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Review Article

Sperm Sexing in Animals

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Abstract | Nature has gone to extreme lengths to make X- and Y- chromosome bearing spermatozoa phenotypically identical so that mammalian sex is determined randomly, with equal chances of male or female offspring. However, from an owner's point of view the sex of the calf or any other young-one to be born does matter. At present, flow cytometric sorting of sperm based on difference in DNA content of X and Y sperm is the only confirmed commercial method with 90% accuracy but it has certain limitations such as damage of sperm, cost of straw, sorting rate etc. Development of techniques to nullify these limitations is an active area of research. In past, various techniques have been used to separate the X and Y sperm which were based on principle of difference in mass, size swimming pattern, immunological structure and surface charges etc. Some of them showed the encouraging results but lacking scientific validation and some remained yet to be established. It seems inevitable that some techniques will prove to be efficacious. This review is meant to update the knowledge of researchers regarding recent development of sperm sexing in bovine.

Keywords | Sperm sexing, Bovine, Flow cytometry, Immunological, H Y antigen

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The FAO has recognized that production of pre-sexed livestock by sperm or embryo sexing, when combined with other biotechnologies, genomics, proteomics or phenomics, for example sperm-mediated gene transfer (De Cecco et al., 2010; Niemann et al., 2011), offers a promising breeding strategy to help meet the increased demand for food production (Rath et al., 2013). Sex pre-selection also decouples the quantity of dairy replacement heifers from those required for milk production (De Vries et al., 2008).

Commercial dairy farms producing and marketing milk could use sexed semen to produce replacement daughters from genetically superior cows and beef crossbred sons from the remainder of their cow population. It also reduces calving difficulty in first calvers (Seidel, 2007). An increased proportion of female offspring would be desirable in dairy cattle production, whereas an increased proportion of male offspring would be desirable in beef cattle production (Madalena, 2004).

HISTORY OF SPERM SEXING

It's long back when Democritus, 470-402 BC, suggested that the right testis produced males, whereas the left testis produced females. In the first half of the 20th century advances in the biological sciences, especially genetics, resulted in numerous discoveries, including identification of the sex chromosomes. According to Moruzzi (1979), the difference in total length of the bovine chromosomes between those from bulls and cows is approximately 4.2%. Lush (1925) conducted one of the first significant attempts to pre-select sex with semen of pigs and rabbits. But his study, based on centrifugation, failed to attain any significant skewing of the sex ratio. With the advance of time many new theories and methods were developed for the efficient sexing of semen. More than a dozen approaches to sexing sperm have been attempted, but convincing results were not produced. First attempts to separate X and Y bearing sperms were made by Gledhill et al. (1976) through analytical flow cytometry. The major breakthrough was devel-

opment of flow cytometry/ cell sorting in the early 1980s Collaboration between Oklahoma State University (OSU) and Lawrence Livermore National Laboratory (LLNL) demonstrated the potential use of flow cytometry to convincingly identify X- and Y-sperm populations based on their DNA content differences. Highly condensed sperm nucleus with unusual shape of sperm head caused difficult in quantitative fluorescence measurement and thus marginal successful in separation of sperms. Pinkel et al. (1982) overcome sperm heads associated problems through development of flow cytometry precisely for sperm sorting that orient the sperm heads with flattened side. Sperm sorting technology was first developed at Lawrence Livermore National Laboratory where Pinkel et al. (1982) separated the X and O sperm nuclei of the *Microtus oregoni*, which have 9% DNA content difference of its sex determining chromosomes. In 1989, the USDA Beltsville Research Center group reported production of live offspring from sex-sorted, living rabbit sperm. In 1989, a major breakthrough in sperm sexing was reported by Johnson et al. (1989) through production of live offspring from sex sorted live rabbit spermatozoa after surgical insemination in the oviduct. Application to domestic livestock sperm separation was then implemented at Beltsville Agriculture Research Centre, USDA. Flow cytometry for sperm sexing is a patent procedure and patency lies with the M/s X – Y – INC Colorado (USA). Through license of sexing technology to many companies, sexed semen has been produced for nearly 18 different breeds of cattle in USA and in European countries.

METHODS FOR SEPARATION OF X- AND Y- BEARING SPERMATOZOA

Centrifugal Counter Current Distribution based on Density Characteristics

Meistrich (1982) found the difference in density between X-bearing bovine spermatozoa and Y-bearing bovine spermatozoa to be only 0.0007 g/cm^3, hence this feature was also not suitable to be exploited as a characteristic to sex sperm. Ollero et al. (2000) have attempted to sex ram spermatozoa by centrifugal counter current distribution using an aqueous two-phase system.

Albumin Gradient

Moruzzi (1979) reported that Y chromosome is smaller than X chromosome. Successful separation of X and Y-bearing spermatozoa using an albumin gradient was first reported by Ericsson et al. (1973). Maxwell et al. (1984) recorded that though the method was effective in increasing the proportion of spermatozoa with motility and elimination of abnormal forms, there was no much difference in the ratio of X- Y- bearing spermatozoa.

Swimming Patterns under Laminar Flow

This method was based on the observation of Sarkar et al. (1984) that Y-bearing spermatozoa swim differently and more quickly than X-bearing spermatozoa in a column of flowing media. The feasibility of this technique is questionable as only 10 % of the total number of spermatozoa placed in the system could be recovered.

Percoll Density Gradient

Semen is layered on top of a percoll column and spermatozoa are allowed to penetrate the column. Iwasaki (1988) reported that the technique was not effective in separation of X or Y-bearing spermatozoa.

Free Flow Electrophoresis

It is based on the possibility that the electric charge on the surface of X-bearing spermatozoa differs from that of Y-bearing spermatozoa, uses an electric field to separate spermatozoa into the two major classes (Kaneko et al., 1984). Inseminations with semen separated by this technique yielded disappointing results. Blottener et al. (1983) found a birth rate of 50.4% female calves in inseminations carried out on 1185 animals using semen enriched in X-bearing spermatozoa. Another drawback of this technique was an associated reduction in motility of the sperm after being subjected to electrophoresis.

Counter Current Galvanic Separation

Each sperm will have an individual sedimentation velocity that will be influenced by physical forces such as size, shape, mass, specific gravity and difference in density between cell and suspending medium. The selection can be further enhanced by the application of a suitable micro-ampere current that will attract Y-bearing spermatozoa to the anode and X-bearing spermatozoa to the cathode (Bhattacharya, 1977). Foote (1985) emulates the same technique but could not succeed in producing any significant alteration of sex ratio.

Separation on the Basis of Presence of HY Antigen

Attempts to inactivate the Y sperm by immunological methods have been tried on the H-Y antigen (Goldberg et al., 1971). Other reports have suggested the usefulness of H-Y antigen as a means of selecting only the X-bearing rabbit spermatozoa (Zavos, 1985). Hoppe and Koo (1984) suggested that X- and Y-bearing spermatozoa probably share the same surface antigen. Watchel (1983) demonstrated the presence of HY antigen in the membrane of both X and Y spermatozoa under normal circumstances. The observation of Watchel (1983) was confirmed by the experimental data of Hendrikssen et al. (1993) which confirmed that there is no preferential expression. Hence sexing the spermatozoa on the basis of HY antigen is not effective.

Hormonal Manipulation for Sexing of Spermatozoa

Barat and Leger (1979) and James (1980) reported that administration of clomiphene citrate and or/ gonadotrophins resulted in 8.7% lowering of sex ratio.

Sperm Sorting based on Volumetric Differences

Van Munster et al. (1999a) used interference microscopy and subsequent image analysis to demonstrate a difference in sperm head volume that matched differences in DNA content between X and Y-bearing bovine spermatozoa. A method based on this principle has been developed for sorting live spermatozoa by using interference microscopy optics with a flow cytometer (van Munster, 2002). Unfortunately, the potential purity of spermatozoa separated using volumetric measurements cannot exceed 80% purity of either sex based on theoretical considerations (van Munster et al., 1999b)

Genetic Approaches

Seidel (1988) suggested a genetic approach for sperm sexing. Herrmann et al. (1999) demonstrated this concept by placing part of this genetic system on the Y chromosome using transgenic procedures.

Immunological Sexing of Semen

Blecher et al. (1999) carried out a study in this aspect. Immunization of male and female rabbits by injecting sperm preparations with Freund's incomplete adjuvant subcutaneously was done to raise antibodies to sperm membrane proteins. The anti-sperm antisera obtained from the female rabbit were putative "anti-Y" and those obtained from male rabbit were "anti-X" antisera. Sperm doses after suitable treatment were mixed with either of these antisera and incubated for 60 min at 38.5C and 5% CO_2. It was found that only the "anti-X" antisera resulted in agglutination of spermatozoa whereas the "anti-Y" antisera failed to show any agglutinations in the spermatozoa. The agglutinated sperm population was separated from the free-swimming sperm by glass wool filtration and the free-swimming sperm population (potentially Y-bearing spermatozoa) was isolated. Bovine embryos were produced *in vitro* using the isolated sperm population and blastocysts were sexed cytogenetically. The results indicated that 92% of the sexed embryos were male thus marking the technique as one of the potential methods of sperm sorting. This method has to be validated by further experiments and another constraint is that the method was successful in isolating Y-bearing spermatozoa only and attempts to isolate X-bearing spermatozoa by agglutinating Y-bearing spermatozoa was not successful. Beerli et al. (2008) reported that each cellular protein can be recognizable by a set of antibodies, so an immunological approach may be more efficient for detecting proteins with a low concentration. Yang et al. (2014) confirmed that the putative XSSAb contained SSAbs that captured three candidate SSP spots. This provides a potentially more efficient method for sorting sperm and lays a foundation for future search for SSPs. However, additional studies are needed to further confirm the specificity of XSSAb and identify candidate proteins of SSPs.

Flow-cytometric Sorting of Semen

Sexing of X- and Y-chromosome bearing sperm based on the difference in DNA content is the most reliable and repeatable method to produce sex-preselected animals. Since the first report by Johnson et al. (1989) in rabbits, flowcytometric sexing technology has been shown to be efficacious in several species including buffalo (Johnson, 2000; Seidel Jr and Garner, 2002; Maxwell et al., 2004; Lu et al., 2007; Liang et al., 2008). The earlier studies failed to notice difference in the DNA content between X- and Y- bearing sperm because of random orientation of the sperm in the flow-cytometer fluid stream (Johnson and Welch, 1999). The first flow sorting of sperm for purpose of isolating X from Y-bearing spermatozoa was reported by Pinkel et al. (1982). The work was done by using a specially built orienting flow cytometer using sperm from the creeping vole (*Microtus oregoni*).

The difference in DNA content between X and Y sperm of many animals has been quantified (Johnson, 2000; Parrilla et al., 2004; O'Brien et al., 2005; Lu et al., 2013) (Table 1) .

Table 1: Differences in DNA content between X and Y spermatozoa in different species

Species	% of more DNA of X sperm than Y sperm	References
Cattle	3.8	Garner et al., 1983 Garner, 2001, 2006 Johnson and Welch, 1999 Johnson, 2000
Buffalo	3.6	Johnson, 2000 Lu et al., 2006
Sheep	4.2	Johnson, 1995, 2000
Goat	4.4	Parilla et al 2004
Horse	3.7	Johnson, 2000
Swine	3.6	Johnson, 2000
Human	2.8	Johnson, 2000
Rabbit	3.0	Johnson, 2000
Camel	3.3	Johnson, 2000
Bison	3.6	Johnson, 2000
Yak	3.6	Johnson, 2000

AI with sexed sperm for production of sex-preselected offspring has been successful in cattle (Seidel et al., 1999), Sheep (Cran et al., 1997; Hollinshead et al., 2002), Horse (Buchanan et al., 2000) and Pig (Johnson, 1991; Gross-

feld et al., 2005). There were two reports on the application of AI with sexed sperm in buffalo. One was from Presicce et al. (2005) in which a conception rate of 42.8% was observed in Mediterranean Italian buffaloes following Ovsynch protocol and AI with sexed sperm, and another was from the aboratory (Lu et al., 2010). AI with sexed sperm is successful in practice in term of rate of calve born and the sex accuracy (Lu et al., 2013)

Sperm sorting for X/Y separation is dependent on the sperm's orientation (Johnson and Pinkel, 1986) to the laser beam so as to reduce variability sufficiently to distinguish the small difference in DNA content of the sperm. Due to the compactness of sperm chromatin, differential fluorescence is exhibited from the edge of the cell compared to the more transparent flat side of the sperm head. This leads to variable DNA fluorescence that masks the small (3-4%) X/Y DNA differences of many mammals. A significant enhancement in orientation was gained by the use of a new orienting nozzle system. By using this new nozzle, instead of only 20-30% of the sperm being oriented properly, the oriented population was changed to 65-70% of the total. This improvement alone increased the sorting speed from less than 0.4 million per hour to 0.8 to 1.0 million sperm per hour using a standard speed sperm sorter. The other development was the use of a high-speed cell sorter along with the new orienting nozzle. This was made possible by bringing about some minor adjustments to the nozzle so that it could be attached to a high-speed cell sorter. This adaptation to high-speed sorting was successful in increasing the speed again by several folds.

Sperm sexing by flow cytometric sorting (Johnson et al., 1989) requires two stages of extensive semen dilution. Some of the apparently detrimental changes observed after flow-cytometric sorting of spermatozoa may be due to the removal of decapacitation factors present in seminal plasma as a result of extensive dilution (Maxwell and Johnson, 1999). The first stage of dilution involves a 100- to 200 fold, in the case of boars and bulls, or 500- to 1000- fold extension in the case of rams, of the raw semen in preparation for staining with the DNA- permeant Hoechst 33342 flouro-chrome required for differentiation of X- and Y- bearing spermatozoa. The second occurs after passage through the flow cytometer or cell sorter, when the sorted spermatozoa are projected with the sheath fluid into the collection tube. This extension results in a 3,000- to 30,000- fold final dilution (depending on the species) of the original ejaculated spermatozoa. The spermatozoa and sheath fluid are collected in a tube containing test-yolk (2 or 20%), which provides protection from the combined effects of dilution by sheath fluid and potential physical damage to the sperm cells from projection into the collection tube (Johnson, 1995). A large proportion of the sperm are able to reach the lower portion of the tube containing the Test-yolk medium in which they are concentrated.

Sexed bovine semen can be used successfully in *in vitro* fertility systems (Lu et al., 1999). Artificial insemination with sexed sperm has been accomplished successfully in humans (Fugger, 1999).

LIMITATIONS OF SPERM SEXING

Percentages of motile sperm post- thaw are diminished (<10%) by the flow cytometric sorting process.

Higher laser intensities are found to damage sperm more than the lower intensities, though there was no effect of dye concentration on sperm damage (Schenk et al., 1999). Reduced male fertility and differences in fertility in sorted spermatozoa among males could be due, in part, to DNA damage (Libbus et al., 1987). Spermatozoa, like other non-dividing cells, are less susceptible to light radiation damage, but sperm cannot repair themselves after such damage occurs. Sperm possessing damaged DNA are capable of fertilizing an oocyte, and the oocyte can repair DNA damage to some extent after fertilization (Brandriff and Pederson, 1981).

A possible disadvantage of this technique is the use of an UV-excitable DNA specific stain. Although numerous healthy animals have been born using this technique, the risk of cytotoxic and or mutagenic effects cannot be ruled out completely. Reductions in fertility (Johnson, 1991; Mcnutt and Johnson, 1996) and negative effects on the rate of blastocyst formation (Merton et al., 1997) have been reported after sorting sperm cells. Johnson et al. (1989) postulated that fluorochrome dyes reduced embryonic viability and mid-gestation pregnancy rate. AI with flow sorted rabbit sperm resulted in fewer fetuses between days 7 and 14 of gestation than non-sorted sperm (Mcnutt and Johnson, 1996). However, Seidel et al. (1998) found no excess embryonic loss between 1 and 2 months of gestation in heifers inseminated with sorted sperm.

Procedure is relatively slow, potentially invasive and requires specialized, expensive and immobile equipment and highly skilled operators.

The cost of the sexed semen is prohibitively high in the present. However, availability and use of especially designed flow sorters could reduce per unit cost of sexed semen to around $ 9 to $12 (Amann, 1999). Moreover the development of newer techniques of sorting semen like the immunological approach could reduce the final cost of sexed semen making it affordable for the common farmer. The present day methods of sexing are not able to sex semen with 100% purity even though they can attain a high degree of purity of around 90%.

CONCLUSION

Sexing of semen has applications in both large and small scale farming systems. Though many methods of sexing spermatozoa have been reported, most of them need to be scientifically validated. At present, flow cytometric sorting of spermatozoa based on the difference in DNA content of X and Y-bearing spermatozoa is the only confirmed method of sperm sexing. But the method is expensive, time taking and doubts of potential harmful effects on spermatozoa have been raised. Sperm surface markers specific for X and Y spermatozoa may be a used as a tool for sperm sexing. One can also think about killing of unwanted sex bearing spermatozoa either at the production site or development designer bulls that produce only one type (either X or Y) of spermatozoa by knocking out the other type. These newer methods will circumnavigate the past limitations and thus making sperm sexing process viable commercially.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHORS' CONTRIBUTION

All the authors have contributed in terms of giving their technical knowledge to frame the article.

REFERENCES

- Amann RP (1999). Issues affecting commercialization of sexed sperm. Theriogenol. 52: 1441-1457. http://dx.doi.org/10.1016/S0093-691X(99)00229-0
- Ballinger HJ (1970). The effect of inseminations carried out early or late in oestrus on the sex ratio of calves born. Vet. Rec. 86: 631. http://dx.doi.org/10.1136/vr.86.21.631
- Barat J, Leger D (1979). Avenir des grossesses obtenuse apres stimulation de I' ovultion. A propos de 519 grossesses. J. Gynecol. Obslel. Biol. Reprod. 5: 332.
- Bhattacharya BC, Shome P, Gunther AH, Evans BM (1977). Successful separation of X and Y spermatozoa in human and bull semen. Int. J. Fert. 22: 30-35.
- Blecher SR, Howie R, Li S, Detmar J, Blahut LM (1999). A new approach to immunological sexing of sperm. Theriogenol. 52: 1309-1321. http://dx.doi.org/10.1016/S0093-691X(99)00219-8
- Blottener S, Nehring H, Jenichem W, Peter W (1983). Use of carrier free deflection electrophoresis in experiments for separation of sperm genotypes. Arch. Exp. Veterinamed. 37: 641-655.
- Brandriff B, Pederson RA (1981). Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. Science. 211: 1431-1433. http://dx.doi.org/10.1126/science.7466400
- Buchanan BR, Seidel GE Jr, McCue PM, Schenk JL, Herickhoff LA, Squires EL (2000). Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenol. 53: 1333-1344. http://dx.doi.org/10.1016/S0093-691X(00)00276-4
- Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WMC, Evans G (1997). Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human sperm. Human Reprod. 3: 821-825. http://dx.doi.org/10.1093/molehr/3.9.821
- Cran DG, McKelvey WAC, King ME, Dolman DF, McEvoy TG, Broadbent PJ, Robinson JJ (1997). Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted sperm. Theriogenol. 47: 267. http://dx.doi.org/10.1016/S0093-691X(97)82394-1
- De Cecco M, Spinaci M, Zannoni A, Bernardini C, Seren E, Forni M, Bacci ML (2011) Coupling sperm mediated gene transfer and sperm sorting techniques: a new perspective for swine transgenesis. Theriogenol. 74: 856–862. http://dx.doi.org/10.1016/j.theriogenology.2010.04.010
- De Vries A, Overton M, Fetrow J, Leslie K, Eicker S, Rogers G (2008). Exploring the impact of sexed semen on the structure of the dairy industry. J. Dairy Sci. 91: 847–856. http://dx.doi.org/10.3168/jds.2007-0536
- Ericsson RJ, Langevin CN, Nishino M (1973). Isolation of fractions rich in Y spermatozoa. Nature. 246: 421-424. http://dx.doi.org/10.1038/246421a0
- Evans JM, Douglas TA, Renton JP (1975). An attempt to separate fractions rich in human Y sperm. Nature. 253: 352-354. http://dx.doi.org/10.1038/253352a0
- Foote RH (1977). Sex ratios in dairy cattle under various conditions. Theriogenol. 8: 349-356. http://dx.doi.org/10.1016/0093-691X(77)90186-8
- Foote RH (1985). Normal development of fetuses resulting from Holstein semen processed for sex separation. Theriogenol. 24: 197-202. http://dx.doi.org/10.1016/0093-691X(85)90183-9
- Fugger EG (1999). Clinical experience with flow cytometric separation of human X- and Y- chromosome bearing sperm. Theriogenol. 52: 1435-1440. http://dx.doi.org/10.1016/S0093-691X(99)00228-9
- Fulwyler MJ (1965). Electronic separation of biological cells by volume. Science. 150: 910. http://dx.doi.org/10.1126/science.150.3698.910
- Fulwyler MJ (1977). Hydrodynamic orientation of cells. J. Histochem. Cytochem. 25: 781-783. http://dx.doi.org/10.1177/25.7.330728
- Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA, Johnson LA (1983). Quantification of the X- and Y-chromosome bearing sperm of domestic animals by flow cytometry. Biol. Reprod. 28: 312–321. http://dx.doi.org/10.1095/biolreprod28.2.312
- Goldberg EH, Boyse EA, Bennet D, Scheid M, Carswell EA (1971). Serological demonstration of H-Y (male) antigen on mouse sperm. Nature. 232: 478-480. http://dx.doi.org/10.1038/232478a0
- Grossfeld R, Klinc P, Sieg B, Rath D (2005). Production of piglets with sexed semen employing a non-surgical insemination technique. Theriogenol. 63: 2269–2277. http://dx.doi.org/10.1016/j.theriogenology.2004.10.004

- Hendriksen PJM, Tieman M, van der Lende T, Johnson LA (1993). Binding of anti H-Y monoclonal antibodies to separated X and Y chromosome bearing porcine spermatozoa. Mol. Reprod. Dev. 35: 189-96. http://dx.doi.org/10.1002/mrd.1080350213
- Hermann BG, Koschroz B, Wertz K, McLaughlin KJ, Kispert A (1999). A protein kinase encoded by the *t complex responder* gene causes non-mendalian inheritance. Nature. 402: 141-146. http://dx.doi.org/10.1038/45970
- Hohenboken WD (1999). Applications of sexed semen in cattle production. Theriogenol. 52: 1421-1433. http://dx.doi.org/10.1016/S0093-691X(99)00227-7
- Hollinshead FK, O'Brien JK, Maxwell WM, Evans G (2002). Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen-thawed sorted X- or Y-chromosome-bearing spermatozoa. Reprod. Fertil. Dev. 14: 503–508. http://dx.doi.org/10.1071/RD02034
- Hoppe PC, Koo GC (1984). Reacting mouse sperm with monoclonal H-Y antibodies does not influence sex ratio of eggs fertilized *in vitro*. J. Reprod. Immunol. 6: 1-9. http://dx.doi.org/10.1016/0165-0378(84)90036-6
- Iwasaki S, Shioya, I, Masuda H, Hanada A, Akahara T (1988). Sex ratio of early embryo fertilized *in vitro* with spermatozoa separated by percoll. Theriogenol. 30: 1191-1198. http://dx.doi.org/10.1016/0093-691X(88)90295-6
- James W (1980). Gonadotrophins and the human secondary sex ratio. Br. Med. J. 281: 711. http://dx.doi.org/10.1136/bmj.281.6242.711
- Johnson LA (1991). Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. Reprod. Dom Anim. 26: 309. http://dx.doi.org/10.1111/j.1439-0531.1991.tb01546.x
- Johnson LA (1995). Sex pre selection by flowcytometric separation of X and Y- chromosome bearing sperm based on DNA difference: a review. Rep. Fert. Dev. 7: 893-903. http://dx.doi.org/10.1071/RD9950893
- Johnson LA (2000). Sexing mammalian sperm for production of offspring: the state of- the-art. Anim. Reprod. Sci. 61: 93–107. http://dx.doi.org/10.1016/S0378-4320(00)00088-9
- Johnson LA, Pinkel D (1986). Modification of a laser based flow cytometer for high resolution DNA analysis of mammalian spermatozoa. Cytometry. 7: 268-273. http://dx.doi.org/10.1002/cyto.990070307
- Johnson LA, Welch GR (1999). Sex-preselection: high speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenol. 52: 1323-1341. http://dx.doi.org/10.1016/S0093-691X(99)00220-4
- Johnson LA, Flook JP, Hawk HW (1989). Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol. Reprod. 41: 191-203. http://dx.doi.org/10.1095/biolreprod41.2.199
- Johnson LA, Flook JP, Look MV (1987). Flow cytometry of X and Y chromosome bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. Gamete Res. 17: 203-212. http://dx.doi.org/10.1002/mrd.1120170303
- Kamentsky LA, Melamed RR (1967). Spectrophotometric cell sorter. Science. 156: 1364-1365. http://dx.doi.org/10.1126/science.156.3780.1364
- Kaneko S, Oshiro S, Kobayashi T, Itzuka R, Mohri H (1984). Human X- and Y- bearing sperm differ in cell surface sialic acid content. Biochem. Biophys. Res. Commun. 124: 950-955. http://dx.doi.org/10.1016/0006-291X(84)91050-7
- Killian GD, Cash E, Griel L, Allen CH, Gilbert G, Kaproth M, Lenz R, Marshall CE (1996). Conditions which affect the abundance of fertility associated proteins in seminal plasma. Proc. 16th NAAB Tech. Conf. On artificial insemination and Reproduction, National Association of Animal Breeders, Columbia, MO. Pp. 57-60.
- Liang XW, Lu YQ, Chen MT, Zhang XF, Lu SS, Zhang M, Pang CY, Huang FX, Lu KH (2008). *In vitro* embryo production in buffalo (*Bubalus bubalis*) using sexed sperm and oocytes from ovum pick up. Theriogenol. 69: 822–826. http://dx.doi.org/10.1016/j.theriogenology.2007.11.021
- Libbus BL, Perreault SD, Johnson LA, Pinkel D (1987). Incidence of chromosome aberrations in mammalian sperm stained with Hoechst 33342 and UV-laser irradiated during flow sorting. Mutation Res. 182: 265-274. http://dx.doi.org/10.1016/0165-1161(87)90011-2
- Lu KH, Cran DG, Seidel GE Jr (1999). *In vitro* fertilization with flow cytometrically sorted bovine sperm. Theriogenol. 52: 1407-1420. http://dx.doi.org/10.1016/S0093-691X(99)00225-3
- Lu YQ, Liang XW, Zhang M, Wang WL, Kitiyanant Y, Lu SS, Meng B, Lu KH (2007). Birth of twins after *in vitro* fertilization with flow-cytometric sorted buffalo *(Bubalus bubalis*) sperm. Anim. Reprod. Sci. 100: 192–196. http://dx.doi.org/10.1016/j.anireprosci.2006.09.019
- Lu Y, Zhang M, Lu SS, Xu DX, Huang WH, Meng B, Xu HY, Lu KH (2010). Sex-preselected buffalo (*Bubalus bubalis*) calves derived from artificial insemination with sexed sperm. Anim. Reprod. Sci. 119: 169-171. http://dx.doi.org/10.1016/j.anireprosci.2010.01.001
- Lu Y, Lu SS, Zhang M, Xiaogan Y (2013). Sperm sexing in buffalo using flow cytometry Buffalo Bull. 32: 22-30.
- Lu YQ, Zhang M, Meng B, Lu SS, Wei YM, Lu KH (2006). Identification of X- and Y-chromosome bearing buffalo (*Bubalus bubalis*) sperm. Anim. Reprod. Sci. 95: 158–164. http://dx.doi.org/10.1016/j.anireprosci.2005.11.005
- Lush JL (1925). The possibility of sex control by artificial insemination with centrifuged spermatozoa. J. Agri. Res. 30: 893-913.
- Maxwell WMC, Johnson LA (1999). Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenol. 52: 1353-1362. http://dx.doi.org/10.1016/S0093-691X(99)00222-8
- Maxwell WMC, Meddoa G, Shite IG (1984). Post thawing survival of motile ram sperm after isolation by layering on protein columns. Theriogenol. 21: 601-606. http://dx.doi.org/10.1016/0093-691X(84)90445-X
- Maxwell WMC, Evans G, Hollinshead FK, Bathgate R, De Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK (2004). Integration of sperm sexing technology into the ART toolbox. Anim. Reprod. Sci. 83: 79–95. http://dx.doi.org/10.1016/j.anireprosci.2004.04.013
- McNutt TL, Johnson LA (1996). Flow cytometric sorting of sperm: influence on fertilization and embryo fetal development in rabbit. Mol. Reprod. Dev. 43: 261-267. http://dx.doi.org/10.1002/(SICI)1098-2795(199602)43:2<261::AID-MRD16>3.0.CO;2-6
- Meistrich M (1982). Potentials and limitations of physical methods for separations of sperm bearing an X or Y chromosome prospects for sexing Mammalian sperm. Colorado Associated Univ. Press, Boulder, Colorado. Pp. 145-163.

- Merton JS, Haring RM, Stap J, Hoebe RA, Aten JA (1997). Effect of flow cytometrically sorted frozen/thawed semen on success rate of *in vitro* bovine embryo production. IETS Annual Meeting, Nice, France. http://dx.doi.org/10.1016/s0093-691x(97)82422-3
- Moruzzi JF (1979). Selecting a mammalian species for the separation of X- and Y-chromosome bearing spermatozoa. J. Reprod. Fert. 57: 319-323. http://dx.doi.org/10.1530/jrf.0.0570319
- Muller W, Gautier F (1975). Interactions of heteroaromatic compounds with nucleic acids. Eur. J. Biochem. 54: 358. http://dx.doi.org/10.1111/j.1432-1033.1975.tb04138.x
- Niemann H, Kuhla B, Flachowsky G (2011). Perspectives for feed efficient animal production. J. Anim. Sci. 89: 4344–4363. http://dx.doi.org/10.2527/jas.2011-4235
- O'Brien JK, Stojanov T, Heffernan SJ, Hollinshead FK, Vogelnest L, Maxwell WMC, Evans G (2005). Flow cytometric sorting of non-human primate sperm nuclei. Theriogenol. 63(1): 246–259. http://dx.doi.org/10.1016/j.theriogenology.2004.04.013
- Ollero M, Perez-Pe R, Gargallo I, Morlanes S, Osada J, Muino-Blanco T, Cebrian-Perez J (2000). Separation of ram spermatozoa bearing X and Y chromosome by centrifugal countercurrent distribution in an aqueous two phase system. J. Androl. 21: 921-928.
- Parrilla I, Vazquez JM, Roca J, Martinez EA (2004). Flow cytometry identification of X- and Y-chromosome bearing goat sperm. Reprod. Dom. Anim. 39: 58–60. http://dx.doi.org/10.1046/j.1439-0531.2003.00480.x
- Pinkel D, Gledhill BL, Lake S, Stephenson D, Ven Dilla MA (1982). Sex preselection in mammals? Separation of sperm bearing Y and 'O' chromosome in the vole *Microtus Oregoni*. Science. 218: 904-906. http://dx.doi.org/10.1126/science.6753153
- Presicce GA, Verberckmoes S, Senatore EM, Klinc P, Rath D (2005). First established pregnancies in Mediterranean Italian buffaloes (*Bubalus bubalis*) following deposition of sexed spermatozoa near the utero tubal junction. Reprod. Domest. Anim. 40: 73–75. http://dx.doi.org/10.1111/j.1439-0531.2004.00560.x
- Rorie RW (1999). Effect of timing of artificial insemination on sex ratio. Theriogenol. 52: 1273-1280. http://dx.doi.org/10.1016/S0093-691X(99)00192-2
- Russel FL (1891). Breeding statistics. Maine Agricultural Statistic, Annual report for 1891. Pp. 208-209.
- Sarkar S, Jolly DJ, Friedman T, Jones OW (1984). Swimming behaviour of X and Y human sperm. Differentiation. 27: 120-125. http://dx.doi.org/10.1111/j.1432-0436.1984.tb01417.x
- Schenk JL, Suh TK, Cran DG, Seidel GE Jr (1999). Cryopreservation of flow sorted bovine spermatozoa. Theriogenol. 52: 1375-1391. http://dx.doi.org/10.1016/S0093-691X(99)00224-1
- Seidel GE Jr (1988). Sexing spermatozoa and embryos – state of the art. In: Proceedings of XI international congress of animal reproduction and artificial insemination (Dublin) Vol. **5**, University College, Dublin. Pp. 136-144.
- Seidel GE Jr, Herichkoff LA, Schenk JL, Doyle SP, Green RD (1998). Artificial insemination on heifers with unfrozen sexed semen. Theriogenol. 49: 365. http://dx.doi.org/10.1016/S0093-691X(98)90718-X
- Seidel GE Jr, Schenck JL, Herichkoff LA, Doyle SP, Brink Z, Green RD, Cran DG (1999). Insemination of heifers with sexed spermatozoa. Theriogenol. 52: 1407-1420. http://dx.doi.org/10.1016/S0093-691X(99)00226-5
- Seidel GE Jr, Garner DL (2002). Current status of sexing mammalian spermatozoa. Reproduction. 124: 733–743. http://dx.doi.org/10.1530/rep.0.1240733
- Stovel RT, Sweet RG, Herzenberg LA (1978). A means of orienting flat cells in flow systems. Biophys. J. 23: 1-5. http://dx.doi.org/10.1016/S0006-3495(78)85427-7
- Van Munster EB (2002). Interferometry in flow to sort unstained X- and Y- bearing bull spermatozoa. Cytometry. 47: 192-199. http://dx.doi.org/10.1002/cyto.10064
- Van Munster EB, Stap J, Hoebe RA, Te Meerman GJ, Aten JA (1999a). Difference in sperm head volume as a theoretical basis for sorting X- and Y-bearing spermatozoa: potentials and limitations. Theriogenol. 52: 1281-1293. http://dx.doi.org/10.1016/S0093-691X(99)00217-4
- Verme LJ, Ozoga JJ (1981). Sex ratio of white-tailed deer and estrus cycle. J. Wildl. Manage. 45: 710-715. http://dx.doi.org/10.2307/3808704
- Watchel SS (1983). Sex specific transplantation antigens in species other than mouse. In: HY antigen and the biology of sex determination, Gruene and Stratton, New York. Pp. 22-37.
- Wehner GR, Wood C, Teague A, Barker D, Hubert H (1997). Efficiency of Ovatec unit for estrus detection and calf sex control in beef cows. Anim. Rep. Sci. 46: 27-34. http://dx.doi.org/10.1016/S0378-4320(96)01604-1
- Yang WC, Sang L, Xiao Y, Zhang HL, Tang KQ, Yang LG (2014).Tentative identification of sex-specific antibodies and their application for screening bovine sperm proteins for sex-specificity. Mol Biol Rep. 41(1):217-23. http://dx.doi.org/10.1007/s11033-013-2854-2
- Zavos P (1985). Sperm separation attempts via the use of albumin gradients in rabbits. Theriogenol. 23: 875. http://dx.doi.org/10.1016/0093-691X(85)90005-6