Assessment of Sperm Quality

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1. Introduction
During spermatogenesis, spermatozoa lose many of the organelles that most somatic cells possess (such as endoplasmic reticulum, lysosomes, and most of the cytoplasm) reducing the “baggage” they need to carry en route to the oocyte. The primary function of a spermatozoon, fertilizing an oocyte, is an integrated set of processes, which require multiple cell attributes. Therefore, although the spermatozoon contains few organelles, it remains a complicated cell, having multiple cell compartments, membrane compositions, and subcellular structures, all of which must be functioning properly for the spermatozoon to fertilize an oocyte. To complicate matters further, and of particular interest to the practitioner, during spermatogenesis the DNA of the cell is condensed in such a manner that genes cannot be expressed, hence no proteins can be made by the spermatozoon. This means the cell cannot repair itself from any cellular damage that occurs naturally or due to human interventions (semen handling, cooling, or cryopreservation). Improper handling of spermatozoa can permanently damage spermatozoa, making them infertile.

The stallion spermatozoon consists of 3 major anatomical regions, the head, the middle piece, and the principle piece or tail, all of which are surrounded by a plasma membrane (for a detailed discussion of spermatozoal anatomy and function see Amann and Graham). The head contains the cell nucleus consisting of the cell's chromosomes tightly packaged by protamine proteins which replace histones during spermatogenesis; and the acrosome, a membrane bound bag of enzymes at the anterior portion of the head which are necessary for the sperm to penetrate the zona pellucida of the oocyte. Disruption of the acrosome prior to the sperm being already bound to the zona pellucida renders a spermatozoon incapable of penetrating the zona pellucida and subsequently fertilizing the oocyte.

The middle piece of the spermatozoon contains the mitochondria of the cell and provides the means of converting sugars, glucose primarily, into ATP which is utilized by the fibers of the principle piece to cause the movement of the sperm. Destruction of the mitochondria, either by physical damage (from improper cooling or freezing) or chemical damage (improper pH or osmolality) can render the spermatozoon incapable of producing the ATP necessary to fertilize an oocyte. Likewise, damage to the principle piece, will alter the ability of the spermatozoa to swim to the oocyte, penetrate the zona pellucida and fertilize the oocyte.

In light of the complex nature of the stallion spermatozoon, analysis of sperm function is also more complicated than we generally acknowledge. In this article, I will discuss what analyses can be used
to evaluate the various sperm functions and how these analyses can be used by practitioners.

2. What do Semen Analyses Measure?

Differences in the fertility of semen from different sires exist, as well as differences between fresh and frozen-thawed semen from the same sire. Laboratory assays for semen samples have been developed to evaluate the various characteristics of a semen sample, with the expectation that the analysis will provide some indication of the fertilizing potential of the sample. However, a review of the literature indicates that no laboratory assay reliably correlates with fertility. The correlations between the fertility of bull semen samples and results from laboratory assays range tremendously, for the various studies reviewed (correlations between fertility and morphological assays ranged from 0.06 to 0.86; and correlations between fertility and cell viability ranged from 0.33 to 0.66).

Part of the problems in correlating laboratory results with fertility is that fertility is affected by the number of spermatozoa that are inseminated into females. As the number of sperm inseminated increases, fertility increases until it reaches a maximum. Unfortunately, not only does the ultimate maximum fertility for each sire differ, but the rate at which fertility increases for each sire also differs. Therefore, the fertility ranking of a sire is dependant upon the number of sperm used for insemination. Part of the problem in correlating laboratory results with fertility also results from inherent problems in the laboratory assays themselves. For a laboratory assay to be useful it must be: 1) objective—have little error due to human judgment or bias; 2) repeatable—produce similar results when the assay is repeated; 3) accurate—evaluate a sperm attribute precisely; 4) rapid; and 5) inexpensive. Unfortunately, many of the laboratory assays used do not fit these criteria. Finally, because spermatozoa are complex cells, which need to possess many attributes (motility, an intact acrosome, ability to bind to the zona pellucida, ability to bind to the oolemma, etc.) in order for them to fertilize an oocyte, sperm may be infertile for a variety of reasons (one sperm may be infertile due to a lack of motility, while another due to damaged acrosomal membranes, while another due to nuclear aberrations), therefore, results from any laboratory which measures a single sperm attribute (i.e., motility) will produce results that do not correlate well with fertility. Although results from laboratory assays may not be able to evaluate actual fertility of semen samples, they do permit us to detect samples that are likely to possess low fertility. The remainder of this article will discuss some of the laboratory assays available, and what the results from these assays can tell us.

3. Sperm Motility

Visual estimates of the percentage of motile sperm in a semen sample is the most common laboratory assay utilized on a routine basis, and very likely the only assay conducted on the vast majority of semen samples. Although useful, the percentage of motile sperm in a semen sample is not highly correlated with the fertilizing ability of the sample.

The recent availability of computer assisted sperm analysis (CASA) systems to evaluate sperm samples have reduced some of the variability in analyses and permit the accurate evaluation of not only the percentage of motile sperm, but also percentages of progressively motile cells and the velocities of the motile sperm. However, these analyses still evaluate only a single sperm attribute and results are not highly correlated with fertility.

4. Sperm Viability (Plasma Membrane Integrity)

The percentage of viable sperm in a semen sample, defined as the percentage of sperm that possess intact plasma membranes and can therefore keep large molecules (stains) out of the cell, can be evaluated using a variety of stains. Classical stains, such as eosin, have been used for decades to analyze dried smears. These stains are often used in combination with nigrosin or aniline blue, used as “background” stains to provide contrast for “live” sperm not stained with eosin. More recently, fluorescent stains have been developed, such as propidium iodide, Hoechst 33258, and diamidino-phenylindole (DAPI), which provide greater contrast between “live” and “dead” cells, but require a microscope with fluorescent capabilities to analyze the spermatozoa. Recent developments in flow cytometry and fluorometry make viability assessments rapidly and inexpensively.

5. Acrosomal Integrity

Even though a spermatozoon may be viable and possess progressive motility, if its acrosome is damaged or lost, it will not be able to fertilize an oocyte. The acrosomal membranes are preprogrammed to undergo membrane fusion and are therefore, the most labile membranes of the sperm. This means that they are also the most susceptible to damage due to mishandling of the sperm, cooling and freeze-thaw procedures. The size and shape of the stallion sperm acrosome make it difficult to analyze using conventional microscopy, but fluorescently labelled lectins from the pea (Pissum sativum agglutinin) or the peanut (Arachis hypogea agglutinin) can be used to visualize the stallion sperm acrosome.

6. Sperm Morphology

Several classification systems have been developed for the analysis of stallion sperm morphology. Light microscopic evaluation of unstained or stained sperm can be used to assess sperm. High percentages of abnormal spermatozoa in a semen sample
have been correlated with lower fertility in the bull.\textsuperscript{20}

Recently, Fourier harmonic analysis was used to analyze the shape of the nuclei of bull sperm.\textsuperscript{21} In this technique, sperm are stained with the fluorescent DNA probe YOYO-1, digital fluorescent images of the sperm collected, and the shape of the nuclei of the cells analyzed using Fourier harmonics. Using this objective analysis procedure, a population of bulls was detected that had a high probability for low fertility.\textsuperscript{21}

In addition to evaluating the shape of sperm nuclei, the susceptibility of chromatin to denaturation when sperm are incubated under denaturing conditions, can also be evaluated. This sperm chromatin structure assay (SCSA) was first developed by Evenson and coworkers\textsuperscript{22} using the stain acridine orange. They found that bull, mouse, and human sperm from males with lower fertility exhibited greater amounts of chromatin denaturation than did sperm from high fertility males.\textsuperscript{22} Subsequent studies using bull sperm\textsuperscript{23–25} and stallion sperm\textsuperscript{26} showed similar results. Therefore, the SCSA provides another measurement of a sperm attribute, which will help us to more completely assess the number of sperm in a semen sample that are capable of fertilizing an oocyte.

7. Sperm Capacitation and Induction of the Acrosome Reaction

Several recent studies indicate that the ability of spermatozoa to undergo capacitation and the acrosome reaction in vitro may be related the fertilizing potential of the male.\textsuperscript{26–29} However, data are preliminary at this point and these assays are difficult to perform, making such assays impractical for the general practitioner at this time.

In vitro assays, which evaluate the ability of spermatozoa to bind to the zona pellucida, to bind to homologous or heterologous oocytes, or to fertilize homologous or heterologous oocytes can also be conducted. These assays require significant expertise and can be relatively expensive to conduct. Some of the pitfalls involved with these assays are that they do not evaluate the motility of sperm or their ability to survive in in vivo conditions. In addition, few sperm, usually less than 10, are actually evaluated in these assays.

8. Simultaneous Evaluation of Sperm Attributes

In addition to monitoring cell viability and acrosome integrity using fluorescent probes, we can also evaluate mitochondrial activity in the sperm. Thomas et al. recently showed that the mitochondrial stain JC-1 can differentiate between poorly functional mitochondria in sperm and highly functional mitochondria.\textsuperscript{30} JC-1 is actively transported from the extracellular fluid into the interior of functioning mitochondria. The higher the metabolic activity of the mitochondria, the more JC-1 is internalized. JC-1 can differentiate between poorly functional mitochondria (staining green) and viable mitochondrial activity in the sperm. Thomas and coworkers\textsuperscript{22} using the stain acridine orange. JC-1 is actively transported from the extracellular fluid into the interior of function- ing mitochondria. The higher the metabolic activity of the mitochondria, the more JC-1 is internalized. In the monomeric state, JC-1 fluoresces green, how- ever, as the concentration of JC-1 inside the mitochondria increases, the stain forms aggregates which fluoresce orange.\textsuperscript{30} Therefore, using JC-1, we can distinguish between cells having highly functional mitochondria (staining orange) from cells having weakly functional mitochondria (staining green).

Using propidium iodide, fluorescently labeled lectins and JC-1, several sperm attributes can be evaluated simultaneously. This permits cells to be categorized into more refined populations, which may be useful in differentiating between semen samples. For example, examining the data for only 4 of the 12 stallions that were analyzed to develop this assay,\textsuperscript{31} the stallions can be separated into 2 groups based upon differences observed in the percentages of motile cells (Table 1). When these same semen samples were evaluated for the percentages of live-acrosome intact sperm in each sample, using flow cytometry, they could be separated into three distinct groups. Finally, when these same samples were evaluated for the percentage of live-acrosome intact sperm having high mitochondrial function in each sample, the samples into 4 separate catagories.

9. Conclusion

Laboratory assays have been developed which evaluate multiple sperm parameters on large numbers of sperm in a semen sample.\textsuperscript{10,11,31} These assays may prove useful in evaluating semen prior to insemination, or at the beginning of the breeding season. They will also be invaluable in developing new techniques for preserving stallion semen. Although the ability of these assays to predict fertility has not been established, recent data indicate that utilizing several assays that evaluate different attributes of the spermatozoa can be highly correlated with stallion fertility. Although the correlation of fertility with the percentage of motile cells is not very high, when this sperm attribute is utilized with the percentage of live-acrosome intact cells (measured using flow cytometry), the correlation with fertility approaches 0.8. In addition, when mitochondrial function is added into the equation, along with sperm motility and viability, the correlation

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<th>Stallion</th>
<th>Percent Motile</th>
<th>Percent Live-Acrosome Intact</th>
<th>Percent Live-Acrosome Intact with High Mito. Function</th>
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<td>D</td>
<td>15\textsuperscript{b}</td>
<td>28\textsuperscript{c}</td>
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\textsuperscript{a,b,c,d}Column means with different superscripts are different.
with fertility is even better. It should be pointed out that correlating sperm attributes with the fertility of semen samples is very different than predicting the fertility of semen samples, and studies need to be conducted to determine if measuring these attributes can be used to accurately predict the fertilizing potential of semen samples. The question might be asked, which assay or assays are the best to use, when evaluating a semen sample? My answer would be, ones that practitioners will use. For even if a series of assays is developed that accurately predicts the fertility of a semen sample, if no one will conduct those assays, nothing will be accomplished to increase the quality of semen being used in industry. It is my opinion that visual motility estimates should be made on every semen sample, at the very least, as this will identify semen samples that are likely to have low fertility. Evaluating the percentage of viable sperm in samples will be the next likely most important assay to conduct. Additional assays evaluate the mitochondrial function of cells and spermatozoal velocity will likely help the practitioner in identifying semen samples that are likely to have poor fertilizing potential.

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References and Footnote

*Kirk, unpublished results.