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Bovine semen quality control in artificial insemination centers

P. Vincent[1], S.L. Underwood[1], C. Dolbec[1], N. Bouchard[1], T. Kroetsch[2], P. Blondin[1,3]

[1]L'Alliance Boviteq, Saint-Hyacinthe, Quebec, Canada.
[2]The Semex Alliance, Guelph, Ontario, Canada.

Abstract

Quality control (QC) is a fundamental area of management for semen production centers (SPCs) supplying bovine semen to breeders and producers. Semen production centers are moving away from subjective semen assessment, that is largely uncorrelated to field fertility, to objective semen analyses that incorporate computer assisted sperm analysis (CASA) and flow cytometry. A multiparametric approach to semen analysis using a combination of CASA and flow cytometry can provide SPCs with the highest QC for all semen production. In this paper we review probes used for labelling spermatozoa for viability, acrosomal integrity, mitochondrial activity, DNA integrity and calcium release. Limitations of CASA and flow cytometry when analyzing spermatozoa, especially frozen-thawed samples, are discussed. Finally, we described how a multiparametric approach using CASA and flow cytometry could be applied in SPCs to establish QC of production before the release of the product in the field.

Keywords: bovine, CASA, fertility, flow cytometry, quality control.

Introduction

Fertility is a multiparametric phenomenon that relies on the use of semen of sufficient quality and quantity, accurate timing and method of insemination, and appropriate herd management. When using artificial insemination, the dairy producer must manage a range of these factors, including heat detection, timing of insemination in relation to estrus, and correct handling of the frozen straws. However, it is the onus of the semen production centers (SPCs) to supply straws containing spermatozoa of good viability that produce acceptable conception rates if all other variables are managed correctly.

To ensure acceptable fertility after artificial insemination, frozen-thawed spermatozoa must be present in sufficient number in each straw (concentration), and possess a number of characteristics important for fertilization. Accordingly, spermatozoa must survive the thawing procedure with normal morphology, an intact acrosome, DNA integrity, active mitochondria, and maintain forward progressive motility to traverse the female reproductive tract. Some or many of these characteristics are measured during post-thaw quality control procedures undertaken by SPCs prior to distribution. QC is the assurance that each batch of straws has undergone semen analysis to verify that the sample is likely to be fertile.

Although semen analysis may seem easy to perform, meticulous attention to detail and technique is essential in order to obtain an accurate and reproducible analysis. Manual semen analysis using a light microscope has been the standard method for analysis in most SPCs. However, manual analyses can be very subjective and prone to within and between technician errors. Similarly, the use of fluorescence microscopy to assess spermatozoa for acrosome, membrane and DNA integrity is markedly slow and limited due to the low number of spermatozoa analyzed from each sample and the incapacity for an extensive multiparametric analysis.

To maximise accuracy in QC, SPCs are realizing the benefit of a multiparametric approach and have increased the rigor of their semen testing, moving from time-consuming basic subjective assessment of a few hundred spermatozoa for concentration, motility and morphology using microscopy, to the use of computer-assisted tracking to assess motility, and flow cytometry to analyse thousands of cells within seconds for characteristics such as viability, mitochondrial activity, acrosome, DNA and capacitation status. The topics for discussion within this review are the various tools and assays in use in cattle SPCs to determine QC values, factors to consider when using these tools and how the efficacy of QC procedures may be maximized in order to predict field fertility.

Objective assessment of sperm motility

Computer assisted sperm analysis (CASA) is a powerful tool for the objective assessment of sperm motility and is hence now frequently used for evaluating semen quality. The basic components of this technology consist of a microscope to visualize the sample, a digital camera to capture images and a computer with specialized software to analyze the movement of the spermatozoa. The essential principle behind most microscopy-based CASA systems is that a series of successive images of motile spermatozoa within a static field of view are acquired by computer software algorithms, which then scan these image sequences to

[3]Corresponding author: blondinpa@boviteq.com
Phone: +1(450)774-7949; Fax: +1(450)774-1740
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identify individual spermatozoa and trace their progression across the field of view. This involves recognising the same cell in each image by its position, and inferring its next position by estimating the likelihood that it will only have moved a certain maximum distance between frames. Computer assisted sperm analysis can also provide information about sperm concentration, morphology, viability and index of DNA fragmentation of frozen-thawed sperm. However, these more specialized techniques are not routinely applied for regular analysis of frozen-thawed semen in SPCs.

With the use of CASA, several motility parameters describing the specific movements of spermatozoa can be obtained in greater detail than what is possible in subjective assessment. These computerized measurements can be useful to assess various sperm characteristics simultaneously and objectively, and are valuable for the detection of subtle changes in sperm motion that cannot be identified by conventional subjective semen analysis as reviewed elsewhere (Davis and Katz, 1993; Mortimer, 2000; Kathiravan *et al*., 2011). The parameters typically collected using CASA systems are motility, velocity, linearity and lateral displacement of spermatozoa as they progress along their trajectories in a sample. The percentages of total and progressive motility are the most important motility parameters in the evaluation of spermatozoa. Total motility refers to the fraction of spermatozoa that display any type of movement, whereas progressively motile spermatozoa swim forward in an essentially straight line. Spermatozoa that swim, but with an abnormal path, such as in tight circles, are not included in the proportion of progressively motile sperm. In addition to evaluation of sperm motility, the software calculates the kinetic values of each spermatozoon, which covers the velocity of movement, the width of the sperm head's trajectory and frequency of the change in direction of the sperm head. The velocity values that are determined by CASA are the curvilinear velocity, straight-line velocity and average path velocity (Mortimer, 2000). The amplitude of lateral head displacement and beat cross frequency are two other characteristics measured with CASA instruments (Mortimer, 2000).

Limitations of CASA instruments

Despite the power of an objective evaluation by CASA, there are some constraints associated with this technology. Many factors are known to affect CASA results. The type of specimen chamber used for analysis can affect the movement of sperm, the accuracy of the cell count number, and therefore the percentage of motile spermatozoa (Massányi *et al*., 2008). The temperature at which semen is analyzed is also an important factor that may affect CASA results. Independent studies showed that analyzing semen below 37^{o}C significantly affected results (Iguer-Ouada and Verstegen, 2001; Brito, 2010). These groups performed CASA on spermatozoa maintained at 37^{o}C with a stage warmer and compared the results with spermatozoa analyzed at room temperature or at 30^{o}C. The data demonstrated a decrease in the motility parameters (percentage of motile spermatozoa and track speed) when spermatozoa were not analyzed at 37^{o}C. These experiments suggest that a simple variation introduced in the analysis of sperm motility can have a considerable effect on the results. The concentration at which semen is analyzed is an essential aspect that influences CASA results. It has been established that at low semen concentrations (less than 20 million per ml) an overestimation of the concentration and thus an underestimation of the percentage of motile cells can occur due to the acquisition of non-spermatic particles (debris). On the other hand, at a higher concentration (above 50 million per ml), a large proportion of the fast moving cells will be excluded from analysis because of cell collisions, spermatozoa exiting the analysis area or excluded on the basis of the nearest-neighbour effects, leading to an underestimation of the motility (Iguer-Ouada and Verstegen, 2001; Contri *et al*., 2010).

Sampling condition is a source of error when acquiring data with CASA. Computer and video camera equipment are continuously evolving and different CASA systems use various models of video camera. Most of the CASA systems allow 30 Hz or 60 Hz as a frame rate to analyze sperm tracks and speed. Studies have shown the importance of the frame rate for reliability of the analysis (Mortimer and Swan, 1999a; Brito, 2010; Castellini *et al*., 2011). It is generally accepted that a higher frame rate is required to render an evaluation closer to the real path for a fast non-linear sperm cell. To study a hyperactivated sperm cell, Mortimer and Swan (1999b) suggested using the highest frame rate available on the system in order to have the most accurate evaluation.

The type of extender in which semen is diluted is another aspect that should be taken in consideration when evaluating spermatozoa with CASA. Some extenders contain debris of size similar to a sperm head, causing CASA software to include them in the analysis. Egg-yolk and milk based diluents are examples of extenders containing such particles. In addition, when observing semen diluted with milk extender, the globular lipids mask the spermatozoa thus rendering CASA analysis impossible. To assess motility analysis in these conditions, samples could be washed to remove extender debris from semen. However, it has been

established that washing the semen affects thereafter the motility of the spermatozoa (Fernández-Santos *et al*., 2009) making correct evaluation more difficult. To overcome this problem, fluorescence technology allows discrimination of sperm cells from particles in the extender by staining sperm heads with a DNA binding fluorochrome. Under fluorescent light, only DNA-containing objects will be detected by the CASA software, thus omitting the need for washes. This technique improves the accuracy of the concentration (Zinaman *et al*., 1996) as well as motility analysis (Tardif *et al*., 1998) when working with semen diluted in these extenders. Therefore, standardizing the type of chamber, the temperature, the concentration and the type of extender is crucial to assure repeatable standard QC in a SPC.

Motility is one of the most important characteristics believed to be associated with the fertilizing ability of spermatozoa. Several groups have reported a significant correlation between total (Wood *et al*., 1986; Kjaestad *et al*., 1993; Correa *et al*., 1997; Gillan *et al*., 2008) and progressive (Kathiravan *et al*., 2008) motility of bull semen and its associated field fertility. However other groups reported that the subjective analysis of semen motility did not correlate with fertility (Farrell *et al*., 1998; Januskauskas *et al*., 1999). Computer assisted sperm analysis instruments collect a wide range of sperm motility parameters, allowing a more detailed and accurate analysis of sperm movements and track speed. Researchers have also tried to correlate the kinetic parameters with the field fertility of semen, with some groups able to show a positive correlation between straight line velocity of spermatozoa and field fertility (Budworth *et al*., 1988; Farrell *et al*., 1998; Januskauskas *et al*., 1999; Gillan *et al*., 2008; Kathiravan *et al*., 2008). Another study used a combination of several motility parameters to reach a very high correlation with bull fertility (Farrell *et al*., 1998). Taken together, these studies show the high potential of CASA to estimate the quality of the semen, therefore becoming a powerful tool to measure sperm characteristics and predict bull fertility compared to standard semen evaluation. However, as mentioned above, standardization of conditions and parameters of all CASA analyses are key to obtain repeatable and valid correlations with fertility.

Several models of CASA instruments are now available to evaluate the quality and the motility of spermatozoa. Each system operates on similar principles but they differ in their parameter settings and use different algorithms to determine speed and trajectories. Parameter settings, threshold settings, video frame rate and other variables will affect CASA results as reviewed by Davis and Katz (1993). As mentioned above, new technologies and CASA software evolve quickly. Our lab undertook a small study to measure the aptitude of the CEROS (Hamilton-Thorne, USA) and the Sperm Class Analyzer (SCA; Microptics, Spain) in evaluating the motility and the concentration of frozen-thawed bovine spermatozoa diluted in an egg yolk-based extender (unpublished data). A total of 18 different frozen-thawed ejaculates were analyzed with both systems and the mean total and progressive motility percentages, concentration, average path velocity, curvilinear velocity, straight-line velocity, beat cross frequency and amplitude of lateral head displacement were compared (Fig. 1). Among all parameters analyzed, only the percentage of total motile cells was not significantly different between the systems. The discrepancies can be explained by the better capacity of the SCA to exclude egg yolk particles from the analysis. The SCA discriminated non-spermatic particles based on size in microns while the CEROS used pixels to estimate the size of the cells. Differences in the algorithms to calculate slow, medium and fast spermatozoa may also explain the variation in the motility and kinetic parameters observed between each system. Overall, this mini study indicates high variability between CEROS and SCA systems in estimating sperm motility parameters.

Analysis of sperm function by flow cytometry

Flow cytometry analyzes cells suspended in a stream of fluid passing at high velocity in front of one or several lasers. The light emitted by fluorochrome-bound cells is captured by photomultiplier tubes and converted into an electronic signal subsequently digitalized by cytometry software. Key features of flow cytometry are the acquisition and analysis of thousands of cells within seconds and the multiparametric potential of the technology. The most modern cytometers are routinely equipped with 3 lasers and at least 10 photomultiplier tubes allowing cell labelling with several probes at the same time thus enabling analysis of numerous parameters simultaneously. In the last few years, the multiparametric aspect of flow cytometry allowed this technology to become a popular tool to evaluate sperm attributes (Gillan *et al*., 2005; Martínez-Pastor *et al*., 2010; Hossain *et al*., 2011). A wide range of fluorochromes has been developed to assess numerous characteristics of sperm cells. In this paper, we will review some of the fluorochromes used to study sperm cells with flow cytometry.

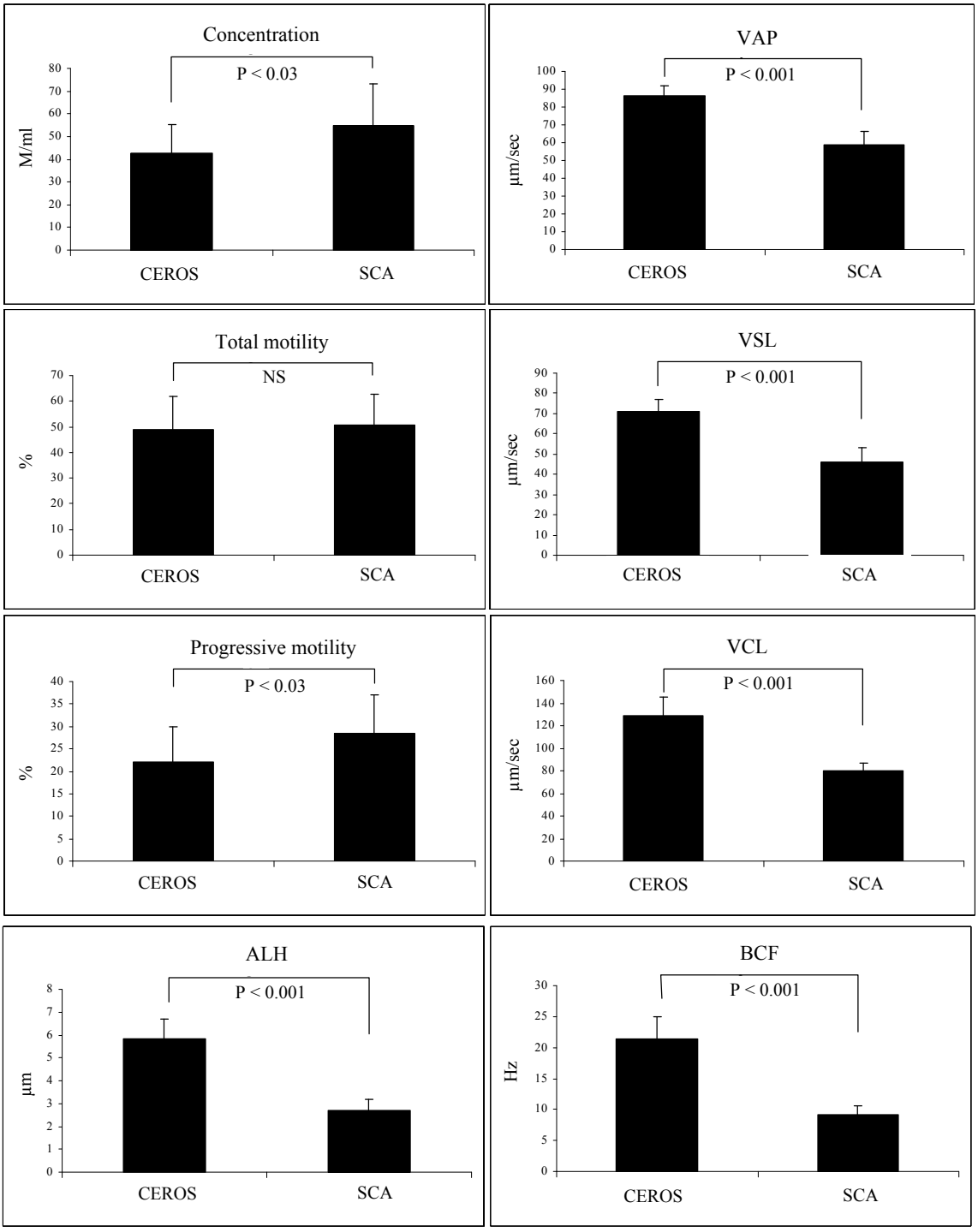

Figure 1. Comparison of CEROS (Hamilton-Thorne) and SCA (Sperm Class Analyser) to determine concentration, percentage of total and progressive motility, lateral head displacement (ALH), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL) and beat cross frequency (BCF) from 18 different frozen-thawed bovine ejaculates. Bars represent mean ± S.E.M. $P < 0.05$ indicates a significant difference between CEROS and SCA, and NS indicates absence of significance, Student's paired T-test.

Sperm attributes analyzed by flow cytometry

Viability/mortality

Propidium iodide is the most popular dye used to identify dead cells. This membrane permeable fluorochrome enters spermatozoa with damaged cellular membranes and binds to DNA where it can be excited with a 488 nm laser present on most cytometers

(Graham *et al*., 1990; Partyka *et al*., 2010; Oldenhof *et al*., 2011). Propidium iodide is often used in combination with SYBR-14, another DNA-labeling probe (Garner *et al*., 1994; Garner and Johnson, 1995). SYBR-14 is also excited by the 488 nm laser and is a permeant probe staining all cells. Added to the cells simultaneously, propidium iodide displaces or quenches the SYBR-14 fluorescence in damaged cells. A new fixable dye commercialized by Invitrogen under the name Live/Dead® fixable dead cell kit is now available to evaluate the viability of cells (Marchian *et al*., 2011). This dye reacts with cellular amines on the surface of cells or inside the cytoplasm of cells with damaged membranes. Cell surface staining of amines of viable cells will result in relatively dim staining compared to the bright staining of dead cells. This fixable dye belongs to a large family available in different wavelength of excitation/emission, allowing its use on most cytometers.

Acrosome integrity

Evaluation of acrosomal status is mainly assessed by using plant lectins recognizing acrosomal ligands. *Pivum sativum* agglutinin binds mannose and galactose moieties of the acrosomal matrix. As *Pivum sativum* agglutinin cannot penetrate the intact acrosomal membrane, only spermatozoa with a reacted or damaged acrosome will be stained (Maxwell *et al*., 1996; Januskauskas *et al*., 1999; Nagy *et al*., 2003). However, it has been shown that *Pivum sativum* agglutinin has an affinity for egg yolk and non-specific binding sites on the sperm cell surface (Purvis *et al*., 1990; Lybaert *et al*., 2009). This aspect could become a problem when analysing semen frozen in egg yolk-based extender and result in misinterpretation of the acrosomal status of sperm. *Arachis hypogaea* (peanut) agglutinin binds galactose moieties of the outer acrosome membrane and is the most popular lectin used to study the integrity of the acrosomal membrane with flow cytometry (Carvalho *et al*., 2010; Anzar *et al*., 2011; Yi *et al*., 2012). In addition, *Arachis hypogaea* (peanut) agglutinin seems the most reliable probe to identify spermatozoa with a damaged acrosome as it displays less non-specific binding to other areas of spermatozoa (Carver-Ward *et al*., 1997). *Pivum sativum* agglutinin and *Arachis hypogaea* (peanut) agglutinin are usually labeled with FITC fluorochromes, allowing them to be used by all cytometers.

Mitochondrial activity

Mitochondria are very important organelles involved primarily in the generation of the energetic substrates for the motility of the sperm cell. Rhodamine 123 was one of the first probes to monitor mitochondrial activity (Evenson *et al*., 1982; Garner *et al*., 1997). Rhodamine 123 is sequestered in active mitochondria and washed out from the cell when the membrane potential is lost. This characteristic limits its use when quantification is needed or when fixation of spermatozoa is required. To overcome the fixation problem, Mitotracker® dye could become a solution. This fixable dye accumulates and stains active mitochondria and has the advantage of availability in different ranges of excitation and emission fluorescence (Garner *et al*., 1997; Hallap *et al*., 2005; Sousa *et al*., 2011). The most popular probe to evaluate mitochondrial activity is JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide; Thomas *et al*., 1998; Garner and Thomas, 1999; Gillan *et al*., 2005; Guthrie and Welch, 2008). In spermatozoa with mitochondria having a high membrane potential, JC-1 enters the mitochondrial matrix where it accumulates and forms J-aggregates and become fluorescent red. In spermatozoa having mitochondria with low membrane potential, JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm in a green fluorescent monomeric form. JC-1 has the advantage to quantify the mitochondrial burst of the cell compared to Rhodamine 123 and Mitotracker. A disadvantage of JC-1 probe is its dual fluorescence emission that limits its combination with other probes emitting in the green and red fluorescence.

DNA integrity

Assessment of chromatin status is important in the determination of the fertility potential of spermatozoa. In recent years, the sperm chromatin structure assay developed by Evenson and Jost (Evenson and Jost, 2000) is the main technique used to evaluate chromatin integrity in spermatozoa by flow cytometry (Januskauska *et al*., 2001, 2003). The sperm chromatin structure assay uses the dual fluorescence emission of acridine orange depending whether it binds to single strand DNA (red fluorescence) or double strand DNA (green fluorescence). Following a denaturation step, the sperm sample is incubated with acridine orange then analyzed by flow cytometry. Denaturation will induce single strand DNA formation when DNA breaks are present and generate a heterogeneous population of red and green fluorescence depending on the integrity of the chromatin. The most important data derived from sperm chromatin structure assay is the ratio red/green + red fluorescence called DNA fragmentation index where a high DNA fragmentation index correlates with high DNA damage. The DNA fragmentation index has shown correlation with fertility in different species (Karabinus *et al*., 1990; Love and Kenney, 1998; Evenson *et al*., 1999). The large luminal spectrum covered by acridine orange and the denaturation step required to induce single strand DNA are two main inconveniences of sperm chromatin structure assay for a multiparametric analysis. Acridine orange fluoresces in the green and red spectrum; that leaves few possibilities to add other fluorochromes in these spectral areas and the denaturation step is performed with an acid/detergent solution not

compatible with all probes. Another assay to assess DNA integrity developed for flow cytometry is the TUNEL assay (terminal transferase dUTP nick end labelling), which can identify DNA strand breaks (Anzar *et al*., 2002; Sutovsky *et al*., 2002; Waterhouse *et al*., 2006). Transferase enzyme incorporates fluorescent or modified nucleotides at the sites of DNA breakage and labelled cells can then be analyzed by flow cytometry. The TUNEL assay allows quantification of labelled nucleotides incorporated into fragmented DNA reflected by the increase of fluorescence, which gives an appreciable advantage over sperm chromatin structure assay.

Calcium influx

Calcium influx is one of the primary steps involved in the sperm capacitation process. The rise in intracellular calcium ultimately leads to the phosphorylation of tyrosine and serine residues from proteins regulating the signalling cascade. The most popular dye used to determine the intracellular calcium concentration in sperm cells is the Fluo-3/4 family probe excited by the 488 nm laser line (Colás *et al*., 2009; Guthrie *et al*., 2011; Kumaresan *et al*., 2011). Calcium-unbound Fluo-3 is a non-fluorescent molecule but when calcium ions enter the cell and bind Fluo-3 the latter becomes fluorescent. Fluo-4 is a derivative of Fluo-3 bearing higher fluorescence intensity. Fura red is a probe also excited by the 488 nm laser where its fluorescence emission decreases upon calcium binding. Dual labeling of spermatozoa with Fluo-3/Fura red allows a calculation of the ratio of unbound to bound calcium. The ratio between the two mean fluorescence intensities (Fluo-3/Fura red) is proportional to the intracellular calcium concentration of the spermatozoa. This experimental approach has been used to assess dog semen by Peña *et al.* (2003). One critical aspect when using these dyes is cell loading. Because mean fluorescence intensity is the parameter used to indicate the level of intracellular calcium, errors in pipetting of the probe will change the mean intensity resulting in misinterpretation of the results. The drawback of Fluo-3/Fura red combination is the need of two different fluorescence detectors, which decreases the scope of a multiparametric approach. Penta acetoxymethyl ester (Indo-1 acetoxymethyl) is a membrane-permeable calcium sensor dye used to monitor changes in intracellular calcium in the cell. Once Indo-1 enters the cell, esterases cleave the acetoxymethyl group, yielding a membrane-permeable dye. Unbound Indo-1 has a peak emission at 485 nm. Upon binding calcium, the peak emission shifts down to 410 nm. Measurement over time can be represented as a ratio of the two emission wavelengths. As Indo-1 acetoxymethyl is a ratiometric probe, cell-loading concerns (as for Fluo-3/4) are less important. One restriction with this probe is that not all laboratories are equipped with an instrument comprising the ultra-violet laser needed to excite Indo-1 acetoxymethyl. For those with this instrument, Indo-1 acetoxymethyl becomes a very good probe for a multiparametric approach because it is one among few probes using the UV laser, thus leaving the 488 nm and 633 nm lasers available to study other parameters.

Limitations of flow cytometry

Several factors influence the choice of the cytometer to use for the analysis of sperm cells. The price of the instrument remains a major factor that will influence this choice. Multiparametric analysis is usually obtained with instruments containing more than one laser and many photomultiplier tubes, which increases substantially the price of the equipment. Indeed, the type of analyses to be performed is also a factor that will determine the choice of the flow cytometer. Depending on the objectives of the breeding center and the experimental design, the combination of lasers (number and wavelength) and the number of photomultiplier tubes included in the instrument must be taken into account. An instrument with only one laser and 3 photomultiplier tubes allows detection of a maximum of 3 parameters on each cell while a multiparametric analysis including 4 and more parameters will usually require an instrument having at least 2 lasers and 4 photomultiplier tubes. The software operating the flow cytometer is another important aspect in the choice of the instrument. Most software products available are fairly easy and straightforward to operate for a novice user in flow cytometry. However, some software require certain knowledge of flow cytometry concepts, making the instrument more difficult to operate. As an example, samples stained with a cocktail containing several probes are subjected to subpopulation gating analysis. In order to obtain representative results, gates need to be associated to the proper population in the correct hierarchy, a perspective that is difficult to handle with some software for a novice user. Moreover, some programs have gaps in export and data compilation, making it more difficult to analyze the data, and these shortcomings are time consuming for the user.

As mentioned in section "Analysis of sperm function by flow cytometry", flow cytometry is a relatively new avenue for the SPCs. The unique characteristics of spermatozoa must be considered when selecting an instrument. The paddle shape of the head and presence of the flagellum make spermatozoa very different in size and cellular complexity compared to most cells studied by flow cytometry. Indeed, the majority of cells studied with this technology have a round shape and passage in front of the laser of the instrument leads to a neat forward scatter vs. side scatter plot. However, when a sperm cell hits the laser, it could be on the thick or on the thin side of the head. This unique feature of sperm cells will lead to a scatter plot of different size/complexity. A very important aspect we found when studying frozen-thawed spermatozoa with flow cytometry, and which has been discussed in this

review is the extender in which semen is diluted. As stated, different types of extenders are used to dilute semen, and some contain particles of a similar size to spermatozoa. This aspect of particle contamination of the target population is a concern when considering the purchase of a cytometer for multiparametric analyses. An apparatus powerful enough to discriminate sperm cells from particles based only on the size and complexity of the cells allows gating of the sperm cell population without using any fluorochromes. As a result, more photomultiplier tubes are left available for cell characterization with fluorochromes. Our lab compared several flow cytometers, and only half of them were sensitive enough to accomplish this discrimination between foreign particles and spermatozoa without having to use any fluorochromes. A research lab possessing a cytometer that cannot discriminate debris from spermatozoa will have to either wash the cells to remove particles or stain the sperm cells with a fluorochrome, which leaves fewer photomultiplier tubes to study other parameters. Not all fluorochromes are suitable with semen extenders, especially egg yolk-derived extenders known to quench some fluorescent dyes. Hoechst 33342 is routinely used to stain the nucleic acid of spermatozoa with a cytometer equipped with a UV or violet laser. This approach allows elimination of the particles by gating them out, resulting in a more accurate analysis (Fig. 2). Unfortunately, not all systems are equipped with such lasers to use Hoechst 33342 as a cell tracking dye, but a cell permeant dye like SYBR-14 could be added to sperm cells and this fluorescent population gated for further analysis.

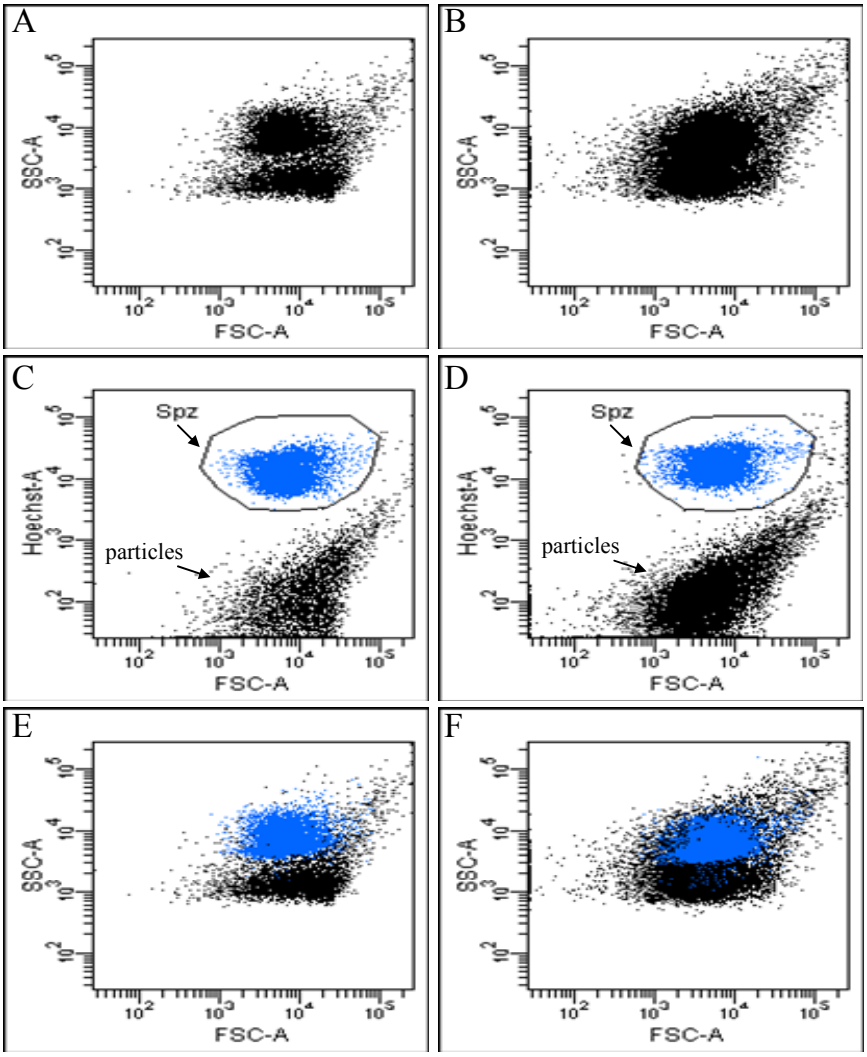

Figure 2. Comparison of good (A) and bad (B) resolution of spermatozoa from non-spermatic particles in frozen-thawed ejaculates. Labelling cells with Hoechst 33342 (blue cells; C-D) allows exclusive staining of spermatozoa and discrimination from cellular debris and egg yolk-derived non-spermatic particles. The positive population can be used for further characterizations in a multiparametric approach (E-F). Spz = spermatozoa; SSC = Side scatter plot; FSC = Forward scatter plot.

A multiparametric approach for standardization and QC among SPCs

Use of CASA and flow cytometry as QC tools

Subjective evaluation of semen by conventional microscopy is still used by numerous SPCs. As discussed earlier, this type of semen evaluation results in variation on the final decision whether the semen lot will be accepted or rejected during QC. Even though CASA is used in some SPCs, fertility is a multiparametric phenomenon; thus motility parameters of spermatozoa are not sufficient to evaluate the global fertility potential of a semen sample. Introduction of flow cytometry in SPCs will allow a better characterization of the spermatozoa because by itself, flow cytometry has the potential for a multiparametric analysis of spermatozoa. Combining CASA and cytometry will provide SPCs with a powerful multiparametric approach to evaluate the quality of the semen produced and allow the establishment of standardized procedures to make accurate and repeatable decisions on the outcome of the semen.

We have evaluated the potential of these tools to accurately estimate the quality of semen produced in SPCs across Canada. The percentage of total and progressive motility and the percentage of viability, acrosome integrity and high mitochondrial activity were evaluated with CASA and flow cytometry. We obtained and re-analyzed 660 lots of semen diluted in egg yolk produced by different SPCs that had previously been processed by standard subjective QC, where 58% (n = 385) were accepted and 42% (n = 275) were rejected. Semen evaluation was performed at two different time points: immediately after thawing and 2 h later after a thermoresistance stress. Cutoff values (Table 1) were established for each parameter analyzed with CASA/flow cytometry at each time point. Semen lots that did not reach our cutoff standard would have been rejected and discarded. On the other hand, if the quality of the semen was good and met our cutoff values, the lot was considered as accepted using CASA and flow cytometry and would have been distributed.

Table 1. CASA/flow cytometry cutoff values used to determine pass/fail rates during quality control of frozen-thawed semen immediately after thawing and after 2 h thermoresistance stress. Semen lots meeting or exceeding these cutoffs values passed the evaluation.

Parameter	Post thaw	After 2 h stress
Total motility (%)	40	35
Progressive motility (%)	15	10
Intact acrosome (%)	66	61
Membrane intact cells (%)	40	40
Mitochondrial activity (%)	40	45

Table 2 highlights the percentage of lots rejected after analyzing the 660 lots with CASA and flow cytometry. CASA analyses revealed that 14% (94/660) unique lots failed to meet the cutoff immediately after thawing compared to 27% (178/660) unique lots after 2 h incubation. The same observation could be made from flow cytometry analyses where 24% (156/660) unique lots were rejected after thawing compared to 32% (214/660) unique lots after 2 h incubation. This analysis demonstrates the need for a thermoresistance test to be included in QC to increase precision where the majority of the lots were discarded based on the 2 h incubation. In addition, CASA evaluation alone resulted in the discard of 28% of the lots whilst flow cytometry analysis alone discarded 34% (224/660) of the lots. When CASA and flow cytometry were used in combination, a total of 41% (268/660) unique lots were rejected resulting in an increase of 13% of rejection compared to CASA alone (28%). Overall, these results demonstrate the importance of the multiparametric approach that provides a very high power to the rejection/acceptance decision.

Detailed analyses of accepted and rejected lots with CASA/flow cytometry *vs*. standard QC showed 77.4% agreement in the decision made using the two different methods of QC assessment, but 22.6% of the samples were discordant between them (Fig. 3). This discrepancy would be considered as the "precision impact" of the multiparametric technique over the standard subjective evaluation. This 22.6% consists of 10.8% of the accepted lots by standard methods that would have been rejected with our multiparametric tools and 11.8% of the rejected lots by standard method that would have been accepted with the multiparametric approach. At first sight, 22.6% of discrepancies between objective and subjective evaluation would appear high. However CASA/flow cytometry would have discarded 268 lots (Table 2) whilst accepting 392 lots compared to the original 385 accepted lots and 275 rejected by the SPCs. This represents a 1% difference of the semen production and would be considered as "production impact" of CASA/flow cytometry over standard QC. This study demonstrates that the production impact would be negligible but the precision impact would be quite considerable.

To estimate the impact of this multiparametric

approach on semen fertility in the field, we applied this analysis on semen lots of known fertility that were released in the field after using standard QC. Computer assisted sperm analysis and flow cytometry were performed on 192 lots with at least 250 first inseminations. The fertility associated to each lot was obtained from the Canadian Dairy Network and was expressed as FERTSOL which represents the 56-day non-return rate, adjusted for multiple parameters including season of insemination, inseminator, number of inseminations etc. Using our multiparametric approach 9.4% (18/192) of the semen lots would have been discarded before distribution. These lots corresponded to 5.3% of the low fertility semen (< -1 FERTSOL), 3.6% of average fertility semen (between -1 and +1 FERTSOL) and 0.5% of high fertility semen (> +1 FERTSOL). Again, this multiparametric approach would have increased the overall fertility of semen released in the field.

Table 2. Number (%) of samples rejected (of 660 total) for at least one QC parameter based on CASA alone, flow cytometry alone or CASA + flow cytometry.

Parameter	Post-thaw (0 h)	After 2 h stress	0 h + 2 h*
CASA alone			
Total motility %	76 (12)	157 (24)	163 (25)
Progressive motility %	70 (11)	137 (21)	151 (23)
Total CASA	94 (14)	178 (27)	188 (28)
Flow cytometry alone			
Intact acrosome %	104 (16)	97 (15)	117(18)
Membrane intact cells %	132 (20)	144 (22)	159 (24)
Mitochondrial activity %	133 (20)	208 (32)	212 (32)
Total flow cytometry	156 (24)	214 (32)	224 (34)
Total CASA + flow cytometry	176 (27)	260 (39)	268 (41)

*unique lots rejected at 0 h + unique lots rejected at 2 h.

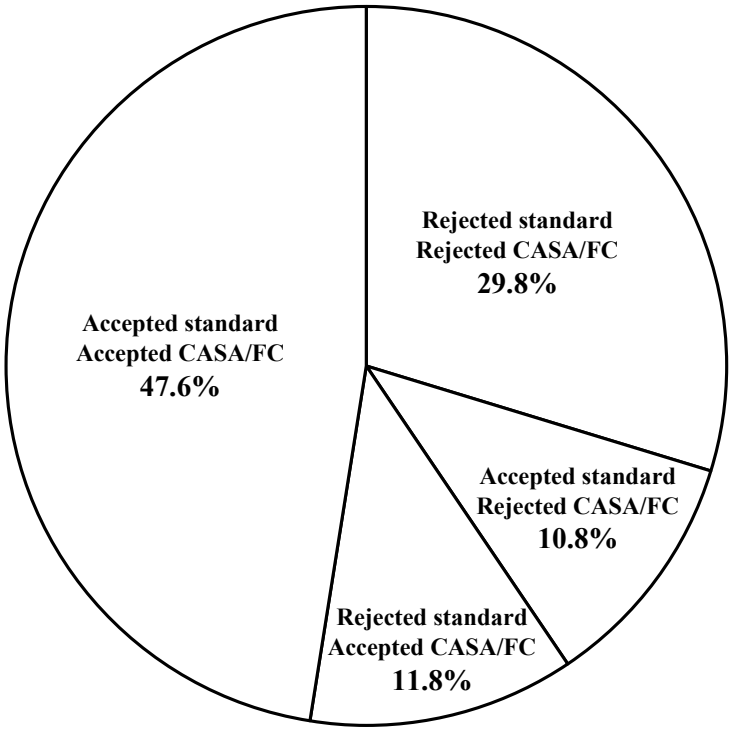

Figure 3. Comparative analysis of pass/fail percentage between standard QC and CASA/flow cytometry during quality control of 660 frozen-thawed semen lots.

Specific applications of multiparametric approach in semen evaluation

The multifactorial aspect of fertility can result in misevaluation of semen quality when assessing only a few parameters. Even semen that went through rigorous QC steps may have low fertility once distributed in the field. Such lots would have passed all of our CASA/flow cytometry cutoffs values but still resulted in few calves despite the high number of inseminations. A more detailed analysis of this semen is then required to understand its low fertility. However, deeper analyses of semen samples are time consuming and expensive, so they are performed only on high value semen. Here, we report two cases where flow cytometry helped us to target a putative cause of low fertility-associated semen.

The first case involved a young bull (bull A), in which one of its semen lots produced very few embryos after IVF compared to the average embryo production. This lot of semen was compared (in triplicate) to one from an average bull (bull C) used as an internal control for most of our experiments. Motility and viability parameters estimated with CASA/flow cytometry showed average quality spermatozoa from both bulls (average of 36% total motility and 15% progressive motility). Evaluation of the level of intracellular calcium and the integrity of the acrosome membrane was carried out immediately after thawing and after 5 h of post-thaw incubation with heparin at 38.5°C. At both time points, an aliquot of spermatozoa was incubated with Indo-1 AM and FITC-peanut agglutinin, and then challenged with thapsigargin to induce the intracellular cascade involved in the capacitation process. The percentages of live spermatozoa with high intracellular levels of calcium and reacted acrosomes were determined by flow cytometry. Analysis immediately after thawing before the heparin incubation and thapsigargin challenge showed a similar percentage of acrosome reacted cells and cells with high intracellular calcium between bull A and bull C. Following thapsigargin stimulation, a similar percentage of spermatozoa from each bull was acrosome reacted, but semen from bull A showed an increase of cells with high calcium compared to bull C (23.3% ± 6.3 *vs*. 13.3% ± 2.5, respectively, P = 0.14; Table 3). After 5 h of incubation with heparin at 38.5°C and without thapsigargin challenge, spermatozoa from bull A had fewer (10.5% ± 2.3 *vs*. 24.1% ± 0.9; P < 0.01) acrosome reacted cells compared to bull C, but a similar percentage of cells possessing elevated calcium (5.3% ± 0.5 *vs*. 5.7% ± 0.1, respectively P > 0.05; Table 3). After the thapsigargin challenge, bull A still had fewer acrosome reacted cells compared to Bull C (10.5% ± 2.0 *vs*. 23.1% ± 0.5; P < 0.01), but also had a proportionally smaller increase in the percentage of cells having high calcium. These observations suggest that bull A spermatozoa could not capacitate in heparin-containing medium, demonstrated by fewer acrosome reacted cells and high intracellular calcium sperm. Altogether, this analysis suggests that this specific lot of semen from bull A was not mature enough to reach its full capacitation process and fertility potential. Saacke *et al.* (2000) described some compensable and uncompensable seminal deficiencies related to subfertility. Compensable factors included functional or molecular traits reflected in this case study by the low percentage of cells having reacted acrosome or elevated calcium. This case could benefit from increasing the number of spermatozoa per straw of bull A to compensate its subfertility.

Table 3. Comparison of acrosome integrity and calcium level of live spermatozoa from bulls A and C before and after a thapsigargin challenge, both post-thaw and after 5 h incubation with heparin.

Bull	Immediately post-thaw				After 5 h incubation with heparin			
	- Thaps		+ Thaps		- Thaps		+ Thaps	
	AR	HighCa	AR	HighCa	AR	HighCa	AR	HighCa
	%	%	%	%	%	%	%	%
A	1.8 ± 0.7	3.1 ± 1.2	16.3 ± 3.8	23.3 ± 6.3	10.5 ± 2.3[a]	5.3 ± 0.5	10.5 ± 2.0[a]	25.3 ± 2.3
C	1.5 ± 0.2	1.1 ± 0.2	17.8 ± 4.0	13.3 ± 2.5	24.1 ± 0.9[b]	5.7 ± 0.1	23.1 ± 0.5[b]	36.6 ± 8.8

AR, acrosome reacted; HighCa, elevated calcium level; Thaps, thapsigargin.
Values are mean percentages from 3 replications ± S.E.M. Different letters on the same column represents significant differences (a, b), P < 0.01, Student's paired t-test.

The second case studied was an adult bull (bull B) that exhibited extremely low fertility in the field (FERTSOL <-2) based on several different semen lots released from a SPC after standard QC tests. We firstly performed the basic CASA/flow cytometry tests explained previously on five different batches and compared the results with an average bull (bull C). All the lots studied would have passed our QC cutoffs. Surprisingly, total and progressive motility percentages were not significantly different in bull B compared to bull C (Table 4). On the other hand, acrosomal reaction, cell death and mitochondrial activity were significantly different in some lots analyzed compared to bull C (Table 4). As these results could not explain the very

low fertility of bull B, we conducted a sperm chromatin structure assay analysis as discussed before on the same batches and compared results with those obtained by bull C. The DNA fragmentation index percentage was approximately two to four times higher in bull B than in bull C ($P < 0.001$; Table 4). Mostly in human, high DNA fragmentation index percentage has been correlated with subfertility (Evenson *et al*., 1980, 1999). Therefore, the very low fertility observed for bull B could be explained by his high level of DNA denaturation as measured by sperm chromatin structure assay. As opposition to the first case study, chromatin aberration is a noncompensable deficiency in subfertility (Saacke *et al*., 2000). Increasing the number of spermatozoa per straw in this case cannot compensate for the extremely low fertility of bull B observed in the field.

Table 4. Comparison of sperm functions based on CASA and flow cytometry analysis of bull B and C immediately after thawing.

Bull	Batch	Total motility %	Progressive motility %	Acrosome reacted %	Dead %	Mitochondrial activity %	DNA fragmentation index %
B	1	57.5 ± 4.5	32.5 ± 2.5	19.1 ± 0.2	43.5 ± 0.1*	55.3 ± 0.2*	7.05 ± 0.09**
B	2	44.5 ± 3.5	24.5 ± 3.5	19.0 ± 0.2	51.7 ± 1.2**	46.7 ± 1.1**	5.76 ± 0.12**
B	3	63.5 ± 1.5	34.5 ± 2.5	17.9 ± 0.1	39.8 ± 0.7	60.0 ± 0.6	4.24 ± 0.01**
B	4	56.0 ± 0.0	32.5 ± 0.5	16.1 ± 0.5	38.7 ± 1.3	60.3 ± 2.1	4.83 ± 0.11**
B	5	61.0 ± 5.0	34.5 ± 1.5	14.0 ± 0.3*	35.0 ± 0.3	64.6 ± 0.4	3.85 ± 0.03**
C	1	60.5 ± 0.5	29.5 ± 0.5	17.6 ± 1.0	38.6 ± 0.9	62.7 ± 0.2	1.61 ± 0.16

Values are mean percentages from 2 replications ± S.E.M.
*P < 0.05 and **P < 0.001 indicate significant differences from control; Dunnett's multiple comparison test.

Conclusions

Multiparametric analyses of the semen produced by SPCs with CASA and flow cytometry demonstrates a very high predictive potential for semen quality and fertility. As fertility has multiparametric aspects, research and development of new markers to identify high fertility semen needs to be extensive. Incorporation of new markers in a multiparametric approach will lead to a better evaluation of semen quality and fertility. In addition, the implementation of these tools in SPCs will help to standardize the QC procedures by eliminating the subjective aspect of semen evaluation. This will standardize semen produced within a SPC and within multiple SPCs for artificial insemination companies that have semen produced in multiple labs. Another application of these tools in the artificial insemination industry is the characterization of semen with high genetic value. In the artificial insemination industry, most of the semen produced arises from a few high genetic value animals. These highly demanded semen samples should be very well characterized to optimize the production in the number of straws produced and in the quality of the semen available for the market. Overall, the artificial insemination industry will benefit from the implementation of CASA and flow cytometry in SPCs.

References

Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol Reprod*, 66:354-360.

Anzar M, Kroetsch T, Boswall L. 2011. Cryopreservation of bull semen shipped overnight and its effect on post-thaw sperm motility, plasma membrane integrity, mitochondrial membrane potential and normal acrosomes. *Anim Reprod Sci*, 126:23-31.

Brito LFC. 2010. Variations in laboratory semen evaluation procedures and testing. In: Proceedings of the 23rd Technical Conference on Artificial Insemination and Reproduction, 2010, Milwaukee, WI, USA. Milwaukee: NAAB. pp. 61-67.

Budworth PR, Amann RP, Chapman PL. 1988. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. *J Androl*, 9:41-54.

Carvalho JO, Sartori R, Machado GM, Mourão GB, Dode MA. 2010. Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in in vitro embryo production. *Theriogenology*, 74:1521-1530.

Carver-Ward JA, Moran-Verbeek IM, Hollanders JM. 1997. Comparative flow cytometric analysis of the human sperm acrosome reaction using CD46 antibody and lectins. *J Assist Reprod Genet*, 14:111-119.

Castellini C, Dal Bosco A, Ruggeri S, Collodel G. 2011. What is the best frame rate for evaluation of sperm motility in different species by computer-assisted sperm analysis? *Fertil Steril*, 96:24-27.

Colás C, Grasa P, Casao A, Gallego M, Abecia JA, Forcada F, Cebrián-Pérez JA, Muiño-Blanco T. 2009. Changes in calmodulin immunocytochemical localization associated with capacitation and acrosomal exocytosis of ram spermatozoa. *Theriogenology*, 71:789-800.

Contri A, Valorz C, Faustini M, Wegher L, Carluccio A. 2010. Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa.

Theriogenology, 74:424-435.

Correa JR, Pace MM, Zavos PM. 1997. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology*, 48:721-731.

Davis RO, Katz DF. 1993. Operational standards for CASA instruments. *J Androl*, 14:385-394.

Evenson DP, Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, 210:1131-1133.

Evenson DP, Darzynkiewicz Z, Melamed MR. 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. *J Histochem Cytochem*, 30:279-280.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. 1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod*, 14:1039-1049.

Evenson DP, Jost L. 2000. Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci*, 22:169-189.

Farrell PB, Presicce GA, Brockett CC, Foote RH. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology*, 49:871-879.

Fernández-Santos MR, Martínez-Pastor F, Matias D, Domínguez-Rebolledo AE, Esteso MC, Montoro V, Garde JJ. 2009. Effects of long-term chilled storage of red deer epididymides on DNA integrity and motility of thawed spermatozoa. *Anim Reprod Sci*, 111:93-104.

Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. *J Androl*, 15:620-662.

Garner DL, Johnson LA. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod*, l53:276-284.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol Reprod*, 57:1401-1406.

Garner DL, Thomas CA. 1999. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. *Mol Reprod Dev*, 5:222-229.

Gillan L, Evans G, Maxwell WM. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology*, 63:445-457.

Gillan L, Kroetsch T, Maxwell WM, Evans G. 2008. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. *Anim Reprod Sci*, 103:201-214.

Graham JK, Kunze E, Hammerstedt RH. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol Reprod*, 43:55-64.

Guthrie HD, Welch GR. 2008. Determination of high mitochondrial membrane potential in spermatozoa loaded with the mitochondrial probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) by using fluorescence-activated flow cytometry. *Methods Mol Biol*, 477:89-97.

Guthrie HD, Welch GR, Theisen DD, Woods LC 3rd. 2011. Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and plasma membrane integrity in striped bass (Morone saxatilis) sperm. *Theriogenology*, 75:951-961.

Hallap T, Nagy S, Jaakma U, Johannisson A, Rodriguez-Martinez H. 2005. Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. *Theriogenology*, 63:2311-2322.

Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl*,13:406-419.

Iguer-ouada M, Verstegen JP. 2001. Evaluation of the "Hamilton Thorn computer-based automated system" for dog semen analysis. *Theriogenology*, 55:733-749.

Januskauskas A, Gil J, Söderquist L, Håård MG, Håård MC, Johannisson A, Rodriguez-Martinez H. 1999. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. *Theriogenology*, 52:641-658.

Januskauska A, Johannisson A, Rodriguez-Martinez H. 2001. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology*, 55:947-961.

Januskauskas A, Johannisson A, Rodriguez-Martinez H. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology*, 60:743-758.

Karabinus DS, Evenson DP, Jost LK, Baer RK, Kaproth MT. 1990. Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. *J Dairy Sci*, 73:2364-2371.

Kathiravan P, Kalatharan J, Edwin MJ, Veerapandian C. 2008. Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. *Anim Reprod Sci*, 104:9-17.

Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. 2011. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system: a review. *Reprod Domest Anim*, 46:165-172.

Kjaestad H, Ropstad E, Berg KA. 1993. Evaluation of

spermatological parameters used to predict the fertility of frozen bull semen. *Acta Vet Scand*, 34:299-303.

Kumaresan A, Siqueira AP, Hossain MS, Bergqvist AS. 2011. Cryopreservation-induced alterations in protein tyrosine phosphorylation of spermatozoa from different portions of the boar ejaculate. *Cryobiology*, 63:137-144.

Love CC, Kenney RM. 1998. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology*, 50:955-972.

Lybaert P, Danguy A, Leleux F, Meuris S, Lebrun P. 2009. Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa. *Histol Histopathol*, 24:999-1007.

Marchiani S, Tamburrino L, Giuliano L, Nosi D, Sarli V, Gandini L, Piomboni P, Belmonte G, Forti G, Baldi E, Muratori M. 2011. Sumo1-ylation of human spermatozoa and its relationship with semen quality. *Int J Androl*, 34:581-593.

Martínez-Pastor F, Mata-Campuzano M, Alvarez-Rodríguez M, Alvarez M, Anel L, de Paz P. 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod Domest Anim*, 45(suppl 2):67-78.

Massányi P, Chrenek P, Lukáč N, Makarevich AV, Ostro A, Živčak J, Bulla J. 2008. Comparison of different evaluation chambers for analysis of rabbit spermatozoa motility parameters using casa system. *Slovak J Anim Sci*, 41:60-66.

Maxwell WM, Welch GR, Johnson LA. 1996. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. *Reprod Fertil Dev*, 8:1165-1178.

Mortimer ST, Swan MA. 1999a. Effect of image sampling frequency on established and smoothing-independent kinematic values of capacitating human spermatozoa. *Hum Reprod*, 14:997-1004.

Mortimer ST, Swan MA. 1999b. The development of smoothing-independent kinematic measures of capacitating human sperm movement. *Hum Reprod*, 14:986-996.

Mortimer ST. 2000. CASA-practical aspects. *J Androl*, 21:515-524.

Nagy S, Jansen J, Topper EK, Gadella BM. 2003. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol Reprod*, 68:1828-1835.

Oldenhof H, Blässe AK, Wolkers WF, Bollwein H, Sieme H. 2011. Osmotic properties of stallion sperm subpopulations determined by simultaneous assessment of cell volume and viability. *Theriogenology*, 76:386-391.

Partyka A, Nizański W, Lukaszewicz E. 2010. Evaluation of fresh and frozen-thawed fowl semen by flow cytometry. *Theriogenology,* 74:1019-1027.

Peña AI, López-Lugilde L, Barrio M, Becerra JJ, Quintela LA, Herradón PG. 2003. Studies on the intracellular Ca2+ concentration of thawed dog spermatozoa: influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. *Reprod Domest Anim*, 38:27-35.

Purvis K, Rui H, Schølberg A, Hesla S, Clausen OP. 1990. Application of flow cytometry to studies on the human acrosome. *J Androl*, 11:361-366.

Saacke RG, Dalton JC, Nadir RL, Nebel RL, Bame JH. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim Reprod Sci*, 663-667.

Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, Freitas A, Paiva A, Almeida-Santos T, Ramalho-Santos J. 2011. Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. *PLoS One*, 6:e18112.

Sutovsky P, Neuber E, Schatten G. 2002. Ubiquitin-dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. *Mol Reprod Dev*, 61:406-413.

Tardif AL, Farrell PB, Trouern-Trend V, Simkin ME, Foote RH. 1998. Use of Hoechst 33342 stain to evaluate live fresh and frozen bull sperm by computer-assisted analysis. *J Androl*, 19:201-206.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE. 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. *Biol Reprod*, 58:786-793.

Waterhouse KE, Haugan T, Kommisrud E, Tverdal A, Flatberg G, Farstad W, Evenson DP, De Angelis PM. 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. *Reprod Fertil Dev*, 18:781-788.

Wood PD, Foulkes JA, Shaw RC, Melrose DR. 1986. Semen assessment, fertility and the selection of Hereford bulls for use in AI. *J Reprod Fertil*, 76:783-795.

Yi YJ, Zimmerman SW, Manandhar G, Odhiambo JF, Kennedy C, Jonáková V, Maňásková-Postlerová P, Sutovsky M, Park CS, Sutovsky P. 2012. Ubiquitin-activating enzyme (UBA1) is required for sperm capacitation, acrosomal exocytosis and sperm-egg coat penetration during porcine fertilization. *Int J Androl*, 35:196-210.

Zinaman MJ, Uhler ML, Vertuno E, Fisher SG, Clegg ED. 1996. Evaluation of computer-assisted semen analysis (CASA) with IDENT stain to determine sperm concentration. *J Androl*, 17:288-292.