@SPRA Asociación Peruana de Reproducción Animal

SPERMOVA

Spermova. 2013; 3(2): 134 - 141

Artículo original:

NEGATIVE BIOMARKERS OF SPERM QUALITY AND MALE FERTILITY: THE GOOD, THE BAD AND THE UGLY

Marcadores de la calidad de semen y la fertilidad del macho: Lo bueno, lo malo y lo feo

C.E. Kennedy(1); P. Sutovsky (1,2)

(1) Division of Animal Sciences, and
(2) Department of Obstetrics, Gynecology and Women's Health, University of Missouri-Columbia, Columbia, MO 65211-5300

Email:
SutovskyP@missouri.edu

Palabras Clave:
sperm, fertility, biomarker, flow cytometry, ubiquitin, lectin

ABSTRACT

Semen evaluation via traditional light microscopy provides useful information about sperm motility and morphology, yet it has a limited utility in predicting reproductive performance due to its subjective nature and inconsistency among technicians and laboratories. Furthermore, not all spermatozoa abnormalities are detectable with standard light microscopy. As a result, flow cytometric evaluation using biomarkers to detect specific spermatozoon characteristics is growing in popularity in both andrology laboratories and agricultural studs. Fluorescently labeled biomarkers can be used to assess a variety of structural and functional properties, including sperm chromatin integrity, acrosomal status, mitochondrial membrane potential and cell viability. A variety of vital stains, DNA and mitochondrial dyes and lectins are best used as fluorescent conjugates f in conjunction with sperm flow cytometry. Antibodies are used to detect and quantify proteins that are up- or down-regulated in defective spermatozoa. Biomarkers, uniquely found in defective spermatozoa are indicative of poor semen quality and decreased fertility in breeding. Thus, such biomarkers are described as "negative" biomarkers of fertility. Inversely, defective spermatozoa may lack proteins associated with normal sperm structure and function. In conjunction with the availability of sperm-specific probes and biomarkers, new instrumentation is being implemented to disseminate the flow cytometry based semen analysis among andrologists. The advent of sperm quality biomarkers and the implementation of flow cytometry are expected to benefit the cattle industry through cost reduction of sire evaluation and increase of reproductive performance in artificial insemination service.

INTRODUCTION

Semen evaluation via traditional light microscopy provides useful information about sperm motility and basic morphology. However, due to its subjective nature and inconsistency among technicians and laboratories, semen evaluation done in this manner has limited utility in predicting reproductive performance or fertilization ability in an assisted reproduction setting [1]. Furthermore, not all spermatozoa abnormalities are detectable with standard light microscopy. Semen samples with cryptic sperm defects and reduced fertilization ability may appear normal by conventional standards and be deemed suitable for insemination. In-depth analysis carried out quickly and with repeatable precision on a large number of sperm cells is of paramount importance to farm animal biotechnology and human assisted reproductive therapies (ART). As a result, flow cytometric evaluation using biomarkers to detect specific spermatozoon characteristics is growing in popularity in both andrology laboratories and agricultural studs.

Fluorescently labeled biomarkers can be used to assess a variety of structural and functional properties, including sperm chromatin integrity, acrosomal status, mitochondrial membrane potential and cell viability. Antibodies are used to detect and quantify proteins that are up- or down-regulated in defective spermatozoa [2]. The goal of ongoing research is to validate the candidate biomarker proteins or ligands associated with defective spermatozoa, even if defects are subtle or unnoticed during evaluation using standard light microscopy. The central hypothesis of this research is that these biomarkers, uniquely found in defective spermatozoa, are indicative of poor semen quality and decreased fertility. Thus such biomarkers are described as "negative" biomarkers of fertility.

The use of negative biomarkers to detect infertility, anticipate negative outcomes when using assisted reproductive techniques, or predict fertility parameters in maturing sire prospects could potentially save time and money. One such negative biomarker, ubiquitin, has already been validated and .

shown to correlate with various sperm quality parameters; the amount of ubiquitinated sperm in a sample has been positively correlated with poor quality or infertility in humans, stallions, bulls, and boars [3]. Furthermore, increased levels of sperm ubiquitination were found in samples from men with idiopathic, unexplained infertility[4]. The sperm chromatin structure assay (SCSA) is able to predict even small changes in bull fertility [5] , and sperm DNA damage as detected by SCSA has been implicated in having a role in offspring mortality[6]. The validation of additional negative biomarkers for detection of sperm quality and their relationship to fertility parameters is a necessary component in the improvement of semen analysis.

FLOW CYTOMETRY

Flow cytometry is a process in which fluorescently labeled cells (in this instance spermatozoa) travel individually at high speed (hundreds or thousands per second) through a flow cell, where they are illuminated by one or more lasers. This causes light scattering and fluorescence excitation of markers located on specific parts of the sperm, which is then detected by photo-detectors and routed to a computer program. The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms [7]; (Figure 1). The scatter plots and histograms can be analyzed and various sperm populations can be separated to produce information regarding fluorescence intensity, percentage of sperm population with certain fluorescence characteristics within a total sample, median fluorescence intensity, etc. One of the main concerns with analyzing spermatozoa by flow cytometry is the presence of other material in the sample such as immature forms of spermatogenic cells, bacteria, blood cells, tissue, and in the case of frozen-thawed or fresh-extended semen, extender contaminants such as egg yolk particles. During the data analysis, these contaminants can be eliminated from the evaluation by gating of the scatter diagram/histogram.

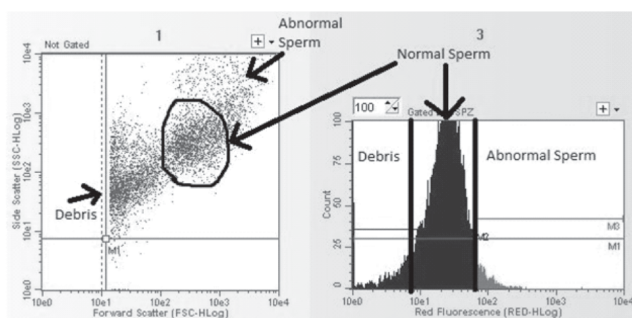

Figure 1: Typical scatter diagram of visible light (left) and a histogram of PAWP-induced fluorescence (right) yielded by flow cytometric measurement of a bull sperm sample. Normal spermatozoa are gated based on their size (forward and side scatter) in a scatter diagram, and based on their relative fluorescence in a histogram.

BIOMARKERS USED FOR BULL SEMEN ANALYSIS

Sub-fertile bulls can cost producers a significant amount of money – they may contribute to delayed conception, prolonged calving season, reduced calf weaning weight, and increase the number of female culls. When used in a multiple-sire or low breeding pressure situation, a sub-fertile bull may be less evident, but in a single sire, high breeding pressure, or artificial insemination (AI) service, the fertility of the bull is of extreme importance [8]. Breeding soundness evaluation of yearling bulls may give some indication of fertility, but conventional semen analysis is less than ideal –it is time consuming; only a few hundred spermatozoa per sample are analyzed, and the agreement between microscopic assessments of sperm motility for different lab technicians can be low. The lack of precision in conventional semen analysis, coupled with the subjective nature of such an assessment, implies that some acceptable semen may be erroneously rejected, and at the same time semen of unacceptable quality may be used for inseminations [1]. A quick, precise, and accurate method for semen evaluation has been a goal of the cattle industry, and flow cytometry using fluorescent biomarkers may be part of the answer. Flow cytometry is fast, accurate, highly repeatable, and can analyze significantly more spermatozoa per sample (up to 10,000) than standard semen analysis [9]. In addition to the speed, repeatability, and accuracy, flow cytometry allows close examination of numerous sperm characteristics, including sperm viability/membrane integrity [10-12], mitochondrial function and membrane potential [10,13,14] chromatin structure [5,15], and acrosomal status [14,16, 17].

VITAL DYE BASED SPERM FLOW CYTOMETRY

When coupled with specific fluorochromes conjugated to biomarker or reporter molecules, flow cytometry can be used to analyze a variety of structural and functional characteristics of spermatozoa, including plasma membrane integrity, mitochondrial activity/mitochondrial membrane potential, acrosome integrity, chromatin structure, changes in the sperm surface induced by sperm capacitation, and certain forms of morphological abnormalities present in a sperm sample [5]. Some of these biomarkers are still in their infancy but are showing promise and are correlating well with standard fertility parameters. Numerous biomarkers are available or are in development for use in sperm evaluation with flow cytometry,as will be discussed below. Some, such as ubiquitin, are present only in poor quality sperm and are generally referred to as negative biomarkers. Others, such as those used for mitochondrial membrane potential [10,14,17], viability [10,12,17], and PAWP protein [18], are present in varying amounts depending on the quality of the spermatozoa.

The fluorescent probes JC-1 and MitoTracker are used for mitochondrial membrane potential and can reveal damage to the mitochondria that may occur after ejaculation or as a result of cryopreservation. The MitoTracker probe is taken up only by active mitochondria, and the reduced intensity of labeling in spermatozoa may also be reflective of malformations of the sperm tail midpiece/mitochondrial sheath. The JC-1 probe is transported into the interior of functioning mitochondria and senses the potential of the inner mitochondrial membrane. JC-1 emits green fluorescence when it exists as a monomer, but when the concentration of JC-1 inside the active mitochondria increases, the stain forms aggregates which fluoresce orange [19]. Therefore, the mitochondria with high membrane potential fluoresce orange and those with medium to low membrane potential fluoresce green [13]. Depolarization of mitochondrial membrane impairs the electron transport chain, the proton gradient and

aerobic ATP production in the midpiece of the spermatozoa, thereby rendering its motility characteristics inadequate for fertilization [16]. A correlation between mitochondrial fluorescence intensity and sperm motility has been established [13].

Viability is another parameter that can be measured by flow cytometry. Sperm viability of both raw and frozen-thawed bull semen correlates with non-return rates [1]. Currently, one of the most commonly used viability stain combinations is SYBR-14 and Propidium Iodide (PI), sold commercially as LIVE/DEAD® Sperm Viability Kit. Both SYBR-14 and PI dyes target the same cellular component, DNA, eliminating the ambiguity that can arise when different components are targeted. With these biomarkers, the nuclei of live spermatozoa display green fluorescence due to the integration of SYBR-14, and dead/dying cells with compromised membrane integrity stain orange because of passive PI-uptake through damaged plasma membrane. By combining the viability stains with other biomarkers, additional sperm functions such as acrosomal integrity and mitochondrial function can be assessed [20]. To date, the SYBR-14/PI combination has been used to reliably identify live and dead sperm populations in bulls, boars, rams, rabbits, mice, rats, and men, though it has also been used to stain spermatozoa of more exotic species such as tigers and chinchillas [11].

Sperm capacitation includes a process of plasma membrane destabilization which may lead to physiological acrosome reaction upon sperm-egg binding, or to sperm cell death in the absence of sperm-zona interaction, both of which are dependent on an influx of calcium ions in the sperm interior [21]. Therefore, the capacitation status of spermatozoa can be detected using the fluorescent antibiotic chlortetracycline (CTC), which traverses the sperm plasma membrane and enters intracellular compartments containing free calcium. Once inside, the CTC becomes negatively charged and binds to the calcium, increasing the CTC fluorescence [21]. This CTC-calcium complex proceeds to bind to hydrophobic regions of plasma/acrosomal membranes and produces distinct staining patterns based on capacitation status: capacitated (B-pattern), non-capacitated (F-pattern), and acrosome reacted (AR-pattern) [22]. The CTC staining has been routinely used with fluorescence microscopy to visualize capacitation and AR in several species, including mouse [22], bull [23], boar [24], stallion [25], and men [26] and has been adapted for use in flow cytometry by Maxwell and Johnson [21].

The collection of semen in livestock species is not a sterile procedure. Despite rigorous attempts at cleanliness, bacterial contamination from the penis and prepuce, collection equipment, and handlers is possible [27]. Consequently, bacteria may compromise semen quality and contaminate the receiving female's reproductive tract. Though more frequently studied in humans, a variety of bacteria have been identified via culture of semen samples, and certain bacteria have been shown to have detrimental effects on semen quality in several domestic species [28, 30]. Fluorescent markers such as SYBR-Green 1 have been adapted to identify bacteria in semen, and the use of flow cytometry for bacterial counts is becoming more routine [31]. This enables the producer to inspect bacterial counts in collected semen prior to cryopreservation and to use an extender containing antibiotics, if necessary [27].

The sperm chromatin structure assay (SCSA) detects abnormal chromatin structure consisting of sperm DNA fragmentation index (%DFI) and abnormal nuclear proteins (%HDS). The DNA fragmentation index is the proportion of sperm containing fragmented DNA and is calculated from a histogram obtained from the ratio between red and total (red+green) fluorescence. High DNA stainability (% HDS) is calculated based on the percentage of spermatozoa with high levels of green fluorescence, representing immature spermatozoa with incomplete chromatin decondensation [32]. In a study by Bochenek *et al.*, the sperm chromatin structure assay was performed on mature bulls considered qualified for AI. The results showed that ejaculates positively evaluated at the AI stud via microscope contained as much as 23.8% chromatin-defective spermatozoa [5]. Furthermore, SCSA values were shown to correlate with fertility parameters and the percentage of spermatozoa with chromatin defects varied over several weeks, suggesting that defective chromatin structure could be a variable trait that can be affected by disturbances in the spermatogenetic process or external factors such as semen extender, heat stress, or chemicals [5].

LECTINS

The visualization of acrosomal status is an important parmeter to consider when evaluating semen quality, as some instances of male infertility could be the result of a lack of spermatozoa with functional acrosomes at the time of ejaculation [33]. Acrosomal integrity of spermatozoa can be measured using fluorescently labeled plant lectins, proteins that recognize and bind glucosidic residues in different parts of the acrosomal membrane. Pisum sativum agglutinin (PSA) derived from the pea plant and Arachis hypogaea agglutinin (PNA) derived from the peanut plant are the most commonly used because of their specificity [19]. However, PSA has a tendency to bind to the egg yolk particles in the extenders and has slightly less specific binding, so in agreement with other scientists [19,20], the author's preference is to use peanut agglutinin (PNA). PNA shows a high affinity and strong specificity for disaccharides with terminal galactose, especially the D Gal α (1,3) D GalNac disaccharide, and binds to the outer acrosomal membrane, which becomes exposed during the acrosome reaction. Spermatozoa with reacted, damaged, or abnormally formed acrosomes acquire green fluorescence after PNA labeling, while spermatozoa with intact, normal acrosomes have no fluorescence [16]. We have found that PNA values correlate with the conventionally established parameters of sperm morphology and sperm concentration, and also with flow cytometric measures of sperm ubiquitin [33]. A study by Thomas *et al* (1997) compared acrosomal integrity as determined by PNA via flow cytometry to the standard microscopic morphology assessments and found the percentage of spermatozoa with normal acrosomes, relative to the values obtained using fluorometric methods, appeared to be understated in bulls producing semen of low quality and overstated in bulls producing semen of high quality, further illustrating the subjective nature of microscopic semen analysis and the tendency to bias visual examinations [17]. A third lectin, LCA from the lentil plant (Lens culinaris) has also been used to evaluate bull sperm quality. LCA shows a strong specificity to D-glucose and D-mannose residues and binds to the entire surface of defective spermatozoa, but only to the acrosomal surface in normal spermatozoa. Consequently, distinct histograms of LCA-induced fluorescence are seen in normal vs. defective spermatozoa. In our unpublished data, we

have found a positive correlation between LCA and ubiquitin staining and a negative correlation of LCA with % normal sperm morphology. Thus, it appears LCA can be useful in the detection of abnormal spermatozoa via flow cytometry (PS& CEK, unpublished data).

PROTEIN BIOMARKERS OF SPERM QUALITY

Candidate sperm quality/fertility biomarkers include proteins that are exclusively, or predominantly associated with morphological or molecular sperm defects ("negative" fertility biomarkers) and proteins more abundant in morphologically and functionally normal spermatozoa ("positive" biomarkers of sperm quality/fertility). One of the protein biomarkers of bull sperm quality that has been studied in depth is ubiquitin. Abnormal spermatozoa are tagged by ubiquitination of the plasma membrane/sperm surface during epididymal passage [34, 35]. Though some of these ubiquitin tagged spermatozoa may disintegrate and be removed in the epididymis, many abnormal spermatozoa appear in the ejaculate, and their increased content is indicative of poor semen quality [36], or even infertility [37]. Increased binding of fluorescently-labeled anti-ubiquitin antibodies to the sperm surface reflects the occurrence of sperm abnormalities, which is then detected by the flow cytometer as an increase in the relative fluorescence induced by the presence of ubiquitin on the sperm surface. Ubiquitin as a sperm biomarker has been assessed in numerous species including men [38], stallions [37], bulls [36], and boars [39], and has been found to correlate with infertility and indications of poor sperm quality, including primary and total morphological defects [3]. Though not yet published, our data indicate ubiquitin may be correlated with non-return rate as well. Similarly, ubiquitin is associated with human male infertility. In a retrospective analysis of infertile couples treated by ART, it was found that higher levels of ubiquitin in the sperm sample also correlated negatively with the percentage of cleaved embryos and the percentage of embryos with two pronuclei after infertility therapy by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [4]. If the decreased embryo cleavage and pronuclei-formation rates also apply to cattle AI, it can be inferred that early embryo loss could account for an increased non-return rate.

As mentioned above, sperm ubiquitin levels in bulls correlate positively with acrosomal damage or malformations revealed by fluorescent PNA lectin [33]. This observation inspired our efforts to develop a nanoparticle based magnetic purification protocol for bull semen purification [40]. In this protocol, freshly collected semen is incubated for a short period of time with ferritin nanoparticles coated with PNA lectin or anti-ubiquitin antibodies. Such particles bind exclusively to the defective spermatozoa, allowing for their removal using a magnet placed under the tubes with semen. The procedure is rapid and has no effect on sperm viability. So far, this effort resulted in two successful field AI trials with a total of 800 inseminated cows/heifers. We found that nanopurification removes only about 30% of spermatozoa but allows using the collected purified spermatozoa at half of the usual AI dose, thus producing more straws per ejaculate without reducing fertility (Sutovsky *et al.*, in preparation).

Ubiquitin has also been used for validation of other candidate biomarkers of sperm quality, further illustrating the utility of multiple stains during flow cytometric analysis. A 2007 study examined the correlation between semen platelet activating factor-receptor (PAFr) and ubiquitin labeling, showing a relationship between those two biomarkers [41].

Platelet activating factor (PAF) is an important phospholipid mediator in reproduction and, its sperm plasma membrane receptor PAFr, has a positive association with sperm motility and high fertility history in boars [42]. Sutovsky *et al.* examined the relationship between ubiquitin and PAFr via flow cytometry during breeding soundness evaluations in yearling bulls. Contrary to the association shown in swine, increased PAFr content was indicative of increased white blood cells (WBC) in bull semen samples, and PAFr-induced fluorescence correlated negatively with several BSE parameters (palpation, scrotal circumference, and satisfaction of evaluation). A positive correlation was found at the same time with semen ubiquitin content [41]. Without the additional parameter of ubiquitin expression, an increased PAFr expression could have resulted in an incorrect assumption of high semen quality.

One biomarker that correlates negatively with high levels of ubiquitin is PAWP, the post-acrosomal, ww-domain binding protein. PAWP is a novel protein found only in the post-acrosomal sheath (PAS) of the spermatid, within which it resides in the protective capsule enveloping the sperm nucleus, the perinuclear theca. PAWP first appears during spermatid elongation, coinciding with the time frame in which spermatids acquire their egg-activating ability [18]. Though the downstream elements of PAWP-dependent oocyte activation cascade are currently under investigation, PAWP triggers meiotic resumption and pronuclear development. A proper integration of PAWP in the sperm PAS is thought to be reflective of bulls' sperm quality, and sperm head morphology in particular. When flow cytometry is performed on anti-PAWP antibody labeled spermatozoa, a threshold of acceptable PAWP content can be established. Anything above or below the threshold amount would be considered abnormal. In normal spermatozoa, PAWP fluorescence formed a regular band around the proximal PAS. Defective spermatozoa displayed various anomalies of PAWP labeling including a jagged or abnormally wide PAS-band, an irregular spot or completely absent labeling [43] . Depending on which population is analyzed, PAWP can have a positive or a negative correlation with secondary sperm morphology, conception rate, number of services, non-return rate and residual value reflective of bulls' performance in AI service, and has also been found to have a negative correlation with the ubiquitin biomarker (Figure 2 and unpublished data/in preparation).

Heparin binding proteins (HBP) secreted by the seminal vesicles, prostate, and bulbourethral glands are present in the seminal fluid and bind to spermatozoa after ejaculation. The presence of a specific heparin binding protein, HBP-30, on the sperm plasma membrane has been correlated with increased fertility in bulls, inspiring a synonym the fertility-associated antigen (FAA) [44]. High semen content of FAA was predictive of high fertility in bulls of several breeds in trials grouping bulls based on the presence or absence of FAA [44,46]. Despite the fluctuation of absolute fertility values, groups of FAA-positive bulls were consistently more fertile (by 9 to 40 percentage points) than groups of FAA-negative bulls [44]. Furthermore, cows covered by FAA positive bulls were impregnated earlier in the breeding season, resulting in increased numbers of older and heavier calves at weaning

[44]. Based on this information, an artificial insemination trial on beef cows inseminated using semen from FAA positive or FAA negative bulls was performed [47]. The results of this insemination trial support the usefulness of the fertility associated antigen as a biomarker of sperm quality: pregnancy rates in females inseminated with FAA-negative bull semen ranged from 2.8-69.6%, while females inseminated with FAA-positive bull semen had a pregnancy rate of 22.1-91.3% [47]. The presence or absence of FAA can only be determined via biochemical analysis—it has no relation to breeding soundness or serving capacity [47].

Figure 2: Dual immunofluorescence labeling of PAWP and ubiquitin (UBB) in bull spermatozoa. A spermatozoon displaying UBB labeling lacks PAWP on its postacrosomal sheath. Parfocal transmitted light image was acquired by using differential interference contrast (DIC) optics.

Clusterin, an acidic glycoprotein produced in the testis and epididymis, has been associated with sperm quality in several species, including ram [48], rat [49, 50] , bull [51], and men [52]. In human semen, one type of clusterin was detected only on abnormal spermatozoa, with another type only being detected on normal spermatozoa [52]. Bull and ram spermatozoa that exhibited morphological defects strongly reacted with anti-clusterin antibody [51]. Clusterin is associated with cell damage in several disease conditions [53], but the reason for clusterin accumulation in abnormal spermatozoa is unknown. The result of a scrotal insulation trial in rams [51] suggests that the accumulation of clusterin on abnormal spermatozoa may indicate unfavorable testicular conditions or individual germ cell aberrations [53]. The incidence of clusterin-positive bull spermatozoa, as determined via flow cytometry, negatively correlates with non-return rate and estimated relative conception rate [53]. In addition, an inverse relationship between clusterin-positive bull spermatozoa and pre/post-thaw motility has also been established, suggesting that clusterin is another potential biomarker of bull fertility.

SPERM CHROMATIN STRUCTURE EVALUATION

The sperm chromatin structure assay (SCSA) is used to detect spermatozoa with abnormal chromatin structure using the sperm DNA fragmentation index (%DFI) and high stainability due to abnormal nuclear proteins (%HDS). In this flow cytometric assay, acridine orange stained spermatozoa fluoresce red or green, depending on their chromatin packaging. The ratio between red and total (red + green) fluorescence represents %DFI, and the percentage of spermatozoa with high levels of green fluorescence represents %HDS. SCSA parameters are more repeatable than conventional semen parameters such as sperm motility and, in humans, can be indicative of subjects' exposure to industrial toxicants, smoking, or environmental pollutants [54]. Numerous studies have evaluated the usefulness of SCSA in a human infertility clinic setting and have found that SCSA results can predict the degree of treatment success in assisted reproductive techniques such as IVF, IUI, and ICSI [55]. Furthermore, high levels of sperm DNA fragmentation (DFI $\geq$30%) may cause a significant decrease in blastocyst formation rates and an increase in spontaneous abortions [54].

Aniline blue (AB) staining is an indirect approach used to detect superfluous histones and thereby decreased amounts of protamines in the sperm nucleus [56]. During spermiogenesis, protamines replace DNA histones to form ahypercondensed, tightly packed sperm chromatin structure. By detecting the presence of residual histones, the degree of histone-protamine replacement can be deduced. Normal spermatozoa have no staining or may appear grey, spermatozoa with a moderate amount of histones are stained light blue, and spermatozoa with increased amounts of histones are stained a bright blue color [57]. Though not as commonly used as SCSA, AB staining correlates with spontaneous recurrent abortion, progressive sperm motility, and abnormal morphology in humans [57].

Chromomycin A3 (CMA3) detects protamine deficiency in loosely packed chromatin by competing with protamines for binding to the minor groove of DNA[57]. Also correlated to DNA nicks, CMA3 staining intensity corresponds to the type of chromatin packaging: abnormally packaged chromatin causes the head of the spermatozoon to fluoresce bright green, while spermatozoa containing normally packaged chromatin faintly fluoresce dark green [57]. The number of brightly stained CMA3 labeled spermatozoa is increased in infertile patients. Patients with >30% CMA3 labeled spermatozoa and >10% DNA nicks had more than double the number of unfertilized oocytes containing spermatozoa that had remained condensed after ART [58]. A study by Kazerooni *et al.* found an increased number of CMA3 and AB stained spermatozoa in men with recurrent spontaneous abortion, compared to fertile subjects [57].

SPERM PROTEOMICS: SEARCHING FOR NEW BIOMARKERS

In the United States, roughly 70% of dairy cows are bred via artificial insemination [59]. Of that 70%, only about 50% of inseminations result in a full term pregnancy, in part as a result of a lack of thorough understanding of the molecular events and mechanisms that determine the fertilizing potential of a semen sample [60]. In order to develop new biomarkers to assess the quality of a semen sample, we must first develop an understanding of these mechanisms through proteomics. Proteomics refers to the qualitative and quantitative comparisons of proteomes to identify cellular mechanisms which are involved in biological processes. However, proteomics is not only about the identification of such mechanisms; it also involves the study of protein structure, localization, post-translational modifications, protein-protein interactions, biological activities, and function [61]. Sperm

proteomics can be used to discover new biomarkers of fertility, such as those described above. The proteomics of seminal plasma and epididymal fluid could be arguably just as important as sperm proteomics because spermatozoa acquire numerous sperm surface proteins that convey fertilization potential in the epididymis [62]. Comparison of bull sperm proteomes between fertile and sub/infertile bulls [60] as well as proteomic characterization of bovine seminal plasma [63, 64] have given some insight into which proteins at what levels are indicative of fertility or infertility.

NEW INSTRUMENTATION

As new technology is developed, instrumentation is updated or created to make use of the new biomarker-based methodology. The development of biomarkers is no exception, and new state of the art flow cytometers, including dedicated sperm flow cytometers have been introduced recently. These include the EasyCyte Plus 'bench-top' micro-capillary flow cytometer (IMV Technologies,L'Aigle, France) and the ImageStream high speed quantitative imaging cytometer (Amnis Corp., Seattle, WA).

In a study performed by Odhiambo *et al,* the EasyCyte Plus flow cytometer was used to validate a dual ubiquitin-PNA based bull sperm assay. The EasyCyte Plus proved easy to use and can be operated by anyone who has completed the IMV Technologies web-based training program, which takes less than four hours—no previous expertise is needed. Setup is fast and the instrument can be fully operational in less than 15 minutes [33]. In addition to the quick setup, EasyCyte Plus has an automated self-cleaning cycle without the large-volume fluidics found in conventional flow cytometers. When the results of the ubiquitin-PNA trial were compared between the EasyCyte Plus and the conventional flow cytometer (Becton Dickinson FACScan), both instruments produced robust evaluations of the semen samples [33]. The relative affordability, compact 'bench-top' design, dedicated sperm analysis software and reliable results make the EasyCyte Plus an attractive choice for andrologists and Ai technicians.

The ImageStream high speed quantitative imaging cytometer was evaluated in another sperm biomarker trial by Buckman *et al.* (2009). The ImageStream combines the standard fluorescence intensity measurements with bright field and epifluorescence imaging, enabling it to directly correlate biomarker intensity with individual cell morphology, something standard flow cytometers cannot accomplish. This instrument captures up to six different images (four channels of fluorescence, side scatter, and bright field) of each passing cell at approx. 100 cells/second with a resolution comparable to that of a 40x microscope-lens magnification. It is equipped with image analysis tools that can measure parameters related to fluorescence intensity, cell size, shape, texture, and localization [65]. In addition, the ImageStream results correlate well with conventional flow cytometry results. However, the cost of this instrument makes it more suitable for core facilities shared by basic researchers rather than for commercial or clinical andrology laboratories.

CONCLUDING REMARKS

The advent of sperm quality biomarkers and the implementation of flow cytometry should benefit the cattle industry greatly. Being able to identify markers of good fertility as well as poor fertility in a semen sample in a fast and objective manner could reduce the need for multiple inseminations and prevent expenses covering offspring testing of sub-fertile bulls producing poor pregnancy rates in AI service. While the idea of using flow cytometry is relatively new, and some of the biomarkers are still being developed, an increase in speed, accuracy, and precision in the assessment of fertility in bulls should be welcomed by the cattle industry. Even if flow cytometry were only used to determine sperm viability and concentration, it could potentially still lead to more straws of semen being produced, and better quality control overall, which leads to an increase in profits. Finding the sub-fertile bulls that "fly under the radar" could potentially save producers considerable amounts of money, especially if sub-fertility could be determined early on before the bulls reach breeding age. Furthermore, flow cytometry could be used to aid in the development of new cryopreservation techniques, as some scientists are testing the effect of cryopreservation on various sperm characteristics, such as organelle function and viability , acrosomal integrity and viability , and mitochondrial function and viability .

ACKNOWLEDGEMENTS

Authors would like to thank their collaborators including Richard Oko, PhD, of Queen's University, Tom Geary, PhD, of USDA Fort Keogh, Eric Schmitt, DVM, PhD, and Ludivine Chevrier, PhD, of IMV Technologies, Mel De Jarnette, PhD, of Select Sires and Kari Beth Krieger, PhD, of Genex Cooperative, for their support of original research discussed in this article. Collegial support from Miriam Sutovsky, Shawn Zimmerman, Young-Joo Yi, PhD, and Edward (TJ) Miles is much appreciated. Original research reviewed in this article was in part supported by National Research Initiative Competitive Grant no. 2007-35203-18274 and grant no. 2011-67015-20025 from the USDA National Institute of Food and Agriculture, to P.S.

REFERENCES

1. Christensen, P., *et al.*, 2005. Implementation of flow cytometry for quality control in four Danish bull studs. *Anim Reprod Sci*, 85(3-4): p. 201-8.
2. Sutovsky, P. and K. Lovercamp. 2010. Molecular markers of sperm quality. *Soc Reprod Fertil Suppl*, 67: p. 247-56.
3. Purdy, P.H. 2008. Ubiquitination and its influence in boar sperm physiology and cryopreservation. *Theriogenology,* 70(5): p. 818-26.
4. Ozanon, C., J. Chouteau, and P. Sutovsky. 2005. Clinical adaptation of the sperm ubuquitin tag immunoassay (SUTI): relationship of sperm ubiquitylation with sperm quality in gradient-purified semen samples from 93 men from a general infertility clinic population. *Hum Reprod*, 20(8): p. 2271-8.
5. Bochenek, M., Z. Smorag, and J. Pilch. 2001. Sperm chromatin structure assay of bulls qualified for artificial insemination. *Theriogenology,* 56(4): p. 557-67.
6. Ruiz-Lopez, M.J., et al. 2010. Paternal levels of DNA damage in spermatozoa and maternal parity influence

offspring mortality in an endangered ungulate. *Proc Biol Sci,* 277(1693): p. 2541-6.
7. Martinez-Pastor, F., *et al*., 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod Domest Anim.* 45 Suppl 2: p. 67-78.
8. Kastelic, J.P. and J.C. Thundathil. 2008. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim,* 43 Suppl 2: p. 368-73.
9. Christensen, P., J.P. Stenvang, and W.L. Godfrey, 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. *J Androl,* 25(2): p. 255-64.
10. Evenson, D.P., Z. Darzynkiewicz, and M.R. Melamed,1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. *J Histochem Cytochem*, 30(3): p. 279-80.
11. Garner, D.L. and L.A. Johnson, 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod,* 53(2): p. 276-84.
12. Garner, D.L., et al., 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. *J Androl,* 15(6): p. 620-9.
13. Garner, D.L., *et al.,* 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol Reprod,* 57(6): p. 1401-6.
14. Graham, J.K., E. Kunze, and R.H. Hammerstedt, 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol Reprod,* 43(1): p. 55-64.
15. Evenson, D.P., Z. Darzynkiewicz, and M.R. Melamed, 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science,* 210(4474): 1131-3.
16. Nagy, S., *et al.,* 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(5):1828-35.
17. Thomas, C.A., *et al.,* 1997. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. *Biol Reprod,* 56(4): 991-8.
18. Wu, A.T., et al., 2007. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem,* 282(16): 12164-75.
19. Graham, J.K.,2001. Assessment of sperm quality: a flow cytometric approach. *Anim Reprod Sci,* 68(3-4): 239-47.
20. Gillan, L., G. Evans, and W.M. Maxwell, 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology,* 63(2): p. 445-57.
21. Maxwell, W.M. and L.A. Johnson, 1997. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. *Mol Reprod Dev,* 46(3): p. 408-18.
22. Saling, P.M. and B.T. Storey, 1979. Mouse gamete interactions during fertilization in vitro. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. *J Cell Biol,* 83(3): p. 544-55.
23. Fraser, L.R., L.R. Abeydeera, and K. Niwa, 1995. Ca(2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Mol Reprod Dev,* 40(2): p. 233-41.
24. Wang, W.H., *et al.,* 1995. Functional analysis using chlortetracycline fluorescence and in vitro fertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. *J Reprod Fertil,* 104(2): p. 305-13.
25. Bergqvist, A.S., *et al.,* 2011. Single layer centrifugation of stallion spermatozoa through Androcoll-E does not adversely affect their capacitation-like status, as measured by CTC staining. *Reprod Domest Anim,* 46(1): p. e74-8.
26. Li, H.W., *et al.,* 2009. Effect of leptin on motility, capacitation and acrosome reaction of human spermatozoa. *Int J Androl*, 32(6): p. 687-94.
27. Yaniz, J.L., *et al.,* 2010. Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15 degrees C. *Anim Reprod Sci,* 122(1-2): p. 142-9.
28. Akhter, S., *et al.,* 2008. Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo (Bubalus bubalis) bull semen. *Reprod Domest Anim,* 43(3): p. 272-8.
29. Althouse, G.C., *et al.,* 2000. Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology,* 53(5): p. 1167-76.
30. Aurich, C. and J. Spergser, 2007. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology,* 67(5): p. 912-8.
31. Tripp, H.J. 2008. Counting marine microbes with Guava Easy-Cyte 96 well plate reading flow cytometer. Protocol Exchange Volume, DOI: 10.1038/nprot.2008.29
32. Giwercman, A., *et al.,* 2010. Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl, 2010. 33(1): p. e221-7.
33. Odhiambo, J.F., *et al.,* 2011. Adaptation of ubiquitin-PNA based sperm quality assay for semen evaluation by a conventional flow cytometer and a dedicated platform for flow cytometric semen analysis. *Theriogenology,* 2011.
34. Baska, K.M., *et al.,* 2008. Mechanism of extracellular ubiquitination in the mammalian epididymis. *J Cell Physiol,* 215(3): p. 684-96.
35. Sutovsky, P., *et al.,* 2001. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci,* 114(Pt 9): p. 1665-75.
36. Sutovsky, P., E. Neuber, and G. Schatten, 2002. Ubiquitin-dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. *Mol Reprod Dev,* 61(3): p. 406-13.
37. Sutovsky, P., *et al.,* 2003. Differential ubiquitination of stallion sperm proteins: possible implications for infertility and reproductive seasonality. *Biol Reprod,* 68(2): p. 688-98.
38. Sutovsky, P., Y. Terada, and G. Schatten, 2001. Ubiquitin-based sperm assay for the diagnosis of male factor infertility. *Hum Reprod,* 16(2): p. 250-8.
39. Kuster, C.E., R.A. Hess, and G.C. Althouse, 2004. Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. *J Androl,* 25(3): p. 340-7.
40. Sutovsky, P. and C.E. Kennedy, 2013. Biomarker-based nanotechnology for the improvement of reproductive performance in beef and dairy cattle. *Industrial Biotechnology,* 9(1): p. 24-30.
41. Sutovsky, P., *et al.,* 2007. Relative levels of semen platelet activating factor-receptor (PAFr) and ubiquitin in yearling bulls with high content of semen white blood cells: implications for breeding soundness evaluation. *J Androl*, . 28(1): p. 92-108.

42. Roudebush, W.E. and J.R. Diehl, 2001. Platelet-activating factor content in boar spermatozoa correlates with fertility. Theriogenology, 55(8): p. 1633-8.

43. Sutovsky, P. and R. Oko, 2011. Spermatozoa: the good, the bad and the ugly. *Mol Reprod Dev,* 78(2): p. 67.

44. Bellin, M.E., *et al.,* 1998. Fertility-associated antigen on bull sperm indicates fertility potential. *J Anim Sci,* 76(8): p. 2032-9.

45. Bellin, M.E., H.E. Hawkins, and R.L. Ax, 1994. Fertility of range beef bulls grouped according to presence or absence of heparin-binding proteins in sperm membranes and seminal fluid. *J Anim Sci,* 72(9): p. 2441-8.

46. Bellin, M.E., *et al.,* 1996. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *J Anim Sci,* 74(1): p. 173-82.

47. Sprott, L.R., *et al.*, 2000. Artificial insemination outcomes in beef females using bovine sperm with a detectable fertility-associated antigen. *J Anim Sci,* 78(4): p. 795-8.

48. Tung, P.S. and I.B. Fritz, 1985. Immunolocalization of clusterin in the ram testis, rete testis, and excurrent ducts. *Biol Reprod,* 33(1): p. 177-86.

49. Sylvester, S.R., *et al.*, 1991. Localization of sulfated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. *Biol Reprod,* 45(1): p. 195-207.

50. Sylvester, S.R., M.K. Skinner, and M.D. Griswold, 1984. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol Reprod,* 31(5): p. 1087-101.

51. Ibrahim, N.M., *et al.,* 1999. Reproductive tract secretions and bull spermatozoa contain different clusterin isoforms that cluster cells and inhibit complement-induced cytolysis. *J Androl,* 20(2): p. 230-40.

52. O'Bryan, M.K., *et al.,* 1990. Human seminal clusterin (SP-40,40). Isolation and characterization. *J Clin Invest,* 85(5): p. 1477-86.

53. Ibrahim, N.M., *et al.*, 2000. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. *J Androl,* 21(6): p. 887-94.

54. Virro, M.R., K.L. Larson-Cook, and D.P. Evenson, 2004. Sperm chromatin structure assay (scsa®) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility,* 81(5): p. 1289-1295.

55. Bungum, M., *et al.,* 2004. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod,* 19(6): p. 1401-8.

56. Hofmann, N. and B. Hilscher, 1991. Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. *Hum Reprod*, 6(7): p. 979-82.

57. Kazerooni, T., *et al.,* 2009. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *Journal of Assisted Reproduction and Genetics,* 26(11): p. 591-596.

58. Sakkas, D., *et al.,* 1998. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum Reprod,* 13 Suppl 4: p. 11-9.

59. Killian, G. 1999. High-Fertility Proteins Enhance Reproduction Rates in Dairy Cattle. NRI *Research Highlights Volume,*

60. Peddinti, D., *et al.,* 2008. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst Biol,* 2: p. 19.

61. Strzezek, J., *et al.,* 2005. Proteomics of boar seminal plasma - current studies and possibility of their application in biotechnology of animal reproduction. *Reprod Biol,*. 5(3): p. 279-90.

62. Sutovsky, P., 2009. Proteomic analysis of mammalian gametes and sperm-oocyte interactions. *Soc Reprod Fertil Suppl,* 66: p. 103-16.

63. Kelly, V.C., *et al.,* 2006. Characterization of bovine seminal plasma by proteomics. *Proteomics,* 6(21): p. 5826-33.

64. Moura, A.A., *et al.,* 2007. A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. *Anim Reprod Sci,* 98(3-4): p. 169-88.

65. Buckman, C., *et al.,* 2009. High throughput, parallel imaging and biomarker quantification of human spermatozoa by ImageStream flow cytometry. *Syst Biol Reprod Med*, 55(5-6): p. 244-51.

66. Thomas, C.A., *et al.,* 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. *Biol Reprod,* 58(3): p. 786-