

Cellular associations and the differential spermogram: Making sense of stallion spermatozoal morphology

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Abstract

Morphologic assessment of spermatozoa is one of the most objective measures in a Breeding Soundness Examination of a stallion. There are different systems for morphologic assessment of spermatozoa. The objectives of this article are to review spermatogenesis, describe clinical sample preparation, discuss previous methods of morphologic classification and explain the use of a differential spermogram. The advantages of the differential spermogram method of analysis are discussed, along with its use in delineating intrinsic and extrinsic disturbances in spermatogenesis. Case examples of specific cellular associations and their diagnostic relevance are discussed.

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1. Introduction

The morphologic assessment of spermatozoa has a long history that begins with the scientist Van Leeuwenhoek in 1677. He noted “animalacula” in seminal fluid, and was the first to record heterogeneity in spermatozoal head shapes in men and other animal species. It has been shown that specific morphologic abnormalities of spermatozoa are related to male sub- or infertility. Systematic studies of spermatogenesis and spermatozoal morphology resulted in the development of a number of classification systems with the goal of finding an efficient and accurate way to evaluate the potential fertility of a male.

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2. Review of spermatogenesis in the stallion

In the process of spermatogenesis, cells replicate and mature from diploid germinal cells termed spermatogonia, to haploid, mature spermatozoa. Billions of spermatozoa are produced each day (16 million spermatozoa per gram of stallion testicular tissue per day). Many of the cells produced are defective and some are eliminated through apoptosis and phagocytosis by the Sertoli cells, whereas others are passed into the ejaculate [1]. Long convoluted tubules called seminiferous tubules, contain the spermatogenic epithelium and Sertoli cells. Cross-sections of the seminiferous tubules show that four or five specific cell types are consistently found together, and these are present in different combinations called cellular associations. In the stallion, each successive progression in the cellular associations requires 12.2 days. Repetitions of the 12.2-day-period includes a progression of the cells through eight stages. These stages are numbered I–VIII. The time frame to pass through each stage is not equal. The stages can be differentiated by a detailed examination of the spermatocytes, spermatogonia and spermatids in a cross-section. The succession is evident when viewing the rings of the germinal cells in the tubule. The cells located in the outer ring of the tubule replicate, so that over time more differentiated cells are formed, which through successive generations, results in the most differentiated cells being located closer to the lumen. The 12.2 day time frame, multiplied by the five specific cellular associations results in a rough estimate of the total duration of spermatogenesis [2,3].

2.1. Time frame

The time period from a committed spermatogonium to a spermatozoan entering the lumen of the seminiferous tubule has been shown experimentally to be 57 days in the stallion, whereas other authors quote this interval as 55 days. This 55–57-day-period may be divided into three phases, including: spermatocytogenesis, where mitosis and differentiation of spermatogonia occur (19.4 days); meiosis, the period of replication (primary spermatocytes) and then reduction of genetic material into haploid spermatids (19.4 days); and spermiogenesis, development and differentiation (18.6 days). Spermiation is the release of spermatids as spermatozoa into the lumen of seminiferous tubule. Approximately 9 days are required for transportation of spermatozoa through the excurrent duct system. Therefore, a new population of spermatozoa are ejaculated every 64–66 days. There is no seasonal influence on this interval; in other words, in winter or summer spermatozoa take the same amount of time to produce. There is a seasonal influence and an influence of age on total sperm production, spermatozoal motility and spermatozoal morphology. An ejaculate, therefore, is a composite of the events that occurred in the past 2 months that influenced spermatogenesis, when the spermatozoa were being formed, and their subsequent transport and maturation through the excurrent duct system [2,3].

3. Preparation of slides

Slides are prepared using prewarmed Eosin Nigrosin stain (Society for Theriogenology, Montgomery, AL, USA) and glass slides. The most common error in slide preparation is to

use raw, unfiltered semen. The gel must be filtered or aspirated from a stallion's ejaculate before obtaining a sample for staining, because the gel interferes with the ability to visualize the spermatozoa. A 5–6 mm drop of stain is placed on the slide first, followed by a 3–4 mm drop of the sample. This method prevents contamination of the stain bottle with spermatozoa. A larger amount of stain is used than the sample amount; a 60:40 ratio of stain to sample is common when the sample is diluted with extender. The stain and sample are mixed together and the mixture is spread across the slide using a wooden stick and a shaking or rolling motion or alternatively, another slide is used to pull the sample across the slide. The slide is then quickly dried. Placing the stained slide on a warming tray is conducive to rapid drying of slide preparations. Quick drying prevents hyposmotic shock from the stain. The slide is scanned under low power to be sure a representative area on the slide is being evaluated. A progressive increase in magnification is then used to achieve 1000 $\times$ bright-field oil immersion. A minimum of 100 spermatozoa are counted under oil immersion (1000 $\times$); increasing the number to 300 cells is recommended to increase accuracy. Generally, all the spermatozoa in a field are counted and then a new field is viewed and counted. If the microscope has a powerful light source, phase contrast may be used under oil to assess the sample; however, most clinical microscopes do not have sufficient power and bright-field microscopy is used. Lack of adequate illumination of the spermatozoa on the specimen slide often results in errors, e.g. failure to observe nuclear vacuoles or segmental aplasia of the mitochondrial sheath. Adequate stain–spermatozoa contrast is important so the background is dark, but the stain should not be so thick that it cracks. Proper staining technique and adequate light penetration of the specimen are of paramount importance in a clinical setting to assess spermatozoal morphology. Phase contrast microscopy is a very useful tool to assess spermatozoal motility in wet mounts and is preferred over light microscopy; however, the opposite is true for morphologic assessment using a typical clinical microscope. A sample sent for a referral opinion on morphology should include a few Eosin Nigrosin stained slides, a few raw air-dried semen slides, and a portion of the sample preserved with glutaraldehyde (as little as a few drops) or 1% formol buffered saline (1:4 ratio). Prior to morphologic assessment, the Eosin Nigrosin stained slide may be used for a separate live/dead count on 100 spermatozoa. Eosin Nigrosin is a supravital stain; cells staining red are considered dead or devitalized and whereas white (unstained) cells are considered alive and intact. A sample where a large proportion of the spermatozoa stained red suggests membrane damage (impaired viability).

4. Classification systems

Blom describes two morphological categories for spermatozoal defects: Primary, those that occur during spermatogenesis, representing a failure of spermatogenesis; and Secondary, those that occur in excurrent ducts, representing a failure of maturation. A Primary defect is therefore testicular in origin and includes such defects as nuclear vacuoles. An example of a Secondary defect is a proximal droplets [4]. The Primary–Secondary system is one of the most widely used systems of morphologic classification of spermatozoa in North America. Another classification system reported by Blom labels defects as Major or Minor [5,6]. Major defects include abnormal heads, midpieces and

proximal droplets which are thought to have a greater impact on fertility. Minor defects such as distal droplets, have an unknown role or no consequence for fertility. These classification systems are effectively categorical yes/no methods of assessing spermatozoa into defects that are likely or not likely to influence fertility. Another concept regarding spermatozoal defects involves determining if the defect is compensable or non-compensable. Spermatozoal defects may be classified as compensable and non-compensable [7]. A compensable defect is one where the defective spermatozoa either do not reach the site of fertilization, or if the defective spermatozoan reaches the oocyte the cortical reaction is not induced. Providing there is a population of normal spermatozoa available for fertilization, the compensable defect has no effect on fertility. In other words, the fertility of the individual is not affected by defects that can be effectively overcome by having adequate levels of normal spermatozoa; the normal spermatozoa compensate for the abnormal spermatozoa. A non-compensable defect is a defect that does not interfere with the spermatozoa reaching the site of fertilization, or the induction of the cortical reaction on the oocyte by the spermatozoan, but the defective spermatozoan is not capable of supporting embryonic development. These non-compensable spermatozoal defects interfere with fertility and compete with normal spermatozoa. An example of a non-compensable defect is a defect in chromatin condensation.

5. Differential spermogram

The present Society for Theriogenology forms available for stallion BSE have the following categories: normal spermatozoa, abnormal acrosomal regions/heads, tailless head, proximal droplets, distal droplets, abnormally shaped/bent midpieces, and bent/coiled tails [8]. There is also a place to note the presence of premature round germ cells and other cells (WBC, RBC, etc.). These forms are suited to the use of a system of morphologic evaluation termed the differential spermogram [9]. A frequency distribution of all defects is performed. Both live and dead spermatozoa are evaluated for morphology during the count. Detached heads, but not headless midpieces, are counted. The order of frequency of the type of head defects and midpiece defects is recorded. Head defects include microcephalic, macrocephalic, pyriform, detached and vacuolated. Knobbed acrosomes may be counted separately. Premature germ cells are counted if more than just an occasional cell is present. Midpieces defects include: segmental aplasia of the mitochondrial sheath, swollen, fractured, pseudodroplet and distal midpiece reflexes. Abaxial insertion of the midpiece is not considered an abnormality in the stallion. Therefore, if a spermatozoan has a macrocephalic head and a midpiece defect, both are enumerated. To enumerate both defects simultaneously, one presses both keys down at the same time on a cell counter. This will only advance the counter by one number in the total counted column. Using the example where 100 spermatozoa are counted and all the abnormalities are enumerated on each spermatozoan, only one number is added to the total count when more than one defect are identified by pressing keys representing multiple categories of defects. The percentages for the categories of defects when added to the percentage of normal spermatozoa will not tally to 100% using this example of the differential spermogram system.

The differential spermiogram system is similar to what occurs when we analyze the white cells in a leukogram. A total white count and a differential cell count is performed on a blood sample. The differential white blood cell count gives us the percentage of lymphocytes, neutrophils, monocytes, eosinophils and basophils. The changes in the percentages of neutrophils and lymphocytes are evaluated because they give us valuable clinical information. The neutrophils are scored for toxic changes (impending cell death). Daily and weekly changes in the absolute cell numbers, changes in percentages of cells such as neutrophils, and changes in the condition of the cells (toxic change) in the leukogram help us to determine if our patient is improving, staying the same or deteriorating. This same principle applies to use of the differential spermiogram in the stallion. The percentages of the spermatozoal defects are determined. Live/dead spermatozoal ratios are determined, similar to the use of identifying toxic changes in neutrophils.

6. Advantages and disadvantages of classification systems

Most examiners will perform a differential count on a semen sample yielding a percentage of spermatozoa with Primary or Secondary defects, or a percentage of spermatozoa with Major or Minor defects, and a percentage of normal spermatozoa. Each spermatozoan is assigned one category. The major drawback of both of those schemes is that spermatozoa are produced with both Major and Minor defects or Primary and Secondary defects on the same spermatozoan. There are also spermatozoa produced with more than one type of Major or Minor defect on a single spermatozoan. An example of this would be a spermatozoan with a macrocephalic head and swollen midpiece. In the situation where a spermatozoan has more than one defect, the Society for Theriogenology guidelines suggests the most proximal defect is identified on each spermatozoa [10]. In the Primary and Secondary defect system, if a spermatozoa has three defects such as a knobbed acrosome, a swollen midpiece and a proximal droplet, a primary defect would be enumerated even though there is another primary (swollen midpiece) and a secondary (proximal droplet) defect present. Using the Major–Minor system where a spermatozoan has a pyriform head, segmental aplasia of the mitochondrial sheath and a distal droplet, only one major head defect would be enumerated. If a system is used where the defects are prioritized so that only one is chosen per spermatozoan, the examiner will achieve a percentage distribution that will total to 100%. They do not however have a record of the distribution of the spermatozoal defects in the ejaculate. It is not possible to track changes in individual defects. Primary–Secondary, Major–Minor or differential spermiogram systems will identify the percentage of normal spermatozoa in the sample, which is a constant in all systems. The differential spermiogram system allows investigation of changes in the frequency or the prevalence of the defects recorded over time. When more than one defect is identified and counted per spermatozoan, it is not assumed that one defect is more important than another. Enumerating all defects for each spermatozoan allows the examiner to determine the potential of the defect to interfere with fertility, and to chronicle the changes in the patterns or cellular associations (increase, decrease and stay the same) of the defects over time. Different abnormalities when found together represent more severe disturbances in spermatogenesis and influence the prognosis [3].

7. Disturbances in spermatogenesis

A perturbation in spermatogenesis is generally manifested by morphologic changes in the spermatozoa characterized using a differential spermogram. The changes occur concurrent with all of the other factors that influence spermatozoal production, including: age of the stallion, season, environmental conditions, and the general stress the horse is experiencing. The nature, duration and sensitivity of the spermatozoal cell types to the perturbing insult determines the consequence in the differential spermogram. The prognosis for recovery depends, in part, on the susceptibility of the progenitor spermatogonia to the insult. Note that all of the spermatozoal cell types, stages, and cellular associations are present in all stallions. By integrating the information on age, season and ejaculatory characteristics such as concentration, total number of spermatozoa, progressive motility and the features of the differential spermogram, the status of the stallion's fertility and progression through the breeding season may be followed. The nature of the disturbance in spermatozoal morphology is assessed by evaluating changes in the cellular associations in the differential spermogram. It is possible to determine if the disturbance was the result of the stallion's intrinsic fertility, a point-source stress (fever), or a long-standing alteration (testicular degeneration) by serial examination of ejaculates. In a stallion with intrinsically low fertility, the morphologic defects are: present consistently at a young age, found in each ejaculate and show only minor seasonal variation. A point-source insult creates damage to the cells present at the time, and by 64 days, an entirely new population of cells is formed which is ejaculated. Scrotal insulation for 36 h increased the percentage of abnormal spermatozoa 8 days after the onset. Histologic evaluation of testes showed that late primary spermatocytes were most severely influenced by insulation [1]. The susceptibility of the epididymides or epididymal spermatozoa to injury determines if abnormal spermatozoa are passed immediately. The epididymal spermatozoal reserve will clear in 9 days, and damaged spermatozoa from the testes will then start to enter the ejaculate. Defects may be observed in the head, midpiece and acrosome, which are formed in the testes. By evaluating the history and morphology in the ejaculates an examiner may determine if they are looking at: fresh evidence of a problem, a sample during the recovery phase or stable spermatozoal production. If the percentage of normal spermatozoa increases and the other categories of defects, decrease this would indicate recovery, but a lack of change or a decline in the percentage of normal spermatozoa indicates the problem is either ongoing or is not yet resolved. Long-standing alterations in spermatogenesis consistently result in the production of abnormal spermatozoa. Changes in cellular associations are associated with a variety of physiological states, stresses and pathologic conditions.

8. Cellular associations and the differential spermogram

The average stallion has approximately 50% morphologically normal spermatozoa. There is a lack of experimental evidence on the tolerance level of various defects, and their effect on fertility, however general guidelines for concern include: >10% premature germ cells, greater than 30% head and/or midpiece defects, >25% proximal droplets and the

percentage of morphologically normal spermatozoa is <30%. The present Society for Theriogenology BSE Stallion Guidelines have no standard for percentage of normal spermatozoa in the ejaculate, but state that approximately 2 billion progressively motile morphologically normal spermatozoa should be present in two collections spaced 1 h apart. Stallions with less than 40% morphologically normal spermatozoa may achieve acceptable pregnancy rates if breeding pressure is low or spermatozoal numbers are increased per breeding dose so a minimum threshold number of normal spermatozoa are present. The normal spermatozoa compensate for many of the abnormal spermatozoa. Stallions with $\geq 60\%$ morphologically normal spermatozoa frequently require fewer spermatozoa per insemination dose.

8.1. Puberty

Increased numbers of germinal epithelial cells, increased numbers of proximal droplets, head and midpiece defects, low spermatozoal concentration, and numbers of spermatozoa.

8.2. Spermiostasis/ampullary blockage

High numbers of detached heads and supraphysiological concentrations of spermatozoa may be produced intermittently, along with ejaculates with low sperm numbers. Treatment may ameliorate the problem.

8.3. Testicular degeneration

Characterized by increased numbers of premature germ cells, increased numbers of head and midpiece defects, declining total spermatozoal numbers.

8.4. Poor intrinsic fertility

Spermogram shows high numbers of a specific defect (>30%) such as dag-like fractured midpieces, or consistently poor morphology with multiple categories of defects (Clydesdale, Friesian) consistent subfertility at a young age (per cycle pregnancy rate <30%) frequently accompanied by low spermatozoal concentration and total number.

8.5. Point insult

There is an increased number of proximal droplets, followed by head and midpiece defects, the defects are present over the next 2 months, and then decrease over time until baseline morphologic parameters are reached.

8.6. Long-standing stressor

A history of decline in spermatozoa morphologic characteristics (e.g. before and after chronic laminitis) and a general increase in morphologic defects.

8.7. Summary clinical interpretation

The challenge is to determine which abnormalities are constitutive and reflect an individual stallion's intrinsic genetic ability, and which changes are extrinsic due to a disturbance (nutritional, hormonal, infectious, toxic, degenerative, neoplastic and idiopathic) in spermatogenesis. This is a complicated process because intrinsic and extrinsic factors concurrently influence spermatogenesis. Intrinsic factors alone that influence spermatogenesis result in a spermogram with only seasonal fluctuations, and the features of the spermogram do not change or improve substantially over time. In any population of males examined, there will be below average, average and above average levels of intrinsic fertility, which will be reflected in their testicular size/location, sperm number/concentration and motility/morphology parameters. Extrinsic factors influencing spermatogenesis either changes over time (point-source insult), are long-standing stressors (the development of laminitis) or historically were not present in the past (testicular degeneration). Serial evaluations of ejaculates and a comparison of the changes in the proportions or prevalence of the various spermatozoal morphologic defects provide the best information on the nature of and subsequent recovery from a problem.

8.8. Examples of clinical assessments of semen samples and cellular associations

8.8.1. Puberty versus low intrinsic fertility

Young stallion (2-year-old), total scrotal width (82 mm marginal), with soft testes. Sample is watery in appearance (volume, 35 mL). Low concentration (20 million/mL) 10 million/mL premature germ cells (germinal epithelial cells also called spheroids). For both collections, progressive motility is 20% raw, 25% extended, 30% normal spermatozoa, 25% head defects (microcephalic, pyriform and macrocephalic) and 28% midpiece defects (segmental aplasia, fractures, distal midpiece reflexes), 20% proximal droplets. The second ejaculate is similar, except the concentration is 5 million/mL. Progressively motile morphologically normal sperm in two ejaculates is 65.5 million.

This ejaculate is typical of a stallion that either has poor intrinsic fertility or is slow to mature and may have not yet completed puberty. A total of 100 million spermatozoa per ejaculate are often used to indicate a stallion is pubertal. Stallions reaching 18 months of age in the winter may have a delayed onset of puberty related to photoperiod. The process of puberty takes approximately 2 months to complete and involves the attainment of patent seminiferous tubules. This explains why germinal epithelial cells appear in the ejaculate at this time. The process of spermatogenesis is not highly efficient when spermatozoal production first begins and there are increased numbers of abnormal spermatozoa. Four types of defects are common in pubertal stallions; germinal cells, head defects, midpiece defects and proximal droplets. In addition, young performance stallions may be under stress or are receiving anabolic steroids that will negatively influence spermatozoal morphology. The ejaculatory characteristics and differential spermogram of this stallion fit mostly closely with puberty.

A typical ejaculate of a pubertal stallion has a low concentration, low motility, high percentage of germinal cells and other defects such as head, midpiece and proximal droplets. A pubertal horse should be classified as a questionable breeding prospect. Suggest

a re-evaluation in 2–4 months. At re-evaluation, you would expect to find increases in: total sperm numbers, sperm concentration, motility and percentage of morphologically normal sperm, with a decrease in germinal epithelial cells, proximal droplets and head/midpiece defects.

8.8.2. *Stress*

Twelve-year-old retired thoroughbred actively breeding (natural service) 150 mares/year. Last year, he achieved a per cycle pregnancy rate of 35%. Symmetric testes, with a firm consistency. Ejaculate 1: volume 70 mL, concentration 100 million/mL. Ejaculate 2 was 50 mL, with a concentration of 55 million/mL. Progressive motility was 55% in both collections. Differential spermogram shows 30% normal spermatozoa, 28% head defects (microcephalic, pyriform and vacuoles), 50% midpiece defects (segmental aplasia, fractures, distal midpiece reflex and swollen), 18% proximal droplets, 10% distal droplets, and 2% germinal epithelial cells. Progressively motile morphologically normal sperm in two ejaculates is 1.736 billion. Questionable breeding potential, relatively low percentage of normal spermatozoa, may be due to stress. Increased ejaculatory frequency is not reported to increase epididymal passage of spermatozoa. Increased percentages of proximal droplets may relate to the effects of stress-induced cortisol release on epididymal transport or spermatozoal maturation, rather than a depletion of spermatozoal reserves. Health issues or fatigue from a large book may cause stress and result in an increase in immature spermatozoa being passed in the ejaculate. Recommend investigating medical issues and decreasing breeding frequency, schedule re-examinations in 2 and 4 months.

8.8.3. *Testicular degeneration*

Mature stallion (6-year-old). History per cycle pregnancy rate of 30% last year, but this year 0/12 cycles. Asymmetric testes. Left testis is firm, right testis is soft and is located high near the inguinal canal. Ejaculate 1: volume 60 mL, concentration is 33 million/mL, 12 million/mL germinal epithelial cells. The second ejaculate 40 mL, concentration is 15 million/mL, 10 million germinal epithelial cells. Progressive motility is 15% in both ejaculates. Differential spermogram: 10% normal, 40% head defects (microcephalic, pyriform and vacuoles), 50% midpiece defects (segmental aplasia, fractures, distal midpiece reflex and swollen) and 10% proximal droplets. Progressively motile morphologically normal sperm in two ejaculates is 38.7 million. Potential for improvement is low, based on history, testicular consistency and location. The low motility has a basis in the morphologic problems of the midpiece. Increased numbers of germinal epithelial cells, high percentages of head and midpiece defects suggests testicular degeneration [11]. The presence of premature germ cells and abnormal head and midpiece morphology is typical of testicular degeneration in stallions; this condition is often irreversible and carries a guarded to poor prognosis. The cause of this condition is not usually identified. Classification: unsatisfactory breeding potential, recommend monitoring for change.

8.8.4. *Subfertility knobbed acrosomes*

Six-year-old Arabian stallion. History of 20% per cycle conception rate. First ejaculate: 30 mL, 75 million/mL and 50% progressive motility. Second ejaculate 45 mL, 35 million/mL, 60% progressive motility. Differential spermogram (both ejaculates): 24% normal,

30% knobbed acrosomes, 40% head defects (microcephalic, pyriform, vacuoles), 25% midpiece defects (distal midpiece reflex, segmental aplasia, fractures, swollen), 10% proximal droplets, 10% distal droplets and a few germinal epithelial cells. Progressively motile morphologically normal sperm in two ejaculates is 496 million.

Spermatogenesis is a complex process, which includes maturational changes in spermatozoal chromatin in the testes and epididymides. The formation of the acrosome occurs at the same time some chromatin maturation is occurring. The presence of a substantial percentage of acrosomal defects suggests that additional tests may be required (chromatin assays) to detect if the spermatozoa with knobbed acrosomes have immature chromatin. Spermatozoa with acrosomal defects are a heterogeneous population. Mild knobbing or folding of the acrosome may not influence fertility, but a severely knobbed acrosome with retained vesicular material in the matrix is a more serious form of the defect. The knobbed acrosome defect may be associated with underlying problems in chromatin maturation. The knobbed acrosome defect has a heritable basis in bulls and may be a constitutive problem in some stallions. Therefore, there may be two problems; an acrosome defect and a chromatin defect. Subsequent evaluation of this stallion showed no change in the spermiogram, suggesting the knobbed acrosomes were an intrinsic defect. Classification: unsatisfactory breeding potential, suggest re-examination to confirm status.

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