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Constraints Affecting Fertility of Sex Sorted Semen: An Overview

Roshan Krishna M S, Babulal Natvarlal Suthar*, Hareshbhai Chhotalal Nakhashi and Karan Faljibhai Chaudhary

Department of Gynecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Sardakrushinagar-385506 (Gujarat), India

ABSTRACT

In most mammals, the male to female sex ratio of offspring is about 50% because half of the sperm contains either the Y chromosome or the X chromosome. Desired sex either male or female which is produced from semen having X or Y bearing sperm is known as sexed semen. Flow cytometry is the only proven method for semen sexing to be commercially viable with more than 90% accuracy to produce calves of desirable sex. The sexed semen technology has several advantages, the production of more female calves and more milk, and lowered risk of dystocia. Increased efficiency of progeny testing, embryo transfer and *in-vitro* fertilization program has proposed a demand of feasible technology for sex sorting of spermatozoa. In India, this revolutionary technology is in its budding stage. This paper aimed to review the constraints affecting the fertility of sex sorted semen.

Key words: Chromosome, Flow cytometry, sexed semen

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INTRODUCTION

Sex sorting of semen is an assisted reproductive technology involving the separation of X and Y chromosomes bearing spermatozoa. For the production of sex sorted semen, the proportion of X-bearing (female) and Y-bearing (male) spermatozoa are separated from the natural mix through certain sorting techniques with 85 to 95 % accuracy (Cerchiaro *et al.,* 2007; De Vries *et al.,* 2008; Garner and Seidel, 2008; Gaddam *et al.,* 2022). Controlling the sex ratio permits faster genetic progress, and higher productivity, improves animal welfare and helps to reduce environmental impact due to the elimination of the unwanted sex, the male (Rath et al., 2009). The first attempt was made in 1976, to separate X and Y sperm by analytical flow cytometry in eutherian species (Gledhill *et al.,* 1976). From then on, the sex sorted semen was reported in various species like Equine (Lindsey *et al.,* 2005), Porcine (Knox, 2016), Ovine (de Graaf *et al.,* 2007), Cervine (Kjelland *et al.,* 2011), Caprine (Bathgate

*Corresponding author.
E-mail address: bnsuthar2@kamdhenuuni.edu.in (Babulal Natvarlal Suthar)
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et al., 2013), Elephant (Hermes *et al.,* 2009), and Canine (Merlo *et al.,* 2015). Sex sorting by flow cytometry causes chemical and mechanical stress on the sperm cells due to dilution, nuclear staining, high pressure centrifugation and other factors (Vishwanath and Moreno, 2018). The first sex-selected calf was born in 1999 by using frozen sexed semen through AI. This process became commercially available in 2004 through 'Sexing Technologies' with labs in Texas, Ohio, Wisconsin and Brazil. In India, a government of West Bengal organization, Paschim Banga Go Sampad Bikash Sanstha (PBGSBG), installed high speed semen sorter or flow cytometer (Influx, Becton Dickinson, Biosciences, San Jose, CA, USA) reported the first male calf Shreyas born through sex sorted semen in 2011. The one aspect that has remained constant until recently and perhaps most concerning is fertility. In cattle and possibly other species it is believed that the fertility of sexed semen is compromised by about 10 percentage points. This paper affords have been made to elucidate the factors influencing the fertility rate of the sex sorted semen and its managemental strategies to improve fertility in bovine. In India, there are a total of 7 units presently functioning on sexed semen production these are depicted in table no. 1.0

Table 1: Sexed semen institutes in India

State	Organization	Manufacturing company
Gujarat	State frozen semen station - Patan	Genus – ABS India Pvt Ltd.
	Frozen semen station - Jagudhan (Dudhsagar dairy)	Genus – ABS India Pvt Ltd.
Maharashtra	BAIF Development research foundation, Urulikanchan	ST Genetics (Sexed ULTRA™)
	BG Chitale dairy farm Brahmanand nagar, Sangli	Genus – ABS India Pvt Ltd.
Madhya Pradesh	Bhopal	ST Genetics (Sexed ULTRA™)
Uttar Pradesh	Livestock development board, Babugarh	Genus – ABS India Pvt Ltd.
Uttarakhand	Deep frozen semen production centre shyampur, Rishikesh	ST Genetics (Sexed ULTRA™)

In Patan and Jagudhan of Gujarat, there are eight sex sorting semen units installed that have been used in their institutes producing nearly 15,000 sexed semen straws per month. The unsorted fresh semen of a reputed bull is provided to the sorting manufacturer, where they sort the spermatozoa and provide straws of sex sorted semen back to the organization. The approximate cost of each sexed semen straw in Gujarat varies from 650-750 per straw

CONCEPTION RATE

Sex-sorted semen is desirable by many livestock farmers for its ability to determine the sex of the offspring at the point of conception. It has a major disadvantage; the conception rates are lower than conventional semen. Reports vary on the conception rates of sexed semen when compared to conventional semen ranging from 60 % to 90 % (Cerchiaro *et al.,* 2007; Galli and Balduzzi, 2009; De Jarnette *et al.,* 2010; De Jarnette *et al.,* 2011; Norman *et al.,* 2011; Healy *et al.,* 2013). A comparison of fertility rate between sex sorted and conventional semen is depicted in table 2.0

Table 2: Fertility rates of bovine sex sorted and conventional semen

Conventional semen	Sex sorted semen	References
-	51 %	Cerchiaro *et al.* (2007)
58%	47 %	DeJarnette *et al.* (2009)
55%	38%	DeJarnette *et al.* (2011)
39.6%	31.6%	Healy *et al.* (2013)
54.85%	42.65%	Razmkabir (2018)
-	63.4%	Aubuchon *et al.* (2022)
70%	50%	Jadhav *et al.* (2022)
-	39.92 %	Joshi *et al.* (2022)

Various researchers used different artificial insemination protocols like GPG + G protocol (Jadhav *et al.,* 2022), melengestrol acetate plus prostaglandin F2α (Aubuchon *et al.,* 2022), modified 5-d CO-Synch protocol (Macmillan *et al.,* 2021) with conception rates 70%, 63% and 69.5% respectively. Magopa *et al.* (2022) studied the effect of body condition score (BCS) and lactation status of dairy and beef cows on pregnancy rate with sex sorted and unsorted semen and observed the higher (64.3%) proportion of pregnancy in dairy cows having BCS ≥3.5 in compared to beef (40.0%) cows inseminated with conventional semen. However, with sex-sorted semen, the beef cows having BCS of 3 had a higher (41.9%) pregnancy rate compared to dairy (31.6%) cows. Lactating dairy cows inseminated with sex-sorted (42.5%) or unsorted (50.0%) semen had higher pregnancy rates compared to beef cows (sex-sorted; 31.2%; unsorted; 34.4%). BCS and Lactation status of dairy cows do affect negatively on pregnancy rate

Sorting Process: A major constrain for sex sorted semen fertility

During sorting, about 78 % of all sperm prepared for sorting are lost because of different technical and biological factors associated with the sorting process before they are inseminated (Seidel and Garner, 2002). Therefore, special handling of the semen during sorting is required to

maintain the fertilizing capacity of sex-sorted sperm. During the sorting process, sperm undergo time, temperature, mechanical and chemical stress (Seidel and Garner, 2002; Sharpe and Evans, 2009; Lenz *et al.,* 2017; Vishwanath and Moreno, 2018), pressure from the collection process, and finally, centrifugation to purify the sample (Cerchiaro *et al.,* 2007). The analysis of a single sperm requires extensive dilution, electrical charging and electrostatic deviation (Klinc and Rath, 2006), post-sorting centrifugation to concentrate the highly diluted sexed sperm, incubation at 34–37 °C, nuclear staining with Hoechst 33342, high pressure passage through the flow cytometer, and exposure to UV laser light before collection at the base of the flow cytometer and being cooled to 5 °C (Seidel and Garner 2002; Sharpe and Evans, 2009; Lenz *et al.,* 2017; Vishwanath and Moreno, 2018). Thus, sex-sorting results in numerous sperm alterations including reduced progressive motility (Holden *et al.,* 2017; Steele *et al.,* 2020), reduced velocity (Suh *et al.,* 2005; Steele *et al.,* 2020), reduced hyperactivation (Steele *et al.,* 2020) and abnormal movement patterns (Pirez *et al.,* 2020; Steele *et al.,* 2020). Additionally, reduced chromatin integrity (Boe *et al.,* 2005), reduced mitochondrial potential (Carvalho *et al.,* 2018), increased reactive oxygen species levels (Leahy *et al.*, 2010; Balao *et al.,* 2013), decreased number of sperms with acrosome integrity, destabilized plasma membrane (Carvalho *et al.,* 2018), increased membrane permeability and reduced intracellular ATP levels (Carvalho *et al.,* 2010; Balao *et al.,* 2013; Holden *et al.,* 2017) as well as a shortened time to acrosome reaction have been reported. Further, the binding in the oviductal sperm reservoir is also reduced (Spinaci *et al.,* 2006; Pirez *et al.,* 2020). Sex-sorted spermatozoa reveal deformations in the head, sharp bends in the tail and a significantly increased prevalence of damaged mitochondria (Pirez *et al.,* 2020; Gaddam *et al.,* 2022) Also, there are altered motility characteristics, velocity and amplitude of lateral head displacement as assessed by computer-assisted sperm analysis in sorted sperm of bull (Suh *et al.,* 2005) and sheep (de Graaf *et al.,* 2006), and ability to penetrate cervical mucus (de Graaf *et al.,* 2006) compared with non-sorted spermatozoa has been reported.

Sperm cells for Insemination

Conception rates in sex-sorted semen are also affected by the number of sperm cells per straw. The standard dose for a straw of sexed semen is approximately 2 x 10^6 sperm cells (Garner and Seidel, 2008; Healy *et al.,* 2013). Conventional straws have approximately 15-20 x 10^6 sperm cells per standard dose (Garner and Seidel, 2003; Healy *et al.,* 2013). The lower number of sperm cells in sexed semen straws is because of the cost of the equipment and expertise required for the sorting process, the time needed to create a dose of sexed semen, and the variability in bull semen viability to survive the sorting process. Seidel *et al.* (1997) established the concept of low dose insemination with sexed sperm. They obtained a 22.4 % calving rate in heifers that were inseminated with 1-2×10^5 non-frozen, sexed sperm. Pregnancy rates were 48/118 (41%), 56/l 11 (50%), and 35/57 (61%) for 1 x 10^5, 2.5 x 10^5 and 2.5 x 10^6 sperm per insemination (Seidel *et al.,* 1997). Further DeJarnette *et al.* (2010) found that by increasing the number of sexed semen cells from 2.1 x 10^6 to 3.5 x 10^6 did not increase conception rates. Both dosages of sex-sorted semen had conception rates that were approximately 75 % of conventional semen. When semen doses were doubled or tripled (4 x 10^6 or 6 x 10^6), pregnancy rates only increased slightly (5-7%). Increasing the number of sexed sperm cells present does not compensate for the damage that occurs during the sorting process (Hall, 2011). The expense and efficiencies dictate that commercial application of sex-sorted sperm is only economically viable through the use of extremely low sperm numbers per dosage (Frijters *et al.,* 2009).

Site of insemination

During mating, the bull deposits several billions of spermatozoa into the anterior vagina. However, because the cervix is a major obstacle for sperm transport, the number of spermatozoa that finally reach the uterine body usually is very little. In artificial insemination, semen is generally deposited directly into the uterine body, thus bypassing the cervix and permitting the use of a considerably reduced number of sperm (López, 2000). Insemination with sex-sorted sperm into the body (54.0%; 54/100) of the uterus achieved pregnancy/AI values similar to those achieved with insemination into the horn of the uterus (50.0%; 50/100) (Filho *et al.,* 2012). Grossfeld *et al.,* (2011) developed a device to transfer a very small volume (30 µl,10^5 sperm) of sperm in the bovine oviduct and achieved 41.2 % pregnancy in field conditions. However, the current recommendation is to deposit them into the uterine body as is done conventionally, because there has been little advantage to uterine horn insemination.

TECHNOLOGICAL ADVANCEMENT IN SEX SORTING OF SPERMATOZOA

In the following decades, the technology underwent further improvements. As the low sorting throughput rate was a main barrier to commercial success, the flow cytometric system, the MoFlo™ cytometer, first underwent

modifications to its nozzle so that an increased number of sperm were orientated correctly by the fluidic system pressure (Johnson 1999). Further improvements of the nozzle led to an increased analytic capacity exceeding 20,000 sperm/s and sorting up to 6,000 of each X and Y bearing sperm with 90% accuracy (Garner & Seidel 2008). Reduction in fluidic pressure from 50 to 40 psi resulted in an increased number of recoverable viable sperm (Suh, *et al.,* 2005). The addition of further photodetectors, (at the angles of 45° and 135° relative to the detector at 0°) enabled to measure diagonally orientated sperm (Sharpe, & Evans, 2009). For improving the accuracy of determining the X and Y bearing sperm gas-based argon ion lasers were replaced by diode-pumped solid-state systems (Sharpe, & Evans, 2009). Apart from flow cytometry, the Lumisort sperm sexing method couples a pioneering optical system for the detection of sperm cell sex, with a fast and effective laser-based killing method that eliminates the sperm cells that are not of the desired sex. Raman spectroscopy allows for the analysis of various components sex associate dated membrane proteins in X-bearing or Y-bearing sperm. More precisely the spectra of Y-sperm show an increased intensity as compared to X-bearing sperm. 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. Gamrad *et al.*, (2017) identified triplex-forming oligonucleotides (TFOs) as ligands to specifically bind specific regions of Y chromosome-bearing spermatozoa of bull semen samples. These TFOs were conjugated with gold nanoparticles for the selection of Y chromosome-bearing spermatozoa. In a study of differential proteins of X and Y-sorted bovine spermatozoa, four 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). Quelhas *et al.* (2021) proposed a sperm sexing method that involves the identification of plasma membrane proteins and the subsequent development of antibodies against sex-specific proteins. This method also suggested that the conjugation of antibodies would allow sperm sexing with less damage.

MANAGEMENT STRATEGIES TO IMPROVE FERTILITY

Sex-sorted semen has been recommended for use in first and second AI services in virgin heifers, because of increased fertility rates in heifers compared to lactating cows (Seidel and Schenk, 2008; Garner and Seidel, 2008; De Vries and Nebel, 2009; De Jarnette *et al.,* 2010). The suggested time of AI with conventional semen (>12 hrs after the onset of estrus) may not be compatible with sexed semen (Sales *et al.*, 2011). In a trial by Schenk *et al.* (2009), a team of researchers found that delaying AI to 18-24 hrs after the onset of estrus increased pregnancy rates per AI, when compared to females that received AI 0-12 hrs after the onset of estrus, whereas in another study Guner *et al.* (2020) observed sex-sorted semen resulted in 60.9 % pregnancy rate on 29-35 days when delayed the time of insemination (20.1-24 hrs) after estrous detection was used. They have also observed that timed AI and experienced technicians were also found to be critical factors in increasing fertility with the use of sex-sorted semen in Holstein heifers. The next recommendation is to handle sex-sorted semen with extreme care, including thawing at the proper temperature and inseminating the female in a timely manner. Researchers recommend using a proven and successful inseminator to increase conception rates. Above all, optimal use of sexing technology requires excellent and careful animal management like nutrition, disease control, estrus detection, semen handling, and insemination technique (Manzoor, 2017).

CONCLUSIONS

Sex-sorted semen is a powerful tool, but heterogenous fertility is causing the farmers and researchers its decreased usage. Reasons for this are varied, but the sorting process does clear out much dead and compromised sperm, and if conditions are right and the timing of insemination is accurately matched with ovulation, there is a real opportunity to improve the fertilization outcome. In Indian conditions, there is a need to standardize the lower dosage of spermatozoa, the site of deposition for AI with a good conception rate in our conventional system. There is also an imminent requirement to train skilled manpower in the above area to achieve good results. The main focus is to use sex-sorted spermatozoa in good quality heifers and cows with excellent reproductive and productive performance to achieve good results and study actual economic benefits in the long-term application of sexed semen. Enough fertility rates with reduced cost of sex sorted semen are the pre-requisite in India for acceptance of this technology as a most popular breeding tool.

CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

Aubuchon, K. W., Odde, J. A., Bronkhorst, C., Bortoluzzi, E. M., Goering, M. J., Fike, K. E., and Odde, K. G. (2022). Field trial assessing the use of sex-sorted semen in beef cattle. *Kansas Agricultural Experiment Station Research Reports*, **8**(1), 14.

Balao da Silva, C. M., Ortega-Ferrusola, C., Morrell, J. M., Rodriguez Martinez, H. and Pena, F. J. (2013). Flow cytometric chromosomal sex sorting of stallion spermatozoa induces oxidative stress on mitochondria and genomic DNA. *Reprod. Domest. Anim*., **51**, 18-25.

Bathgate, R., Mace, N., Heasman, K., Evans, G., Maxwell, W.M.C. and de Graaf, S.P. (2013). Birth of kids after artificial insemination with sex-sorted, frozen-thawed goat spermatozoa *Reprod. Domest. Anim*., **48**, 893-898.

Boe-Hansen, G. B., Morris, I. D., Ersboll, A. K., Greve, T. and Christensen, P. (2005). DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology.,* **63**(6), 1789-1802.

Carvalho, J. O., Sartori, R., Machado, G. M., Mourao, G. B. and Dode, M. A. N. (2010). Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in in vitro embryo production. *Theriogenology.,* **74**(9), 1521-1530.

Cerchiaro, I., Cassandro, M., Dal Zotto, R., Carnier, P. and Gallo, L. (2007). A field study on fertility and purity of sex-sorted cattle sperm. *J. Dairy Sci*., **90**(5), 2538-2542.

de Graaf, S.P., Evans, G., Maxwell, W.M.C. and O'Brien, J.K. (2006). *In vitro* characteristics of fresh and frozen-thawed ram spermatozoa after sex-sorting and re-freezing. *Reprod. Fertil. Dev*., **18**(8), 867-874.

de Graaf, S.P., Evans, G., Maxwell, W.M.C., Downing, J.A. and O'Brien, J.K. (2007). Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep. *Reprod. Domest. Anim*., **42**(6), 648–653

de Oliveira Carvalho, J., Sartori, R., Rodello, L., Mourão, G. B., Bicudo, S. D. and Dode, M. A. (2018). Flow cytometry sex sorting affects bull sperm longevity and compromises their capacity to bind to oviductal cells. *Livest. Sci*., **207**, 30-37.

De Vries, A. and Nebel, R. (2009). National heifer supply and the effects of sexed semen. In Proceedings Western Dairy Management Conference, Reno, NV, March (pp. 9-13).

De Vries, A., Overton, M., Fetrow, J., Leslie, K., Eicker, S., and Rogers, G. (2008). Exploring the impact of sexed semen on the structure of the dairy industry. *J. Dairy Sci.,* **91**(2), 847-856.

DeJarnette, J. M., Leach, M. A., Nebel, R. L., Marshall, C. E., McCleary, C. R. and Moreno, J. F. (2011). Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? *J. Dairy Sci.* **94**(7), 3477-3483.

DeJarnette, J. M., Nebel, R. L. and Marshall, C. E. (2009). Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology.,* **71**(1), 49-58.

DeJarnette, J.M., Cleary, C.R., Leach, M.A., Moreno, J.F., Nebel, R.L. and Marshall, C.E. (2010). Effects of 2.1 and 3.5 x 10^6 sex-sorted sperm dosages on conception rates of Holstein cows and heifers. *J. Dairy Sci.,* **93**(9), 4079-4085.

Frijters, A. C. J., Mullaart, E., Roelofs, R. M. G., van Hoorne, R. P., Moreno, J. F., Moreno, O. and Merton, J. S. (2009). What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? *Theriogenology.,* 71(1), 64-67.

Fujita, Y., Mihara, T., Okazaki, T., Shitanaka, M., Kushino, R., Ikeda, C. and Shimada, M. (2011). Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. *Hum. Reprod.,* **26**(10), 2799–2806.

Gaddam, N., ST, V. R., Putty, K., Sakaram, D. and Ganji, V. K. (2022). Quantitative and qualitative assessment of sex sorted semen of *Bos indicus* and *Bos taurus* breeds. Research Square (preprint).

Galli, A. and Balduzzi, D. (2009). Fertility of sexed bovine semen. In Proceeding: 44 International Symposium of Animal Production "Impact and perspectives of biology and technology of Animal Reproduction" (Eds GF Greppi, S. Mura) (p. 77).

Gamrad, L., Mancini, R., Werner, D., Tiedemann, D., Taylor, U., Ziefuß, A. and Rath, D. (2017). Triplex-hybridizing bioconjugated gold nanoparticles for specific Y-chromosome sequence targeting of bull spermatozoa. *Analyst*, **142**(11), 2020–2028.

Garner, D. L. and Seidel Jr, G. E. (2008). History of commercializing sexed semen for cattle. *Theriogenology*, **69**(7), 886-895.

Gledhill, B.L., Lake, S., Steinmetz, L.L., Gray, J.W., Crawford, J.R. and Dean, P.N. (1976). Flow micro fluoro metric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution. *J. Cell. Physiol*., **87**(3), 367- 376.

Grossfeld, R., Strothmeyer, M. and Rath, D. (2011). Low dose sperm intra fallopian transfer (SIFT) in dairy cows. *Reprod. Domest. Anim.,* **46** (Suppl 3): 109 (Abstr).

Guner, B., Erturk, M., Yilmazbas-Mecitoglu, G., Keskin, A., Karakaya-Bilen, E., Cakircali, R. and Gumen, A. (2020). Effect of delaying the time of insemination with sex-sorted semen on pregnancy rate in Holstein heifers. *Reprod. Domest. Anim.,* **55**(10), 1411-1417.

Hall, J. B. 2011. Sexed Semen – The newest reproductive technology for the beef industry NCBA Pfizer Cattlemen's College, Denver, CO.

Healy, A. A., House, J. K. and Thomson, P. C. (2013). Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. *J.Dairy Sci*., **96**(3), 1905-1914.

Hermes, R., Behr, B., Hildebrandt, T. B., Blottner, S., Sieg, B., Frenzel, A. and Rath, D. (2009). Sperm sex-sorting in the Asian elephant (*Elephas maximus*). *Anim. Reprod. Sci*., **112**(3-4), 390-396.

Holden, S. A., Murphy, C., Moreno, J. F., Butler, S. T., Cromie, A. R., Lonergan, P. and Fair, S. (2017). *In vitro* characterization of fresh and frozen sex-sorted bull spermatozoa. *Reprod. Fertil. Dev.,* **29**(7), 1415-1425.

Jadhav, S., Patil, A., Razzaque, W., Jadhav, R., Gaikwad, N. and Dongre, V. (2022). Fertility Response of the Sex Sorted and Conventional Semen in Cattle. *J. Anim. Res.,* **12**(2), 279-283.

Johnson, L.A., Flook, J.P. and Hawk, H.W. (1989). Sex pre-selection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod*., 41, 199-203**.**

Johnson, L. A., Welch, G. R. and Rens, W. (1999). The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. *J. Anim. Sci.,* **77**(suppl_2), 213-220.

Joshi, S., Bhave, K., Potdar, V., Gaundare, Y., Punde, N., Shirsath, T. and Swaminathan, M. (2021). Performance of sex sorted semen under Indian Small Holder Dairy Farming Systems. *Int. J. Curr. Microbiol. and Appl. Sci.,* **10**(02), 1335-1343.

Kjelland, M., Gonzalez-Marin, C., Gosálvez, J., Lopez, C.W., Lenz, R., Evans, K.M. and Moreno, J.F. (2011). DNA fragmentation dynamics and post thaw motility of white-tailed deer sperm. *Reprod. Fertil. Dev.,* **23**(1), 239-240.

Klinc, P. and Rath, D. (2006). Effects of processing procedures after flow sorting to sex bovine spermatozoa and cryopreservation on sperm quality and fertility. *Slov. Vet. Res.,* **43**(2), 67-114.

Knox, R. (2016). Artificial insemination in pigs today. *Theriogenology,* **85**(1), 83-93.

Laxmivandana, R., Patole, C., Sharma, T. R., Sharma, K. K. and Naskar, S. (2021). Differential proteins associated with plasma membrane in X-and/or Y-chromosome bearing spermatozoa in indicus cattle. *Reprod. Domest. Anim.,* **56**(6), 928–935.

Leahy, T. celi, P., Bathgate, R., Evans, G., Maxwell, W.M.C. and Marti, J.I. (2010). Flow-sorted ram spermatozoa are highly susceptible to hydrogen peroxide damage but are protected by seminal plasma and catalase. *Reprod. Fertil. Dev.,* **22**(7), 1131-1140.

Lenz, R. W., Gonzalez-Marin, C., Gilligan, T. B., DeJarnette, J. M., Utt, M. D., Helser, L. A. and Vishwanath, R. (2017). 190 SexedULTRA™, a new method of processing sex-sorted bovine sperm improves conception rates. *Reprod. Fertil. Dev.,* **29**(1), 203-204.

Lindsey, A.C., Varner, D.D., Seidel, G.E., Bruemmer, J.E. & Squires, E.L. (2005). Hysteroscopic per rectally guided, deep-uterine insemination of mares with spermatozoa stored 18 h at either 5 degrees C or 15 degrees C prior to flow-cytometric sorting. *Anim. Reprod. Sci.,* **85**(1-2), 125-130.

López-Gatius, F. (2000). Site of semen deposition in cattle: A review. *Theriogenology,* **3**(7), 1407-1414.

Macmillan, K., Gobikrushanth, M., Mapletoft, R. J., and Colazo, M. G. (2021). The effect of altering the timing of GnRH administration and artificial insemination in a modified 5-d CO-Synch protocol using sex-sorted semen in dairy heifers. *Theriogenology,* **159**, 53-59.

Magopa, T. L., Mphaphathi, M. L., Ledwaba, M. R., Thema, M. A., Mulaudzi, T. and Nedambale, T. L. (2022). Influence of body condition score and lactation status on oestrus response and pregnancy rate in dairy and beef cows inseminated with sex-sorted or non-sex-sorted semen. *Anim. Reprod.,* **19**(2), e22221.

Manzoor, A., Patoo, R.A., Akram, T., Shah, A.A. and Nazir, T. (2017). Sperm sexing and its utility in commercial cattle production: A review. *Adv. Anim. Vet. Sci.,* **5**(7), 293-298.

Merlo, B., Zambelli, D., Cunto, M., Iacono, E., Nasi, L., Giaretta, E. and Spinaci, M. (2015). Sex-sorted canine sperm cryopreservation: limits and procedural considerations. *Theriogenology*, **83**(7), 1121-1127.

Norman, H. D., Hutchison, J. L., and VanRaden, P. M. (2011). Evaluations for service-sire conception rate for heifer and cow inseminations with conventional and sexed semen. *J. Dairy Sci.,* **94**(12), 6135-6142.

Pirez, M. C., Steele, H., Reese, S. and Kolle, S. (2020). Bovine sperm-oviduct interactions are characterized by specifc sperm behaviour, ultra-structure and tubal reactions which are impacted by sex sorting. *Sci. Rep.,* **10**(1), 1-19.

Quelhas, J., Santiago, J., Matos, B., Rocha, A., Lopes, G., and Fardilha, M. (2021). Bovine semen sexing: Sperm membrane proteomics as candidates for immunological selection of X-and Ychromosome-bearing sperm. *Vet. Med. Sci*.

Rath, D., Moench-Tegeder, G., Taylor, U., and Johnson, L. A. (2009). Improved quality of sex-sorted sperm: a prerequisite for wider commercial application. *Theriogenology*, **71**(1), 22-29.

Razmkabir, M. (2018). A field study on the reproductive efficiency of sex-sorted semen in Holstein heifers. *J. Livest. Sci. Technol*., **6**(2), 41-46.

Sá Filho, M. F., Girotto, R., Abe, E. K., Penteado, L., Campos Filho, E. P., Moreno, J. F. & De Vries, A., and Nebel, R. (2009). National heifer supply and the effects of sexed semen. In Proceedings Western Dairy Management Conference, Reno, NV, March (pp. 9-13).

Sales, J., Neves, K., Souza, A. H., Crepaldi, G. A., Sala, R. V., Fosado, M. And Baruselli, P. S. (2011). Timing of insemination and fertility in dairy and beef cattle receiving timed artificial insemination using sex-sorted sperm. *Theriogenology,* **76**(3), 427-435.

Schenk, J. L., Cran, D. G., Everett, R. W. and Seidel Jr, G. E. (2009). Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology,* **71**(5), 717-728.

Seidel Jr, G. E., and Schenk, J. L. (2008). Pregnancy rates in cattle with cryopreserved sexed sperm: effects of sperm numbers per inseminate and site of sperm deposition. *Anim. Reprod. Sci.,* **105**(1-2), 129-138.

Seidel Jr, G. E., Allen, C. H., Johnson, L. A., Holland, M. D., Brink, Z., Welch, G. R. and Cattell, M. B. (1997). Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. *Theriogenology,* **48**(8), 1255-1264.

Seidel, Jr. G.E., and Garner, D.L. (2002). Current status of sexing mammalian spermatozoa. *Reproduction*, **124**(6), 733-743.

Sharpe, J. C., and Evans, K. M. (2009). Advances in flow cytometry for sperm sexing. *Theriogenology*, **71**(1), 4-10.

Spinaci, M., Volpe, S., Bernardini, C., De Ambrogi, M., Tamanini, C., Seren, E. and Galeati, G. (2006). Sperm sorting procedure induces a redistribution of Hsp70 but not Hsp60 and Hsp90 in boar spermatozoa. *J. Androl.*, **27**(6), 899-907.

Steele, H., Makri, D., Maalouf, W. E., Reese, S. and Kolle, S. (2020). Bovine sperm sexing alters sperm morphokinetics and subsequent early embryonic development. *Sci. Rep.,* **10**(1), 6255.

Suh, T. K., Schenk, J. L. and Seidel, G. E. Jr. (2005). High pressure flow cytometric sorting damages sperm. *Theriogenology,* **64**(5), 1035-1048.

Vishwanath, R. and Moreno, J. F. (2018). Review: Semen sexing - Current state of the art with emphasis on bovine species. *Animals.,* **12**(1), 85-96.