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Review

Use and evolution of sperm sexing in cattle. Review

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Abstract:

Since the commercial beginning of sperm sexing in artificial insemination, the adoption of this technology by the livestock industry (producers, veterinarians, and genetics companies) has been a reality in cattle production, mainly in dairy cattle. This review describes the beginnings of sperm sexing, its development, commercial application, and evolution to the present. The most significant events were undoubtedly the determination of the difference in DNA content between spermatozoa carrying the “Y” or “X” chromosome, the flow of these in the cytometer, and their separation into the so-called “Y” and “X” spermatozoa. The subsequent achievements that favored the application of this technology commercially were the determination of the optimal concentration and the successful cryopreservation of sexed semen; since then, research to try to reduce the deleterious effects of the sexing process has

not stopped, leading to the emergence of new sperm sexing technologies where this effect is minimal. The most widely used technique commercially is the ultrasexing of 4 million spermatozoa (SexedULTRA-4M™), in which the method, media, and cytometers were completely modified so that this technology has results very similar to those obtained with unsexed semen (conventional semen). There is another sperm sexing technology called Sexcel™ that is offered commercially, in which they have obtained similar results to those obtained with conventional semen, but only in heifers. With these advances, sperm sexing is shown to be a technology in constant development and of high impact on cattle farming.

Keywords: DNA, Sperm sexing, Sex chromosomes, Flow cytometry.

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Introduction

As far as reproductive biotechnologies in cattle are concerned, sex preselection has a long history, in which attempts have been made to separate spermatozoa with "X" and "Y" chromosomes by various techniques based on principles of difference in mass and motility, sperm kinetics, changes in sperm surface, and differences in volume; however, none of these methods (sedimentation, centrifugation, and antiserum Y) was able to produce an effective separation of fertile sperm populations[1].

Sex predetermination was achieved thanks to advances in computation, biophysics, cell biology, and applied reproductive physiology, among others. From 1980 onwards, a technique called flow cytometry began to be applied, which allowed spermatozoa to be separated according to their sex chromosomes. It took 20 yr for this technology to be commercialized for use in artificial insemination (AI) in cattle. This technique is based on differentiating "X" and "Y" spermatozoa in terms of their DNA content. In the case of cattle, the "X" spermatozoa that produce females contain an average of 3.8 % more DNA than the "Y" spermatozoa that produce males[2]. Sperm sexing by flow cytometry is a valuable tool that undoubtedly had a beneficial impact on the genetic improvement of the livestock industry. This technology had an efficiency of 85 to 95 % regarding the birth of offspring with the preselected sex; nevertheless, it was not completely perfected[3].

The first commercial production of sexed semen was carried out by the Cogent company in the United Kingdom[4]. Although it had a relatively slow start, the production of bovine-sexed semen increased exponentially, with an estimated 4 million doses in 2008[4]. The sexed semen was marketed in 0.25 ml straws at a concentration of 2.1 million spermatozoa[5]. A minimum efficient concentration was used because there were losses of approximately 80 % of the ejaculate between the spermatozoa of the unwanted sex and the spermatozoa that could not be differentiated[6]. This semen was less fertile and more delicate than conventional semen since the spermatozoa were subjected to several processes for the separation of spermatozoa with an “X” chromosome and those with a “Y” chromosome, in addition to the freezing and thawing process[7]. Despite the limitations of sexed semen, there was clearly good acceptance[4]. Acceptable gestation percentages were achieved with the reduced dose (2.1 x 10^6 spermatozoa) of sexed semen in heifers, but few studies were carried out with lactating cows[2]. Nowadays, sperm sexing technology has evolved, modifying techniques, increasing the speed of sexing, reducing stress, increasing sperm concentration, and therefore improving sperm viability parameters. At the moment, three sexing techniques are applied commercially, all through flow cytometry: SexedULTRA™[8], Sexcel™[9], and Lumisort™[10]; however, there are other promising techniques other than flow cytometry: sperm sexing using gold nanoparticles[10] and sperm sexing using magnetic nanoparticles[11], but which are not yet used commercially.

Historical overview of sperm sexing

Quantification of sperm DNA

Undoubtedly, the determination of the DNA content in the sperm opened the doors to sperm sexing technology. In 1976[12], the sperm DNA content in different animal species (hamster, mouse, rabbit, bull, pig, horse, oysters, abalone, and octopus) was evaluated by flow cytometry. In this work, they found that the distribution of the populations depended on the shape of the sperm head and how they were oriented. In the case of abalone spermatozoa (cylindrical heads), they presented a symmetrical fluorescence pattern; nevertheless, in the case of eutherian spermatozoa (elongated heads), the fluorescence pattern was asymmetrical, which presented a problem for reproductive biology applications; however, the authors concluded that this problem could be solved using a flat flow given the shape of the sperm head (Figure 1).

Figure 1: Morphology and morphometry of the sperm head in different species[13]

Dimensions and profiles of sperm heads and flow cytometric sorting indices for some domestic mammals and man

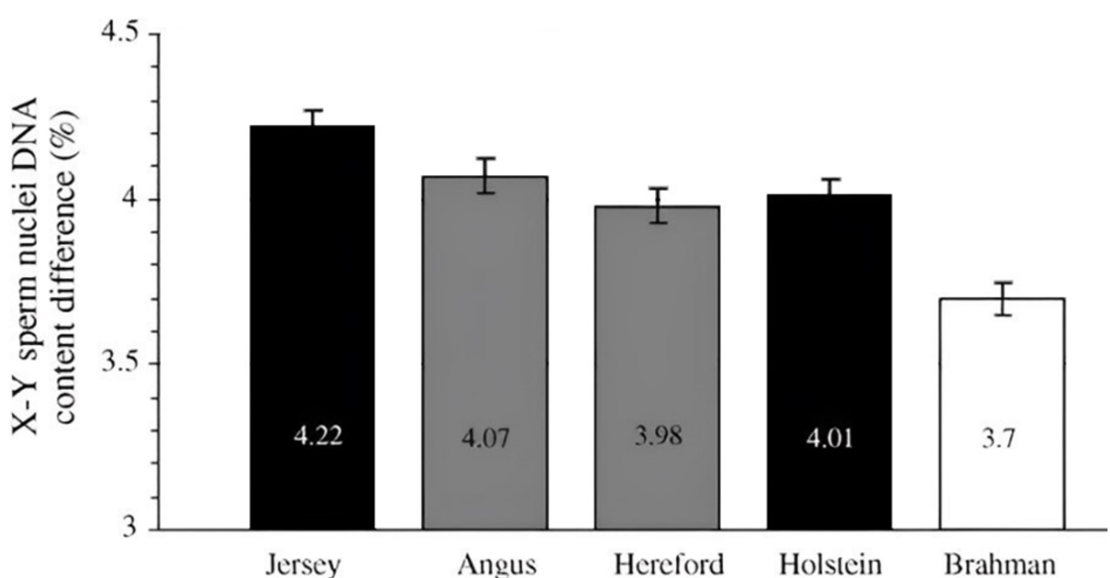
Dimension	Bull	Boar	Ram	Rabbit	Cat	Dog	Horse	Man
Length (µm)	9.1	9.0	8.1	7.7	7.7	7.0	6.5	4.6
Head sagital section								
Width (µm)	4.7	5.0	4.0	4.5	3.2	3.5	3.4	3.2
Head profile								
Area (μm^2)	34.5	37.5	26.6	28.0	19.0	20.9	15.2	10.8
X-Y difference (%)	3.8	3.6	4.2	3.0	4.2	3.9	3.9	2.8
Sorting index [a]	131	115	112	84	80	82	59	31

[a] An approximation of the ability to flow cytometric sort sperm consisting on the head profile area (μm^2) x X-Y Sperm DNA difference (%).

"X" and "Y" chromosomes in productive species

Since flow cytometry opened up the possibility of separating spermatozoa based on their DNA content, the next step in the development of sperm sexing technology was the quantification of DNA from "X" and "Y" spermatozoa from domestic species. In 1983[14], the difference in DNA content between spermatozoa with "X" and "Y" chromosomes from domestic animals was evaluated, where a difference of 3.9 % was found in the case of bulls, 3.7 % in pigs, 4.1 % in sheep, and 3.9 % in rabbits. In the case of bulls, they used 25 bulls representing five breeds (Jersey, Holstein, Hereford, Angus, and Brahman), and they observed that the average among the population of spermatozoa with the "Y" chromosome was in the range of 49.5 to 50.5 % for all breeds. The differences between spermatozoa with "X" and "Y" chromosomes did not vary within each breed but significantly differed when compared between breeds. The Jersey breed had the largest difference between "X" and "Y" chromosomes, and the Brahman breed had the smallest difference (Figure 2); this indicates that the Jersey breed is easier to sex than the Brahman breed.

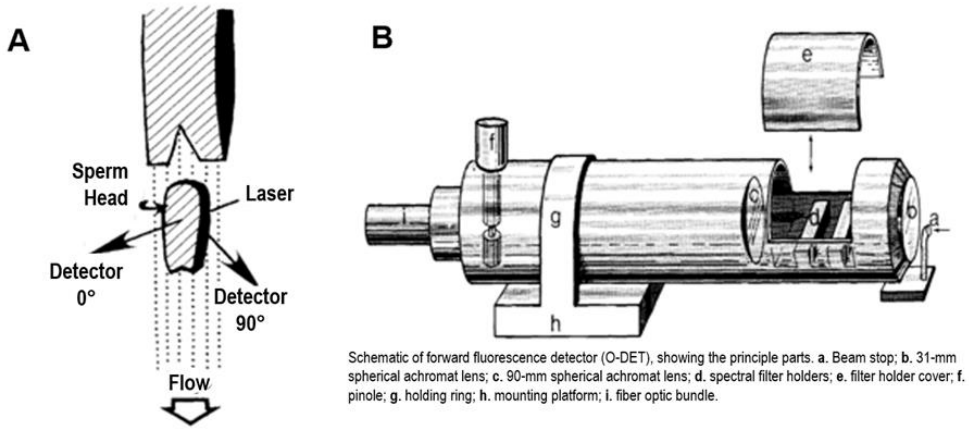
Figure 2: Difference between the DNA content of “X” and “Y” spermatozoa between different cattle breeds[13]



First modifications to flow cytometers for sperm sexing

Once it was possible to differentiate the “X” and “Y” spermatozoa based on their DNA content, work began on the flow cytometer to be able to make more efficient readings. In 1986[15], the first modifications to the flow cytometer were made to separate sperm populations. In this work, they used an EPICS V cytometer (Coulter Corporation, FL, USA), which they adapted to improve orientation. These adjustments consisted of modifying the bevel of the sample injection tube and the addition of a second fluorescence detector at 90° (Figure 3A) along the axis of the laser beam, directing the fluorescence collected by a beam of optical fibers toward the photomultiplier tubes (Figure 3B).

Figure 3: Bevel and injection tube used in the EPICS V cytometer. **A.** Beveled tip and fluorescence detectors. **B.** Photomultiplier tubes[15]



Subsequently, the sample injection tube modifications were continued, making a beveled tip. This beveled tip (25°) caused a sample flow of a flat shape, so the hydrodynamic force in the sperm nuclei caused them to be preferentially oriented in the plane of the flow. The fluorochrome-stained nuclei were excited by a laser beam perpendicular to the sample flow plane. The laser hit the flat side of the oriented sperm nuclei, and the fluorescence was simultaneously detected from the flat side by a fluorescence detector at 0°; in addition, a standard detector at 90° was added. To generate the fluorescence, a Coherent Innova 90-5 Argon-ion laser (Coherent Inc, CA, USA) was used, operating in ultraviolet light (351, 364 nm) at 150-200 mW of power. The fluorescence emitted individually by each sperm nucleus was collected by both detectors (0° and 90°) and stored as frequency distributions (histograms) in a multiparameter data acquisition display system. The separation process was carried out by droplet formation using a drop flow through ultrasonic vibration. Each droplet contained a sperm nucleus that emitted fluorescence, which was detected and electrostatically charged in one of two containers for each population, “X” or “Y”. With these modifications and working with chinchilla spermatozoa, it was possible for the nuclei of the spermatozoa to be separated into “X” and “Y” at a rate of 55 nuclei/sec for each population, with a purity of 95 %[16].

Progeny obtained from spermatozoa with “X” and “Y” chromosomes

Until now, invasive techniques have only been used for both staining and selecting spermatozoa with “X” and “Y” chromosomes; therefore, the next step would be selecting viable spermatozoa with which AI could be performed. In 1989[17], the first AI test was carried out using sexed sperm in rabbits. For this test, fresh semen from two rabbits was used,

which was sexed in an EPICS V flow cytometer with previous modifications[15,16]. This test resulted in sperm populations with an “X” chromosome with a purity of 86 % and 81 % for spermatozoa with a “Y” chromosome. With the semen obtained, surgical inseminations were performed on previously synchronized females with unsexed semen, with semen with an “X” chromosome, and with semen with a “Y” chromosome. Of the females inseminated with semen with an “X” chromosome, 94 % of the offspring were female; in the case of females inseminated with semen with a “Y” chromosome, 81 % of the offspring were males. This work demonstrated the accuracy of the sperm sexing technique employing flow cytometry.

Subsequently, there were reports of the first bovine offspring born from embryos produced *in vitro* with sexed semen, which had a purity of 79 % for spermatozoa with the “X” chromosome and 70 % for the “Y” chromosome. The embryos produced *in vitro* were sexed by PCR; the analysis indicated that 73 % were female and 69 % were male, with no statistically significant difference compared to what was obtained in the flow cytometry analysis. In this research, it was observed that sexed semen continued to have its fertilizing capacity and that it had an acceptable purity; however, the number of selected spermatozoa was too low to be used in AI, but it was feasible to be used in the case of *in vitro* embryo production (IVP)[18].

In 1996[19], a field trial was carried out in which Holstein heifers were inseminated (deep insemination, ipsilateral to the ovary with a larger follicle) with sexed semen (90 % purity, 1 x 10^5 spermatozoa) refrigerated at 5 °C. In this work, approximately 18 h passed from the time the semen was collected until the heifers were inseminated. Twenty-two inseminations were performed, of which 11 females were diagnosed pregnant at 60 d; of these pregnancies, the sex was determined by ultrasonography (between 60 and 70 d of gestation); one of the 11 fetuses was not of the predicted sex.

Creation of the company XY Inc.

The results obtained in the AI test in cattle[19] encouraged the USDA (United States Department of Agriculture) to license the Colorado State University Research Foundation (CSURF), Fort Collins, CO, USA, to proceed with the commercialization of the Beltsville sperm sexing technology for sex selection in non-human mammalian spermatozoa. With the issuance of this license in 1996, the company XY Inc. was formed, which was a collaboration between CSURF, Cytomation Inc. (CO, USA) and private investors. This company acquired the rights to high-speed flow cytometry and marketed it as the MoFlow™ cytometer (CO, USA). This cytometer included the modifications made to the injection needle[15,16] and it was improved by adding a selection nozzle that oriented 70 % of the spermatozoa through

the pressure of the hydrostatic fluid system. With this improvement, around 20,000 spermatozoa/sec could be analyzed, and up to 6,000 or more spermatozoa/sec from each of the “X” or “Y” populations could be sorted with 90 % accuracy. In 2003, Cytomation Inc. was bought by the Danish biotechnology company Dako, and it became Dako A/S; the company continued to produce the cytometer for sperm sexing, which they renamed MoFlow SX™ (CO, USA). Subsequently, the flow cytometry instrumentation division was acquired by Beckman Coulter, located in Fullerton, CA, USA[5].

Artificial insemination with low doses of sexed semen

In 1997, a study was carried out with two objectives: 1) to evaluate pregnancy rates of heifers that were synchronized and inseminated (in the uterine horn, ipsilateral to the ovary with a larger follicle) with very low doses of semen (1 x 10^5; 2.5 x 10^5; 2.5 x 10^6 spermatozoa/0.21 ml) refrigerated at 5° C under ideal conditions at the field level; 2) to evaluate the pregnancy rates of heifers that were synchronized and inseminated (in the uterine horn, ipsilateral to the ovary with a larger follicle) with low doses of sexed semen (1-2 x10^5 spermatozoa/0.1 ml) refrigerated at 5 °C. In the first experiment, the pregnancy rates at 40 d were 41 %, 50 %, and 61 % for 1 x 10^5, 2.5 x 10^5, and 2.5 x 10^6 spermatozoa/insemination, respectively. Regarding the second experiment, 22 % of 67 inseminated heifers were pregnant, and 82 % of the offspring were of the selected sex[20].

Successful cryopreservation of sexed semen

Later, in 1999, another research was carried out with the aim of evaluating the process of freezing the sexed semen; this could be done because the semen was processed in a MoFlow SX™ flow cytometer, with which it was possible to have a sufficient number of spermatozoa unlike when working with the EPICS V flow cytometer. In this work, it was determined that the use of the laser at a power of 100 mW had a lower impact on the progressive motility of the post-thawed semen than when it was used at 150 mW. It was also observed that the post-thawed progressive motility was higher when using a TRIS-based diluent than when using citrate-egg yolk or TEST. Regarding the equilibrium time at 5 °C before freezing, it was concluded that the progressive motility after thawing from 3 to 6 h was better than when it lasted 18 h. On the other hand, it was determined that it was better to keep raw semen (freshly collected, undiluted semen) at 22 °C than to dilute it with TALP medium added with Hoechst 33342 fluorochrome (ICN Biomedicals Inc., OH USA). With these new procedures for sperm sexing, slightly lower results were achieved than with conventional semen in terms of

motility and acrosomal integrity, and it was considered that the use of sexed semen for artificial insemination on a commercial basis would be available in approximately 2 yr[21].

Beginnings of the commercialization of sexed semen

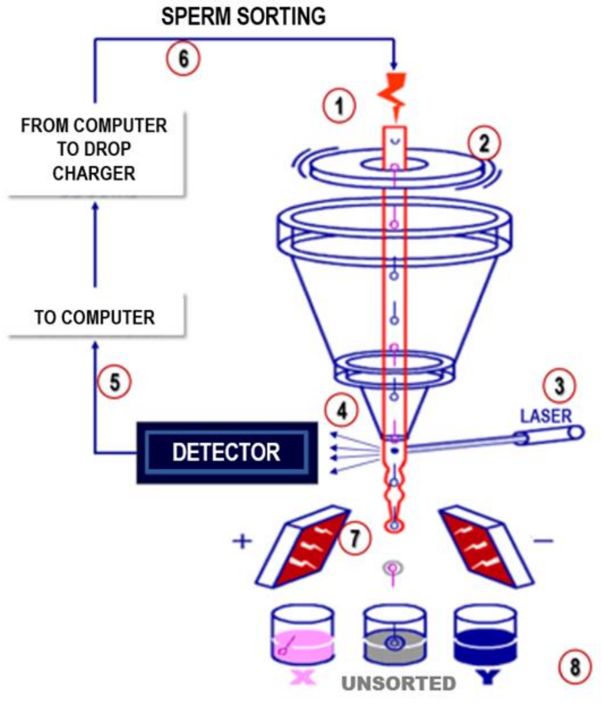
The Monsanto company, located in St. Louis, Mo, USA, developed a one-of-a-kind sperm selection system, which used 16 selection nozzles instead of just one, as in the case of the MoFlow SX™ cytometers. This equipment was intended to be commercialized, but apparently, due to problems with low conception percentages that were detected in its first tests, the company gave up[5]. In 2003, Genetic Resources International / Sexing Technologies in Navasota, TX, USA, purchased the intellectual property and sperm sexing equipment developed by Monsanto and the entire infrastructure of XY Inc[5]. The company has now changed its name to STgenetics®[22].

Sperm sexing with the conventional technique (Legacy or XY)

Legacy sperm sexing overview

The Legacy sperm sexing system used a MoFlow SX™ flow cytometer, which consisted of a closed-loop high-speed fluid flow that allowed spermatozoa to be aligned and read individually in microdroplets. The fluorescence produced by each stained sperm was processed by software that allowed the operator to select the sperm population with minimum and maximum luminosity according to the sex to be separated. The chosen spermatozoa were electrically charged, diverted from the original flow in a magnetic field, and finally collected[6] (Figure 4). They were then concentrated by centrifugation and frozen, leaving only half of the total alive[4].

Figure 4: Flow cytometry sperm classification system

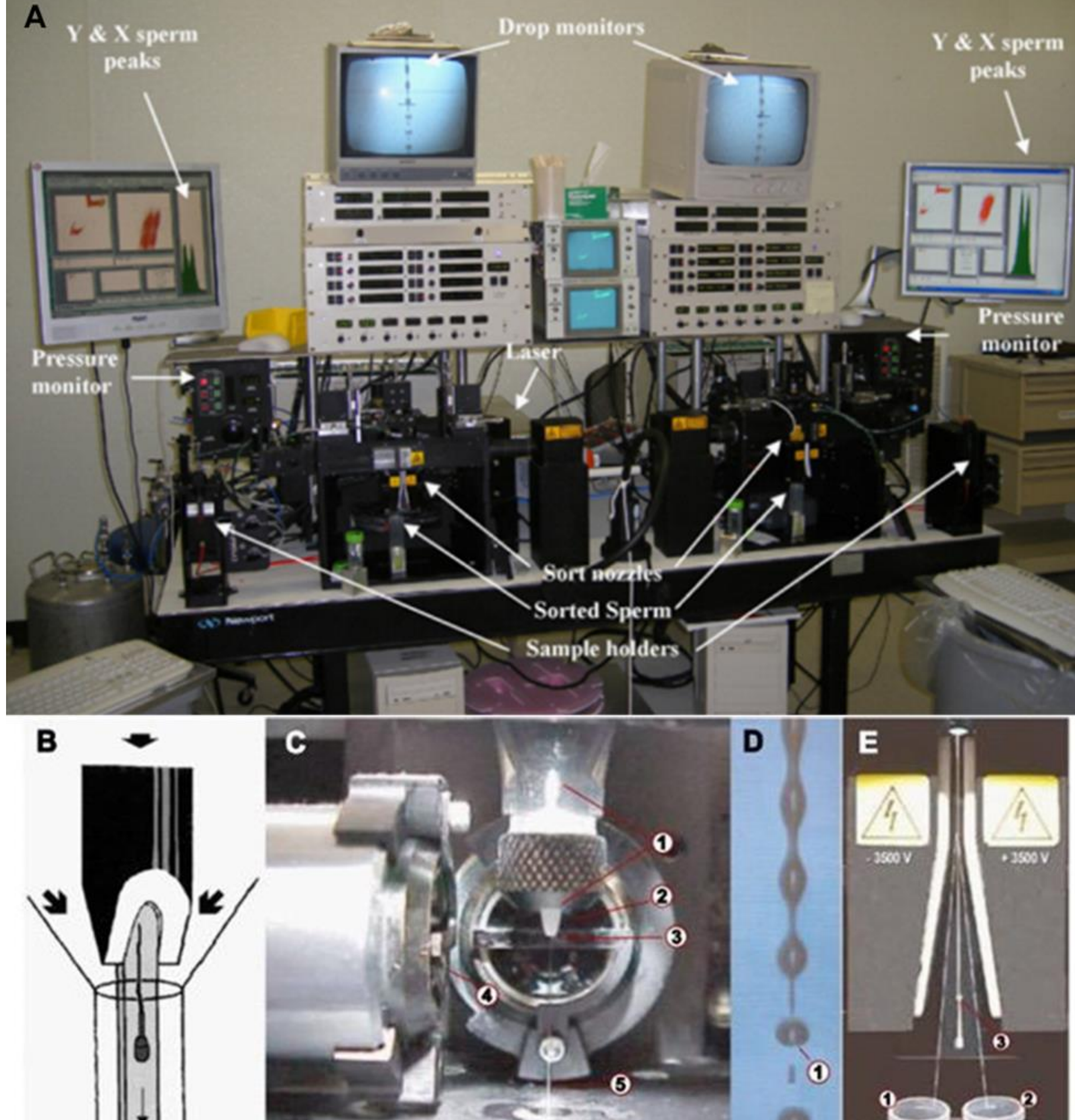


1) Spermatozoa are injected through the system after being stained with a DNA-binding fluorochrome, 2) A vibrating ring of piezoelectric crystal causes 90,000 droplets per second to form as the stream exits the system, 3) A UV laser illuminates the spermatozoa as they flow through the beam, 4) X spermatozoa fluoresce with 4 % more intensity than Y spermatozoa, 5) The signal detected with a photomultiplier tube is sent to a computer that processes the detected fluorescence and categorizes whether the sperm is X, Y, or unoriented, 6) Negative, positive or uncharged, it is applied to the droplets that emerge from the flow, 7) As the charged droplets pass between continuously charged plates, they are diverted, 8) The spermatozoa are collected in three containers: X, Y, unoriented or sperm-free[(2)].

The sperm quality and concentration of the ejaculates were perhaps the most important factors in obtaining a good separation of the two populations since a high correlation between motility, concentration, and separation of the populations was demonstrated with high-speed flow cytometers. Therefore, the separation of “X” and “Y” spermatozoa was usually carried out in ejaculates with more than 50 % progressive motility and 75 % normal spermatozoa[(6)].

With the high-speed cytometers, the MoFlow SX™ (Figure 5), spermatozoa passed through the cytometer at a speed of 80 km/h, approximately 20,000 total spermatozoa/second[(3)] and it took 9 min to sex a straw of 2 x 10^6 spermatozoa, approximately seven straws per hour[(5)].

Figure 5: MoFlow SX™ cytometer for XY sperm sexing



A. Sperm sorter and computer. **B.** Beveled tip. **C.** Optics and hydrodynamics, 1) Nozzle with X Y orienting tip, 2) Side fluorescence objective: cell orientation, 3) Locking bar, 4) Front fluorescence objective: cellular DNA quantifier, 5) Flow output. **D.** Formation of microdroplets, 1) Last drop united. **E.** Deflection plates, 1) Spermatozoa with Y chromosome, 2) Spermatozoa with X chromosome, 3) Residual stream[1,2,5].

In the sexing process, of 100 % of the spermatozoa, approximately 20 % ended up collected in the "X" fraction and 20 % in the "Y" fraction; the remaining 60 % consisted of

spermatozoa that could not be detected by the cytometer, dead spermatozoa, and droplets without spermatozoa[3,6].

The sperm characteristics and survival of sexed sperm were poor compared to unsexed spermatozoa (the increase in dead spermatozoa reached 18.6 %); this was attributed to the sexing process[7] that began with many hours of maintenance from semen collection until the semen was sexed[1].

Factors affecting Legacy sexed semen outcomes in AI

Viability

The damage due to the sexing process with the Legacy technique had a direct impact on the pregnancy percentages. The lower fertility of sexed semen was mainly due to exposure to mechanical forces during the sexing process and, to a lesser extent, due to staining and exposure to lasers[3]. After the sexing process, the spermatozoa were partially capacitated, reducing the lifespan and consequently reducing fertility[23]. For all of the above, the straws had a minimum of 35 % spermatozoa with progressive motility and a minimum of 85 % sex certainty to reach the approval standards[6].

Concentration

In addition to the damage caused by the sexing process, another cause of the decrease in fertility of Legacy sexed semen was due to the low number of spermatozoa contained in the dose[24,6]. A dose of 2.1 million spermatozoa is a low dose for AI[25]; however, it was observed that for most bulls, the concentration of spermatozoa to obtain a percentage of 80 % of normal conception is approximately 2 million spermatozoa per dose[23]. In cows inseminated (12 h after natural heat) with doses of 2 million spermatozoa of sexed and conventional semen, the pregnancy rates were less than 30 % and did not differ between sexed and conventional semen, indicating that the total number of inseminated spermatozoa seems to have a more significant impact on conception than the use of sexed or conventional semen[23]. On the other hand, no difference (P=0.64) was found when inseminating (12 h after natural heat) Holstein cows with 2.1 and 3.5 million sexed spermatozoa, obtaining percentages of 23 % and 25 %, respectively. Nonetheless, under ideal insemination conditions and with doses of 3 million sexed spermatozoa in lactating beef cows, the pregnancy rates were similar to those of

heifers[27]. Another study found that pregnancy rates were virtually identical with 1, 1.5, and 3 million spermatozoa per dose (54 %, 56 %, and 51 %, respectively)[28]. Based on the work carried out, it can be seen that the low sperm concentration of the sexed semen doses was sufficient to obtain adequate pregnancy rates.

Differences between bulls

Differences have been reported between bulls regarding sperm tolerance to the sexing process[3,27]. In addition, a difference of up to 18 % of gestation was found according to the bull used[23,27], which indicates that the fertility of the sexed semen seems to differ between bulls. This implies that field tests cannot accurately predict the fertility of sexed semen as with conventional semen[24]. Therefore, care should be taken when interpreting the results obtained with sexed semen since there is a strong influence of the bull used with the gestation percentages[23]. Thus, monitoring the results of sexed semen and keeping bulls (Holstein) with the highest fertility for sexing is the best way to increase its fertility[24].

Other applications of Legacy sexed semen

Reverse-sorted semen

Reverse-sorted semen (RSS), also known as reverse semen, is a technique that allows spermatozoa with “X” and “Y” chromosomes to be obtained from conventionally frozen semen. An advantage of this technology is that it is possible to obtain sexed semen from bulls of high genetic merit that have died[29]. RSS has been associated with other biotechnologies, such as AI[30] and IVP[31]. In tests carried out with AI, pregnancy rates were low, from 4 to 10 %[30], with 14.2 % of offspring born[32]. Therefore, this technology is primarily used with IVP[29].

In vivo production of embryos with Legacy sexed semen

The use of Legacy sexed semen for multiple ovulation of donors has had very variable results, generally poor or very low compared to conventional semen, where between 1.4[33] and 2.3 transferable embryos[34] per collection are reported. Some promising results using heifers

report that there is no significant difference between sexed and conventional semen[35]. For all of the above, the use of Legacy sexed semen in multiple ovulation programs has been limited.

In vitro production of embryos with Legacy sexed semen

Historically, it has always been considered that the most economical method of using sexed semen in breeding programs in cattle is through IVP since, with this reproductive biotechnology, a very small number of spermatozoa is required. Combined with ultrasound-guided follicular aspiration, large quantities of embryos generated from both “X” and “Y” spermatozoa are obtained. Many studies have been carried out using Legacy sexed semen to produce embryos *in vitro*, and many aspects related to the *in vitro* production of bovine embryos with this semen have been described; among these are the low rates of fertilization, divisions, blastocyst production, gestation, and variation between bulls[36]. When evaluating fresh sexed semen compared to fresh conventional semen and frozen sexed semen compared to frozen conventional semen for IVP, it was found that, in the case of fresh semen, the results seemed to be similar in terms of motility parameters; nevertheless, in the percentage of divisions, they were lower ($P<0.001$) for fresh sexed semen compared to fresh conventional semen (66 *vs* 76 %, respectively). When using frozen sexed semen and frozen conventional semen, they had no differences in the percentage of divisions. Another aspect observed with the sexed semen was that there was a delay of half to one day in development to the blastocyst stage. These authors found that blastocyst production with sexed semen was ~30 % lower compared to conventional semen[37]. In another study, it was reported that the percentage of blastocyst production obtained from oocytes collected by ultrasound-guided follicular aspiration was lower ($P<0.05$) when sexed semen was used compared to conventional semen[38]. In general, blastocyst production with conventional semen is around 30 to 40 % and 10 to 20 % with sexed semen[36].

On the other hand, in the case of IVP with RSS, no significant difference ($P>0.1$) was found between the percentage of blastocysts obtained using sexed semen and RSS[31,39]. Commercially, in *Bos taurus* and *Bos indicus* breeds, the use of RSS for IVP had an average percentage of 30 % blastocyst production[29]. A relevant aspect is that it has been found that offspring produced from IVP with RSS have significantly higher birth weights ($P=0.028$), higher postnatal growth ($P=0.001$), higher mortality percentages (in the first 6 mo of age; $P=0.008$), and reduction in milk ($P=0.001$), fat ($P=0.007$), and protein ($P=0.031$) production, compared to offspring born from AI[40].

SexedULTRA™ sexed semen

Technique overview

The causes of lower fertility of sexed semen have been attributed to the various biochemical changes to which spermatozoa are subjected during the sexing process. There are about 20 different subprocesses involved in sperm sexing; among the most critical and important are the maintenance time before staining, exposure to the laser to generate fluorescence, and achieving separation between spermatozoa (with “X” and “Y” chromosomes), and finally, exposure to an electric field for the separation of relatively pure populations in a container[1,3,13]. According to the above, the challenge was to find new ways to control these events using new hardware, software, and new processing techniques during, before, and after the sperm separation stages[13].

The Legacy or XY technology described in previous publications[41,21] has been modified and has now changed to an entirely new sexing system called ultrasexing or after its brand, SexedULTRA™ (Navasota, TX, USA). Ultrasexing technology has been designed to be less aggressive for the sperm during the most critical points of the process, particularly improving changes in pH (buffer system) and oxidative stress[42].

Modifications to the technique

Although there is currently very little data about this new technology (due to intellectual property issues), it has been reported that, in this new technique, the sperm physiology was altered to facilitate the entry of the Hoechst 33342 fluorochrome and to retain it within the cell, which allows for greater fluorescence and thus better discrimination between the “X” and “Y” populations. On the other hand, the cryopreservation process is another very stressful step for the sperm cell, which is why the SexedULTRA™ technology was designed to simplify and optimize the media and control these stressors for the sperm. The protocol was modified with a treatment before the staining process and the use of a new staining medium that maintains the pH stable for a more extended period of time. The freezing medium was also modified, considering the dose of sexed semen[42].

The success of the ultrasexing process was mainly influenced by two factors: modifications in the media and equipment for sexing. MoFlow SX™ cytometers (Cytomation Inc, Fort Collins, CO, USA) were very expensive, bulky, had low performance, and required highly

trained personnel to operate them (Figure 4). The modern Genesis cytometers developed by Cytonome ST™ (Boston, MA, USA) have advanced and automated electronic features with multiple heads in one machine for parallel separation. The Genesis III™ cytometer (Figure 6) uses a solid-state laser, two orthogonal detectors (0° and 90° to the laser), an orientation nozzle, and a subpopulation separation of ~8000 spermatozoa/second with ~90 % purity, reaching a maximum separation of 500 million spermatozoa/hour[42].

Figure 6: Genesis III™ cytometer for sperm sexing[42]

Laboratory tests of SexedULTRA™ technology

With these changes, in laboratory tests, sperm motility and the integrity of the acrosome were increased compared to XY Legacy technology (conventional sexing), considering the same sperm concentrations (Figure 7)[8].

Figure 7: Comparison of SexedULTRA™ and XY Legacy (Control) sexing methods on *in vitro* semen quality assessment

Sperm motility and progressive motility were assessed using computer-aided semen evaluation, and the percentage of intact acrosomes was determined by differential interference contrast microscopy (n=12 bulls). Bars with two asterisks differ significantly (P<0.001)[8].

In addition, in *in vitro* fertilization tests, ultrasexed semen had a higher number of freezable embryos compared to Legacy sexed semen, with 13.2 % and 9 %, respectively[8].

On the other hand, in 2018[43], the considered sperm quality, plasma membrane integrity, percentage of intact acrosomes, and DNA fragmentation index (DFI) of SexedULTRA™ semen compared to conventional semen were evaluated. In SexedULTRA™ semen at 3 h post-thawed, the percentage of intact acrosomes was significantly higher than in conventional semen (Table 1). In terms of DFI, SexedULTRA™ semen had a significantly lower DFI at all evaluation points compared to conventional semen. The authors conclude that the SexedULTRA™ technology maintains semen quality and, in many cases, has greater longevity *in vitro* compared to conventional semen.

Table 1: Comparison of characteristics of SexedULTRA™ semen and frozen-thawed conventional semen

Value	Time	Least square means		Tukey		
		Conventional	SexedULTRA™	Mean difference	SE	*P*
Visual motility	0 h	61.0	63.8	2.8	2.4	0.250
	3 h	50.1	51.0	0.9	2.4	0.709
Total motility	0 h	60.6	63.8	3.2	2.2	0.157
	3 h	49.6	50.0	0.4	2.2	0.862
Progressive motility	0 h	49.8	53.0	3.3	2.5	0.198
	3 h	28.5	29.4	1.0	2.5	0.698
Intact plasma membrane	0 h	55.6	56.7	1.1	1.6	0.502
	3 h	40.7	43.4	2.6	1.6	0.121
Intact acrosomes (%)	0 h	72.6	76.0	3.3	2.1	0.126
	3 h	55.6	62.3	6.7	2.1	**0.004**

SE= standard error. The differences were considered significant with a value of $P<0.05$ (underlined and bold values), n=10[43].

Evaluation and standardization of SexedULTRA™ technology in the field

In the first field-level evaluation using SexedULTRA™ technology for AI (Table 2)[44,45], there was a 7.4 % increase in heifer conception rates compared to XY Legacy technology. The second test was carried out in collaboration with the commercial company Select Sires (OH, USA); in this test, eight Holstein bulls were used, from which semen was collected and processed using both SexedULTRA™ technology and XY Legacy technology, with which 6,930 heifers were inseminated. The results showed that SexedULTRA™ semen increased conception rate by 4.5 % ($P<0.001$) compared to XY Legacy semen (46.1 and 41.6 %, respectively[44,45].

Table 2: Results of field fertility tests of heifers inseminated with SexedULTRA™ semen[44,45]

	Number of inseminations	Conception rates
Sexing Technologies test		
XY Legacy	1,166	47.3 [a]
SexedULTRA™	957	54.7 [b]
Mean difference		7.4
Select Sires test		
XY Legacy	3,384	41.6 [a]
SexedULTRA™	3,546	46.1 [b]
Mean difference		4.5

[ab] Within the test, rows with different superscripts differ (P<0.01).

With these tests, it was observed that the deleterious effects of the XY Legacy technology were partially lessened with the new SexedULTRA™ technology, so the next logical step was to increase the sperm concentration per dose; however, in the past, the increase in sperm concentration did not improve fertility. The following test was carried out in collaboration with the company German Genetics International, for which they used five Holstein bulls, from which semen was collected, and each ejaculate was divided into four parts to be processed with XY Legacy technology of 2.1 million spermatozoa, SexedULTRA™ of 2.1, 3 and 4 million spermatozoa per dose; in addition, semen from these same bulls of contemporary ejaculate frozen conventionally was used, with a concentration of 15 million spermatozoa per dose. The rates of non-return to estrus at 65 d were calculated from 7,855 inseminations with sexed semen and 62,398 inseminations with conventional semen. Overall, XY Legacy semen of 2.1 million spermatozoa per dose resulted in lower rates of non-return to estrus compared to all SexedULTRA™ and conventional semen treatments. SexedULTRA™ treatments of 2.1 and 3 million spermatozoa per dose were similar to but lower than conventional semen; nevertheless, the SexedULTRA™ treatment of 4 million spermatozoa per dose had non-return rates to estrus similar to conventional semen of 15 million spermatozoa per dose (Table 3)[45]. With the data obtained, the effect of the dose-response using sexed semen was demonstrated for the first time and the SexedULTRA-4M™ technology ($4x10^6$ spermatozoa/straw) emerged.

Table 3: Effect of increasing sperm dose with SexedULTRA™ semen on the rates of non-return to estrus at 56 days[45]

Treatment	Number of inseminations	Rate of non-return to estrus at 56 days (%)
Legacy 2.1 millions	1,953	55.9 [a]
SexedULTRA™ 2.1 millions	1,999	59.9 [b]
SexedULTRA™ 3.0 millions	2,013	60.0 [b]
SexedULTRA™ 4.0 millions	1,890	66.7 [c]
Conventional 15.0 millions|	62,298	66.5 [c]

[abc] Different literals in the same column differ (P<0.001).

In the case of multiple ovulation, the use of SexedULTRA™ semen was evaluated in lactating Holstein embryo donors. In this study, they used three doses of FSH for multiple ovulation and inseminated with SexedULTRA™ semen. With the highest doses of FSH, four point five embryos were obtained, with no difference between the qualities (Table 4)[46].

Table 4: Percentages of all recovered structures, transferable and non-transferable embryos of lactating dairy cows superovulated with three protocols[46]

	F700	F1000	F700 P300
Total structures	4.7 ± 3.0 [a]	8.1 ± 3.8 [b]	8.5 ± 6.4 [b]
Transferable embryos (%)	1.9 ± 1.7 [a] (41.2)	4.4 ± 2.6 [b] (54.7)	4.5 ± 3.3 [b] (52.9)
Non-transferable embryos (%)	2.8 ± 3.2 (58.8)	3.6 ± 2.9 (45.3)	4.0 ± 5.4 (47.1)
Grade 1* (%)	19/33 (57.6)	96/150 (64.0)	66/117 (56.4)
Grade 2* (%)	13/33 (39.4)	46/150 (30.7)	43/117 (36.8)
Grade 3* (%)	1/33 (3.0)	8/150 (5.3)	8/117 (6.8)
Grade mean*	1.45 ± 0.5	1.41 ± 0.6	1.50 ± 0.6

F700= Folltropin 700 IU, F1000= Folltropin 1000 IU, F700P300= Folltropin 700 IU+Pluset 300 IU.
* Quality grades (IETS 1-3) of transferable bovine embryos recovered from lactating dairy cows superovulated with three protocols.
[ab] Different literals in the same column differ (P<0.05).

SexedULTRA-4M™ technology and its application in the field compared to conventional semen

In the case of the SexedULTRA-4M™ technology ($4x10^6$ spermatozoa/straw)[47], the use of SexedULTRA-4M™ semen in fixed-time artificial insemination was evaluated using cows and heifers. Its results show that there was no significant difference (*P*=0.61) in terms of the pregnancy rates between conventional semen (61.9 %) and SexedULTRA-4M™ semen (63.8 %) when females were in heat before fixed-time artificial insemination.

Another experiment[48] compared the use of conventional semen and SexedULTRA-4M™ semen in AI using three different bulls (Angus) and beef cows. In this study, it was found that fertility is influenced by the bull since only one out of three bulls had no differences in terms of the percentage of pregnancies when comparing conventional semen and SexedULTRA-4M™ semen, which shows that there is a difference between bulls, as is the case with Legacy sexed semen.

In the case of dairy cattle, through AI of grazing Holstein cows, they evaluated conventional semen and SexedUltra-4M™ semen from 10 bulls and concluded that SexedULTRA-4M™ semen has a lower conception rate compared to conventional semen; however, this depends on the bull, the fertility of the cow, and the herd[49].

In vitro production of embryos with SexedULTRA-4M™ semen

To date, there is very little information about the use of SexedULTRA-4M™ sexed semen in IVP. In one study, this semen was evaluated in IVP, and it was found that the SexedULTRA-4M™ semen generated a bigger number of freezable embryos compared to the Legacy sexed semen (13.2 and 9.2 %, respectively; *P*>0.05)[8]. In two other studies, IVP was evaluated using conventional semen and SexedULTRA-4M™ semen from the same bull, using oocytes from adult animals[50] and using oocytes from 6-mo-old prepubertal females[51] and no significant differences (*P*>0.05) were found between blastocysts produced with conventional semen and those produced with SexedULTRA-4M™ semen in both studies; nonetheless however, in the case of adult animals, there was a higher number of blastocysts with SexedULTRA-4M™ semen (43.6 and 37.8 %, respectively; *P*>0.05). In another study, IVP was evaluated using conventional semen and SexedULTRA-4M™ semen from four bulls of the Angus breed; in this study, it was found that two bulls were significantly superior for blastocyst production with SexedULTRA-4M semen compared to conventional semen [24.2 and 20.4 %; 14.2 and 10.4 %, respectively (*P*<0.05)]. In this study,

it was also concluded that the results of IVP with SexedULTRA-4M semen were similar to those obtained with conventional semen[52].

Other sperm sexing techniques

Lumi sort™

Lumisort™ (Microbix Biosystems Inc., ON, Canada) is a next-generation sperm sexing technology for the livestock industry. The Lumisort method combines an optical system for detecting the sex of spermatozoa with a fast and effective laser that destroys spermatozoa that are not of the desired sex. The spermatozoa do not suffer damage due to hydrostatic pressure; it does not use droplets, so it does not require vibrations to align the sperm, it does not require electrical charges, and the selected spermatozoa are gently separated. It was first started in 2005 and later introduced in the dairy industry in 2013[10]; nevertheless, there are no studies published in scientific journals evaluating this technology.

SexCell™ (Gender ablation)

This technology is very recent, like Lumisort technology; sexing is performed by flow cytometry, and the spermatozoa of the unwanted sex are destroyed[9]; nonetheless, the sexing process is not described in detail. This technology has been developed by the company Genus-IntelliGen Technology[53] and is marketed by the company ABS (WI, USA)[54]. There is only one publication in which they evaluated the conception rate in beef cows and heifers inseminated with conventional semen and semen sexed by gender ablation. Conventional semen had statistically superior results compared to sexed semen in cows; however, in heifers, there was no significant difference between conventional semen and sexed semen[9].

Techniques in development

Sperm sexing using gold nanoparticles

This technique uses functionalized gold nanoparticles (AuNPs) to detect specific sequences of the “Y” chromosome in morphologically and functionally intact spermatozoa. The first step consists of the entry of AuNPs through the sperm membrane. Subsequently, there is a non-invasive coupling of a specific DNA sequence with the double strand of sperm DNA. Once mated, the specific signal pattern of the “Y” chromosome is recognized to identify the sperm population[10].

Sperm sexing using magnetic nanoparticles

This technique has only been reported in donkeys; however, it could later be used in other species. Magnetic nanoparticles (MNPs) have a diameter of 50 nm, are composed of an iron magnetite core covered with silica, and are negatively charged. MNPs are mixed with semen and exposed to a magnet for 20 min. The interaction between the negative charge of MNPs and the electrical potential of spermatozoa is different for spermatozoa with an "X" chromosome (20 mV) and those with a "Y" chromosome (16 mV). In this way, spermatozoa with a "Y" chromosome will be kept closer to the MNPs and will form an accumulation of spermatozoa, and in this way, the populations can be separated[11].

Prospects

The advancement of the different technologies involved in sperm sexing is remarkable. This shows that sperm sexing is in continuous evolution and with increasingly better results, both for artificial insemination and for other biotechnologies, such as the *in vivo* and *in vitro* production of embryos in cattle, which could be applied to other species, such as sheep, goats, horses, and pigs. For this reason, it is envisaged that in the not-too-distant future, this technology will displace conventional semen, or even that expensive and sophisticated equipment will not be required to carry it out.

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