The association between sperm function and field fertility in bulls

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Introduction

A thorough evaluation of bulls to predict their fertility potential is critical for viable cattle production. The importance of fertility prediction is even more important when the bull is used for single-sire mating or artificial insemination (AI). Traditional semen analysis may identify semen or bulls that are grossly abnormal, but cannot identify sub-fertile bulls that are producing apparently normal semen. It has been clearly documented that bulls classified as satisfactory differ in their field fertility, necessitating development of new approaches to precisely document potential variation in the fertility of these bulls. In that regard, various submicroscopic approaches such as computer-assisted sperm analysis (CASA) and flow cytometry-based evaluation of sperm characteristics, are widely used. These improvements in semen evaluation have led to several studies correlating various end points to fertility of semen used for AI.[1,2] However, the desired level of accuracy in fertility estimations based on in vitro assays has not been achieved, due to the inadequacy in fertility measurements and lack of suitable in vitro tests. Number of females used for insemination and their reproductive health, number of sperm in the inseminate, and insufficient variations in fertility of males can all influence associations between in vitro tests and fertility. Every sub-compartment of sperm must be intact and functional in order to fertilize an oocyte; furthermore, the association between fertility and almost any fertility assay depends on the efficacy of the assay to measure multiple sperm traits. For example, sperm must have normal motility, morphology and plasma membrane integrity. Each ejaculate contains a heterogeneous population of sperm, with variations in the functional attributes needed for fertilization. Unfortunately, most laboratory assays give a population mean for each attribute, which confounds the true number of fertile sperm in the population. In addition, assays are often prone to error due to judgement or bias. Current methods, including genomics and proteomics, are still confined to the research community because of their sophisticated instrumentation and advanced bioinformatics tools. Regardless, it is predicted that these "omics" methods will play a major role in future diagnostics. The present review describes the current laboratory methods available for semen analysis in bulls as an aid for predicting their fertility following AI.

Traditional breeding soundness evaluation

Fertility of a bull is predicted by breeding trials (breeding many normal, fertile females and assessing pregnancy rates) or conducting a breeding soundness evaluation (BSE). Since no single measurement or criterion reliably predicts fertility, several criteria are usually evaluated. The standards of the Society for Theriogenology (www.therio.org) are intended to assess the likelihood of a bull establishing pregnancy in >25 healthy, cycling females in a 65-70 d breeding season. The BSE classifications are based on a physical evaluation and acceptable thresholds for testicular development, sperm motility, and normal sperm morphology. Although libido is a major determinant of bull fertility, this aspect of fertility is not routinely evaluated during the BSE. In addition, evaluation of bulls for venereal diseases is also not a common practice during BSE. A standard BSE includes assessment of size, muscling, body condition, and freedom from disease and physical defects (i.e. abnormal angularity of legs, hoof problems, etc.). The scrotum should have a distinct neck, testes should be freely moveable, similar in size, firm and resilient, with normal epididymides and spermatic cords. Scrotal circumference (SC) is measured by forcing the testes to the bottom of the scrotum and using a flexible tape to apply moderate tension at the largest circumference. The SC is highly correlated with paired testis weight, which is correlated with daily sperm production and semen quality.[3]

Sperm motility is estimated by examining semen on a clean, warm slide and is reduced by extreme temperatures and environmental contaminants. Mass motility (affected by both individual sperm motility and sperm concentration) is detectable at low power, but progressive motility should be assessed

under medium power (~400 x); a cover slip is applied and concentrated samples are diluted with warm, fresh saline.

Sperm morphology should be evaluated under oil immersion. Semen is diluted in eosin-nigrosin (smear) or 10% neutral buffered formalin (wet mount) and at least 100 sperm cells (up to 300 if there are multiple abnormalities) are evaluated. In general, fertility will be decreased with >30% morphologically abnormal sperm or >20% head defects.[3] A bull that is healthy and sound, with an adequate SC, >70% morphologically normal sperm, and >30% progressive motility, is designated a Satisfactory Potential Breeder.[3] Furthermore, a bull with temporary conditions (likely to resolve) is designated Classification Deferred; this typically includes recent puberty, an injury or lameness that is likely to resolve, or temporary testicular degeneration (e.g. due to hot weather). Finally, a bull with undesirable heritable defects, small SC, debilitating injury or disease, or permanent testicular degeneration, is designated an Unsatisfactory Potential Breeder.

Although BSE can identify bulls that are producing grossly abnormal semen, this approach cannot identify variations in fertility among bulls that are classified as satisfactory potential breeders. Consequently, the use of bulls that meet minimum standards in natural breeding or their semen for AI can result in pregnancy rates that differ by 20-25 %.[4] Therefore, it is important to develop fertility tests to detect this variation in fertility. The following sections of this manuscript are a review of recent advances in evaluation of submicroscopic characteristics of sperm that are apparently normal.

Sperm kinematic parameters

Computer assisted sperm analysis is the preferred system for objectively evaluating sperm motility in a commercial setting. With the advent of CASA, it is possible to identify various sperm kinematic characteristics simultaneously that cannot be identified by subjective semen analysis. As sperm progress along a trajectory, the CASA system calculates motility, velocity, linearity and lateral displacement of the sperm head (ALH). Several studies have highlighted the importance of total and progressive motility and its association to field fertility.[5,6] Farell et al[7] also reported high correlation between several CASA parameters (linearity, average path velocity, curvilinear velocity, total motility and beat cross frequency[BCF]) and fertility, as determined by their 59d non-return rate to first service. Kasimanickam et al[8] reported that ALH and BCF in conjunction with either straight line velocity (VSL) or average path velocity (VAP) were significantly correlated to fertility. We recently reported that ALH was significantly higher in low- (LF) versus high-fertility (HF) bulls.[9] This finding suggested that sperm from HF bulls have higher vigor just after thawing in comparison to those from LF bulls. Consistent with these results, significantly higher percentage of hyperactivated sperm were recorded in HF bulls. Three types of sperm motility patterns have been described: 1) forward progressive motility; 2) transition phase to hyperactivated motility; and 3) hyperactivated motility. Based on these criteria, we inferred that post-thaw sperm from HF bulls represent a "transition phase" from forward progressive to hyperactivated motility. Sperm from LF bulls at post-thaw had a lower ALH and numerically higher linear motility representing the "forward progressive" motility pattern, which drives the sperm in a more-or-less straight line. "Transition phase" is a motility pattern usually exhibited by physiologically normal sperm that are in progress to hyperactivation. These differences in motility between HF and LF bulls may reflect submicroscopic differences at the structural (plasma membrane) and/or functional level in the sperm among these bulls. Based on these findings, we postulated that frozen-thawed sperm from HF bulls are more efficient in undergoing hyperactivation compared to LF bulls, influencing their ability to cross the barriers of the female reproductive tract and reach the site of fertilization. To test this hypothesis, we subjected post-thaw sperm from both LF and HF bulls to a swim-up procedure that provided a barrier between post-thaw semen and the swim-up medium and evaluated the concentration of post swim-up sperm and their motion characteristics under capacitating conditions. Interestingly, based on CASA analysis of post-swim-up sperm, ALH in LF bulls was significantly lower than that of HF bulls, whereas linearity (LIN), straightness (STR),and wobble (WOB) were significantly higher, suggesting that post-swim-up sperm from HF bulls were in transition to hyperactivation. Concentration of sperm recovered after swim-up (expressed as a percentage of viable sperm present in the post-thaw sample) and the

proportion of viable sperm in post-swim-up sperm were significantly different between HF and LF bulls and positively correlated with fertility.[9] Although a significant correlation of sperm concentration after swim-up and fertility have been reported,[10] our experimental approach provided an opportunity to normalize concentration of sperm recovered after swim-up for the concentration of viable sperm used for the swim-up procedure. Based on low recovery of sperm in the post-swim-up preparations from LF bulls, we inferred that viable sperm from LF bulls were inherently compromised to pass through sodium hyaluronate, indirect evidence for their reduced ability to pass through the barriers of the reproductive tract and populate the site of fertilization. Based on these results and observed differences in sperm kinematic parameters, we postulated that sperm from LF bulls have a reduced ability to undergo hyperactivation under appropriate physiological conditions, which affects their ability to pass through the female reproductive tract, and interact with the oocyte, thereby contributing to reduced fertility.[11]

Sperm plasma membrane integrity and viability

The integrity of the sperm plasma membrane is often linked to the viability of sperm; consequently, most sperm viability tests assess the intactness or leakiness of the cell plasma membrane.[12] Cell viability is often assessed after staining sperm with membrane-impermeable fluorescent dyes with affinity for the DNA. Viable cells are able to exclude these dyes and prevent the staining of the nucleus. Some of the membrane-impermeant probes that have been successfully used to assess sperm viability include Hoechst 33528, YoPro-1, and propidium iodide (PI).[13] Another method to assess membrane integrity includes the use of membrane-permeant DNA dyes such as SYBR-14.[14] These probes can enter the living cell by virtue of their amphipathic nature, but are immediately deacylated by intracellular esterases, thereby rendering the dye membrane-impermeable. Thus, viable cells will have the entrapped SYBR-14 whereas in dead cells, the dye easily leaks out due to the damaged membrane.[13] Recently, combinations of stains are available, which can simultaneously label the viable and non-viable cells. The LIVE/DEAD® Sperm Viability Kit (Invitrogen, Carlsbad, CA) employs both SYBR-14 and propidium iodide, which is routinely used for evaluating viability of frozen-thawed semen. This kit is reliable and user friendly, as it does not involve any laborious processing steps that might affect cell viability. Another added advantage is that egg-yolk particles in the extender will not interfere with the flow cytometer, as they will remain unlabelled for both the dyes used in the kit.[15] Sperm viability, evaluated by fluorescent labelling of sperm coupled with flow cytometry, had significant correlations between viability and fertility in the stallion,[16] bull,[17] and boar.[18] We recently demonstrated that the percentage of viable sperm at post-thaw was higher in HF versus LF bulls.[9] Furthermore, there were a significantly lower percentage of the dead and moribund cells (stained with propidium iodide and SYBR-14, indicating compromised viability) in HF vs LF bulls. In addition, the rate of conversion of live to moribund sperm was lower in HF compared to LF bulls. It is noteworthy that the proportion of dead and moribund sperm and also rate of conversion of live to moribund sperm were negatively correlated with field fertility.[11]

Sperm mitochondria

Several fluorescent dyes such as rhodamine 123 (R123), Mitotracker (MITO), and JC-1 have been used to evaluate the functional status of sperm mitochondria. Both R123 and MITO dyes are transported into actively respiring mitochondria, which then fluoresces green. All actively functioning mitochondria stain green with R123 and MITO and therefore one cannot distinguish sperm that exhibits different respiratory rates.[14,19] More recently, JC-1 has been used to distinguish sperm with poor and highly functional mitochondria.[19,20] This fluorescent dye stains green in normally functioning mitochondria but when the respiratory rate increases, the stain forms aggregates that fluoresce orange.[20] Therefore, JC-1 not only differentiates between functional and non-functional mitochondria, but also permits us to assess various levels of mitochondrial function. Studies have highlighted the importance of functional mitochondria to sperm motility, irrespective of whether R123[21,22] or JC-1[19,20] is used to evaluate mitochondrial function.

Sperm DNA damage

The quality of the sperm DNA transmitted during fertilization is essential to support embryo development and fertility. Programmed cell death or apoptosis is a major factor that has been implicated in DNA damage in sperm and is negatively associated with fertilization rate, implantation, and successful pregnancy[23,24] and also related to the field fertility of bull semen.[25] Increased percentages of apoptotic sperm decrease bull fertility by decreasing the percentage of viable sperm.[26,27] The structure of the mature sperm DNA is extremely compact due to the presence of the protamines in the sperm chromatin and can display various abnormalities, such as damage to the DNA in the form of single- or double-stranded breaks, protein defects which interfere with histone or protamine conversion and thereby affecting DNA compaction, and defective tertiary chromatin configuration. All of these defects could impair nuclear decondensation and jeopardize embryonic development, since the oocyte would not be able to overcome these forms of damage.[28] Moreover, damaged paternal DNA could be incorporated into the genome of the embryo, leading to errors in transcription or translation in embryo development, ultimately having a trans-generational effect.[29] Due to its importance, evaluation of DNA integrity of the semen has increased over the past years. For example, a single-cell DNA gel electrophoresis assay (COMET) can distinguish between normally condensed sperm nuclei (minimal migration on an agarose gel) and more loosely packed DNA (tailing of DNA). Mice with low expression of protamine 2 demonstrates tailing of DNA in this assay, highlighting the dependence of DNA condensation on protamines.[30] Chromomycin A3 (CMA3) is a fluoroprobe, which binds to deprotaminated DNA (at GC specific regions) in a loosely packed chromatin structure.[31] Lolis et al reported that low CMA3 staining in human sperm correlated with higher in vitro fertilizing rates than samples with high CMA3 staining. The second classical method to detect the intactness of the sperm DNA is by taking advantage of the metachromatic properties of acridine orange (AO) in the sperm chromatin structure assay (SCSA). In that regard, normal intact DNA is fully resistant to acid denaturation (green fluorescent in double stranded DNA), whereas damaged DNA (red fluorescent in single stranded DNA) is susceptible to denaturation, with the extent of damage detected using flow cytometry. Several studies have reported a high correlation of the SCSA tests with fertility in several species, including bull,[32,33] human,[34] stallion[35] sperm; HF males had less chromatin denaturation compared to those with LF sperm samples. Furthermore, DNA fragmentation can also be assessed by using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays, which identify single stranded DNA areas by labeling free 3-OH termini with modified nucleotide analogs. A combined TUNEL/PI procedure is now available, which takes advantage of the accuracy of TUNEL and differentiation of viable and dead sperm populations based on PI intensity.[36]

Sperm acrosome integrity

Acrosome integrity is traditionally measured during frozen semen evaluation by evaluating a wet film of semen under the microscope, ideally with differential interference contrast (DIC)-capabilities, which provides a clear view of the acrosomal ridge. However, fluorescent staining of acrosome coupled with flow cytometry can be used to objectively evaluate acrosomal integrity on a larger number of sperm, thereby improving reliability of this sperm function test. Acrosome integrity is usually measured with fluorescently labeled plant lectins. For example, *Pisum sativum* agglutinin (PSA), a pea plant agglutinin that binds to α-mannose and α-galactose moieties of the acrosome matrix,[37] whereas a lectin from peanut, *Arachis hypogaea* agglutinin (PNA) binds to β-galactose moieties of the acrosome.[38,39] However, it is believed that PNA has less non-specific binding to other areas of the sperm and thereby it is favored over PSA.[40] In both the cases, the lectins are not inherently fluorescent, but can be labeled with fluoroprobes (e.g. FITC). Acrosome-intact sperm emit bright fluorescence throughout the acrosomal region, whereas partial, patchy or focal fluorescence from the acrosomal region indicates partial acrosome reaction. Finally, an acrosome-reacted sperm, sperm will be fluorescence-free, except for a fluorescent band at the acrosome-postacrosomal region.[41]

Membrane fluidity and capacitation status

The capacitation status of sperm is usually determined with the fluorescent antibiotic chlortetracycline (CTC), which binds to the plasma membrane in Ca^{2+}/Mg^{2+} dependent manner.[13] Uncomplexed CTC (neutral) binds with free calcium in the intracellular compartments and becomes negatively charged and highly fluorescent. In non-capacitated sperm, an overall staining of the sperm head is apparent (F pattern). More prominent staining near the apical area and decreased staining in the posterior regions is evident in capacitated sperm (B pattern).[42] Loss of CTC staining from the sperm head indicates that the cell has undergone an acrosome reaction.[43] This capacitation-associated B pattern has also been noticed in frozen-thawed sperm (cryo-capacitation like changes).[44,45] In AI bulls, the percentage of non-capacitated sperm was significantly related to fertility.[46,47] Another method to assess capacitation is to stain the sperm with merocyanine 540 (M540). In capacitated sperm, a decrease in the packing order of the phospholipids was reported in the outer leaflet of the plasma membrane, which allowed the dye to intercalate into the hydrophobic core of the membrane.[48] Under capacitating conditions, membrane fluidity changes detected by M540 staining preceded the calcium influx measured by CTC staining.[49] Therefore, M540 and CTC could be used to monitor calcium dependent and independent events associated with capacitation. Fluorescent phospholipid binding proteins such as annexin-V (binds phosphatidylserine) can also be used to monitor changes in the phospholipid asymmetry of the plasma membrane. In uncapacitated sperm, phosphatidylserine (PS) is confined to the cytoplasmic side of the phospholipid bilayer, whereas in capacitated sperm, it is exteriorized to the outer leaflet (owing to changes in membrane fluidity).[50] Annexin-V binding is considered more sensitive than other probes in detecting membrane changes associated with cryopreservation, since translocation of PS usually precedes the loss of membrane integrity.[51] However, correlation between this assay and the AI bull semen fertility was variable.[26,52]

In vitro fertilization and related techniques

Sperm is also tested for its ability to bind to homologous oviductal epithelium in vitro. This binding prolonged their life and kept them in an uncapacitated state,[53] thus providing insight into the fertilizing capabilities of a semen sample.[54] However, only a marginal correlation was identified between the outcome of these tests and fertility.[55] Effective binding of the sperm to either homologous or heterologous zona pellucidae (ZP) is yet another in vitro test to evaluate the fertilizing ability of sperm. Zona binding tests use either whole or hemi-ZP. Fertility is also evaluated using zona-free hamster oocyte penetration tests; ability of a sperm sample to fertilize oocytes is deduced based on the ability of these sperm to penetrate zona-free oocytes and develop into normal male pronuclei.[56] A positive correlation was reported between this test and sire fertility.[57,58] It is noteworthy that in vitro fertilization (IVF) is the gold standard test to determine the relative fertility of semen sample across several domestic species, as it is the only method that closely resembles sperm-egg interaction in vivo during fertilization. Furthermore, a statistically significant relationship between in vitro and in vivo fertility was documented in several studies.[59-61]

The 'omics' revolution

The use of different "omics" approaches such as genomics (genes), transcriptomics (RNA) and proteomics (proteins) have widened our knowledge, not only on the regulatory networks that govern gamete function, but also indicated that subtle differences in their expression (genes and proteins) could positively or negatively influence the fertility of the animal. High-throughput technologies such as DNA and RNA sequencing, DNA microarray, and mass spectrometry, along with proper bioinformatics tools, may provide better cues for annotation of genes and proteins in the context of gamete biology.

Sperm genomics

Recent advances in the field of genome sequencing have provided great insight into genetic differences among sires. These genetic factors may contribute partly to variations in fertility among sires and hence bull fertility could be improved by genetic selection. For example, Druet et al[62] reported that

moderate (0.15-0.3) and high (close to 0.6) heritabilities exist for semen parameters such as volume and concentration, motility and percentage of abnormal sperm, respectively. The most abundant genomic variation, single nucleotide polymorphisms (SNPs), have been linked to economically important traits in the horse, pig and cattle[63-66] Information about specific SNPs related to fertility could facilitate earlier and accurate prediction of fertility and would speed genetic selection programs. Khatib et al[67] reported that SNPs in the FGF2 and STAT5A gene are related to male fertility. Feugang et al[68] reported that polymorphisms in the integrin beta 5 gene (ITB5B) may have a role in Holstein bull fertility. Functional studies on ITG5B suggested that it might have a role in sperm-egg interaction. Dai et al[69] reported that SNPs in the follicle stimulating hormone beta subunit (FSHB) were associated with lower semen quality, poor freezability and ultimately a lower non-return rate. Thus, identifying individual genes that are responsible for differences in fertility among sires would give us opportunities to improve bull fertility through selective breeding. Ultimately, this SNP technology would pave the way for marker-assisted selection in choosing young bulls as sires for the next generation.

Sperm mRNA profiles

At the time of fertilization, sperm delivers coding and non-coding RNA to the oocyte. Studies have highlighted the presence and subcellular localization of a specific population of RNAs in mature sperm, yet very little is known about its purpose and function. For example, Kumar et al[70] localized c-myc RNA in the tail mid-piece, whereas there are other reports[71] that the mRNAs for the transcription factors nuclear factor-kappa B (NFκB), interferon consensus sequence binding protein (ICSB), protein kinase c-jun n-terminal kinase 2 (JNK2), growth factor heparin-binding EGF-like growth factor (HBEGF) and epidermal growth factor receptor (ErbB3) are present in the sperm head. It is noteworthy that recent developments in global gene expression analysis have allowed us to evaluate the functional diversity of RNAs present in ejaculated sperm. Various transcripts profiling platforms have identified a myriad of diverse transcripts.[72-74] Furthermore, semen quality was associated with diverse transcripts that are differentially expressed between bulls of different fertility status. In a study by Kasimanickam et al,[75] there was a strong association between the mRNA abundance for adenylate kinase (AK) 1, integrin β (IB) 5, tissue inhibitor of metalloproteinases (TIMP) 2, lactate dehydrogenase c, outer dense fiber 2, phospholipase C zeta (PLCζ) 1 and sire conception rates. Lalancette et al74 reported that mitogen-activated protein kinase kinase, testis-specific serine/threonine protein kinase 3, LIM/homeobox protein, and SH3 protein transcripts (along with several others) were differentially expressed in sperm from HF bulls. Thus the heterogeneous RNA content of the ejaculated sperm could be used as a fingerprint for the genomic analysis to assess semen quality, in terms of both spermatogenesis quality and fertility outcome.[71,76-78] Therefore, development of a reliable method for the routine isolation of RNA from sperm will serve as an important step to develop novel non-invasive procedures to evaluate bull fertility.

Sperm proteomics

By focusing on analysis of expression profiles, proteomics could lead to novel biomarkers that could be linked to bull fertility. In the past decade, mass spectrometry (MS) approaches have enabled global identification and quantification of proteins. In that regard, 2D-PAGE and 2D-DIGE techniques coupled with MS have not only facilitated individual protein identification, but also their post-translational modifications. Technologies such as isobaric tags for relative and absolute quantitation (iTRAQ) enable relative quantification of the number of peptides (and thus proteins) between two or more samples. As proteins define a cell phenotype, changes at the proteome level could lead to differences in phenotypes influencing economically important traits. For example, fertility-associated antigen (FAA), a heparin binding protein was greater in the sperm membranes of beef bulls with greater fertility potential.[79] High fertility bulls also had greater abundance of two seminal plasma proteins, osteopontin and prostaglandin-D-synthetase in their semen sample as compared to LF bulls[80,81] Another protein, clusterin was also in higher concentration in LF bulls compared to HF bulls.[82] In addition, several studies have highlighted the differential expression of proteins in bulls with known fertility records.[83-85] Five proteins, enolase 1 (ENO1), ATP synthase, H^+ transporting mitochondrial F1 complex

(ATP5B), apoptosis-stimulating of p53 protein 2 (ASPP2), alpha-2-HS-glycoprotein (ASHG), phospholipid hydroperoxide glutathione peroxidase (Gpx4) were highly expressed in the HF bulls, whereas voltage dependent anion channel 2 (VDAC 2), ropoporin-1, ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2) were more represented in LF bulls.[83] In a study by Soggiu et al,[85] alpha enolase was significantly down-regulated in the estimated relative conception rate (ERCR)- group, whereas two other proteins, isocitrate dehydrogenase, and triosephosphate isomerase were upregulated in ERCR- (LF) group in comparison to ERCR+ (HF) group. D'Amours et al[84] reported that T-complex protein 1 subunits (CCT5 and CCT8), epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type 6 and binder of sperm 1 (BSP1) were more expressed in LF bulls than in HF bulls. Adenylate kinase 1 (AK1) and phosphatidylethanolamine-binding protein (PEBP1) were more expressed in the HF group compared to the LF group. A two-fold enrichment was noticed in epidermal growth factor (EGF) and platelet-derived growth factor (PGDF) signaling pathways in HF versus LF sperm. In addition, peroxisome proliferated activator receptor (PPAR) signaling, interleukin (IL)-4 signaling, chemokine signaling, insulin growth factor (IGF)-1 signaling was identified only in HF sperm.[86] In the same study, two proteins, ATP synthase, H^+ transporting, mitochondrial F1 complex (ATP5B) and cytochrome c oxidase subunit III (COX3) were higher in the HF group in comparison to the LF group. Shojaei et al[87] demonstrated that a cohort of sperm proteins was differentially expressed in normal versus pyriform sperm derived from the same bulls. Several proteins involved in sperm capacitation, sperm-egg interaction and sperm cytoskeletal structure were decreased in pyriform sperm, whereas proteins, which regulate antioxidant activity, apoptosis and metabolic activity were increased. Contents of reactive oxygen species and ubiquitinated proteins were higher in pyriform sperm. We concluded that comparing normal versus morphologically abnormal sperm was a suitable experimental model for identifying important sperm functional proteins, which may serve as novel markers for fertility predictions.

Epigenetics

The epigenome represents specific marks around the chromatin that can influence gene expression.[88,89] These epigenomic marks are influenced by environmental conditions (e.g. toxicants, nutrition, temperature, etc.). Although these environmental triggers do not alter the DNA sequence, they induce chemical modifications to DNA and influence DNA compaction, which in turn controls gene expression. Some of these epigenetics modifications are transient modulators of the chromatin structure, influencing access of transcription machinery to particular stretches of DNA. For example, histones can be modified by acetylation, phosphorylation and methylation, thereby modifying the affinity of these proteins to DNA. However, changes in methylation patterns (addition of methyl groups to the cytosine residues) of the DNA are considered permanent epigenetic marks. Therefore, evaluating changes in epigenetic marks and associated changes in phenotypes may have implications for identifying epigenetic contributions to regulation of fertility.

Summary

Traditional BSE remains as the first-line of approach to identify bulls or their semen that are grossly abnormal. However, bulls classified as satisfactory based on a BSE still differ in fertility. Identification of these variations in fertility may allow us to eliminate bulls or their semen from breeding programs, or allow AI centers to make necessary adjustments in the semen doses to maximize sale of elite sires while preserving optimal fertility. Since fertility is a trait influenced by a multitude of factors, no single fertility test is sufficient to precisely document fertility. Therefore, a combination of fertility tests to specifically evaluate ability of sperm to pass through barriers of the female reproductive tract and reach the site of fertilization, undergo successful capacitation, bind with the oocyte, undergo an acrosome reaction, penetrate zona pellucidae, fuse with the oocyte plasma membrane, initiate zona and vitelline blocks, induce resumption of meiosis of the oocyte by releasing sperm factors, undergo DNA decondensation and form a male pronucleus, fuse with the female pronucleus, initiate cleavage and effectively contribute to embryo-specific gene expression to support developmental competence of resulting embryos. In addition to existing sperm function assays, emerging genetic approaches may

enable us to improve fertility predictions and facilitate genetic screening of bulls for fertility at an early age. Such advancements in fertility predictions will substantially improve the economic viability of the livestock industry.

References

1. Ostermeier GC, Sargeant GA, Yandell BS,et al: Relationship of bull fertility to sperm nuclear shape. J Androl 2001;22:595-603.
2. Kirk ES, Squires EL, Graham JK: Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology 2005;64:1422-1439.
3. Barth AD: Evaluation of potential breeding soundness. In: Youngquist RS, Threlfall WR, editors. Current therapy in large animal theriogenology. St. Louis: W.B. Saunders; 2007. p. 228-240.
4. Larson JL, Miller DJ: Can relative spermatozoal galactosyltransferase activity be predictive of dairy bull fertility? J Dairy Sci 2000;83:2473-2479.
5. Gillan L, Kroetsch T, Maxwell WM, et al: Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 2008;103:201-214.
6. Kathiravan P, Kalatharan J, Edwin MJ, et al: Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. Anim Reprod Sci 2008;104:9-17.
7. Farrell PB, Presicce GA, Brockett CC, et al: Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998;49:871-879.
8. Kasimanickam R, Nebel RL, Peeler ID, et al: Breed differences in competitive indices of holstein and jersey bulls and their association with sperm DNA fragmentation index and plasma membrane integrity. Theriogenology 2006;66:1307-1315.
9. Shojaei H, Kroetsch T, Wilde R, et al: Moribund sperm in frozen-thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. Theriogenology 2012;77:940-951.
10. Hallap T, Jaakma U, Rodriguez-Martinez H: Changes in semen quality in Estonian Holstein AI bulls at 3, 5 and 7 years of age. Reprod Domest Anim 2006;41:214-218.
11. Shojaei H, Kroetsch T, Wilde R, et al: Moribund sperm in frozen-thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. Theriogenology 2012;77:940-951.
12. Graham JK, Moce E: Fertility evaluation of frozen/thawed semen. Theriogenology 2005;64:492-504.
13. Silva PF, Gadella BM: Detection of damage in mammalian sperm cells. Theriogenology 2006;65:958-978.
14. Gillan L, Evans G, Maxwell WM: Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 2005;63:445-457.
15. Nagy S, Jansen J, Topper EK, et al: 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 2003;68:1828-1835.
16. Kavak A, Johannisson A, Lundeheim N, et al: Evaluation of cryopreserved stallion semen from tori and estonian breeds using CASA and flow cytometry. Anim Reprod Sci 2003;76:205-216.
17. Nagy S, Hallap T, Johannisson A, et al: Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4 h incubation as measured by multicolor flow cytometry. Anim Reprod Sci 2004;80:225-235.
18. Saravia F, Hernandez M, Wallgren M, et al: Controlled cooling during semen cryopreservation does not induce capacitation of spermatozoa from two portions of the boar ejaculate. Int J Androl 2007;30:485-499.
19. Garner DL, Thomas CA, Joerg HW, et al: Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57:1401-1406.
20. Thomas CA, Garner DL, DeJarnette JM,et al: Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod 1998;58:786-793.
21. Evenson DP, Darzynkiewicz Z, Melamed MR: Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 1982;30:279-280.
22. Auger J, Ronot X, Dadoune JP: Human sperm mitochondrial function related to motility: A flow and image cytometric assessment. J Androl 1989;10:439-448.
23. Henkel R, Hajimohammad M, Stalf T, et al: Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 2004;81:965-972.
24. Simon L, Castillo J, Oliva R, et al: Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. Reprod Biomed Online 2011;23:724-734.
25. Waterhouse KE, Haugan T, Kommisrud E, et al: Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod Fertil Dev 2006;18:781-788.
26. Anzar M, He L, Buhr MM, et al: Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002;66:354-360.
27. Martin G, Sabido O, Durand P, et al: Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 2004;71:28-37.
28. Johnson KM: Fertility clinic, egg donation agency, and sperm bank policies. Fertil Steril 2011;96:877-879.

29. Katari S, Turan N, Bibikova M, et al: DNA methylation and gene expression differences in children conceived in vitro or in vivo. Hum Mol Genet 2009;18:3769-3778.
30. Cho C, Jung-Ha H, Willis WD, et al: Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 2003;69:211-217.
31. Bianchi PG, Manicardi GC, Bizzaro D, et al: Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod 1993;49:1083-1088.
32. Ballachey BE, Evenson DP, Saacke RG: The sperm chromatin structure assay. relationship with alternate tests of semen quality and heterospermic performance of bulls. J Androl 1988;9:109-115.
33. Karabinus DS, Evenson DP, Jost LK, et al: Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. J Dairy Sci 1990;73:2364-2371.
34. Evenson DP: Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. Reprod Fertil Dev 1999;11:1-15.
35. Love CC, Kenney RM: Scrotal heat stress induces altered sperm chromatin structure associated with a decrease in protamine disulfide bonding in the stallion. Biol Reprod 1999;60:615-620.
36. Muratori M, Forti G, Baldi E. Comparing flow cytometry and fluorescence microscopy for analyzing human sperm DNA fragmentation by TUNEL labeling. Cytometry A 2008;73:785-787.
37. Farlin ME, Jasko DJ, Graham JK, et al: Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. Mol Reprod Dev 1992;32:23-27.
38. Mortimer D, Curtis EF, Miller RG: Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J Reprod Fertil 1987;81:127-135.
39. Flesch FM, Voorhout WF, Colenbrander B, et al: Use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. Biol Reprod 1998;59:1530-1539.
40. Dalimata AM, Graham JK: Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. Theriogenology 1997;48:831-841.
41. Mendoza C, Carreras A, Moos J, et al: Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. J Reprod Fertil 1992;95:755-63.
42. Ward CR, Storey BT: Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 1984;104:287-296.
43. Saling PM, Storey BT: Mouse gamete interactions during fertilization in vitro. chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. J Cell Biol 1979;83:544-555.
44. Kaneto M, Harayama H, Miyake M, et al: Capacitation-like alterations in cooled boar spermatozoa: assessment by the chlortetracycline staining assay and immunodetection of tyrosine-phosphorylated sperm proteins. Anim Reprod Sci 2002;73:197-209.
45. Collin S, Sirard MA, Dufour M, et al: Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. J Androl 2000;21:938-943.
46. Thundathil J, Gil J, Januskauskas A, et al: Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artificial insemination. Int J Androl 1999;22:366-373.
47. Gil J, Soderquist L, Rodriguez-Martinez H: Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. Theriogenology 2000;54:93-108.
48. Harrison RA, Gadella BM: Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology 2005;63:342-351.
49. Rathi R, Colenbrander B, Bevers MM, et al: Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod 2001;65:462-470.
50. Gadella TW Jr, Moritz A, Westerman J, et al: Enzymatic synthesis of pyrene-labeled polyphosphoinositides and their behavior in organic solvents and phosphatidylcholine bilayers. Biochemistry 1990;29:3389-3395.
51. Rodriguez-Martinez H: Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domest Anim 2003;38:312-318.
52. Januskauskas A, Johannisson A, Rodriguez-Martinez H: Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 2003;60:743-758.
53. Lefebvre R, Suarez SS. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. Biol Reprod 1996;54:575-582.
54. Fazeli A, Duncan AE, Watson PF, et al: Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod 1999;60:879-886.
55. Waberski D, Magnus F, Ardon F, et al: Binding of boar spermatozoa to oviductal epithelium in vitro in relation to sperm morphology and storage time. Reproduction 2006;131:311-318.
56. Thundathil J, Palasz AT, Barth AD, et al: The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. Anim Reprod Sci 2001;65:181-192.
57. Henault MA, Killian GJ: Effects of sperm preparation and bull fertility on in vitro penetration of zona-free bovine oocytes. Theriogenology 1995;43:739-749.
58. Brahmkshtri BP, Edwin MJ, John MC, et al: Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim Reprod Sci 1999;54:159-168.

59. Zhang BR, Larsson B, Lundeheim N, et al: Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-programme. Int J Androl 1999;22:253-260.
60. Selles E, Gadea J, Romar R, et al: Analysis of in vitro fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. Reprod Domest Anim 2003;38:66-72.
61. Larsson B, Rodriguez-Martinez H: Can we use in vitro fertilization tests to predict semen fertility? Anim Reprod Sci 2000;60-61:327-336.
62. Druet T, Fritz S, Sellem E, et al: Estimation of genetic parameters and genome scan for 15 semen characteristics traits of Holstein bulls. J Anim Breed Genet 2009;126:269-277.
63. Amills M, Vidal O, Varona L, et al: Polymorphism of the pig 2,4-dienoyl CoA reductase 1 gene (DECR1) and its association with carcass and meat quality traits. J Anim Sci 2005;83:493-498.
64. Brym P, Kaminski S, Wojcik E: Nucleotide sequence polymorphism within exon 4 of the bovine prolactin gene and its associations with milk performance traits. J Appl Genet 2005;46:179-185.
65. Haegeman A, Williams JL, Law A, et al: Mapping and SNP analysis of bovine candidate genes for meat and carcass quality. Anim Genet 2003;34:349-353.
66. Heaton MP, Harhay GP, Bennett GL, et al: Selection and use of SNP markers for animal identification and paternity analysis in U.S. beef cattle. Mamm Genome 2002;13:272-281.
67. Khatib H, Monson RL, Huang W, et al: Short communication: Validation of in vitro fertility genes in a Holstein bull population. J Dairy Sci 2010;93:2244-2249.
68. Feugang JM, Kaya A, Page GP, et al: Two-stage genome-wide association study identifies integrin beta 5 as having potential role in bull fertility. BMC Genomics 2009;10:176.
69. Dai L, Zhao Z, Zhao R, et al: Effects of novel single nucleotide polymorphisms of the FSH beta-subunit gene on semen quality and fertility in bulls. Anim Reprod Sci 2009;114:14-22.
70. Kumar G, Patel D, Naz RK: C-MYC mRNA is present in human sperm cells. Cell Mol Biol Res 1993;39:111-117.
71. Dadoune JP, Pawlak A, Alfonsi MF, et al: Identification of transcripts by macroarrays, RT-PCR and in situ hybridization in human ejaculate spermatozoa. Mol Hum Reprod 2005;11:133-140.
72. Mao XM, Ma WL, Feng CQ, et al: An initial examination of the spermatozoal gene expression profile. Di Yi Jun Yi Da Xue Xue Bao 2004;24:1033-1036.
73. Gilbert I, Bissonnette N, Boissonneault G, et al: A molecular analysis of the population of mRNA in bovine spermatozoa. Reproduction 2007;133:1073-1086.
74. Lalancette C, Thibault C, Bachand I, et al: Transcriptome analysis of bull semen with extreme nonreturn rate: use of suppression-subtractive hybridization to identify functional markers for fertility. Biol Reprod 2008;78:618-635.
75. Kasimanickam V, Kasimanickam R, Arangasamy A, et al: Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. Theriogenology 2012;78:2007-2019.
76. Ostermeier GC, Dix DJ, Miller D, et al: Spermatozoal RNA profiles of normal fertile men. Lancet 2002;360:772-777.
77. Steger K, Fink L, Failing K, et al: Decreased protamine-1 transcript levels in testes from infertile men. Mol Hum Reprod 2003;9:331-336.
78. Lambard S, Galeraud-Denis I, Martin G, et al: Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. Mol Hum Reprod 2004;10:535-541.
79. Bellin ME, Oyarzo JN, Hawkins HE, et al: Fertility-associated antigen on bull sperm indicates fertility potential. J Anim Sci 1998;76:2032-2029.
80. Moura AA, Chapman DA, Killian GJ: Proteins of the accessory sex glands associated with the oocyte-penetrating capacity of cauda epididymal sperm from Holstein bulls of documented fertility. Mol Reprod Dev 2007;74:214-222.
81. Gerena RL, Irikura D, Urade Y, et al: Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. Biol Reprod 1998;58:826-833.
82. Ibrahim NM, Gilbert GR, Loseth KJ, et al: Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. J Androl 2000;21:887-894.
83. Park YJ, Kwon WS, Oh SA, et al: Fertility-related proteomic profiling bull spermatozoa separated by percoll. J Proteome Res 2012;11:4162-4168.
84. D'Amours O, Frenette G, Fortier M, et al: Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. Reproduction 2010;139:545-556.
85. Soggiu A, Piras C, Hussein HA, et al: Unravelling the bull fertility proteome. Mol Biosyst 2013;9:1188-1195.
86. Peddinti D, Nanduri B, Kaya A, et al: Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. BMC Syst Biol 2008;2:19.
87. Shojaei Saadi HA, van Riemsdijk E, Dance AL, Proteins associated with critical sperm functions and sperm head shape are differentially expressed in morphologically abnormal bovine sperm induced by scrotal insulation. J Proteomics 2013;82:64-80.
88. Rajender S, Avery K, Agarwal A: Epigenetics, spermatogenesis and male infertility. Mutat Res 2011;727:62-71.
89. Jenkins TG, Carrell DT: The sperm epigenome and potential implications for the developing embryo. Reproduction 2012;143:727-734.