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Aspects related to the technique and the utilization of sexed semen *in vivo* and *in vitro*

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Abstract

The development of methods capable of selecting the sex of animals has always been a great challenge for humankind. Separating X chromosome-bearing and Y chromosome-bearing sperm based on DNA difference using flow cytometry is the only technique that has achieved a useful level of progress, especially in cattle. This allowed the technique to be commercially applicable in this species. Thorough this review, aspects related to the sexing technique (flow cytometry) and to the sperm will be discussed along with the *in vitro* use of sexed semen. Besides, number of sperm used and semen deposition location, the influence of reproductive status (heifers *vs.* cows), time of insemination and production of embryos in superovulated donors will be reviewed.

Keywords: flow cytometry, IVF, IVEP, sexed semen, sex-sorted sperm.

Introduction

For many years, humankind has tried to develop methods to select the sex of animals. Various experimental techniques using sperm have been reported, such as density differences (Ericsson *et al*., 1973), weight and electric charge (Kaneko *et al*., 1984; Engelmann *et al*., 1988), differing protein content (Blecher *et al.*, 1999, Hendriksen *et al.*, 1999), immunological properties (Welch *et al*., 1995), and chromosome volume (Van Munster *et al*., 1999) among others.

Some techniques have been described with the objective of sexing the embryo. The invasive techniques include the polymerase chain reaction method (PCR) and cytogenetic analysis, and non-invasive techniques include quantification of X chromosome related enzymes and immunological tests (Gardon *et al*., 2004).

Separating X and Y bearing sperm based on DNA differences using flow cytometry has been largely accepted as a major breakthrough in reproduction technology (Blondin *et al*., 2009). Larry A. Johnson developed this technique in 1989, at the USDA Beltsville Agricultural Research Center (Seidel *et al*., 1999; Johnson, 2000; Garner and Seidel, 2003; Weigel, 2004; Garner, 2006), and it is the only method validated so far for sex selection before birth (Graaf *et al*., 2009). However, only in the bovine species has this technology progressed sufficiently to allow commercial use (Rath and Johnson, 2008). Nevertheless, numerous publications on semen sexing using flow cytometry on other species are being reported in order to allow commercial use (Morris, 2005; O'Brien and Robeck, 2006; Karabinus, 2009; O'Brien *et al*., 2009; Rath *et al*., 2009; Vasquez *et al*., 2009; Leahy *et al*., 2010; Gibb *et al*., 2011; Clulow *et al*., 2012).

The use of sexed semen provides an increase in beef and dairy cattle productive efficiency by the production of an ideal proportion of males and females to obtain advantages from characteristics that are limited or influenced by sex, and facilitates economically flexible and practical management (Rath and Johnson, 2008). Additionally, this allows selection of potentially superior females, producing replacement heifers specifically from these animals (Hohenboken, 1999; Weigel, 2004; Mocé *et al*., 2006). A 15% increase in genetic gain is expected compared to conventional semen, besides the reduction of progeny testing costs and embryo transfer and genetic markers cost (Hohenboken, 1999; Johnson, 2000; Weigel, 2004; De Vries *et al*., 2008).

With *in vitro* production (IVP) of embryos, more male embryos are produced than females, and using sexed semen would revert this situation. This inversion is beneficial as females often have a higher market value than males (Wheeler *et al*., 2006; Rath and Johnson, 2008; Rath *et al*., 2009). In human health, one major application is the prevention of sex linked genetic diseases (Seidel, 2003; Maxwell *et al*., 2004; Mocé *et al*., 2006).

It is important to highlight that populations of calves resulting from sexed semen do not differ from those derived from the use of conventional semen, including gestational length, birth weight, mortality rate and weight gain (Tubman *et al*., 2004). No genotoxic effects on sexed semen were detected to date from using Hoechst 33342 (Parrilla *et al*., 2004; Garner, 2006).

Aspects related to the flow cytometry and to sperm

Several studies have shown that the process of sexing semen using flow cytometry involves a series of steps that can cause alterations in membrane functionality, motility characteristics and sperm morphology. These steps are the use of DNA stain (Hoechst 33342), rewarming and incubation, high pressure (40-50 psi), vibrational mechanical forces to form the drop during the sperm

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cell passage through the nozzle-tip, exposure to the UV laser, electric charge, high force during sperm deceleration inside the collecting tube, high dilution. Besides, sperm must resist changes in media, centrifugation, cooling and the cryopreservation process (Garner and Seidel, 2003; Garner, 2006; Mocé *et al*., 2006; Garcia *et al*., 2007; Graaf *et al*., 2007; Arruda *et al*., 2011).

Therefore, a great number of studies report decreased fertility using sexed semen compared to conventional frozen semen (Lu *et al*., 1999; Seidel *et al*., 1999; Sartori *et al*., 2004; Seidel and Schenk, 2008). However, more studies are needed to evaluate this subject. In this respect, our research group has developed experiments with the objective of evaluating morphofunctional alterations on bovine sperm submitted to sexing using flow cytometry compared to conventional semen and the differences among the subspecies (Tanno, 2009; Mejia-Gallego, 2010). Semen was obtained from 6 bulls, 3 *Bos taurus* (Holstein) and 3 zebu (Dairy Gir). Five treatments were used: conventional semen using L extender (Lagoa); conventional semen using S extender (Sexing) and sexed semen (on times 0, 3 and 6 h after ejaculation to evaluate if there was an effect of time on membrane alterations). Flow cytometry was used to examine plasma membrane integrity and acrosomal reaction, lipid peroxidation and sperm capacitation, by analyzing protein tyrosine phosphorylation and changes in plasma membrane fluidity and disorganization. The semen quality of the *Bos taurus* subspecies was more negatively affected than semen from zebu. Sperm from the sexed semen groups with intact plasma membrane had more lipid peroxidation and more phosphorylated proteins on the surface of the plasma membrane than unsexed sperm, indicating sperm capacitation. There was no decrease in semen quality due to ejaculate waiting time for up to 6 h before sexing. Using the same experimental design, Mejia-Gallego (2010) studied motility parameters using computer-assisted sperm analysis (CASA), plasma membrane functionality using the osmotic resistance test (HOST), and sperm morphology using differential interference contrast (DIC). The majority of sexed sperm had motility patterns superior to unsexed sperm (around 60 *vs.* 40%), indicating a possible selection by flow cytometry. There was a decrease of sperm linearity during the time that semen waited before sexing (0 h: 46.7%; 3 h: 46.5%; 6 h: 45.4%), compared with conventional sperm (50.1%). Linearity was statistically different at 6 h. On the other hand, plasma membrane functionality (0 h: 45.3%; 3 h: 42.7%; 6 h: 41.5%) did not show difference among different times that semen waited before sexing. An increase in minor defects was detected in sexed semen compared to conventional semen, probably because of plasma membrane alterations in sperm tail (conventional semen: 2.2% *vs.* sexed semen at 0 h: 3.4%; 3 h: 6.0%; 6 h 4.3%). Zebu semen was more sensitive to plasma membrane alterations in the tail than *Bos taurus*. Finally, strong evidence exist that a population of sperm subjected to flow cytometry enters a state of hyperactivation (Mejia-Gallego, 2010).

All parabiological and mechanical interactions between media and sperm can, theoretically, be a potential inductor of cellular structural changes, including to DNA. When all these stressors are taken in account, low fertility rates can be related not only to low sperm concentration but also to DNA damage (Gosálvez *et al*., 2011a, b). Thus, Gosálvez *et al*. (2011b) examined the dynamic response of DNA fragmentation on bull sperm after sexing. The comparison of the dynamics of loss of DNA quality of sexed and conventional semen, when analyzed over time, showed a lower fragmentation level initially in sexed semen than conventional semen. Samples of conventional semen maintained low levels of DNA fragmentation during the first 48 h of incubation, and there was a slight increase between 48 and 72 h. In contrast, thawed sexed semen had a reduced DNA longevity, with damage occurring between 24 and 48 h. The level of DNA damage obtained in the end of the 72 h period of incubation was six times greater than conventional semen samples.

The above-mentioned result allowed the establishment of a DNA fragmentation indicator, which is the threshold point between dynamic tendencies of DNA fragmentation in sexed and conventional semen. This indicator can be used as the fragmentation rate for bulls after sperm sexing. Moreover, it was evident that this DNA fragmentation dynamics was not the same for all bulls. In certain cases, sperm from some animals were more resistant to damage than others (Gosálvez *et al*., 2011a, b).

In vitro methods aimed to improve sexed semen efficiency

Klinc *et al*. (2007b) believe that stressor effects imposed on sperm by the sexing procedure cause production of oxygen reactive species, culminating in plasma membrane damage, as observed by Tanno (2009) also. Hence, Klinc *et al*. (2007b) have added antioxidants to the extender traditionally used in the sexing technique to counterbalance the negative effects.

Marked benefits were obtained using the antioxidants catalase and sodium pyruvate, especially in acrosome morphology protection and improvement in sperm cell longevity. However, besides adding antioxidants, procedures such as correct semen manipulation and the use of selected bulls are valuable for improvement of the final results using sexed semen (Klinc *et al*., 2007a).

Another recent protocol has shown positive results, including a whole set of modification that are summarized as a new procedure called Sexcess®. Among others, it includes the temporary inhibition of

sperm metabolism and mobility using fluoride during sorting, supplementation with different antioxidants, and a protocol of balance adaptation in three steps at 4°C. Data from field studies initially indicated that birth rates were practically the same comparing AI in heifers with sexed semen (73.6%) and non-sexed semen (76.7%; Rath *et al*., 2009).

The main effect of the Sexcess® procedure on treated sperm is an increase in sperm viability after thawing. In a recent study, 18 sexed semen samples from 3 bulls were processed using Sexcess® and compared to controls (non-sexed semen) from the same ejaculates. Results revealed similar quality of the semen submitted to Sexcess® compared to control (Rath *et al*., 2009).

As indicated by the thermotolerance test, Sexcess® extended the lifespan for all parameters tested and improved the chance of fertilization significantly. Benefits of Sexcess®, measured by incubation for 6 h, include maintenance of motility, viability and acrosomal integrity (Rath *et al*., 2009).

Several steps through the sexing process affect the productivity and efficiency of the technique. Consequently, since its implementation 20 yr ago, research and improvements in these critical points have been pursued (Sharpe and Evans, 2009).

A recent and important advancement in flow cytometry technology that directly affects the outcome of sperm sexing is the introduction of a digital pulse processing circuity replacing time-gated analogue system. The greatest advantage of digital technology is decreasing "dead time" during measurement (temporary inability of processing and solving photodetector pulses due to the proximity of sperm cells) plus coincidence events are rare, and almost completely eliminated. This is because these systems are able to perform sampling continuously, almost in real-time, enabling multiple photodetectors to be precisely distinguished and measured, causing an increase of 33% in throughput and 30% in sorting efficiency compared to analogue systems (Sharpe and Evans, 2009; Arruda *et al*., 2011).

Utilization of sexed semen *in vivo*

Sperm number and site of semen deposition

For most bulls, fertility is maintained if each insemination dose contains $\geq 10 \times 10^6$ motile sperm (Sullivan and Elliott, 1968; Pace *et al*., 1981). Andersson *et al*. (2004), working with sex-sorted sperm in dairy cows found that lower doses (2×10^6 spermatozoa/dose) significantly reduced pregnancy rates compared to higher doses (15×10^6). Similarly, Borchersen and Peacock (2009) found 5-10% conception rate differences in heifers of all breeds between 2×10^6 sperm/dose of sexed semen compared to 15×10^6 sperm/dose of conventional semen. In another study, Seidel and Schenk (2008), using several insemination doses ($1.0\text{-}6.0 \times 10^6$ sperm/dose), found no increase in pregnancy rates in heifers inseminated 12-24 h after the occurrence of estrus when an inseminating dose greater than 2×10^6 sperm was used. DeJarnette *et al*. (2008) found no significant increase in pregnancy rates using 3.5 or 5×10^6 sperm/dose, compared to 2.1×10^6 sperm/dose in heifers or cows. Likewise, Sá Filho *et al*. (2010) found that an increase in the sperm number per insemination after heat detection from 2.1 to 4.2×10^6 and the execution of two AI with an interval of 12 h did not alter the conception rate in Jersey heifers. Bodmer *et al*. (2005) also found similar pregnancy rates for both sexed sperm and non-sexed sperm in dairy cattle when a low dose was used for insemination (2×10^6 sperm). Using semen with and without sorting, Frijters *et al*. (2009) analyzed the effects of low sperm numbers per dose on non-return rates after 56 days (NRR56). A significant decline in NRR56 was found with sexed semen (13.6 percentage points) compared to the use of conventional semen. When they evaluated the effects of low dosage and sorting in the sexing process separately, it was estimated that about two-thirds of the decrease detected was caused by the low dose, and a third was due to sorting (8.6 and 5.0%, respectively).

The majority of previous results support the idea that site of semen deposition would play little to no role in pregnancy rates. However, due to the presumably reduced viability of sexed sperm, insemination closer to the site of ovulation would potentially provide better results (Pallares *et al*., 1986; Seidel *et al*., 1997). Thus, Brogliatti *et al*. (2009), using sex-sorted sperm, found an increase in pregnancy rates in dairy heifers when the insemination was performed in the ipsilateral horn to the dominant follicle compared to insemination in the uterine body, and these results were obtained when inseminations were performed 58 h after the intravaginal progesterone device removal. However, Sá Filho *et al*. (2012) found no difference in pregnancy rates in beef cows using insemination in the uterine body or uterine horn ipsilateral to the dominant follicle, in agreement with previous researchers comparing different deposition sites (Andersson *et al*., 2004; Seidel and Schenk, 2008). Kurykin *et al*. (2007) obtained comparable results in dairy heifers, with similar pregnancy rates between the different deposition sites of sexed semen.

Reproductive status of heifers vs. cows

The reduction in fertility when sexed semen is used has been attributed to damage of spermatozoa caused by the sexing process (Seidel and Garner, 2002). Also fertility in heifers is generally higher than in cows (Bodmer *et al*., 2005). Therefore some researchers are studying these differences in fertility between the two groups. Bodmer *et al*. (2005) used a dose of 2×10^6 sperm in heifers and cows and found similar pregnancy

rates with sex-sorted sperm, although they were lower than reported by other studies (Seidel *et al*., 1997). Schenk *et al*. (2006) suggested that excellent reproductive management of heifers appears to be the key to achieve comparable pregnancy rates between sexed and unsexed inseminations.

In order to increase the probability of acceptable conception rates, Dejarnette *et al*. (2009) recommend restricting the use of sexed semen to 1st or 2nd service heifers showing estrus, in agreement with Sá Filho *et al*. (2010), who found decreased pregnancy rates in 3rd service heifers compared to 1st and 2nd service heifers. In cows, however, there appeared to be little influence of service number on conception rates using sexed semen and on overall conception. The use of sexed semen in lactating cows, fixed time AI or problem breeders was specifically discouraged (Dejarnette *et al*., 2009). In superovulated Holstein cows the results were poor, resulting in lower production of transferable embryos compared to heifers. Hence, the use of sexed semen in superovulation programs is not economically viable, and its applicability in this situation becomes questionable (Hayakawa *et al*., 2009). Similarly, Peippo *et al*. (2009) have reported that the fertilization rate was significantly decreased in donor cows inseminated with 6 or 8 x 10^6 total sexed sperm into the uterine horns compared with conventional semen. These results suggest that cows show less predictable and inconsistent embryo production than heifers, possibly because of postpartum uterine conditions and/or physiological transitions. Schenk *et al*. (2006) recovered, on average fewer transferable embryos in heifers than cows, but the difference was not significant. Additionally, more ova were recovered from cows than heifers; however, the percentage of fertilized oocyte was greater in heifers than cows, more oocytes were fertilized, and more transferable embryos were recovered with conventional than sexed semen.

A higher percentage of embryonic loss occurred between 30-40 days and 70-90 days of pregnancy when sex-sorted sperm was used compared to non-sorted sperm (Bodmer *et al*., 2005), but the incidence of dystocia and stillbirths in both cows and heifers decreased (especially in heifers) due to the increase in births of females (Norman *et al*., 2010).

Time of artificial insemination (AI)

Despite the increased use of sex-sorted semen, pregnancy rates remain lower than those achieved with conventional semen (DeJarnette *et al*., 2008). The time of AI has an important influence on these rates, and longer intervals from the induction of ovulation to the AI (*i.e*., closer to the expected moment of ovulation) have increased the likelihood of pregnancy in females inseminated with sex-sorted sperm (Schenk *et al*., 2009; Sá Filho *et al*., 2010; Sales *et al*., 2011). Sá Filho *et al*. (2010) evaluated three treatments: AI with one dose (2.1 x 10^6 sperm) 12 h after estrus detection; two doses 12 h after estrus detection; one dose at 12 h and one dose at 24 h after estrus detection. No differences in conception rate between the groups were detected. In another study, Baruselli *et al*. (2011) compared timed insemination with non-sexed sperm (40 x 10^6 sperm/dose) or sexed sperm (2.1 x 10^6 sperm/dose) at two different times after the removal of Crestar (ear implant containing norgestomet), 54 h (16-18 h before ovulation) or 60 h (10-12 h before ovulation). No difference in conception rates was observed when AI was performed 54 or 60 h after Crestar removal using sexed semen. Furthermore, no differences were found between the use of conventional semen, sexed-X and sexed-Y. Moreover, Sales *et al*. (2011) evaluated different intervals relative to ovulation to perform the TAI with sexed semen. In this study, lactating cows received timed AI with sexed semen at 36, 48 or 60 h after progesterone device withdrawal. Ovulations occurred on average 71.8 ± 7.8 h after device removal. Pregnancy rates were higher in cows inseminated close to ovulation. However, Souza *et al*. (2008) used Nelore cows in the postpartum period (50 days) and found a reduction in conception rates when TAI was performed 64 h after implant removal, compared with TAI 60 h after removal of the progesterone source. Thus, the results suggest that the most appropriate time to perform the TAI with sexed semen can be 60 h after removal of the source of progesterone (10-12 h prior to ovulation), similar to what Sales *et al*. (2011) observed. Interestingly, this effect was not observed for non-sexed sperm. Thus, according to Baruselli *et al*. (2011) better results are obtained when the insemination is performed between 16-24 h after the onset of estrus (6-14 h prior to ovulation).

Baruselli *et al*. (2011) suggested that using synchronization techniques would reduce the variation in time of ovulation, increasing the efficiency of AI programs using sexed sperm. In beef and dairy cows synchronized with progesterone and estradiol, ovulations occur between 70-72 h after device removal (Baruselli *et al*., 2006; Souza *et al*., 2006; Sales *et al*., 2008). Thus, another possibility to try to increase the conception rate of heifers inseminated with sexed semen would be the use of GnRH on the day of artificial insemination. However, in a recent study, Sá Filho *et al*. (2010) observed that treatment with GnRH on the day of AI, when two doses of prostaglandin were used to synchronize estrus, did not improve the conception rate in dairy heifers inseminated with sexed semen.

Using different artificial insemination times in superovulated cows, Baruselli *et al*. (2008) reported an increase in the number of transferable embryos using sexed semen at 18 and 30 h after estrus detection, both in Holstein and Nelore cattle, compared to the protocol using two inseminations at 12 and 24 h after estrus detection. Similar results were observed by Soares *et al*. (2011); numbers of freezable/transferable embryos were

lower when inseminating at 12 and 24 h compared to 18 and 30 h after ovulation induction, suggesting that the 6 h delay of the insemination may increase the production of embryos in superovulation protocols. Also, the period between insemination and ovulation is an important factor in success of the protocol.

Embryo production in vivo with superovulation

Sexed sperm doses are more expensive than conventional semen; however, it leads to a greater genetic gain, making its use in embryo transfer programs of great interest. Embryo production efficiency is an important factor to breeders, and the implementation of insemination with sexed semen would increase the number of heifers for replacement or sale and trading of valuable embryos (Hayakawa *et al*., 2009). The limited number of sperm along with the alterations imposed by cytometry and freezing-thawing are challenging factors for sperm transport, resulting in reduced sperm in the oviducts at the time of fertilization (Seidel and Garner, 2002). Multiple inseminations or increased sperm per dose may be advantageous for embryo production when using a single fixed time insemination protocol (Schenk *et al*., 2006).

Authors reported that the use of sexed sperm resulted in more unfertilized oocytes (Sartori *et al*., 2004; Schenk *et al*., 2006; Baruselli *et al*., 2007) and lower number of transferable embryos (Soares *et al*., 2011) compared to non-sexed sperm. Additionally an increased percentage of fertilized embryos that were degenerating was observed (Sartori *et al*., 2004). Conversely, Hayakawa *et al.* (2009) achieved similar rates of transferable embryos in heifers inseminated twice (12 and 24 h after estrus detection) with the same dose (5 x 10^6 sperm/dose) with both sexed and non-sexed sperm. More transferable embryos were obtained when higher numbers of sperm per dose of sexed semen were used, but fewer transferable embryos were recovered compared with non-sexed control semen (Schenk *et al*., 2006; Hayakawa *et al*., 2009)

These differences may be due to interacting factors that likely influence the success of superovulation programs using sexed sperm, including sperm quality, quantity, and fertilization potential, ovulatory response of donors, embryo recovery and viability, and number and timing of AI (Schenk *et al*., 2006).

Utilization of sexed semen *in vitro*

The sex-sorting procedure by flow cytometry affects some structural characteristics of bovine sperm, but does not eliminate their capacity to produce embryos *in vitro* (Carvalho *et al*., 2010). Despite this sperm ability, some aspects of *in vitro* production (IVP) using sex-sorted semen remain to be examined to overcome difficulties that compromise results.

In vitro production employing sex-sorted semen has been investigated in several studies. However, differences have been reported, and causes are attributed to a number of factors. Some studies found lower cleavage and blastocyst rates (Merton *et al*., 1997; Bermejo-Alvarez *et al*., 2008; Palma *et al*., 2008; Stinshoff *et al*., 2012) while other studies have not observed differences in blastocyst development between sorted and non-sorted sperm (Zang *et al*., 2003; Lu and Seidel, 2004; Carvalho *et al*., 2010; Underwood *et al*., 2010). Blondin *et al*. (2009) reported a higher cleavage rate using sexed semen, although lower blastocyst formation. The reported differences may be due to varying methods of oocyte selection, quality of sorted sperm, or type of culture medium (Underwood *et al*., 2010). Moreover, it is important to highlight that individual differences between bulls were observed, showing that some sires lead to better cleavage rates (Zang *et al*., 2003), and semen from some sires is more negatively affected than others by the cytometry process (Blondin *et al*., 2009). When Lu and Seidel (2004) tested sorted sperm from different bulls, different heparin concentrations were required for optimal capacitation to be achieved. Sorted sperm from some bulls did not benefit from heparin as an additive reagent to induce capacitation. Effective sperm concentrations likely differ between sorted and unsorted sperm from the same bull.

Differences in IVF procedures like smaller fertilization drop (Cran *et al*., 1995), concentration of 1 x 10^6 sperm/ml and lower sorting pressure from 50 to 40 psi (Barceló-Fimbres *et al*., 2011) were required to achieve better results. As the IVP technique is being refined, the use of sex-sorted sperm in IVF is increasing. Embryo cryopreservation also plays an import role in this progress. Vitrification works very well with *in vitro*-produced embryos in several different species (Xu *et al*., 2009). Vitrified sexed IVF embryos were produced by Xu *et al*. (2006), and no difference was found in post-thaw viability of sexed (96%) and control (92%) blastocysts.

Regardless of the reduced membrane integrity and progressive motility of sexed sperm, Carvalho *et al*. (2010) did not find lower embryo production in IVP. However embryo ultrastructure showed a lack in number and structure of organelles like mitochondria and rough endoplasmic reticulum (Palma *et al*., 2008). This discrepancy in compromised embryonic development may be due to DNA fragmentation in the sperm of some bulls used in these experiments. Gosálvez *et al*. (2011a) showed that some bulls have more sperm with DNA damage than others; thus, identifying resistant individuals to the rigorous process of sex-sorting would allow an increase of this technology in ART.

In order to explain the lower embryonic development and ultrastructural damage of embryos produced *in vitro* with sexed semen observed by some

authors, gene expression has been studied. Morton *et al.* (2007) showed that embryos derived from sex-sorted sperm display a differential expression of developmentally important genes compared with their counterparts derived from unsorted sperm. In contrast, Carvalho *et al*. (2012) examined the DNA methylation patterns of the IGF2 and IGF2R genes and observed no alteration, although individual variation in their patterns among bulls was detected. Likewise, Stinshoff *et al*. (2012) confirmed that the process of sorting the sperm used for IVF does not influence early embryonic quality at the molecular level.

Interestingly, the ability of reverse sorting is also possible. It consists of thawing conventional semen doses for subsequent sex sorting and refreezing for future AI or IVF. It allows the sorted product to be shipped to an IVF facility, distant from a sorting laboratory, removing the current requirement to have bulls housed at the sorting facility or for frozen-thawed, sex-sorted sperm to be used immediately after the sorting procedure. This technique could offer new possibilities to the use of sex-sorted sperm in IVP (Underwood *et al*., 2010; Gosálvez *et al*., 2011a). Another promising ART is Intracytoplasmic Sperm Injection (ICSI). This technique offers the ultimate solution to sexed IVF, because one sperm can be used to fertilize one oocyte (Xu *et al*., 2009). Sexed sperm could be used for ICSI with similar efficiency as non-sorted sperm (Hamano *et al*., 1999). However, ICSI in cattle faces difficulties: there is limited ability to initiate calcium oscillations to induce oocyte activation and this appears to compromise the success of ICSI in this species (Malcuit *et al*., 2006). Besides that, recent research promoted a step toward the optimization of ICSI in cattle. Bevacqua *et al*. (2010) achieved high blastocyst development using oocytes chemically activated. ICSI could, in the future, circumvent the higher dose costs and the lower sperm concentration problems.

Final considerations

A series of reports indicate that sexed semen lowers fertility rates compared to non-sexed semen (around 50 to 80%). However, recent data shows 80-90% fertility compared to conventional semen (Schenk *et al*., 2009). Furthermore, several studies indicate that bulls with good quality semen and correctly managed females can have similar fertility rates with sex sorted or conventional semen. This discrepancy can be true or there might be flaws in the laboratories where freezing is done to provide needed biological conditions to sperm, mainly due to large-scale production. Nevertheless, it is not realistic at the present moment to consider that the quality of sexed semen is comparable to conventional semen (Seidel and Schenk, 2008, Arruda *et al*., 2011).

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