

Sperm Function Tests in the Stallion

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Introduction:

There are many tests that describe semen quality in the stallion which include sperm motility, morphology, and longevity of motility and determination of total sperm numbers. In addition, recently, the flow cytometer (FC) has been used to evaluate various sperm compartment following staining with specific fluorescent probes that bind to different compartments of the sperm. Our lab is interested in developing sperm function tests that evaluate sperm compartments as well as determining how these tests can be applied clinically to the diagnosis of stallion subfertility. The sperm compartments that we are currently evaluating include the sperm DNA, the membrane integrity of the head and midpiece mitochondria, and the ability of the acrosome to react. The evaluation of these compartments allows the clinician additional diagnostic latitude when faced with a stallion that has a history of subfertility.

Evaluation of sperm morphologic features:

Evaluation of sperm morphologic features is an integral part of the stallion breeding soundness examination. The evaluation is usually accomplished by counting the percentage of normal sperm as well as identifying those sperm that exhibit abnormalities. A fixed semen sample is the most representative sample the referring practitioner can provide because it is preserved immediately after collection and is therefore less subject to handling and processing artifacts that may affect sperm motility. A recent study in our lab determined the relationship between the specific sperm morphologic features (abnormal head, detached head, etc) and fertility in 17 Thoroughbred Stallions.

How we use this assay:

We are interested in all of the morphologic features (normal and abnormal) that are present in the ejaculate particularly those associated with a decrease in fertility (abnormal heads, detached heads, abnormal midpieces, coiled tails, and premature germ cells). For example, two stallions may have 40% normal sperm, one because of a high percentage of distal droplets and the other because of a combination of abnormal heads, detached heads, abnormal midpieces, and coiled tails. The fertility status of the latter stallion would be expected to be worse than the former.

We are very cautious about individual abnormalities that occur in large numbers and are not associated with increases in other abnormalities. Examples of this include samples that have high percentages of sperm heads with subtle abnormalities, detached heads, hairpin tails, proximal or distal droplets. When these abnormalities occur by themselves it may indicate a non-testicular origin or an artifactual change.

A **high percentage of abnormal heads** is unlikely to occur by itself in the absence of other abnormalities. Formation of head shape occurs in the testicle and disruption of this process commonly results in the presence of other abnormalities. The stallion tends to have more heterogeneity in their head shape than the bull or ram and the variability can be interpreted as abnormal rather than simply normal variation. **Detached heads** in large numbers are more commonly associated with blockage of the ampullae rather than testicular pathology. Conversely, small percentages of detached heads tend to be associated with testicular disruptions. The significance of **proximal droplets** in large numbers is equivocal. It is unclear the effect this abnormality might have on the longevity of sperm that is cooled or frozen. **Distal droplets** tend to be associated with stallions that have not been ejaculated for a period

of time and are at sexual rest. Once these stallions are ejaculated for several days in a row this abnormality tends to get “flushed out”. Initially, this abnormality can occur in large numbers thereby lowering the percent of normal sperm. There are unusual cases where this abnormality persists despite frequent ejaculations. This has the potential to reduce the longevity of cooled semen because of the tendency of the tail to reflect around the droplet, resulting in the hairpin tail defect. These sperm may be motile, but will swim backwards.

Evaluation of sperm morphologic features is an essential part of the stallion breeding soundness evaluation. The interpretation relies on the fact that all abnormalities are not equal with respect to their effect on fertility, therefore, it is important to identify specific abnormalities and base your interpretation on the types of abnormalities identified rather on just percent of normal sperm present.

How is it used?

Sperm morphology may be the single most important method for evaluation of sperm quality. Sperm motility is an important measure of sperm quality, but should not be used alone when routine sperm evaluations are performed. There are sperm abnormalities that will reduce fertility, but can nevertheless be motile, resulting in a situation where sperm motility may appear good, but actual sperm quality is reduced when all sperm abnormalities are identified. Morphologic samples also have the advantage that the raw semen can be fixed on the farm and can be evaluated at a later time and place or sent to a referral institution for consultation. These samples are used for general evaluation of sperm quality as well as quality control evaluation of cooled-shipped semen. In the case of the latter, one of the primary concerns with the use of shipped semen is the initial handling and proper preparation of the sample to be shipped. Motility is the primary endpoint affected by improper handling, but the percent of morphologically normal sperm does not change. Therefore, if motility is low but the percent normal sperm is high, it suggests that modifications can be made to the handling technique to maximize motility.

Sperm Chromatin Structure Assay (SCSA)

How does the test work?

The Sperm Chromatin Structure Assay (SCSA) uses a metachromatic (fluoresces two colors depending on what type of DNA it is attached to) dye, acridine orange to evaluate the ratio of single (abnormal) and double-stranded (native) DNA present in individual sperm. The dye attaches to the DNA and fluoresces red when bound to single-stranded DNA and green when bound to double-stranded DNA.

Since double-stranded DNA is the normal state of sperm DNA, single-stranded DNA is considered abnormal. Individual sperm emit a certain amount of both green and red fluorescence, with DNA from normal sperm emitting mostly green and little if any red. The results are represented in the form of a scatterplot (Figure 1) with green fluorescence on the Y-axis and red along the X-axis. Since normal sperm (main population) emit mostly green fluorescence they form an elliptical shape on the left side of the plot. Sperm that lose green fluorescence and gain red (single-stranded DNA) are to the right of this population and are considered abnormal. Those sperm are termed the **Cells Outside the Main Population (% COMP)**. Further analysis creates the term alpha-t which is the amount of red fluorescence divided by the amount of red + green for each sperm (i.e. the percentage of red emitted).

This value allows for the two dimensional description of the sperm on the scatterplot as well as the determination of the mean and standard deviation of alpha-t.

What do the results mean?

An increase in the %COMP is associated with reduced fertility in stallions.

How is it used?

Based on previous research we categorize stallion fertility in one of three categories based on the %COMP. This is used clinically to determine whether a stallion whose fertility is in question has a %COMP that is consistent with the level of fertility that is reported. Many stallions that are presented with a history of subfertility are subfertile due to causes unrelated to sperm quality, therefore, it is critical for sperm function tests to reliably describe and identify sperm samples that are of high quality to allow the clinician to focus on other areas that might be the source of the subfertility such as management and mare quality. In effect, we want to determine whether the stallion's level of sperm quality is consistent with the level of fertility that he is exhibiting.

Quality control of shipped semen:

Stallions that have a normal level of %COMP in their fresh semen may exhibit an elevation when their sperm is chilled for an extended period of time. Previous work in our lab indicates that semen that is processed correctly (adequate semen/extender ratio, no exposure to toxic substances, etc.) for shipment will have essentially no change in the DNA after 24 hours of storage at 5°C. When we evaluate shipped samples that exhibit a high degree of change in DNA quality after storage it strongly suggests that the semen has not been handled in an optimal manner at the time of processing. One reason for this decline in DNA quality is due to the level of seminal plasma in the extended sample. We have found that as the level of seminal plasma increases the quality of DNA declines in an almost linear fashion. Therefore, seminal plasma level by itself is sufficient to reduce the level of DNA quality in a shipped semen sample. If a skim-milk based extender is used we recommend that no more than 10% seminal plasma is included in the extended sample. If semen is diluted based on a ratio (i.e. 1:1; 1:2, etc) the level of seminal plasma is 50 and 33%, respectively. Some stallions consistently give dilute ejaculates and in these cases semen can be centrifuged and seminal plasma can be reduced or removed.

Some stallions that exhibit higher levels of compromised DNA in fresh semen also have an *accelerated rate* of decline in DNA quality when the sperm are chilled and stored over time, which is independent of the level of seminal plasma present. The DNA from these stallions appears to have an *inherent sensitivity* to storage and other environmental conditions that results in a decline in the DNA quality. The sperm from these stallions will also be inherently less tolerant to poor semen handling technique and other influences that the sperm from more fertile stallions are resistant to.

This test is useful for describing the expected fertility level for stallions that are presented as suspected subfertile cases. In addition, we also recommend this test for use in determining the suitability of a particular stallion's sperm for cooling and evaluating the semen handling practices that are used and whether they maintain a high level of sperm quality after storage.

Sample collection and processing for the SCSA:

Practitioners that are interested in submitting a sperm sample for evaluation can freeze either a *raw* or *extended* sample *immediately* after collection. The sample can be frozen either on dry ice or liquid nitrogen and then shipped to the laboratory for evaluation by flow cytometry. Samples should *not*

be stored in *frost-free type freezers* that go through freeze-thaw cycles, which will damage and induce lesser quality DNA.

If shipped semen samples are to be evaluated a sample should be frozen immediately after ejaculation, as described above. In addition, a sample should be sent to the laboratory using the conditions (i.e. similar extender, shipping container, etc.) routinely used by the practitioner. Upon arrival at the lab an aliquot of this sample is frozen and evaluated using the SCSA and compared to the sample that was frozen immediately after collection. We also recommend that a sample fixed in buffered formol saline (BFS) also be shipped, at ambient temperature, as well.

Evaluation of membrane integrity (viability)

How does the test work?

This test uses three fluorescent probes (propidium iodide, JC-1 and SYBR-14) to evaluate the membrane integrity of the sperm head and midpiece mitochondria. The **SYBR-14** acts by penetrating only an *intact* sperm head membrane and then attaches to DNA and fluoresces green, therefore, identifying an intact sperm head membrane. **Propidium iodide** penetrates only *disrupted* membranes and then also attaches to the DNA and fluoresces red. The **SYBR-14/propidium iodide** combination, therefore identifies two sperm populations, one that is membrane intact and one that has a disrupted membrane. **JC-1** evaluates the *membrane potential* of the mitochondrial membrane and does so by fluorescing red/orange when the potential is high and green when the potential is low.

How is it used?

This assay can be used for the evaluation of fresh, cooled, and frozen-thawed sperm. A recent study in our lab has determined the applicability of these dyes for use with cooled semen to determine the relationship between sperm motility and membrane integrity. We have found that sperm from highly fertile stallions have similar levels of membrane integrity and total sperm motility after 24 hours of storage, however, when these same samples are evaluated for up to 7 days of storage the motility declines, but membrane integrity is maintained for a longer period of time. These results suggest that motility and membrane integrity exhibit a certain degree of independence after storage for long periods of time and further suggests that membrane integrity is a more resistant sperm compartment than is sperm motility.

This test has particular application for evaluation of cooled semen and samples that exhibit low motility, to determine whether these samples maintain membrane integrity or have lost their membrane integrity along with their motility. Clinically, it appears that many shipped samples with lower than expected motility retain a high level of membrane integrity which suggests that improved processing of the sample after collection could also improve the motility. The retention of membrane integrity in the face of lowered motility could also explain why some stallions appear to have good fertility even though their sperm does not cool well and motility appears poor if evaluated after 24 hours. The sperm from these stallions could simply be temporarily inactivated in terms of their motility rather than being dead.

Sample collection and processing for the membrane integrity:

These samples should be processed (cooled and shipped) as they normally would to the client. Sperm can be evaluated in the fresh state (immediately or after cooling and shipping) or in the frozen

state (after addition of egg yolk and glycerol). Raw samples cannot be frozen similar to the handling for the SCSA since sperm membranes are disrupted and damaged after the freeze-thaw process.

Evaluation of acrosome function

How does the test work?

This test relies on the specific binding of the lectin, *Pisum sativum* agglutinin (PSA) to the acrosome contents and the green fluorescence of the fluorescein isothiocyanate (FITC). The FITC-PSA combination allows for a qualitative and quantitative evaluation of the presence of the acrosome contents. An acrosome intact sperm will emit maximal green fluorescence because of the presence of the acrosome contents. As the acrosome reaction occurs these contents are released and the amount of green fluorescence decreases. Therefore, a sperm that has completely reacted has lost most of its contents and will exhibit minimal green fluorescence compared to the intact sperm.

How is it used?

Our lab uses the calcium ionophore (A23187), a potent inducer of the acrosome reaction, to evaluate the ability of the acrosome to react. So we are able to determine the *initial percentage* (before exposure to A23187) as well as determine the capability of intact sperm to undergo the acrosome reaction. Sperm populations from fertile stallions respond to ionophore stimulation by reacting almost completely (80% acrosome reacted) after a 2-3 hour incubation time. In contrast, there is a subset of subfertile stallions whose sperm do not react. These stallions are unique because when a routine sperm evaluation is performed, motility, morphology tend to be very normal, making it difficult to determine the cause of their subfertility based exclusively on the routine measures of semen quality.

Sample collection and processing for the membrane integrity and acrosome function:

Samples that have been collected and extended to 25 million sperm/ml with a skim milk based extender can be cooled and shipped for evaluation.

Conclusion:

This presentation gives the practitioner an overview of recently developed sperm function tests that use flow cytometry to identify and evaluate the functional status of specific sperm compartments. These compartments include the sperm DNA, sperm head and mitochondrial membranes, and the acrosome. Evaluation of these compartments allows the clinician to more critically identify the source of reduced fertility in the stallion by describing the functional status of multiple sperm compartments from an individual stallion. The intent of these studies is to describe the sperm compartments from fertile stallions and thereby be able to evaluate that same compartment from suspected subfertile stallions and diagnose the compartmental defect or isolate a non-sperm source of the problem.