Techniques for evaluating selected reproductive disorders of stallions

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Abstract

Numerous techniques may be used for evaluation of the different reproductive disorders of the stallion. Approaches may vary from real-time ultrasonography and biopsy for evaluating testicular tumors to use of special assays for evaluating sperm or plasma for presence of antisperm antibodies. This communication addresses techniques used to evaluate five relatively uncommon, but perplexing, disorders of breeding stallions: (1) seminal vesiculitis, (2) hemospermia associated with idiopathic urethral defects, (3) acrosomal dysfunction, (4) abnormal spermatozoal chromatin, and (5) azoospermia. © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Seminal vesiculitis

The stallion has four distinct accessory genital glands: (1) the paired seminal vesicles or vesicular glands, (2) the paired ampullae, which constitute a specialized portion of the terminal segment of each deferent duct, (3) the bilobed prostate gland, (4) and the paired bulbourethral glands (Fig. 1). The vesicular glands are slightly pyriform sacs which are situated on either side of the bladder neck, and extend craniolaterally into the posterior abdomen. Each gland cones into a singular excretory duct which communicates with the
Fig. 1. Illustration of the accessory genital glands of the stallion (dorsal view). (a) Genital fold, (b) bladder, (c) ampulla, (d) vesicular gland (seminal vesicle), (e) lobe of prostate gland, (f) isthmus of prostate gland, (f) pelvic urethra (enclosed by urethralis muscle), (g) bulbourethral gland, (h) retractor penis muscle.
lumen of the proximal urethra via a protuberance on its dorsal surface, the colliculus seminalis (Fig. 2).

Seminal vesiculitis is quite uncommon in stallions; however, it is a noteworthy disorder because of its persistent nature and interference with fertility. Reported bacterial
etiolologic agents include *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus* spp., *Staphylococcus* spp., and *Brucella abortus*. We recently identified a stallion with unilateral erosive seminal vesiculitis associated with *Acinetobacter calcoaceticus*.

Characteristically, stallions with seminal vesiculitis do not manifest overt clinical signs. In general, affected horses tend to be afebrile and do not exhibit signs of pain at the time of urination, defecation, or breeding. Presumptive diagnosis of seminal vesiculitis is usually made through gross and microscopic analysis of semen. Upon cytological examination, variable numbers of neutrophils may be present. Grossly, the semen sample may be blood-tinged and flocculent, with clumps of purulent material. Large numbers of bacteria may also be detectable microscopically. Spermatozoal quality may appear unaffected when examined immediately post-ejaculation, but spermatozoal longevity is usually reduced.

It can be difficult to determine the site of inflammation if neutrophils are detected in semen, because they may arise from lesions involving the exterior of the penis, urethra, upper urinary tract, deferent ducts, epididymides, testes, or any of the accessory genital glands. An internal source of infection is suspected if no lesions are observed on the penile integument or urethral process.

A comparison of bacterial types and numbers recovered from the exterior of the penis, urethra, and semen can be used to help localize an infectious process. Culture swabs of the unwashed penis and preputial cavity are first obtained to determine the resident bacterial flora in these areas. This step is important because many cases of suspected “seminal vesiculitis” have an external source of infection by potentially pathogenic bacteria that reside on the skin of the penis and prepuce. In preparation for further cultures, the penis is washed with a bactericidal soap and thoroughly rinsed and dried. Pre- and post-ejaculate swabs are taken from the distal urethra. Culture swabs of the semen are also procured. Semen is collected using an artificial vagina that has been sterilized prior to use to prevent possible contamination. A heavy pure growth of bacteria in the post-ejaculate urethra and semen is consistent with an internal genital infection.

Both culture and cytologic evaluation of expressed secretions of the vesicular glands aid in diagnosis of seminal vesiculitis. To fill the vesicular gland lumina with secretions, the stallion is first aroused sexually (teased) for approximately 15 min by exposure to a mare in estrus. The distal penis is washed, rinsed and dried. A sterile 100-cm rubber catheter with an inflatable cuff is then passed into the urethra until its tip is immediately caudal to the colliculus seminalis, where the ducts of the vesicular glands enter the urethra (Cooper, 1979). Proper placement of the catheter tip is aided by palpation per rectum. After the cuff is inflated, each vesicular gland is identified, and the contents are removed by manual compression of the gland. Fluid is collected for bacterial culture and cytology, using a sterile container attached to the catheter opening at the urethral orifice.

Palpation of the vesicular glands is usually not rewarding diagnostically in stallions with seminal vesiculitis. Typically, no changes are evident in size, shape or consistency of affected glands, although on occasion they may be enlarged, firm, and lobulated. Pain may be elicited upon palpation in acute cases of seminal vesiculitis. Abscessation of the vesicular glands has been reported in stallions, but occurs rarely. Such lesions may be detectable by palpation per rectum.
Transrectal ultrasonography has been used to evaluate the accessory genital glands of normal stallions (Little and Woods, 1987). This procedure may not detect seminal vesiculitis, but can be used to support a diagnosis made by traditional means (Malmgren and Sussemlach, 1992).

We have used a flexible endoscope for definitive diagnosis of seminal vesiculitis in four stallions. A small-diameter (7–9 mm) fiberoptic or video endoscope can be passed directly into the vesicular gland lumina, via the duct openings at the seminal colliculus (Fig. 2). Care should be taken to prevent undue trauma to the duct openings when the endoscope is inserted into each vesicular gland. The gland lumina can be directly visualized to facilitate sampling for bacterial culture and cytology analysis. Culture instruments or catheters designed for endoscopes can be passed into position via the biopsy channel for acquisition of samples. Using this urethral endoscopic approach, the character of fluid expressed manually per rectum from the vesicular glands, prostate gland, and bulbourethral glands can also be visualized.

2. Hemospermia associated with urethral defects

Hemospermia refers to contamination of ejaculates with blood. Blood-laden ejaculates are pink to reddish in hue on gross examination. Cytological examination may be

Fig. 3. Illustration of pelvic urethra, as viewed by urethral videoendoscopy. A urethral defect which communicates with the corpus spongiosum penis is illustrated on the right.
required to identify less obvious blood contamination. Hemospermia can result from a variety of conditions. Trauma to the penis may result in injuries such as bruising, lacerations, and hemorrhage, and most commonly involves the urethral process. Lacerations or contusions of the proximal penis, either prescrotal or in the area of the ischial arch, can lead to hemospermia. The application of stallion rings or brushes to prevent masturbation may lead to penile/urethral bruising, lacerations, pressure necrosis, and subepithelial vessel prolapse through splits in the urethral epithelium. Tumors of the penis may also result in hemospermia.

Profuse arterial hemorrhage in ejaculates usually results from an idiopathic defect in the urethra that communicates with the surrounding corpus spongiosum penis (Schaumacher et al., 1995). The lesion is typically longitudinal, and is almost always located on the posterior surface of the urethra near the pelvic ischium. Diagnosis of the urethral defect requires urethral endoscopy. The urethral defect can be identified near the pelvic ischium (Fig. 3). The defect can be small and difficult to identify. Endoscopy immediately following ejaculation may aid the examiner in locating the defect because blood can often be detected within the defect at this time. Bleeding is profuse because the lesion communicates with the underlying corpus spongiosum penis. As such, bleeding only occurs when blood pressure within the corpus spongiosum penis increases with erection or micturition. The cause of the lesion has not been determined.

3. Acrosomal dysfunction

In some cases, results of a routine breeding soundness examinations are unable to provide a primary etiology for a stallion’s reduced fertility. Despite proper mare management, and normal semen evaluations, some stallions are still unable to impregnate an acceptable proportion of mares bred. Determining the cause for subfertility in stallions with normal semen parameters can be frustrating. For such stallions, additional tests are required to help explain their condition. These may include assays for sperm chromatin structure (described below), antisperm antibodies or the acrosome reaction. Subfertile stallions have a higher incidence of elevated antisperm antibody titers than fertile stallions (Kenney et al., 1998; Love, 1997). In human males with unexplained infertility, the presence of antisperm antibodies has been shown to interfere with the induction of the acrosome reaction by the zona pellucida (Francavilla et al., 1997). A direct correlation has been demonstrated between the acrosome reaction and fertilization rate in human males. This parameter may be a better predictor of IVF success than the more traditionally evaluated semen parameters. (Calvo et al., 1994a,b).

The acrosome is an membrane-bound organelle that covers the anterior portion of the mammalian spermatozoal nucleus (Fig. 4). The term, acrosome reaction, refers to the fusion and vesiculation of the outer acrosomal and plasma membranes (Fig. 5). During the acrosome reaction, hydrolytic enzymes are released from this organelle which facilitate penetration of the investments of the ovum. Whether or not the functions of these enzymes go beyond aiding in development of the acrosome reaction, penetration of the cumulus oophorus and binding of the zona pellucida to actually lysing a path through the zona pellucida is a subject of some debate (Bedford, 1998). Regardless of
whether or not penetration of the zona is a lytic event, a normally functioning acrosome is essential to successful in vivo fertilization.
The acrosome reaction in equine spermatozoa can be induced by exposing capacitated spermatozoa to progesterone (Meyers et al., 1995) or calcium ionophores such as ionomycin or A23187 (Varner et al., 1987, 1993). The acrosome reaction can occur in uncapacitated spermatozoa when high molar concentrations of calcium ionophore are used. Although induction of the acrosome reaction with progesterone is more physio-
logic, it is also more time consuming. Incubation in a capacititating medium for up to 5 h may be required prior to exposing spermatozoa to progesterone. Results of one study suggested spermatozoa from fertile stallions may be more responsive to progesterone-induced acrosome reactions than those of subfertile stallions (Meyers et al., 1995). Similarly, lower levels of both spontaneous and progesterone-stimulated acrosome reactions have been reported to occur in semen from infertile men with severe teratozoospermia (Oehninger et al., 1994).

Our laboratory has identified four stallions with previously unexplained infertility, whose spermatozoa do not respond to A23187 to the same degree as fertile stallions. Exposure of fertile stallion semen to the calcium ionophore resulted in > 80% of the spermatozoa becoming acrosome reacted, whereas < 20% of spermatozoa from the stallions with unexplained subfertility or infertility underwent the acrosome reaction under the same conditions. Acrosomal status was evaluated by transmission electron microscopy (TEM). Among human patients with acceptable levels of spermatozoal numbers, motility and morphology, 20% produce ejaculates which fail to acrosome react normally (Calvo et al., 1994a). The prevalence of this condition in the population of breeding stallions is unknown, but may mimic that in the human population.

Fig. 6. Transmission electron micrographs of stallion spermatozoa, demonstrating (a) intact acrosome, (b) vesiculated acrosome, and (c) fragmented acrosomal and plasma membranes.
Briefly, the procedure we use for induction and assessment of the acrosome reaction is as follows: Semen is collected in a routine manner using an artificial vagina. Aliquots of gel-free semen are exposed to 10 μM A23187 in a milk-based extender for 2 h at 37°C, then fixed and processed for TEM (Varner et al., 1992). TEM is expensive, time consuming and requires sophisticated laboratory facilities. Consequently, alternative methods of assessing the acrosome reaction are being investigated. The use of fluorescent probes is the most commonly employed alternative to TEM for evaluating the acrosome reaction. Chlortetracycline can be used to assess both capacitation and the acrosome reaction. Unfortunately, it does not appear to be reliable when semen has been diluted with milk-based extenders. Conjugated lectins such as FITC-PNA (Arachis hypogea) and FITC-PSA (Pisium sativum) are commonly used fluorescent acrosomal probes. These probes can be used with milk-based extenders. Initial studies in our laboratory revealed that the fluorescent lectin, PSA, produced a high incidence of false acrosome reactions when used with a stallion exhibiting failure of the acrosome reaction following spermatozoal exposure to A23187. In this stallion, fragmentation of acrosomal and plasma membranes following exposure to A23187 created a fluorescent pattern on the spermatozoa which simulated that of a true acrosome reaction (Fig. 6). At this time, TEM remains the “gold standard” for evaluating acrosomal integrity.

4. Abnormal spermatozoal chromatin

Sperm chromatin implies sperm DNA and associated nucleoproteins. The relationship between sperm chromatin stability and male fertility has been reported for several species. Repeated abortions in women impregnated by men with large numbers of easily denatured sperm is an example of how abnormal sperm chromatin can result in subfertility (Ibrahim and Pedersen, 1988). Recently, the relationship between sperm chromatin stability and fertility in stallions had been described (Kenney et al., 1995; Love, 1993; Love and Kenney, 1998).

All stallions have some level of abnormal spermatozoal chromatin. However, the degree of the abnormality is what determines the severity and how it relates to the fertility status of the stallion. Therefore, the higher the percentage of abnormal chromatin, the greater the reduction in the fertility of the stallion that can be expected.

While there are many potential causes for disruption of sperm chromatin structure, clinically there appear to be two manifestations in the stallion. The first is a result of a traumatic or stressful incident to the testes, such as a kick or any event that would result in fluid accumulation around the testes. This has the effect of insulating the testes, resulting in a heat stress condition. In this case, changes in spermatozoal motility and morphology can be seen, as well and changes in sperm chromatin structure (Love, 1993). Depending on the severity of the heat stress, the morphology and motility will be affected first, while the chromatin appears more resistant to mild forms of heat stress. As the adverse effects resolve, the chromatin returns to normal. Therefore, it appears that chromatin disruption is reversible when the inciting cause is removed.

The second form of chromatin disruption involves those stallions which appear to have a high percentage of chromatin that is inherently abnormal with no known cause.
Sperm chromatin may deteriorate progressively as stallions age, or may be manifest as an inherent trait at the onset of their breeding career.

Diagnosis can be performed using a flow cytometric procedure, the sperm chromatin structure assay (SCSA), to evaluate the structural integrity of sperm chromatin. The flow cytometer offers precise detection of fluorescent labels and assessment of several thousand spermatozoa in only a couple of minutes. Raw semen samples can be collected, immediately frozen, and stored in either liquid nitrogen or in a $-20^\circ C$ freezer until analysis. The assay provides a measurement of the relative amounts of double-stranded and single-stranded DNA in spermatozoal populations following exposure to heat- or acid-induced denaturing conditions. Using the metachromatic DNA stain,
acridine orange, double-stranded DNA appears green and single-stranded DNA appears red under special fluorescing conditions. Quantitation of DNA denaturation is determined by the term, alpha-t, which is defined as the ratio of red/red + green fluorescence, and is measured for each spermatozoan represented in a scatter plot (Fig. 7) or histogram (Fig. 8). Several parameters can be created using this scatter plot or histogram to describe the susceptibility of the DNA to denaturation. The mean of alpha-t ($\alpha_t$) reflects the degree of DNA denaturation of the whole spermatozoa population. The SD $\sigma_t$ indicates the extent of denaturation or how far individual spermatozoa may deviate from the main population. The percentage COMP $\alpha_t$ is the percentage of cells outside the main population, and it is determined by selecting those cells to the right of the main

Fig. 9. Sperm chromatin structure assay (SCSA) scattergrams representing varying levels of fertility among stallions from highest (A) to lowest (D).

<table>
<thead>
<tr>
<th>Stallion</th>
<th>Seasonal pregnancy rate (%)</th>
<th>First-cycle pregnancy rate (%)</th>
<th>Mean $\alpha_t$</th>
<th>SD $\sigma_t$</th>
<th>COMP $\alpha_t$</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>94</td>
<td>80</td>
<td>222</td>
<td>79</td>
<td>11</td>
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<tr>
<td>B</td>
<td>81</td>
<td>46</td>
<td>266</td>
<td>124</td>
<td>28</td>
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<tr>
<td>C</td>
<td>67</td>
<td>40</td>
<td>279</td>
<td>99</td>
<td>68</td>
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<tr>
<td>D</td>
<td>12</td>
<td>8</td>
<td>288</td>
<td>78</td>
<td>16</td>
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population and represents the number of cells outside the main population as a percentage of the total number of spermatozoa evaluated (Figs. 7 and 8). Fig. 9 demonstrates the progressive decline in fertility of stallions as deterioration of chromatin increases. Stallions of high fertility (stallion A) have low values for all SCSA variables, indicative of chromatin that is structurally sound. As chromatin deteriorates, the SCSA values increase, as indicated for stallions B–D. Stallion B is of lesser fertility than stallion A (seasonal pregnancy rate of 81 vs. 94%) and this is represented by a higher mean, SD and COMP of $\alpha_s$. Stallion C has a very high COMP $\alpha_s$ (68%) and a lower seasonal pregnancy rate than stallions A and B. Stallion D is an extreme example of a highly subfertile stallion, in which the entire spermatozoal population has shifted off the “normal” axis, resulting in essentially 100% COMP $\alpha_s$. Stallions exhibiting this type of chromatin can be easily misdiagnosed unless the axial shift is recognized.

5. Azoospermia

Azoospermia implies a complete absence of spermatozoa in ejaculates. Causes include spermatogenic failure, obstruction of extracurrent ducts, failure of emission (release of sperm and accessory gland fluids into the pelvic urethra) or ejaculation (forceful expulsion of the combined fluids from the urethra), and retrograde ejaculation. Severe testicular hypoplasia or degeneration can cause azoospermia due to failure of spermatozoal production by the seminiferous epithelium. Complete obstruction of the epididymal or deferent ducts also results in azoospermia, as sperm produced by the testes cannot pass through the excurrent duct system to enter the urethra for ejaculation. Spermatozoa occasionally become impacted within the epididymal/deferent duct lumina to the point that they are not released by the strong muscular contractions of the ducts at the time of ejaculation. The condition is often referred to as “plugged ampullae” when ampullary lumina become obstructed (Love et al., 1992). The size and glandular nature of the ampullae of the stallion provides a potential for excessive accumulation of spermatozoa, occasionally leading to inspissation and blockage of the lumina. With complete bilateral blockage, azoospermia occurs. Blockage can be sporadic, with affected stallions having poor spermatozoal morphology and motility due to a high percentage of tailless heads when being bred infrequently. Densely packed spermatozoal casts may also be present in the semen. Diagnosis is based on and ultrasonic physical examination and characteristics of semen quality (Love et al., 1992). Azoospermic stallions with this underlying etiology will likely have negligible alkaline phosphatase concentrations in semen (Turner, 1996). Palpation per rectum may reveal enlarged, tense ampullae, and occasionally narrowing or loss of the echolucent lumen that is characteristic of a patent ampulla when examined by ultrasonography.

A major cause of azoospermia in stallions is anejaculation due to an ejaculatory disorder. In this instance, little to copious amounts of pre-ejaculatory fluid can be produced during the act of coitus, but sperm are not present. Many of these cases are psychogenic in nature (McDonnell, 1992a,b, 1986), but physical disorders can also cause ejaculatory failure. Examples include musculoskeletal disorders (e.g., back pain, degenerative joint disease, laminitis, or foot abscesses), neurologic diseases (e.g., equine
protozoal myeloencephalitis or nerve root injury), vascular disorders (e.g., aorto-iliac thrombosis), gastrointestinal problems (e.g., abdominal adhesions or integument scarring secondary to colic surgery, or inguinal herniation) or lesions specific to the reproductive tract (e.g., spermatic cord torsion, orchitis/epididymitis, seminal vesiculitis, or penile injury) (Martin et al., 1998; McDonnell, 1992a,b, 1999; Varner et al., 1991). Retrograde ejaculation (i.e., ejaculation into the bladder) has also been documented in a stallion.

Azoospermia can be a diagnostic dilemma, as the most common cause of this condition is failure to ejaculate (i.e., that associated with psychogenic or physical ailments). Diagnostic efforts are directed first toward determining if the stallion is actually ejaculating. In the stallion with normal libido and breeding behavior, indirect evidence for ejaculation includes engorgement of the glans penis, urethral pulsations, flagging of the tail, and the presence of gel in the sample collected. After removal of the gel portion, the gel-free liquid is examined, using a phase-contrast microscope at 200–400× magnification, for the presence of spermatozoa or premature germ cells. If no spermatozoa are present, assay of the gel-free liquid for alkaline phosphatase is performed to confirm presence of epididymal/testicular fluids (Turner, 1996). High alkaline phosphatase values confirm the presence of epididymal/testicular secretions in the seminal fluid. If necessary, a testicular biopsy can be procured to determine if spermatogenesis is proceeding to completion (Fig. 10). Both incisional (DelVento et al., 1992; Threlfall and Lopate, 1993) and needle (Faber and Roser, 1999; Hillman et al., 1994; Threlfall and Lopate, 1993) biopsy techniques have been reported recently for use in stallions. Although detrimental effects have been associated with testicular biopsy, these studies suggest that it is a relatively safe procedure. The technique is used routinely in subfertile men (Carpi et al., 1999; Chen, 1997; Craft et al., 1997; Ezeh et al., 1998; Morey et al., 1994).

To illustrate the diagnostic approach for azoospermia, we present the data from three stallions. A 5-year-old stallion with slightly small testes appeared to ejaculate normally with gel being present in his ejaculates, but the semen contained only premature (round) germ cells (0.5 million/ml). Assay of seminal plasma from this stallion yielded 1130 U/l alkaline phosphatase, indicating testicular/epididymal contribution to the ejaculate. Testicular biopsy revealed spermatogenesis was not proceeding to completion (seminiferous tubules of smaller than normal diameter with no elongating or elongated spermatids, and only a few round spermatids were present). Another 7-year-old stallion with small testes ejaculated gel and gel-free semen containing only premature germ cells (6.5 million/ml). This horse was castrated and histomorphometric analyzes of fixed testicular parenchyma (Johnson et al., 1991) revealed disruption of spermatogenesis between meiosis and spermiogenesis (counts of late primary spermatocyte counts yielded a daily sperm production estimate of 30 million/g, while greatly reduced numbers of round spermatids yielded an estimate for daily sperm production of less than 2 million/g). Only occasional elongating spermatids were seen in the seminiferous tubules of this horse. Finally, a 3-year-old stallion with slightly small testes was examined that ejaculated gel and gel-free semen with no sperm or germ cells in the ejaculate. Assay of seminal plasma from this stallion yielded only 43 U/l alkaline phosphatase, confirming that testicular/epididymal secretions were absent from the ejaculate. The epididymides were quite small, particularly where the body (corpus epididymis) joins the tail (cauda
epididymis), and were uniformly hyperechoic (i.e., without the “Swiss cheese” appearance attributed to the convoluted, fluid-containing epididymal duct) on ultrasound examination (Love, 1992). Palpation per rectum revealed normal size and location of the paired vas deferens and ampullae, seminal vesicles and lobes of the prostate. A testicular biopsy was taken (Hillman et al., 1994) and revealed seminiferous tubules of normal appearance. The finding that spermatogenesis proceeded to completion, in conjunction with the azoospermia and low concentration of alkaline phosphatase in the gel-free semen, suggested obstruction of the epididymal ducts preventing sperm from entering the ejaculates. The presence of the extremely small epididymides in a young stallion likewise suggested epididymal hypoplasia (failure of epididymides to develop to normal size).

References


