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Several insights on evaluation of semen

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Abstract

There are many procedures available for evaluating semen, and except for culling obviously inferior samples, none of the procedures is highly correlated with fertility. The most commonly used method is estimating sperm motility subjectively, and this method is appropriate for culling substandard semen; this approach also can be used for more stringent quality control and for experiments if done "blindly" by well trained persons. However, computer-assisted sperm analysis is more objective. Currently, the bovine AI industry frequently uses flow cytometry to measure sperm cell membrane integrity; this is an excellent procedure for evaluating sperm that is rapid, precise, objective, and reasonably correlated with fertility. *In vitro* fertilization would seem to be an excellent functional assay of spermatozoa, but results are not always well correlated with *in vivo* fertility, probably in part due to artifacts of *in vitro* capacitation. A rarely used method of evaluating sperm is competitive *in vivo* fertilization, which is very sensitive for detecting treatment or bull-to-bull differences in fertility. However, it requires genetically marking sperm and then evaluating embryos, fetuses, or offspring. The competitive fertilization approach is particularly useful for evaluating sexed semen because sex can be used as the genetic marker, treatments can be evaluated within the same male, and fetuses of cattle and horses are easily sexed non-invasively via ultrasound.

Keywords: cattle, morphology, motility, sperm.

Introduction

All methods of evaluating semen are imperfect, but semen requires evaluation primarily in three situations: 1) culling semen that does not meet some minimum standard, 2) evaluating whether semen that met the minimum standard is suitable for a specific application, and 3) evaluating semen to test hypotheses in experiments. Examples of applications are in Table 1.

I will concentrate on bovine semen, but most principles apply to semen of the majority of mammals. More research has been done with bovine semen than with that of any other species, and bull semen has one other advantage in that most measures of bovine semen quality correlate more reliably with fertility than similar measures in other species, in which it is not unusual, for example, for sperm to have excellent motility but very poor fertility.

Table 1. Applications of bovine semen.

Application	Comments
Natural mating	Breeding soundness examinations
AI with fresh semen	Can be stored at various temperatures for various times
AI with frozen semen	Thawing procedures can greatly affect end quality
AI with sexed semen	Sorting sperm causes some damage to sperm
Superovulation	Quality of semen much more critical than without superovulation
Estrus synchronization with timed AI	Timing of ovulation and insemination less synchronized – excellent quality semen essential
IVF	Can be with fresh, cooled, frozen, sexed, etc.
ICSI	Motility less critical
Refrozen semen	Requires special freezing procedures
Use of substandard semen for any of the above	For genetic reasons lowered fertility may be acceptable
Genotyping	Dead but not decomposed sperm sufficient
Combinations of the above – e.g. frozen, sexed semen, superovulation, timed AI	Semen of some bulls will not tolerate multiple insults
Experiments	Precision via replication required

Measuring sperm motility

There are literally dozens of ways to evaluate semen (Table 2), and it is desirable to evaluate semen at a number of timepoints (Table 3). The most used method of evaluating semen is to measure sperm motility, which would seem to be relatively easy to do, but in practice can be quite complicated to do correctly. For some purposes, such as a breeding soundness examination to determine if a bull is suitable for natural

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service, measurement of motility need not be particularly precise, while for most research, precision is important. Even if precision is not needed, attention to certain details is important. For example, conditions need to be standardized for any measurement of motility including temperature, sufficient dilution of semen with a suitable extender or other medium so that individual sperm can be observed easily (about 10 x 10^6 sperm/ml works well), thickness of the sample under the coverslip, etc. A phase contrast microscope is ideal, although for sperm in some extenders such as milk, fluorescence microscopy after labeling the sperm with a fluorescent dye is an excellent alternative. In my opinion, progressive motility, i.e. the sperm's proceeding in a forward progression at a reasonable speed, is the most appropriate form of motility to evaluate. While other forms of motility (such as circular movement, tails moving but with little or no forward movement, total motility, etc.) may be important for various research purposes, or for selecting sperm for ICSI, non-progressive motility is generally not a good measure of potential fertility.

Table 2. Selected procedures for evaluating semen.

Method	Comments
Subjective motility	Unreliable unless done "blindly"
Computer-assisted sperm analysis (CASA)	Objective, but means are highly dependent on which CASA parameters are selected
Morphology	Excellent for screening, values above 70% normal not highly correlated with fertility
Cell membrane integrity (live/dead)	Measured via flow cytometry or with a microscope
Acrosome status	Measured via flow cytometry or with a microscope
DNA integrity	Evaluation of single and double stranded DNA breaks
Osmotic swelling	Stress test
Zona binding	Oocytes from slaughterhouse ovaries ideal
*IVF - pronuclei	Timing critical
*IVF - cleavage	Best to control for parthenogenesis
*IVF - blastocyst development	Robust measure of sperm quality
ICSI	Abnormal sperm can produce embryos
Pregnancy rates	Requires hundreds of matings per treatment for reasonable experimental power
In vivo competitive fertilization	Requires genetic markers; especially useful for sexed semen

*While IVF can be very useful for evaluating semen, results do not always correlate with in vivo fertility, especially because of inefficient capacitation of sperm *in vitro*.

Table 3. Critical time points for evaluating semen.

Timepoints
After semen collection from the bull
After cryopreservation
Upon receiving a semen shipment, especially if not from a major bull stud
At the time of breeding valuable animals such as superovulated cows
Upon initiating IVF or ICSI
At the end of co-incubation with oocytes for IVF
For experiments, ideally 15-30 min post-treatment or post-thaw, and again after incubation at 35-38°C for 90 to 150 min.

For experiments, there often is a concern, which I share, about subjective motility estimates. However, if done correctly, such estimates are in my opinion as valid as those done objectively with computer-assisted sperm analysis (CASA), and in some cases may be superior. Choosing CASA parameters needs to be done carefully (Brito, 2010). Evaluating subjective motility correctly absolutely requires 1) an experienced and well-trained evaluator, and 2) evaluating samples "blindly." The well-trained evaluator knows not to evaluate sperm sticking to debris or in clumps, or along edges of coverslips, etc. When samples are not evaluated "blindly," they are suspect. Ideally, subjective motility is evaluated by two persons, and the average used after checking that overall treatment mean differences are similar in magnitude for both evaluators, even if absolute means differ somewhat. I want to emphasize that CASA also is an excellent approach to measuring sperm motility, because it is objective, plus many aspects of sperm motion can be measured simultaneously (Brito, 2010). However, the most important motility parameter, progressive motility, also

can be evaluated subjectively with validity.

I will not discuss the myriad of issues involved in obtaining valid data with most other methods in Table 2, as appropriate procedures can be obtained from the scientific literature. I do point out, however, that semen collection procedures are important, such as standardizing sexual preparation of the male, examining semen for contamination with urine or feces, recognizing that electroejaculation produces more heterogeneous samples than collection with a properly prepared artificial vagina, etc. Also, although not strictly a method of evaluating semen quality, ejaculate volume, sperm concentration, the interval since the last semen collection, accurate identification of the bull, etc. should usually be recorded.

Use of flow cytometry to measure cell membrane permeability

The newest method of evaluating semen that has been widely adopted is use of flow cytometry to evaluate cell membrane integrity/permeability (Christensen *et al*., 2005). This procedure has two huge advantages: 1) thousands of sperm can be evaluated in a few seconds, and 2) it is objective, and results are correlated with fertility. Bull studs worldwide have adopted this procedure for routine semen evaluation.

Competitive fertilization for assessing fertility *in vivo*

I will explain the method of competitive fertilization in more detail. This involves mixing semen of two (or more) males prior to insemination, and determining via genetic marking, which male sired embryos or offspring. Note that this also can be done by marking sperm in other ways and using in vitro fertilization, but I will not go into those details.

Competitive fertilization can be used for two distinctly different objectives: 1) comparing fertility among bulls, and 2) studying treatment differences. There are two major advantages to this approach: 1) the sperm of the two animals or treatments are competing in an identical environment, so which sperm fertilize the oocyte should be due to the sperm characteristics, not some environmental effect, and 2) many fewer animals are needed to test hypotheses than without competitive fertilization, perhaps only 1/10 as many animals. What occurs is that small differences in fertility among treatments or bulls become large differences with competitive fertilization. For example, Schenk *et al*. (2009) reported an experiment comparing two pressures in the flow cytometer for sexing sperm. Sperm at the lower pressure (30 psi) were responsible for 82% of the pregnancies, with only 18% of the pregnancies due to the equal number of sperm in the inseminates at the higher pressure (50 psi). These data were obtained from only 67 pregnant heifers. Competitive fertilization resulted in a huge difference compared to the approximately 10%-point difference, that one might expect with homospermic insemination (Schenk *et al*., 2009). In other words, a 10%-point difference was turned into a 64%-point difference with this approach. This does illustrate a major constraint with competitive fertilization, which is that although efficient in determining which treatment or bull has the highest fertility, the magnitude of the difference is almost meaningless. Another constraint of the technology is the need to mark the embryos, fetuses, or calves genetically to determine which sperm fertilized the ovum. The genetic marker can be determined with molecular biology approaches from recovered embryos, amniocentesis (or allantocentesis) of the pregnancy or the resulting calves, or calves can be genetically marked by coat color. Of course, waiting for calves to be born delays obtaining results. For experiments with sexed semen, there is a huge advantage because conceptuses can be genetically marked by sex - X-sperm for one treatment and Y-sperm for the other (and the reciprocal for half the inseminations), and experimental treatments can be done within bulls. Sexing the fetuses at 2-3 months of gestation then provides the information about which treatment was best (Schenk *et al*., 2009).

If there is no significant treatment difference, that also is valuable information obtained economically. Note that it always is necessary to do reciprocal bull X treatment matings (except for sexed semen where only one male is used and X and Y sperm have equal fertility; Barcelo-Fimbres *et al*., 2011) because there usually are male-to-male differences with competitive (heterospermic) fertilization (Robl and Dziuk, 1988), so bull A, treatment X and bull B, treatment Y would be used for half of the inseminations, and the reciprocal for the other half.

For evaluating fertility of bulls, it is easy to compare two bulls but more complicated if more bulls are involved, when an index needs to be calculated (Saacke *et al*., 1980). The strategy that I suggest is to have a reference bull of known average fertility and compare that bull to those to be tested by using half of the sperm from the reference bull and half from any bull to be tested for each inseminate. The outcomes (statistical significance determined by χ^2) then would be that the test bull would be superior, about the same, or inferior in fertility to the reference bull. Depending on the objectives, the reference bull might be chosen for fertility slightly below average (likely most appropriate for most situations), average, or slightly above average.

One other potential problem with competitive fertilization is that a particular treatment may simply result in a shorter capacitation time, and therefore fertilization may occur sooner than for sperm given a different treatment. Faster capacitation might not be associated with higher fertility. This problem has not been seen to date with competitive fertilization, but could lead to incorrect conclusions under some circumstances.

In vitro fertilization

A final assay for sperm quality that I want to expand upon briefly is *in vitro* fertilization. Procedures for testing bull or treatment fertility for IVF purposes is relatively straightforward and inexpensive – one simply tests the semen with oocytes from slaughterhouse ovaries. The problem is that IVF fertility often is not highly correlated with *in vivo* fertility. One of the main reasons for this is that current methods of capacitating bull sperm *in vitro* are not very efficacious, so hundreds to thousands of sperm per oocyte are used for routine IVF, whereas *in vivo*, the best evidence is that there are only a few sperm in the vicinity of the oocyte at the time of fertilization, and essentially all of those sperm are capacitated (Hunter, 1993; Guidobaldi *et al*., 2012). It is likely that sperm of some bulls capacitate more readily *in vitro* than sperm of other bulls, but that this characteristic is not closely related to *in vivo* fertility.

Conclusions

There are many procedures available for evaluating semen quality, and the method chosen will depend on the objective of evaluating the sperm and the resources available. I have emphasized four procedures, motility, membrane integrity, competitive fertilization, and IVF. Estimating progressive motility is the most used procedure, and it is an excellent approach, that, however, must be done "blindly" for valid experimental results. Measuring membrane integrity by flow cytometry recently has been widely adopted by the AI industry. Competitive fertilization is a new approach to estimating fertility, and while it has many limitations, many fewer animals are needed to test hypotheses or screen bulls for low fertility. IVF is a good approach to measuring fertility for IVF purposes, but less reliable for predicting *in vivo* fertility.

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