sperm and 3 mL for bull sperm for 60 min, followed by mouse sperm upper layer (400 μL) and 1 mL for bull sperm were collected separately. Sperm were further suspended in ligand-free IVF medium after washing by centrifugation for use in IVF. Results showed upper layer sperm formed 90% of the XY embryos in both mice and cattle, and precipitated sperm formed >80% of the XX embryos. Total duration taken for separation of X sperm and Y sperm was within 2 h (Umehara et al, 2019; Nakao et al., 2020).

The evolutionary conundrum caused by TLR7/8

While most of the researchers agreed with the role of TLR7/8 and its limited expression to X chromosome bearing sperms only. Regardless, there were others who argued that presence of an X linked gene that would impair the fitness and fertilization ability of X chromosome bearing sperms is not evolutionarily fit. They argued that this could lead to distorted sex ratios. They even referred to such phenomena as suicidal and that such alleles are evolutionarily unfit and are rapidly deleted from the environment by natural selection. However, the only argument that answered these questions is that TLR7/8 present in the sperm cells has no role in in vivo conditions and they remain inactive under physiological conditions. The ligand responsible for activation of these receptors is also absent in female reproductive system and these receptors have no role in fertilization and development stages (Navarro-Costa et al., 2020).

Improving fertility of sex-sorted semen using SexedULTRA™

In order to simplify and optimize the sex sorting media prescribed SexedULTRA™ was devised. This was devised to remove the stressors retaining the sperm in a more benign medium. The modified protocol included pre-treating the sperm before the staining step. A new staining medium for balancing the pH and maintaining stability to extend it for a long period was used. Modification of the sheath fluid and the freezing medium was done in order to accomplish low dose freezing which is required for sex-sorted semen. All these changes were reflected in the initial laboratory evaluations and in vitro semen quality tests reflected changes in processed semen using the SexedULTRA™ using improved media for sperm motility as well as acrosome integrity as compared to the XY Legacy technology (González-Marín et al., 2018). SexedULTRA™ semen used in trials of IVF resulted in freezable embryos in a greater number when compared with the XY method (13.2% and 9.2%, respectively, González-Marín et al., 2018). SexedULTRA™ small scale trials involved an improvement of 7.4 percentage points (15.6% relative) in conception rates of heifer as compared with the XY Legacy technology. Large field trials in collaboration with select sires from eight Holstein bulls undergone sorting by either the SexedULTRA™ or XY processing methods. This method leads to insemination of 6930 Holstein heifers across 41 commercial herds in the USA and resulted in an improvement of 4.5 percentage points (10.8% relative).

Difference in reproductive performances in sexed versus conventional semen studied in Holstein breed of cattle

For comparative analysis between sexed versus conventional semen, a total of 3573 heifer insemination was compared for investigate the differences in their reproductive performances (Joezy et al., 2017). It was investigated in five herds in four provinces of Iran. Studied provinces include hot semiarid (Tehran and Alborz provinces), temperate semiarid (Khorasan Razavi province) and cold semiarid (Zanjan province). Conception rate, number of services per conception, calf sex ratio, calf birth weight, gestation length, calving ease score, abortion and stillbirth were the parameters used in this investigation. The regression method deployed for the analysis of categorical variables was logistic and analysis of continuous variables was carried out using GLM procedure. Conception rate average between sex sorted and conventional semen was found to be 48.3 and 63.8% and sex sorted semen resulted in 91.1% female calves. Results obtained were highly re-affirm based on previous findings of the sex sorted group reproductive performances. A special climate was considered while deciding for utilisation of sex sorted semen in a dairy farm and it was concluded that the selection of sex sorted semen was highly related to herd management practices (Bhalakiya et al., 2018; Brito et al., 2019).

Application of microfluidics and nanoparticles in semen sexing- A major game changing approach

Sperm sex sorting is one of the most studied fields for over past three decades and there is no reason to doubt the fact that some worthwhile progress has been made over these years. Yet we face the issues like low productivity and lower conception rates. With the increasing population, the livestock industries are facing an immense pressure to increase their productivity without compromising their quality. This raises the need for development of an alternative modern approach for solving the unmet issues pertaining to the increasing market demand. The increasing popularity of nanoparticle and microfluidics based approaches have proved to be the front runner in this race. A study was conducted and identified a triplex sequence specific to Y chromosome and proposed a novel approach based on this sperm sequence specific gene targeting and detection using laser generated gold nanoparticle bio-conjugates (Gamrad et al., 2017). The triplex binding sequence binds only with only poly purine binding sites. Previously, nanoparticles have been used for separation of dead sperm cells from live ones (Bisla et al., 2020).

Zeta potential refers to the membrane charge that develops between a solid and liquid surface. Domínguez et al., 2018 showed that the Y spermatozoa have a difference in membrane potential called the zeta potential of -16mV whereas the X spermatozoa have a zeta potential of -20mV. As a result of this, the Y spermatozoa form complexes with the magnetic nanoparticles more easily than X chromosome. The Y chromosome complex with magnetic nanoparticle beads was pulled towards the inner wall of the test tube whereas the X chromosome bearing sperm cells remain suspended in the media and can be collected separately (Fig 6). Zeta potential based methods have been used for development of magnetic nanoparticles methods. These nanoparticles can also be used for isolation of X spermatozoa. This difference between membrane potential has also been used for the development of microfluidic dielectrophoretic-based chip. Domínguez et al. (2018) showed the application of magnetic nanoparticles in X sperm cell separation in donkey. They reported an efficiency of 90% using this approach without affecting any sperm functionality. Wongtawan and colleagues (2020) recently proposed the use of microfluidic dielectrophoretic-based chip as the new and effective approach for separation of X chromosome bearing sperm cells. In this technique, Y sperm cells are trapped by the electric probes and the X sperm cells can pass through the chip (Fig 7).

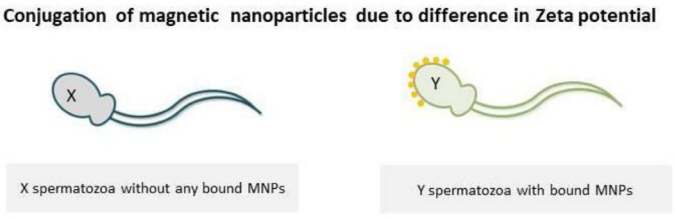

Fig. 6. Conjugation of magnetic nanoparticles using the difference between sperm cells based on surface charge or zeta potential. (Neculai-Valeanu et al., 2021)

A recent report by Chalinee et al., (2021) involved the use of magnetic beads conjugated with Y sperm specific monoclonal antibodies, 1 F9, were used in a using carbon walled nanotubes in microfluidics based system for separation of sperm cells. They highlighted that the MCNT-microfluidic chip successfully sorted magnetic particle beads, monoclonal antibody-conjugated magnetic particle beads, and sperm-conjugated monoclonal antibodies with magnetic particles, with success rates of 100%, 98.84%, and 80.12%, respectively. They reported a success rate of 80%

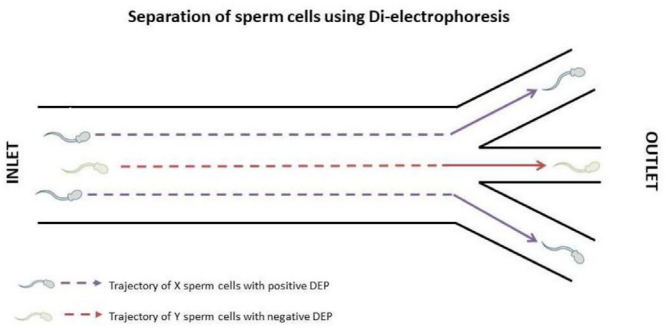

Fig. 7. Use of Di-electrophoresis (DEP) for separation of X and Y chromosome bearing sperm cells using difference between sperm cells based on surface charge or zeta potential. (Neculai-Valeanu et al., 2021)

in X and Y sperm separation using the specially designed multi- walled carbon nanotube microfluidic device which resulted in separation of Y sperm cells towards the negative electrical fluid channel.

Joana Quelhas and colleagues in their review paper titled "Bovine semen sexing: Sperm membrane proteomics as candidates for immunological selection of X and Y chromosome-bearing sperm" discussed the emerging importance of immunological approaches in the field of semen sexing (Quelhas et al., 2021). They also highlighted the use of sperm surface proteins in conjugation with antibodies and magnetic beads based approaches (Fig 8) shows a representation of a method for separation of sperm cells using antibodies conjugated with magnetic beads.

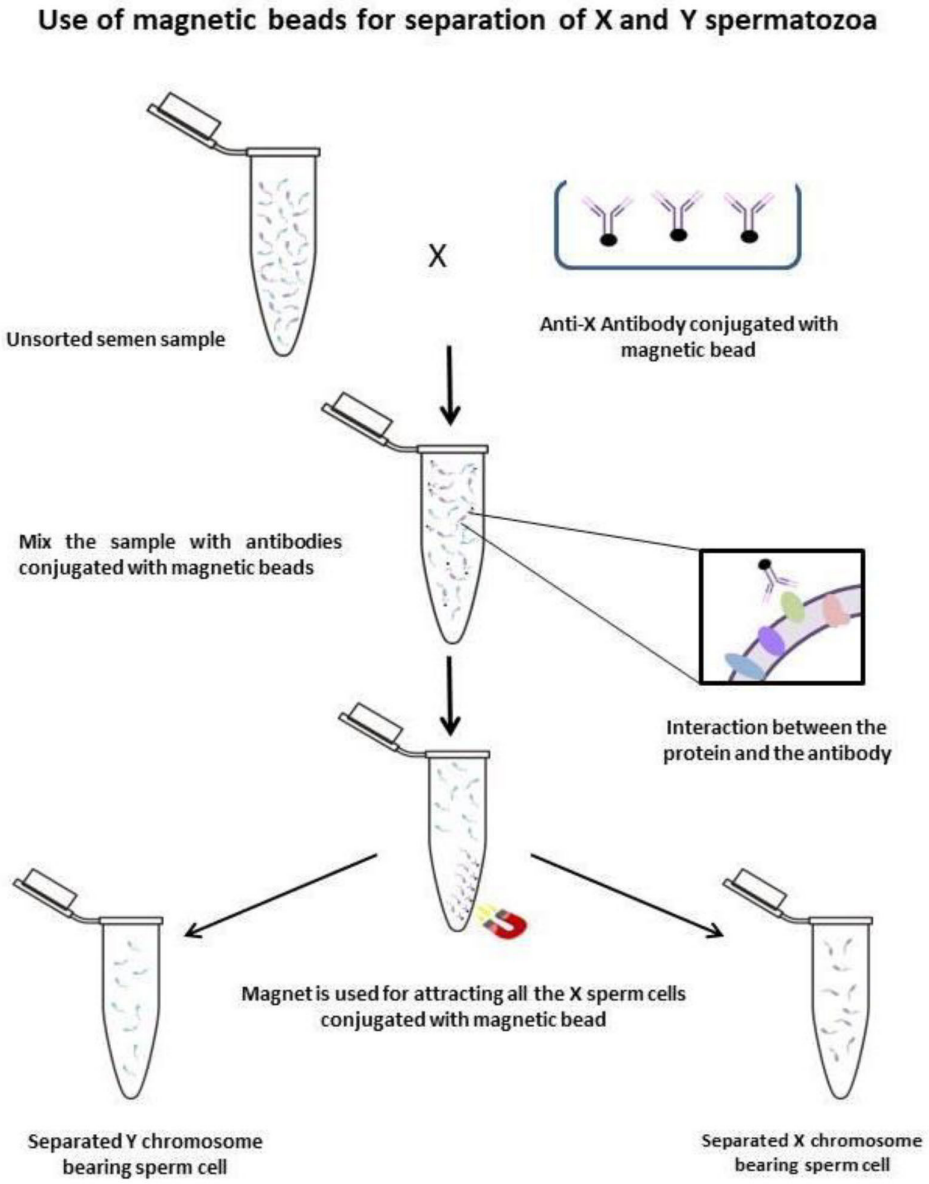

Fig. 8. Separation of X and Y spermatozoa using antibodies conjugated with magnetic beads using magnetic force (Quelhas et al., 2021)

Status of semen sexing in India

Production of sexed semen and artificial insemination is one of the major steps towards achieving the bigger goal of increasing milk production. The Indian dairy industry is growing at a rapid rate as well. The use of sorted semen allows farmers to achieve higher profits with reduced maintenance costs of unwanted male calved. Over years several attempts have been made by the Government of India (GOI) in promoting the import as well as production of indigenous sorting technologies. The government adopted the approach of sex sorted semen and artificial insemination to support the farmers by increasing the number productive animals available to them.

In India, several state governments and government supported organizations have also taken initiatives for promoting the use of sexed semen. By 2009, a Bengal government led organization named Paschim Banga Go-Sampad Bikash Sanstha (PBGSBS) initiated semen sorting using high speed flow cytometers at Frozen Semen Bull Station, Haringhata. The first a male calf named Shreyas was born on January 1, 2011. In Kerala, the livestock development board imported 650 sexed semen straws from Canada. They also reported the birth of 2 calves using sexed semen to Jersey crossbred heifers and Holstein crossbred cattle respectively. The responsibility of sex sorting of sperm cells in cattle was assigned to NDRI-Karnal by the Government of India. The aim of this project was to provide farmers with sexed semen with the aim of increasing the indigenous and crossbred cattle populations in India. Haryana and Punjab have emerged as the key players in import and use of sexed semen. Other than these, ABS India is a provider of HF and Jersey sexed semen and prime bovine genetics in collaboration with sexing technologies also provide sexed semen for HF, Gir, Brown swiss and Jersey crossbreds (Kumar et al., 2016; Yadav et al., 2018).

Bharatiya Agro Industries Foundation (BAIF) development Research foundation was established in 1967 in Pune. It produces about 10 million doses of deep- frozen bovine semen and artificial insemination. BAIF also inaugurated its state of the art semen sex sorting laboratory for cattle, in Uruli kanchan, Maharashtra in November, 2018. The facility was established in collaboration with Sexing Technologies, Texas, USA. The centre focuses majorly on production of sorted semen doses of indigenous breeds of cattle including Sahil, Gir, Tharparkar, Red Sindhi and buffalo breeds of Murrah and Jaffarabadi.

The government of India aims to sanction install 12 semen stations. Some of these stations have been completely set up and are now fully functional. The deep frozen semen production centre (DFSPC), set up in Uttarakhand, was the first centre producing sex sorted semen (under Rashtriya Gokul Mission) and became operational since March 2019. Another similar facility will be developed in Bhopal, Madhya Pradesh

In 2017, Genus ABS India deployed IntelliGenTM technology for production of sexed semen for all breeds in a centre located near Pune, Maharashtra under the brand name SEXCELTM. Genus ABS also established a deep frozen semen centre (UPLDB) in Babugarh, Ghaziabad, Uttar Pradesh.

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Advantages and disadvantages of sexed semen

With the increase in the demand of animal based products like milk and meat, the need to focus more production efficiency in different animal husbandry industries has also increased. This led to increase in use of sexed semen at farm and industry levels. This helps in achieving higher profit levels and also reduces management practices and costs in farms. In dairy based industries, this approach provides a way to overcome the production of unwanted male calves and the need to divert resources for their care even though they have low economic value. It also helps in reducing the

cases of dystocia that were observed during birth of male calves in dairy farms. In milk as well as meat industries, the use of sexed semen from superior bulls allows the farmers to raise high quality heifers that can directly improve the production quality and yields. Since only a particular type of off-spring is being reared, the maintenance and labour costs are also reduced.

The main disadvantage, however, with the use of sexed semen is its lowered quality in terms of motility, sperm count, sperm viability which ultimately led to reproduction rates. Due to lower sperm count, an increased number of inseminations as well as higher doses are required for successful conception. Moreover, the lower embryonic development rates do not always support the desired outcome and also leads to lower profit levels. These disadvantages prompted the need to improvise the currently available methods of separation, as well as develop more efficient methods that will not hinder the quality of sperm cells.

Effect of semen sexing on sperm morphology

Sex sorted semen has proved its mettle as a valuable tool in animal husbandry industries by empowering the farmers with the ability to preselect the sex of the off-springs. Over years, semen sexing has been at the centre stage in reproductive biotechnology researches. Despite making valuable progress over the previous decades, the outcomes observed are still under expectations. AI using the sexed bovine sperms results in lower than expected conception rates and lower embryonic development. Sexed semen showed a fertility of 75-80% of the conventional non-semen. Conception rates were 55% for conventional semen and only 44% in case of sexed semen (Manzoor et al., 2017). Steele et al. (2020) evaluated the effect of sexing procedures on sperm function and embryo development. They used CASA for demonstrating that the progressive motility was reduced in case of sexed semen, whereas sperm cells before sexing showed faster progressive motility. By using time-lapse video-microscopy for embryos produced using sexed semen showed evidences that such embryos have a greater tendency of getting arrested at 4-celled stage. They also noted that the risk of shrinkage and reduced blastomere development rates were also observed in case of embryos developed using sexed semen. Ultimately it was concluded that this decline in conception rate and reduced embryo development was due to change in sperm morphokinentics, which was a result of semen sexing.

Semen sexing technology: Worldwide development and applications

Sex sexing technology development was done by the United States Department of Agriculture researchers in Livermore, California, and Beltsville, Maryland. Semen sexing technology was patented as "Beltsville Sperm sexing technology". Commercialization of the technology was started in United States in 2001 and a license was granted to Sexing Technologies (ST), Texas. Presently, semen sexing technology commercially produces sexed semen in many countries of Europe, USA, Canada, Mexico, Brazil, China, Japan, etc. Flow cytometry based semen sexing has emerged to be the most efficient, refined through the decades and producing a purity of more than 90% sexed semen, well standardized, patented and commercialized in USA, Europe and other countries.

Cost and regulation of sexed semen in India

In USA per straw sexed semen cost is around $20-$61 whereas, in Indian its cost come around INR 1500-4500/ per dose as compared to INR 15-20/ per dose for conventional semen. Purchasing sexed semen require approvals from Central Government (Dept. of Animal Husbandry, Dairying and Fisheries) and State Governments (State AH department). This allows import permit from Director General of Foreign Trade (DGFT). Complete records of its use and progeny born out of imported semen are mandatory and should be maintained from time to time. Currently, sexed semen is commercially available mainly from Sexing Technology, USA. Many other breeding companies in USA, Canada and Europe are producing sexed semen commercially using licence from Sexing Technology, USA.

Sexed semen technology-Commercialisation difficulty in developing countries

Commercialisation of sexed semen is so far found to be difficult in developing countries like India and currently no agency is producing sexed semen in India and states/ agencies are using imported sexed semen. The reason behind this difficulty in commercialisation is conception rate which is 10-15% less in sex sorted semen as compared to the conventional semen. This factor was found

to be more critical in Indian condition considering low AI coverage (20-25%) and low conception rate with AIs (25-35%). Unavailability of standard operating procedure to perform insemination with sexed semen is an important factor which leads to inefficient development of sexing technology in developing countries. This is another area of concern as the sperm concentration of sexed semen ranges between 2 and 4 million/dose whereas it is 20 million/dose in conventional semen. Managing lower sperm concentration will be a challenge in the field under Asian countries including India.

Future perspectives

Increasing demand of milk production by 2020 was estimated to be 191.3 MT and in order to meet this increasing demand, substantial increase in the number of elite female which can achieved by shifting the sex ratio towards females. Sexed semen bearing X sperm could be used in elite cows in order to produce superior high yielding cows at a faster rate than the conventional unsexed semen and this can also lead to production of superior breeding bulls. Proteomics/bioinformatics data and its implication on in vitro trials with conventional semen can lead to identification of several potential proteins/biomarkers expressed differentially or more specifically in X or Y bearing sperm and lead to manipulation in sperm parameters leading to their separation in terms of manipulated physiological and individual motility.

Conclusion

Separation of X and Y chromosome bearing sperm has been practiced for selection of offspring of desired sex to increase the profit in livestock industries. Inexpensive, convenient, and non-invasive approaches for sperm sexing including H Y antigen, sex-specific antigens, and differentially expressed proteins for sperm sexing can be used for producing less compromised, high fertility rate semen. Proteomics approach does not require any special equipment or professional skills and can lead to bulk production of sexed semen with minimum cost involvement.

Conflict of interest

No conflict of interest.

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