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INTRODUCTION

When selecting stallions for `breeding soundness', actual fertility data is often absent or unreliable. As an alternative, a combination of a thorough physical examination and semen evaluation has become accepted as a way of determining whether a stallion has an acceptable chance of adequate fertility (Kennedy et al., 1983). In fact, evaluating semen quality has some advantages over in-field fertility data since the latter can easily be biased by factors extrinsic to the stallion, such as quality of the mares or of the breeding management (van Buiten et al., 1998; Morris and Allen, 2002). Pre-breeding semen evaluation is also a useful way of establishing a baseline for subsequent use in the event of fertility problems. Nevertheless, while poor semen quality is a good indicator of sub-fertility, ‘good’ semen quality in terms of the conventional parameters (sperm number, motility and morphological normality), or indeed in terms of any single test currently available, is no guarantee of acceptable fertility (Colenbrander et al., 2003). For this reason, considerable efforts are currently being made to identify tests or markers that more accurately predict a stallion’s fertility (Graham, 2001). This is no easy task given that any one test is likely to measure only one, or a limited range, of the many attributes that a sperm must possess if it is to reach, bind to, penetrate and successfully fertilize an oocyte, and give rise to a viable embryo that develops into a healthy foal.

Given the difficulties in accurately predicting fertility, disqualifying stallions from breeding on the basis of poor semen quality may seem undesirable, particularly if we accept that many would have “selected themselves out” by way of poor fertility. However, selection of stallions on the basis of fertility potential is crucial if we consider it important to protect mare (and stallion) owners against the disappointment and losses of fruitless breeding and, moreover, if we want to maintain a fertile horse population; at least some semen quality parameters (e.g. motility, morphological normality) are heritable (van Eldick et al., 2005). Moreover recently developed tests have considerably improved our ability to identify abnormalities inconsistent with ‘normal’ fertility and it is hoped that a combination of tests will be developed that identifies most sub-fertile stallions or, even better, gives a reliable indication of expected fertility. This paper will examine the utility of existing semen quality tests for identifying sub-fertility or predicting fertility in stallions.

THE STANDARD BREEDING SOUNDNESS EXAMINATION

The aim of a breeding soundness examination (BSE) is to identify stallions that do have the attributes necessary for “adequate” fertility. In most cases, the BSE is performed before the start of that stallion’s breeding career and covers all aspects of fertility potential including libido, mating ability, testicle...
size, sperm production and semen quality (percentages of motile and morphologically normal sperm). If available, past reproductive performance is taken into account. In terms of the usefulness of the information produced, while sperm production has little influence on the ability to establish pregnancy in a mare, unless it is extremely low, it gives valuable information about the number of mares that a stallion should be able to cover, or number of insemination doses he can produce, during a given period of time.

Exceeding that number may negatively affect ‘apparent’ fertility.

The most comprehensive study of the relationship between conventional semen quality parameters and fertility was performed by Jasko et al. (1992) and, although they found reasonable correlations between the percentages of motile (r=0.40), progressively motile (r=0.46) and morphologically normal (r=0.36) sperm with fertility, they also reported that variation in these characteristics accounted for only 20% of the total variation in fertility. Thus, the standard BSE remains strictly a means for selecting out obviously unsatisfactory breeding prospects, where the general rule of thumb is that fertility will almost certainly be compromised when percentages of progressively motile or morphologically normal sperm drop below 35-40%.

### ADDITIONAL TECHNIQUES FOR ANALYZING SEMEN QUALITY

Given the limitations of the standard BSE for predicting fertility or even for identifying all seriously sub-fertile stallions, many alternative approaches have been investigated. However, since any single test may identify stallions with an obvious problem for that particular parameter, it may fail to recognise stallions with a catastrophic abnormality in another compartment. For this reason, combinations of tests are still necessary if we are to have a reasonable chance of identifying most sub-fertile animals. On the other hand, the more test performed, the more expensive the examination will be. Nevertheless, test now in use include:

**Computer-assisted semen motility analysis (CASA):** In theory, computerized analysis of sperm motility should be more objective and repeatable than assessing sperm motility by eye (Figure 1). Moreover, it allows much more in depth analysis of the way in which a sperm moves, where this may relate either to the sperm’s activation status (e.g. capacitated) or its ability to reach and penetrate an oocyte.

In practice, computerized analysis of sperm motility has proved to correlate no better than by-eye assessment with pregnancy rates achieved with either fresh (Jasko et al., 1992) or frozen-thawed semen (Samper et al., 1991).

![Figure 1 - CASA analysis of stallion sperm. The computer categorizes sperm on the basis of motility characteristics as (1) progressively motile – green; (2) motile, but not progressive - blue; (3) immotile – red. While the analysis is objective, the accuracy is dependent on whether the computer can recognize all sperm and reliably differentiate them from other particles.]
However, Quintero-Moreno et al. (2003) have suggested that the failure to find a significant relationship between CASA motility characteristics and fertility is due to inappropriate use of the data rather than to a true absence of a relationship. They used multivariate analysis of CASA data to differentiate sperm into 4 different sub-populations and tentatively concluded that the category with the highest degree of progressive motility represented sperm with the highest fertilizing potential; unfortunately, the hypothesis that there is a relationship between different sperm motility sub-populations and fertility has yet to be properly tested.

**Computer assisted sperm head morphometry:** Computers can also be used to analyze the shape of a sperm’s head (sperm head morphometry). Indeed, Gravance et al. (1996) reported that subfertile stallions had a significantly lower percentage of sperm with heads that conformed to the morphometric norm (19% versus 52% for fertile stallions), and suggested that sperm head morphometry was a useful adjunct to routine semen analysis for the prediction of fertility. On a cautionary note, experience in our laboratory suggests that there are breed differences in sperm head size and shape. For example, some Friesian stallions have sperm heads that differ considerably in size and shape to other stallions. As yet it is not clear whether this relates in any way to fertility, but it does mean that the settings on any computerized sperm analysis system may need to be altered for particular groups or breeds of horse.

**Hypo-osmotic swelling test:** The hypo-osmotic swelling (HOS) test is a means of investigating membrane integrity in sperm and, in this respect, can be considered an alternative to supra-vital (live-dead) staining. In fact, the HOS test has the advantage of indicating not only whether the membrane is intact but also whether it is osmotically active. When exposed to a hypo-osmotic solution, sperm with a functional plasma membrane swell to establish an osmotic equilibrium, this is visible as a characteristic swelling of the sperm tail (Figure 2). The HOS test is easy to perform and, in man, has been reported to correlate highly with fertility following IVF (Van der Venn et al., 1986). In boars, Pérez-Llano et al. (2001) found that a modified HOS test correlated better with pregnancy rates (r=0.43) than either motility or acrosome integrity. However, while the HOS test has been validated for use on stallion sperm (Nield et al., 1999) and proved to be simple and repeatable, it is not yet clear how closely the results correlate with fertility. Nevertheless, it seems likely that, as in boars, the HOS test will identify a small population of sub-fertile stallions that would not have been identified by conventional semen analysis, but will offer little extra predictive value in other animals. Moreover, the HOS test may be more appropriate for predicting fertilizing capacity of frozen-thawed than fresh semen, because membrane damage is a more important limiting factor in the former.

**Capacitation and the acrosome reaction:** To develop the ability to fertilize an oocyte, sperm must be activated in a process called capacitation. In practice, sperm capacitation takes place after their arrival in the female genital tract and involves a delicate modification of the plasma membrane molecules that
enables the sperm to bind to the zona pellucida (ZP). In turn, sperm-ZP binding stimulates the sperm to undergo the acrosome reaction (AR) and release enzymes previously stored within the acrosomal cap that help the sperm penetrate the ZP; only capacitated sperm are able to bind to the ZP in the manner required to trigger the AR and the subsequent events of fertilization. The changes that occur during capacitation of stallion sperm include ‘scrambling’ of the phospholipids between the inner and outer leaflets of the plasma membrane (Rathi et al., 2001), loss of membrane cholesterol (Gadella et al., 2001) and exposure of membrane-bound progesterone receptors (Cheng et al., 1998a); all of these events precede and enable the AR. In addition, capacitation is associated with a change in the pattern of sperm motility known as ‘hyperactivation’ and characterized by vigorous tail movements, marked lateral displacement of the head, and a non-progressive trajectory.

In essence, there are two ways of using capacitation and AR analysis to assess sperm quality. The first is to assess the number of capacitated or acrosome reacted sperm in unstimulated semen; the latter are no longer capable of fertilization, while the former are thought to suffer from reduced longevity (Watson, 1995). The alternative is to examine the effect of capacitation or AR inducing agents (e.g. bicarbonate and calcium ionophore, respectively), and thereby determine whether the sperm are able to undergo these essential biological changes. The latter approach (i.e. inducing sperm activation) seems to be most useful when analysing the fertilizing capacity of fresh semen, while the former (i.e. staining unstimulated sperm) is particularly appropriate when assessing frozen-thawed semen. The best described methods for examining capacitation and AR status are:

**Chlortetracycline (CTC) staining:** Chlortetracycline is a fluorescent antibiotic that binds to the surface of sperm in a calcium-distribution dependent fashion. CTC is considered useful because it distinguishes 3 different stages of sperm activation, namely non-capacitated, capacitated-acrosome intact and capacitated-acrosome reacted. However, there is some debate as to how closely the various patterns follow the biological events of sperm activation. Furthermore, because CTC analysis is not amenable to flow cytometric quantification, it is laborious. For these reasons, CTC staining has been largely superseded by stains that track changes in sperm activation status more specifically, and can be analysed flow-cytometrically.

**Flow cytometric detection of capacitation and the AR:** The best described assays for the flow cytometric detection of capacitation in stallion sperm measure the increase in plasma membrane fluidity or disorder using probes that intercalate in a more fluid membrane, e.g. merocyanin 540 (Rathi et al. 2001), or that recognise phospholipids only exposed in the outer phospholipid bilayer of capacitated sperm e.g. Annexin V; however, a number of laboratories have encountered difficulties in validating these assays for stallion sperm. A more repeatable measure of capacitation that can be assessed by either fluorescence microscopy or flow cytometry is the immunodetection of tyrosine phosphorylation of surface proteins. Capacitated sperm show an increase in tyrosine phosphorylated proteins and therefore fluorescence, particularly in the tail region. For the AR, the most commonly used marker is fluorescein-isothiocyanate conjugated peanut agglutinin (FITC-PNA), a lectin that binds specifically to the outer acrosomal membrane (Figure 3: Cheng et al., 1996).

Flow cytometry offers the advantages of objectively assessing the capacitation or AR status of many thousands of sperm in a matter of seconds, while simultaneously allowing evaluation of sperm viability by including a membrane impermeant DNA stain such as propidium iodide. Essentially, analysis then allows separation within the viable sperm of non-capacitated versus capacitated or acrosome intact versus acrosome reacting/reacted.

In recent years, we have examined a number of stallions referred for the investigation of poor fertility despite apparently good semen quality, and many have shown a failure of sperm to capacitate and/or acrosome react in response to priming with bicarbonate or calci-
um ionophore. Thus, the ability to AR in response to an inducing agent appears to be an important indicator of fertilizing capacity in a stallion, although it is not yet clear whether this is only useful for identifying the source of the problem in specific sub-fertile stallions, or whether it offers further predictive value.

**Sperm progesterone receptor exposure:** It is thought that progesterone present in follicular fluid is involved in the induction of the AR in stallion spermatozoa, and that it exerts its effects via specific progesterone receptors on the plasma membrane of those sperm (Meyers et al., 1995). Physiologically, it appears that progesterone enhances both sperm-zona binding and ZP-mediated AR induction (Cheng et al., 1998). Furthermore, sub-fertility in some stallions has been correlated to an inability of their sperm to undergo the AR in response to progesterone (Meyers et al., 1995). Rathi et al. (2000) similarly reported that the percentage of sperm in an ejaculate in which the progesterone receptors were ‘exposed’ on the plasma membrane after incubation in capacitating conditions was highly correlated with the fertility of the donor stallion (r=0.73-0.84). These authors suggested that the ability of sperm to expose the progesterone receptor after capacitation is related to ZP-binding capacity and, therefore, fertility.

**Figure 3** - Stallion sperm stained with FITC-PNA to examine acrosome status. Acrosome intact sperm do not exhibit green fluorescence. Sperm in which the acrosome reaction has been initiated, or which have sustained damage to the acrosome, show staining over the whole acrosomal cap (A). Sperm that have completed the acrosome reaction show green fluorescence only along the equatorial band (B). Dead sperm take up the accompanying membrane impermeant viability stain (e.g. ethidium homidimer) and red fluorescence (C).

**The sperm chromatin structure assay (SCSA):** The chromatin (DNA) of a sperm is more highly condensed than that in the nucleus of a normal somatic cell. It has been suggested that this chromatin condensation helps protect the DNA from environmental stress and mutagenesis (Ward and Coffey, 1991). In man, reduced sperm chromatin stability has been related to recurrent abortion (Ibrahim and Pedersen, 1988). DNA stability can be assessed using the sperm chromatin structure assay (SCSA). The SSCA uses the metachromatic dye acridine orange to distinguish between undamaged (green fluorescence = double stranded) and denatured (red fluorescence = single stranded) sperm DNA, after exposure of the sperm to an acid environment. Strong correlations have been demonstrated between sperm chromatin stability (resistance to denaturation) and fertility rankings in both bulls (r = 0.94) and boars (r ≤ 0.93: Evenson et al., 1994). In addition, Evenson et al. (1999) reported that men with ≥ 30% sperm with denatured DNA were subfertile/infertile; using this threshold, the proportion of men predicted to have a fertility problem that actually did have one, was 52%. The SCSA has also been tested on stallions; Kenney et al. (1995) reported higher rates of sperm chromatin denaturation in semen from sub-fertile than fertile stallions (32% vs 16%) and a negative correlation be-
tween denaturation score and seasonal pregnancy rate. In a group of fertile stallions, Love and Kenney (1998) were able to demonstrate variations in SCSA that correlated moderately with both seasonal and per cycle fertility rates; they suggested that the SCSA could be used to prospectively rank stallion fertility and to identify changes associated with impending fertility loss. Certainly, the SCSA seems to be a useful addition to the armoury of tests available for examining fertility in stallions since it investigates a property, normality of the genetic material, not tested by any other conventional tests (except pregnancy rates).

CONCLUSIONS

Breeding stallions vary greatly in their per cycle fertility rates and there is a need/desire for tests that can predict future fertility with a reasonable degree of certainty before the stallion embarks on a breeding career. Given the complexity of the attributes a sperm needs to successfully fertilize an oocyte, it appears that a combination of tests that covers the most important aspects of sperm function is the best way to identify stallions with defects likely to affect fertility. Since it is also desirable to limit the complexity and costs of semen evaluation to keep it practical, flow cytometrically assessable tests are favoured. Stains are now available for assessing sperm viability, capacitation status, acrosome status, and chromatin stability while future research is likely to focus on what combination of sperm properties correlates best with fertility, bearing in mind that this will differ depending on whether the semen is fresh, frozen-thawed or treated in some other fashion (e.g. sex-sorted).

BIBLIOGRAPHY


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