

SEMEN EVALUATION

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Introduction

Semen may be evaluated in bulls as part of the routine breeding soundness evaluation, for investigation of fertility problems and for use in A.I.

Semen Collection and Handling

Semen is usually collected from bulls by use of an artificial vagina (AV) or by electro-ejaculation (EE). Semen collected by EE is often more dilute and may appear to be less motile than that collected by AV, although total sperm per ejaculate and sperm viability should be essentially similar (assuming the EE collection has been successful and complete). As fresh semen is very susceptible to environmental influences, procedures should ensure that it does not come into contact with excess heat, cold or toxic substances. Initial assessments that require live sperm (eg motility) should be done as quickly as possible after collection.

Initial Impressions

Volume, density, and gross characteristics of the ejaculate are not generally "front-line" assessments for breeding soundness because they have not been highly repeatable or predictive. However, all such information should be recorded, as well as spermatozoal concentration if done. Other gross characteristics which may be noted include evidence of contamination, hemorrhage or inflammatory material.

Sperm Movement

Methodology

- ❖ **Bright & Dark-Field Microscopy (100X, 400X)**
- ❖ **Phase Microscopy**
- ❖ **Time-lapse micro-photography**
- ❖ **Optical-Path**
- ❖ **IVOS/CASA**

Individual progressive motility of spermatozoa can be assessed under a brightfield or phase-contrast microscope preferably equipped with a warm stage or other means of preventing cold shock of spermatozoa. Coverslipped specimens are usually examined at a total magnification of 400x, although gross motility (or mass activity) can be assessed in non-coverslipped samples at

100X. In dense samples (milky or creamy) the sample should be diluted for proper observation of individual sperm movement. Sodium citrate or skim milk based semen extenders are serviceable diluents; physiological sterile saline (PSS) may be used although readings should not be delayed. With individual motility, an estimate is made of the percentage of active, progressively motile cells.

In practice, considerable observer variation occurs with conventional methods of assessing sperm movement. This has led, over the years, to a variety of more objective alternatives, including time-lapse micro-photography, optical path devices and computerized automated sperm analysis systems (CASA). The latter represents the current “gold standard” for sperm motility assessment, although its cost and complexity present problems for use except in dedicated andrology laboratories.

Sperm Morphology

Methodology

- ❖ **Bright-Field Microscopy (1000X)**
- ❖ **Ordinary Phase Microscopy**
- ❖ **Differential Interference Contrast (DIC)**
- ❖ **Electron Microscopy**
- ❖ **Computerized Image (Fourier) Analysis**

In practice, routine morphological assessment of spermatozoa (differential counts of normal and abnormal cells) is usually conducted either with phase microscopy (using preparations "fixed" in e.g. formol-buffered-saline or PBS-glutaraldehyde) or by using bright-field microscopy of stained smears. Common stains used for this purpose include nigrosin-eosin, William's stain and modified Giemsa, while Dif-Quik and Papanicolaou (“Pap”) stains are serviceable. The Society for Theriogenology (SFT) recommends the nigrosin-eosin stain for its combination of ease and utility. Although this stain is a "supra-vital" stain (i.e. sperm which are "alive" at staining will not exhibit staining while those that are "dead" will partially or completely stain with the red eosin color), here it is used for its ability to depict sperm morphology only.

Improvements in sperm morphology assessment have occurred with the use of different forms of phase-contrast microscopy. Here, differential-interference phase contrast (DIC) microscopy is regarded as the “gold standard” for certain types of abnormalities, particularly those involving the acrosome (Garner 1997), and for depicting subtle abnormalities of the sperm head or midpiece (Chenoweth et al 1994). Phase microscopy has an advantage that it may be used with “fixed” semen samples, thus avoiding possible structural damage to sperm, which may occur with traditional stained smears. The electron microscope (EM) represents the ultimate method for depiction of individual sperm abnormalities. However, this option is not regarded as practical for routine use as the process is complex, expensive, and difficult to quantify.

Automated high resolution image analyzers (computer-automated sperm head morphometry analysis) can identify subtle differences in head shape and size, with the added advantage they can process sperm at high speed, and thus provide easily quantifiable results (Gravance et al 1998). Despite this, their applicability for routine diagnostic use in domestic animals has yet to be established (Boersma et al 2000).

Categorization of Sperm Abnormalities

Systems

- ❖ **Primary/Secondary**
- ❖ **Specific Abnormalities**
- ❖ **Major/Minor**
- ❖ **Compensable/Uncompensable**

The current SFT system for evaluating bulls relies upon a threshold level (70%) of normal sperm, with no direct reference to types of abnormalities or their classification. This is partly because the practice of separately classifying different sperm abnormalities based upon underlying assumptions concerning their etiology and their significance is under challenge. The case for using a single threshold for "normal" sperm receives support from a number of sources (e.g. Barth and Oko 1989). It is reinforced by knowledge many sperm abnormalities previously considered as discrete entities, may in fact reflect stages within a spectrum of standardized responses to stress by the spermatogenic epithelium (Larsen and Chenoweth, 1990; Vogler et al, 1993). This approach is not inconsistent with the pioneering conclusions of Lagerlof (1934) who argued damage to spermatogenesis might well influence many more sperm than those with easily discernable faults, and a threshold level for satisfactory fertility appeared to exist. The threshold of 70% normal sperm is consistent with the observations of Lagerlof (1934) and, more recently, work by Wiltbank and Parrish (1986). However, the categories of "primary" and "secondary" sperm abnormalities are often used to assist in the mechanics of collating totals, prognosticating and monitoring progress. The categories "major" and "minor" (Blom 1972) are used to distinguish between sperm abnormalities proven to be associated with infertility and those which were regarded as being less harmful. In practice, this represents a moving target, while many similarities occurred with the primary/secondary system. The "compensable/non-compensable" sperm anomaly classification system, as described by Saacke et al. (1991), shows great promise but probably requires further refinement and testing before widespread field use is recommended.

Sperm Concentration

Methodology

- ❖ **Visual Approximation**
- ❖ **Hemocytometer**
- ❖ **Spectrophotometer**
- ❖ **IVOS/CASA**
- ❖ **Cell Counter (Coulter)**

Sperm concentration, while not a "front-line" assessment for bull breeding soundness evaluation, remains an important part of semen assessment, especially for A.I. centers. A variety of techniques exist, ranging from "eye-balling" the fresh ejaculate through to the use of electronic cell counters. Even though other technologies exist, such as the spectrophotometer and

CASA, the “gold standard” for sperm concentration assessment remains the humble hemocytometer, used in conjunction with volumetric dilution. If electronic devices are used, they should be regularly calibrated e.g. using Accu-Beads. Some andrology laboratories employ specialized counting chambers such as the Makler chamber, while accurate, disposable chambers are available from several manufacturers.

Acrosome Assessment

A variety of techniques exist to detect either the presence of the acrosome (percent intact acrosomes (PIA), or aspects of its functionality, e.g. acrosin assay). While standard sperm morphology bright-field preparations, such as nigrosin-eosin staining, can help to depict the acrosome, they are not ideal for accurate classification. Better results are obtained with specific

Methodology

- ❖ **Modified Giemsa/Brightfield Microscopy**
- ❖ **Ordinary Phase Microscopy**
- ❖ **Differential Interference Phase Microscopy**
- ❖ **Acrosin assay**
- ❖ **Lyso-Tracker**
- ❖ **ZP Binding/Penetration**
- ❖ **Acrosin Assay**

stains (e.g. modified Giemsa, Wells-Awa, Casarrets), or use of phase-contrast microscopy (ordinary or DIC). Acrosome-specific fluorochromes (e.g. Lectins, FITC-PSA, CTC) may also be employed, as may antibodies directed against acrosome-associated antigens (Cross and meizel 1989). As the acrosome is essential for sperm binding and penetration of the zona pellucida, indirect tests of acrosome functionality include such measures as acrosin assay.

Chromatin Structure/ Genetic Content

Methodology

- ❖ **Phase/DIC Microscopy**
- ❖ **DNA specific stains**
 - **Aniline blue**
 - **Fuelgen**
 - **Acridine Orange**
 - **Hoechst**
- ❖ **SCSA**
- ❖ **FISH**

Disturbances in sperm chromatin structure and condensation have been associated with infertility in different species. Although poor condensation may be detected using phase or DIC microscopy alone, this is not an appropriate method for accurate quantification. Aniline blue staining depicts poor chromatin condensation in the sperm head via increased staining intensity,

while acridine orange can distinguish between native (double-stranded) DNA, which stains green, and single-strand DNA which stains orange-red. This stain is the basis for the sperm chromatin structural assay (SCSA) test, which has been useful in diagnosing bull infertility.

Sperm Membrane Integrity

The functional integrity of sperm membranes can be assessed using a variety of systems. An indirect method is to use supra-vital stains which rely on plasma membrane damage to stain underlying structures. The hyperosmotic swelling test (HOS) relies on the physiological phenomenon that membrane-intact sperm will swell when placed into a moderately hypoosmotic environment, whereas membrane-damaged sperm do not. The induced swelling produces a characteristic coiling of the flagellum inside the swollen membrane which is easily observed under phase-contrast microscopy.

Methodology

- ❖ **Supra-Vital Stains**
 - Eosin/nigrosin
 - Trypan-blue
- ❖ **HOS test**

Other Procedures

A number of other procedures exist for the evaluation of semen and sperm; many are more appropriate for research while others are used mostly in human andrology work. Of particular current interest are a number of proteins that have been associated with either male fertility or infertility, with one of the best characterized being heparin binding protein (HBP).

Methodology

- ❖ **Cervical mucus penetration**
- ❖ **Heparin-binding protein**
- ❖ **Mitochondrial assessment**
- ❖ **Semen enzymes**
- ❖ **Semen proteins**
- ❖ **ATP Content**

Selected Sperm Abnormalities

Certain types of sperm abnormality are heritable. A high proportion of certain abnormalities can cause infertility. Abnormal sperm can adversely affect sperm that appear to be normal (Amann 1990). Constraints to the detection of some abnormalities include lack of knowledge and observational ability, as well as by technical limitations (*better microscopy detects more sperm abnormalities*). Sperm abnormalities can cause infertility in a number of ways. One group of abnormalities may not successfully complete the journey to the fertilization site. These are generally characterized by abnormalities which adversely affect sperm movement, such as tail and midpiece abnormalities and misshaped heads. Barriers in the female tract act to constrain

such abnormalities which have been termed *compensable sperm* factors (Saacke et al. 2000). Other abnormal sperm may succeed in fertilizing the ovum, but result in reproductive wastage (either early embryonic death or abnormal development). Such problem sperm (*uncompensable sperm*) are less easy to characterize, but include more subtle forms of the diadem defect, as described below.

Diadem/Crater Defect

The spermatogenic epithelium generally reacts in a stereotyped manner to thermal insult. A characteristic feature of this response is development of the diadem/crater defect of sperm. The severity and length of insult determine the incidence of malformed sperm, and their degree of malformation (Fig 1).

The diadem crater defect has been recognized in most species, and has been induced by a variety of causes including pyrexia, scrotal insulation, ethylene dibromide, sulfasalazine, corticosteroids, low T levels and perhaps viruses. An important aspect of this concept is a number of sperm abnormalities previously thought to occur independently are now considered to be linked as part of the same process of spermatogenic response to stress. The earliest lesions may be detected in the round spermatid (Larsen and Chenoweth 1990). Theories concerning the etiology of the defect include the deleterious actions of reactive oxygen species (ROS) during a critical phase of development.

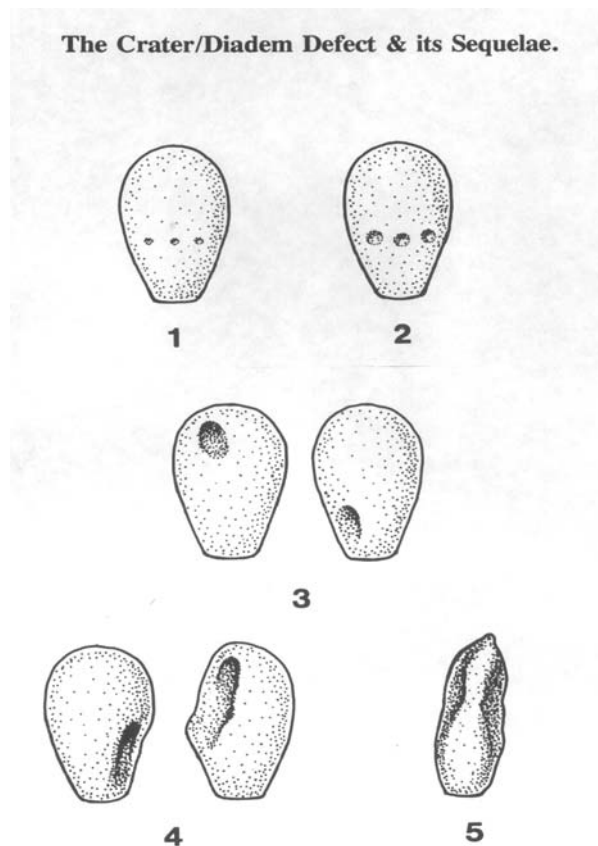


Fig 1. The Crater/Diadem Defect and its Sequelae

This response provides a predictable temporal pattern of sperm abnormalities following scrotal insulation in the bull (Fig. 2).

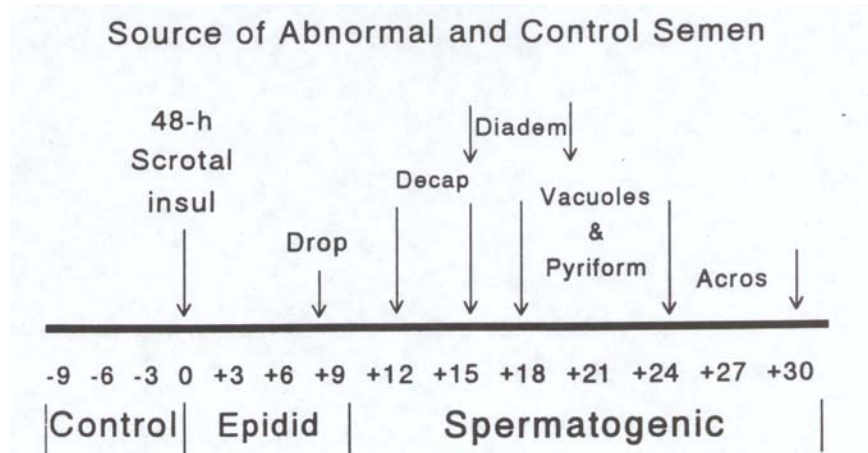


Fig. 2. Temporal Appearance of Sperm Abnormalities following 48h Scrotal Insulation. after Vogler et al 1993

Midpiece Defects

Gossypol/Respiratory Disease. Gossypol, a phenolic compound produced by the cotton plant (*Gossypium spp.*) appears to exert unique and selective effects upon the male reproductive system. Earlier work showed chronic administration of gossypol acetic acid (GAA) caused a consistent and marked reduction in the number of ejaculated spermatozoa, usually preceded by lowered sperm motility. In nonruminants, this reduction in spermatozoal numbers was associated with damage to the spermatogenic epithelium. In bulls, gossypol effects have included degeneration and reduction of spermatogenesis, damage to the basement membrane of spermatogenic tubules and an increase in abnormal sperm. In all species studied, the spermatotoxic effects of gossypol appear to be both time and dose dependent.

A specific lesion of the sperm midpiece, namely segmental aplasia of the mitochondrial sheath, has been identified as a consistent feature of gossypol spermatotoxicity in both nonruminants and ruminants. This lesion may be difficult to discern with ordinary light microscopy. Although gossypol causes specific sperm midpiece abnormalities, other factors associated with abnormalities of sperm midpiece include ethylene dibromide, genetics and respiratory disease.

Gossypol appears to cause damage to the mitochondrial sheath of cells during the latter phases of spermatogenesis, with lesions first detected in elongating spermatids. An apparent association between lesion type and extragonadal passage led to the conclusion the primary sperm lesion was induced during spermatogenesis, with subsequent damage being secondary (Chenoweth et al., 2000). The types of lesion first observed at extragonadal sites suggests structural failure in already weakened structures, possibly exacerbated by the onset of sperm motility.

Although gossypol has been shown to cause spermatogenic dysfunction in bulls, many factors influence the possibility of this actually occurring when cotton seed products are fed..

Gossypol levels vary between strains of cotton and regions, between cotton seed meal (CSM) and other forms of cottonseed, between methods of production of CSM and between gossypol isomers. In addition, the effects can be modified by the rate of ingesta passage, and by the feeding of elevated levels of vitamin E (Velasquez-Pereira et al 1998).

Primary Ciliary Dyskinesia. (previously termed immotile cilia syndrome) is seen in humans with Kartagener's syndrome, and also in conjunction with viral respiratory diseases which damage all ciliated cells, including sperm. Here, affected sperm (and respiratory tract cilia) have perturbed axonemal structures such as part or complete absence of dynein arms, microtubule disorganization or absent radial spokes. This suggests a genetic link between these structures or that they are coded by the same gene. Similar sperm aberrations exist in the animal world, although the link with respiratory diseases has not been adequately pursued.

Knobbed Acrosomes. Knobbed acrosomes have been associated with infertility in a number of species. In the bull, the term is perhaps misleading as the usual form is either a folded knob, or an abnormally shaped, thickened and optically dense apex of the acrosome. Although good bright-field microscopy may detect these abnormalities, greater success is achieved using phase or DIC with oil immersion (1000X plus). The cause may be either genetic or environmental (e.g. following scrotal insulation). Differentiation between the 2 causes may require sequential examinations as well as interpretation of the spermogram. With the genetic form, the abnormality is often predominant, with relatively few other sperm defects, and it is persistent. In beef cattle, the genetic form has been detected in Friesians, Charolais, Horned Herefords, Salers, Simmental, Maine Anjou, Chianina and Angus (Chenoweth 2000). Affected bulls may be undetected by routine BSE and even by AI center screening tests. However, if present in significant numbers (>20-30%) then infertility and even sterility will be the result, with a number of apparently normal sperm also being compromised (Thundathil et al 2000).

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