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Developments in stallion semen evaluation

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Abstract

The conventional approach to evaluation of stallion semen dates back several decades, and includes evaluation of spermatozoal concentration, semen volume, spermatozoon morphological characteristics, and spermatozoal motility patterns initially and following in-vitro storage. While an analysis performed in this manner does have predictive value, incorporation of some more newly developed techniques may improve the predictive value of the examination. This communication addresses some newer tests that can be applied today for evaluation of semen, as well some tests that may be available in the coming years.

Keywords: Stallion; Semen; Semen evaluation; Diagnostic tests; Diagnostic assays

1. Overview

Conventional laboratory tests for assessment of spermatozoal quality have included light microscopic evaluation of spermatozoal morphologic characteristics and estimation of spermatozoal motility (including percentages of motile and progressively motile sperm; velocity of spermatozoal movement; and longevity of spermatozoal motility following in vitro storage) [1–5]. Although other features of semen quality are also considered (including spermatozoal concentration, semen volume, and the presence of blood, urine, or potentially pathogenic bacteria), the basic features of this examination process date back several decades. The value of the examination is predicated, to a large extent, on reliable equipment, and personnel with good observational power. Even when these stipulations are in effect, the predictive value of the examination can be limited [6–21]. The same holds true for spermatozoa of other mammalian species [22–25]. As such, expanded analysis of equine spermatozoal populations might improve the predictive value of the testing process. In fact, additional tests shown to be of diagnostic value are presently incorporated into the spermatozoal examination process in some reference laboratories.

2. Sperm chromatin integrity

One method of assessing sperm chromatin integrity is the Sperm Chromatin Structure Assay (SCSA). This assay, introduced by Evenson in 1980 [26], has been applied to spermatozoa from a number of species, including horses [27–29]. The SCSA tests a compartment of spermatozoa that is not monitored by conventional methods, i.e., nuclear chromatin. The SCSA is a flow cytometric procedure that utilizes the metachromatic fluorochrome, acridine orange, and tests the denaturability of spermatozoal chromatin challenged with acid treatment. The literature provides variable results regarding the relationship of stallion

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spermatozoal chromatin denaturation to the extent of disulfide bonding within and between protamine molecules [30,31]; however, chromatin susceptibility to denaturation is correlated with the level of actual DNA strand breaks [29]. The DNA strand breaks can be associated with a myriad of factors, including idiopathic apoptosis, oxidative stress, heat stress, radiation injury, or protamine deficiency [31–38], and may involve double-stranded or single-stranded DNA fragmentation, or oxidized nucleosides [32–39]. Such lesions could create genetically defective spermatozoa, leading to germ-line mutations. Interestingly, spermatozoa affected by such damage may appear to be normal, based on laboratory parameters such as spermatozoal motility and membrane integrity, but may induce post-fertilization embryonic failure [40]. Owing to the highly condensed nature of the spermatozoal chromatin, mature spermatozoa are known to be transcriptionally inactive [41], so it is logical that DNA damage might not be expressed until mitosis occurs at the time of spermatozoon–oocyte fusion. This becomes quite important clinically, as it represents a potential noncompensible defect, i.e., affected spermatozoa in an ejaculate may not be impaired for fertilization, so increasing the insemination number will not increase pregnancy rate. Ejaculated spermatozoa are known to retain a cohort of cytoplasmic mRNAs and as well as translational ability [41,42], so it is also possible that fragmentation of mRNA could have a negative impact on some other spermatozoal functions, leading to reduced fertilization potential. Assays other than the SCSA are available to measure spermatozoal DNA fragmentation/chromatin disruption, including a TdT-mediated-dUTP nick end labeling (TUNEL) assay, an in situ nick translation (NT) assay, a sperm chromatin dispersion (SCD) assay, and an electrophoresis-based Comet Assay [32,33,43–48]. Although these assays have not been used to the same extent as the SCSA in the equine arena, they are commonly applied in the human field. An immunofluorescence assay has also been developed for evaluation of human spermatozoal protamine levels [34]; a similar assay for equine spermatozoa could have diagnostic value.

3. Acrosomal reaction and capacitation

Another assay that commonly employed in our battery of tests is the Acrosomal Responsiveness Assay (ARA). This assay is directed at testing the functionality of the spermatozoal acrosome, i.e., its ability to acrosome reacts when challenged with a potent inducer, the Ca^{2+} ionophore, A23187. Conventional tests are unable to predict the fertility of a subset of stallions with acrosomal dysfunction, because these stallions present with normal spermatozoal morphology and motility, as well as normal chromatin quality (based on SCSA testing) and normal acrosomal structure (based on fluorescent light microscopy and transmission electron microscopy). Meyers and co-workers first reported that the acrosomes of subfertile stallions with poor spermatozoal motility did not react readily in response to progesterone exposure. Fertile stallions averaged a 17% acrosomal reaction rate following 5 h of incubation in capacitating conditions followed by exposure to progesterone, whereas subfertile stallions averaged only a 6% response rate. They concluded that progesterone was capable of stimulating the acrosome reaction in equine spermatozoa exposed to capacitating conditions, and that the response in subfertile stallions was reduced [49]. Others have reported that the plasma membrane of stallion spermatozoa contains progesterone receptors, and indicate that this may be a pathway for induction of the acrosome reaction [50]. In this regard, the percentage of spermatozoa with exposed progesterone receptors was highly correlated with fertility of stallions [51].

Other workers identified a subset of subfertile stallions whose only distinguishing spermatozoal characteristic was the drastically reduced ability to acrosome react when exposed to A23187 for up to 3 h (i.e., mean reaction rate of 84% in fertile stallions versus mean reaction rate of 6% in subfertile stallions [52]); they inferred that the spermatozoal defect may be isolated to the acrosome. The cholesterol–phospholipid ratio in semen of these stallions was significantly greater in both seminal plasma and whole sperm from the subfertile stallions when compared to fertile stallions [53]. Further studies are required to elucidate the underlying etiology of this condition and to determine appropriate treatment strategies.

Although transmission electron microscopy remains a definitive means for detecting the acrosome reaction in stallion spermatozoa [54], acrosomal status can be assessed by bright-field microscopy using Commassie blue stain [55,56]. Fluorescence-tagged lectins or acidotrophic probes can be used with light microscopy or flow cytometric methods to assess the acrosome reaction or acrosomal damage [49,57–64]. Although the more popular fluoresceinated lectin markers have generally replaced chlortetracycline-based assays for detecting the acrosome reaction, the latter assay remains useful because of its ability to assess capacitation, as well as the acrosome reaction [54,56,65]. Another marker demonstrated to be useful

for detection of capacitation in stallion spermatozoa is merocyanine 540, an impermeant lipophilic probe which permits evaluation of the architecture and disorder of lipids in the outer leaflet of the plasma membrane bilayer [66–69]. Other probes, such as fluorochrome-conjugated Annexin V or Ro-09-0198, are being used increasingly to monitor membrane asymmetry. These two probes bind with phosphatidylserine and phosphatidylethanolamine, respectively. Surface exposure of these phospholipids is considered to represent spermatozoal capacitation, because they generally reside in the cytoplasmic leaflet of the lipid bilayer in uncapacitated spermatozoa [70–72].

4. Evaluation of other spermatozoal compartments

A spermatozoon is laden with special molecular domains that are designed and modulated, for a multitude of functions within the female reproductive tract. In some respects, however, the spermatozoon might be considered a relatively simple structure, as it is only composed of a head and tail, with a dramatic reduction in cellular organelles in comparison with somatic cells or its homolog, the ovum. As such, a spermatozoon might be subdivided into: (1) a nucleus, (2) an overlying acrosome, (3) the fibrous and microtubular components of the flagellum, (4) a mitochondrial sheath, and (5) an enveloping plasma membrane. With this context, one can appreciate that a battery of tests devised to evaluate these various compartments might aid in improved localization of cellular dysfunction and might also improve the predictive value of a laboratory-based semen evaluation. Assays developed for the nucleus and the acrosome were discussed above. In addition, techniques specific for mitochondrial function, flagellar substructure, and plasma membrane integrity are available. Whereas the importance of mitochondrial-based oxidative metabolism for ATP production in spermatozoa has been challenged [73,74], a key role of the mitochondria in sustenance of spermatozoal functions remains quite likely [75]. Mitochondria-selective fluorescent markers abound, and include rhodamine 123; an assortment of MitoTracker® probes (which differ in spectral characteristics, adaptability to permeabilization and fixation, and reliance on respiration state of the mitochondria); and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine (JC-1). These reporter molecules passively diffuse across the plasma membrane and accumulate in the mitochondria. Rhodamine 123 was one of the original mitochondrial probes, but has largely been replaced by other markers, due to its reduced photostability and tendency to lose retention in the mitochondria following a loss in membrane potential. Some of the MitoTracker® probes do not fluoresce until oxidized, so these markers permit easy assessment of the respiration state of the mitochondria. However, JC-1 may be optimally suited for evaluation of the energetic state of mitochondria because it produces a membrane potential-sensitive shift in color pattern, i.e., the color changes from green to red–orange as membrane polarization increases. This is due to the reversible formation of *red–orange* J-aggregates in a state of high membrane potential, as compared to *green* monomeric JC-1 in a state of low to medium membrane potential [64,76–79].

The semi-permeable plasma membrane, which is composed of a wide assortment of lipids, proteins, and carbohydrates, envelops the entire spermatozoon. This structure is vital to regulation of spermatozoal functions by establishing ion gradients, facilitating cytosolic entry of larger molecules, and orchestrating various cell-signaling events, to name a few. Thus, assessment of its integrity would seem an important component of a semen evaluation. To this end, a variety of laboratory procedures have been used to evaluate the integrity of the plasma membrane. One method is to evaluate the ability of spermatozoa to exclude extracellular dyes, such as eosin Y, which are nonpermeable when the membrane is intact. Another approach is to expose spermatozoa to hypotonic media (range, 50–100 mOsm) to test their osmoregulatory function; this is defined as the hypo-osmotic swelling test (HOST) [80,81]. With this assay, membrane-intact spermatozoa theoretically permit excessive water entry into the cytosol, resulting in a variety of morphological changes in the flagellum associated with the cytosolic swelling. Conversely, osmoregulation-incompetent spermatozoa will not experience noticeable changes in flagellar shape. Although these tests can serve an ancillary role in semen evaluation, they are not widely applied in the clinical setting, due to the potential for misinterpretation. For instance, humidity impacts the cellular permeability of eosin Y [82]. The incidence of spermatozoa with a bent flagellum prior to HOST will also confound the interpretation of results following exposure of a spermatozoal population to hypo-osmotic media.

A broad array of membrane-impermeable fluorescent dyes is available commercially and can be used to test membrane intactness. Examples include the DNA dyes, propidium-iodide, bis-benzimide (Hoechst 33258), YO-PRO®-1, TOTO®-1, and ethidium homo-

dimer-1. Alternatively, spermatozoa can be bathed in cell-permeable probes that become hydrolyzed to form membrane-impermeant fluorescent products in the cytosol. Examples include carboxyfluorescein diacetate (hydrolyzed by nonspecific cytosolic esterases to form carboxyfluorescein); calcein AM or dihydrocalcein AM (hydrolyzed by esterases to form calcein, which, in turn, forms fluorescent complexes with Ca^{2+}, and other metals) and SYBR®-14 (deacylated in the cytosol, with the resulting product expressing strong fluorescence when complexed with nucleic acids). Of interest, staining of porcine spermatozoa with SYBR®-14, which complexes with DNA, does not affect their ability to fertilize oocytes [83]. Certain membrane-impermeable and membrane-permeable dyes can be combined in solution prior to spermatozoal exposure in an effort to provide a more accurate reflection of membrane integrity. For instance, a combination of SYBR®-14 and propidium-iodide yields three populations of stained spermatozoa: (1) membrane-intact, SYBR®-14-stained cells (green); (2) membrane-damaged, propidium-iodide-stained cells (red), and (3) moribund cells (double-stained) [84,85]. In one study, the percentage of motile stallion spermatozoa (based on computerized motility analysis) was highly correlated ($r = 0.98$) with the percentage of spermatozoa with intact plasma membranes, based on staining with SYBR®-14 and propidium-iodide [86]. Some fluorescent plasma-membrane dyes can also be combined with certain mitochondrial dyes [86,87] or acrosomal dyes [88–92] to provide more thorough compartmental coverage in the assay. The literature reports triple-stain fluorescent techniques for use with stallion spermatozoa: propidium-iodide/SYBR®-14/JC1 [86], and propidium-iodide/FITC-PNA (an acrosomal lectin probe)/carboxy-SNARF-1 (an intracellular pH indicator) [88,93]. Although images can be ascertained with the various fluorophores described above by using fluorescence microscopy, flow cytometry is typically applied because of the high-throughput and objectivity associated with this approach [61,62,79,92,94]. Utilization of a fluorescence microplate reader assay is also reported for use with JC-1 [95].

5. Potential biochemical markers

Considerable effort has been directed toward identification of biochemical markers of spermatozoal function that might aid in laboratory-based detection of fertility by targeting specific subcellular compartments or domains. Many of these methods have been devised for use with non-equine subjects, but several have been proposed for potential use with stallion spermatozoa. Although the value of such tests requires further scrutiny and standardization, the following table provides examples of assays that may prove valuable as diagnostic tools:

Biochemical marker	Potential value of test
Acrosin/amidase activity [96–98]	Acrosome integrity
SNARE proteins [99,100]	Acrosome reaction
Caspases [101–104]	Apoptosis
Heparin-binding proteins [105,106]	Capacitation
Protein phosphotyrosine activity [107–109]	Capacitation
Superoxide anion [110]	Capacitation
Chromomycin A_3 [111–114]	Chromatin packing
Acetylcarnitine/carnitine	Motility/morphology
Phospholipase Cζ [115,116]	Oocyte activation
C_{11}-BODIPY(581/591) [117–122]	Oxidative injury
Malondialdehyde [123–130]	Oxidative injury
Glutathione peroxidase [131–135]	Oxidative injury
Reactive oxygen species [23,130,134,136–143]	Oxidative injury
Lactate dehydrogenase [144–147]	Spermatozoal motility
Creatine kinase/heat shock protein A2 [148–151]	Spermatozoal maturity
CRISP proteins [105,152–157]	Sperm–oocyte binding
SP20/hyaluronidase [158–160]	Sperm–oocyte interaction
AWN spermadhesion protein [105,161–163]	Sperm–zona binding
P34H protein [164–167]	Sperm–zona binding
Zonadhesin [168–174]	Sperm–zona binding
SP22 protein [175–178]	Spermatozoal fertility

6. Spermatozoal motility

Incorporation of in-depth tests, such as those above, for semen evaluation does not replace, nor diminish the value of, the classical measurements of spermatozoal motility or morphology; therefore, these two methods are likely to remain the hallmarks of semen evaluation. Evaluation of spermatozoal motility in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of spermatozoa in an ejaculate. Evaluation of raw (undiluted or neat) semen provides an indication of how well spermatozoa perform in their natural fluid milieu. Determining motility in the raw form can be hampered by higher sperm concentrations and spermatozoal agglutination to the cover glass, making it difficult to discern individual motility patterns. To overcome this limitation, an aliquot of semen can also be appropriately diluted (e.g., to 25×10^6 sperm/mL) in a good-quality semen extender that is free of debris when visualized microscopically. The extender may

slightly alter motility pattern, usually by increasing the velocity measures. After initial extension, a high percentage of spermatozoa may exhibit a circular motility pattern; however, this behavior usually resolves following 5–10 min of exposure time in the extender. Spermatozoal motility is exceptionally susceptible to environmental conditions (e.g., excessive heat or cold, lubricants, light, disinfectants, and osmolarity/pH of semen extender), so it is necessary to protect the semen from injurious agents or conditions prior to analysis. To enhance the reliability of motility estimation, the procedure should be performed by an experienced person using a properly equipped microscope, i.e., one with a built-in stage warmer and properly adjusted phase-contrast optics. Estimations of the percentages of motile and progressively motile spermatozoa are generally determined, in addition to an estimation of spermatozoal velocity (based on an arbitrary scale of 0–4; stationary to fast, respectively). Subjective assessment of motility is generally quite acceptable, provided personnel are experienced in analysis of spermatozoal motility.

Several different techniques and instruments have been developed in an effort to secure an objective (i.e., unbiased) evaluation of spermatozoal motility; however, these methods (e.g., time-lapse photomicrography, frame-by-frame playback videomicrography, spectrophotometer, or computerized analysis) are generally considered too tedious or expensive for routine use. Computerized systems are currently in place in many reference laboratories, with the intent to objectively assess motion characteristics of spermatozoa. Despite the commercial availability of various generations of computer-assisted spermatozoal analysis (CASA) systems for >20 y, their presence has not provided the definitive assay for measuring spermatozoal fertilizing potential [179]. Such an expectation, however, is unrealistic, given the numerous independent spermatozoal attributes that are required for a spermatozoon to possess fertilization competence. What these systems do provide, however, is the prospect of objective measurement and protocol standardization. These instruments permit customized selection of various features, including frequency and length of frame capture; threshold demarcations for the presence of spermatozoal motion, progressivity of motion, and velocity measures, and gating freedom for both size and luminosity representative of spermatozoal heads, as a means to maximize capture of spermatozoa while minimizing capture of non-spermatozoal material in the sample of interest. Such manipulations are important for improving measurement accuracy and repeatability within a given laboratory, but make it virtually impossible for reliable comparisons among laboratories or among different CASA brands [180,181]. Computerized analysis of spermatozoa is primarily reserved for the research setting, where standardization, accuracy, and precision are a prerequisite to measurement of experimental end points. A distinct value of CASA instruments in the commercial environment (at a veterinary hospital or an equine breeding operation) is the ability to garner objective results for a variety of motility end points. Confusion arises, however, regarding the relationship of the myriad of obtainable CASA variables to fertility of the sample. As an example, we recently conducted a fertility trial with a subfertile stallion whose semen was subjected to density-gradient centrifugation in an effort to improve semen quality prior to insemination. Values for % motility (MOT), % progressive motility (PMOT), and mean curvilinear velocity (VCL) prior to, and after, semen processing for the subfertile stallion and a fertile control stallion are listed below:

Stallion	Semen treatment	MOT (%)	PMOT (%)	VCL (μm/s)
Fertile	Before	80	63	205
Fertile	After	91	78	209
Subfertile	Before	63	48	251
Subfertile	After	90	79	259

Based on these results, we inferred that semen treatment for the subfertile stallion yielded a spermatozoal population with quality similar to, or exceeding (based on velocity values), that of the fertile control stallion. Nonetheless, when fertile mares were inseminated hysteroscopically with 20×10^6 progressively motile spermatozoa (100 μL-volume), the resulting pregnancy rates were 15/20 (75%) for the fertile stallion, as compared to 7/20 (35%) for the subfertile stallion ($P = 0.01$). This demonstrates that spermatozoal motility does not provide absolute discrimination power, again emphasizing that spermatozoal attributes other than motility play critical roles in spermatozoal fertilizing ability. Some CASA systems can be outfitted with fluorescence optics, and the predictive value of CASA might be improved by incorporation of fluorescent dyes in the media, such that motility variables can be segregated by presence or absence of membrane intactness [182]. Further studies are required to determine if the predictive value of spermatozoal motility can be improved by this approach. A study involving boars suggests that CASA analysis might have an improved relationship with fertility if the

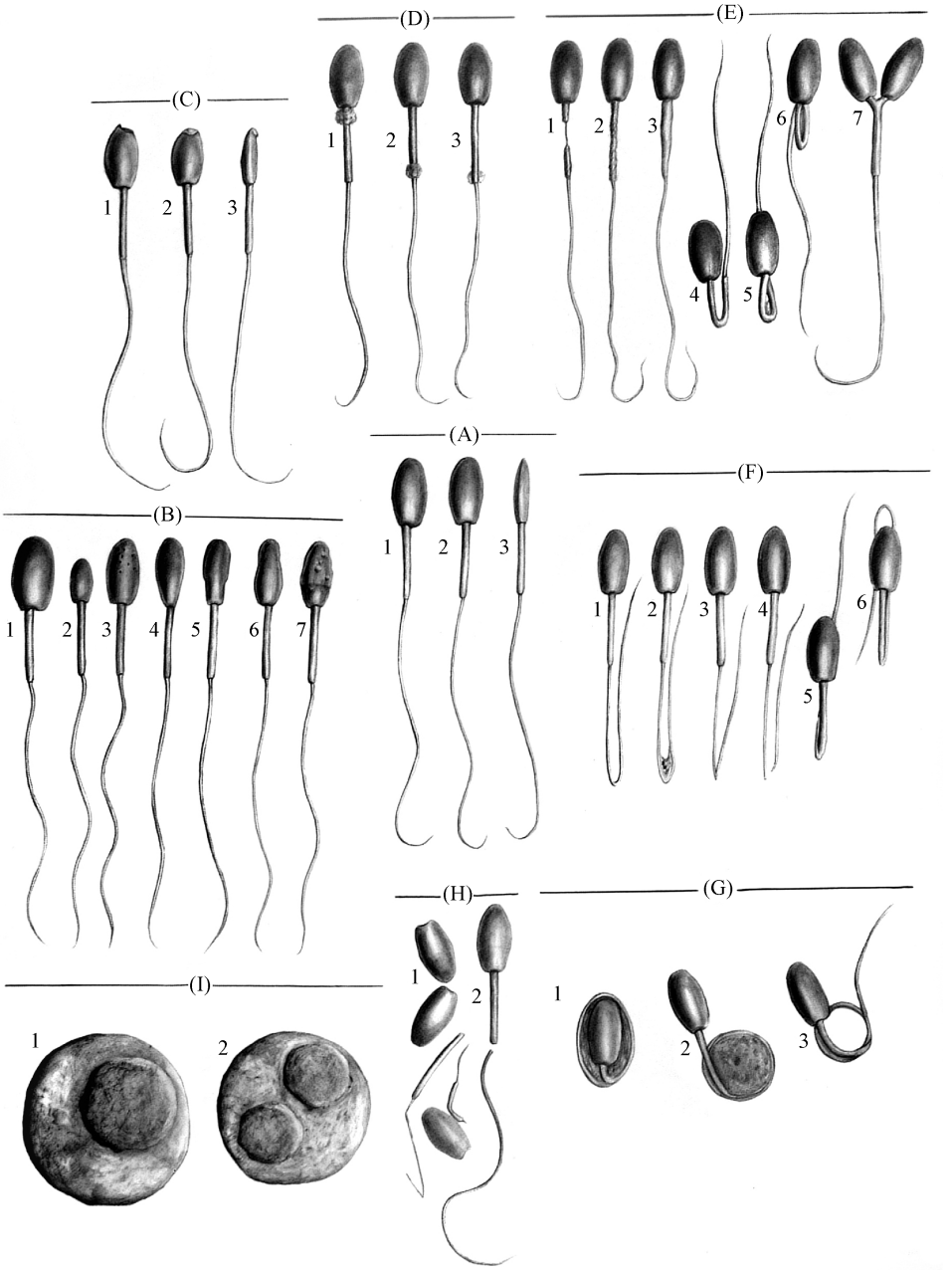

Fig. 1. Drawings of spermatozoa to resemble images of normal and abnormal equine spermatozoal morphology, as viewed by differential-interference contrast (DIC) microscopy of a fixed and unstained wet-mount semen specimen. The DIC optics provides a three-dimensional image of spermatozoa. (A) Normal spermatozoal morphology, in dorsoventral (A1, A2) and side (A3) views. Abaxial midpieces (A1) are considered to be morphologically normal. (B) Variations in abnormal head morphology, including macrocephalic (large head) morphology (B1), microcephalic (small head) morphology (B2), nuclear vacuoles (or crater defects; considered by our laboratory to be normal if low in number and size, and located randomly over acrosomal region; B3), tapered head (B4), pyriform head (B5), constricted or hour-glass head (B6), and degenerate head (B7). (C) Acrosomal defect in dorsoventral (C1, C2) and side (C3) views. This morphologic defect is termed "knobbed acrosome." (D) Proximal (D1) and distal (D2, D3) cytoplasmic droplets. (E) Abnormalities of the midpiece, including segmental aplasia of the mitochondrial sheath (E1), roughed midpiece from uneven distribution of mitochondria (E2), enlarged mitochondrial sheath (E3), bent midpiece (E4, E5, E6), and double midpiece/double head (E7). (F) Bent tail (or hairpin tail), involving the mid-region of the principal piece (F1–F4) or with a singular bend (F5) or proximal bend (F6) involving the midpiece-principal piece junction. (G) Coiled tail, with the tail tightly encircling the head (G1) or the tail coil not encircling the head (G2, G3). (H) Fragmented sperm, including head detachment (termed detached heads or tailless heads; H1) or fragmentation at the level of the annulus (H2). Fragmentation can also occur at other sites, as demonstrated in F4. (I) Premature germ cells with a single nucleus (I1) or multiple nuclei (I2).

spermatozoa are also incubated under capacitating conditions to observe changes in spermatozoal velocity over time [183].

7. Spermatozoal morphology

The morphology or structure of spermatozoa is typically examined with a light microscope at 1000× magnification. Standard bright-field microscope optics can be used to examine air-dried semen smears, provided appropriate stains are used in slide preparation. Specific sperm stains include those developed by Williams [184] and Casarett [185]. General-purpose cellular stains (e.g., Wright's, Giemsa, hematoxylin-eosin) also have been used to accent both germinal and somatic cells in semen smears. Background stains (e.g., eosin-nigrosin, India ink) probably are the most widely used stains because of their ease of application. Visualization of the structural detail of spermatozoa can be greatly enhanced by fixing the cells in buffered formol saline or a similar fixative, then viewing the unstained cells as a wet-mount with either phase-contrast or, preferably, differential-interference contrast (DIC) microscopy [82]. In addition, the incidence of artifactual changes is reduced in comparison with stained smears.

At least 100 spermatozoa should be evaluated for evidence of morphological defects. The type and incidence of each defect should be recorded. Abnormalities in spermatozoal morphology traditionally have been classified as primary, secondary, or tertiary. Primary abnormalities are considered to be associated with a defect in spermatogenesis and, therefore, are of testicular origin. Secondary abnormalities are created in the excurrent duct system. Tertiary abnormalities, as opposed to the previous two types, develop in vitro as a result of improper semen collection or handling procedures.

The current trend is to record the numbers of specific morphologic defects, such as knobbed acrosomes, proximal protoplasmic droplets, swollen midpieces and coiled tails. This method of classification is considered superior to the traditional system because it reveals more specific information regarding a population of sperm, while avoiding erroneous assumptions about the origin of these defects. The origin of some spermatozoal morphologic defects is unknown. Additionally, some morphologic abnormalities like detached heads can be primary, secondary or tertiary in nature, thereby introducing the possibility of error when using the traditional classification system exclusively. Drawings of normal and abnormal sperm morphologic features, as they appear with DIC microscopy, are shown (Fig. 1).

The percentage of morphologically normal spermatozoa is positively correlated with spermatozoal motility. Moreover, close morphological inspection provides additional information about characteristics of individual spermatozoa. This information is important because semen can possess good spermatozoal motility, yet have a relatively high incidence of spermatozoal morphologic abnormalities. Furthermore, stallions can have many spermatozoal abnormalities yet display normal fertility. Some morphologic defects (e.g., cytoplasmic droplets, bent tails) appear to have a minor effect on fertility of stallions bred by natural cover, whereas other defects (e.g., detached heads, abnormally shaped heads, abnormally shaped midpieces, coiled tails, and premature germ cells) have a deleterious effect on fertility [8]. In general, morphologically abnormal spermatozoa do not exert a direct negative influence on normal spermatozoa. Therefore, the total number of morphologically normal sperm in ejaculates may provide more information regarding the fertility of a stallion than the percentage or absolute number of morphologically abnormal spermatozoa. In general, the percentage of morphologically normal sperm in a semen sample is similar to the percentage of progressively motile spermatozoa. If spermatozoal motility is low and the percentage of morphologically normal sperm is high, it suggests that laboratory errors occurred which led to a lowering of spermatozoal motility. One cannot discount, however, a potentially negative effect of seminal plasma on spermatozoal motion characteristics.

8. New horizons

As discussed above, a variety of techniques and protocols are available for evaluation of the spermatozoon. Application of newly developed assays can be cumbersome and expensive. Nonetheless, incorporation of some of these diagnostic tools into a standard semen analysis may yield improved discrimination ability regarding the competence of these complexes, yet intriguing, cells. Similar applications would be valuable for critical evaluation of laboratory techniques applied to liquid preservation or cryopreservation of spermatozoa.

Although a plethora of scientific information surrounds spermatozoal structure and function, many unresolved issues remain, even with human and rodent spermatozoa where the largest body of information has been assimilated. Only when we know precisely the

specific molecular interactions required for attaining full spermatozoal fertilizing potential, including the spatial and temporal changes, energetics, and gaseous environment involved, will we be able to reliably manipulate spermatozoa to meet the growing needs within the equine breeding industry. Goals might include devising methods for long-term cooled semen preservation, improving the fertility of cryopreserved semen, and incorporation of in vitro fertilization (both conventional in vitro fertilization and intracytoplasmic sperm injection) into commercial programs. While these might appear to be lofty goals, a more absolute understanding of spermatozoal structure and function would certainly take a lot of the ''guess work'' out of current approaches to analysis and manipulation of equine spermatozoa.

Attempts to gain a better understanding of equine spermatozoa solely from extrapolation of data acquired from other mammalian species are likely destined to failure, due to well-known species differences in spermatozoal attributes and physiology. Nonetheless, much information derived from other species may have relevance to equids, and should be investigated for applicability. Examples include identification of candidate genes for specific spermatozoal traits, targeted mutation of genes for specific proteins to study the resulting effect on reproductive function, and use of gene silencing agents for regulatable ablation of gene function. Incorporation of molecular techniques would appear to be the key to elucidation of mechanisms that control spermatozoal development and function in stallions.

Indeed, the ''omics'' era (e.g., genomics, toxicogenomics, transcriptomics, metabonomics, and proteomics) is upon us. Technological progress in the field of molecular genetics has been remarkable, and has opened the door to new paradigms for the study of spermatozoal function and dysfunction. Substantial inroads have been made in horse genome mapping in recent years, including availability of synteny, linkage, radiation-hybrid, cytogenetic, and human–horse comparative maps [186–191]. The National Institutes of Health recently reported (www.nih.gov/news/pr/feb2007/nhgri-07.htm) that the 2.7-billion DNA base-pair horse genome has been sequenced and partially assembled, and the second draft of the sequence is now accessible through public databases; hence, the importance of bioinformatics to biological research. Continued progress in genomic research will lead to isolation and identification of genes responsible for spermatozoal development and function, as well as genetic factors that underlie various types of reproductive failure. High-throughput microarray technology (cDNA and oligonucleotide) is being used with increased intensity to study differential gene expression [192]. Reverse transcription and amplification steps are being applied to quantify gene activity in targeted cells or processes, and to unravel the complexity of genes [193,194]. The large-scale information obtained from these emergent technologies is being applied to male fertility [195], including that of horses [196–198]. Interpretation of such expression profiling and its biologic significance, however, can be a quagmire and continued effort is required to hurdle this obstacle [199]. Given the intensity of investigation in this area, the basic mechanisms that control gene expression will emerge in the years ahead, and genetic markers for stallion fertility may one day abound [200]. Robust mass-spectrometry proteomics methodologies allow inventory of proteins in cells, organelles, and body fluids [201–203], and offer the potential for identification of biomarkers for diagnostic tests [204–206].

As with humans and other animal species, the impacts that environmental chemicals have on stallion fertility deserve attention. An apparent decline in spermatozoal quality and quantity of men has been described in recent years [207–209]. Increased rates of other reproductive tract problems, such as cryptorchidism and testicular cancer, have also been reported [210,211]. Environmental reproductive toxicants are considered to be a major contributor to these patterns [212–214]. Both cDNA microarray and real-time RT-PCR approaches have revealed differential expression profiles in some spermatogenesis-related genes (i.e., *heat shock protein 70-2, insulin growth factor binding protein 3, and glutathione S transferase pi*) following toxicant exposure, so genes such as these may serve as useful biomarkers when screening for testicular toxicity [215].

A better understanding of the molecular mechanisms regulating spermatozoal development and function will likely lead to new diagnostic techniques and therapeutic strategies for reduced fertility in stallions and may possibly translate to methodologies designed to curb gonadal aging. Such progress can only be curtailed by funding deficits or diverted interests of investigators in the years ahead. Undoubtedly, future generations will look beyond descriptive morphology and motility in characterizing structural and functional features and deficiencies of spermatozoa. In addition, they will likely use the spermatozoon as a vehicle for production of transgenic horses [216,217], as well as spermatogonial stem cell transplantation or testicular grafting techniques to study testicular function or to propagate a genetic line [218–225].

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