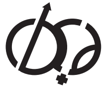

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Different ways to evaluate bovine sexed sperm *in vitro*

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Abstract

Over the years, many techniques for *in vitro* evaluation of sperm have been developed. Those assessments allow to perform structural, functional and molecular evaluations of the sperm cell. A combination of laboratory tests used simultaneously can provide more accurate information on sperm function and quality because sperm have multiple compartments with different functions. Many of those analyses have been used to assess the effect of sexing by flow cytometry on sperm cellular and molecular levels such as DNA methylation pattern, sperm shape, sperm morphology and capacity to remain viable after thawing. Considering that sexed sperm are submitted to a variety of adverse conditions during sorting, evaluation and identification of the possible damages caused by the sexing process are needed. It is expected that those information will help to develop procedures to improve results when sexed sperm is used. This review is focused on the recent results using structural, functional and molecular tests to evaluate sperm viability after sexing by flow cytometry.

Keywords: flow cytometry, *in vitro* assessment, sexing process, sperm.

Introduction

Sperm sexing has the potential to influence the birth rate of the desired gender, allowing greater production efficiency and flexibility in herd management. Moreover, the possibility to choose the gender of offspring, even before embryo production or pregnancy, according to the needs of the livestock and/or market demands, results in greater economic gain (Wheeler *et al*., 2006).

Although several methods have been developed for sperm sex determination (Koo *et al*., 1973; Kaneco *et al*., 1984; Johnson *et al*., 1987), the only method effective for routine use is the fluorescence-activated cell sorting using flow cytometry. This method is based on differences on DNA content of X and Y chromosome-bearing sperm cells (Garner *et al*., 1983), and usually has 90% of accuracy (Seidel and Garner, 2002). Bovine sperm prepared by this method (for method description see Seidel and Garner, 2002) is available commercially in Brazil since 2006, and since then its use has increased markedly. Part of this growth can be attributed to its wide use in the *in vitro* embryo production (IVF), which is one of the most advantageous combinations of reproductive biotechnologies. In other words, the sexed sperm is gaining more and more space and, like other reproductive biotechnologies, has become almost an indispensable procedure for those who want to keep high production and economically performance, especially in the dairy industry. This makes a real economic sense because recipient resources would not be wasted to produce calves of the unwanted gender (Butler *et al*., 2014).

Although sexed sperm is currently used, the high cost and the reduced pregnancy rates compared to conventional sperm, have been limiting its application in cattle breeding. This suggests that sexing process may induces sperm damages, which can be due to exposure to the laser, to the high velocity inside the collecting tube, to electric charges, and to room temperature before being processed (Garner, 2006; Wheeler *et al*., 2006). Considering that sexed sperm are submitted to a variety of adverse conditions during sorting, an evaluation of the possible structural and functional damages caused by the sexing process is needed.

Undoubtedly, the best way to assess the quality of sperm sample is through the pregnancy rate and/or birth after artificial insemination (AI). However, the high cost and time required to obtain the results, make those types of analysis almost unfeasible. Therefore, other techniques for *in vitro* evaluation of sperm have been developed in order to better predict fertility of those cells and to estimate most accurately quality of a sperm sample (Amann and Hammerstedt, 1993). Therefore, this review aims to present several analyses made in sexed sperm that can estimate its structural and functional viability.

Effect of sexing process on sperm structure and function

Assessment of motility

According to Malmgren (1997), motility is an important factor to be considered in the analysis of sperm viability. Among the characteristics affected by the sexing process, decreased motility has been reported

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by several authors (Hollinshead *et al*., 2004; Blondin *et al*., 2009; Carvalho *et al*., 2009, 2010). Moreover, Carvalho *et al*. (2009) found lower straight-line velocity, beat-cross frequency and linearity assessed by computer-assisted semen analysis (CASA), in sexed than non sexed sperm. This change in the sexed sperm motility, could have been caused by exposure to Hoechst 33342 stain, the laser light, or exposure in the droplets to electric charges (Watkins *et al*., 1996). According to Smith (1993), the effect of exposure to dye and then, the laser, may reduce mitochondrial activity, causing a decrease in the production of ATP. According to Alomar *et al*. (2006), motility is one of the most important sperm characteristics for the maintenance of fertility, being its evaluation, either subjective or by computerized analysis, essential in any sperm analysis.

Assessment of DNA, plasma membrane and acrosome integrity

Besides motility, several other sperm features can be evaluated after sperm sexing procedure, such as changes in DNA. However, studies have shown that sexing does not affect sperm DNA integrity (Blondin *et al*., 2009; Carvalho *et al*., 2010; Gosálvez *et al*., 2011). It is well known that chromatin stability is related to the proportion of protamine: histone present in sperm chromatin which varies from 1% in the mouse (Balhorn *et al*., 1977) to 15% in the human (Gatewood *et al*., 1990) and over 50% in some marsupial species (Soon *et al*., 1977). Then, the high proportion of protamine: histone present in DNA of bovine sperm can be responsible by the high stability of the chromatin which protects the DNA against the possible damages of the sexing procedure. Additionally, it is important to indicate that the sexing process has improved in recent years (Sharpe and Evans, 2009) due to modifications on the process, such as decrease of sexing pressure, and the use of dye to exclude dead sperm, among others. Those changes made the process more efficient and less harmful to the sperm. This is supported by recent studies that reported similar structural and/or functional quality in sorted and non sorted bull sperm (Blondin *et al*., 2009; Peippo *et al*., 2009; Carvalho *et al*., 2010).

Another physical characteristic that may be affected by sexing process is plasma membrane integrity. Results from different studies have shown that the sexing procedure increases the percentage of sperm with plasma membrane damaged (Blondin *et al*., 2009; Carvalho *et al*., 2010; Villamil *et al*., 2012; Spinaci *et al*., 2013). These effects may be due to mechanical stress (Garner, 2006), since it has been shown that a decrease in pressure during cell sorting increases the percentage of sperm with intact membrane, increasing fertilization (Suh *et al*., 2005) and pregnancy rates (Schenk *et al*., 2009). However, despite the fact that lower pressure minimize damage to sperm, it may compromise the efficiency of the sexing process (Garner, 2006).

Besides plasma membrane, the acrosome can also be affected by the sexing process, which can substantially impair the ability of sperm cells to fertilize the oocyte since an acrosome intact is necessary to bind to the zona pellucida and fertilize the oocyte. Using different techniques, such as fluorescent probe used to assess acrosome integrity by fluorescent microscope or flow citometry, studies have shown a higher (Mocé *et al*., 2006; Carvalho *et al*., 2010) or similar (Klinc and Rath, 2007; Blondin *et al*., 2009) percentage of cells with acrosome reacted in sexed sperm than in non sexed. This variation can be due to the different techniques used to evaluate the acrosome integrity (Brito *et al*., 2003). Moreover, the variation found in those results can also be attributed to individual sensibility of each bull to the sexing process.

There are large numbers of possibilities to evaluate structural characteristics of sperm cell using different staining methods and different types of equipments. A simple assessment can be performed with vital dyes such as Eosin/nigrosin, Trypan blue and Giemsa, which can be assessed by light microscopy or phase contrast. However, this type of procedure tends to underestimate the percentage of damaged sperm. Therefore, to have a more accurate detection of the different structural characteristics of sperm, the use of a variety of fluorescent probes was established. For sperm evaluation with fluorescent probes, a fluorescence microscope or flow cytometer is required. Although the fluorescence microscope is a cheaper option, the evaluation of spermatozoa by flow cytometry is recommended. This technique allows to evaluate about 10.000 sperm cells for several sperm features simultaneously (Cheuqueman *et al*., 2012), in a fast and accurate way.

Assessment of sexed sperm morphology

The assessment of sperm morphology by phase contrast microscopy is a very simple method to assess the effect of sexing on sperm morphology. Until recently, no effect of sexing process on percentage of cells with normal morphology had been reported (Carvalho *et al*., 2010). Because sexed sperm are submitted to a variety of adverse conditions during sorting, more detailed evaluation is needed. In this way, we used an atomic force microscope (AFM) to investigate if small changes could occur in the sperm head due to cell sorting (Carvalho *et al*., 2013). The AFM gives detailed three-dimensional information of a cell, with image of the surface of sperm at nanometer resolution (Berdyyeva *et al*., 2005). We acquired images from approximately 1800 sperm to measure 23 morphometric characteristics of the sexed and non sexed sperm head, such as volume, means radio, perimeter and surface area. Those measurements

provided the features of *Bos indicus* sperm head (Fig. 1 A-C). The authors observed that non sexed sperm presented a higher minimum height, elongation and membrane roughness and a lower form factor, circularity ratio and degree of circularity than the sexed sperm. Moreover, simultaneous evaluation of all the measured features using discriminant analysis differentiated the sexed and the non sexed sperm with 100% accuracy. The differences in head shape of sexed and no sexed sperm may be related to modifications in the plasma membrane, such as loss of some proteins and sperm capacitation. These modifications may cause decrease in longevity of the sperm in the female reproductive tract and compromise sperm binding in the oviduct to form the sperm reservoirs.

Although AFM is an interesting and accurate tool for *in vitro* evaluation of the sperm head shape, their use as a routine is still limited due to the high cost of the equipment and the long time required acquiring the images.

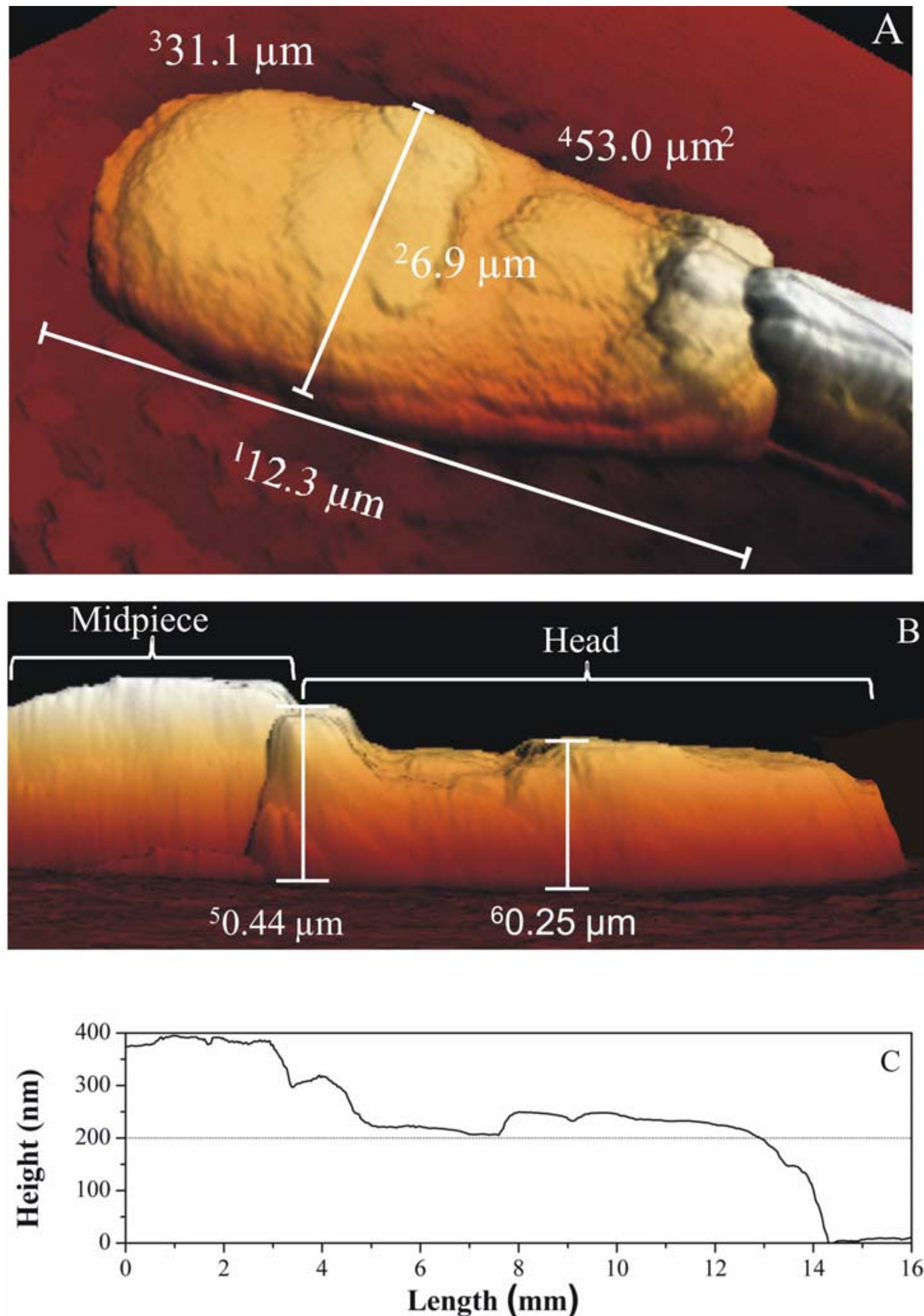

Figure 1. Atomic force microscopy (AFM) 3D view images (A and B) and line profile (C), showing different dimensional parameters of the bovine sperm cells containing an X-chromosome. 1. Maximum diameter; 2. Width; 3. Perimeter; 4. Surface area; 5. Maximum height; 6. Average height.

Effect of sexing process on sperm biochemical features

Assessment of plasma membrane proteins

Sperm plasma membrane is composed of protein, phospholipid, cholesterol and other components (Eddy and O'Brien, 1994). These proteins have an important function in protection and capacitation of sperm, as well as are necessary to fertilize the oocyte correctly. Therefore, any event that modifies the plasma membrane proteins, such as removing or cleaving these proteins can change the correct sperm function (Flesch and Gadella, 2000). It has been shown that intense manipulation of sperm during the sexing procedure, induced damage in plasma membrane and premature capacitation (Carvalho, 2013). Modification in protein profile of plasma membrane (McNutt and Johnson, 1996; Leahy *et al.*, 2011), assessed by two-dimensional polyacrylamide gel electrophoresis has also being reported. According to McNutt and Johnson (1996), the sexing procedure can remove cleavage or change the glycosylation of membrane proteins. However, despite this evidence, it is not known exactly which proteins can be altered by the sexing process, or even if these proteins are directly related to the maintenance of sperm viability and the fertilization process. Because the membrane proteins have an important function, especially for events related to *in vivo* fertilization, it is possible that the lower fertility rate found when sexed sperm are used *in vivo* has correlation with modification on membrane protein profile of those sperm.

Assess of methylation pattern

As previously mentioned, assessment of DNA fragmentation has been the only method used for evaluating the effect of sexing on chromatin integrity (Blondin *et al.*, 2009; Carvalho *et al.*, 2010; Gosálvez *et al.*, 2011). However, sperm DNA damage may result from DNA fragmentation, abnormal chromatin packaging and epigenetic defects (Tavalaee *et al.*, 2009). DNA methylation is the most well characterized example of epigenetic contribution of the sperm nucleus to the developing embryo (Carrel and Hammound, 2010). In addition, changes in DNA methylation can alter regulation of gene expression (Bird, 2002; Jaenisch and Bird, 2003).

Changes in methylation pattern of two important imprinted genes, insulin-like growth factor 2 (IGF2) and insulin-like growth factor-2 receptor (IGF2R), has been related to assisted reproduction technologies (ARTs), and may produce problems of embryo development and placentation (Curchoe *et al.*, 2005; Long and Cai, 2007). To investigate the effect of sexing process in sperm methylation, Carvalho *et al.* (2012), using the quantitative bisulfite sequencing method, evaluated the methylation of distinct regions of the IGF2 and IGF2R gene for non sexed, sexed for X-sperm and sexed for Y-sperm. The authors have not found changes in methylation pattern between the different groups evaluated for both genes (Fig. 2). Although both genes evaluated in that study are imprinted, it is not known whether these regions are packaged by histones or protamines, making these regions more or less susceptible to changes. Moreover, it is important to point out that we only assessed two regions of the genome, and it cannot be assumed that other regions do not have altered patterns of methylation due to sexing. It should also be considered that epigenetic changes may be related not only to changes in the DNA, but also to histone modifications. Therefore, an assessment of other genes or a new approach allowing an evaluation of a larger number of genes in sexed sperm, as well as studies of the effect of sexing process to another epigenetic mechanisms, could demonstrate if the sex-sorting affect other sperm epigenetic characteristics.

Assessment of the longevity of sexed sperm

Currently, changes in IVF protocols, such as sperm preparation and co-incubation time between sperm and oocyte, have been used to increase the blastocyst rate when sexed sperm are used (Blondin *et al.*, 2009; Carvalho *et al.*, 2010; Villamil *et al.*, 2012). However, the reduced fertility rates after AI or embryo transfer program (Seidel *et al.*, 1999; Sartori *et al.*, 2004; Bodmer *et al.*, 2005; Andersson *et al.*, 2006; Peippo *et al.*, 2009; Dejanette *et al.*, 2010, 2011; Mellado *et al.*, 2010; Underwood *et al.*, 2010a, b; Sales *et al.*, 2011; Healy *et al.*, 2013) remain a problem for the use of sexed sperm *in vivo*. These results associated with the differences between *in vitro* and *in vivo* conditions necessary for fertilization suggest that the sexing process can compromise parameters that, although are not important to *in vitro* fertilization, could be essential for *in vivo* fertilization. Among them, the time of sperm survival on the female reproductive tract can be highlighted. A study by Ijaz *et al.* (1994) showed that the sperm can remain viable up to 30 h after thawing. Therefore, assessment of sperm characteristics, such as motility, plasma membrane and acrosome, over different times of incubation (Mocé *et al.*, 2006; Carvalho, 2013), could identify the effect of sperm sexing on longevity. In a recent study (Carvalho, 2013), using flow cytometry to assess several sperm characteristics, we showed that the sexing process has negative effect on motility, mitochondrial membrane potential and integrity of plasma and acrosome membrane. Moreover, it was identified higher level of plasma membrane destabilization and less mitochondrial membrane potential of sexed sperm. Such difference was kept even at 12 h of incubation at 3°C in 5% CO_2 in air. Although those studies do not reflect the actual condition of spermatozoa in the female

reproductive tract, this is an indication that sexed sperm have lower resistance, and subsequently remain viable for a shorter period of time after thawing than the non sexed sperm.

Considering the differences found in the viability of sexed and non sexed sperm, it could be suggested that increasing sperm concentration in the dose used for AI could compensate the reduced quality of sexed sperm, increasing their *in vivo* fertility. However, Dejarnette *et al.* (2011), using 2.1 or 10 x 10^6 sperm per dose of sexed or non sexed sperm in AI, observed that the non sexed sperm had higher conception rates than sexed sperm, regardless the concentration used. According to these authors, factors other than concentration may be responsible for the lower conception rates obtained when sexed sperm is used. One of these factors could be the sperm ability to bind to oviduct epithelial cells, to form the sperm reservoir.

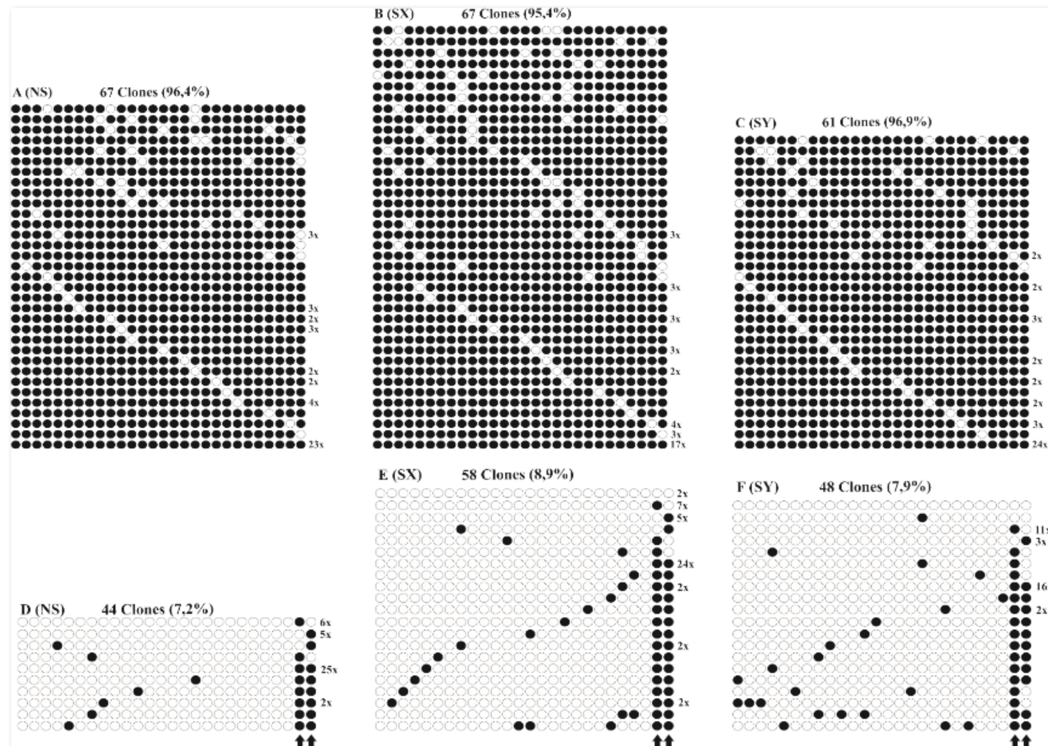

Figure 2. Methylation patterns in the DMR of the last exon of the *IGF2* gene (A-C) and the second imprinting control region (ICR2) of the *IGF2R* gene (D-F) in non-sexed (A and D), sexed X (B and E) and sexed Y sperm (C and F) from four Nellore bulls. The arrow indicates the very specific methylation patterns observed in the 25th and 26th CpG sites, which had high methylation. White and dark circles represent unmethylated and methylated CpGs, respectively; horizontal lines of circles represent one clone, and the number of clones with the same methylation patterns is indicated at the right end of the lines. The data are the average of four bulls (three replicates per bull for the IGF2 gene and two replicates per bull for the IGF2R gene.

Assessment of sexed sperm ability to bind to oviduct epithelial explants

The oviduct sperm reservoir regulates the timing of sperm capacitation (Fazeli *et al*., 1999; Tienthai *et al*., 2004), helps to maintain sperm viability in female reproductive tract, and synchronizes the release of a fertile sperm with ovulation (Pollard *et al*., 1991). The formation of the sperm reservoir is dependent on the presence of sugars and proteins in sperm membrane (Green *et al*., 2001; Gwathmey *et al*., 2003; Foye-Jackson *et al*., 2011; Kadirvel *et al*., 2012). Therefore, the beginning of capacitation (Carvalho, 2013), as well as changes in the protein profile of the plasma membrane after sexing process could alter the sperm binding to the oviduct cells. Thereby, it is possible that the lower *in vivo* fertility obtained when sexed sperm is used, could be related to changes in the formation and release of the sexed sperm from sperm reservoir. Based on that, Carvalho (2013), using epithelial oviduct explants, assessed the capacity of the sexed sperm to bind to oviduct cells after 30 min and 24 h of co-incubation. The number of sperm bound per mm of the explants was similar between sexed and non sexed sperm after 30 min (67.1 ± 9.0 and 70.3 ± 8.0, respectively) of co-incubation. However, after 24 h of co-incubation, there were less sexed sperm (6.7 ± 2.0) bound per mm of oviduct explants than non sexed sperm (23.6 ± 7.2). This suggests that sexed sperm has the ability to bind and to form the oviduct reservoirs, but

has a reduced ability to remain attached to the reservoirs compared to non sexed sperm. This could be responsible by the lower *in vivo* fertility of sexed sperm since the higher number of the sperm bound to the explants after 24 h of co-incubation has positive correlation with cattle non-return rate (De Pauw *et al*., 2002).

Another aspect to be considered is the evidence that a correct sperm-oviduct cell communication is needed to ensure the correct environment for fertilization and early embryonic development, since this interaction induces changes in gene expression (Fazeli *et al*., 2004; Kodithuwakku *et al*., 2007; Foye-Jackson *et al*., 2011) and protein secretion by those cells (Georgiou *et al*., 2005, 2007). Therefore, changes in the sperm reservoir, could compromise the oviduct environment, with higher number of unfertilized oocytes (Sartori *et al*., 2004; Schenk *et al*., 2006; Peippo *et al*., 2009) or embryos with low quality (Schenk *et al*., 2006; Peippo *et al*., 2009; Larson *et al*., 2010) reported when sexed sperm was used.

Final consideration

This review presented the most recent results related to the effect of sexing in the structure and function of bovine sexed sperm. Although several studies have already evaluated the effect of sexing process in structural and functional characteristics of sperm, the real cause of lower fertility of those sperm, especially when used *in vivo* has not yet been identified. Possibly, the lower fertility of sexed sperm has a multifactorial cause, since the sperm are complex cells that need the integrity and functionality of multiple attributes to successfully fertilize the oocyte.

Regarding changes in sperm caused by the sexing process, discussed in this review, we can list altered sperm motility, membrane integrity and acrosome, premature capacitation with modification of their membrane proteins and less ability to remain viable after thawing. This reduced viability of sexed sperm observed after thawing is responsible for the shorter longevity and consequently, less ability to remain bound to the oviduct cells after formation of sperm reservoirs. Those information lead us to suggest that the best moment for AI using sexed sperm is near ovulation time. This delay in the moment of AI may reduce the waiting time of the sperm in the female reproductive tract, ensuring larger numbers of viable cells at the time of ovulation.

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