

REVIEW ARTICLE
METHODS FOR SEMEN AND ENDOCRINOLOGICAL EVALUATION
OF THE STALLION: A REVIEW

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Abstract

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Breeding soundness examination of stallions includes the examination of reproductive organs, behavior, quantity and quality of the ejaculate and function of the reproductive endocrine system. The paper reviews the current knowledge of this field. First, the morphology of the equine spermatozoa is described in details. Semen examination is divided into routine and advanced laboratory procedures and new techniques. During routine laboratory examination, the quantitative (volume, concentration, total sperm number) and the qualitative (appearance, motility, morphology) parameters of the ejaculate are determined. Owing to different evaluation systems and interpretation, data on morphology from different laboratories are difficult to compare. Following routine laboratory tests, morphological and functional membrane integrity and acrosome integrity can be determined. Several non fluorescent and fluorescent staining techniques have been developed to determine the viability and/or the acrosomal status of spermatozoa which can be used separately or in combination. Hypo-osmotic swelling (HOS) test is a relatively simple test to evaluate the functional integrity of the spermatozoal membrane. New automated techniques, such as computer assisted sperm analysis (CASA) and flow cytometry have been developed recently. These new methods allow us to evaluate different motility parameters, morphology, different compartments and functions of individual cells, including the nucleus, plasma membrane, mitochondria, capacitation, acrosomal status. The determination of baseline concentration of reproductive hormones helps to evaluate the breeding soundness of stallions. GnRH and hCG challenge-tests are also available to monitor the function of the hypothalamus-pituitary-testis axis. The accuracy of all these methods has to be confirmed on a large number of stallions and their correlation with fertility has to be determined. The content of the paper is the following:

1. Introduction
2. The equine spermatozoon
 - Plasma membrane
 - Head
 - Tail
3. Semen examination
- 3.1. Routine laboratory tests
- 3.1.1. Quantitative parameters
 - Volume
 - Concentration
 - Total sperm number
- 3.1.2. Qualitative parameters
 - Appearance
 - Motility
 - Morphology
- 3.2. Advanced laboratory tests: evaluation of membrane integrity
 - Morphological integrity: viability
 - Functional integrity: hypo-osmotic swelling
 - Acrosome integrity

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- 3.3. New techniques
 - Mitochondrial activity
 - Capacitation of spermatozoa and calcium influx
 - Binding activity
 - Morphometric analysis
 - Chromatin analysis
4. Diagnostic endocrinology in stallions
 - GnRH challenge
 - HCG challenge
5. References

Stallion, spermatozoa, semen evaluation, GnRH and hCG challenge tests

1. Introduction

The proper breeding soundness examination of a stallion includes examination of the reproductive organs, the behavior, the quantity and quality of ejaculate and the function of the reproductive endocrine system. Wide range of clinical and laboratory methods are used for this purpose. Semen quality and endocrine function are estimated by various laboratory procedures, which must be designed, performed and evaluated in accordance with the species-specific functional characteristics of genitals. Due to the high individual value of stallions and the relative incidence of fertility disorders, there is a constant interest in these methods from practitioners working on stud farms all over the world. The aim of this paper was to update and overview the current knowledge on this field in stallions.

2. The Equine Spermatozoon

The spermatozoon consists of head and tail, and is entirely surrounded by plasma membrane. Length of the spermatozoon is $87.85 \pm 7.02 \mu\text{m}$ (Bielanski and Kaczmarek 1979; Amann and Graham 1993; Johnson et al. 1997).

Plasma membrane. The plasma membrane is composed of lipids, mainly phospholipids and cholesterol, and proteins. Lipids are arranged as a bilayer, locating their hydrophilic ends externally, and the hydrophobic fatty acyl chains internally. Proteins are intermingled with the lipids as integral or peripheral proteins. Some of them serve as channels through the membrane or are surface receptors. Many proteins on the external surface contain carbohydrate side chains, which can loosely bind other proteins from the medium. The ratio of cholesterol to phospholipids and the nature of phospholipids determine the flexibility of the membrane, which is "fluid" at body temperature (Hammerstedt et al. 1990).

Head. The head of the equine spermatozoon is elliptical shape, slightly thicker at the posterior part. Its length is $6.62 \pm 0.02 \mu\text{m}$, width is $3.26 \pm 0.19 \mu\text{m}$ and area is $16.28 \pm 0.80 \mu\text{m}^2$. The head includes the nucleus, the acrosome, the postacrosomal lamina and the implantation fossa. The nucleus contains the highly condensed chromatin, the DNA, and is enclosed by the double-layer nuclear membrane. The rostral 2/3 portion of the nucleus is covered by the acrosome, which is a special vesicle, containing glycolipids and hydrolytic enzymes. The acrosome is covered by the inner and outer acrosomal membrane and can be divided into apical, principal and equatorial segments. The equatorial segment does not contain enzymes and is not involved in the acrosomal reaction, but the plasma membrane in this area fuses with the plasma membrane of the oocyte. The postacrosomal lamina may have a role in attachment of the spermatozoa to the oocyte. The implantation fossa is the place, where the tail attaches to the head, and its position is often abaxial in the stallion (Amman

and Graham 1993, Johnson et al. 1997).

Tail. The length of the tail is 80.45–6.16 μm . The tail of the spermatozoon includes the neck, the middle piece, the principal piece and the end piece. The *neck* is the connection between the head and the middle piece. It contains the connecting piece and the centriole, which are the base of the dense fibers and the axoneme. This region of the spermatozoon is fragile, but likely this is the site, where beat of the tail is initiated. The *middle piece* is the widest part of the tail, extends from the caudal end of the neck to the annulus. It is characterized by the helically arranged mitochondria, which contain enzymes and cofactors for production of ATP. Central to the mitochondria the nine dense fibers are located, which extend from the neck, through the entire middle piece and most of the principal piece. Dense fibers do not contract, however, they provide rigidity and flexibility at the same time for the flagellar movement. Central to the dense fibers the axoneme is located, which is the contractile element of the spermatozoon. The axoneme consists of a central pair of microtubules surrounded by nine microtubular doublets. The axoneme extends from the neck, continues through the mid piece, principal piece and end piece. The annulus lies between the middle and the principal piece, and at this point the plasma membrane is firmly attached to the underlying structures. The *principal piece* is the longest part of the spermatozoon. It contains the axoneme, the dense fibers and a fibrous sheath, which is characteristic of this part. This rib-like structure also provides support and flexibility during movement. The *end piece* is only composed of the axoneme or single microtubules which are surrounded by the plasma membrane (Bielanski and Kaczmarek 1979; Amman and Graham 1993; Johnson et al. 1997).

3. Semen Examination

After semen collection, seminal characteristics related to quantity and quality of the spermatozoa are evaluated. The methods and assays, described below, give more and more information about the spermatozoon, its morphology, compartment and function. The accuracy of the new tests has to be confirmed on a large number of stallions and the correlation of these and other well known seminal characteristics, with stallion fertility still to be determined. Values for the most frequently used parameters of fresh equine semen are presented in Table 1.

3.1. ROUTINE LABORATORY TESTS

3.1.1. Quantitative parameters

Volume. Total and gel-free volume are determined from the ejaculate (Table 1). The gel should be removed either during collection or immediately thereafter.

Concentration. Determination of the sperm concentration in the gel-free portion of the ejaculate is important, because this parameter is used to determine the correct insemination dose, or to calculate the volume of the sperm samples for different assays (Table 1). Concentration can be determined by hemocytometer or by spectrophotometer.

Total sperm number. Total sperm number per ejaculate is calculated from the volume and the concentration (Table 1). Sperm production of the stallion depends on age, testicular volume, sperm reserve capacity of the epididymis, breeding season, frequency of ejaculation (Pickett 1993).

3.1.2. Qualitative parameters

Table 1
Quantitative and qualitative parameters of fresh equine semen

Parameter	Mean \pm SD	Number of Stallions	Reference
Gel free volume (ml)	65 \pm 26	398	Parlevliet et al. 1994
	45 \pm 30	417	Pickett 1993b
	33.7 \pm 2.13	165	Dowset and Knott 1996
	51.6 \pm 31.5	8	Long et al. 1993
	45.3 \pm 30.9	47	Dowsett and Pattie 1982
Concentration (10^6 / ml)	206.1 \pm 168.5	398	Parlevliet et al. 1994
	335 \pm 232	417	Pickett 1993b
	164.13 \pm 39.35	165	Dowset and Knott 1996
	223 \pm 148	8	Long et al. 1993
	178 \pm 168	47	Dowsett and Pattie 1982
Total sperm number (10^9)	11.29 \pm 7.13	398	Parlevliet et al. 1994
	11.9 \pm 9	417	Pickett 1993b
	6.34 \pm 1.93	165	Dowset and Knott 1996
	9.1 \pm 4.7	8	Long et al. 1993
	7.21 \pm 6.87	47	Dowsett and Pattie 1982
Total motility (%)	53 \pm 15	417	Pickett 1993b
	76.43	165	Dowset and Knott 1996
	72.1 \pm 16	47	Dowsett and Pattie 1982
	70.3 \pm 17.4	64	Jasko et al. 1991
Progressive motility (%)	68 \pm 9	398	Parlevliet et al. 1994
	53.1 \pm 16.2	8	Long et al. 1993
	52.7 \pm 23.8	64	Jasko et al. 1991
Live spermatozoa (%)	65 \pm 16	398	Parlevliet et al. 1994
	82.56	165	Dowset and Knott 1996
	78.8	47	Dowsett and Pattie 1982
Morphologically normal spermatozoa (%)	66 \pm 15	398	Parlevliet et al. 1994
	51 \pm 15	417	Pickett 1993b
	67.82	165	Dowset and Knott 1996
	47.5 \pm 12.4	8	Long et al. 1993
	58.2	47	Dowsett and Pattie 1982
	52.5 \pm 20.1	64	Jasko et al. 1991
HOS (%)	49.6 \pm 4.6 (50 mOsm)	15	Neild et al. 1999
Maximum swelling in hypo-osmotic solution	50 (18 – 153 mOsm)	3	Caiza de la Cueva et. al. 1997
	67 (50 mOsm)	4	<i>Vidament, personal comm.</i>

Appearance. Fresh stallion semen has a grayish-white opaque appearance. Flocculent debris, urine or blood in the ejaculate severely affect the spermatozoa and indicate disorder in the stallion reproductive or urinary tract.

Motility. Motility evaluation of the ejaculate is one of the most important assays carried out on the routine basis (Table 1). The results are subjective and good experience is required from the examiner to perform reliable evaluation. However, the percentage of motile sperm, evaluated visually, is not highly correlated with fertility (Graham 1996). Computer assisted sperm analysis (CASA) was introduced more than ten years ago (Jasko et al. 1988), and since then, it is used regularly in the semen evaluation process in most laboratories. This technique is objective and evaluates the motility according to the given criteria. The most important parameters measured are velocity, linearity, percentage of motile spermatozoa above a certain velocity, lateral oscillatory movement (Palmer and

Magistrini 1992). The relationships between fertility and different motility parameters of spermatozoa analysed by CASA need to be determined (Magistrini et al. 1996).

Morphology. Motility and morphology of the spermatozoa have been used as indicators of the quality, although their true relationships to fertility have not been determined (Bielanski 1975; Voss et al. 1981; Jasko et al. 1988; Jasko et al. 1990). A number of morphological evaluation methods and classification systems are used. Features of sperm morphology can be evaluated by examining unstained, buffered-formol saline fixed suspensions or stained smears (Malmgren 1992). The most frequently used staining techniques are the eosin-nigrosin (Dott and Foster 1972), the eosine-anilin blue (Van der Schaaf 1952), the Spermac (Oettlé 1986) and the Giemsa (Graham 1996). Unstained samples can be analyzed using differential interference contrast or phase contrast microscopy at 1 000 × magnification, while in case of stained samples only bright-field microscopy is suitable. More detailed analysis can be done using transmission and scanning electron microscopy (Dott 1975; Bielanski and Kaczmariski 1979; Dowsett et al.). Classification system varies among laboratories, and everyone uses his or her own categories based on the following methods separately or in combination. In some of the systems, spermatozoa are classified according to the presumed origin of the abnormalities (Bielanski 1950; Dott 1975). Primary abnormalities are considered to originate during spermatogenesis, and are divided into subgroups of abnormalities of the head, mid-piece and tail. Secondary abnormalities develop in the excurrent ducts, and classified into subgroups of abnormalities of the head, mid-piece and tail. Blom (1973) simplified the classification into major defects and minor defects. Major defects include certain types of abnormal heads, acrosome defects, loose abnormal heads, mid-piece defects, proximal droplets and pouch formation. Minor defects include certain types of abnormal heads, loose normal heads, detached acrosomes, abaxial implantation, distal droplet and single bent tails (Blom 1973; Dowsett and Pattie 1982; Jasko et al. 1990). Nishikawa used a more simple classification of abnormalities which includes seven basic categories of abnormalities: abnormal head, abnormal neck, abnormal connecting piece, abnormal tail, duplication of any parts, isolation of head and tail, deficiency of head and tail (Nishikawa 1959). Bretschneider classified the abnormalities into six groups: abnormal acrosome, abnormal sperm head, abnormal connecting piece, abnormal mid-piece, cytoplasmic droplet, abnormal tail and double spermatozoa (head or tail) (Bretschneider 1948; Parlevliet et al. 1994). Lagerlof used the categories of abnormal head, detached head, nuclear pouches, abnormal acrosome, proximal cytoplasmic droplet, distal cytoplasmic droplet, abnormal mid-piece and abnormal tail (Malmgren 1992). The system used in France divides the abnormalities into abnormal head, abnormal flagellum, abnormal mid-piece, abnormal principal piece, abnormal end piece and distal cytoplasmic droplet. These categories are further divided into subgroups (Vidament, personal communication). Data from different laboratories are difficult to compare because of the large variation in categories and the difference in interpretation of certain features of stallion spermatozoa such as head shapes, craters on the head, knobbed head, abaxial position of the tail, distal and proximal cytoplasmic droplets (Dowsett et al. 1984; Johnson and Hurtgen 1985).

3.2. ADVANCED LABORATORY TESTS: EVALUATION OF MEMBRANE INTEGRITY

Morphological integrity: viability. Plasma membrane integrity is essential for the maintenance of sperm viability. Several staining methods have been developed to detect disruption in the plasma membrane. The principle of these techniques is dye exclusion,

which means that these stains can not penetrate through the intact membrane, but they color the spermatozoa if the plasma membrane is damaged, due to the affinity of the stains to bind to the nucleus (Graham 1996; Magistrini et al. 1997). First, eosin-nigrosin staining was used to differentiate “live” and “dead” mammalian spermatozoa (Dott and Foster 1972). This technique is easy to use, no special microscope is required and it gives reliable results. Since fluorescent microscopy and flow cytometry become more frequently used in semen evaluation, new fluorescence supravital staining techniques have been developed. The most commonly used fluorescence staining methods are the followings: Hoechst 33258 (H258) (Eliason and Treichl 1971); dual DNA stain consisting of propidium iodide (PI) combined with carboxyfluorescein diacetate (CFDA) (Garner et al. 1986; Harrison and Vickers 1990) or with SYBR-14 (Garner et al. 1994; Magistrini et al. 1996), or carboxydimethylfluorescein diacetate (CDMFDA) (Ericsson et al. 1993; Magistrini et al. 1997). Propidium iodide is a DNA-specific stain, which enters the dead or moribund sperm cells. CFDA and CDMFDA are nonspecific esterase substrates, which are non fluorescent and can penetrate through the membranes. Entering the sperm cell, nonspecific esterases readily hydrolyse them, resulting in a highly fluorescent, membrane impermeable green fluorescent stain. Dual staining techniques classify the sperm population into three categories: (1) live spermatozoa emitting green fluorescence resulting from positive staining by CFDA or SYBR-14, (2) dead spermatozoa with nuclei emitting red fluorescence resulting from positive PI and negative CFDA or SYBR-14 staining, (3) moribund spermatozoa with nuclei emitting green and red fluorescence resulting from positive staining by PI and CFDA or SYBR-14 in the same time (Garner et al. 1986).

Functional integrity: hypo-osmotic swelling (HOS) Test. This assay is a relatively simple test to evaluate the functional integrity of the spermatozoal membrane. Biochemically active sperm membrane is required for the process of capacitation, acrosome reaction and the binding of the sperm cell to the oocyte surface. Jeyendran developed this assay for human spermatozoa (Jeyendran et al. 1984), and recently HOS has been used in bulls (Correa and Zavos 1994; Correa et al. 1997), in boars (Vazquez et al. 1997), in dogs (Kumi-Diaka 1993) and in stallion (Caiza de la Cueva et al. 1997; Vidament et al. 1998; Neild et al. 1999). First, Drevious and Eriksson observed that spermatozoa exposed to hypoosmotic conditions undergo morphological alteration and their size is increased (Drevious and Eriksson 1996). During the HOS test, the biochemically active spermatozoa, due to the influx of water, will undergo swelling and increase in volume to establish an equilibrium between the fluid compartment within the spermatozoa and the extracellular environment. This volume increase is associated with the spherical expansion of the cell membrane covering the tail, thus forcing the flagellum to coil inside the membrane. Coiling of the tail begins at the distal end of the tail and proceeds towards the mid-piece and head as the osmotic pressure of the suspending media is decreased (Drevious and Eriksson 1966; Jeyendran et al. 1984). The plasma membrane surrounding the tail fibers appears to be more loosely attached than the membrane surrounding the head. The optimal hypo-osmotic medium should exert an osmotic stress large enough to cause an observable increase in volume, but small enough to prevent the lysis of the sperm membrane. Hypo-osmotic mediums contain mainly fructose and sodium citrate mixed in equal proportions (Jeyendran et al. 1984; Kumi-Diaka 1993; Correa and Zavos 1994; Correa et al. 1997; Vazquez et al. 1997), or fructose, sodium citrate, lactose and sucrose alone (Neild et al. 1999). Optimal results can be obtained in the range of 50 to 150 mOsm/L osmolality (Jeyendran et al. 1984). Results of HOS test give good correlation with progressive motility (Jeyendran et al. 1984; Kumi-Diaka 1993; Correa and Zavos 1994; Neild et al. 1999).

Acrosome integrity. To fertilize an oocyte, spermatozoa must possess an intact acrosome. Shortly before fertilization, sperm cell undergoes acrosome reaction which is required for penetration through the zona pellucida and for fusion with the oocyte plasma membrane (Yanagimachi 1994). Several techniques have been used to differentiate the acrosome-intact from acrosome-reacted spermatozoa (Gros and Meizel 1989). The physiological acrosome reaction is a well coordinated process that only occurs in living spermatozoa and is called "true acrosome reaction". In contrast, the loss of acrosome may also occur due to degenerative changes in the membrane (freezing), which is called "false acrosome reaction" (Bedford 1970). To differentiate these two reactions, acrosomal stainings are combined with supravital stains (Cross et al. 1986; Varner et al. 1987; Casey et al. 1993; Meyers 1996). Acrosomal staining alone can be used for (1) evaluation of semen for acrosomal abnormalities, (2) evaluation of the effect of cryopreservation on the acrosome, (3) evaluation of acrosomal status during *in vitro* capacitation. The acrosomal status of sperm from species with large acrosome (e.g. bull, hamster) can be assessed by phase-contrast or differential interference microscope without staining. In other species, including mouse, human, and stallion, the unstained acrosome is too small to be visualized accurately. For this reason, specific acrosomal labeling techniques have been used to enhance the visualization of the acrosome with bright-field or fluorescence microscopy (Cross and Meizel 1989). For bright-field microscopy, the triple stain technique (trypan blue, Bismark brown and rose bengal) is used most frequently (Talbot and Chacon 1981; Varner et al. 1987; Cross and Meizel 1989). For fluorescence microscopy, there are two kinds of fluorescent probes (Cross and Meizel 1989). The first type detects intracellular acrosome-associated materials, and requires cell permeabilization before labeling. This category consists of lectins (Talbot and Chacon 1980) and antibodies to intracellular antigens (Wolf et al. 1985). The second type of fluorescence probe consists of reagents that are used without permeabilization of cells, such as chlortetracycline (CTC) (Varner et al. 1987) and antibodies to externally exposed antigens (Saling et al. 1985). Lectins bind to the glycoconjugates of the acrosomal matrix or outer acrosomal membrane. First, a highly toxic lectin (*Ricinus communis* agglutinin-II), labeled with fluorescein isothiocyanate (FITC), was used (Talbot and Chacon 1980), but later the technique was modified and *Pisum sativum* agglutinin (PSA) became the most widely used lectin since then (Cross et al. 1986). A number of staining methods to assess acrosomal changes in stallions have been described. These include the use of chlortetracycline (Varner et al. 1987), monoclonal antibodies (Zhang et al. 1990), FITC-PSA (Farlin et al. 1992, Casey et al. 1993) and fluorescein isothiocyanate conjugated *Arachis hypogea* agglutinin (FITC-PNA) (Cheng et al. 1996). Spermatozoa stained with FITC-PSA have different staining patterns, depending on the acrosomal status. Bright fluorescence over the acrosomal cap indicates "*acrosome intact*" sperm. No fluorescence at all or limited fluorescence at the equatorial segment indicates complete loss of acrosomal cap, either due to complete acrosomal reaction, called "*acrosome reacted*", or due to cell death caused by membrane degeneration. These staining patterns do not give precise information about the process of acrosome reaction. However, the staining patterns of FITC-PNA clearly demonstrate the different stages of acrosome reaction. Appearance of the "*acrosome intact*" and "*acrosome reacted*" spermatozoa are the same as with PSA staining, but "*acrosome reacting*" cells can be differentiated by patchy disrupted fluorescence over the acrosomal cap (Cheng et al. 1996). FITC-PSA and FITC-PNA staining can also be used without permeabilization of cells. This method allows the evaluation of acrosomal membrane injuries of spermatozoa. While spermatozoa with damaged plasma and acrosomal membranes become fluorescent over the acrosomal cap and the equatorial region, spermatozoa with intact membranes, impermeable to these lectins, do not show fluorescence after exposure (Farlin et al. 1992; Szász 1999).

3.3. NEW TECHNIQUES

New automated techniques, such as computer assisted sperm analysis (CASA), flow cytometer are now available to evaluate sperm cells *in vitro*, besides traditional standard methods. These new techniques allow us to evaluate different motility parameters, morphology (Jasko et al. 1988), different compartments and functions of the individual cell, including the nucleus, plasma membrane, mitochondria, capacitation, acrosomal status (Graham et al. 1990). All the sperm staining methods using fluorescence probe can be evaluated by flow cytometry. This new technique increases the objectivity of the analysis, large number of spermatozoa can be evaluated in a short period of time and the results are reliable. Since combined fluorescent probes can be used in one assay, flow cytometry allows the evaluation of multiple characteristics simultaneously, providing more precise description of the sperm population.

Spermatozoal function

Mitochondrial activity. Mitochondria are located in the middle piece of the spermatozoa, and they contain enzymes and cofactors necessary for the production of ATP. Good energy supply is essential for the sperm to maintain its motility. Mitochondrial activity is evaluated by Rhodamine 123 (Rh123) that is accumulated primarily by the mitochondria within the spermatozoa (Graham et al. 1990; Ericsson et al. 1993). Rh123 is a cationic fluorescent molecule and its fluorescence is emitted only from functioning mitochondria. The intensity of Rh123 fluorescence depends on the total amount of functional mitochondria in the flagellum. This staining method is usually used together with one of the supravital probes (Graham et al. 1990).

Capacitation of spermatozoa and calcium influx. Capacitation is defined as all the physical and biological changes in the spermatozoon, which enable the sperm cell to undergo the acrosome reaction (Bedford 1983). During this process, calcium enters the sperm cell. Ionophore A23187 and heparin treatment can induce capacitation leading to the acrosome reaction, characterized by calcium influx into the cell (Magistrini and Palmer 1991; Fleet et al. 1995). This influx is measured by flow cytometry using fluorescent probe fluo3-AM (Magistrini et al. 1997).

Binding ability. At the end of the fertilizing process, sperm must contact and penetrate the oocyte. This requires intact receptor proteins on the sperm to bind to the zona pellucida, trigger the acrosome reaction and bind to the plasma membrane of the oocyte (Yanagimachi 1994). Different techniques are used to evaluate the binding ability of the sperm cell. Hamster zona-free oocyte binding assay is widely used (Graham et al. 1987; Padilla et al. 1991). Homologous equine zona pellucida-sperm binding assay (Pantke et al. 1995) and homologous equine hemizona assay have been developed recently (Fazeli et al. 1993). In the zona pellucida binding assay, equal numbers of spermatozoa from a control stallion and from the test stallion are labeled with different fluorescent stains, and incubated with the oocyte. Number of spermatozoa bound to the zona pellucida are counted and the binding ratio is calculated. In the hemizona assay, sperm from the control and test stallions are labeled with the same fluorescent stain and incubated separately with the two hemizona.

Morphometric analysis. Morphometric analysis is used for measuring the length, width, area, perimeter and nuclear density of the sperm head performed on stained smears, either manually with an ocular-mounted micrometer or by computer assisted microscopy. For staining, Papanicolau (Kruger et al. 1995) or Feulgen stains (Davis et al. 1993) are used.

Chromatin analysis. Subfertility can be related to defects of the chromatin, involving increased susceptibility to DNA denaturation in situ (Ballachey et al. 1987; Evenson et al. 1994; Kenney et al. 1995). The etiology of this type of defect may be induced by various toxic agents, or there could be a genetic predisposition. The assay is based on the estimation of the susceptibility of sperm nuclear DNA to acid denaturation in situ, and the test is called sperm chromatin structure assay (SCSA). The degree of denaturation is determined by flow cytometry measuring the ratio of green (native, double-stranded DNA) and red (denatured, single-stranded DNA) fluorescence emitted from acid treated spermatozoa stained with acridine orange.

4. Diagnostic endocrinology in stallions

Stallions with a hormonal imbalance that is associated with testicular degeneration are difficult to diagnose and manage. Hormone therapy, including GnRH, eCG, hCG, FSH has been clinically implemented, but with limited success (Douglas and Umphenour 1992; Nett 1993). Identifying and diagnosing these stallions as early as possible, is essential to prepare a careful management system, which is the only solution to get the most out of a subfertile stallion. Measuring baseline concentrations of reproductive hormones helps to evaluate the breeding soundness of stallions. Recent studies suggest, that plasma levels of FSH and estrogens could be better markers to predict future changes in fertility than testosterone and LH (Douglas and Umphenour 1992; Roser and Hughes 1992a). A stallion with declining fertility usually shows changes in hormone levels including increasing levels of LH and FSH, decreasing levels of estradiol and inhibin, and finally decreasing level of testosterone. These changes may take few months or a few years depending on the testicular disorder (Roser 1997). In a recent study including 107 stallions, increased basal LH and decreased basal total estrogen concentrations resulted in decreased fertility during the subsequent breeding season (Palmer et al. 1998). Besides measuring basal levels of reproductive hormones, there are challenge-tests to evaluate the function of the hypothalamus-pituitary-testes axis in stallions.

GnRH challenge. This test monitors the responsiveness of the pituitary gland to a bolus or repetitive doses of exogenous GnRH, with the subsequent release of LH and FSH. It also determines the testosterone secretion of the testes in response to the increased level of endogenous gonadotropins (Roser and Hughes 1992ab). In fertile stallions, the pituitary and testis appear to be more responsive to pulsatile administration of low dose (5 µg/stallion) GnRH in the non-breeding season than in the breeding season (Roser and Hughes 1991). In subfertile stallions, the pituitary responsiveness is significantly reduced during the nonbreeding season compared to normal stallions, but it is not affected in the breeding season (Roser and Hughes 1992a). Applying non-pulsatile bolus of GnRH, pituitary responsiveness does not differ between fertile and subfertile stallions. However, the testicular response to a discrete dose of GnRH is significantly reduced in subfertile stallions (Roser and Hughes 1992b). This low testicular response may be due to a dysfunctional hypothalamus-pituitary axis producing bio-inactive LH, or to a primary testicular disorder (Whitcomb et al. 1991; Roser 1997).

HCG challenge. This test is used to investigate the testicular function of stallions. Application of hCG is a diagnostic tool to directly test the secretory capacity of the Leydig cells (Roser 1995). In stallions, it is also used for the diagnosis of cryptorchidism (Cox 1975). Testosterone and estrone sulphate response to a single dose of hCG (10,000 IU) appears to be biphasic. A short-term increase occurs within 2 hours after treatment, while a marked and prolonged increase occurs between days 1 and 5 post-treatment (Cox and Redhead 1990). Stallions, having fertility problems, have lower testosterone response to

hCG challenge than fertile ones (Roser 1995). Decreased testosterone response to hCG was associated with decreased fertility during the subsequent breeding season. It was suggested that the determination of basal LH, basal total estrogens and testosterone response to hCG in a group of stallions could be used as a diagnostic tool to select stallions with high risk for fertility problems (Palmer et al. 1998).

Metody hodnocení spermatu a endokrinologických ukazatelů u hřebců: přehled

Posouzení hřebců vhodných pro plemenitbu zahrnuje vyšetření reprodukčních orgánů, kvantitu a kvalitu ejakulátu, funkci reprodukčního endokrinního systému a zhodnocení chování. Práce se zabývá současnými poznatky v oboru. Úvodem je detailně popsána morfologie spermie. Vyšetření spermatu zahrnuje rutinní a pokročilé laboratorní postupy včetně nových technik. Během rutinního laboratorního vyšetření, kvantitativního (objem, koncentrace, celkový počet spermií) a kvalitativního (vzhled, motilita, morfologie) byly stanoveny parametry ejakulátu. Vzhledem k odlišným evaluačním systémům a k interpretaci výsledků bylo nesnadné srovnat údaje týkající se morfologie spermatu. Na základě rutinních laboratorních testů lze stanovit morfologickou a funkční integritu membrán a integritu akrozómu. Několik nefluorescenčních a fluorescenčních pokrovovacích technik bylo vyvinuto ke stanovení životnosti a akrozómů spermií, které mohou být použity zvlášť nebo společně. Test na základě hypoosmotického otoku (HOS) je relativně jednoduchý test, používaný ke zhodnocení funkční integrity spermatické membrány. V poslední době byly vyvinuty nové automatizované techniky, např. počítačová asistovaná analýza spermatu (CASA) a průtoková cytometrie. Tyto nové metody umožňují posoudit různé parametry motility, morfologii, různé části a funkce jednotlivých buněk včetně jádra, plazmatické membrány, mitochondrií, kapacity a stav akrozómu. Stanovení koncentrace základních hodnot (baseline) koncentrace reprodukčních hormonů umožňuje posoudit vhodnost hřebců pro plemenitbu. Čelení testy pomocí GnRH a hCG jsou rovněž vhodné pro monitorování funkce hypotalamo-hypofyzárně-testikulární osy. Přesnost všech těchto metod však musí být ověřena na velkém počtu hřebců a na jejím vztahu k fertilitě. Práce je rozdělena takto:

1. Úvod
2. Spermie hřebců
 - Plazmatická membrána
 - Hlavička
 - Ocas
3. Vyšetření spermatu
 - 3.1. Rutinní laboratorní testy
 - 3.1.1. Kvantitativní parametry
 - Obsah
 - Koncentrace
 - Celkový počet spermií
 - 3.1.2. Kvantitativní parametry
 - Vzhled
 - Motilita
 - Morfologie
 - 3.2. Pokročilé laboratorní testy: posouzení integrity membrán
 - Morfologická integrita: životnost
 - Funkční integrita: hypoosmotický otok
 - Integrita akrozómů

- 3.3. Nové techniky
 - Mitochondriální aktivita
 - Kapacitace spermií a influx kalcia
 - Vazebná aktivita
 - Morfometrická analýza
 - Chromatinová analýza
4. Diagnostická endokrinologie u hřebců
 - GnRH čelenž
 - HCG čelenž
5. Reference

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