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Ancillary semen tests for stallions: which ones to use and what do they mean

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Introduction

Actual fertility trials are the best gauge of a stallion’s fertility, be it under natural-cover or artificial-insemination conditions. Nonetheless, use of such trials to assess the intrinsic fertility of a stallion can be complicated by factors such as mare fertility, management issues, time concerns, and associated expenses. Laboratory based examination of semen generally has good predictive value regarding the fertility of a stallion, at least from the perspective of determining if a given stallion will be highly subfertile or have commercially acceptable fertility. Such an examination is generally done for one of four reasons: 1) as part of a pre-purchase examination; 2) as a quality control measure prior to the onset of a breeding season; 3) when reduced fertility is apparent or suspected; or 4) prior to acquisition of some types of fertility-related insurance.

Conventional laboratory tests typically include assessment of total sperm number in ejaculates and assessment of semen quality. Assessment of sperm quality has included light microscopic evaluation of sperm morphologic characteristics and estimation of sperm motility (including percentages of motile and progressively motile sperm; velocity of sperm movement; and longevity of sperm motility following in-vitro storage), as well as presence of blood, urine, or potentially pathogenic bacteria in the semen.1-5

The value of semen evaluation, as in any type of diagnostic approach, is centered around reliable equipment, good laboratory technique, and personnel with good observational power. Even when these stipulations are thought to be met, the predictive value of the examination has been reported to be limited.6-21 The same holds true for sperm of other mammalian species.22-25 As such, more critical and expanded analysis of equine semen might improve the predictive value of the testing process.

The use of the term, ancillary, for describing additional/alternative methods for evaluating semen is quite vague. The term can imply newly developed assays that impart novel information regarding sperm fertilizing potential; however, it can also denote better ways to measure specific features of semen than are commonly examined by more conventional methods. Both contexts of the term will be addressed in this communication.

Keywords: Stallion, semen evaluation, fertility prediction,

Measurement and prediction of sperm number

Determination of total sperm number in an ejaculate would seem to be a relatively simple procedure, but accurate measurement can be fraught with error. Total sperm number is the product of semen volume and sperm concentration. A first recommendation as an ancillary semen test is to use weight of gel-free semen to determine volume. Semen has a specific gravity (1.0085-1.010) that approaches that of deionized water (1.00);26 as such, 1 gram ≈ 1 mL. Measurement of volume in cups with marked gradations is inaccurate, as is measurement of volume in graduated cylinders that are oversized for the specimen to be measured. For example, use of a 100-mL capacity graduated cylinder would be less accurate than a 25-mL capacity graduated cylinder for measurement of semen volume in the 10-25-mL range. The authors prefer to use a scale with a tare function and a sensitivity of 0.01 g to measure volume of semen. For the measurement, the semen container (prior to insertion of an in-line filter, but with a sterile disposable liner in place, if used) can be weighed prior to attachment to an artificial vagina. The tare function is then activated to enable one to account for the weight of the container; thereby, subsequent placement of the same container with semen on the scale will allow one to account only for the additional weight of the semen. If semen extender is added to the semen container prior to semen collection, this can also be included in the tare activation such that one can accurately determine the volume of gel-free semen in the ejaculate while providing more immediate protection for the semen at the time of semen collection.
Sperm concentration has typically been measured in the clinical arena by hemacytometry or photometric methods. Both of these methods can be inherently inaccurate. Hemacytometer counts have the advantage of directly identifying sperm, but the proclivity for technician mistakes and relatively few sperm in counts can lead to a high margin of error. Photometric methods can also be inaccurate. These methods measure light transmission through a sample and are generally calibrated with hemocytometer counts of sperm. While photometric methods tend to have high repeatability, accuracy is affected negatively by contamination of the ejaculate with debris, urine, or blood. Similarly, sperm concentration cannot be correctly measured when the semen is pre-mixed with an opaque extender, such as a yolk- or milk-based extender. These photometric methods also tend to underestimate sperm concentration when actual sperm concentration is high (i.e., over 300 x 10^6 sperm/mL) and tend to overestimate sperm concentration when actual sperm concentration is relatively low (i.e., less than 100 x 10^6/mL).

Introduction of a fluorescence-based instrument (NucleoCounter® SP-100, Chemometec, A/S, Allerod, Denmark) for the purpose of measuring sperm concentration has, in the view of the authors, revolutionized our procedural methods for analyzing and processing stallion semen. The instrument determines sperm concentration, based on intercalation of the fluorescent dye, propidium iodide, with DNA of sperm after permeabilizing the sperm membranes with a detergent solution to expose the DNA to the dye. The treated sperm emit fluorescence when excited with green light from the fluorescence microscope within the instrument. Studies have demonstrated that this instrument can reliably evaluate sperm concentration, even when semen is pre-mixed in a variety of opaque and non-opaque media. This, in itself, has significant applications in laboratories that process semen for cooled transport or frozen storage. Additionally, the instrument readings compared favorably with hemacytometer and flow cytometer measurements over a large range of sperm concentrations.

Determination of total sperm number in ejaculates is important when proportioning semen for insemination of mares; however, it is also an important part of a breeding soundness evaluation. In this capacity, it may be most important when semen is collected from stallions on a daily basis for several days to determine actual daily sperm output (DSO). This approach can be troublesome for some stallion owners/managers because of the time and costs involved. An alternative method for predicting daily sperm output is to obtain accurate measurements of testicular dimensions and calculate total testicular volume. Testicular volume is highly correlated with daily sperm output in stallions, and provides a more accurate reflection of DSO than does measurement of total scrotal width. The formula for testicular volume is as follows: Testicular volume (TV) = 4/3πabc, where a=height/2, b=width/2, c=length/2, and measurements are made in cm; therefore, TV = 0.52 (height x width x length). Predicted DSO is determined as follows: Predicted DSO (x10^9) = 0.024x – 0.76, where x=total testicular volume.

If spermatogenic efficiency is normal, as would be expected if sperm morphologic and motility features are normal, then predicted daily sperm output generally approximates actual DSO. Our laboratory also compares actual DSO to predicted DSO in some stallions with reduced fertility to determine spermatogenic efficiency ([Actual DSO/Predicted DSO] x 100). In aging breeding stallions with deteriorating testicular function, a decline in spermatogenic efficiency will oftentimes precede declines in testicular size or semen quality. This can also be the case in some stallions with a transitory decline in fertility. As a case in point, Table 1 provides information on predicted and actual daily sperm output, as well as measures of sperm motility and sperm morphology in a middle-aged Thoroughbred stallion with a history of high fertility in previous years (> 90% seasonal pregnancy rate when covering over 150 mares per season with a spermatogenic efficiency that approached 100%). The stallion underwent general anesthesia to correct a nephrosplenic entrapment (by rolling) the November prior to the breeding season and was treated medically for iliac thrombosis in January prior to the breeding season. Per-cycle pregnancy rate for February-April was 22% (31/141), and per-cycle pregnancy rates for May and June were 1.5% (1/67) and 5% (2/41) respectively.

As shown in Table 1, minimal changes were detected in testicular size and sperm quality (as judged by percentages of total sperm motility, progressive sperm motility, and morphologically normal sperm) during the examination period. Circulating hormonal values and sperm chromatin values were within normal limits during this time. Spermatogenic efficiency was low in April, dropped precipitously...
in May, began to rebound in June, and increased markedly by November. In mid-November, 7 mares were bred by natural cover and 5 became pregnant (71% per-cycle pregnancy rate). The stallion had normal fertility (>90% seasonal pregnancy rate) the following commercial breeding season. With this clinical case, spermatogenic efficiency appeared to be the most sensitive indicator of fertility.

**Measures of sperm quality**

Sperm quality is considered to be important to fertility of males, including stallions, when fertilization occurs under *in vivo* conditions. Nonetheless, no single measure, or combination of measures, for semen quality has an exact correlation to fertility. In fact, the strength of the relationship for traditional measures, such as sperm motility and sperm morphology, is relatively low, with these measures singularly accounting for less than 50% of variability in pregnancy rate per cycle for groups of commercial breeding stallions.\(^{33-35}\) Relatively low correlations can be attributed, in part, to mare and management factors that could confound one’s ability to use laboratory measurements to predict intrinsic stallion fertility; however, if pregnancy data are corrected to help remove such potential confounders (by measuring first-cycle pregnancy rate, i.e., the number of mares pregnant in their first breeding cycle divided by the number of mares pregnant at the end of the breeding season), singular laboratory measures of sperm quality still do not account for the majority of variation stallion fertility.\(^{35}\) Given this information, it is logical to consider development/implementaton of a battery of laboratory assays that could more clearly define fertilizing potential of stallion sperm. Fertilization *in vivo* requires an array of highly coordinated cellular and molecular events so it would seem intuitive to evaluate various sperm compartments and characteristics in an effort to determine if a sperm population has the attributes necessary for efficient fertilization. While this approach has been considered by others in years past,\(^{36}\) a decade later we still do not have the “magical formula” for explicitly predicting the fertility of stallions. That said, it remains important to pursue development of laboratory assays that can be useful for more effectively predicting stallion fertility.

**Sperm motility**

Measurement of sperm motility is likely to remain a hallmark of semen evaluation, and it is relatively easy to perform. Evaluation of sperm motility in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of sperm in an ejaculate.\(^1\) Dissimilarity in scores among examiners is inevitable when the evaluation is done subjectively, but this variation can be minimized when a high-quality microscope with phase contrast optics and a warming stage is used. Evaluation of raw (undiluted or neat) ejaculated semen gives one an idea of how well sperm perform in their natural milieu, i.e., seminal plasma. Determining motility in the raw semen can be hampered by higher sperm concentrations and sperm agglutination to the cover glass, making it difficult for the evaluator to discern individual motility patterns. To overcome this limitation, an aliquot of semen can also be appropriately diluted (e.g., to 25-30 x 10^6 sperm/ml) in a good quality semen extender that is free of microscopic debris. The extender may slightly alter motility pattern, usually by increasing the sperm progressivity and velocity measures. After initial extension, a high percentage of sperm may exhibit a circular motility pattern; however, this behavior oftentimes resolves following 5-10 minutes of exposure time in warmed (37°C) extender. A downside of sperm motility as a laboratory measure of sperm function is that sperm motility is susceptible to environmental conditions (e.g., excessive heat or cold, lubricants, light, disinfectants, and osmolarity/pH of semen extender), so it is necessary to protect the semen from injurious agents or conditions prior to analysis. Estimations of the percentages of motile and progressively motile sperm are generally determined, in addition to an estimation of sperm velocity (based on an arbitrary scale of 0 [stationary] to 4 [fast]). Subjective assessment of motility is generally quite acceptable, provided personnel are experienced in analysis of sperm motility.

Several different techniques and instruments have been developed in an effort achieve an objective (i.e., unbiased) evaluation of sperm motility; however, these methods (e.g., time-lapse photomicrography, frame-by-frame playback videomicrography, spectrophotometry, or computerized analysis) are generally considered to be too tedious or expensive for routine use. Computerized systems...
are currently in place in many reference laboratories with the intent to objectively assess motion characteristics of sperm. Despite the commercial availability of various generations of computer-assisted sperm analysis (CASA) systems for over two decades, their presence has not provided the definitive assay for measuring sperm fertilizing potential.\textsuperscript{37} Such an expectation, however, is unrealistic given the numerous independent sperm attributes that are required for a spermatozoon to possess fertilization competence. What these systems do provide is the prospect of objective measurement and protocol standardization. These instruments permit customized selection of various features, including frequency and length of frame capture; threshold demarcations for sperm motion, progressivity of motion, and velocity measures; and gating freedom for both size and luminosity representative of sperm heads, as a means to maximize capture of sperm while minimizing capture of non-sperm material in the sample of interest. Such manipulations are important for improving measurement accuracy and repeatability within a given laboratory, but make it virtually impossible for reliable comparisons among laboratories or among different CASA brands.\textsuperscript{38,39} Computerized analysis of sperm is primarily reserved for the research setting, where standardization, accuracy, and precision are a prerequisite to measurement of experimental end points. A distinct value of CASA instruments in the commercial environment (at a veterinary hospital or an equine breeding operation) is the ability to garner objective results for a variety of motility variables. Confusion arises, however regarding the relationship of the myriad of obtainable CASA variables to fertility of the sample. As an example, we conducted a fertility trial with a subfertile stallion whose semen was subjected to density-gradient centrifugation in an effort to improve semen quality prior to insemination. Values for percent total motility, percent progressive motility, and mean curvilinear velocity prior to, and after, semen processing for the subfertile stallion and a fertile control stallion are listed in Table 2.

Based on these results, it would appear that semen treatment for the subfertile stallion yielded a sperm population with quality similar to, or exceeding (based on velocity values), that of the fertile control stallion. Nonetheless, when fertile mares were inseminated hysteroscopically with 20 x 10\textsuperscript{6} progressively motile sperm (100 µl-volume), the resulting pregnancy rates were 15/20 (75\%) for the fertile stallion, as compared to 7/20 (35\%) for the subfertile stallion (P<0.05). This demonstrates that sperm motility does not provide absolute discrimination power, again emphasizing that sperm attributes other than motility play critical roles in sperm fertilizing ability. Others have reported that sperm populations possess several clusters (subpopulations) of sperm with specific sperm motion characteristics;\textsuperscript{40} however, the specific relationships of these semen characteristics to fertility have not been described. Some CASA systems can be outfitted with fluorescence optics, and the predictive value of CASA might be improved by incorporation of fluorescent dyes in the media such that motility variables can be segregated by presence or absence of plasma membrane intactness (integrity).\textsuperscript{41} Further studies are required to determine if the predictive value of sperm motility can be improved by this approach. This topic is addressed further below under the section regarding plasma membrane integrity.

Sperm morphology

The morphology of sperm is typically examined with a light microscope at 1000X magnification. Standard bright-field microscope optics can be used to examine air-dried semen smears, provided appropriate stains are used in slide preparation. Specific sperm stains include those developed by Williams\textsuperscript{42} and Casarett.\textsuperscript{43} General-purpose cellular stains (e.g., Wright's, Giemsa, hematoxylin-eosin) also have been used to accent both germinal and somatic cells in semen smears. A recent study described the use of Diff Quik (a commercially available modification of the Wright-Giemsa formula and containing triarymethane, xanthene and thiazine dyes) for morphologic assessment of stallion sperm.\textsuperscript{44} Background stains (e.g., eosin-nigrosin, India ink) probably are the most widely used stains because of their ease of application. In the authors’ view, visualization of the structural detail of sperm can be greatly enhanced by fixing the cells in buffered formol saline or a similar fixative, then viewing the unstained cells as a wet mount with either phase-contrast or, preferably, differential interference contrast (DIC) microscopy.\textsuperscript{1} In addition, the incidence of artifactual changes is reduced in comparison with stained smears.
In our laboratory, at least 100 sperm per specimen are evaluated for evidence of morphological defects. The type and incidence of each defect is recorded. Abnormalities in sperm morphology traditionally have been classified as primary, secondary or tertiary. Primary abnormalities are considered to be associated with a defect in spermatogenesis and, therefore, are of testicular origin. Secondary abnormalities are created in the excurrent duct system. Tertiary abnormalities, as opposed to the previous two types, develop in vitro as a result of improper semen collection or handling procedures. The current trend is to record the percentages of specific morphologic defects, e.g., abnormal heads, knobbed acrosomes, proximal protoplasmic droplets, swollen midpieces, coiled tails, etc. The authors consider this method of classification to be superior to the traditional system because it reveals more specific information regarding a population of sperm, while avoiding erroneous assumptions about the origin of these defects. The origin of some sperm morphologic defects is unknown. Additionally, some morphologic abnormalities like detached heads can be primary, secondary or tertiary in nature, thereby introducing the possibility of error when using the traditional classification system exclusively.

In one study, the percentage of morphologically normal sperm had a relatively high correlation to percentages of total and progressive sperm motility and to measures of sperm velocity. Similarly, the percentages of several sperm morphologic abnormalities (i.e., midpiece abnormalities, detached heads, bent tails, coiled tails, and premature germ cells) were negatively correlated with percentage of morphologically normal sperm.\textsuperscript{35} Such correlations cannot be considered exact and can vary among individual stallions and ejaculates. For instance, some semen samples can possess good sperm motility, yet have a relatively high incidence of certain sperm morphologic abnormalities, such as proximal or distal cytoplasmic droplets. Generally speaking, the percentage of morphologically normal sperm in a semen sample is similar to the percentage of progressively motile sperm in fresh extended semen. If sperm motility is low and the percentage of morphologically normal sperm is high, it suggests that laboratory errors ( mishandling of semen) occurred which led to a lowering of sperm motility. One cannot discount, however, a potentially negative effect of seminal plasma on sperm motion characteristics (discussed below under the section on seminal plasma toxicity) or abnormalities of sperm ultrastructure that are not perceptible when using light microscopy. A distinct advantage of sperm morphology over sperm motility for judgment of sperm quality is that sperm morphological features are less disrupted by environmental disturbances than are measures of sperm motility.

Sperm structure is undoubtedly related to fertility; however, the impact of specific sperm morphologic features on fertility remains unclear. For instance, investigators have reported that the incidence of proximal cytoplasmic droplets can negatively impact fertility,\textsuperscript{35} has a questionable impact on fertility,\textsuperscript{8} or has no significant impact on fertility.\textsuperscript{8} Significant intra-stallion variation in sperm morphology typically occurs in commercial breeding stallions without impacting fertility;\textsuperscript{8} as such, isolated examinations of sperm morphology may not be as reliable an indicator of fertility as would more frequent sperm morphologic examinations. Investigators are generally in agreement that the percentage of morphologically normal sperm is positively correlated with fertility.\textsuperscript{8,33-35} Increasing percentages of defects considered to be major, i.e., abnormal heads or abnormally shaped midpieces, are associated with a corresponding reduction in fertility.\textsuperscript{8,35} Increasing percentages of coiled tails and premature germ cells may also be associated with reduced fertility.\textsuperscript{8} Conversely, percentages of bent tails and distal droplets do not appear to have any predictive value for fertility.\textsuperscript{8,33,35} If the morphologically abnormal sperm do not exert a direct negative influence on normal sperm, it is possible that the total number of morphologically normal sperm in ejaculates may provide more information regarding the fertility of a stallion than the percentage or absolute number of morphologically abnormal sperm.

Transmission electron microscopy can provide striking detail of sperm ultrastructure and other constituents of semen. Others have recommended this technique as a means of more critically assessing sperm morphology at the ultrastructural level and identifying other components of semen that may not be detectable with light microscopy.\textsuperscript{45} The authors contend that insufficient information can be gleaned from transmission electron microscopy of sperm on a routine basis to rationalize the additional time and expense associated with this method of evaluation. Nonetheless, transmission electron microscopy may be well justified with selected cases.
Plasma membrane integrity (intactness)

The semi-permeable plasma membrane, which is composed of a wide assortment of lipids, proteins, and carbohydrates, envelops the entire spermatozoon. This structure is vital to regulation of sperm functions by establishing ion gradients, facilitating cytosolic entry of larger molecules, and orchestrating various cell-signaling events, to name a few. Thus, assessment of its integrity would seem an important component of a semen evaluation. To this end, a variety of laboratory procedures have been used to evaluate the integrity of the plasma membrane. One method is to evaluate the ability of sperm to exclude extracellular dyes, such as eosin Y, which are nonpermeable when the membrane is intact.46 Another approach is to expose sperm to hypotonic media (50-100 mOsm range) to test their osmoregulatory function (termed the hypo-osmotic swelling test; HOST).47-49 With this assay, membrane intact sperm theoretically permit excessive water entry into the cytosol, resulting in a variety of morphological changes in the flagellum associated with the cytosolic swelling. Conversely, osmoregulation-incompetent sperm will not experience noticeable changes in flagellar shape. While these tests can serve an adjunctive role in semen evaluation, they are not widely applied in the clinical setting because of the potential for misinterpretation. For instance, the incidence of sperm with a bent flagellum prior to HOST will also confound the interpretation of results following exposure of a sperm population to hypo-osmotic media.

In recent years, fluorescent dyes have generally replaced non-fluorescent dyes for evaluation of plasma membrane integrity. A broad array of membrane-impermeable fluorescent dyes is available commercially and can be used to test membrane intactness. Examples include the DNA dyes, propidium iodide, bis-benzimide (Hoechst 33258), YO-PRO-1, TOTO-1, and ethidium homodimer-1. As an alternative, sperm can be bathed in cell-permeable probes that become hydrolyzed to form membrane-impermeant fluorescent products in the cytosol. Examples include carboxyfluorescein diacetate (hydrolyzed by nonspecific cytosolic esterases to form carboxyfluorescein); calcein AM or dihydrocalcein AM (hydrolyzed by esterases to form calcein, which, in turn, forms fluorescent complexes with Ca²⁺, and other metals) and SYBR®-14 (decayed in the cytosol, with the resulting product expressing strong fluorescence when complexed with nucleic acids). Of interest, staining of porcine sperm with SYBR®-14, which complexes with DNA, does not affect their ability to fertilize oocytes.50 Certain membrane-impermeable and membrane-permeable dyes can be combined in solution prior to sperm exposure in an effort to provide a more accurate reflection of membrane integrity. For instance, a combination of SYBR®-14 and propidium iodide yields three populations of stained sperm: 1) membrane-intact, SYBR®-14-stained cells (green); 2) membrane-damaged, propidium-iodide-stained cells (red), and 3) moribund cells (double-stained).51,52 Some fluorescent plasma-membrane dyes can also be combined with certain mitochondrial dyes or acrosomal dyes to provide more thorough compartmental coverage in the assay.53-59 The literature reports triple-stain fluorescent techniques for use with stallion sperm: propidium iodide/SYBR®-14/JC1,54 and propidium iodide/FITC-PNA (an acrosomal lectin probe)/carboxy-SNARF-1 (an intracellular pH indicator).55,60 Although images can be ascertained with the various fluorophores described above by using fluorescence microscopy, flow cytometry is typically applied because of the high throughput (i.e., many more sperm are counted) and objectivity associated with this approach.59,61-64 Utilization of a fluorescence microplate reader assay is also reported for use with JC-1.55

The relationship between plasma membrane integrity and sperm motility remains unclear. In one study, the percentage of motile stallion sperm (based on computerized motility analysis) was highly correlated (r=0.98) with the percentage of sperm with intact plasma membranes, based on staining with SYBR®-14 and propidium iodide.54 Others have reported that sperm motility declines at a more rapid rate than plasma membrane integrity in extended, cool-stored semen, and that separation between these endpoints is more pronounced when the extended semen contains a high percentage of seminal plasma.66,67 Interestingly, daily centrifugation and resuspension of sperm in fresh extender containing only 10% seminal plasma aids in protecting sperm from declines in sperm motility and membrane intactness following cooled storage for up to 96 h.67 From these studies, it would appear that sperm
motility is more negatively impacted by cooled storage than is plasma membrane integrity. Recently, Kiser et al. reported that percent total sperm motility was significantly lower in extended semen stored for four days in 50% seminal plasma (6%), as compared to 10% seminal plasma (55%) from a single stallion; however, percent viable sperm was similar between the two treatment groups (75 and 74%, respectively). One-cycle pregnancy rates for mares bred with stored semen were similar between semen-treatment groups (45% [5/11] and 58% [7/12], respectively). These results suggest that sperm plasma membrane viability may be a better predictor of fertility than sperm motility for predicting the fertility of cool-stored semen.

Sperm chromatin quality

Assessment of sperm chromatin quality addresses a compartment of sperm that may not be assessed by light microscopic evaluation of sperm motility or sperm morphology, and such assays are routinely incorporated into sperm evaluations in some laboratories. One of these tests is the sperm chromatin structure assay (SCSA). This assay, introduced by Evenson in 1980, has been applied to sperm from a number of species, including horses. This assay has been shown to be a useful predictor of fertility in stallions. The SCSA is a flow cytometric procedure that utilizes the metachromatic fluorochrome, acridine orange, and tests the denaturability of sperm chromatin challenged with acid treatment. The literature contains variable results regarding the relationship of stallion sperm chromatin denaturation to the extent of disulfide bonding within and between protamine molecules, however, chromatin susceptibility to denaturation is correlated with the level of actual DNA strand breaks. The DNA strand breaks can be associated with a myriad of factors, including idiopathic apoptosis, oxidative stress, heat stress, radiation injury, or protamine deficiency, and may involve double-stranded or single stranded DNA fragmentation or oxidized nucleosides. Such lesions could create genetically defective sperm, leading to germ-line mutations. Interestingly, sperm affected by such damage may appear to be normal, based on laboratory parameters such as sperm motility and membrane integrity, but may induce post-fertilization embryonic failure. Owing to the highly condensed nature of the sperm chromatin, mature sperm are known to be transcriptionally inactive, so it is logical that DNA damage might not be expressed until mitosis occurs at the time of spermatozoon-oocyte fusion. This becomes quite important clinically as it represents a potential noncompensible defect, i.e., affected sperm in an ejaculate may not be impaired for fertilization, so increasing the insemination number may not increase pregnancy rate, but may contribute to early embryonic death. Ejaculated sperm are known to retain a cohort of cytoplasmic mRNAs with translational ability, so it is also possible that fragmentation of mRNA could have a negative impact on some other sperm functions leading to reduced fertilization potential.

Assays other than the SCSA are available to measure sperm DNA fragmentation/chromatin disruption, including a TdT-mediated-dUTP nick end labeling (TUNEL) assay, an in-situ nick translation (NT) assay, a sperm chromatin dispersion (SCD) assay, and an electrophoresis-based Comet Assay. While such assays have been applied only on a limited basis in stallions, they are commonly used in the human field. An immunofluorescence assay has also been developed for evaluation of human sperm protamine levels, and a similar assay for equine sperm could have diagnostic value.

Acrosomal responsiveness

Our laboratory has identified a subset of highly subfertile Thoroughbred stallions whose sperm acrosomes do not undergo the acrosome reaction effectively when the sperm are exposed to a potent inducer of this reaction. The underlying mechanism of this defect remains unclear; however, further studies have revealed that the cholesterol-to-phospholipid ratio in sperm of these stallions is significantly higher than that of fertile control stallions. Recently, a susceptibility locus for impaired acrosome reaction (FKBP6) has been discovered in this group of stallions and the frequency of the genotype for impaired acrosomal reaction was estimated to be 7% in Thoroughbreds. Conventional tests are unable to predict the fertility of stallions with this form of acrosomal dysfunction, because these stallions present with normal sperm morphology and motility, as well as normal chromatin quality (based on SCSA
testing) and normal acrosomal structure (based on fluorescence microscopy and transmission electron microscopy). Our laboratory routinely performs an acrosomal responsiveness assay for stallions suspected of having this condition. This assay is directed at testing the functionality of the sperm acrosome, i.e., its ability to acrosome react when challenged with a potent inducer of the event, the Ca\(^{2+}\) ionophore, A23187.\(^{95,96}\) While the acrosome reaction can be identified readily using transmission electron microscopy, fluorescence-based microscopy or flow cytometry using lectin agglutinin isolated from the pea, *Pisum sativum*, or the peanut, *Arachis hypogaea*, can also be used successfully for this purpose.\(^{99-101}\) Although the more popular fluoresceinated lectin markers have generally replaced chlortetracycline-based assays for detecting the acrosome reaction, the latter assay remains useful because of its ability to gauge capacitation, as well as the acrosome reaction.\(^{102-104}\) Others report that acrosomal status can be assessed by bright-field microscopy using Commassie blue stain.\(^{105,106}\)

Meyers and co-workers first reported that the acrosomes of subfertile stallions with poor sperm motility did not react readily in response to progesterone exposure.\(^{107}\) Fertile stallions averaged a 17% acrosomal reaction rate following five hours of incubation in capacitating conditions followed by exposure to progesterone, while subfertile stallions averaged only a 6% response rate. This study indicated that progesterone was capable of stimulating the acrosome reaction in equine sperm exposed to capacitating conditions, and the response in subfertile stallions was reduced. Others have reported that the plasma membrane of stallion sperm contains progesterone receptors, and indicate that this may be a pathway for induction of the acrosome reaction.\(^{108}\) In this regard, the percentage of sperm with exposed progesterone receptors has been shown to have a high correlation with fertility of stallions \((r=0.70)\).\(^{109}\)

Other assays for sperm quality

Considerable effort has been directed toward identification of biochemical markers of sperm function that might aid in laboratory-based prediction of fertility by targeting specific subcellular compartments or domains. Many of these methods have been devised for use with non-equids, but several have been proposed for potential use with stallion sperm. Although the value of such tests requires further scrutiny and standardization, Table 3 provides examples of assays that may one day prove valuable as diagnostic tools:

**Seminal plasma toxicity**

Seminal plasma represents the non-sperm portion of semen, with contributions from the rete testis, epididymis, deferent ducts, and the accessory genital glands. Seminal plasma is of very complex composition, containing a rich assortment of both organic and inorganic constituents.\(^{195-197}\) While it is considered “an essential attribute” to sperm,\(^{196}\) with functions ranging from sperm transport in the female, to modulation of uterine inflammation, oxidative injury, and sperm capacitation, to nutritive support and other protective roles, seminal plasma, as it exists in ejaculated semen, is not essential to fertilization. This statement can be supported by the fact that mares (and females of other species) can become pregnant when inseminated with epididymal sperm that have not been exposed to contributions from the accessory genital glands. Possibly, the contributions of seminal plasma which arise from the rete testis and epididymis are essential to fertilization. Under natural conditions, sperm are exposed to a high concentration of ejaculated seminal plasma for a relatively short period of time. Studies have demonstrated that seminal plasma can adversely affect some properties of sperm following prolonged exposure at elevated concentrations.\(^{67,198-201}\) Significant inter-stallion variation also exists regarding seminal plasma effects on semen quality following either short-term or long-term exposure.\(^{202}\)

Some stallions possess seminal plasma that appears “toxic” to their sperm, as evidenced by a reduction in sperm motility percentage or sperm velocity, as compared to sperm from the same ejaculate following exposure to seminal plasma from another stallion.\(^{203}\) Other stallions ejaculate sperm that are negatively impacted by their own seminal plasma or seminal plasma from other fertile control stallions.\(^{31}\) Stallions with normal semen quality in fresh ejaculates, but rapidly reduced semen quality following cooled storage in extender can be subjected to testing for susceptibility to seminal plasma. Extended semen of the stallion in question is subjected to centrifugation, followed by removal of seminal plasma,
and resuspension of sperm in homologous seminal plasma, seminal plasma of a fertile control stallion, or extender free of seminal plasma. While most commonly used semen extenders do no support good sperm motility if no seminal plasma is present, milk-based extender containing modified Tyrodes medium can be used effectively for extended semen where no seminal plasma is present.200,204 Following such experimentation, one can determine if a stallion’s own seminal plasma is problematic to sperm function, or if all seminal plasma imparts a negative effect of sperm function. Management strategies can then be implemented to improve the reproductive performance of these stallions.31,203

Concluding remarks

Although a plethora of scientific information surrounds sperm structure and function, many unresolved issues remain, even with human and rodent sperm where the largest body of information has been assimilated. Only when we know precisely the specific molecular interactions required for attaining full sperm fertilizing potential, including the spatial and temporal changes, energetics, and gaseous environment involved, will we be able to reliably manipulate sperm to meet the growing needs within the equine breeding industry. While these might appear to be lofty goals, a more absolute understanding of sperm structure and function would certainly take a lot of the “guess work” out of current approaches to analysis and manipulation of equine sperm. Attempts to gain a better understanding of equine sperm solely from extrapolation of data acquired from other mammalian species are likely destined to failure because of the well-known species differences in sperm attributes and physiology. Nonetheless, much information derived from other species may have relevance to equids, and should be investigated for applicability. Examples include identification of candidate genes for specific sperm traits, targeted mutation of genes for specific proteins to study the resulting effect on reproductive function, and use of gene silencing agents for regulatable ablation of gene function. Incorporation of molecular techniques would appear to be the key to elucidation of mechanisms that control sperm function in stallions.

Table 1. Measures of testicular volume, predicted daily sperm output, actual daily sperm output, spermatogenic efficiency, total sperm motility, progressive sperm motility, and morphologically normal sperm in a stallion with a transient, but profound, reduction in fertility.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>27 Apr</th>
<th>19 May</th>
<th>24 Jun</th>
<th>24 Sep</th>
<th>15 Nov</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total testicular volume (mL)</td>
<td>435</td>
<td>459</td>
<td>471</td>
<td>480</td>
<td>427</td>
</tr>
<tr>
<td>Predicted daily sperm output (x 10^9)</td>
<td>9.7</td>
<td>10.3</td>
<td>10.5</td>
<td>10.8</td>
<td>9.5</td>
</tr>
<tr>
<td>Actual daily sperm output (x 10^9)</td>
<td>4.6</td>
<td>2.1</td>
<td>5.0</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Spermatogenic efficiency (%)</td>
<td>47</td>
<td>20</td>
<td>48</td>
<td>70</td>
<td>79</td>
</tr>
<tr>
<td>Total sperm motility (%)</td>
<td>65</td>
<td>55</td>
<td>65</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td>Progressive sperm motility (%)</td>
<td>40</td>
<td>40</td>
<td>30</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Morphologically normal sperm (%)</td>
<td>56</td>
<td>49</td>
<td>42</td>
<td>49</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 2. Evaluation of sperm motility values from a one fertile and one subfertile stallion before, and after, subjecting the semen to density-gradient centrifugation.

<table>
<thead>
<tr>
<th>Stallion</th>
<th>Semen treatment</th>
<th>Total sperm motility (%)</th>
<th>Progressive sperm motility (%)</th>
<th>Curvilinear velocity (μm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile</td>
<td>Before</td>
<td>80</td>
<td>63</td>
<td>205</td>
</tr>
<tr>
<td>Fertile</td>
<td>After</td>
<td>91</td>
<td>78</td>
<td>209</td>
</tr>
<tr>
<td>Subfertile</td>
<td>Before</td>
<td>63</td>
<td>48</td>
<td>251</td>
</tr>
<tr>
<td>Subfertile</td>
<td>After</td>
<td>90</td>
<td>79</td>
<td>259</td>
</tr>
</tbody>
</table>
### Table 3. Examples of biochemical markers that have been used to assess sperm function in various mammalian species.

<table>
<thead>
<tr>
<th>Biochemical marker</th>
<th>Potential value of test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrosin/amidase activity</td>
<td>Acrosome integrity</td>
</tr>
<tr>
<td>SNARE proteins</td>
<td>Acrosome reaction</td>
</tr>
<tr>
<td>Caspases</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Heparin-binding proteins</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Protein phosphotyrosine activity</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Superoxide anion</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Chromomycin A3</td>
<td>Chromatin packing</td>
</tr>
<tr>
<td>Phospholipase Cε</td>
<td>Oocyte activation</td>
</tr>
<tr>
<td>C11-BODIPY(581/591)</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Sperm motility</td>
</tr>
<tr>
<td>Creatine kinase/heat shock protein A2</td>
<td>Sperm maturity</td>
</tr>
<tr>
<td>CRISP proteins</td>
<td>Sperm-oocyte binding</td>
</tr>
<tr>
<td>SP20/hauluronidase</td>
<td>Sperm-oocyte interaction</td>
</tr>
<tr>
<td>AWN spermadhesion protein</td>
<td>Sperm-zona binding</td>
</tr>
<tr>
<td>P34H protein</td>
<td>Sperm-zona binding</td>
</tr>
<tr>
<td>Zonadhesin</td>
<td>Sperm-zona binding</td>
</tr>
<tr>
<td>SP22 protein</td>
<td>Sperm fertility</td>
</tr>
</tbody>
</table>

### References


75. Makhlouf AA, Niederberger C: DNA integrity tests in clinical practice: it is not a simple matter of black and white (or red and green). J Androl 2006;27:316-323.


