Vinod Kumar Yata

Sperm Sexing and its Role in Livestock Production

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Vinod Kumar Yata
Animal Biotechnology Centre
National Dairy Research Institute (NDRI)
Karnal, Haryana, India

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Foreword

The book, *Sperm Sexing and its Role in Livestock Production* authored by Dr Vinod Kumar Yata, covers the principles, methods, economic and ethical issues of the sperm sexing. The field of sperm sexing is of great interest to the livestock industry, and the number of groups involved in the commercialization of sexed sperm is increasing gradually. Although the book chapters cover a different aspect of sperm sexing, I want to highlight two of them. The first refers to the characterization of animal spermatozoa which provides the clues to the interdisciplinary researchers to develop novel methods of spermatozoa. The second relates to the immunological methods of sperm sexing which highlights the identification of sex-specific markers on the surface of animal spermatozoa. Apart from this, the book discussed other issues having significant importance in the sperm sexing, for example, FACS-based sperm sexing and latest developments of sperm sexing, among others.

In summary, there is no doubt about the interest of the contents displayed in this book. I am sure that this book will provide the scientific community and animal industry with great benefits for the coming years.

Joanna Maria Gonçalves de Souza Fabjan

Department of Pathology and
Veterinary Medicine,
School of Veterinary Medicine,
Universidade Federal Fluminense
Niterói, RJ, Brazil

Preface

In recent years, sperm sexing has made an impressive progress as one of the important animal reproduction technologies for livestock production. The successful commercial exploitation of sexed semen requires knowledge on the principles, applications, ethical and economic issues of sperm sexing. A major purpose of the book is to depict the basic and critical aspects of sperm sexing which may be used directly to increase the production of livestock. This book will act as an important means of information to various interdisciplinary researchers working on sperm research.

The book focuses on describing the conventional and state-of-the-art methods of sperm sexing. Characterization of the animal spermatozoa is one of the major areas for the development of novel methods of sperm sexing, and this book provides an enhanced description of this research area. A separate chapter is devoted to discussing the commercially available sperm sexing based on fluorescent-activated cell sorting. The possibility of sperm sexing by immunological methods discussed in this book to an expanded level. Recent advances in sperm sexing methods, based on microfluidics, nanoparticles, and genetic engineering, were discussed in this book. This book provides insight into the various cryopreservation methods of semen which is very crucial for the storage and transport of sexed semen. Ethical issues raised by animal welfare societies and the common public are discussed in this book. Patent issues and commercial aspects of sperm sexing are also discussed in this book.

The book briefs the readers on various novel and innovative ideas of emerging sperm sexing methods. This comprehensive book will be of great value to the livestock industry, veterinarians, researchers as well as the food industry.

Karnal, Haryana, India — Vinod Kumar Yata

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About the Author

Vinod Kumar Yata is an interdisciplinary researcher working at the National Dairy Research Institute, Karnal, India. Previously, he worked as an Assistant Professor at the Department of Biotechnology, Dr B R Ambedkar National Institute of Technology Jalandhar, Punjab, India. He received his PhD in Biotechnology from the Indian Institute of Technology Guwahati. He specializes in interdisciplinary research which includes Nanotechnology, Microfluidics, Animal Biotechnology, Cancer biology, and Bioinformatics. He has developed a microfluidic device for the separation of live and motile spermatozoa from cattle semen samples. His research interests have been focused on the development of nanocarriers, understanding prodrug enzyme therapy, and targeted drug delivery. He elucidated the structural features and binding interactions of several biomolecules by *in silico* methods. He has published four books as an editor and one book as an author with Springer Nature publisher. He has published several research papers in peer-reviewed international journals and presented papers in several international conferences.

Introduction to Sperm Sexing

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Contents

Abstract

Sperm sexing is an assisted reproductive technology that involves the sorting "X" and "Y" chromosome-bearing live sperm cells of semen samples. Insemination of sexed semen would help to produce animals of predetermined sex, and the use of sexed semen is an essential solution for the animals with sex-linked diseases. Animals of the desired sex are of great interest for animal farmers to increase the economic benefits. Furthermore, production of genetically superior quality animals could be possible by the use of sexed semen. Use of sexed semen is a feasible solution to maintain the optimum sex ratio of the animals.

Potential differences of "X" and "Y" chromosome-bearing sperm cells form the basis for sperm sexing. It is evident from previous reports that Y chromosome-bearing sperm cell contains relatively less DNA, and it is smaller in size and swims faster than X chromosome-bearing sperm cell. Based on the surface charge, Y chromosome-bearing sperm moves slower than "X" chromosome-bearing sperm cells toward the cathode. Absence of HY antigen

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on the surface of X chromosome-bearing sperm cells also forms the basis for immunological sexing. So far, Flow cytometric sorting of semen is proven to be successful in sperm sexing on a commercial scale. This technology is based on the staining of the sperm cells with a fluorescent dye and sorting according to the DNA content. Real-time polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), and Raman microspectroscopy are being developed to validate sperm sexing. Sexed semen is in high demand for the breeding of production animals like cattle, sheep, and pigs. Applications of sexed semen may expand to sports and companion animals like horses, dogs, and cats. Nanoparticle-based sperm sexing, genome editing, and microfluidics are some of the emerging new strategies being developed for safe and efficient sperm sexing. Even though the animal welfare societies raised some ethical issues, sexed semen can be used with ethical policies laid down by recognized national and international animal welfare agencies.

Keywords

Sperm sexing · Animal breeding · Artificial insemination · Sexed semen · Farm animals

1.1 Introduction

Sperm sexing technology has been contributing immensely to increasing livestock productivity, particularly in the bovine industry. It can help out to reduce the animal food scarcity and to improve the economy of animal farmers (Garner & Seidel Jr, 2008; Rath & Johnson, 2008; Holden & Butler, 2018). World human population is expected to increase and reach some 9.7 billion people by 2050. While population growth increases, the animal food product availability needs to be increased proportionately (Gilland, 2002). As per The Food and Agriculture Organization (FAO) of the United Nations statistics, there is more than 26 billion livestock (cattle, buffaloes, horses, pigs, chickens, sheep, goats, and ducks) available worldwide. Milk, meat, eggs, and hides are the major animal products appreciated by the human population. As per the FAO, meat demand in middle-income countries will rise by 80 percent by 2030 and by over 200 percent by 2050. Livestock contributes to the livelihood of large numbers of rural poor people and generates income for the livestock keeping households. World milk production had increased from 522 million tonnes in 1987 to 828 million tonnes in 2017, and approximately 150 million households across the globe are engaged in milk production (www.fao.org). Livestock can improve productivity through selective breeding and control of Production (Stear et al., 2001). Artificial insemination (AI) is a regular practice of large-scale animal farms and desired sex animals are in high demand in such animal forms (Robertson, 1954). For example, Dairy animal keepers demand female cattle, whereas male calves are required for the meat products. The ideal method for sex control in farm animals is the separation of "X" and "Y" chromosome-bearing live sperm cells of semen

samples. Some sectors of livestock benefit from the sexed X chromosome-bearing sperm cells, and it could be useful for producing high milk yielding animals. In contrast, sexed Y chromosome-bearing sperm could help produce elite male animals of good quality breeding. Manipulating sex ratio in large-scale animal forms would help in reducing the number of animals of unwanted sex, and this will greatly reduce the maintenance cost of the animal farm (Seidel Jr, 2007). The main challenge in sperm sexing involved in the separation of X- and Y-bearing spermatozoa, which are almost phenotypically similar to differences in the nanoscale (Carvalho et al., 2013). Sperm damage, low accuracy, and lack of reproducibility are the major problems associated with conventional sperm sexing methods like density gradient centrifugation (Ericsson & Ericsson, 1999), swim up (Awan et al., 2017), Sephadex column (Steeno et al., 1975), and HY antigen-based sperm sexing (Bennett & Boyse, 1973) methods. The invention of Beltsville sperm sexing technology and subsequent developments of this technology revolutionized the use of sexed semen in small- and large-scale animal farms (Johnson et al., 1999). This technology is based on the flow cytometric separation of sperm cells stained with a DNA-binding fluorescent dye. X chromosome bearing sperm were separated from Y chromosome bearing sperm based on differences in their DNA content. This method uses a UV laser beam having a wavelength of 351–364 nm and Bright fluorescence emitted by illuminated spermatozoa is measured by a photomultiplier tube (Johnson & Welch, 1999). An orientation nozzle tip has been developed to make sure the proper orientation of sperm for fluorescent measurements, and this invention improved the accuracy of flow cytometric-based sperm separation up to more than 90%. (Rens et al., 1998). The flow cytometry-based sperm sexing set up at Sexing Technologies, Navasota, TX, USA was shown in Fig. 1.1. Even though this method has achieved substantial success in sperm sexing, sperm damage by this method has not been addressed, and this could lead to decreased fertility (Amann, 1999; Frijters et al., 2009). Alternative approaches to flow cytometric sperm sexing need to be developed for safe and efficient sperm sexing. Sex-specific binding of gold nanoparticles functionalized DNA probes, sex-specific triplex-forming DNA probes, genome editing for the sex selection and microfluidics-based sperm separation are the advanced research areas which shown promising results for the development of novel sperm sexing methods (Rath & Maxwell, 2018). Apart from the livestock productivity, prevention of sex-linked diseases (Johnson et al., 1993), reducing the risk of dystocia by preventing the production of male animals, maintaining the optimum sex ratio and conservation of endangered species are the other potential applications of sperm sexing (Durrant, 2009). Sperm sexing has gained importance in the bovine industry due to the high demand for milk and meat by the growing population of the world (Rath et al., 2009). Sperm sexing in other farm animals like sheep, pig, and horses attracted the researchers to develop and commercialize sperm sexing methods.

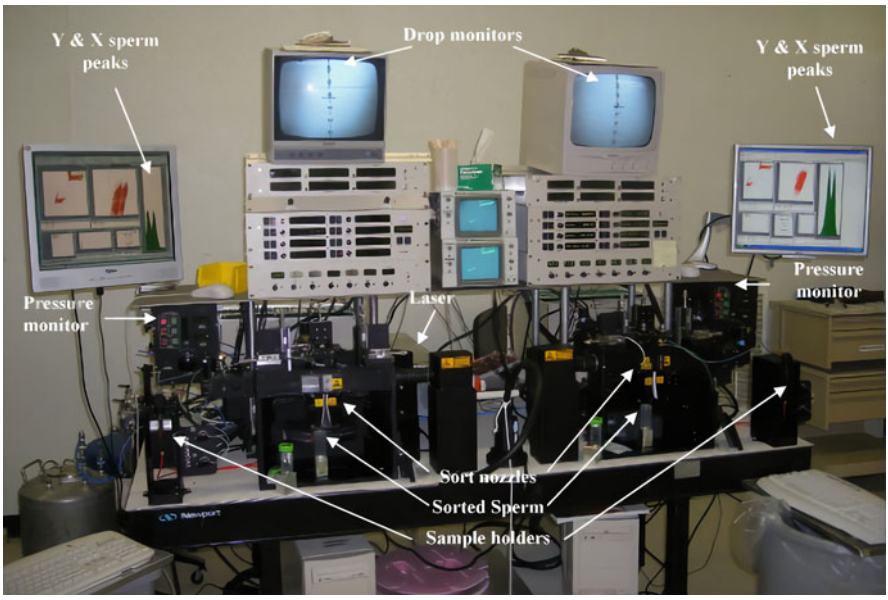

Fig. 1.1 Dual-headed sperm flow cytometer/sperm sorter (Dako MoFlo® SX) as currently used to commercially sort sexed sperm at Sexing Technologies, Navasota, TX, USA. The system has been redesigned from the original MoFlo® SX by K. Michael Evans so that the solid-state UV laser beam could be split and directed into two sorting heads (nozzles). Reproduced with permission from Garner & Seidel Jr (2008)

1.2 History of Sperm Sexing

There is a long history of inquisitiveness of humans for selecting the sex of living beings. The earliest record is of ancient Greek philosophers like Democritus, around 470–402 BC, who proposed that the right testicle produces males, whereas the left testicle produces females. Hippocrates (460–377 BC) and Aristotle (384–322 BC) used the term "sperma" for the semen. Then, after centuries of no recorded development, Anton Van Leeuwenhoek sent a letter to The Royal Society, dated November 1677, stating that he had observed several "small animals" which he named "animalcules" in the semen of sick patients (Van Leeuwenhoek, 1677). These animalcules were the moving sperms. In 1889, Geddes and Thomson published a book on the "Evolution of Sex" in which they stated that nutrition plays a major role in determining sex. They elaborated that catabolic conditions favor the production of males and anabolic conditions tend to produce more females (Geddes & Thomson, 1889). The invention of advanced microscopy and works of Gregor Mendel paved the way for the curiosity to find the causes of sex determination. In 1902, McClung suggested that the accessory chromosomes, later came to known as sex chromosomes, are in some way responsible for sex determination (McClung, 1902). Subsequently, the discoveries during the first quarter of the twentieth century

triggered other major advances like sorting of X and Y spermatozoa by differential centrifugation (Lush, 1925). Eventually, differences in physical parameters of sperms like motility, density, electrical surface charge, immunologically relevant surface proteins, volume, semen deposition site in the uterus, and insemination time have been propounded for identification and separation of X and Y sperms.

Human X and Y bearing sperms were separated based on progressive motility by layering over the BSA or ovalbumin containing medium by some of the researchers successfully (Ericsson et al., 1973; Dmowski et al., 1979; Beernink & Ericsson, 1982), while others reported unsuccessful separation of sperms (Ross et al., 1975; Beal et al., 1984; Flaherty et al., 1997). The difference in density of X and Y sperms was employed to separate the fractions of sperms by density gradient sedimentation in egg yolk and glycocoll solution (Bhattacharya, 1962; Bhattacharya et al., 1966), sucrose solution (Rohde et al., 1975), bovine serum albumin (Schilling & Thormaehlen, 1977), Ficoll–Sodium metrizoate (Shastry et al., 1977), Percoll (Kaneko et al., 1983; Kobayashi et al., 2004), and simple centrifugation technique (Koundouros & Verma, 2012). While several researchers showed the separation of X and Y sperms based on density differences, others observed no significant separation of the sperms (Vidal et al., 1993). Based on variation in surface charge, X and Y spermatozoa were first separated using artificial electric field by Koltzoff and Schröder (1933) obtaining a ratio of 100% male and 80% female from cathode and anode migrating sperms, respectively (Koltzoff & Schröder, 1933). While Schröder reported 80% success in the separation of spermatozoa by electrophoresis (Schröder, 1934; Kordts, 1952) was unable to repeat Schröder's work. Many other research groups also confirmed two-way migration of X and Y spermatozoa under electric field (Lewin, 1956; Gordon, 1957; Shishito et al., 1974; Shirai et al., 1974; Uwland & Willems, 1975; Engelmann et al., 1988; Blottner et al., 1994; Manger et al., 1997). There is a hypothesis that sex ratio in mammals could be controlled using immunologically relevant structures and surface proteins of spermatozoa came into consideration when Eichwald and Silmser proposed the existence of a Y-linked histocompatibility antigen (Eichwald & Silmser, 1955). Others suggested that serum from inbred females immunized by the skin from males of the same strain might selectively mutilate the fertilizing capacity of Y-bearing sperms (Feldman, 1958; Sachs & Heller, 1958). Various immunological techniques, like complement-dependent cytotoxicity (Bennett & Boyse, 1973). flow cytometry/cell sorter (Pinkel et al., 1985; Ali et al., 1990; Hendriksen et al., 1993), magnet-assisted cell sorting (Sills et al., 1998), and the researchers have also applied column chromatography (Blecher et al., 1999). In 1999, it was discovered that the difference in DNA content matches with the difference in volumes between X- and Y-bearing sperm heads (Van Munster et al., 1999a, b). This property was employed by Van Munster to obtain purities of 60–66% in both X- and Y-bearing sperm enriched fractions (van Munster, 2002). Zobel and co-workers led a field trial to study the effects of different semen deposition sites on the fertility and sex ratio in cattle. They observed a higher ratio of female calves on Intracornual semen deposition, whereas deposition in uterine body resulted in a higher male calves ratio (Zobel et al., 2011). Various studies (Ballinger, 1970, Guerrero, 1974, Gebicke-Härter et al., 1977, Harlap, 1979, Verme & Ozoga,

1981, Huck et al., 1990, Hedricks & McClintock, 1990, Krackow & Burgoyne, 1997, Rorie et al., 1999) in different mammalian species also suggested that the sex ratio of offspring may depend on the timing of artificial insemination or mating about ovulation. Whereas in 2006, Roelofs et al. reported that the varying the period between insemination and ovulation does not influence the sex ratio of embryos (Roelofs et al., 2006).

Over the past nine decades, several techniques have been proposed which claim to alter the sex ratio. Statistically significant separation of fertile X and/or Y sperms was achieved by none of these methods. However, quantitative methods, which are based on total DNA, distinguish between X and Y sperms followed by separation through flow cytometric sorting, have been able to separate the two sperm populations precisely.

1.3 Methods of Sperm Sexing

Numerous techniques have been developed for sperm sexing based upon the difference in various parameters of the X and Y chromosomes bearing spermatozoa such as mass, immunological structure, size, swimming pattern, and the charges on the sperm surface. Flow cytometry method of sperm sexing is most efficient, and commercial sexed semen is being produced by this method. The important techniques used in sexing of X and Y chromosomes bearing sperms are summarized in Table 1.1.

1.4 Validation of Sperm Sexing

Validation methods of sperm sexing are summarized in Table 1.2. Real-time PCR method of sperm sexing is the most reliable method so far, and it depends on the presence of sex-specific genes in X and Y chromosome-bearing spermatozoa. In a study, biochemical analysis of single spermatozoon directly collected from bulls was done by Raman spectroscopy. X and Y bovine sperm cells showed a change in the intensity of DNA Raman bands at 726, 785, 1095, and 1581 cm^{-1} wavenumbers (De Luca et al., 2014). In a recent study, a change in the intensity of DNA Raman bands was observed on Raman spectroscopic analysis of bull semen samples, which contains mixed populations of X and Y chromosome-bearing spermatozoa (Yata et al., 2020). Further studies of Raman spectroscopy X and Y chromosome-bearing spermatozoa could lead to the development of novel sperm sexing methods.

1.5 Importance of Sperm Sexing in Farm Animals

Livestock farms require either male or female animals on the farm for economic benefits and farm management issues. Applications of sperm sexing in farm animals are summarized in Table 1.3.

Table. 1.1 Summary of the important methods in sexing of X and Y chromosomes-bearing sperms

S. no.	Method	Principle	Remarks	References
1	Albumin gradient method	Sperms are separated based upon the difference in swim down ability across albumin gradient because of their size difference between Y-bearing sperm and the X-bearing sperm	1. Not reproducible	Ericsson et al. (1973), Ericsson and Ericsson (1999)
2	Percoll gradient method	Percoll method exploits the difference in the sedimentation density where Y sperm swims faster during swim-up procedure based upon which the X- and Y-bearing sperms were separated	1. It is very difficult to obtain enough number of spermatozoa for in-vitro fertilization after the procedure 2. Not reproducible	Iizuka et al. (1987), Parrish et al. (1995)
3	Free-flow electrophoresis	Electric charge differences on the surface of X and Y chromosome-bearing sperms	1. Motility of sperm is significantly reduced 2. Not reproducible	Mohri et al. (1986), Kaneko et al. (1984)
4	Swim up procedure	Y-bearing swim faster than X-bearing spermatozoa due to its smaller size	Not reproducible	Orsztynowicz et al. (2014)
5	Sperm sorting based on volumetric differences	The difference in the sperm head volume, optical thickness, and head volume are used in separating X- and Y-bearing sperm using differential interference contrast microscopy or digital holographic microscopy	1. Not reproducible 2. Purity of spermatozoa of either sex did not exceed 80%	Van Munster et al. (1999a), Van Munster et al. (1999b), Van Munster (2002)
6	Centrifugal counter current distribution	Chromatographic process that partitions cells based on the size	Not reproducible	Ollero et al. (2000)

(continued)

Table. 1.1 (continued)

S. no.	Method	Principle	Remarks	References
7	Immunological methods	(a) HY antigen is a male-specific protein and is found in male tissues. Monoclonal antibodies are prepared against this antigen and differential binding is used in identifying Y sperm	Not reproducible	Goldberg et al. (1971), Blecher et al. (1999), Hendriksen et al. (1996), Hendriksen (1999)
		(b) Development of antibodies against sex-specific proteins (SSP) present in X and Y sperms	Further research is required	Blecher et al. (1999), Sang et al. (2011)
8	Flow cytometric method	Sperms are prepared by staining with DNA-specific stain (specific to adenine thymine region). Fluorescence obtained when this stain is excited by approximately 335 nm and is detected by photodetectors. Based upon DNA content, sperms are either positively or negatively charged. Depending on charge over the sperm, X and Y sperms are separated	(1) Measurement of individual sperm DNA content is difficult (2) High cost of equipment (3) Low pregnancy rate (4) Requires skilled manpower (5) Half of sperm sample is un sexable	Johnson (2000), Seidel and Garner (2002), Maxwell et al. (2004), Lu et al. (2007), Liang et al. (2008)

1.5.1 Cattle

In initial studies by Cran (1993) and Cran et al. (1995), sexed semen of cattle was produced by flow cytometry cell sorting and produced 90% males from Y-sorted sperm cells by in vitro fertilization followed by embryo transfer. In a preliminary experiment, semen was collected from 3 Holstein bulls of above-average fertility and spermatozoa were sorted based on the DNA content of the X and Y chromosomes by flow cytometry over 10 months. They achieved 82% accuracy in sex selection, where 14 out 17 calves were of selected sex (Seidel Jr et al., 1997). In another study, 90% accuracy on sperm sexing has been achieved using a MoFloQ flow

Table 1.2 Summary of validation methods of sperm sexing

S. no.	Methods	Principles	References
1	Polymerase chain reaction (PCR)	**X chromosome-specific genes**: Zinc finger protein X (ZFX), proteolipid protein (PLP), amelogenin **Y chromosome-specific genes:** SRY gene	Parati et al. (2006), Khamlor et al. (2014), Tan et al. (2015)
2	Fluorescence In situ hybridization (FISH)	Using specific probes conjugated with fluorescence molecule for the X and Y sperms	Han et al. (1993), Parrilla et al. (2003)
3	Raman microspectroscopy	Intensity of specific DNA Raman bands at 726, 785, 1095, and 1581 cm-1 for X- and Y-bearing bovine sperms	De Luca et al. (2014)

Table. 1.3 Summary of the applications of sperm sexing in farm animals

	Farm animals	Applications of sperm sexing	References
1	Cattle	Production of female cattle in the dairy industry and production of males in the beef cattle industry	Hohenboken (1999), Garner and Seidel Jr (2008).
2	Sheep	Development of improved germplasm for mutton and wool production	Evans et al. (2004)
3	Pigs	Production of female pigs to avoid the boar taint	Vazquez et al. (2009)
4	Horses	Production of female offspring for sports activities such as polo mares	Panarace et al. (2014)

cytometer/cell sorter (Seidel Jr et al., 1999). Lu et al., 1999 achieved 90% accuracy in sperm selection by in vitro fertilization with flow cytometrically sorted bovine sperm. Hamano et al. (1999) achieved 80% on sex selection by flow cytometrically sorted bull sperm heads. In their experiments, in vitro matured bovine oocytes intracytoplasmically injected with flow cytometrically sorted bull sperm heads. In a field study, semen of seven bulls of different ages and breeds (five Brown Swiss, two Red Holstein) was collected, and Sperm sorting was performed with a MoFlo1 SX (Cytomation Inc., Fort Collins, USA). A total of 27 heifers and 105 cows were inseminated with sex-sorted sperm and 27 heifers and 64 cows with non-sorted sperm. This study concluded that conception rates of sorted and non-sorted semen are almost similar, using an insemination dose of 2×10^6 (Bodmer et al., 2005). Hayakawa et al., 2009 concluded that the use of sexed frozen-thawed sperm might be economically viable for multiple commercial ovulation and embryo transfer programs in Holstein heifers. In a study on Holstein heifers, Conception rates of sex-sorted semen were improved by the 10×10^6 sperm dosage and conception rates were not comparable to either dosage of conventionally processed sperm (DeJarnette et al., 2011). Reduced pregnancy rates were reported after inseminating beef cows with sexed semen (Cooke et al., 2014).

1.5.2 Sheep

In a study, semen was collected from three Merino rams, and Spermatozoa were sorted using a modified high-speed flow cytometer (MoFlo_ SX; Dako Colorado, Fort Collins, CO, USA) with a sorting rate of 600 to 13,000 spermatozoa/s. This study has shown that sex-sorted ram spermatozoa can have almost equal fertility to non-sex-sorted spermatozoa (De Graaf et al., 2007a). In another study by De Graaf et al. (2007b), ejaculates were collected from two White Dorper rams, and the X and Y chromosome-bearing spermatozoa were separated using a modified high-speed flow cytometer (MoFlo1 SX; Dako Colorado Inc., Fort Collins, CO, USA) 0.92.5% sorting purity was observed in this study, and they produced female lambs with sorted X chromosome-bearing spermatozoa by multiple ovulation and embryo transfer method. In another study, semen was collected by artificial vagina from three Merino rams and X and Y chromosome-bearing spermatozoa were sorted using a modified high-speed flow cytometer (MoFlo1; Dako Colorado Inc., Fort Collins, CO, USA). Births of offspring after the insemination of sex-sorted spermatozoa were observed in this study (De Graaf et al., 2007c). In a study, semen was collected by artificial vagina from three Merino rams, and sexed semen was obtained by a high-speed cell sorter (SX MoFlo1, Cytomation, Inc., Fort Collins, CO, USA). This study achieved 93% predicted sex lambs after determining the optimum timing of insemination and minimum effective dose rate of sex-sorted ram sperm (Beilby et al., 2009).

1.5.3 Pigs

Preselection of sex is one of the important criteria for reproductive management in the pig farming industry. Boar taint is a major problem for pork eaters as it results from sexually matured male pigs. Boar taint causes an unpleasant smell when it is cooked due to the accumulation of chemical compounds like skatole and androstenone in the meat of matured male pigs. Pig producers prefer female piglets for commercial, economic, and farm management reasons. In an initial breakthrough study, semen was collected from mature Yorkshire, and Landrace boars and sperm were sorted on a modified Epics V flow cytometer cell sorter (Coulter Corporation, Hialeah, FL). Crossbred gilts of 7–9 months of age were inseminated surgically with sorted X- and Y-bearing sperms. Gilts inseminated with sorted X-bearing sperm produced litters of offspring that were 74% female whereas assorted Y-bearing sperm produced offspring that were 68% male (Johnson, 1991). In another study, semen was collected from 2 boars which were the result of a gilt and boar mating. Sperm cells were sorted using a modified EPICS V/750 series flow cytometer cell sorter (Coulter Corporation, Miami, FL; 8) at 150 mW ultraviolet light and sorting flow rate of 2300 sperm/sec with the sorting rate of 70–110 sperm/s. Eighteen prepuberal gilts, 165 days of age and averaging 76 kg, were chosen for in vitro fertilization. Fifteen piglets were produced in this study, and 13 of the were predicted gender (Rath et al., 1997). In a study by Abeydeera et al. (1998), semen was

collected from a mature boar, and MoFIo high-speed cell sorter (Cytomation inc., Fort Collins, CO) modified for sperm sorting was used to sort the X and Y chromosome-bearing spermatozoa. Sperm sexing efficiency was examined on pig embryos derived from in vitro fertilization of in vitro matured oocytes by sorted X and Y chromosome-bearing spermatozoa. Transfer of embryos derived from X-bearing spermatozoa to 18 recipients resulted in 95% female piglets and Y chromosome-bearing sperm cells to 10 recipients resulting in 100% male piglets (Abeydeera et al., 1998). In a study by Probst and Rath, Semen was collected from fertility proved boar and sorted X and Y chromosome-bearing spermatozoa with a high-speed Cell Sorter (MoFlo® Cytomation, Fort Collins, USA) with an average sorting rate of 3000–4000 sperm/s. Artificially activated in vitro matured oocytes were fertilized using flow cytometrically sorted Y chromosome-bearing spermatozoa by intracytoplasmic sperm injection (ICSI). Fertilized oocytes were transferred surgically into four recipients and produced a total of 13 male piglets (Probst & Rath, 2003). FACS-based sperm sexing is not suitable for the swine as the insemination dose should be 1.5–5 billion sperm. Whereas in cattle, normal insemination dose is 10 to million spermatozoa and FACS-based sperm sexing is more suitable for animals.

1.5.4 Horses

In an early study by Buchanan et al., semen was collected from Two Arabian breed stallions of known high fertility, and sperm sexing was done by MoFlo@ flow cytometer with a rate of 900 live cells/sec. In their preliminary experiments, they observed one phenotypically normal female fetus when mare inseminated with X chromosome-bearing spermatozoa (Buchanan et al., 2000). Poor fertility is reported with low doses of sex- or non-sorted spermatozoa semen was collected from two Hannovarian stallions and X and Y chromosome-bearing spermatozoa were separated by a high-speed flow cytometer (SX MoFlo®, Dako Colorado Inc., Fort Collins, USA) with sorting rates of 20,000–25,000 events/sec. Poor fertility was observed in the low dose insemination of frozen-thawed rather than sex-sorted spermatozoa (Clulow et al., 2008). In a study, X and Y chromosome-bearing sperm were sorted on a high-speed flow cytometer (SX MoFlo; DakoCytomation, Fort Collins, CO, USA) using stallion semen samples. It is proved in their study that a diluent containing 1% BSA instead of skim milk improved the sorting efficiency and kinetic parameters of stallion sperm after storage for 18 h (Gibb et al., 2011). In a large-scale field study, Nine hundred and eighty-five semen samples from 40 different Polo breed stallions were collected and sorted X-bearing sperms using a flow cytometer (Moflo SX; DakoCitomation, Fort Collins, CO., USA) with an assorting rate of 2.500 and 3.500 events per second. A commercial embryo transfer program evaluates the efficiency of sexed semen, and sex accuracy of the sex determination was found to be 90.3% (Panarace et al., 2014).

1.6 Advances in Sperm Sexing

Engender, a startup company at the Auckland University campus developed a sperm sexing technology by moving sperm from a flowing stream in a microfluidic chip and changing the orientation and direction of sperm by using radiation pressure. Inventors of this method claim that this technology would significantly decrease sperm damage and reduce the cost of sexed semen (Simpson & Rohde, 2017). Microbix Biosystems Inc., a biotechnology company, located in Mississauga, Canada, developed a laser-based sperm sexing flow cytometric method and it offers fast and effective sperm separation by eliminating the sperm cells that are not of the desired sex (Luscher, 2011). In a study, bovine Y chromosome triplex target sites were identified by SequenceFinder1.0, and triplex-forming oligonucleotides (TFOs) were used as functional ligands. Conjugation of gold nanoparticles with TFOs was performed by ex situ conjugation. Hybridization of TFO–AuNP conjugates on sorted sperm nuclear matrix was tested by incubation and results confirmed the hybridization of these conjugates to sex-specific bovine genomic DNA in a qualitative and significant manner (Gamrad et al., 2017). Even though the transition of AuNPs through the intact sperm membrane has not been successful yet, it could lead to the invention of novel strategies in sperm sexing. Gene editing nucleases, such as the transcription activator-like effector nucleases (TALENs), the zinc finger nucleases (ZFNs), and the CRISPR/Cas system, have been employed to modify the genome in mammals (Kurtz & Petersen, 2019). SRY gene is the sex-determining gene in mammals and knockout of this gene by genome editing methods resulted in female phenotypes in mice (Bergstrom et al., 2000) and rabbits (Kato et al., 2013). In a recent proteomic analysis, researchers identified 17 differentially expressed proteins in sperm cells of sexed bovine semen (De Canio et al., 2014). In this study, 15 proteins were upregulated in X sperm, whereas the other two were in Y sperm. These biomarkers could be potential biomarkers for the development of new sperm sexing methods. In a recent study, the treatment of mouse sperm with Toll-like receptors 7/8 ligands (Resiquimod and Imiquimod) lead to the selective suppression of the mobility of the X chromosome-bearing sperm without altering sperm viability. The difference in sperm motility of Y sperm from X sperm allowed the sperm sexing (Umehara et al., 2019). Raman-activated cell sorting (RACS) is an emerging technology of cell sorting that can be operated in solution (Raman tweezers), inflow (microfluidic-based RACS), and on a surface (Raman activated cell ejection—RACE). RACS could be the potential alternative to FACS-based cell sorting if these methods can improve the speed of cell sorting (Song et al., 2016).

1.7 Conclusions

Sperm sexing has emerged as an effective and commercial method to produce desired sex animals, which ultimately benefits the animal farmers. Sperm sexing for medical reasons is unprejudiced, and it also helps to maintain the optimum sex ratio of the animals and conserve the endangered species. Conventional sperm

sexing methods like Percoll and albumin gradient centrifugation, swim up method, Sephadex columns-based sperm sexing and HY antigen-based sperm sorting methods are unsuccessful due to poor reproducibility. So far, Flow cytometric sperm sorting method is proven to be a successful commercial method for mammalian sperm sexing, especially for bovine sexed sperm. Even though there have been substantial developments to improve the purity and accuracy of the flow cytometric-based sperm sexing method, the cost of sexed semen is very high in comparison with non-sexed semen. Apart from the high price, sperm damage is one of the major drawbacks of flow cytometric sperm sexing. Sperm sexing validation methods like real-time PCR and Raman spectroscopic methods have been developed, which ensures the accuracy of the commercially available sexed semen. Genome editing, microfluidics, and nanoparticle-based methods have shown encouraging results of sperm sexing and development of these methods could lead to the invention of safe, efficient, and cheaper sperm sexing methods. Ethical aspects related to the use of sexed semen for nonmedical reasons should not be neglected, and stringent national policies are required for the proper use of sexed semen.

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Semen and Sperm Characteristics of Farm Animals

2

Contents

Abstract

The semen characterization favors the development of more precise and efficient methods of semen storage, transportation, and animal reproduction methods. The conventional and advanced semen evaluation methods ensure the quality of the semen for use in in vitro fertilization or artificial insemination. The conventional semen parameters such as color, pH, and volume, and sperm characteristics such as concentration, morphology, and motility, provide the initial quality assessment of the semen samples. The differences between the X and Y chromosome-bearing spermatozoa have gained importance in recent years to develop novel sperm sexing methods. The existing commercially successful Flow cytometry-based sperm sexing method is dependent on the difference between DNA content of

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X and Y chromosome-bearing spermatozoa. This method is limited due to high cost and sperm damage due to harsh conditions applied in the sperm separation method.

Recently, semen biologists were interested in differentiating the X and Y chromosome-bearing spermatozoa based on the motility, charge, total DNA content, and sex-specific immunological markers. The motility of X and Y chromosome-bearing spermatozoa can be altered by using specific markers that affect the metabolism of either X or Y chromosome-bearing spermatozoa. Advanced methods such as microfluidics and nanotechnology allowed to separate the cells based on charge, velocity, and other minute differences in X and Y chromosome-bearing spermatozoa. The sperm characterization by advanced methods would help semen biologists to develop novel sperm sexing methods.

Keywords

Semen evaluation · Sperm motility · Sperm morphology · Semen rheology · HY antigen · Sperm charge

2.1 Introduction

Semen is a cell suspension consisting of spermatozoa and seminal plasma. The spermatozoa consist of oval-shaped head and tail. The tail region of the spermatozoa consists of neck, middle, principal, and end pieces. The spermatozoa are shielded by a plasma membrane or plasmalemma. The anterior portion of the sperm head is covered by double-layered membranous sac and it is called acrosome or acrosomal sac (Garner & Hafez, 2000). The seminal plasma consists of carbohydrates lipids, proteins, amino acids, and other nitrogen-containing compounds. The main functions of seminal plasma involve the transportation of spermatozoa and metabolism of spermatozoa. The components present in the seminal plasma play a critical role in the metabolism of spermatozoa until it reaches and fertilizes the egg (Setchell, 2014). Semen characteristics such as ejaculation volume, sperm concentration, percentage of motile sperm, pH, specific gravity, concentration of carbohydrates, lipids, and other components, vary greatly among the farm animals (Garner & Hafez, 2000). Based on the characteristics of the semen, the samples are categorized and the basic semen terminologies are summarized in Table 2.1. Semen characterization is an essential practice in semen laboratories for the semen processing, extending, and artificial insemination of the animals (Cseh et al., 2012). Freshly ejaculated semen maintains competent spermatozoa only for a few hours after collection. Therefore, the fresh semen ejaculates are immediately mixed with extenders to improve the lifespan of spermatozoa during storage (Morrell, 2006). The sperm sexing involves the separation of X and Y chromosome-bearing sporozoan from the semen samples. Most of the available sperm sexing methods rely on the minute differences on X and Y chromosome-bearing spermatozoa. The spermatozoa undergo morphological and physiological changes during the sperm sexing process. The sperm handling

Table 2.1 Summary of terminology of semen characteristics

S. no.	Terminology	Definition
1	Aspermia	Following ejaculation, there is no seminal fluid discharge
2	Hyperspermia	Unusual high production of semen during ejaculation
3	Hypospermia	Unusual low production of semen during ejaculation
4	Azoospermia	Complete lack of sperm in semen/samples
5	Oligozoospermia	Reduced sperm count in the ejaculate
6	Normozoospermia	Normal semen volume
7	Polyzoospermia	Abnormal high sperm concentrations
8	Asthenozoospermia	Sperm with low motility (more than 40%)
9	Necrozoospermia	Dead/nonviable sperm in semen
10	Teratozoospermia	Abnormal morphology of sperm (more than 40%)

detrimental effects will lead to undesired effects on functionality of spermatozoa. The semen and sperm characterizations would help the researchers to develop novel methods in sperm sexing with improved longevity of spermatozoa.

Semen characterization is an essential process to evaluate the quality of animal ejaculates for successful artificial insemination. The chapter discusses the various semen evaluation parameters suck as color, volume, pH, motility, and morphological characteristics, the second part of this chapter focused on the differences between the X and Y chromosome-bearing spermatozoa.

2.2 Semen Evaluation Parameters

2.2.1 Color

The correlation between the semen color and other semen parameters such as, semen volume, consistency, mass activity, initial motility, and dilution rate, were studied in crossbreed cattle bulls. This study observed various semen colors such as, cream, light cream, thick cream, dirty, cream, yellow, light yellow, white, dirty white, and milky, consistency in more than 9000 cattle bulls. This study suggested that color of the semen can be used as one of the preliminary screening indicators for the assessment of quality of the semen (Matharoo, 2015). In this study, the semen samples were categorized based on their color. The concentration of the samples decreases in the order of creamy >milky opaque>opalescent >watery colors (Rekwot et al., 1987). the color of the goat semen samples was changed with season. For example, during September–December the semen was observed in yellow or whitish-yellow color whereas the semen was in creamy white color in the remaining season (Ahmad & Noakes, 1996). The color of the camel semen samples varied from camel to camel. It was observed that the color of the camel semen samples varies among creamy white, milky white, and white colors (Agarwal et al., 2004).

2.2.2 Volume

The volume of bull ejaculates varied with age and season. In a study, Bhakat et al. (2011) observed that about 5% of the ejaculate volume of bulls increased during the hot and humid summer season. This study also observed a gradual increase in semen volume up to 5 years of age and also noticed gradual decrease in the ejaculate volume after 5 years of age (Bhakat et al., 2011). In another study, it was observed that ejaculate volume was increased with age but total sperm number decreased with increasing age of the bull (Fuerst-Waltl et al., 2006). The semen volume varies among the breeds, for example, the lower ejaculate volume was correlated with high concentration in Pietrain boars whereas high ejaculate volume correlated with low sperm concentration in Large White boars (Ciereszko et al., 2000). In previous studies, the ejaculate volumes were higher in *B. taurus* bulls than in B. *indicus* bulls (Mathevon et al., 1998; Brito et al., 2002). In a study, seasonal effect was not observed on *B. taurus* ejaculate volume (Schwab et al., 1987).

2.2.3 pH

The pH of the whole ejaculate in animals is alkaline and it ranges from 7.05 to 7.80 (Chaudhari et al., 1990). The increase in the seminal pH resulted in decrease in density and decrease in the fertility rate (Mann, 1954). The motility and velocity of Turkey and quail spermatozoa were found to be increased in alkaline pH (Holm & Wishart, 1998). In a study, alkaline pH improved the motility of human spermatozoa (Zhou et al., 2015). In a study, optimum pH for bull semen was found to be 7–7.5 in terms of sperm kinetic parameters and viability (Contri et al., 2013). In an early study, reversible acidic pH inhibition of semen improved the life span of bovine sperm at room temperature (Norman et al., 1958). Bartoov et al. (1980) studied the effect of pH on Ram semen samples and suggested that the relation between pH and motility could be mediated by metabolic process.

2.2.4 Sperm Motility and Concentration

The Sperm motility and Concentration are the two important parameters that need to be evaluated to determine the quality of semen samples for successful artificial insemination (Nallella et al., 2006). The motile spermatozoa increase the fertility rates in vivo (Nel-Themaat et al., 2006). The sperm concentration is dependent on frequency of semen collection and the sperm concentrations are reported to be approximately 1.2 and 2.0 billion/ml for bull and semen, respectively (Bearden & Fuquay, 1984). The optimized sperm concentrations in semen samples result in improved fertility rates (Ward et al., 2002). The velocity of human spermatozoa showed a significant effect on fertilization rates (Bongso et al., 1989). David et al. (2015) proposed that Mass sperm motility is an indicator of fertility in sheep. In a study, sperm concentration has shown significant effects on motility, morphology,

membrane, and DNA integrity along with oxidative stress parameters of ram semen samples (Gundogan et al., 2010).

2.2.5 Morphology

The sperm morphological defects such as, acrosome defects, head defects, abnormal head shape/size, nuclear vacuoles, midpiece defects, bent/coiled midpiece, rough/swollen midpiece, principal piece defects, detached head proximal droplets, and distal droplets, are negatively related with bull fertility. Morphometric traits of spermatozoa in ruminants and other mammalian species are summarized in Table 2.2 and Table 2.3, respectively. A study by Al-Makhzoomi et al. (2008) suggested periodical evaluation of the morphology of sperm cells upon storage of semen samples. Morphological defects in spermatozoa occurred due to chromatin aberrations, leading to reduced fertility rates (Saacke, 2008). In a study, sperm morphological abnormalities are related to DNA fragmentation. The DNA fragmentation index was higher in abnormal spermatozoa of bull semen samples (Enciso et al., 2011). In a study, abnormalities in the sperm head shape were negatively correlated with fertility, via artificial insemination in bulls (Al-Makhzoomi et al., 2008). In a similar study, sperm nuclear shape is proposed as an indicator for bull fertility (Ostermeier et al., 2001). The abnormalities such as abnormal heads, nuclear pouches, and proximal cytoplasmic droplets were also correlated with the fertility of bulls by artificial insemination (Söderquist et al., 1991). The variations in sperm head morphology were shown in Fig. 2.1.

2.2.6 Rheology

In an early study on human semen rheology, apparent viscosity at a maximum shear rate, thixotropy were found to be 4.3 ± 0.4 cp; 47.1 ± 7.6 dynes/cm^2 (Mendeluk et al., 1996). In a study, human semen rheology was studied and an apparent viscosity at 230 s^{-1} and 25 °C (ηa) was found to be in the range of 4.3 ± 0.2 cp and 5.4 ± 0.4.; these semen samples exhibited pseudoplastic behavior in power law model (Mendeluk et al., 2000). In a recent rheology study on bull semen samples, the pseudoplastic behavior of the semen was observed. This study also observed that nonlinear increase in the shear stress with increasing rate of shear (Yata et al., 2020).

Table 2.2 Morphometric traits of spermatozoa in different species of ruminants. Reproduced with permission from Yániz et al. (2015)

Species/sperm structure	Staining method	Area (m^2)	Perimeter (m)	Length (m)	Width (m)	References
Bull Head	H. Hem*	26.07	24.55	8.5	4.2	Gravance et al. (1997)
	MGZIN	27.01	25.08	8.66	4.37	Idem
	MGZIN	29.45	23.8	8.72	4.46	Gravance et al. (1999)
	Farelly	38.35 ± 2.06		9.4 ± 0.28	5.17 ± 0.23	Boersma et al. (1999)
	Papanicolaou	31.48 ± 1.93		8.59 ± 0.29	4.56 ± 0.24	Idem
	H. Hem*	35.1 ± 1.8	24.9 ± 0.8	9.1 ± 0.5	4.7 ± 0.2	Boersma et al. (2001)
	MGZIN	34.7 ± 2.2	25.2 ± 0.9	9 ± 0.9	4.7 ± 0.2	Idem
	Papanicolaou	35.5 ± 2.0	25.1 ± 0.8	9.1 ± 0.4	4.7 ± 0.3	Idem
	Farelly	40.2 ± 2.1	26.6 ± 0.8	9.6 ± 0.3	5.1 ± 0.2	Idem
	Toluidine blue	32.9 ± 3.6	22.3 ± 1.3	8.9 ± 0.4	4.6 ± 0.5	Beletti et al. (2005)
	H. Hem	38.08 ± 2.59	25.73 ± 2.85	9.49 ± 0.79	4.68 ± 0.32	Vicente-Fiel et al. (2013)
Nucleus	Hoechst	32.11 ± 2.42	23.74 ± 0.93	8.99 ± 0.41	4.54 ± 0.24	Vicente-Fiel et al. (2013)
Head	Head	H. Hem*	28.12	22.08	7.89	4.65
	MGZIN	29.05	24.31	8.06	4.79	Idem
	Hemacolor	35.02 ± 3.17	26.8 ± 2.16	8.90 ± 0.49	4.79 ± 0.33	Maroto-Morales et al. (2010)
	H. Hem	27.43 ± 1.57	21.9 ± 2.11	7.93 ± 0.26	4.23 ± 0.2	Marti et al. (2011a, 2011b)
	Hemacolor	35.38 ± 2.55	24.13 ± 1.32	8.90 ± 0.59	4.73 ± 0.23	Yániz et al. (2012)
	Hemacolor	35.37 ± 2.37	24.12 ± 1.28	8.91 ± 0.48	4.73 ± 0.30	Yániz et al. (2013)
	Sperm blue	34.13 ± 2.71	23.68 ± 1.29	8.76 ± 0.59	4.70 ± 0.27	Idem
	H. Hem*	34.91 ± 2.65	23.68 ± 1.53	8.58 ± 0.62	4.85 ± 0.29	Vicente-Fiel et al. (2013)
Nucleus	Hoechst	30.18 ± 2.44	22.33 ± 1.10	8.23 ± 0.37	4.65 ± 0.26	Vicente-Fiel et al. (2013)
Acrosome	Hemacolor	22.34				Yániz et al. (2013)
	SpermBlue	22.03				Idem
	PI-PSA*	26.14	22.06			Yániz et al. (2014)
Goat head	H. Hem*	19.67	18.62	6.99	3.77	Gravance et al. (1997)

	H. Hem*	25.03 ± 1.48	20.62 ± 0.65	8.03 ± 0.29	3.71 ± 0.17	Hidalgo et al. (2006)
	Hemacolor	28.31 ± 1.7	22.21 ± 0.7	8.38 ± 0.3	4.03 ± 0.19	Idem
	DiffQuick	29.02 ± 1.5	22.2 ± 0.6	8.47 ± 0.27	4.16 ± 0.18	Idem
	Phase-contrast	23.89	20.81	8.16	3.48	Marco-Jiménez et al. (2006)
	H. Hem*	29.80 ± 1.73	22.13 ± 1.39	8.18 ± 0.44	4.28 ± 0.17	Vicente-Fiel et al. (2013)
Nucleus	Hoechst	24.72 ± 1.82	20.45 ± 0.80	7.72 ± 0.28	3.99 ± 0.21	Vicente-Fiel et al. (2013)
Red deer Head	Hemacolor	32.57 ± 2.4	22.83 ± 0.9	8.2 ± 0.44	4.7 ± 0.26	Soler et al. (2005)
	H. hem.*	30.67 ± 2.04	22.2 ± 0.83	8.04 ± 0.45	4.51 ± 0.21	Idem
	DiffQuick	28.38 ± 2.54	21.11 ± 1.08	7.67 ± 0.47	4.42 ± 0.25	Idem
Alpaca Head	Hemacolor	15.95 ± 2.25	15.11 ± 1.22	6.1 ± 0.63	3.62 ± 0.34	Buendia et al. (2002)
	Tincion-15	20.09	18.54	6.6	4.14	Casaretto et al. (2012)
Llama	Hemacolor	13.75 ± 2.82	14.8 ± 1.60	5.51 ± 0.69	3.38 ± 0.42	Soler et al. (2014)

Values are expressed as mean ± SD (when available)
**H. hem.* Harris hematoxylin. **PI-PSA* propidium iodide-pisum sativum agglutinin

Table 2.3 Morphometric traits of spermatozoa in other mammalian species. Reproduced with permission from Yániz et al. (2015)

Species/sperm structure	Staining method	Area (m^2)	Perimeter (m)	Length (m)	Width (m)	References
Boar head	Farelly modified	35.40		9.07	4.69	Hirai et al. (2001)
	Eosin-Gentian H. Hem*	40.93 ± 2.63		9.36 ± 0.4	4.87 ± 0.31	Kondracki et al. (2005)
	Eosin-Gentian H. Hem*	25.06	20.67	7.61	3.85	Garcia-Herreros et al. (2006)
	Hemacolor	29.8	22.62	8.22	4.21	Idem
	Panoptic	29.86	22.56	8.21	4.25	Idem
	H. Hem* Eosin-Gentian	34.85 ± 1.76 27.43	23.93 ± 0.71 21.90	8.82 ± 0.38 7.93	4.59 ± 0.18 4.23	Vicente-Fiel et al. (2013) Kondracki et al. (2011)
	Hoechst	27.65 ± 2.19	22.0 ± 0.88	8.29 ± 0.39	4.14 ± 0.23	Vicente-Fiel et al. (2013)
	Hemacolor	29.8	22.62	8.22	4.21	Idem
Nucleus	Hoechst	27.65 ± 2.19	22.0 ± 0.88	8.29 ± 0.39	4.14 ± 0.23	Vicente-Fiel et al. (2013)
Dog Head	Giemsa	20.57 ± 2.15		6.79 ± 0.49	4.08 ± 0.31	Dahlbom et al. (1997)
Head	Eosin-Methylene blue	15.4 ± 1.34	15.1 ± 0.85	5.4 ± 0.32	3.5 ± 0.21	Núñez-Martinez et al. (2005)
Midpiece	Idem	2.88 ± 0.73			0.80 ± 0.34	Idem
Acrosome Stallion	Idem	9.27				Idem
Head	Diff-Quik	14.72 ± 1.72	15.64 ± 0.92	5.87 ± 0.39	3.07 ± 0.27	Hidalgo et al. (2006)
	Hemacolor	14.29 ± 1.85	15.61 ± 1	5.90 ± 0.41	2.97 ± 0.3	Idem
	H. Hem*	13.42 ± 1.72	15.00 ± 0.89	5.67 ± 0.36	2.85 ± 0.31	Idem
Rabbit head	Phase contrast	31.35 ± 2.53	22.30 ± 1.01	8.35 ± 0.43	4.55 ± 0.31	Marco-Jiménez et al. (2010)
	Phase contrast	31.9	23.9	8.5	4.7	Lavara et al. (2013)
Peccari head	Rose bengal			6.34	4.2	Sousa et al. (2013)
Midpiece	Idem			13.29		Idem
Tail	Idem			32.25		Idem
Mouse	H. Hem*	22.39	24.17	9.13	4.80	Tablado et al. (1998)

Values are expressed as mean ± SD (when available). *H. hem.: Harris hematoxylin

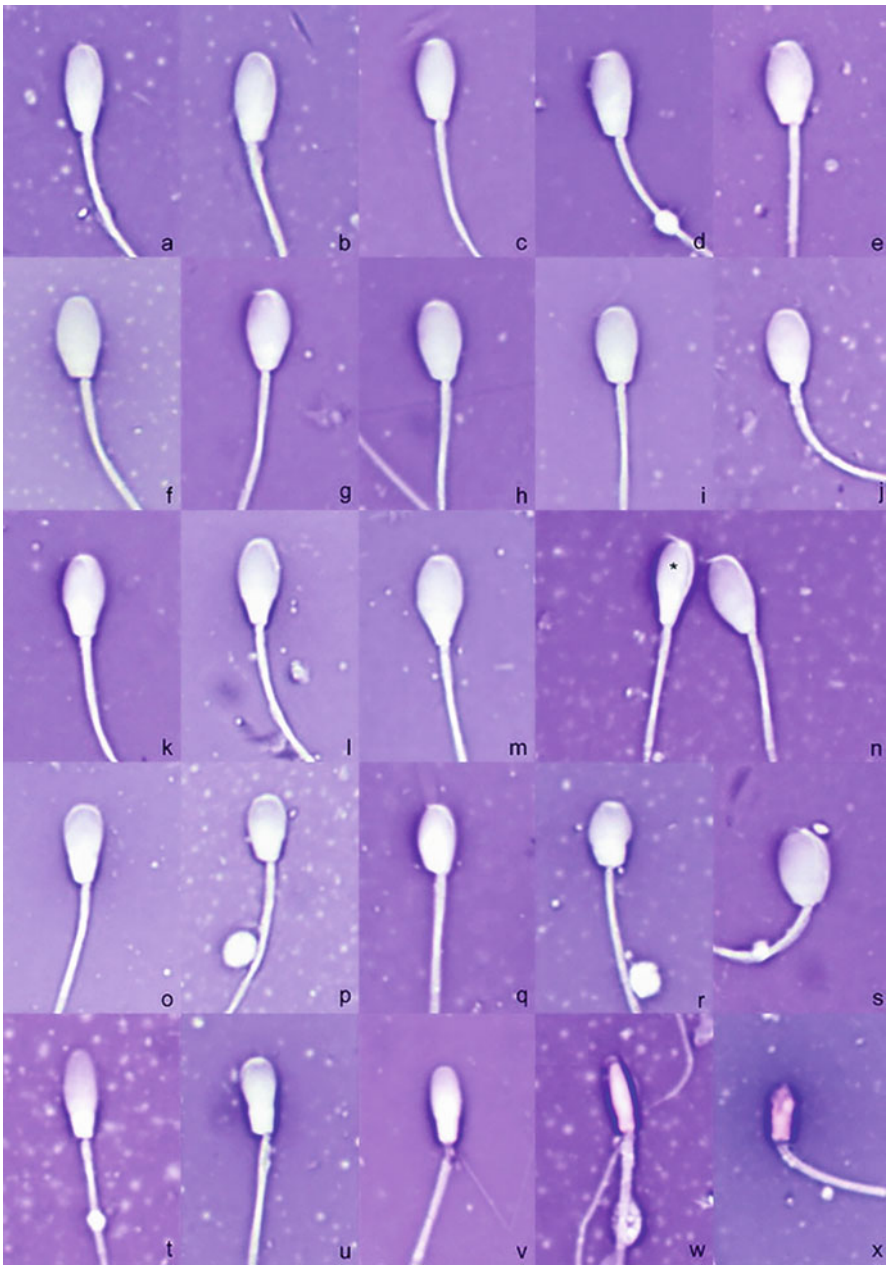

Fig. 2.1 Sperm head morphology. There is considerable variation in the shape of normal stallion sperm heads, from somewhat thinner and elongated to shorter and broader forms (**a**–**j**). Comparison among several sperm to establish the "normal" sperm head shape for an individual stallion is necessary for correct classification of sperm with extreme head shapes. Although sperm heads with narrowness of the entire post acrosomal region are difficult to classify (**k**, **l**), pyriform (or narrow base) sperm heads are common defects of the sperm head shape (**m**, **n**; asterisk indicates pyriform head). Microcephalic sperm heads may have normal shape (**o**–**q**), rounder shape (**r**, **s**), tapered

2.3 Differences in the X and Y Chromosome-Bearing Spermatozoa

2.3.1 Size and Shape

In a study, the difference in the head volume of the X and Y chromosome-bearing spermatozoa was observed and it was correlated with the DNA content (van Munster et al., 1999). An atomic force microscopic study on bull spermatozoa indicated that there was no difference between the X and Y chromosome-bearing spermatozoa (Carvalho et al., 2013). Chandler et al. (1999) demonstrated that there is no difference between the head area of the spermatozoa between the breeds and between bulls. In the case of human studies, Cui, 1997 observed more length, head perimeter, length of the neck and tail in X chromosome-bearing spermatozoa. Geraedts (1997) observed more surface area in X chromosome-bearing spermatozoa. In contrast, Hossain et al. (2001) and Zavaczki et al. (2006) observed no differences in the X and Y chromosome-bearing spermatozoa in human semen samples.

2.3.2 Motility and Swimming Pattern

Previous studies on albumin gradient sperm sexing method on human semen samples indicated that Y sperm has a higher velocity than X spermatozoa (Erickson, 1976). Contrary to this study, other studies showed no significant difference in the velocity of X and Y chromosome-bearing spermatozoa (Evans et al., 1975, Ross et al., 1975, Quinlivan et al., 1982, Brandriff et al., 1986, Ueda & Yanagimachi, 1987). The different motilities of X and Y chromosome-bearing spermatozoa in human semen samples was observed using the Percoll gradient separation method (Iizuka et al., 1987), but other studies have reported no significant differences (Wang et al., 1994, van Kooij & van Oost, 1992). The conventional swim-up method showed that 85% of the X chromosome-bearing spermatozoa were enriched in human semen (Check et al., 1989) whereas other studies demonstrated no significant sperm sexing using the swim up method (Han et al., 1993; Lobel et al., 1993; Yan et al., 2006). Pinkel et al. (1985) observed no significant sperm sexing by motility-based sperm sexing methods such as albumin gradient and density gradient methods on bull semen samples. Similar results were also observed on albumin gradient method of sperm sexing (Beal et al., 1984). A recent study on dog sperm sexing by density gradient method was found to be not effective in the separation of X and Y chromosome-bearing spermatozoa (Mothe et al., 2018).

Fig. 2.1 (continued) shape (**t**–**v**), or extremely abnormal shape (**w**, **x**). Eosin–nigrosin-stained smears. Reproduced with permission from Brito, 2007

2.3.3 DNA Content

In an early study, the total DNA content of X and Y chromosome-bearing spermatozoa of bulls, boars, rams, and rabbits was measured using flow cytometry method. The X–Y peak differences for bulls, boars, rams, and rabbits were found to be 3.9%, 3.7%, 4.1%, and 3.9%, respectively. This study also found significant differences in DNA content in X and Y chromosome-bearing spermatozoa in Jersey bulls Holstein, Hereford, Angus bulls and Brahman bulls (Garner et al., 1983). Johnson and Welch (1999) also observed significant DNA content difference in the range of 2–7% in X and Y chromosome-bearing spermatozoa of various mammals such as possum, human, rabbit, camel, elephant, boar, stallion, elk, bull, dog, ram, and chinchilla (Fig. 2.2). The DNA content of X and Y chromosome-bearing spermatozoa of Murrah buffalo and Nili-Ravi buffalo were determined using the flow cytometry method. The DNA content difference in X and Y chromosome-bearing spermatozoa of these buffalos was found to be around 3.5% (Lu et al., 2006). In a study, flow cytometry analysis of X and Y chromosome-bearing spermatozoa of goat indicated that 4.4% difference in the fluorescent peaks that correspond to the DNA content difference (Parrilla et al., 2004).

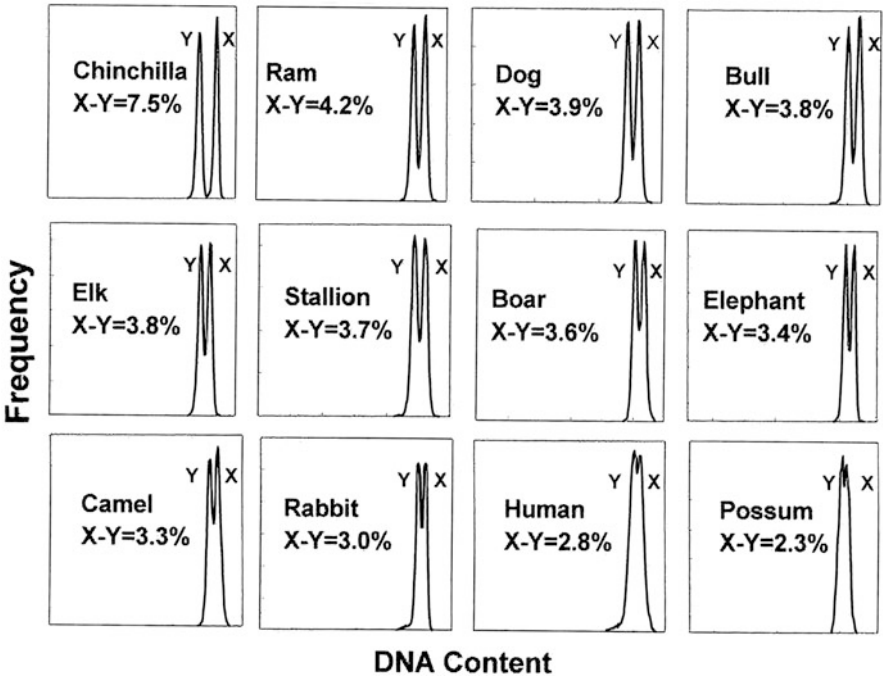

Fig. 2.2 Flow cytometric histograms produced from ejaculated semen from 8 common species illustrating the inherent difference (X-Y) in relative DNA content between X and Y chromosome-bearing sperm. Reproduced with permission from Johnson and Welch (1999)

2.3.4 Immunological Markers

In an interesting study, intravaginal administration of histocompatibility-Y (HY) antisera resulted in the production of 74% female rabbits (Zavos, 1983). In another study, bovine spermatozoa were enriched with Y chromosome-bearing spermatozoa by fluorescent-activated cell sorting. In this study, Y chromosome-bearing spermatozoa was labelled with Monoclonal HY antibody and fluorescein-conjugated goat antibody to mouse $F(ab)_2$. This study suggested that presence of HY antigen on the plasma membrane of male-specific spermatozoa forms the basis for the separation of X and Y chromosome-bearing spermatozoa (Ali et al., 1990). In contrary to this study, Hoppe and Koo (1984) demonstrated that there was no effect on sperm sexing by using monoclonal HY antibodies may be due to the removal of HY antigen on Y chromosome-bearing spermatozoa during epididymal transport and capacitation. In another study, no Y chromosome-bearing spermatozoa binding of HY antibody was observed (Hendriksen et al., 1993).In a study, sex-specific antibodies were raised against sex-specific proteins and this study suggested that sex-specific antibodies could separate X and Y chromosome-bearing spermatozoa (Blecher et al., 1999). In another study, Sang et al. (2011) identified a 30-KDa protein that can bind to X sperm-specific antibody. As these sex-specific antibodies failed to separate X and Y chromosome-bearing spermatozoa, researchers have focused on differentially expressed proteins. In a nano ultra-performance liquid chromatography-tandem mass spectrometry study, 17 differentially expressed proteins were identified in X and Y chromosome-bearing spermatozoa in bovine semen samples (De Canio et al., 2014). Another study observed 42 differentially expressed proteins on X and Y chromosome-bearing spermatozoa of the bull (Chen et al., 2012).

2.3.5 Charge

The zeta potential of the X chromosome-bearing spermatozoa was found to be higher than the Y chromosome-bearing spermatozoa. The sperm sexing was demonstrated on human semen samples by subjecting to free flow electrophoresis. This study observed the movement of X chromosome-bearing spermatozoa toward anode and movement of X chromosome-bearing spermatozoa toward cathode (Kaneko et al., 1983). In a subsequent study, Sialidase-treated X and Y chromosome-bearing spermatozoa lost electrophoretic mobility. This study suggested that the presence of sialic acid on the surface of spermatozoa is responsible for the charge (Kaneko et al., 1984).

2.4 Conclusions

The semen characterization is one of the essential processes needed for proper storage, transportation, and artificial insemination. The semen characterization would help the researchers to develop novel semen handling and sperm sexing methods. The color of the semen sample is a primary indicator to assess the quality of semen. The measurement of semen volume and concentration provide the total number of spermatozoa present in an ejaculate. The microscopic evaluation determines the percentage of spermatozoa with abnormal morphology. The evaluation of sperm motility and kinetic parameters ensure the quality of semen samples. Identification of differences between the X and Y chromosome-bearing spermatozoa is a crucial factor in sperm sexing. There are some contradictory claims regarding the differences in the size, shape, and motility between the X and Y chromosome-bearing spermatozoa. The total amount of DNA is the only measurable difference in X and Y chromosome-bearing spermatozoa. The research has been progressed to identify the sex-specific proteins and differentially expressed proteins on X and Y chromosome-bearing spermatozoa. Further research is needed to identify sex-specific markers to separate X and Y chromosome-bearing spermatozoa.

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Conventional Methods of Sperm Sexing

3

Contents

Abstract

The use of sexed semen for the production of livestock would benefit the animal farmers in terms of choosing either male or female animals. Even though sexed semen is available for the production of desired sex livestock, its use has been limited due to its high cost. Conventional methods of sperm sexing methods have been reported to enrich either X or Y chromosome-bearing spermatozoa. Conventional methods are restricted to the enrichment of either X or Y chromosome-bearing spermatozoa. Some studies achieved up to 80% success rate in the production of desired sex animals by using conventional methods. The reproducibility of the results has not been observed in conventional methods of sperm sexing. Most of the conventional methods are based on the differences in the size shape, motility of the X and Y chromosome-bearing spermatozoa. Recent studies are not convinced with the idea of differences in the size, shape, and motility of X and Y chromosome-bearing spermatozoa. So far, successful sperm sexing methods rely upon the Total DNA content of X and Y chromosome-bearing spermatozoa. The identification of sex-specific surface proteins is another interesting research area for sperm sexing. The conventional methods of sperm sexing based on the presence of HY antigen on Y chromosome-bearing spermatozoa were also failed to reproduce the results of sex-specific enrichment of

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spermatozoa. Integration of multidisciplinary approaches in conventional methods would pave the way to find an efficient method of sperm sexing.

Keywords

Livestock · Sperm motility · HY antigen · Albumin gradient · Percoll gradient · Sex-specific proteins

3.1 Introduction

Recently, Sexed semen has gained importance due to the high demand for sex-selected production of animals (Manzoor et al., 2017). The fluorescent activated cell sorting (FACS) based on the difference in the DNA content of X and Y chromosome-bearing spermatozoa is one of the best commercially successful methods so far (Johnson et al., 1999; Garner et al., 2013)). The slow speed of sorting, sperm damage during the sorting process, and high cost of the sexed semen are some of the factors associated with FACS-based cell sorting (Seidel Jr, 2003; Vazquez et al., 2009). The conventional methods such as Percoll gradient method, albumin gradient method, swim up methods, and immunological methods have been tried for sperm sexing (Resende et al., 2009; Beal et al., 1984). The inconsistency in the efficiency of sperm sexing has been a major problem in the conventional methods (Flaherty et al., 1997; Lucio et al., 2009). Majority of the conventional methods rely upon the difference in the motility of the X and Y chromosome-bearing spermatozoa (Iizuka et al., 1987; Lucio et al., 2012). Previous literature postulated that Y chromosome-bearing spermatozoa move faster than X chromosome-bearing spermatozoa due to its low mass and head volume (Shettles, 1960). In contrary to these claims, recent studies suggested that there is no significant velocity difference between X and Y chromosome-bearing spermatozoa (Grant, 2006). Advanced studies on sperm morphology also suggested that it would be difficult to separate the X and Y chromosome-bearing spermatozoa (Carvalho et al., 2013). Despite these findings, researchers have been trying to modify the conventional methods to achieve the significant enrichment of either X or Y chromosome-bearing spermatozoa. The conventional methods offer simple processing of the sperm and less damage to sperm and these methods can be made available at low cost. Recent advances in research such as nanotechnology, microfluidics, genomics, and proteomics have been implicated to develop conventional methods (Yata, 2021). For example, HY antigen present on the Y chromosome-bearing spermatozoa formed the basis for the separation of X and Y chromosome spermatozoa. Even though this method has been failed to separate X and Y chromosome-bearing spermatozoa, it initiated to find differentially expressed proteins on the surface of X and Y chromosome-bearing spermatozoa (Yadav et al., 2017). The microfluidic methods also have been implicated in sperm sexing to separate the X and Y chromosome-bearing spermatozoa based on the size and shape of the spermatozoa. Even though there is ambiguity in sperm sexing, conventional methods are

successful in the separation of highly motile spermatozoa from dead and not motile spermatozoa (Centola et al., 1998). Recent studies suggest that implications of interdisciplinary approaches in conventional methods would lead to the development of an efficient sperm sexing method at an affordable cost.

The successful and unsuccessful sperm sexing by conventional methods were discussed in the chapter. The conventional gradient separation, swim up methods and immunological methods have been presented with pros and cons.

3.2 Conventional Methods of Sperm Sexing

The conventional methods for sperm separation by density gradient centrifugation and swim up methods are illustrated in Fig. 3.1. This sperm sexing efficiency has been studied by using these methods and inconsistent results have been observed. Apart from these sperm separation methods, HY antigen-based sperm sexing is also discussed in this chapter.

3.2.1 Gradient Centrifugation Method

Percoll gradient centrifugation (PGC) is a colloidal silica particle-based gradient centrifugation (Promthep et al., 2016). An increasingly dense layer from top to bottom is created by Percoll prepared at different concentrations. As Y chromosomes are smaller and lighter (Johnson, 2000) than the X chromosomes, creating a differential density on centrifugation between these two chromosomes bearing sperm. The heavier X chromosome-bearing sperm moves faster to the denser bottom layers while the lighter Y chromosome-bearing sperm migrate faster to the medium layers

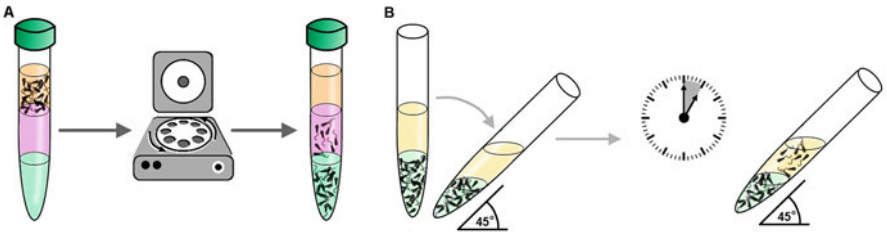

Fig. 3.1 Methods for routine sperm preparation for ARTs. (**a**) Example of sperm selection by density gradient centrifugation (DGC): Semen is placed on top of a gradient of colloid (e.g., silica particles coated with silane) prepared in a centrifuge tube and then subject to centrifugation. Subsequently, motile spermatozoa are recovered from the tube bottom containing the fraction with a higher density of colloid. (**b**) Example of sperm selection by swim up (SU): In a centrifuge tube, a medium free of cells (yellow in the figure) is placed on top of the seminal sample (green in the figure). The tube is then inclined 45° and incubated for about 1 h allowing motile spermatozoa to swim up toward the medium free of cells where they are collected for downstream applications. Reproduced with permission from Oseguera-López et al. (2019) under the terms of the Creative Commons Attribution License (CC BY)

(Ericsson et al., 1973; Hindal et al., 2018; Hossepian de Lima et al., 2000; Henkel & Schill, 2003). It is a widely used method of selection of elite quality sperm from man (Drobnis et al., 1991), bull (Oliveira et al., 2011), boar (Grant et al., 1994; Suzuki & Nagai, 2003; Martecikova et al., 2010; Matás et al., 2011), and ram (Valcárcel et al., 1996) semen.

3.2.1.1 Mice

Mice are polytocous animals producing multiple offspring in single parturition with a very short gestation period of 483 h (20 days) (Murray et al., 2010). This animal model offers a very fast way of elucidating the effect on sex ratio obtained through insemination of PGC semen. The different sperm fractions are prepared by layering whole semen on the top of the increasingly dense layers (45%, 60%, 75%, and 90% from top to bottom) of Percoll. 0.5 ml of each layer are stacked upon another in a conical tube and centrifuged for 20 min at 4000 rpm (Hadi & Mossa, 2020). The PGC semen supports the pregnancy rate of 70% (21 out of 30 female mice) v/s 56.7% (17 out of 30 female mice) in the control group (Hadi & Mossa, 2020). The possible explanation for this can be that only the normal, robust, and motile sperm are filtered out (Swales & Wright, 2000) from the immotile sperm and other junks of viruses (Sheoran et al., 2005), bacteria (Leuschner et al., 1999), and neutrophils (Woldehiwet et al., 2003). The filtering action is done by the polyvinylpyrrolidone coated silica particles of 15–30 nm in diameter that build up specific gravity of 1.13 sg/ml (Samardzija et al., 2006; Makler et al., 1998). However, female-to-male birth ratio remain unaltered (Esmaeilpour et al., 2012), as this method does not favor selection of X or Y chromosome-bearing sperm (Henkel & Schill, 2003).

Still, sperm sample prepared from this method supports the selection of Y chromosome-bearing sperm, probably due to gaining of functional advantage or somehow inactivation or incapacitation of X chromosome-bearing sperm (Schenker, 2002). 72.7% (80 out of 110) male births were reported by this method v/s 52.9% (45 out of 85) of male births in control group on intraperitoneal insemination of female mice (Hadi & Mossa, 2020). Thus, in mice PGC enhances the pregnancy rate with more male births, despite any biases for X or Y chromosome-bearing sperm selection.

3.2.1.2 Ongole Cattle

Cattle are one of the most important livestock and predetermination of their sex will be crucial for food industries, globally (Hodgson, 1979). Their sexed semen can be stored in semen banks and can be used when required by diary or beef industries. Freezing effect on percentage motility and membrane integrity was observed on sexed semen by PGC. X chromosome-bearing sperm were sexed at bottom layers while Y chromosome sperms were sexed at top layers. The control (non-sexed) and sexed groups were replicated 10 times with block design for descriptive analysis. Light microscopy (100 X) revealed percentage motility of $53 \pm 7.93\%$, $47 \pm 6.13\%$, and $31.45 \pm 7.20\%$ for X chromosome-bearing sperm while $48.55 \pm 8.28\%$, $42.1 \pm 7.36\%$, and $27.45 \pm 8.69\%$ for Y chromosome-bearing sperm v/s

64.25 ± 3.94%, 59.75 ± 4.48, and 44.2 ± 2.79 in control group; after sexing, pre-freezing, and post-freezing, respectively.

As manifested by scanning electron microscopy (SEM; 10,000X), sperm of neat semen sample were having intact membrane of head, neck, and tail. Neck membrane integrity is an important factor for sperm motility and its rupture compromises sperm motility. After sexing, leakiness was observed in the head, neck, and tail regions of Y chromosome-bearing spermatozoa while X chromosome-bearing spermatozoa were still intact. Serious membrane damages were observed in all the sexed sperm when compared to the control group. Thus, higher percentage of X chromosome-bearing sperm are motile to freezing shocks yet there was an overall decrease in percentage motility from sexing to thawing in both the sexed groups. Control group is more tolerant to freezing temperature when compared to sexed group for motility. Also, this method is more reliable for sexing of the X chromosome as evident from its intact structure post sexing (Susilawati et al., 2017).

3.2.1.3 Holstein Friesian Cattle

The urge of female animals is always a prerequisite for setting up a dairy industry and the use of seven-layer PGC is at its best for preselection of female Holstein animals (Promthep et al., 2016). The seven layers were formed at concentrations (v/v) of 40%, 50%, 60%, 65%, 70%, 75%, and 80% Percoll from top to bottom in a conical tube (Kobayashi et al., 2004). Two hundred sperm per ejaculate from two bulls were evaluated for parameters such as live sperm (unstained with eosin-nigrosin) (Dott & Foster, 1972), morphologically abnormal sperm (crescent-shaped apical ridge), progressive motility (bright field microscopy), and sex-specific embryos formed through AI and IVF (Promthep et al., 2016).

No significant motile sperm were found at 40% layer and hence discarded. From the remaining layers, sperm were divided into four groups at a gradient of (a) 50%, (b) 60%, (c) 65–70%, and (d) 75–80% with group iii) found to be the best with a concentration of 414.93 ± 30.32 × 106 sperm/ml. Normal morphology, viability, and progressive motility were 39.07 ± 0.44%, 93.57 ± 0.57% and 95.86 ± 0.46% v/s 46.07 ± 0.64%, 92.71 ± 0.69% and 92.50 ± 0.51% in control group (fresh sperm), respectively. This group was taken further in the experimentation and found to have 39.25% of Y chromosome-bearing sperm confirmed by quinacrine mustard staining, the rest (60.75%) being enriched with X chromosome-bearing sperm. However, the concentration, motility, and viability were reduced to 8 × 106 sperm/ml, 65.86% and 69.14% post thawing, respectively. Sixty-nine IVF and 150 AI were performed v/s 120 control (AI with non-sexed semen) that resulted in 28 (40.58%), 60 (40.00%), and 59 (46.09%) pregnancy, respectively. The sex percentage of the offspring showed biasness toward female with 19 (67.86%), 30 (71.43%), and 30 (50.85%) offspring born through IVF, AI, and control.

Thus, the results very clearly show that even though the concentrations of sperm were reduced drastically in post-thawed semen with a significant decrease of motility and viability yet PGC is a reliable method of sexing X chromosome-bearing sperm in HF bull. Also, it is producing a much higher percentage of female animals born through transfer of AI and IVF embryos.

3.2.1.4 Small Ruminants (Goats and Sheep)

Gender selection in case of small ruminants is for sure benefiting milk, meat, wool, and leather industries (Ferreira-Silva et al., 2016) as females are more demanding for dairy farms and males for other industries (Rath et al., 2013). In an experiment conducted by Ferreira-Silva et al. (2017) with four rams and bucks, electroejaculated semen was cryopreserved and sexed by PGC. The X:Y chromosome-bearing sperm ratio was further confirmed by qPCR with CTAB (Cetyl Trimethyl Ammonium Bromide) extracted sperm DNA (Solléro et al., 2004). The primers used were sex gene-specific: SRY and Aml-X for Y and X chromosome, respectively (Phua et al., 2003). No sexing was observed in case of goats' semen, post-PGC when compared with pre-PGC (control). The PGC v/s control sperm percentage was 72 v/s 70, 59 v/s 66, 44 v/s 45, and 60 v/s 58 for X chromosome and 28 v/s 30, 41 v/s 34, 56 v/s 55, and 40 v/s 42 for Y chromosome. In case of sheep, sample from only one biological replicate and that too with compromised motility showed sexing of X chromosome-bearing sperm (62% PGC v/s 46% control). The scenario in rest of the three animals was 34% v/s 37%, 54% v/s 52%, and 70% v/s 60%. For Y chromosome, there was significant decrease in percentage of sample (38% PGC v/s 54% control) with other being not sexed at 66% v/s 63%, 46% v/s 48%, and 30% v/s 40% (Ferreira-Silva et al., 2016). Thus, though one sample from sheep favored sexing of motility exhausted, X chromosome-bearing sperm by PGC yet it needs to be further improved for sexing semen of other small ruminants (Ferreira-Silva et al., 2016).

3.2.1.5 Other Animals

Dogs are polytocous animals with only 2 months of gestation (Concannon, 2000) and when similar type of centrifugation experimentations were performed using Percoll only, Percoll and nycodenz and Percoll and Ficoll dependent gradients in dogs surprisingly opposite result was obtained (Mothe et al., 2018). Percoll only gradients (30%, 40%, 50%, 60%, 70%, 80%, and 90%) and Percoll and nycodenz gradients were prepared as per Andersen and Byskov (1997) while Percoll and Ficoll gradients were prepared as per Davis and Halliday (1977) and Hegde et al. (1977) with slight modifications. The experimental set-up involved digital manipulation of penis-dependent semen collection (Seager & Platz, 1977) from ten males (2–6 years) of different breeds. Semen samples were examined for X or Y chromosome-bearing sperm abundance (after centrifugations) and various sperm-related parameters before and after centrifugations. DNA extraction was done by phenol–chloroform isoamyl-alcohol method in the ratio of 25: 24: 1, respectively (Oliveira et al., 2011) quantified and qualified by nanospectrophotometer for qPCR analysis.

Enrichment of X chromosome-bearing fraction was observed with recovery maxima by Percoll and Ficoll method (55 $\pm$ 1.5%) followed by Percoll only (54.8 $\pm$ 1.9%) and Percoll and nycodenz (53.2 $\pm$ 2.0%) methods. The non-enriched Y chromosome-bearing sperm were at 45.0 $\pm$ 1.5% (by Percoll and Ficoll method), 45.2 $\pm$ 1.9% (Percoll only method) and 46.8 $\pm$ 2.0% (Percoll and nycodenz method). There was a very slight enriched population of Y chromosome-bearing sperm (50.3 $\pm$ 2.3) as compared to X chromosome-bearing sperm (49.8 $\pm$ 2.3) in the control group. The enrichment results were confirmed with

qPCR results of sex-specific genes (SRY for Y chromosome and Factor IX for X chromosome) of spermatozoa from Canis lupus familiaris dog breed. All the sperm-related factors such as concentration, total motility, progressive motility, and morphology were compromised in all the three centrifuged groups when compared with the control group that can be and for sure have detrimental effects on ART-related programs (Mothe et al., 2018). Thus, it is a non-reliable method for commercial applications in breeding programs as it is favoring selection of only up to 50% of X chromosome-bearing sperm and that too with compromised concentration, morphology, and motility.

3.3 Swim Up Method

In a typical swim method, semen sample is placed as a bottom layer and medium is placed as a top layer in a tube. This tube was incubated in a vertical position at 37 °C for about 45 min. It is postulated that Y chromosome-bearing spermatozoa move upward due to its low mass and high velocity than X chromosome-bearing spermatozoa. In human studies, sex-sorted semen by using swim up method showed 81% male offspring (Check et al., 1989). In another study, female child was born with 86% and male child were born with 89% success rate by using sorted semen obtained by swim up method (Khatamee et al., 1999). In a study, 81% of male in vitro ovine embryos were produced using semen sorted by swim up method (Hadi, 2013). In a study, Azizeddin et al. (2014) proposed a modified swim up method and it is called as Pipette-swim up method. This method involves sperm sexing in a 30-cm long 5–10 ml pipette placed on a rubber stopper. The semen sample was injected through the bottom of the pipette and incubated for up to 45 min. After incubation, 1 ml fractions were collected for the bottom of the pipette. This modified swim up method showed 62% enrichment of Y chromosome-bearing bull spermatozoa (Azizeddin et al., 2014). In another study, Nili-Ravi buffalo semen samples were evaluated using a modified swim up method. The results from this study indicated that bottom fractions were enriched X chromosome-bearing spermatozoa and upper fractions were enriched with Y chromosome-bearing spermatozoa (Awan et al., 2017). In contrary to this results, Yan et al. (2006) observed no significant sperm enrichment in the sperm fractions obtained after swim up method. Similarly, no significant sperm sexing was noticed on the production of in vitro bovine embryos by using the semen sorted by swim up method (Wolf et al., 2018).

3.4 Y-Chromosomal Histocompatibility (HY) Antigen

A male minor histocompatibility antigen was detected when male grafts were transplanted on female mice Müller (1996). Gasser & Silvers, 1972 termed this antigen as Y chromosomal histocompatibility (HY) antigen as Y chromosome is the major difference between the male and females. In an early study, Eichwald &

Silmser, 1955 suggested that Y chromosome histocompatibility could help in sperm sexing. Subsequent studies proved that antisera of female mice grafted with male tissues impair the Y chromosome-bearing spermatozoa (Sachs & Heller, 1958; Feldman, 1958). A study on HY antigen confirmed that male graft transplantation in female produce antibodies that are cytotoxic to sperm cells (Scheid et al., 1972). In a study, male sex ratio is slightly altered with HY antibody-treated semen samples. This study observed 45.4% female and 53.4% male offspring upon artificial insemination of mice with HY antibody-treated semen samples Bennett & Boyse, (1973). Later, some other studies proved the presence of HY antigen specifically on Y chromosome-bearing spermatozoa (Silvers & Wachtel, 1977; Krco & Goldberg, 1976; Utsumi et al., 1993). In contrary to these claims, few studies are not convinced with previous results of binding of HY antigen specifically to Y chromosome-bearing spermatozoa possibility of HY antibody-mediated sperm sexing (Sills et al., 1998; Hoppe & Koo, 1984; Hendriksen et al., 1993).

3.5 Conclusions

The development of simple and low-cost sperm sexing methods is still a challenge and it necessitates improvements in the conventional methods to separate the X and Y chromosome-bearing spermatozoa. The existing conventional methods are facing the problem of reproducibility and reliability. The dynamic nature of both X and Y chromosome-bearing spermatozoa do not allow to completely separate them based on velocity. Advance methods with high precisions may be implicated to separate the spermatozoa. The alteration of motility or velocity of either X- or Y-specific spermatozoa by treatment with chemical or biological agents would lead to the sperm sexing with reproducibility. The identification of sex-specific surface antigens on either X or Y chromosome-bearing spermatozoa would lead to the development of efficient sperm sexing method with high accuracy. Recent research has been focused in this direction and some differentially expressed proteins were identified on X and Y chromosome-bearing spermatozoa. The principles of conventional methods are carry forwarded to develop novel sperm sexing methods with reproducibility and reliability.

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Sperm Sexing Using Flow Cytometry: Principles and Applications

4

Contents

Abstract

Sperm sexing has gained importance across the globe for the production of desired sex animals and to reduce the burden of undesired sex animals. Sperm sexing involves the separation of live and dynamic X and Y chromosome-bearing spermatozoa for production of either male or female animals. Conventional methods were failed to differentiate the X and Y chromosome-bearing spermatozoa based on the size, shape, and velocity. The X and Y chromosome-bearing spermatozoa of mammals were found to contain different amount of total DNA and this forms the basis for separation. The DNA staining by fluorescent dyes is able to differentiate X and Y chromosome-bearing spermatozoa based on the relative emission of fluorescent light by the respective spermatozoa. The application of flow cytometry for sperm sexing led to the development commercially successful FACS-based sperm sexing method. The orientation of sperm

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head while measuring fluorescence in flow cytometry was a problem in initial experiments and it was solved by the development of an innovative nozzle that improved the efficiency of FACS-based sperm sexing. The low-speed sorting, sperm damage, low conceptions rates, and high cost of the sexed semen are some of the constraints in FACS-based sperm sexing. Future developments in the FACS-based sperm sexing are needed to improve the sperm sorting speed and to lower the sperm damage during flow sorting of spermatozoa, This chapter reviews the principles, methods, applications, and limitations of FACS-based sperm sexing.

Keywords

Sperm sexing · Fluorescence-activated cells sorter · Animal reproduction · Sex selection

4.1 Introduction

Fluorescence-activated cell sorting of semen is a prevailing method for separating X and Y chromosome-bearing spermatozoa (Garner, 2006). The sorted sperm can be used in various assisted reproductive techniques such as artificial insemination and in vitro fertilization where pre-birth sex determinations of neonatal animals are crucial (Maxwell et al., 2004). Such an importance can be manifested in dairy farms where female animals are preferred. Thus, using FACS-sorted X chromosome bearing sperm can be employed for this purpose that reduces the risk of obtaining animals with random sex by natural mating or using non-sorted semen (Garner & Seidel Jr, 2008). Sperm sexing by conventional methods such as Percoll gradient centrifugation method, albumin gradient method, and swim up method have been failed to achieve reproducible results (Espinosa-Cervantes & Córdova-Izquierdo, 2012). The contradictory previous reports on sperm sexing efficiency by conventional methods may be due to the random movement of X and Y chromosome-bearing spermatozoa (Seidel Jr, 2007). Commercialization of sexed semen needs more than 90% efficiency that should be capable of producing the desired sex (Garner & Seidel Jr, 2008). The main challenge in sperm sexing is separating live and dynamic X and Y chromosome-bearing spermatozoa without any physical damage to the sperm (Amann, 1999). The reported minute differences in the size, shape, and velocity between X and Y chromosome-bearing spermatozoa were also not proven in recent studies (Garner & Seidel Jr, 2003). The difference in the DNA content between the X and Y chromosome-bearing spermatozoa was confirmed by serval studies and it has been the only significant validated difference so far (Xie et al., 2020) (Table 4.1). The staining of nucleic acid with fluorescent dyes and subsequent measurement of fluorescent intensity emitted by X and Y chromosome-bearing spermatozoa forms the basis for separation (Garner, 2006). Measuring fluorescence intensity of dynamic fluorescent activated sperm cells were difficult and application of flow cytometry paved the path for the future development of flow

Table 4.1 Percentage difference in DNA contents between X and Y chromosome-bearing sperm nuclei as determined by flow cytometric analysis of Hoechst 33342-stained samples (Modified with permission from O'brien et al. 2009)

Order	Species (Vernacular name)	Breed	% X-Y DNA difference	References
Artiodactyla	*Bos indicus* (Zebu cattle)		3.7	Garner et al. (1983)
	Bos taurus (Exotic cattle)		3.8, 4.0–4.2	Johnson (1995)
		Jersey	4.24	Garner et al. (1983), Garner (2001, 2006)
		Holstein Friesian	3.98	Garner et al. (1983); Garner (2001, 2006)
		Angus	4.05	Garner et al. (1983), Garner (2001, 2006)
		Hereford	4.03	Garner et al. (1983); Garner (2001, 2006)
	Crossbred cattle species	Brahman	3.73	Garner et al. (1983); Garner (2001, 2006)
	Bubalus bubalis (Buffalo)		3.6	Johnson (2000); Lu et al. (2007)
		Murrah	3.59	Lu et al. (2007)
		Nili Ravi	3.55	Lu et al. (2007)
	Camelus dromedaries (Dromedary camel)		3.3	Johnson (2000)
	Bos bison (Bison)		3.6	Garner and Seidel Jr (2003), Johnson (2000)
	Bos mutus grunniens (Yak)		3.6	Garner and Seidel Jr (2003); Johnson (2000)
	Sus scrofa (Pig)		3.6	Johnson (1995, 2000)
	Hippopotamus amphibius (Hippopotamus)		3.7	O'Brien et al. (2002)
	Cervus elaphus nelsoni (Elk)		3.8	Johnson (2000)
	Ovis aries (Sheep)		4.2	Johnson (1995, 2000)
	Capra aegagrus hircus (Goat)		4.4	Parrilla et al. (2004)
	Gazella dorcas (Dorcas gazelle)		4.3	Johnson (1992)
	Giraffa camelopardalis (Giraffe)		4.4	O'Brien et al. (2002)
	Odocoileus virginianus (White tailed deer)		4.4	Deyoung et al. (2004)
	Muntiacus reevesi (Reeve's muntjack)		6.3	Johnson (1992)

(continued)

Table 4.1 (continued)

Order	Species (Vernacular name)	Breed	% X-Y DNA difference	References
Perissodactyla	*Equus caballas* (Domestic horse)		3.7	Johnson et al. (1999), Johnson (2000)
Rodentia	*Oryctolagus cuniculas* (Rabbit)		3.0	Johnson et al. (1989), Johnson (2000)
	Mus musculus (Mouse)		3.3	Pinkel et al. (1982a, 1982b)
	Chinchilla laniger (Chinchilla)		7.5	Johnson and Clarke (1988)
Carnivora	*Canis lupis familiaris* (Dog)		3.9	Johnson et al. (1999)
	Felis catus (Cat)		4.2	Garner and Seidel Jr (2003)
Diprotodontia	*Trichosurus vulpecula* (Brushtail possum)		2.3	Johnson (1995)
Proboscidea	*Elephas maximus* (Asian elephant)		3.4	Johnson (2000)
	Loxodonta afrincana (African elephant)		3.9	O'Brien et al. (2002)
Cetacea	*Tursiops truncatus* (Bottlenose dolphin)		4.0, 4.1	O'Brien and Robeck (2006), Garner and Seidel Jr (2003)
Primates	*Gorilla gorilla gorilla* (Western lowland gorilla)		2.7–2.8	O'Brien et al. (2005)
	Homo sapiens (Humans)		2.8 2.9	Johnson (1995) Johnson et al. (1994)
	Pan troglotydes (Common chimpanzee)		3.3	O'Brien et al. (2005)
	Callithrix jacchus (Common marmoset)		4.1	O'Brien et al. (2005)

cytometry-based sperm sexing (Welch & Johnson, 1999). Initially, the fluorescence measurements were erroneous because of the different orientations of sperm cells while measuring the fluorescence intensity from live cells (Johnson, 1995). Later, the development of SX Moflo Nozzle to correct the orientation of sperm before the measurement of fluorescence in FACS-based sperm sexing (Rens et al., 1998). This development revolutionized sperm sexing worldwide, and most of the developed and developing countries depend on this technology for the production of desired sex animals (Seidel Jr, 2003). The basic principle of FACS-based sperm sexing is illustrated in Fig. 4.1. Even though there are some limitations such as sperm damage and low conception were reported, this method has been successful in producing desired sex animals (Maxwell et al., 2004).

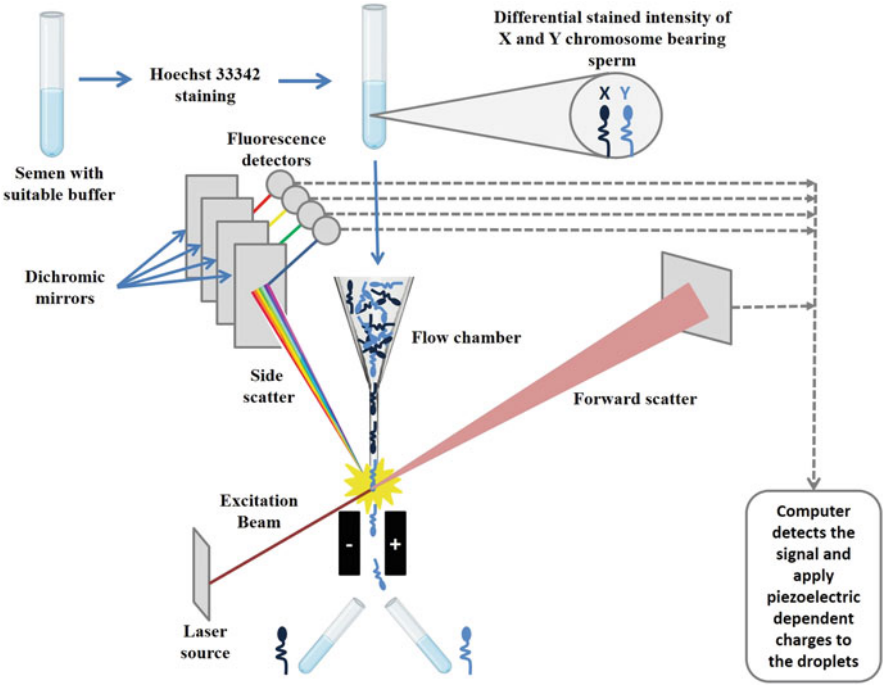

Fig. 4.1 Schematic representation of FACS-based sperm sexing

The FACS-based method has been leading technology since its commercialization and proper use of this method would improve the economy of low income and developing countries. This chapter provides the history, development applications, and limitations of FACS-based sperm sexing method (Maxwell et al., 2004).

4.2 Basis of FACS-Based Sperms Sexing

The technique relies on differential nuclear staining of the sex-specific spermatozoa with nuclear dye such as Hoechst 33342 (Johnson et al., 1987a, b). In bovine's karyotype, there is a total length difference of 4.2% between X chromosome-bearing spermatozoa and Y chromosome-bearing spermatozoa (Garner, 2006). This accounts for the less amount of DNA in Y chromosome-bearing spermatozoa and it gets less nuclear stain intensity as compared to the X chromosome-bearing spermatozoa (Johnson et al., 1999). When these differentially stained sperms are passed as a single cell through the nozzle of the FACS they differentially scatter the laser light (Schenk et al., 1999). They generate forward scatter and side scatter. The excitation and detection of spermatozoa in proper orientation (Fig. 4.2a) and histograms (Fig. 4.2b) produced by flow cytometer were shown in Fig. 4.2. Depending upon the differential signal pattern of the X-bearing chromosome and

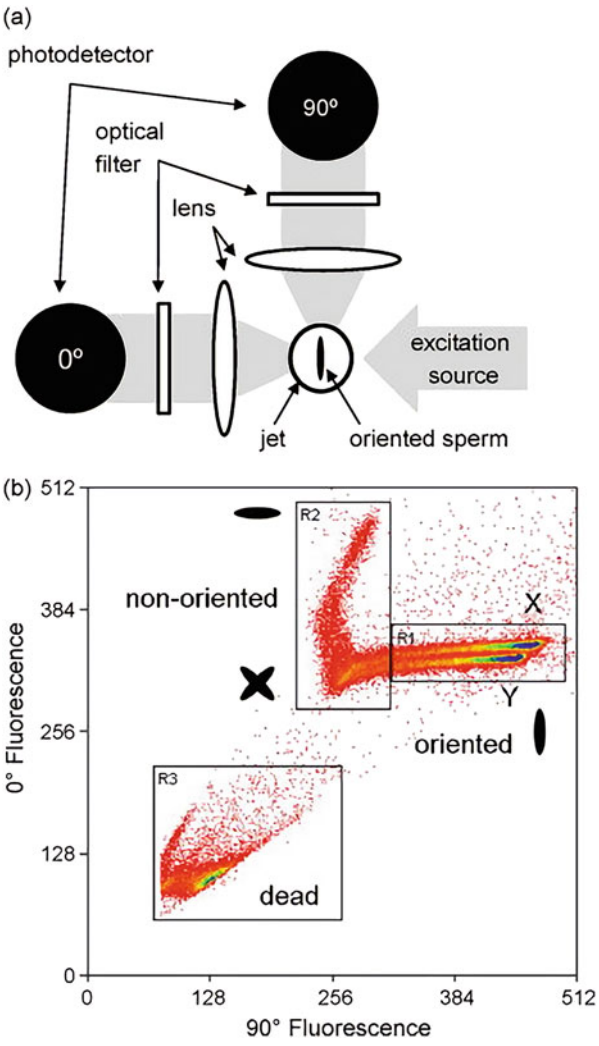

Fig. 4.2 (**a**) Excitation and detection scheme used for flow cytometric sorting of sperm showing preferred sperm orientation (flow axis into page), and (**b**) 0° vs. 90° fluorescence bivariate histogram produced by a flow cytometer showing the effect of approximate 2:1 fluorescence measurement for live oriented sperm that are edge toward the 90° detector (region R1), and non-oriented sperm that are edge toward the 0° detector or somewhere between (R2). X and Y sperm populations can be resolved within R1. Dead (red food dye quenched) sperm are present in R3. Reproduced with permission from Sharpe and Evans (2009)

Y-bearing chromosome, the computer then applies piezoelectric-dependent charges to the emerging drops containing single spermatozoa which are then collected in different tubes.

4.3 DNA Content Measurements in the X and Y Chromosome-Bearing Spermatozoa

In early studies on chromosome staining, it is observed that fluorescent dyes such as quinacrine mustard and quinacrine hydrochloride can bind to the chromosomes (Caspersson et al., 1968, 1969). Based on the fluorescent measurements, Sumner

et al. (1971) distinguished the X and Y chromosome-bearing human spermatozoa by combining quinacrine hydrochloride and Feulgent staining methods. This study observed 2.7% difference in the DNA content (Sumner et al., 1971). In a study, 24 mammalian species were found with more than 6.1% difference in the DNA content in the X and Y chromosome-bearing spermatozoa (Moruzzi, 1979). Even though there is a difference in the fluorescence intensity, flow systems could not distinguish the X and Y chromosomes-bearing spermatozoa due to different orientations of sperm during the measurement of fluorescent intensity (Van Dilla et al., 1977).

The difference in the DNA content among nucleic acid-stained X and y chromosome-bearing spermatozoa forms the basis to sort the sperms by using flow cytometer. In an early study, the relative DNA content in the 4′,6-diamidino-2-phenylindole (DAPI) stained human spermatozoa was measured using flow cytometer (Otto et al., 1979). In another study, the sperm head orientation problem in optical detection was overcome by the use of specially built cell orientating flow cytometer. This study, successfully measured the DNA content from fluorescent stained bull, ram, rabbit, and boar spermatozoa (Pinkel et al., 1982a). The cell orientation plays a key role in measuring the fluorescence in a particular orientation. In a study, cell ordination is manipulated by hydrodynamic methods using sheath liquid fluids (Fulwyler, 1977). In a similar study, DNA content and Cell flatness were measured simultaneously by using a flow cytometer (Dean et al., 1978). Gledhill et al. (1976) demonstrated the fluorescence distribution on acriflavine-stained cells to measure the sperm DNA content. The spherical sperm cell produced symmetric fluorescence distribution and flat cells produced asymmetric fluorescence distribution (Gledhill et al., 1976). The translocation of chromosome 7 on the X chromosome resulted in the DNA content difference in mouse sperms from 3.3% to 4.9% (Cattanach, 1961; Disteche et al., 1981). In a study, X and Y chromosome-bearing spermatozoa the fluorescent peak differences of Jersey bulls, Holstein, Hereford, Angus, and Brahman bulls were compared. This study observed larger and smaller peak differences in Jersey and Brahman bulls, respectively (Garner et al., 1983). Pinkel et al. (1982b) successfully separated Y and O chromosome-bearing spermatozoa of vole *Microtus oregoni.* Even though separated sperm were in this study are not alive, this study paved the way to improve the efficiency of flow cytometry-based sperm sexing (Pinkel et al., 1982b).

4.4 Milestones in the Development of FACS-Based Sperm Sexing

The US Department of Agriculture, Agricultural Research Service, Beltsville, Maryland and Lawrence Livermore National Laboratories, Biomedical Sciences Division, University of California, Livermore, California, combinedly developed modified laser-based flow cytometry to separate X and Y chromosome-bearing spermatozoa. This study used to beveling injection tube to aid the proper orientation of sperm cells during the fluorescence measurements. The bull, boar, and ram X and

Y chromosome-bearing spermatozoa were separated with high resolution (Johnson & Pinkel, 1986). Johnson et al. (1987a, b) stained the spermatozoa of Chinchilla laniger with Hoechst 33342, and separated X and Y chromosome-bearing spermatozoa by using a flow cytometer. This study claimed the 95% purity of sexed semen (Johnson et al., 1987a, b). In another study, Johnson et al. (1987a) suggested that 2.5 or 5.0 micrograms/ml of Hoechst stain resolves X and Y chromosome bearing spermatozoa flowcytometry. The sorting speed of the initial FACS-based sperm sexing instrument is 500 to 600 live sperms per second (Seidel et al., 1998). The sperm orientation is manipulated by replacing beveled needle with a Jet in air nozzle. The nozzle shape is designed such that orientation forces are crated within the nozzle for the better orientation of sperm head. The cattle, swine, rabbit, mouse, and human sperm samples were demonstrated by using this nozzle and exhibited improved orientation of sperm heads (Rens et al., 1998). Subsequently, the sorting rate was increased from 2 to 6×10^6 sperms per hour by using this nozzle (Johnson et al., 1999). Further development of this nozzle into SX MoFlo by XY Inc., improved the sorting efficiency to 15×10^6 sperm per hour (Schenk et al., 1999).

4.5 Evaluation of Sperm Sexing in In Vitro and In Vivo Models

Evaluation of flow cytometry-based sperm sexing was evaluated in rabbit models. This study separated X and Y chromosome-bearing spermatozoa by flow cytometry with purity of 84% and 81% for X and Y chromosome-bearing spermatozoa, respectively. The sex-sorted semen was inseminated in rabbit uterus by a surgical process. The study observed 94% success with X-sorted sperm and 81% success with Y-sorted sperm (Johnson et al., 1989). Later, this technology was evaluated by in vitro fertilization and produces 3 males and 4 females calves of projected sex (Cran et al., 1993). In another study, 90% of male calves were produced on field trial on cattle (Cran et al., 1995). In a study, flow-sorted semen was also evaluated in swine. This study observed 74% success with X chromosome-bearing spermatozoa and 68% success with Y chromosome-bearing spermatozoa (Johnson, 1991). Another study confirmed the superiority of FACS-based sperm sexing over other conventional methods (Johnson, 1992). A commercial FACS-based sperm sexing method, Beltsville sperm sexing technology, achieved 85 to 90% success in repeated experiments. This method used Hoechst 33342 to stain the spermatozoa and sorted the X and Y chromosome-bearing spermatozoa using flow cytometry. The sexed sperm are subjected to in vitro fertilization and subsequent artificial insemination in cattle and pigs (Johnson et al., 1999).

4.6 Applications of Sexed Semen Sorted by FACS

4.6.1 Cattle

The bovine calves of desired sex were successfully produced by in vitro fertilization of oocytes with flow-sorted X and Y chromosome-bearing spermatozoa (Cran et al., 1993). The sexed semen sorted by FACS-based method was usefully applied to cattle on a field trial (Cran et al., 1995). In a study, sexed semen was inseminated into the superovulated cows and embryos were collected by nonsurgical procedures. This study produced embryos by sexed semen and these embryos were found to be competent and transferrable for desired sex animal production (Schenk et al., 2006). In a study, sorting pressure was used between 30 and 50 psi, and sperm number was used between 2×10^6 and 10×10^6 to evaluate the efficiency of cryopreserved sexed semen. This study standardized the parameters for commercialization of FACS-based sperm sorting (Schenk et al., 2009).

4.6.2 Pigs

Sperm sexing of swine spermatozoa by FACS based was demonstrated in several studies. The sexed semen was surgically inseminated into the oviduct of mature pigs and achieved more than 68–74% success (Johnson et al., 1999). In another study, 85% of the piglets with predicted sex were produced by using sexed semen that was sorted by flow cytometry method (Rath et al. 1997). In another study, porcine oocytes were fertilized with sexed semen. This study separated X and Y chromosome-bearing spermatozoa by using Beltsville sperm sexing technology and subsequent in vitro fertilization and embryo transfer resulted in 95% success in the production of piglets (Rath et al. 1999).

4.6.3 Sheep

In a study, ram semen sample was sexed by using FACS-based semen sexing and injected in vitro matured oocyte. This study produced one male lamb using sorted Y chromosome-bearing spermatozoa (Catt et al., 1996). In another study, lambs produced by insemination of flow cytometry-sorted sexed semen through intrauterine insemination (Cran et al., 1997). In a similar study, female lambs were produced with a low number of cryopreserved sexed semen (Hollinshead et al., 2002). The sexed semen of ram samples were evaluated for the embryo production and subsequent transferability of embryos into the female sheep. This study achieved 92.5% of predicted sex of lambs in their experiments (De Graaf et al., 2007a, b). The flow cytometric sorted (MoFlo SX) X and Y chromosome-bearing spermatozoa of ram were found to be equally fertile to that of unsorted spermatozoa. This study used very low insemination doses (1 or five million) of sorted semen to achieve the successful production of desired sex sheep (De Graaf et al., 2007a, b).

4.6.4 Horses

The experimental trials on flow cytometry-sorted sexed semen were conducted on mares indicated that the pregnancy rates were similar to the unsexed semen. This study used 500 × 10^6 unsexed sperm cells and 25 × 10^6 sex-sorted cells to determine the pregnancy rates. Low dose insemination of sexed semen in mares has proven to be successful for the production of mares of desired sex (Buchanan et al., 2000). In a similar study, pregnancy rates were reported to be 37.5% for fresh sex-sorted semen and this pregnancy rate is similar to the unsexed frozen semen (Lindsey et al., 2001). In another study, pregnancy rates in mares inseminated with flow-sorted sexed semen was reported to be 25% (Lindsey et al., 2002).

4.6.5 Buffalo

In an interesting study, Lu et al. (2007) produced female twin buffalos after transplantation of embryos that were in vitro fertilized with flow-sorted X chromosome-bearing spermatozoa (Lu et al., 2007). In a study, flow sorted sexed semen was evaluated in Mediterranean Italian buffaloes (Bubalus bubalis). This study achieved 43.3% conception rates with X chromosome-bearing spermatozoa and 42.8% conception rates were achieved with Y chromosome-bearing spermatozoa (Presicce et al., 2005).

4.7 Limitations of FACS-Based Sperms Sexing

The mean conception rate for heifers was found to be 56% for convention unsexed semen whereas it was 39% for sexed semen. The dystocia and stillbirths were also more for sexed semen than unsexed semen. Difficult births were lower for sexed semen than conventional unsexed semen (Norman et al., 2010). The sexed semen sorted by FACS-based method was evaluated on Holstein bulls and observed that there was a 13.6% decline in the fertility. Among this decline, about 8.6% was due to the low dose and 5% was due to the sorting process (Frijters et al., 2009). Flow cytometry-based sperm sexing has exhibited some detrimental effects on sperm membrane status and fertility. The percentage of capacitated spermatozoa was increased in the spermatozoa of flow-sorted samples. The capacitation during the flow sorting is reduced by the addition of seminal plasma in collection medium but this decreases the fertility (Maxwell et al., 1998). Even though Hoechst33342 staining procedure is set to be 160 μM for 45 min treatment for FACS-based sperm sexing, Hoechst33342 stain causes a decrease in sperm viability (Quan et al., 2015). The longevity of sex-sorted spermatozoa is lesser than the unsexed spermatozoa in bulls and boars (Rath et al., 2003a, b, Rath et al., 2003b). The cost of sexed semen is around $60 whereas unsexed semen cost is around $15–20. The high cost is due to the high cost of instrument (approximately $3000,000) and slow speed. This expensive instrument can sort only sort 8–10 straws per hour (Boneya, 2021).

4.8 Conclusions

Fluorescent activated cell sorting-based sperm sexing emerged as an indispensable tool in sex preselection for desired animal sex production. The minute difference in the total amount of DNA between X and Y Chromosome-bearing spermatozoa facilitated to develop FACS-based sperm sexing method. The DNA contents of both the sperms were stained with fluorescent and nontoxic dyes to measure the difference in the DNA content. The live spermatozoa were allowed pass through the flow cytometry and to measure, identify, and separate X and Y chromosome-bearing spermatozoa. Initial hurdles of sperm improper sperm head orientation were overcome by the development SX Moflo nozzle that helps in the proper orientation of the sperm. So far FACS-based sperm sexing has been proven as the first commercially successful method across the globe. This method has been applied to the separation of X and Y chromosome-bearing spermatozoa of various farm animals such as cattle, sheep, horses, and pigs. Even though conceptions rates of flow-sorted sexed semen are low in comparison to unsexed semen, FACS-based sperm sexing method has been successful in the production of desired sex animals. Another major limitation of this method is the cost of sexed semen, and it is not affordable to poor farmers in low-income countries. The cost of equipment and low speed of sorting are the major challenges in FACS-based sperm sexing need to be addressed for the production of sexed semen.

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5 Recent Advances and Challenges in the Development of Novel Sperm Sexing Methods

Contents

Abstract

Sperm sexing has emerged as an important tool in the sex selection of farm animals. The use of sexed semen is limited due to high cost that cannot be afforded by poor framers in developing and underdeveloped countries. The sperm damage in sexed semen is also an unsolved problem in the existing sperm sexing methods. This necessitated to develop the novel sperm sexing methods for better and efficient sperm sexing of animal semen samples. The minute differences between the X and Y chromosome-bearing spermatozoa pose a challenge to sperm sexing. Despite the challenges, recent advances in research enable interdisciplinary approaches for sperm sexing. Immunological sexing involves the identification of sex-specific protein and subsequent conjugation with signal enhancers to identify the specific sex spermatozoa. Genome editing technologies have been shown encouraging results in altering sex ratio. The use of nanoparticles in sperm sexing proven to be advantageous in sperm sexing. Recently, a microfluidics method of sperm sexing has been already developed and there is a lot of scope for the development of microfluidics-based sperm

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sexing methods. Raman spectroscopy has proven to be highly accurate in differentiating X and Y chromosome-bearing spermatozoa. Sperm sexing by Raman spectroscopy could be a potential sperm sexing method in near future.

Keywords

Immuno sexing · Genome editing · Raman spectroscopy · Microfluidics · Sperm sexing

5.1 Introduction

The Fluorescence-activated cell sorting (FACS)-based sperm sexing method is the only commercially successful method in terms of sorting efficiency and accuracy (Seidel Jr, 2003). The cost of this sexed sperm is high due to high cost involved in the equipment and low speed of sorting (Amann, 1999). Apart from this, sperm damage was also reported on sexed semen sorted by FACS-based method (Carvalho et al., 2018). Even though it is challenging to develop an alternative efficient method, substantial progress in sperm sexing research has been witnessed in the recent past (Quelhas et al., 2021, Neculai-Valeanu & Ariton, 2021). This chapter discusses the sperm sexing approaches using biological, physical, and chemical methods.

Identification of sperm-specific proteins would facilitate the researchers to sort X and Y chromosome-bearing spermatozoa from animal semen samples (Yadav et al., 2017). This would help to enhance the optical signaling of specific sex spermatozoa by various methods such as fluorescent tagging (Ali et al., 1990). The generation of sex-specific spermatozoa antibodies by sex-specific proteins would be helpful in identifying and sorting specific sex spermatozoa (Blecher et al., 1999). Identification of sex-specific protein on the surface of the spermatozoa would be ideal for sorting of live spermatozoa with minimum physical damage (Rahman & Pang, 2020). Recent research has been focused on the identification of X and Y chromosome-bearing spermatozoa to identify the proteins to sort the sperm cells (Mostek et al., 2020). Identification of differentially expressed proteins can facilitate to develop a novel FACS-based method of sperm sexing. Another alternative method is the disruption of sex-specific genes at spermatogenesis stage. There are some specific genes that are present either on X or Y chromosome-bearing spermatozoa. Recent genome editing methods such as CRISPR/Cas system and Zinc finger nucleases (ZFN), Transcription-activator like endonucleases (TALENs) specially have been reported to disrupt the sex-specific genes that lead to skew the sex ratio (Douglas & Turner, 2020, Kurtz & Petersen 2019). Recent research on sperm sexing also focused on some drugs that specifically alter the motility of either X or Y chromosome-bearing spermatozoa. The drug mechanisms for sex-specific spermatozoa motility needs to be studied and this kind of methods offers enrichment of either X or Y chromosome-bearing spermatozoa at an affordable cost (Umehara et al., 2019; Ren et al., 2021). Nanotechnology applications in sperm sexing have been witnessed in the recent past (Gamrad et al., 2017). Due to its size Nanoparticles

can enter inside live cells and these particles can serve as signal enhancers for a specific type of spermatozoa (Feugang et al., 2019). Nanoparticles can be tagged with biomaterials and targeted to sex-specific spermatozoa (Gamrad et al., 2017; Domínguez et al., 2018; Feugang et al., 2015). Microfluidics is an emerging technology and it has exhibited enormous advantages in sperm research (Knowlton et al., 2015; Nosrati et al., 2017; Smith et al., 2015). Specifically, microfluidics can be useful in sperm sexing based characteristics of X or Y chromosome-bearing spermatozoa such as charge, velocity, and size (Koh, 2015. Dararatana et al., 2015, Wongtawan et al., 2020). Raman spectroscopy applications in sperm sexing are its infancy, and a recent study has demonstrated the precise differences in X and Y chromosome-bearing spermatozoa by using Raman spectra (Huser et al., 2009; Meister et al., 2010; Huang et al., 2013; De Luca et al., 2014). The combination of two or more methods could lead to the development of novel sperm sexing methods with more accuracy and less damage to the live sperm cells.

The small differences in the X and Y chromosome-bearing spermatozoa could be magnified using immunological, nanotechnology, and Raman spectroscopic methods. Genome editing methods could efficiently skew the sex ratio. Physical separation of sperms can be achieved by using microfluidics methods. The development of novel sperm sexing technology needs interdisciplinary research and this chapter deals with different approaches to sperm sexing.

5.2 Novel Methods of Sperm Sexing

5.2.1 Immuno-Sexing

In early studies, HY antigen, expressed on plasma membrane of Y chromosome-bearing spermatozoa, was targeted for the separation of X and Y chromosome-bearing spermatozoa. This strategy involves the generation of specific HY monoclonal antibodies, conjugation of fluorescein-conjugated goat antibodies and subsequent separation using a fluorescent activated cell sorter (Ali et al., 1990; Hendriksen et al., 1993). In contrary to these findings, no significant sperm sexing was observed with HY monoclonal antibodies. The reason could be the removal of HY antigen during epididymal transport and capacitation (Hoppe & Koo, 1984). In an interesting study, sex-specific antibodies were developed in rabbits by injecting sex-sorted bovine X and Y chromosome-bearing spermatozoa. This study identified 30-KDa protein against bovine X chromosome-bearing spermatozoa (Sang et al., 2011). Quelhas et al. (2021) proposed a method of sperm sexing method that involves the identification of plasma membrane proteins and subsequent development of antibodies against sex-specific proteins. This method also suggested that conjugation of antibodies would allow sperm sexing with less damage (Fig. 5.1).

Recent research advances such as two-dimensional electrophoresis and mass spectrometry felicitated to separate and identify the differentially expressed sperm proteins. In a study, two-dimensional electrophoresis in combination with MALDI-TOF MS analysis of human normozoospermic spermatozoa revealed 98 different

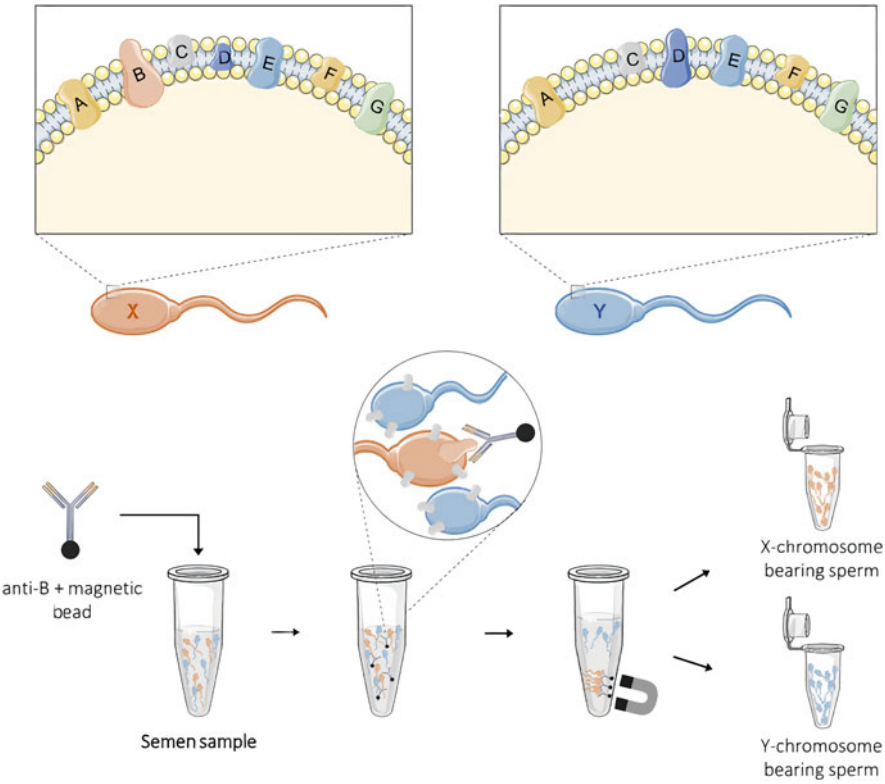

Fig. 5.1 Method proposed for the separation of X- and Y-chromosome-bearing sperm based on differences on plasma membrane protein content. For example, plasma membrane of X-sperm contains protein A, B, C, D, E, F and G, but in Y-sperm plasma membrane, protein B was absent. The development of an anti-B antibody, against protein B exclusively expressed in X-sperm, will allow the recognition of X-sperm only. This anti-B antibody can be coupled to magnetic beads to immunocapture the desired cell population (X-sperm) allowing its separation from Y-sperm, with less damage and potentially less sperm loss than the currently available methods. Reproduced with permission from Quelhas et al. (2021)

proteins (Martínez-Heredia et al., 2006). In another study, 17 different amounts of proteins were observed in asthenozoospermic samples (Martínez-Heredia et al., 2008). In an interesting study, differentially expressed proteins were identified among the bull X and Y chromosome-bearing spermatozoa by using two-dimensional electrophoresis and mass spectroscopy. This study identified 42 differentially expressed proteins and among these spots, 16 proteins were identified by MS analysis (Chen et al., 2012). In another study, nUPLC-MS/MS method was used to identify the differentially expressed proteins of bovine sexed sperm samples. This study observed 17 differentially expressed proteins in X and Y chromosome-bearing spermatozoa. Among these proteins, 15 proteins upregulated in X chromosome-bearing spermatozoa and 2 proteins upregulated in Y chromosome-bearing

spermatozoa (De Canio et al., 2014). In another study, SWATH-MS analysis was performed to identify the differentially expressed proteins on X and Y chromosome-bearing spermatozoa. This study observed four proteins upregulates X chromosome-bearing spermatozoa and four proteins in Y chromosome-bearing spermatozoa Scott et al. (2018). In a recent study, LC/MS MS was used to identify the differential proteins on the X and Y chromosome-bearing spermatozoa of *Bos indicus*. This study noticed 13 differential proteins, and among these, two were found in both X- and Y-sorted bovine spermatozoa, 04 were found or abundant in X-sorted bovine spermatozoa, and seven proteins were found or abundant in Y-sorted bovine spermatozoa (Laxmivandana et al., 2021). The differentially expressed proteins in X and Y chromosome-bearing spermatozoa are summarized in Table 5.1.

In a study, an X chromosome bearing spermatozoa specific gene, extra-embryonic tissue-spermatogenesis-homeobox gene 1 (Esx1), was visualized by confocal laser microscopy. This study used ESX1 antiserum to immunostain the sperm from mixed populations of X and Y chromosome-bearing spermatozoa (Yeh et al., 2005).In another study, Chen et al., 2014 identified the differentially expressed genes in bovine X and Y chromosome-bearing spermatozoa by using suppression subtractive hybridization, cDNA microarray, and sequence homology analysis (Chen et al., 2014). Some of the sex-determining genes in X and Y chromosome-bearing spermatozoa are summarized in Table 5.2.

5.2.2 Genome Editing

The Spermatogenesis development process involves mitotic, meiotic, and post-meiotic phases. During this development process, some genes are expressed that are essential for the development of either X or Y chromosome-bearing spermatozoa (Eddy, 1998). Some of sex-determining genes are summarized in Table 5.1. The sex skewing by disruption of sex-determining genes has been studied in nonmammalians and the results pave the way for implications in mammalians. The Zinc finger nucleases (ZFN), Transcription-activator like endonucleases (TALENs), and the CRISPR/Cas system have been implicated in skewed sex ratio (Xie et al., 2020). In a study, Yano et al., 2014 demonstrated zinc finger nucleases-mediated disruption of sdy (sexually dimorphic on the Y chromosome) gene in *Oncorhynchus mykiss*. Introduction of ZFN mRNA into fertilized eggs resulted in sdy gene mutations in *Oncorhynchus mykiss* (Yano et al., 2014). In another study, sry gene knockout mouse was generated by using TALEN RNA. This study injected TALEN RNA into oocytes at pronuclear stage (Kato et al., 2013). In a recent study, engineered transgenic lines crossed to skew the sex ratio in mouse models. The transgenic cell line with hemizygous g RNA on Y chromosomes and another transgenic cell line with homozygous Cas9 on X chromosomes are mated. This breeding resulted in self-destruction of males (Yosef et al., 2019). In a study, CRISPR/Cas9 system disrupted the high-mobility-group region of the rabbit SRY gene. This study resulted in Hermaphroditism, a disorder that affects the male and female reproductive organs, in rabbit models due to mutations in SRY gene (Song et al., 2018). The Zinc finger X

Table 5.1 Differentially expressed proteins between X- and Y-bovine sperm. Information regarding studies, Uniprot ID, gene name, and protein name were included. Modified from Quelhas et al. (2021) with permission

S. no.	Uniprot ID	Gene name	Characteristics	References
Cell membrane protein in X and Y chromosome-bearing bovine spermatozoa				
1.	F1MY02	N/A	Disintegrin and metalloproteinase domain-containing protein 1b	Laxmivandana et al. (2021)
2.	A4IFP2	KRT4	KRT4 protein	
Cell membrane protein in X chromosome-bearing bovine spermatozoa				
1.	TM190	TMEM190	Transmembrane protein 190	Laxmivandana et al. (2021)
2.	K1C19	KRT19	Keratin, type I cytoskeletal 19	
3.	E1BKT9	DSP	Desmoplakin	
4.	M0QVZ6	KRT5	Keratin, type II cytoskeletal 5	
5.	Q8MJN0	FUNDC2	FUN14 domain-containing protein 2	Scott et al., (2018)
6.	F1MSC3	ACACB	Acetyl-CoA carboxylase, type beta	
7.	P42026	NDUFS7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	
8.	P02784	N/A	Seminal plasma protein PDC 109	De Canio et al., (2014)
9.	P10096	GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	
10.	Q2T9U2	ODF2	Outer dense fiber protein 2	
11.	Q3ZBU7	TUBB4A	Tubulin beta-4A chain	
12.	P19858	LDHA	L-lactate dehydrogenase A chain	
13.	Q29438	ODF1	Outer dense fiber protein 1	
14.	O77797	AKAP3	A kinase anchor protein 3	
15.	Q32LE5	ASRGL1	Isoaspartyl peptidase/L-asparaginase	
16.	Q3MHM5	TUBB4B	Tubulin beta 4B chain	
17.	Q32KN8	TUBA3	Tubulin alpha 3 chain	
18.	Q2TBH0	ODF3	Outer dense fiber protein 3	
19.	Q2KJE5	GAPDHS	Glyceraldehyde 3 phosphate dehydrogenase, testis specific	
20.	Q2YDG7	SPACA1	Sperm acrosome membrane-associated protein 1	
21.	Q5E956	TPI1	Triosephosphate isomerase	
22.	P62157	CALM	Calmodulin	
23.	F1MWY0	NSMAF	Neutral sphingomyelinase activation associated factor	Chen et al., (2012)
24.	P31800	UQCRC1	Cytochrome b–c1 complex subunit 1, mitochondrial	
25.	AAI05544	HIBADH	3-hydroxyisobutyrate dehydrogenase	
26.	Q32KN8	TUBA3	Tubulin alpha-3 chain	

(continued)

Table 5.1 (continued)

S. no.	Uniprot ID	Gene name	Characteristics	References
27.	P41563	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial chain A, the structure of crystalline profilin-beta-actin A chain A, episelection: Novel ki ~ nanomolar inhibitors of serine proteases selected by binding or chemistry on an enzyme surface	
28.	Q3MHM5	TUBB4B	Tubulin beta 4B chain	
Cell membrane protein in Y chromosome-bearing bovine spermatozoa				
1.	EF1A1	EEF1A1	Elongation factor 1-alpha 1	Laxmivandana et al. (2021)
2.	SFP4	N/A	Seminal plasma protein BSP-30 kDa	
3.	E1BC58	RAB2B	RAB2B, member RAS oncogene family	
4.	E1BDP0	LRFN2	Leucine-rich repeat and fibronectin type III domain containing 2	
6.	F1MHK9	LOC524391	Uncharacterized protein	
7.	F1MX68	CPVL	Carboxypeptidase	
8.	E1BKH1	EFHC1	EF-hand domain-containing protein 1	Scott et al., (2018)
9.	E1BPM9	DNAI2	Dynein axonemal intermediate chain 2	
10.	P22439	PDHX	Pyruvate dehydrogenase protein X component	
11.	Q2HJ55	SAMM50	Sorting and assembly machinery component 50 homolog	
12.	P68530	MT-CO2	Cytochrome c oxidase subunit 2	
13.	Q2HJB8	TUBA8	Tubulin alpha 8 chain	De Canio et al., (2014)
14.	Q6B856	TUBB2B	Tubulin beta 2B chain	
15.			Chain A, crystal structure of bovine heart mitochondrial Bc1 with Jg144 inhibitor	Chen et al., (2012)
16.	P00829	ATP5F1B	ATP synthase subunit beta, mitochondrial	
17.	P79136	CAPZB	F-actin-capping protein subunit beta	
18.	Two variants	GSTM3	Glutathione-S-transferase, mu 3 (brain)	

and Zinc finger Y genes involve in the sex determination and RNAi-based disruption of these genes resulted in offspring with an altered sex ratio (Zhang et al., 2017). In a study, siRNA was delivered into pregnant mice through tail vein to silence Sry gene. This study observed the feminization of gonad development in mouse embryos (Wu et al., 2012).

Table 5.2 Some of the important sex-determining genes

S. no.	Name of the gene	References
Y chromosome-bearing spermatozoa-determining genes		
1	Sry	Zhao & Koopman, (2012), Gubbay et al. (1990)
2	Amh	Shahed & Young, (2013)
3	Sox family	Sekido & Lovell-Badge (2008) Polanco et al., (2010), Koopman, (2005)
4	Dmrt1	Matson et al., (2011)
5	Fgf9	Bowles et al., (2010)
6	Gata4	Hu et al., (2013)
X chromosome-bearing spermatozoa-determining genes		
1	Wnt4	Vainio et al. (1999), Ottolenghi et al. (2007), Jeays-Ward et al. (2004)
2	Dax1	Swain et al., (1998)
3	Rspo1	Tomizuka et al., (2008), Chassot et al. (2008)
4	Foxl2	Ottolenghi et al., (2007), Takasawa et al. (2014), Crisponi et al., (2001), Uhlenhaut et al. (2009)

5.2.3 Nanotechnology

Gamrad et al., 2017 identified triplex-forming oligonucleotides (TFOs) as ligands to specifically bind specific region of Y chromosome-bearing spermatozoa of bull semen samples. These TFOs were conjugated with gold nanoparticles for the selection of Y chromosome-bearing spermatozoa. Even though gold nanoparticles could not enter into the intact spermatozoa, gold nanoparticles were found to be powerful tools to enhance signal detection. In a recent study, negatively charged magnetic nanoparticles of 50 nm size were used to separate X and Y chromosome-bearing spermatozoa from Donkey semen samples. Three magnetic nanoparticles per spermatozoa were used to bind the Y chromosome-bearing spermatozoa and unbound X chromosome-bearing spermatozoa separated on magnet exposure for 20 min (Domínguez et al., 2018). The experimental procedure for the magnetic nanoparticle-mediated sperm sexing was illustrated in Fig. 5.2. Apart from sperm sexing, magnetic nanoparticles have been implicated in reproductive improvement. For example, lectin conjugated nanoparticles are used to separate abnormal spermatozoa from pig semen samples (Feugang et al., 2015). In another study, paramagnetic annexin V-conjugated microbeads were used to select the non-apoptotic spermatozoa (Said et al., 2006).

5.2.4 Sex-Specific Alteration of Sperm Motility

In a study, Fujita et al. (2011) identified that Toll-like receptors (TLR) present on the sperm membrane can bind to bacterial endotoxins. This study observed reduced sperm motility and increased apoptosis upon the activation of TLR 2 and TLR 4 by bacterial lipopolysaccharides (Fujita et al. 2011). A subsequent study by the same

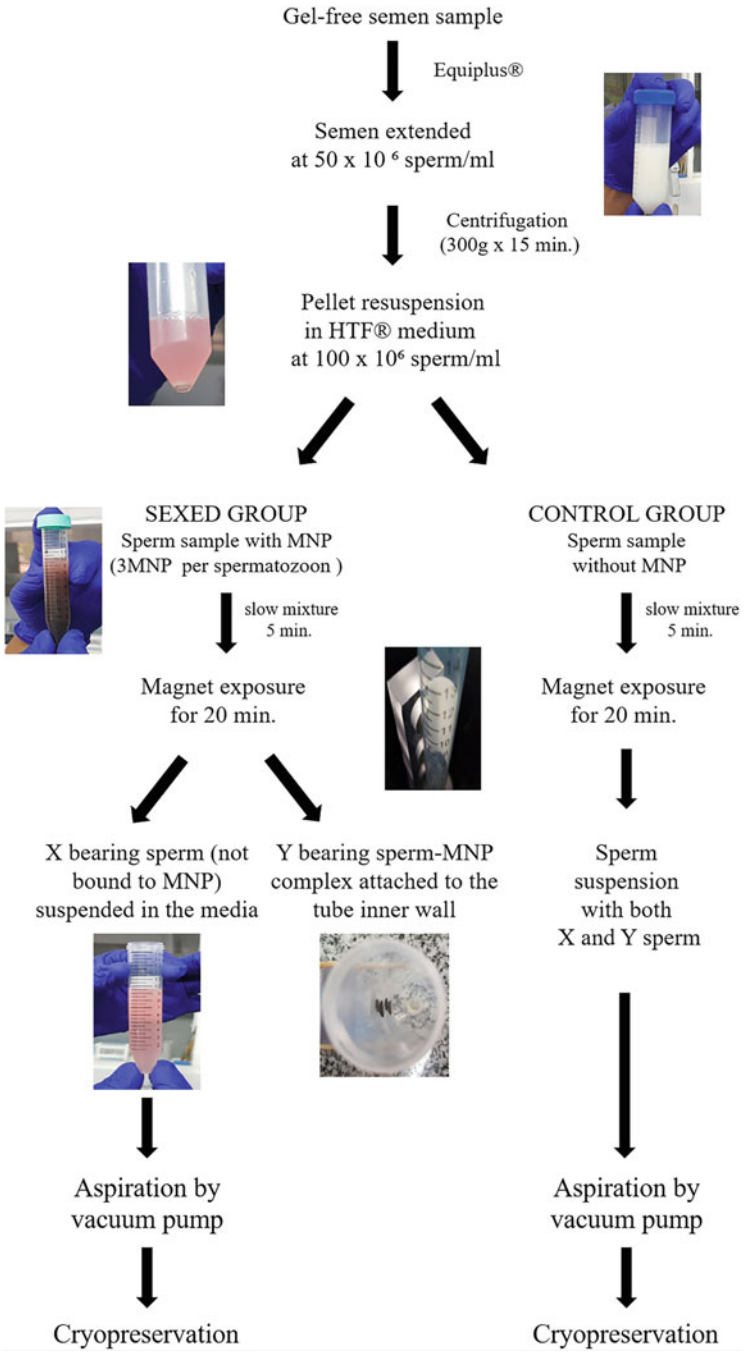

Fig. 5.2 The experimental procedure to obtain sexed spermatozoa by the magnetic nanoparticle technique Reproduced with permission from Domínguez et al., 2018

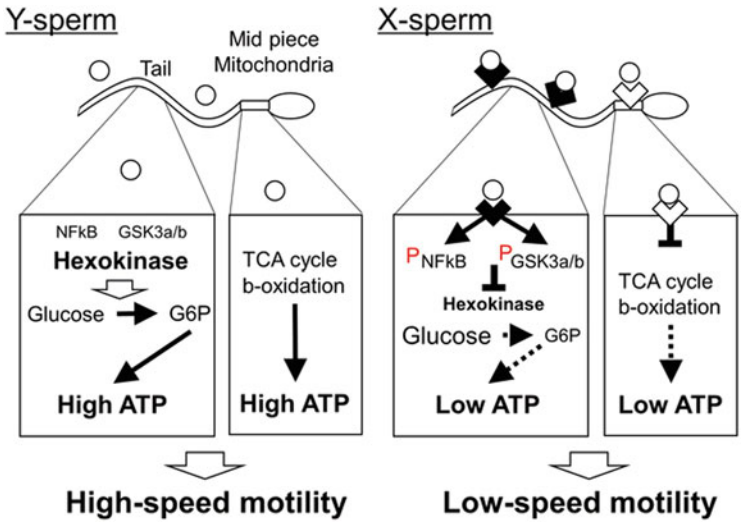

Fig. 5.3 The model of ATP production in Y-sperm and X-sperm under TLR7/8-ligand condition. Reproduced with permission from Umehara et al., 2019

research lab observed that Polymixin B neutralizes the lipopolysaccharides and improved sperm motility (Okazaki et al., 2010). Recently, Umehara et al. (2019) developed a new method of sperm sexing by activating TLR receptors by using TLR-specific ligands. This study observed that TLR7/8 activation by a specific ligand reduced the X chromosome-bearing sperm motility in the mixed semen samples that contain X and Y chromosome-bearing spermatozoa (Umehara et al., 2019). The ATP production in X and Y chromosome-bearing spermatozoa under TLR7/8-Ligand condition is illustrated in Fig. 5.3. After successful demonstration of this strategy in mouse models, bull spermatozoa were treated with resiquimod and produces the cattle embryo by in vitro fertilization method. This method achieved 80–90% of success in sperm sexing in cattle and mice (Umehara et al., 2020). Recently, goat X and Y chromosome-bearing spermatozoa were separated using R848 ligand (Ren et al., 2021). The experimental method is illustrated in Fig. 5.4.

5.2.5 Microfluidics for Sperm Sexing

In a study, the dielectrophoretic force altered the velocities of X and Y chromosome-bearing spermatozoa in various electric fields and viscoelasticity conditions. Further, this study observed that nonuniform dielectrophoresis enhanced sorting efficiency in viscoelastic medium (Koh, 2015). A microfluid device that contains the Tin Oxide (ITO) electrode integrated with microchannel was used to test the effect of dielectrophoresis on the separation of leukocytes and spermatozoa. This study observed 63% efficiency in X chromosome-bearing spermatozoa (Dararatana

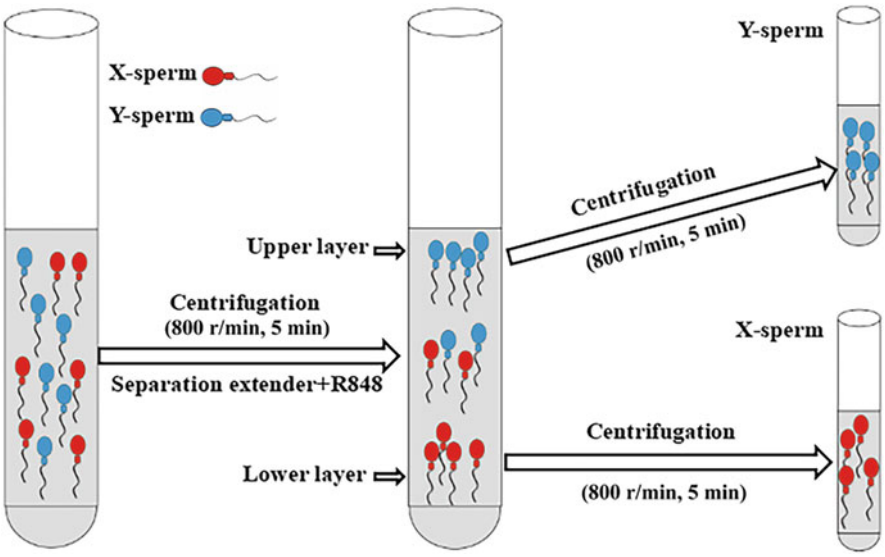

Fig. 5.4 The technical diagram of goat X/Y sperm separation method using R848. The goat semen was centrifuged at 800 r/min for 5 min, resuspended in seperation extender and R848, and incubated on 37 °C. Next, sperm in the upper (Y-sperm) and lower layers (X-sperm) was evaluated. Reproduced with permission from Ren et al., 2021

et al., 2015). The Zeta potential studies revealed that Y chromosome-bearing spermatozoa showed more negative charge than X chromosome-bearing spermatozoa. Furthermore, this study also observes that TALP buffer exhibited more difference in charge of X and Y chromosome-bearing spermatozoa in comparison with other buffers such as HEPES and TRIS buffers (Wongtawan et al., 2018). In a Proof-of-concept study, a microfluidic dielectrophoretic chip is developed to enrich X chromosome-bearing spermatozoa from semen samples. Sperm sexing is achieved on the basis of difference in the dielectrophoresis of X and Y chromosome-bearing spermatozoa upon application of an electric field in the microfluidic device. This device consists of dielectrophoretic and microchannel layers. The dielectrophoretic is connected to power generator and microchannel is connected to silicone tubing. This study observed that during electrophoresis at 4 V 1 MHz, the positive sperm was attached to the electrode and negative sperm was collected at the outlet as it was moved freely in the electric field. This study observed a 30% of reduction of Y chromosome-bearing spermatozoa in a mixed population of spermatozoa (Wongtawan et al., 2020). This study also observed that highest positive dielectrophoresis for X chromosome-bearing spermatozoa at 8 V and 20 MHz, whereas highest positive dielectrophoresis for Y chromosome-bearing spermatozoa at 4 V and one MHz. The experimental setup for this method is illustrated in Fig. 5.5.

Recently, New Zealand-based start company developed a microfluidics-based device to separate X and Y chromosome-bearing spermatozoa. This method uses

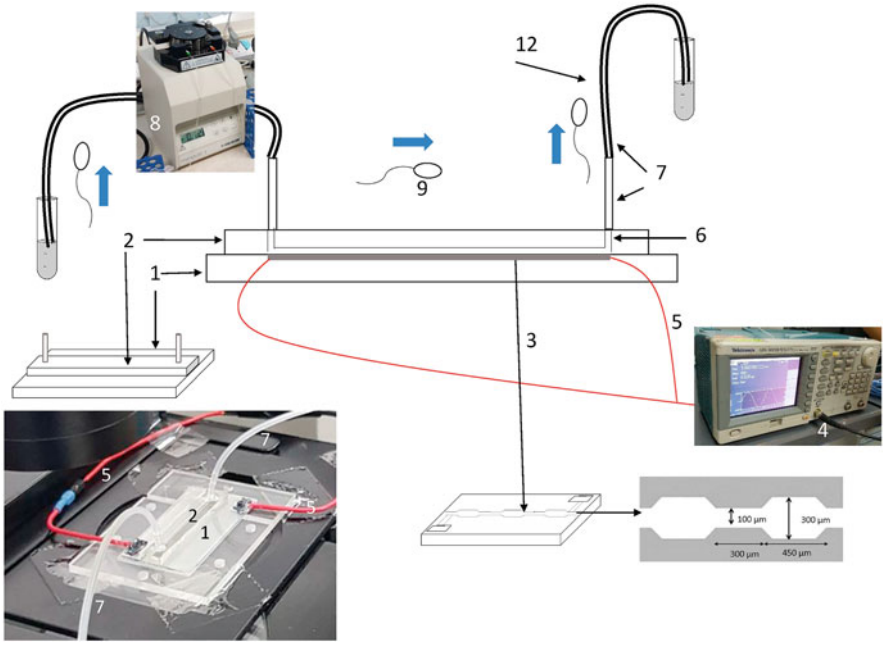

Fig. 5.5 The illustrated image of the microfluidic dielectrophoretic (MF-DEP) system. The sorting chamber includes dielectrophoretic (1) and microchannel layers (2). The dielectrophoretic layer was made from glass and had an electrode pattern (3) which connected to the power generator (4) via electrical wire (5, red line). The microchannel layer was made from silicone and had a microchannel inside (6). the microchannel was connected to silicone tube (7), and the fluid was controlled by a peristaltic pump (8). Sperm (9) moves though the device following the direction represented by thick blue arrows. Reproduced with permission from Wongtawan et al., 2020

radiation pressure to change the direction of movement of a single sperm cell. Similar to FACS-based sperm sexing method. This method detects the X and Y chromosome-bearing spermatozoa by using a DNA-specific fluorescent dye (Simpson & Rohde, 2017 US patent US 9, 784, 663 B2). In comparison to the commercially available FACS-based sperm sexing method, this method would be cheaper and less complex. The commercial success of this method needs to be evaluated in the future.

5.2.6 Raman Spectroscopy in Sperm Sexing

The Raman spectroscopy was first applied to human sperm cells by Huser et al., 2009. This study demonstrated the differences in DNA packaging in normal and abnormal sperm cells (Huser et al., 2009). In another study, nucleus, the neck, and middle piece of a human sperm cell region were analyzed by using Raman spectroscopy (Meister et al., 2010). In a similar study, the sperm head region was envaulted

by using Raman spectroscopy (Mallidis et al., 2011). In another study, micro-Raman spectroscopy was used to improve the sensitivity, specificity, and accuracy in differentiating normal and abnormal sperm cells (Huang et al., 2013). These observations paved the way to differentiate the X and Y chromosome-bearing spermatozoa by using Raman spectroscopy.

In a breakthrough study, the average Raman spectra of 900 bovine sperm cells showed significant differences in X and Y chromosome-bearing spermatozoa. Raman bands at 726, 785, 1095, and 1581 cm^{-1} showed high peak area for X chromosome-bearing spermatozoa than Y chromosome-bearing spermatozoa. This study opened the doors to sperm sexing by using Raman spectroscopy (De Luca et al., 2014). Recent advances in Raman spectroscopy facilitate to sorting the cells in solution and microfluidic flow (Song et al., 2016). Applications of single-cell Raman spectra in sperm sexing would be a noninvasive and potential alternative to existing FACS-based sperm sexing method.

5.3 Conclusions and Future Outlook

The small difference in the DNA content of X and Y chromosome bearing spermatozoa is facilitated the sperm sexing by using FACS. Since the commercialization of this method, none of the alternative methods have proven commercially successful. The recent success of microfluidics-based sperm sexing method has been developed and it needs to be evaluated in the near future. Immunological methods have gained momentum and identification of sex-specific proteins could allow to develop simple and efficient sperm sexing methods. Genome editing is a powerful tool to silence the genes and the implication of genome editing in sperm sexing has started in the recent past. Silencing of sex-specific genes altered the sex ratio in non-mammals and recently it altered the sex ratio in mouse models. Nanoparticles have the potential to invade into live sperms and implications of nanotechnology in sperm sexing would enhance the detection capabilities of specific sex spermatozoa. Raman spectroscopic bands successfully differentiated X and Y chromosome-bearing spermatozoa and this method would be a potential alternative to -based sperm sexing method. In conclusion, multidisciplinary researchers work together to integrate biological, physical, and chemical to successful sperm sexing methods with high accuracy, less sperm damage, and low cost.

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Semen Extenders for Preservation of Sorted Semen

6

Contents

Abstract

Semen extenders play vital role in the storage of semen samples for the storage of spermatozoa that is capable to fertilize the egg in vitro and in vivo conditions. Artificial insemination requires a greater number of viable and motile spermatozoa in the stored or fresh semen samples. The sex-sorted semen samples need long-term storage and commercial sexed semen straws should provide the protection to spermatozoa during transport. Furthermore, sperm sexing process causes mechanical damage to the spermatozoa. The addition of appropriate concentration of additives in existing semen extenders would lead to the long-term maintenance of viable and intact spermatozoa during and before the artificial insemination. The viability and motility of cryopreserved semen can be improved by including various protectives such as antioxidants, cryoprotectants, sugars, and antimicrobials to the conventional and commercial semen extenders.

V. K. Yata, *Sperm Sexing and its Role in Livestock Production*,
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Keywords

Cryopreservation · Semen extenders · Sperm sexing · Cryoprotectants · Artificial insemination

6.1 Introduction

The fresh ejaculate collected from the animals need to be preserved to maintain the viability, motility, and membrane integrity of the spermatozoa until it is used for artificial insemination. The spermatozoa in the semen samples encounter several detrimental factors such as mechanical damage, exposure to dyes, freezing, thawing, microbial growth, and stress-related factors. The success of artificial insemination depends on the number of presences of competent spermatozoa in the stored semen samples. Similarly, utmost care should be taken to preserve the sorted semen to improve the efficiency of sorted semen. The sperm storage leads to release of reactive oxygen species and singlet oxygen species. The enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and nonenzymatic antioxidants such as trehalose and zinc sulfate to semen extenders promote semen quality during the short- and long-term storage. The cryoprotectants are required to prevent the damage that occur due to freezing and to eliminate the ice formation in the cryopreservation of spermatozoa. The cryoprotectants such as glycerol, ethylene glycol, and dimethylformamide are included in the semen extenders to improve the quality of cryopreserved semen samples. The sugars are the main energy source in the semen extenders. The sugars such as fructose, glucose, trehalose, and sucrose are added to the semen extenders. The microbial contamination of preserved semen is a major problem in semen storage. The addition of antibiotics such as Penicillin, Gentamicin, amikacin, tylosin, lincomycin, and spectinomycin semen extenders protects from microbial contamination of semen samples during storage.

Sperm protection is essential during the sperm sorting and storage of the semen samples. This chapter provides an overview of various antioxidants, sugars additives, and antimicrobial agents used in semen extenders.

6.2 Additives in Semen Extenders

The supplements such as antioxidants, cryoprotectants, sugars, antimicrobials, and vitamins have been used for the development of sperm cryopreservation. Some of the important supplements and their functions are summarized in Table 6.1.

6.2.1 Antioxidants

The spermatozoa release reactive oxygen species during cryopreservation and the mechanism is summarized in Fig. 6.1. In a study, the addition of 15 IU/mL each of

Table. 6.1 Extender development for sperm cryopreservation. Reproduced with permission from Ugur et al. (2019)

Supplement	Functions/effects	References
Cryoprotectants		
Egg yolk	Low-density lipoproteins (LDL) in egg yolk bind cell membrane and form an interfacial film during the freezing process	Anton et al. (2003)
Milk	Protein fraction of skim milk protects sperm cells from cryo-injury	Medeiros et al. (2002)
Glycerol	Responsible for membrane lipid and protein rearrangement	Barbas & Mascarenhas (2009)
Ethylene glycol	Reduce intracellular ice formation by increasing dehydration at lower temperature	Holt, (2000)
Dimethyl sulfoxide		
Propylene glycol		
Trehalose	Replace the bound water surrounding macromolecules and protectively hydrate those macromolecules by substituting for water	El-Sheshtawy et al. (2015)
Polyols	Create hydrogen bonds with membrane lipids; thus, lipids of sperm membrane are stabilized at low temperatures	Bailey et al. (2000)
Fatty acids	Increase post-thaw viability, motility, and acrosome integrity by improving plasma membrane fluidity and integrity	Tarig et al., (2017), Kaka et al., (2015), Ejaz et al. (2014)
– Docosahexaenoic acid (fish oil)		
– Lauric acid (coconut oil)		
– α-Linoleic acid		
– Palmitic acid		
– Oleic acid		
Iodixanol	It assumed that reducing ice crystal formation protects the spermmembrane; thus, increases post-thaw sperm motility	Swami et al. (2017), Saragusty et al. (2009)
Butylated hydroxytoluene	Enhances motility, acrosomal integrity, and membrane integrity by increasing membrane fluidity and reducing the activity of the lipid peroxyl radicals	Ijaz et al. (2009), John Aitken (1995)
Antioxidants		
Glutathione	Glutathione supplementation increase motility, plasma membrane integrity, and viability	Ansari et al. (2012)
Resveratrol	Extinguishes superoxide, hydroxyl, and metal-induced radicals. Therefore, it protects sperm Chromatin and membranes from ROS damage	Sarlós et al., (2002)

(continued)

Table. 6.1 (continued)

Supplement	Functions/effects	References
Vitamin E	Affects sperm motility, membrane integrity, and membrane potential positively	Peña et al., (2003)
Bovine serum albumin	Helps to maintain the cell morphology and acrosome integrity, and to increase its catalase (CAT) activity	Sariözkan et al. (2009)
Methionine	Maintain normal sperm morphology	Bucak et al. (2010)
Carnitine	Improve acrosome integrity, sperm motility, and reduce DNA damage	Bucak et al. (2010)
Inositol		
Spirulina maxima extract	Increase the motility and viability of sperm cells, and reduce ROS synthesis and protect DNA structure	Mizera et al. (2019)
Selenium	Improve morphology and integrity of cryopreserved sperm	Zubair et al. (2015)
Vitamins		
Vitamin C	Vitamin C supplementation increase post-thaw motility and percent of intact plasma	Ansari et al. (2012)

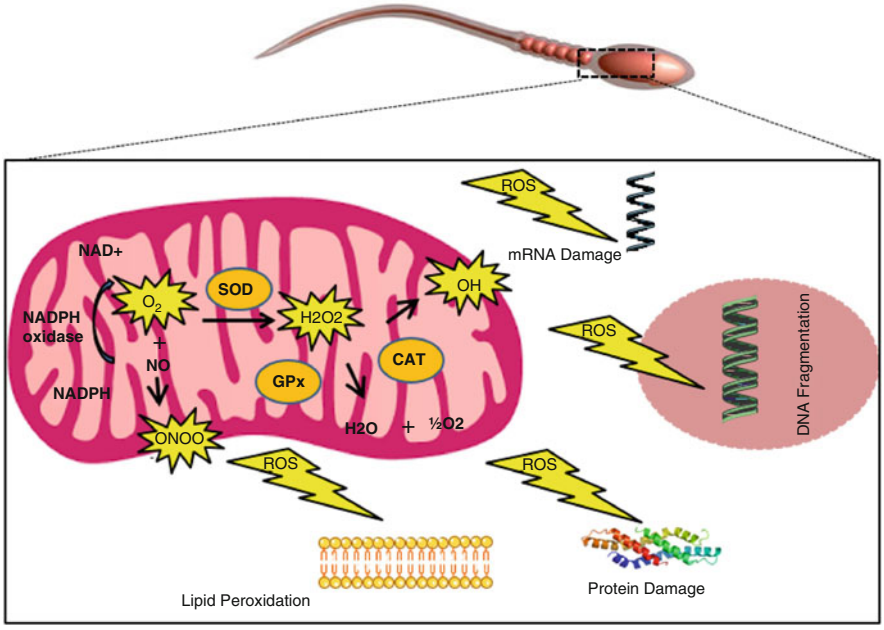

Fig. 6.1 A summary of the production of reactive oxygen species (ROS) in cryopreserved sperm mitochondria that leads to reduced sperm quality. *NADPH* dihydronicotinamide-adenine dinucleotide phosphate, *NAD* nicotinamide adenine dinucleotide, *O2°* superoxide, *H2O2* hydrogen peroxide, *SOD* superoxide dismutase, *ONOO* peroxynitrite, *GPx* glutathione peroxidase, *CAT* catalase. Reproduced with permission from Hezavehei et al. (2018)

superoxide dismutase, catalase, and glutathione peroxidase to the stallion semen samples improves the semen quality by reducing the reactive oxygen species-mediated sperm damage. These enzymes suppress the caspase-3 activation and aids the preservation of sperm viability and motility of stallion spermatozoa during cryopreservation (Del Prete et al., 2019). In another study, the addition of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase to semen extenders resulted in the prevention of reactive oxygen species formation (Kankofer et al., 2005). In a study, nonenzymatic antioxidants such as trehalose and zinc sulfate improve the quality of cryopreserved Arabian stallion spermatozoa (Ghallab et al., 2017). The addition of Coenzyme Q10 as an antioxidant in tris-extender exhibited an advantageous effect on buffalo and cattle semen preservation by improving the motility and viability of cryopreserved spermatozoa (Saeed et al., 2016). In a study, the addition of 5 IU Vitamin E per ml or 1–2 mM glutathione to a semen extender exhibited the improved sperm viability of ram semen samples (Zeitoun & Al-Damegh, 2014). The addition of antioxidants such as glutathione, superoxide dismutase (SOD), ascorbic acid, hypotaurine, 2,2,6,6-tetramethylpeperidine-1-oxyl, and 4-hydroxy-2, 2, 6, 6-tetramethylpeperidine to a whole milk glycerol extender was not effective in improving the quality of bull semen samples except the improvement of 6–10% of motility (Foote et al., 2002). The addition of 100 and 200 IU mL^{-1} catalase to tris-egg yolk semen extender exhibited improved sperm viability in Holstein bull semen samples(Asadpour et al., 2011). The supplementation of manganese (III) tetrakis (4–69 benzoic acid) porphyrin to a semen extender improved the sperm motility and viability of stallion semen samples (Treulen et al., 2019). The addition of 1 mM of glutathione peroxidase a nano lecithin-based extender resulted in improved in vitro fertility of cryopreserved bull semen samples (Mousavi et al., 2019). Andrabi et al. (2008a) suggested the addition of Vitamin E as a nonenzymatic antioxidant in Tris-citric acid extender to improve the quality of cryopreserved buffalo semen samples. In another study, the addition of 30 mM trehalose to an extender resulted in improved fertility in Nili Ravi buffaloes (Iqbal et al., 2016). Da Silva Maia et al. (2010) suggested the use of Trolox and catalase as antioxidants in the semen extenders to improve the cryopreserved ram semen samples. The addition of glutathione and cysteine to a semen extender improved the quality of boar spermatozoa when stored at 10 °C for 29 days (Funahashi & Sano, 2005).

6.2.2 Cryoprotectants

Gil et al. (2003) observed that the addition of glycerol at 5 °C to a milk-based extender with low 5–6% concentrations of egg yolk resulted in the improved quality of cryopreserved semen samples. The addition of 4% glycerol to 50% (v/v) coconut water media was found to be a safe semen extender without the addition of antibiotics. This extender showed high motility and viability of semen samples of cattle bulls (El-Sheshtawy et al., 2017). The Tris-based extender with 20% egg yolk plus 3% glycerol was suggested for the improvement of sperm motility, viability in

cryopreservation of collared peccaries semen samples (Alves et al., 2013). In a study, the effect of glycerol and ethylene glycol were evaluated on an extender containing TRIS-citrate, egg yolk, and glucose and another extender containing skim milk, egg yolk, and fructose. This study observed the higher viability and motility of spermatozoa in alpaca (*Lama pacos*) semen samples in the extender containing skim milk, egg yolk, and fructose with ethylene glycol as a cryoprotectant (Santiani et al., 2005). The effect of cryoprotectants such as glycerol, 1,2-propanediol, sucrose, and trehalose was evaluated in three different extender osmolality levels on various parameters of ram spermatozoa. This study suggested that the detrimental effects of cryoprotectants can be stunned by the use of glycerol in combination with sugar to increase the osmolarity of the extenders (Soylu et al., 2007). The effect of combination of soy milk-based extender with cryoprotectants such as glycerol and dimethylformamide was evaluated on cryopreservation of ram semen samples. This study showed no advantageous effect with the combination of soy milk-based extender with dimethylformamide but significant embryo production rates were observed with the combination of soy milk-based extender with glycerol (Jerez et al., 2016). In a study, tris extender in combination with glycerol and lycopene showed high motility of spermatozoa in bull semen samples. This study also observed low motility of spermatozoa when tris extender was used in combination with glycerol and cysteamine (Tuncer et al., 2014). In a study, a combination of cryoprotective such as glycerol, ethylene glycol, dimethyl sulfoxide, methyl formamide, and dimethylformamide exhibited improved sperm quality in cryopreservation of horse semen samples (Wu et al., 2015). Yang et al. (2016) suggested that the semen extender with glycerol combined with dry ice straw freezing method resulted in high quality cryopreserved boar semen samples. Tonieto et al., 2010 suggested that the use of trehalose and low-density lipoprotein in semen extenders showed similar effects to using conventional cryoprotectants. Herbowo et al., 2019 suggested that 7% glycerol or 5% dimethylformamide in Skim Milk, Yolk-based extender for cryopreservation of Swamp Buffalo Semen samples.

6.2.3 Sugars

In a study, the effect of sugars such as fructose, glucose, trehalose, and sucrose were evaluated on Boer goat semen samples. This study observed improved sperm motility on addition of glucose in semen extender. The combination of glucose and fructose resulted in improved quality of cryopreserved semen samples (Naing et al., 2010). The addition of 100 mM trehalose or 100 mM raffinose in extenders was found to increase more than 30% of the fertility rates than control semen samples (Jafaroghli et al., 2011). The addition of 70 mM of fructose in egg yolk tris extender resulted in high-quality semen after cryopreservation of canine semen samples (Ponglowhapan et al., 2004). In a similar study, the addition of 5 mM and 10 mM fructose in skimmed milk extender improved the cryopreserved semen quality of Nili-Ravi Buffalo (Akhter et al., 2010). The sperm acrosome damage was reduced in dog semen samples cryopreserved with TRIS-citric acid extender

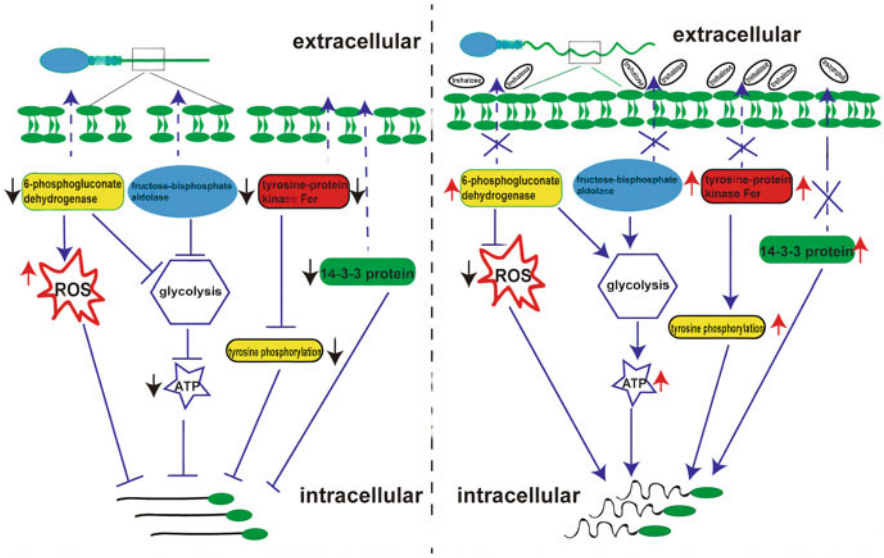

Fig. 6.2 The speculated protective mechanism of trehalose during cryopreservation of ram spermatozoa. Reproduced with permission from Jia et al. (2021)

containing galactose, lactose, trehalose, maltose, and sucrose. This study also observed the improvement of sperm motility by the addition of fructose and xylose in semen extenders. Trehalose, xylose, and fructose improved the sperm activity rates on cryopreserved semen samples (Yildiz et al., 2000). The addition of glucose and fructose in semen extenders aid in the maintenance of the sperm motility and functional integrity of the membrane upon the refrigeration of semen samples at 5 °C (Flores et al., 2010). The addition of trehalose and sucrose to Tris-citric acid-egg yolk-fructose extender resulted in the improved sperm motility, viability, and membrane integrity of cryopreserved bull semen samples (El-Sheshtawy et al., 2015). In a study, the addition of trehalose showed more beneficial effects than sucrose in the cryopreservation of sheep semen samples (Quan et al., 2012).In another study, it was observed that the addition of sucrose and trehalose aid in the maintenance of membrane integrity of sperm cells of Markhoz Goat semen samples (Khalili et al., 2009). In a recent study, the effect of trehalose on the proteome of ram spermatozoa was studied, and this study observed the trehalose-mediated change in the protein profile of cryopreserved ram spermatozoa (Jia et al., 2021). The proposed protective mechanism of trehalose during cryopreservation of ram spermatozoa is illustrated in Fig. 6.2.

6.2.4 Egg Yolk

The lipid-binding proteins present on the surface of spermatozoa induce cholesterol and phospholipid removal from the sperm membrane. This led to detrimental effects on sperm storage. Some of the detrimental proteins interact with low-density lipoproteins of egg yolk extenders and this minimizes the loss of cholesterol and phospholipid removal from sperm membranes. This interaction led to the improved efficiency of cryopreservation of semen samples (Bergeron & Manjunath, 2006). In a study, the effects of egg yolk on sperm motility and acrosome integrity were evaluated in preservation of goat spermatozoa. The egg yolk improved the motility of spermatozoa in cryopreservation of spermatozoa. This study also found that a combination of glycerol and egg yolk reduced the proportion of acrosome-intact spermatozoa in goat semen samples (Aboagla & Terada, 2004). In a study, semen quality and felid fertility were evaluated on various bull semen samples cryopreserved with TRIS-egg yolk extender. This study showed better semen quality of semen samples cryopreserved with TRIS-egg yolk extender than soybean extract (Thun et al., 2002). In another study, egg yolk extender showed less motility than soy lecithin extender (Aires et al., 2003). Forouzanfar et al. (2010) suggested that lecithin can be used as a potential alternative to egg yolk in cryopreservation of ram semen. The skim milk-egg yolk-based semen extender was shown to act as a nonenzymatic antioxidant in the cryopreservation of equine semen samples (Bustamante Filho et al., 2018). Alcay et al. (2015) observed that lyophilized egg yolk-based extender can provide similar cryoprotective effects in cryopreservation of ram semen samples. Van Wagtendonk-de Leeuw et al. (2000) observed fertility when TRIS concentrate extender combined with pasteurized egg yolk for cryopreservation of bovine semen. The duck egg yolk in semen extender was found to be advantageous in cryopreservation buffalo bull spermatozoa in comparison with Guinea fowl egg yolk and Indian indigenous hen egg yolk. The duck egg yolk improves the post-thaw quality of cryopreserved semen (Andrabi et al., 2008a, b, c). In a study, the addition of trehalose sugar in an egg yolk extender enhanced the sperm viability and fertility parameters of boar semen samples (Malo et al., 2010). Pillet et al. (2011) revealed that sterilized egg yolk plasma can be replaced with egg yolk in the extenders and this replacement exhibited slight differences in the motility parameters of stallion semen samples. In a study, egg yolk-based extenders showed more efficiency than soy lecithin extenders in terms of sperm viability and fertility of bovine semen samples (Crespilho et al., 2012). In a study, the addition of butylated hydroxytoluene in Tris-egg yolk extender reduced the oxidative stress of cryopreserved Boer goat semen samples (Memon et al., 2011). The egg yolks of different avian species such as domestic chicken, the goose, turkey, duck, Japanese quail, and chucker, were evaluated on cryopreservation of ram semen samples. This study observed that chucker egg yolk could be potential to replace chicken egg yolk in the extenders (Kulaksız et al., 2010). The use of a combination of egg yolk and glycerol in the semen extender resulted in a synergistic effect in terms of sperm viability (Pace & Graham, 1974). In an interesting study, life span of spermatozoa was increased when the semen extenders containing 10% egg yolk (PH unadjusted)

was cooled in water bath (Ferdinand et al., 2014). In a previous study, it was observed that addition of taurine or trehalose to a Tris-based egg yolk extender reduced the damage of cryopreserved buffalo semen samples (Reddy et al., 2010).

6.2.5 Antimicrobials

The combination of streptomycin and penicillin in sodium citrate-egg yolk extender was shown to be effective in refrigeration of bull semen storage up to 20 days at 4–5 °C (Almquist et al., 1949). The combination or alone of streptomycin and penicillin exhibited improved fertility of bull semen samples of low fertility (Almquist, 1951). The combination of Gentamicin, tylosin, lincomycin, and spectinomycin in tris-citric acid-based extender was tested on Sahiwal bull semen samples. This study showed improved pregnancy rates on one day cryopreservation semen samples at −196 °C (Andrabi et al., 2001). In a study, the addition of Ceftiofur and tylosin to a semen extender exhibited bacterial control in the bull semen preservation for seven days at −145 °C (Gloria et al., 2014). Akhter et al. (2008) observed that the combination of Gentamicin, tylosin, lincomycin, and spectinomycin in Skimmed milk-based semen extender is capable of bacterial control in the preservation of Buffalo (*Bubalus bubalis*) Bull Semen samples at 5 °C for 3 days. In the same study combination of Streptomycin and penicillin G in semen extender was compared but it is less efficient than Gentamicin, tylosin, lincomycin, and spectinomycin combination. The addition of Ciprofloxacin in Tris-citric acid extender showed 55% of fertility rates of cryopreserved water buffalo bull semen samples (Akhter et al., 2013). The combination of Gentamicin, tylosin, lincomycin, and spectinomycin in Tris-citric acid extender for cryopreservation of buffalo (*Bubalus bubalis*) semen samples showed improved in vivo fertility (Andrabi et al., 2016). Moustacas et al. (2010) observed that a combination of penicillin and streptomycin or gentamicin alone can control the *Brucella ovis* and Actinobacillus seminist in cryopreservation of ovine semen samples. The addition of Cefdinir or Cefoperazone sodium in Sodium citrate-fructose-egg yolk extender preserved the ram semen samples when stored at 5 °C for 4 days (Azawi & Ismaeel, 2012). Bryła and Trzcińska (2015) observed higher reproduction rates in boars upon the addition of Gentamicin and florfenicol to Biosolwens Plus extender for storage of boar semen at 15 °C for 10 days. The addition of gentamycin either in Beltsville Thawing Solution or in Androstar Premium extender allows to store the boar semen samples at 17 °C for 3 days (Waberski et al., 2019). The addition of amikacin sulfate to Tris-egg yolk extender allowed to store stallion semen samples at 4 °C for days without affecting the motility of spermatozoa (Arriola & Foote, 1982). The combination of Penicillin G and amikacin or Ticarcillin-clavulanic acid or Ceftiofur in Skim-milk glucose extender controlled the bacteria in the stallion semen samples upon storage at 5 °C for 1 day (Varner et al., 1998). The addition of Gentamicin, Cefquinome in EquiPro© extender allowed to decontaminate the stallion semen samples (Price et al., 2008; Parlevliet et al., 2011). The addition of Ticarcillin-clavulanic acid (Olivieri et al., 2011), Meropenem (Hernández-Avilés et al., 2018), combinations

of Penicillin G and amikacin (Olivieri et al., 2011), Penicillin G and gentamicin (Neto et al., 2015), Penicillin G and amikacin (Hernández-Avilés et al., 2018), to various semen extenders allowed to control stallion semen samples on storage.

6.2.6 Other Additives

The addition of cysteine in Citric-whey extender improved the motility of cryopreserved semen samples of buffalo semen by destruction of spermicidal toxin (Singh et al., 1989). The Iodixanol in Tris-egg yolk extender act as a non-penetrating cryoprotectant to improve the viability and motility of spermatozoa of Thai native bull semen samples (Chuawongboon et al., 2017). The lipid profile of the sperm plasma membrane was restored by the addition of Cholesterol-loaded cyclodextrins in Tris-egg Yolk-Glucose extender and it increased the post thaw motility and viability of spermatozoa of buffalo semen samples (Ezz et al., 2017). Sperm plasma membrane phospholipids were restored by the addition of soy-lecithin in Soya milk-based extender. This additive improved the sperm membrane integrity, viability, and motility of spermatozoa in buffalo semen samples (Singh et al., 2012). The physiological properties of the lipid bilayer of spermatozoa are maintained in the cryopreservation by the addition of docosahexaenoic acid in BioXcell® extender. The sperm morphology, motility, and viability are also maintained with docosahexaenoic acid (Kaka et al., 2017). The addition of regucalcin in tris-citric acid-fructose-egg yolk-glycerol exhibited an advantageous effect in improving the quality of cryopreserved water buffalo semen samples (Pillai et al., 2017). The presence of curcumin in the Tris-citric acid extender promotes the improves the antioxidant property of the extender and this improved antioxidant property led to the better semen quality of cryopreserved buffalo semen (Shah et al., 2017). The addition of Sodium pyruvate in Triladyl® extender exhibited hydrogen peroxide scavenger activity in the cryopreservation of bull semen samples (Korkmaz et al., 2017). The addition of Epidermal growth factor in Bioxcell® extender Inhibited the propagation of the peroxidative chain reaction in cryopreserved buffalo semen samples (Kandiel et al., 2017). The Lycopene act as a scavenger of singlet oxygen and reactive oxygen species in Triladyl® extender to improve the quality of cryopreserved bovine semen (Tvrda et al., 2017). The addition of vitamin B12 in tris-Citrate-Fructose egg yolk prevents the free radical generation in cryopreservation of bovine semen (Hu et al., 2011). The addition of Melatonin in Citrate-egg yolk neutralizes the free radicals and stimulates the antioxidants such as catalase and superoxide dismutase (Ashrafi et al., 2013). The addition of Alpha-tocopherol in Bioxcell® extender scavenges the reactive oxygen species to improve the quality of bull semen samples (Motemani et al., 2017).

6.3 Conclusions

The fresh ejaculates of animals sustain after the addition of diluents or extenders that consists of buffers, antioxidants, cryoprotects, sugars, and antimicrobials. The viability, motility, and membrane integrity are the three major factors evaluated in semen samples. The sorting process involves mechanical damage and semen storage involves freezing and thawing stress. The microbial growth should be prevented during the sorting process and storage of semen samples. The selection of antibiotics plays a vital role in the prevention of microbial contamination. The combination of antibiotics with low concentrations are suggested by the previous to protect the spermatozoa and eliminate the microbes. The additives also showed some detrimental effects during semen storage and selection of suitable additives specific to the particular species of animals would be advantageous. Even though numerous commercial semen extenders are available in the market, the addition of suitable additives would result in improving the quality of cryopreserved semen.

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Commercial and Ethical Aspects of Sperm Sexing

7

Contents

Abstract

Applications of sexed semen in farm animals breeding have been in rise in the last decade majorly due to economic and commercial reasons. The invention of flowcytometric based sorting method of X and Y bearing spermatozoa revolutionized the animal industry for production desired sex animals. In recent years, the development of the orienting nozzle of flow cytometry improved the efficiency of sexed sperm sorting efficiency.

Commercialization of sperm sexing technology leads to the availability of sexed semen in some of the food-producing animals like dairy cows and beef cattle. The cost of sexed semen is high in comparison with non-sexed semen due to the availability of very few sperm sorting methods and high expenditure involved in the invention of current, successful sperm sexing method. A recent patent dispute between ABS Global, INC., *and* Genus PLC.Vs Inguran, LLC, *doing business as* Sexing Technologies *and* XY, LLC also indicates the competition between the firms for the commercialization of animal sexed semen. As there is an increasing demand for sexed semen from animal farmers and the animal farming industry, there are some ethical objections raised by animal welfare societies worldwide. Uncontrolled use of sexed semen can provoke the

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animal sex ratio imbalance, and there is a dangerous threat to biodiversity. It is combined responsibility of the society, veterinarians, and animal welfare societies to ensure the safe and efficient use of sexed semen by considering ethical issues.

Keywords

Sperm sexing · Sexed semen · Animal ethics · Animal breeding · Animal farming industry

7.1 Introduction

Sexed semen has wide applications both in animals and in humans (Garner, 2001; Sills et al., 1998). There are more than 300 human diseases that are sex-linked, and sperm sexing has a great impact on the prevention of genetic disorders (Levinson et al., 1995). Sexed semen helps to preselect the sex of progeny to avoid sex-linked diseases (Johnson et al., 1993). Sperm sexing is a method of separating X-and Y-bearing spermatozoa from the semen samples (Seidel Jr, 2007). In recent times, human dependency has been increased on animals for food products like milk and meat (Speedy, 2003). It is difficult for animal farm owners to maintain the desired sex ratio of animals in animal farms (Johnson, 1998). Dependency on natural mating may not be beneficial for animal farm owners and animal farmers (Trivers & Willard, 1973). Artificial insemination is a regular practice for animal farm owners, and recently, it has been introduced to animal framers (Foote, 1982). Artificial insemination cannot preselect the sex of the offspring, and it is to avoid the delay caused by natural mating of animals (Morrell, 2011). After a breakthrough discovery of sperm sexing based on DNA content difference in X- and Y-bearing spermatozoa by Johnson et al. (1987), initial experiments at Oklahoma State University (OSU) and Lawrence Livermore National Laboratory (LLNL), USA paved the way to the development of Beltsville Sperm Sexing Technology at the United States Department of Agriculture (Johnson et al., 1999). Sperm sexing technology was first commercialized by the Colorado State University Research Foundation (CSURF) and XY, Inc. (Garner & Seidel Jr, 2008).

Now, very few companies leading in the development and commercialization of sexed semen across the world. Continuous development in the flow cytometric sperm sexing resulted in the improvement of the efficiency of sperm sexing (Sharpe & Evans, 2009). More than 100 patents were filed across the globe on methods and apparatus of semen. US Patent Number 8,206,987 on photodamage sperm sexing approach provoked the dispute between leading bull studs, namely, Sexing Technologies, and ABS Global, INC. Sperm damage is the major concern in the existing sperm sexing methods (Suh et al., 2005). Toxicity of the fluorescent dye, being used in the sperm sexing techniques, raises doubts about the implications of sperm sexing methods (Downing et al., 1991). Long-term toxic effects of dye have not been studied in sexed animals. Even though rapid developments are noticed in sperm sexing methods, researchers should find ways to the implication of safe,

efficient use of sexed semen. Animal welfare societies argue that freedom of animals should not be compromised in the artificial breeding methods (Clark et al., 2006). They also suggest that natural mating methods should be preferred to avoid the physical discomfort, pain in the animals, and natural behavior of the animals to be protected (Harrington et al., 2013). Contrary to the animal's societies, researchers argued that there is a dangerous threat of losing the endangered species and sperm sexing is the essential method to protect them (Andrabi & Maxwell, 2007; Kalfoglou et al., 2013). There are biased views on sex ratio imbalance as a result of implementing sperm sexing methods in animals. Animal welfare societies cautions that the implication of sexed semen results in the increase in the number of desired sex animals in the particular region but researchers oppose the view as sperm sexing is the ultimate way to maintain the animal sex ratio if it is used properly (Gledhill, 1983).

This book chapter reviews the historical events of sperm sexing research and commercialization aspects of sperm sexing methods. This chapter also updates the patent-related issues of existing sperm sexing techniques and discusses the safety issues of implementation of sexed semen insemination methods. The final part of this chapter deals with the ethical issues raised by the animal welfare societies and counter-arguments of the animal reproduction biologists.

7.2 History and Commercialization of Sexed Semen Technology

The first successful measurement of DNA content of mammalian sperm was achieved by Otto et al. (1979). In this study, sperms were stained with 4–6 diamidino-2-phenylindole (DAPI) and measurements were performed using the pulse cytophotometer ICP 22 (Phywe, G6ttingen, Germany). High-Resolution DNA Content Measurements of Mammalian Sperm were achieved by DNA staining with 4–6 diamidino-2-phenylindole (DAPI) or an ethidium bromide mithramycin or acriflavine by using specially built cell orientating flow cytometer (OFCM) (Pinkel et al., 1982). The OFCM measured the sperm cells hydrodynamically by controlling the orientation of the sperm about the flow axis. The EBMI and DAPI staining showed good resolution of X and Y sperm in the rabbit, mouse, boar, bull, and ram. In a study, Flow cytometric techniques were used to measure the relative DNA content of the X and Y populations and to flow-sort spermatozoa stained with Hoechst 33342 (Johnson et al., 1987). In breakthrough research, Johnson et al. (1989) separated viable, X and Y chromosome-bearing spermatozoa of rabbits by using a flow cytometer. This technology is popularly known as "the Beltsville sperm sexing technology. In this study, The semen was stained with bisbenzimide H33342 fluorochrome, and Intact sperm were sorted on an EPICS V flow cytometer/cell sorter (Coulter Corporation, Hialeah, FL). The intact stained sperm were excited in the ultraviolet lines of a 5-watt 90–5 Innova Argon-ion laser (Coherent, Inc., Palo Alto, CA). Analysis of the separated Y-bearing sperm data showed that 81% of the sperm were Y-bearing, and data of separated X bearing showed that 86% of the

Table 7.1 Historical events in sperm sexing technology

	Historical events in sperm sexing technology	Institute/company involved
1	Demonstrated the potential use of flow cytometry to convincingly identify X- and Y-sperm populations based on their DNA content differences	Oklahoma State University (OSU) and Lawrence Livermore National Laboratory (LLNL),USA
2	Development of Beltsville Sperm Sexing Technology	United States Department of Agriculture
3	Commercialization of Beltsville Sperm Sexing Technology	Colorado State University Research Foundation (CSURF) and XY, Inc.
4	Development of MoFlo™ Cytometer	Cytomation Inc.

sperm were Y-bearing. The offspring from these inseminations were 81% male and 94% female, respectively, for separated Y-bearing sperm and X-bearing sperms (Johnson et al., 1999). Even though it was a significant advance toward the goal of semen sexing in mammals, several factors lessen against extensive application due to the limitation on several sperms that can be sorted in a reasonable period of and the increased embryo mortality presumed to be related to the presence of the fluorochrome on the DNA. Based on this study, sex ratios were altered in experimental and field studies of swine (Johnson, 1991; Rath et al., 1997), cattle (Cran et al., 1995; Seidel Jr et al., 1997), sheep (Johnson, 1995; Catt et al., 1996; Cran et al., 1997), and humans (Johnson et al., 1993; Fugger et al., 1998). Developments in the Beltsville technology involved the development of a new flow nozzle and the development of a commercial high-speed cell sorter (MoFlo; Cytomation, Inc., Fort Collins, CO). This technology was patented by the United States Department of Agriculture (US Patent #692958) and granted a license to the Colorado State University Research Foundation (CSURF). It set up a company, XY, Inc., in collaboration with Cytomation Inc. Sexing technologies has been developing the Beltsville technology since it acquired the rights from XY, Inc. Historical events of sperm sexing are summarized in Table 7.1.

7.3 Sperm Sexing and Development of Flow Cytometry

Guyer (1910) observed sex chromosomes in mammals and researchers have attempted every so often to separate the spermatozoa into X and Y chromosome-bearing cells. In a previous study, chromatin dissimilarity was estimated between the X and Y chromosome-bearing spermatozoa and their study suggested that calculation of the chromatin difference between X and Y spermatozoa depends much upon the length of the Y chromosome (Moruzzi, 1979). Later, Gledhill et al. (1976) established high-resolution flow fluoro micrometer analysis of sperm for DNA content. In their experiments, sperm cells are stained with a fluorochrome which was excited by the laser light and the emitted fluorescence light is imaged onto a photomultiplier. Sperm with spherical or cylindrical heads showed symmetric

Table 7.2 Historical events in the development of flow cytometry for sperm sexing

	Development of flow cytometry for sperm sexing	References
1	Reported the use of flow cytometry for measuring DNA In sperm to determine changes that might occur with genetic damage. Asymmetric shape was shown to cause differential fluorescence. Coupled with random orientation.	Gledhill et al. (1976)
2	DNA content is measured by differences in chromosome length	Moruzzi (1979)
3	Showed that sperm nuclei sorted using Hoechst 33342 bound to the DNA were capable of fertilization	Johnson and Clarke (1988)
4	Altered sex ratios in offspring following surgical insemination of flow-sorted X– and Y-bearing sperms	Johnson (1991)
5	Development of the orienting nozzle	Rens et al. (1998)

fluorescence distributions and flat sperm heads showed asymmetric distributions consisting of a peak with a lateral extension to higher fluorescence values. In a breakthrough development, flow cytometric techniques were used to calculate the relative deoxyribonucleic acid content of X and Y chromosome-bearing, boar, and ram sperms and to fractionate the two sex-determining spermatozoa (Johnson & Clarke, 1988) Flow Cytometric examination of samples was carried out using an EPICS V flow cytometer cell sorter (Coulter Corp., Hialeah, FL). It consists of a bevelled sample injection tip. This instrument reduced the orientation problem caused by differential emission of fluorescence from the edge of the sperm compared to the face of the sperm. The fluorochrome-stained sperm nuclei were excited in the ultraviolet, and data were collected as histograms. This study showed that the separated spermatozoa of animals could condense and form male pronuclei. Rens et al. (1998) developed a bevelled sample injection needle and an orientation nozzle tip. The nozzle was almost similar to the EPICS nozzle, and the inside is changed to elliptical and tapered in two parts. Cytomation developed state-of-the-art SX MoFLo for flow sorting of spermatozoa and its orienting capability furtherer improved by modifications developed at XY Inc. and sexing technologies. The Historical events in the development of flow cytometry for sperm sexing are summarized in Table 7.2.

7.4 Safety Concerns

In a recent study (de Oliveira Carvalho et al., 2018), bull semen samples were sorted by flow cytometry, and the sorted samples were used to assess the sperm longevity during 12 h in culture media. Sperm motility and progressive motility, acrosome integrity, mitochondrial membrane potential, and plasma membrane stability were evaluated at different time points up to 12 h of culture. The greatest decrease in sperm viability was noted for all groups after the first two hours of incubation. This rapid loss was expected since similar results of sperm viability after thawing has been reported in cattle. It was also shown that the sexing process compromises the capacity of sperm to remain bound to the oviduct cells explants.

Hoechst 33342, fluorescent dye, is being extensively used in flow sorting of X and Y chromosome-bearing mammalian sperm based on the measurement of DNA content. Hoechst 33342 dye consists of two adjacent benzimidazole rings with N-methyl-piperazine and phenolic groups. This dye infuses into the cell membrane of mammalian cells and binds to Adenine and thymine base pairs. Durand and Olive (1982) studied the effect of Hoechst 33342 with cultured Chinese hamster V79 cells and proved that this dye is relatively nontoxic and non-mutagenic to these cells. Hoechst 33342 showed dramatic effects on DNA synthesis and "nontoxic" Hoechst concentrations lead to decreased cell viability at 4 °C or during flow cytometry procedures when laser power is over 100 mW. Chromosome damage, as well as an increase in the degree of damage per sperm nucleus, was reported with Hoechst 33342 and exposure to a UV-laser beam in the course of sperm sorting (Libbus et al., 1987).

7.5 Intellectual Property Issues

Lawrence A. Johnson developed a method for the separation of live and dynamic mammalian sperm into X and Y chromosome-bearing spermatozoa based on deoxyribonucleic acid content as an inventor. In 1992, this method was patented by the United States of America as represented by the Secretary of Agriculture, Washington, DC (Johnson, 1992). This patent consists of 26 claims which include, staining sperm collected from a male mammal with a fluorescent dye, passing the sperm into an electrically conductive and isotonic viability-supporting sheath fluid and collecting the selected sperm in a viability-supporting collecting fluid. Even though this patent was expired; more than 100 other patents related to semen sexing were granted, and some of them are licensed to sperm sexing companies. Some of the important patents are summarized in Table 7.3. In the last decade, there is a sharp rise in global patent applications in this field. As the market of sexed semen in the animal industry has been increased in the past few years, legal disputes were also noticed regarding the patent rights on semen sexing methods.

Recently, there was a patent dispute between ABS Global, INC., *and* Genus PLC. Vs Inguran, LLC, *doing business as* Sexing Technologies *and* XY, LLC. A patent on the photodamage sorting method for sperm separation has been developed, and its rights hold by Sexing Technologies (Durack et al., 2012). ABS Global, INC argued that it generated its photodamage sorter, but Sexing Tech presents litigation on claims 1, 2, and 7 of the US Patent Number 8,206,987. LumiSort™ is a proprietary sperm sexing technology, and it was developed by Microbix Biosystems Inc., a biotechnology company, located in Mississauga, Canada. This technology uses a laser-based sorting method in flow cytometric sperm sexing to determine the sex with high precision and obtained the patent for the same (Luscher, 2011). United States Patent and Trademark Office (USPTO) has upheld the claims of Microbix Biosystems Inc. against the challenges on LumiSort™ sperm sexing technology by a dominant company in livestock sperm sexing. Engender Technologies Limited is a New Zealand company, which has just been bought by multinational genetics

Table 7.3 List of some important patents in the field of sperm sexing

Publication number	Year	Assignee	Title
US5135759A	1992	US Department of Agriculture	Method to preselect the sex of offspring
FR2699678A1	1994	Unceia	Separation of mammalian spermatozoa according to sex
WO1999033956A1	1999	Xy, Inc.	Sex-specific insemination of mammals with a low number of sperm cells
WO1999042810A1	1999	Xy, Inc.	A vibratory system for a sorting flow cytometer
US6149867A	2000	Xy, Inc.	Sheath fluids and collection systems for sex-specific cytometer sorting of sperm
US6263745B1	2001	Xy, Inc.	Flow cytometer nozzle and flow cytometer sample handling methods
WO2001085913A2	2001	Xy, Inc.	High purity X and Y chromosome-bearing populations of spermatozoa
US6489092B1	2002	Vicam, L.P.	Method for sex determination of mammalian offspring
WO2004017041A2	2004	Xy, Inc.	High-resolution flow cytometry
US20040132001A1	2004	Seidel George E	System for in vitro fertilization with spermatozoa separated into X and Y chromosome-bearing populations
US20050003472A1	2005	Monsanto technology Llc	Process for the staining of sperm
US20050130115A1	2005	Abs Global Inc.	Method for altering the gender ratio of offspring in mammals by manipulation of spermatozoa
US20080118908A1	2008	Dmitri Dozortsev	Method of selecting a sperm cell based on its DNA content
US20090208977A1	2009	Androgenix Ltd.	Materials and methods for sperm sex selection
US7713687B2	2010	Xy, Inc.	System to separate frozen-thawed spermatozoa into X and Y chromosome-bearing populations
US7723116B2	2010	Xy, Inc.	Apparatus, methods, and processes for sorting particles and for providing sex-sorted animal sperms
EP2194379A1	2010	Inguran, LLC	Apparatus and methods for providing sex-sorted animal sperms
US20110236923A1	2011	Genetics & Ivf Institute	Method for staining and sorting a small volume of sperm
US8004661B2	2011	Microbix Biosystems Inc	Method and apparatus for sorting cells
US8206987B2	2012	Inguran LLC	Photo damage method for sorting particles
WO2013049631A1	2013	Inguran, Llc	Sperm staining and sorting methods
WO2014055112A1	2014	Inguran, Llc	High-efficiency methods of sex sorting sperm

(continued)

Table 7.3 (continued)

Publication number	Year	Assignee	Title
WO2014142924A1	2014	Inguran, Llc	Apparatus and methods for high throughput sperm sorting
US9027850B2	2015	Inguran, Llc	Nozzle assembly for a flow cytometer system and methods of manufacture
US9222872B2	2015	Inguran, Llc	Flow cytometer nozzle tip
EP3000874A1	2016	Inguran, Llc	Sex sorted sperm demonstrating a dose response and methods of producing sex sorted sperm demonstrating a dose response
US9347040B2	2016	Inguran, Llc	Compositions and methods for processing sperm
US10393644B2	2017	Engender Technologies Ltd. Auckland Uniservices Ltd.	Method and system for microfluidic particle orientation and/or sorting
US9879222B2	2018	Mofa Group Llc	Gender-specific separation of sperm cells and embryos
WO2019043656A1	2019	Genus Plc	Methods and systems for assessing and/or quantifying sex skewed sperm cell populations

company CRV-Ambreed, has developed a technology to separate X- and Y-bearing bull sperm cells by using microfluidic and photonic chips. Recently, Engender's first key patent was allowed in the United States, and it describes a method and system for particles orientation and sorting by using radiation pressure in a microfluidic system (Simpson & Rohde, 2017).

7.6 Ethical Aspects of Sperm Sexing

In human beings, nonmedical sex selection is highly regulated worldwide, and it is acceptable to prevent the vertical transfer of genetic diseases hemophilia, Lesch-Nyhan syndrome, Duchenne-Becker muscular dystrophy, and Hunter syndrome. Some countries like the United States, Cyprus, Ukraine, and Israel are allowing family balancing as a qualifying nonmedical motivation for Sex selection. Human needs animals for food, financial income, and other purposes like accompanying persons or families, amusement activities, protection, and research. It is the society's responsibility to protect the freedom of animals and to control the animal breeding methods as protected as humans. In farm animals, there is a high demand for the sexed semen from the farmers as they want to choose the animals of desired sex for economic and cultural reasons. For example, dairy farmers need more female calves on their farms and consider male calves as a secondary product. In the meat industry, male animals are beneficial for meat production, owing to greater growth than female calves. In India, cows are worshipped by the Hindu religion and cow urine

Table 7.4 Summary of five freedoms defined by the World Organisation for Animal Health (OIE)

Freedom number	Freedom from
1	Hunger, malnutrition, and thirst
2	Fear and distress
3	Heat stress or physical discomfort
4	Pain, injury, and disease
5	Express normal patterns of behavior

is considered sacred. Sexed semen can influence the market environment and management practices of animal farms. It can improve the profits of animal framers compared with using non-sexed semen. It is general practice that a few genotypes of particularly high breeding value animals of a particular region of the world are preferred for breeding with indigenous animals. Adaptability, disease resistance may be declined for future generations of such crossbreeds, and there could be a sharp decline in the number of indigenous varieties. Early pregnancy losses, retarded embryo development, and higher embryo mortality are the major concerns of the sex-sorted spermatozoa (McNutt & Johnson, 1996; Bathgate et al., 2008) and these are still unanswered by researchers and sexed semen marketing companies. Apart from this, in vitro capacitation (Bucci et al., 2012) and inferior motility characteristics (De Graaf et al., 2006; Hollinshead et al., 2003) are evident from the previous studies. The doubts of In vitro embryo production with altered properties by sex-sorted bull spermatozoa have not been answered yet.

The World Organisation for Animal Health (OIE) is an intergovernmental organization responsible for improving animal health worldwide. In 2003, this office became the World Organisation for Animal Health but held its historical acronym OIE. According to the OIE, animal welfare means "the physical and mental state of an animal about the conditions in which it lives and dies." In 1965, OIE developed five freedoms for animal welfare which describe society's expectations for the conditions animals should experience when under human control. OIEs five freedoms are summarized in Table 7.4.

There is a concern that sexed semen technologies could aggravate global animal sex ratio imbalances and others may argue that this could be necessary to maintaining the sex ratio imbalance and increase the number of endangered species.

7.7 Conclusions

Sexed semen has become a boon for the animal industry aiding in the breeding of desired sex animals, which has a direct impact on the economy of the farm owners and animals farmers. The recent decade has seen a rapid rise in the use of sexed semen in the dairy industry, which is evident from the availability of sexed semen across the globe. The existing successful commercial sperm sexing method majorly depends on the DNA content differences in the X-bearing spermatozoa and Y-bearing spermatozoa of animals. Based on this evidence, reproductive biologists developed flow cytometric separation of X-bearing spermatozoa and Y-bearing

spermatozoa, which are commercialized for the use of animal farmers. The high cost of the sexed semen is not stopping the dairy farmers from using the sexed semen even though the long-term side effects of sexed semen have not been studied. Last decade witnessed an unusual rise in the patent applications for sperm sexing across the globe. Animal welfare societies raised objections to global sex ratio imbalance, loss of biodiversity, and animal freedom over the implications of sexed semen in animals. Reproductive animal biologists pacify the fear implications of sexed semen and argue that it helps in a deal with human food security and sperm sexing method will be the ultimate tool for preserving the endangered species. In conclusion, sperm sexing in farm animals is emerging as a powerful tool for maintaining desired sex ratio on animal farms, and it has been successful in providing profits to animal farm owners. Sperm sexing will become a feasible solution to protect endangered species. Consideration of ethical issues raised by animal welfare societies while developing sperm sexing technologies ensures the proper use of sexed semen in animals for the welfare of animals and humans.

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