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SPERM SEXING METHODS AND RECENT DEVELOPMENTS IN THIS FIELD: SYSTEMATIC REVIEW

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Abstract

Importance: Rapid growth in human population is putting a strain on agricultural industry. One of the ways to solving this problem is increasing the yield of farm animals by selecting the sex of the offspring.

Objective: To evaluate the effectiveness, safety and availability of various sperm sex sorting methods.

Evidence review: PubMed search engine was used tofind published works on sperm sex selection methods in the MEDLINE database.

Findings: Search by key words yielded scientific articles describing many sperm sex sorting methods that make use of distinctive features of X- and Y-chromosome bearing sperm cells.

Conclusion and relevance: Many sperm sex sorting methods have been developed over the last half-century, but only some of them had a high level of reproducibility and proved to be reliable. Our review shows that further studies should be performed to eliminate drawbacks of the current methods and solve global problems in the field of animal husbandry and reproductive medicine.

Keywords: sperm sex selection, sex chromosomes, MicroSort, TLR7/TLR8, sex-specific proteins

SYSTEMATIC REVIEW

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Capsule: In this review we set out to paint a complete picture of modern state of sperm sex selection methods, provide comparison of their advantages and disadvantages and pinpoint the most promising techniques.

INTRODUCTION

Sperm sorting is defined as a separation of sperm cells based on certain parameters. The so-called sperm sexing techniques involve separation of sperm cells based on them carrying either an X or Y chromosome. Given the rapid and global growth in population,humanity is facing a threat of food shortage. This has increased the pressure on the agricultural industry where the sperm sexing methods – means of choosing offspring's gender – have become so popular. They give farmers a more flexible method of production management, which in turn helps them provide sufficient resources for growing population [1].

In addition, sperm sexing is also used in medicine in the case of certain medical indications. For example, in order to prevent X-linked diseases that affect boys more often like Duchenne muscular dystrophy. If a couple has a high risk of conceiving a child with such diseases, this technology gives them a chance to have a healthy offspring and avoid abortion if the prenatal screening revealed any genetic defects [2].Microsort sperm sexing technology based on flow cytometry wasfirst used on human sperm in the last decade of the 20th century and allowed a woman who lost two brothers and two sons to an X-linked Hydrocephalus to finally give birth to a girl.Over time, the list of medical indications have been expanded and the FDA as an exception approved the use of Microsort to help couples reach family balance.However, strict requirements were set: 1) only couples that already have at least one child can participate in the program; 2) they can only select the non-dominant sex among their children. Also, couples have to keep in mind the probability of failed sex selection and doctors have to make sure that

a couple will accept a child of either sex in case of failed sex selection [3,4].

In this study we review sperm sexing methods that have been developed over the years taking into account differences discovered between X chromosome bearing and Y chromosome bearing sperm cells (X spermatozoa and Y spermatozoa), compare their effectiveness in sorting spermatozoa and safety of the technique.

Materials and methods

PubMed search engine was used to find scientific articles on sperm sex selection techniques in MEDLINE database with following key words and terms: sperm sex selection, sperm sexing, flow cytometry, MicroSort, albumin gradient, Percoll gradient, swim-up method, H-Y antigen, sex-specific proteins, sperm cell surface proteins, TLR 7/8. We primarily collected data on the reported ratio of X and Y spermatozoa in the semen after sperm sexing was completed, sex ratio among the embryos after fertilization and sex ratio after birth. All types of publications on sperm sexing methods, including original articles and reviews, published before April 2022 were included in the review, but works that contain data on the concentrations of X and Y spermatozoa after the sperm cells have been sorted were prioritized.

Two independent reviewers (A.M and Kh.Zh.) independently performed a search and screening of the articles found based on their title and abstractand excluded irrelevant citations. Any disagreement or uncertainty was resolved by the involvement of a third reviewer (G.B.)

Ericsson method

Scientists have been trying to develop sperm sex sorting methods for a long time by utilizing various qualitative and quantitative differences between X and Y chromosome bearing sperm cells, such as motility, shape, size, etc.

One of the first works on this subject was based on the probable difference in motility between X and Y spermatozoa.

First works describing the difference in motility between X and Y spermatozoa have been published by Ericsson et al. in 1973 using the albumin gradient [5]. It was discovered that a 25% albumin medium has the perfect consistency to separate sperm cells based on their motility by hindering the movement of low motility spermatozoa.Isolation fraction that contains high motility spermatozoa that managed to pass through the albumin gradient then were stained with quinacrine fluorochrome. This dye stains the distal end of the long arm of the Y chromosome,and at the time it was thought that it can be used to identify Y chromosomes. Authors found that 80% of spermatozoa in this fraction contain a Y chromosome [6]. In 1974 Ericsson patented this technique, known to us as the Ericsson method. In his patent, he noted this method's potential in sperm sexing and family planning[7]. Subsequently several authors tried to reproduce these experiments and use Ericsson method for sex selection and family planning but obtained contradicting results. Even though Ericsson describes cases of his method being used to obtain male offspring successfully [8], other authors report inefficiency of this method for Y spermatozoa enrichment or obtained only a slight increase in Y spermatozoa concentration [9-12].

Still there are other works that managed to reproduce the results obtained by Ericsson. Rose et al. reported the live birth of 13 male children, 2 identical twin boys, and 2 twins – one boy and one girl – from 18 couples who wanted a son [13].

Percoll gradient

Discontinuous Percoll gradient has also been used as a potential tool to separate X and Y spermatozoa. Iizuka et al. Used 12-step (25-80%) Percoll gradient and discovered that 94% of sperm cells from 80% Percoll fraction do not stain with quinacrine. Therefore, authors presumed that they were X chromosome bearing sperm cells. All 6 pregnancies after intrauterine insemination (IUI) with sorted sperm lead to the live birth of girls [14].

Later Wang et al. used FISH to assess the effectiveness of 12-step Percoll gradient in sperm sexing and discovered only a slight enrichment of sperm with X spermatozoa (от 51:46 до 57:38), which was a far cry from the 94% reported by Iizuka [15, 16].

This discrepancy was subsequently explained by research done by Van Kooij and Van Oost. They performed Southern blot analysis with DNA probe (pDP34) which can distinguish loci on X and Y chromosome on spermatozoa isolated with 12-step Percoll gradient and found approximatelyequalnumber of X and Y spermatozoa in 80% Percoll fraction. However, 86% of spermatozoa from the same population were stained with quinacrine. These contradicting results made authors question the use of quinacrine staining to estimate X and Y spermatozoa ratio after the sorting [17].

Swim-up method

Following the density gradients, swim-up method has also been used to perform sperm sex selection based on the difference in motility between X and Y spermatozoa. Check et al. report obtaining high enrichment of Y sperm cells (83.6%) and male offspring (81%) after using this method. However, just like the previous authors, they used quinacrine to stain the obtained sperm population [18, 19].Later Han et al. performed double FISH before and after swim-up method and did not discover significant changes in X and Y spermatozoa ratio [20, 21].

Sperm viability

Park et al. tested viability of X and Y spermatozoa in different pH conditions – 6.2, 7.2 and 8.2.They discovered that after storing boar semen at pH 6.2 for 2 and 4 days there are more dead Y spermatozoa than dead X spermatozoa. Ratio of viable X spermatozoa to Y spermatozoa is highest after two days (1.2:1).Moreover, using this sperm for IUI increased the number of female pups. Authors conclude that storing the sperm in acidic solution for 2 days before the IUI might significantly increase the chances of obtaining female offspring [22].

Flow cytometry

Another big dissimilarity between X and Y spermatozoa is the difference in DNA content. It is known

that X spermatozoa contain more DNA than Y spermatozoa (2.8% in humans and 4% in livestock) [23, 24]. If sperm cells are stained with fluorescent DNA dye, then X spermatozoa will have higher fluorescence. Among many DNA dyes the Hoechst 33342 turned out to be the best fit: 1) it is a vital stain (cells remain viable after staining); 2) it can pass through the cell membrane; 3) it is less genotoxic than other similar dyes like DAPI. This difference in fluorescence between X and Y spermatozoa after Hoechst 33342 staining has become the basis for the most conventional sperm selection method – flow cytometry [23]. First attempts to use this method for sperm sexing was made in animals such as bulls, boars, sheep and rabbits [24, 25]. The first breakthrough was achieved in 1989, when the first live offspring was obtained in rabbits [26]. In 1990, Schulman adapted this technology for humans and called it MicroSort [4]. In 1998, Vidal et al. published an article describing the results of using MicroSort technology on human sperm. When used to select X spermatozoa, the effectiveness of this method reached 80-90% and 60-70% - for Y spermatozoa [27].

In their article from 2009,Karabinus et al. provide data on MicroSort from the Genetics & IVF Institute. Between 1994 and 2007, they performed 5370 ART cycles with MicroSort. Among them, 3629 were IUI, 1642 – IVF/ICSI with fresh embryo transfer and 99 – IVF/ICSI with frozen embryo transfer (FET). 5871 sperm samples have been sexed, 74.9% (4399) of which were for X spermatozoa (XSort), while 25.1% (1472) – for Y spermatozoa (YSort). With XSort, an enrichment of87.9% was reached; for YSort, that was equal to 73.4%. Eggs were fertilized in 70.7% of IVF/ICSI cycles and 93.8% of 2 pronuclei (2PN)embryos reached the cleavage stage. Pregnancy rate afterIUI was 15.6% (567); after IVF/ICSI with fresh embryo transfer – 33% (525) and 33.3% (33) after FET. 801 of 1125 pregnancies resulted in the live birth of 943 babies (662 – singleton pregnancy, 272 – twin pregnancy and 9 - triplets) and 167 were ongoing. 92.0% of babies born after XSort were female and 81.5% of babies after YSort were male. Medical records of 760 were reviewed. It was discovered that 20 (2.6%) of them had major congenital malformation and 46 (6.1%) – minor congenital malformation [28].

In 2014,Karabinus et al. published another article with updated data from 2008 and 2012. Overall there has been a slight increase in the effectiveness of MicroSort technology [29].

In 2013, De Geyter et al. reported first successful pregnancy after sperm sexing with MicroSort in Switzerland. Medical indication for using MicroSort in this case was Becker muscular dystrophy. Two brothers of the woman in this couple were diagnosed with this disease. The couple rejected the option of prenatal diagnostics and possible legal abortion. For financial reasons the option of PGD in fertility clinics abroad has been ruled out. After long discussion, doctors suggested sperm sexing for ICSI. After receiving permission from ethics committee of University Hospital of Basel, authors contacted MicroSort representatives. Two samples of semen were obtained and sent to MicroSort lab after cryopreservation for sperm sexing. 14 oocytes in Metaphase stage II were obtained after ovarian hyperstimulation. All the sexed spermatozoa received from MicroSort were found to be immotile after thawing. The morning after the injection of 14 oocytes, 3 fertilized eggs with 2PN were discovered. Only one embryo reached blastocyst stage and was transferred into the uterine cavity. Intrauterine singleton pregnancy was diagnosed 14 days after oocyte retrieval [30].

Flow cytometric sperm sexing is one of the mainstream sperm selection methods, however, it has its shortcomings. One of the biggest drawbacks is Hoechst 33342 and UV radiation's effect on sperm viability and DNA integrity [31, 32].Another downside of flow cytometry is low sorting speed, which prolongs the sperm sexing process to achieve high number of sorted spermatozoa. It may lead to some undesirable outcomes such as decreased motility or complete immobility of the sperm cells and can also affect embryo quality. It is known that the number of dead or damaged spermatozoa increases by 20% after the sorting [23]. Due to these reasons, scientists keep working on improving this technique and developing alternative sperm sexing methods.

One of such methods is the use of aptamers – oligonucleotides that specifically bind target molecules. Characteristics such as mall size, high specificity, high affinity, accessibility and ability to renaturate after denaturation lead to its application in various fields, including sperm sex selection [33, 34].

Sex-specific proteins

Sperm sexing techniques of particular interest to scientists are the methods targeting the so-called sex-specific proteins on spermatozoa.The first discovered sex-specific protein was the H-Y antigen on the spermatozoa surface. It is first mentioned in the article by Eichwald and Silmser who suggest that it is responsible for skin rejection after male-to-female syngeneic transplantation [35]. In 1971, Goldberg et al. discovered that this antigen is present on the surface of 50% of sperm cell population in mice [36]. It led to the suggestion that it is expressed solely by Y-chromosome and could be used for sperm sex sorting. It was later confirmed that the H-Y antigen is encoded by the Y-chromosome gene KDM5D, also known as SMCY. The homologous gene KDM5C, also known as SMCX, is present on the X-chromosome.A number of scientists worked – with varying degree of success – on the development of sperm sexing method with the H-Y antigen [35]. In the work by Ali et al. semen samples from three bulls were sorted and separated into two fractions ($H\text{-}Y^+$ and $H\text{-}Y^-$) by Fluorescence-activated cell sorting (FACS). After incubation with Feulgen stain, the$H\text{-}Y^+$fraction contained 76.1%, 88% and 76.7% Y chromosome in each semen sample, respectively, while X spermatozoa in the$H\text{-}Y^-$ fraction was 74%, 64.6% and 77.8%, respectively [37]. Some articles questioning the effectiveness of this method and the role of the H-Y antigen for sperm sex selection have been published. In 1993, Hendriksen et al. sorted the sperm with flow cytometric method which was new at the time. Then the sorted sperm samples were incubated with anti-H-Y monoclonal antibodies. It was discovered that across all samples, only 20-50% of spermatozoa bound to the antibodies and there was

no significant difference between the X spermatozoa enriched and Y spermatozoa enriched fractions. The authors conclude that this experiment did not discover any proof of H-Y antigen expression on Y-spermatozoa [38]. In 1998, Sills et al. used immunological method with paramagnetic particles that is based on the presence of H-Y antigen on the sperm surface. They processed the sperm with anti-H-Y-antigen monoclonal antibodies (IgM) and then incubated it with anti-antibodies (IgM) bound to paramagnetic particles. Then sperm cells were separated into two fractions with the use of magnetic field - H-Y Ag+ and H-Y Ag-. In total, 1600 cells were analyzed with FISH – 800 from each fraction. In the H-Y Ag+ group, 54.1% of sperm cells contained Y chromosome, while 51% of sperm cells in H-Y Ag- contained X chromosome. Authors reached the conclusion that immunological techniques of the time that are based on H-Y antigen aren't capable of yielding high results when it comes to human sperm sex selection. Nonetheless they suggest that there may exist other sex-specific proteins that can be used for sperm sexing [39].

Among the works on sex-specific proteins, the most notable is research done by Umehara and colleagues. They investigated the effect of ligands on the X sperm cell surface receptors to develop a novel sperm sexing technique. 492 genes are encoded and expressed by X chromosome in mice sperm cells and 18 of them encode a receptor protein. 12 of them have common ligands with receptors encoded by autosomal genes, while the other 6 (Tlr8, Ar, Gpr174, Tlr7, Gpr34 and Edr2a) have unique ligands. Authors examined the presence of mRNA of these receptors in sperm cells with reverse transcription polymerase chain reaction (RT-PCR). Due to authors' previous experience in working with TLR2 and TLR4 ligands that negatively affect viability, motility and capacitation in mammalian sperm cells, they decided to focus on Toll-like receptors (TLR7 and TLR8). They verified the presence of TLR7/TLR8 on the surface of spermatids, epididymal and testicular spermatozoa, Leydig cells and to a lesser extent on the surface of Sertoli cells. After Immuno-FISH with X chromosome probe (X-paint) and anti-TLR7 antibody positive TLR7 signals were only observed in X chromosome bearing cells.After incubating the sperm cells with Resiquimod (R848), TLR7 and TLR8 ligand, Imiquimod (R837), TLR7 ligand, scientists discovered significant decrease in the number of highly active sperm in the upper layer following the swim-up test. They noted that ratio of X spermatozoa to Y spermatozoa in the upper layer had also decreased (X spermatozoa∼20%, Y-spermatozoa∼80%) [40]. It was shown that decreased motility of X spermatozoa and failure to swim to the upper layer during the swim-up test is caused by decreased ATP production by themitochondria via phosphorylation of NF-kappaB (NF-κB) and Glycogen synthase kinase 3α/β (GSK3α/β) [41].

When sperm cells from the upper layer were used for fertilization, 77 embryos at the blastocyst stage were obtained: 68 of them were XY and 9 – XX. After the embryo transfer, 83.1% ± 4.6% of the pups were male. In the case of sperm cells from lower layer, they yielded 83 embryos at blastocyst stage: 58 were XX and 25 were XY. After embryo transfer,81.4% ± 1.9% of the offspring were female [40]. Ren et al. applied the same procedure to goat sperm. After the swim-up test, the upper layer contained 90.5± 2.86 % Y spermatozoa and 9.28% X spermatozoa, while the lower layer contained 80.3± 2.91 % X spermatozoa and 15.7% Y spermatozoa. Due to the goal of the investigation being research into methods of increasing goat milk via increasing the birth rates of does, authors only used the sperm in the lower layer. After IVF they obtained 43 embryos, 35 of them were XX (81.4%). In addition, they used the lower layer sperm for IUI of 5 does, 9 embryos were collected from the two does that conceived. 8 embryos were XX and 1 embryo was XY, but due to the low sample number, these results can only be interpreted as preliminary [41]. In 2020, Umehara et al. published a full protocol of their sperm sex selection method for mice and bull sperm [42].

Toll-like receptors (TLR) are transmembrane proteins that are expressed on the cell surface of many mammalian organisms including mice and humans. At the moment 13 types of Toll-like receptors were identified. TLR1, 2, 4, 5 and 6 are located on the cell surface and mainly bind to bacterial components, while TLR3, 7, 8 and 9 that bind to virus-associated nucleic acids are located in endosomes. Signaling pathways of TLR7 and TLR8 lead to the activation of Interferon-regulating factors 5 and 7 (IRF5 and 7) and expression of Interferon-α,which plays an important role in antiviral immune defense [43]. TLR7 and TLR8 are activated by single-stranded virus RNA such as Hepatitis C, HIV and Zika virus [40]. Perhaps *in vivo* function of TLR7 and TLR8 is an evolutionary defensive mechanism against viral infection which is manifested as decreased spermatozoa motility and prevention of fertilization under adverse environmental conditions. If so, then presence of TLR7 and TLR8 only on X spermatozoa should mean that viral components can't affect Y spermatozoa and prevent fertilization, and therefore affect sex ratio (the ratio of males to females) among the offspring. Robertson and Sheard were first to report the possible connection between hepatitis and sex ratio among the newborns. They noticed unusual sex ratio in newborns after unexpected outbreak of infective hepatitis [44]. Results obtained from investigations in Greece and Greenland suggest that in the presence of Hepatitis B antigen (HBsAg+) in either of the parents,the sex ratio is higher than in parents that don't have Hepatitis B antigen [45, 46].

Table 1 contains a compilation of data on the effectiveness of various sperm sex selection methods based on the principle behind the technique.

Conclusion

Sperm sexing methods should be accurate, highly effective, fast, cheap, accessible by farmers and, most importantly, they shouldn't damage sperm cells. Our review showed many methods developed by relying on various differences between X and Y chromosome bearing sperm cells. But practical validity of some of the methods can be questioned due to inconsistency in their results. Only two sperm sexing methods –flow cytometry and use of ligands for sex-specific proteins – showed consistent and great results among the various

works. Despite being the mainstream sperm sex selection method today, flow cytometric technique still has a lot of shortcomings that we mentioned above. On the other hand, Umehara and colleagues managed to develop a simple, relatively cheap new sperm sex sorting technique that targets sex-specific receptors and doesn't affect quantitative and qualitative parameters of the sperm. It was developed only a few years ago and has already attracted the attention of scientists researching this field. It has a lot of potential to grow and more research is needed to perfect it and adapt it to other species. For example, there is no research describing the use of TLR7/TLR8 ligands for sperm sex selection in humans.Moreover, recent studies revealed a plethora of new potential sex-specific proteins [40, 47, 48]. It opens up new opportunities for researching and developing new methods. We believe that the potential of an immunological approach in sperm sexing hasn't been fully realized, especially when it comes to using antibodies targeting sex-specific proteins and magnetic particles. Therefore, our efforts will be directed to research in this direction.

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Table 1.

Comparison of results of sperm sex selection methods

Sorting method	Authors	Sample	Target sperm cells	Percentage of target sperm cells after the sorting	Method used for determining the X spermatozoa:Y spermatozoa ratio after the sorting
Albumin gradient	Erickson, 1973	Human	Y	80%	fluorochrome quinacrine staining
	Vidal, 1993	Human	Y	49.4%	FISH
	Wang, 1994	Human	Y	46.7%	FISH
	Flaherty, 1997	Human	Y	49.1%	FISH
Percoll gradient	Iizuka, 1987	Human	X	94%	Quinacrine staining
	Van Kooij and Van Oost, 1992	Human	X	50% 86%	FISH Quinacrine staining
	Wang, 1994	Human	X	51-57%	FISH
	Andersen, 1997	Human	X	60.3%	FISH
Swim-up method	Check, 1989	Human	X	81%	Quinacrine staining
	Han, 1993	Human	X	50%	FISH
Viability of sperm cells under different pH conditions	Park, 2020	Boar	X	55%	FISH
Flow cytometry: X-sperm cells contain more DNA	Johnson, 1989	Rabbit	X and Y	X – 86%, Y – 81%	Flow cytometric analysis of sperm nuclei
	Johnson, 1993	Human	X and Y	X – 80% X – 75%	FISH
	Vidal, 1998	Human	X and Y	X – 86.83% Y – 67.43%	FISH
	Karabinus, 2014	Human	X and Y	X - 87.7 ± 5.0% Y - 74.3 ± 7.0%	FISH
Immunologic methods: H-Y antigen on Y-sperm cell surface	Ali, 1990	Bull	X and Y	X – 74% Y – 76.1%	Feulgen staining and scanning microdensitometry
	Sills, 1998	Human	X and Y	X – 51% Y – 54.1%	FISH
Ligand activation of TLR7/TLR8	Umehara, 2019	Mice	X and Y	Y – 91%	RT-PCR
	Ren, 2021	Goat	X and Y	X - 80.30% ± 2.91% Y - 90.50% ± 2.86%	Flow cytometric analysis

References:

1. Holden, S. A., &Butler, S. T. (2018).Review: Applications and benefits of sexed semen in dairy and beef herds. Animal : an international journal of animal bioscience, 12(s1), s97–s103. https://doi.org/10.1017/S1751731118000721

2. Beksac, M. S., Tanacan, A., Aydin Hakli, D., Orgul, G., Soyak, B., BalciHayta, B., Dincer, P., &Topaloğlu, H. (2018). Gestational Outcomes of Pregnant Women Who Have Had Invasive Prenatal Testing for the Prenatal Diagnosis of Duchenne Muscular Dystrophy. Journalofpregnancy, 2018, 9718316. https://doi.org/10.1155/2018/9718316

3. Steinbock B. (2002). Sex selection: not obviously wrong. The Hastings Center report, 32(1), 23–28.

4. Blight, Alysse, "MicroSort". Embryo Project Encyclopedia (2019-07-09). ISSN: 1940-5030 http://embryo.asu.edu/handle/10776/13112.

5. Blight, Alysse, "Ericsson Method of Sperm Separation". Embryo Project Encyclopedia (2019-09-20). ISSN: 1940-5030 http://embryo.asu.edu/handle/10776/13125

6. ERICSSON, R., LANGEVIN, C. & NISHINO, M. Isolation of Fractions rich in Human Y Sperm. Nature 246, 421–424 (1973). https://doi.org/10.1038/246421a0

7. Ericsson, Ronald. "Fractionation of Sperm". US Patent 4009260A filed 11 December 1974, and issued 22 February 1977.

8. Ericsson R. J. (1994). Sex selection via albumin columns: 20 years of results. Human reproduction (Oxford, England), 9(10), 1787–1788. https://doi.org/10.1093/oxfordjournals.humrep.a138331

9. S P Flaherty, J Michalowska, N J Swann, W P Dmowski, C D Matthews, R J Aitken, Albumin gradients do not enrich Y-bearing human spermatozoa., Human Reproduction, Volume 12, Issue 5, May 1997, Pages 938–942, https://doi.org/10.1093/humrep/12.5.938

10. P.M. Zavos, Sperm separation attempts via the use of albumin gradients in rabbits, Theriogenology, Volume 23, Issue 6, 1985, Pages 875-879, ISSN 0093-691X, https://doi.org/10.1016/0093-691X(85)90005-6

11. Vidal, F., Moragas, M., Català, V., Torelló, M. J., Santaló, J., Calderón, G., Gimenez, C., Barri, P. N., Egozcue, J., &Veiga, A. (1993). Sephadex filtration and human serum albumin gradients do not select spermatozoa by sex chromosome: a fluorescent in-situ hybridization study. Human reproduction (Oxford, England), 8(10), 1740–1743.https://doi.org/10.1093/oxfordjournals.humrep.a137926

12. Wang HX, Flaherty SP, Swann NJ, Matthews CD. Assessment of the separation of X- and Y-bearing sperm on albumin gradients using double-label fluorescence in situ hybridization. FertilSteril. 1994 Apr;61(4):720-6. doi: 10.1016/s0015-0282(16)56652-7. PMID: 8150116.

13. Rose, G. A., & Wong, A. (1998). Experiences in Hong Kong with the theory and practice of the albumin column method of sperm separation for sex selection. Human reproduction (Oxford, England), 13(1), 146–149. https://doi.org/10.1093/humrep/13.1.146

14. Iizuka, R., Kaneko, S., Aoki, R., & Kobayashi, T. (1987). Sexing of human sperm by discontinuous Percoll density gradient and its clinical application. Human reproduction (Oxford, England), 2(7), 573–575. https://doi.org/10.1093/oxfordjournals.humrep.a136591

15. Wang, H. X., Flaherty, S. P., Swann, N. J., & Matthews, C. D. (1994). Assessment of the separation of X- and Y-bearing sperm on albumin gradients using double-label fluorescence in situ hybridization. Fertility and sterility, 61(4), 720–726. https://doi.org/10.1016/s0015-0282(16)56652-7

16. Wang, H. X., Flaherty, S. P., Swann, N. J., & Matthews, C. D. (1994). Discontinuous Percoll gradients enrich X-bearing human spermatozoa: a study using double-label fluorescence in-situ hybridization. Human reproduction (Oxford, England), 9(7), 1265–1270. https://doi.org/10.1093/oxfordjournals.humrep.a138692

17. van Kooij, R. J., & van Oost, B. A. (1992). Determination of sex ratio of spermatozoa with a deoxyribonucleic acid-probe and quinacrine staining: a comparison. Fertility and sterility, 58(2), 384–386. https://doi.org/10.1016/s0015-0282(16)55226-1

18. Check, J. H., Shanis, B. S., Cooper, S. O., &Bollendorf, A. (1989). Male sex preselection: swim-up technique and insemination of women after ovulation induction. Archives of andrology, 23(2), 165–166. https://doi.org/10.3109/01485018908986839

19. Check, J. H., &Katsoff, D. (1993). A prospective study to evaluate the efficacy of modified swim-up preparation for male sex selection. Human reproduction (Oxford, England), 8(2), 211–214. https://doi.org/10.1093/oxfordjournals.humrep.a138024

20. Han, T. L., Flaherty, S. P., Ford, J. H., & Matthews, C. D. (1993). Detection of X- and Y-bearing human spermatozoa after motile sperm isolation by swim-up. Fertility and sterility, 60(6), 1046–1051. https://doi.org/10.1016/s0015-0282(16)56408-5

21. Flaherty, S. P., & Matthews, C. D. (1996). Application of modern molecular techniques to evaluate sperm sex selection methods. Molecular human reproduction, 2(12), 937–942. https://doi.org/10.1093/molehr/2.12.937

22. Park, Y. J., Kwon, K. J., Song, W. H., Pang, W. K., Ryu, D. Y., Saidur Rahman, M., & Pang, M. G. (2021). New technique of sex preselection for increasing female ratio in boar sperm model. Reproduction in domestic animals = Zuchthygiene, 56(2), 333–341. https://doi.org/10.1111/rda.13870

23. Neculai-Valeanu, A. S., &Ariton, A. M. (2021). Game-Changing Approaches in Sperm Sex-Sorting: Microfluidics and Nanotechnology. Animals : an open access journal from MDPI, 11(4), 1182. https://doi.org/10.3390/ani11041182

24. D. L. Garner, B. L. Gledhill, D. Pinkel, S. Lake, D. Stephenson, M. A. Van Dilla, L. A. Johnson, Quantification of the X- and Y-Chromosome-Bearing Spermatozoa of Domestic Animals by Flow Cytometry, Biology of Reproduction, Volume 28, Issue 2, 1 March 1983, Pages 312–321, https://doi.org/10.1095/biolreprod28.2.312

25. Johnson, L.A., Flook, J.P. and Look, M.V. (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. https://doi.org/10.1002/mrd.1120170303

26. Lawrence A. Johnson, James P. Flook, Harold W. Hawk, Sex Preselection in Rabbits: Live Births

from X and Y Sperm Separated by DNA and Cell Sorting, Biology of Reproduction, Volume 41, Issue 2, 1 August 1989, Pages 199–203, https://doi.org/10.1095/biolreprod41.2.199

27. Vidal, F., Fugger, E. F., Blanco, J., Keyvanfar, K., Català, V., Norton, M., Hazelrigg, W. B., Black, S. H., Levinson, G., Egozcue, J., & Schulman, J. D. (1998). Efficiency of MicroSort flow cytometry for producing sperm populations enriched in X- or Y-chromosome haplotypes: a blind trial assessed by double and triple colour fluorescent in-situ hybridization. Human reproduction (Oxford, England), 13(2), 308–312.https://doi.org/10.1093/humrep/13.2.308

28. Karabinus D. S. (2009). Flow cytometric sorting of human sperm: MicroSort clinical trial update. Theriogenology, 71(1), 74–79. https://doi.org/10.1016/j.theriogenology.2008.09.013

29. Karabinus, D. S., Marazzo, D. P., Stern, H. J., Potter, D. A., Opanga, C. I., Cole, M. L., Johnson, L. A., & Schulman, J. D. (2014). The effectiveness of flow cytometric sorting of human sperm (MicroSort®) for influencing a child's sex. Reproductive biology and endocrinology : RB&E, 12, 106. https://doi.org/10.1186/1477-7827-12-106

30. De Geyter, C., Sterthaus, O., Miny, P., Wenzel, F., Lapaire, O., De Geyter, M., & Sartorius, G. (2013).First successful pregnancy in Switzerland after prospective sex determination of the embryo through the separation of X-chromosome bearing spermatozoa. Swiss medical weekly, 143, w13718.https://doi.org/10.4414/smw.2013.13718

31. Quan, G. B., Ma, Y., Li, J., Wu, G. Q., Li, D. J., Ni, Y. N., Lv, C. R., Zhu, L., & Hong, Q. H. (2015). Effects of Hoechst33342 staining on the viability and flow cytometric sex-sorting of frozen-thawed ram sperm. Cryobiology, 70(1), 23–31. https://doi.org/10.1016/j.cryobiol.2014.11.002

32. Corral-Baqués, M. I., Rivera, M. M., Rigau, T., Rodríguez-Gil, J. E., &Rigau, J. (2009). The effect of low-level laser irradiation on dog spermatozoa motility is dependent on laser output power. Lasers in medical science, 24(5), 703–713.https://doi.org/10.1007/s10103-008-0606-7

33. Colley, Adam &Buhr, Mary & Golovan, Serguei. (2008). Selection of sex-specific aptamer probes to sperm.Theriogenology. 70. 1384-1384. 10.1016/j.theriogenology.2008.06.039

34. Vinod, S. P., Vignesh, R., Priyanka, M., Tirumurugaan, K. G., Sivaselvam, S. N., & Dhinakar Raj, G. (2021). Generation of single stranded DNA with selective affinity to bovine spermatozoa. Animal bioscience, 34(10), 1579–1589. https://doi.org/10.5713/ajas.20.0235

35. Bradley M. P. (1989). Immunological sexing of mammalian semen: current status and future options. Journal of dairy science, 72(12), 3372–3380.https://doi.org/10.3168/jds.S0022-0302(89)79500-X

36. Goldberg, E. H., E. H. Boyse, D. Bennett, M. Scheid, and E. E. Carswell. 1971. Serological demonstration of H-Y (male) antigen on mouse sperm. Nature 232:476.

37. Ali, J. I., Eldridge, F. E., Koo, G. C., &Schanbacher, B. D. (1990). Enrichment of bovine X- and Y-chromosome-bearing sperm with monoclonal H-Y antibody-fluorescence-activated cell sorter.Archives of andrology, 24(3), 235–245.https://doi.org/10.3109/01485019008987580

38. Hendriksen, P. J., Tieman, M., Van der Lende, T., & Johnson, L. A. (1993). Binding of anti-H-Y monoclonal antibodies to separated X and Y chromosome-bearing porcine and bovine sperm. Molecular reproduction and development, 35(2), 189–196.https://doi.org/10.1002/mrd.1080350213

39. Sills, E. S., Kirman, I., Colombero, L. T., Hariprashad, J., Rosenwaks, Z., & Palermo, G. D. (1998). H-Y antigen expression patterns in human X- and Y-chromosome-bearing spermatozoa. American journal of reproductive immunology (New York, N.Y. : 1989), 40(1), 43–47. https://doi.org/10.1111/j.1600-0897.1998.tb00387.x

40. Umehara, T., Tsujita, N., & Shimada, M. (2019). Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. PLoS biology, 17(8), e3000398. https://doi.org/10.1371/journal.pbio.3000398

41. Ren, F., Xi, H., Ren, Y., Li, Y., Wen, F., Xian, M., Zhao, M., Zhu, D., Wang, L., Lei, A., & Hu, J. (2021). TLR7/8 signalling affects X-sperm motility via the GSK3 α/β-hexokinase pathway for the efficient production of sexed dairy goat embryos. Journal of animal science and biotechnology, 12(1), 89. https://doi.org/10.1186/s40104-021-00613-y

42. Umehara, T., Tsujita, N., Zhu, Z., Ikedo, M., & Shimada, M. (2020). A simple sperm-sexing method that activates TLR7/8 on X sperm for the efficient production of sexed mouse or cattle embryos. Nature protocols, 15(8), 2645–2667. https://doi.org/10.1038/s41596-020-0348-y

43. O'Neill, L. A., & Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nature reviews. Immunology, 7(5), 353–364. https://doi.org/10.1038/nri2079

44. Robertson, J. S., & Sheard, A. V. (1973). Altered sex ratio after an outbreak of hepatitis. Lancet (London, England), 1(7802), 532–534. https://doi.org/10.1016/s0140-6736(73)90341-3

45. Drew, J. S., London, W. T., Lustbader, E. D., Hesser, J. E., & Blumberg, B. S. (1978). Hepatitis B virus and sex ratio of offspring. Science (New York, N.Y.), 201(4357), 687–692. https://doi.org/10.1126/science.566954

46. Drew, J. S., Blumberg, B. S., & Robert-Lamblin, J. (1986). Hepatitis B virus and sex ratio of offspring in East Greenland. Human biology, 58(1), 115–120.

47. Andersen CY, Byskov AG. Enhanced separation of X and Y bearing sperm cells by a combined density gradient centrifugation evaluated by fluorescence in situ hybridization of the Y-chromosome. Acta ObstetGynecol Scand. 1997 Feb;76(2):131-4. doi: 10.3109/00016349709050068. PMID: 9049285.

48. Rahman, M. S., & Pang, M. G. (2020). New Biological Insights on X and Y Chromosome-Bearing Spermatozoa. Frontiers in cell and developmental biology, 7, 388. https://doi.org/10.3389/fcell.2019.00388