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Idiopathic subfertility in stallions. Clinical approaches to a complex problem

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Abstract

At least 30-40% of stallions in commercial breeding programs are moderately fertile and 8-12% are subfertile (0.5-3% with severe subfertility). From the total reported cases of the subfertility, in 2-20% of the stallions the cause is unknown or was not established. The objective of this work is to present the concept of subfertile stallion based on the current state of knowledge and advanced molecular diagnostic technologies. Low pregnancy rates have been reported in stallions with normal semen quality after conventional evaluation. Acrosome reaction (AR) is necessary for natural fertilization and impaired acrosome reaction (IAR) leads to subfertility or infertility in horses, however, AR test is not included in routine semen analysis. Genome-wide association study identified FKBP6 as a strong candidate gene responsible for this failure. The gene encodes for FK506 binding protein 6 (FKBP6) which is involved in sperm development and functions. We could conclude that the evaluation of the acrosomal status is essential in cases of stallions with good motility, concentration, morphology and viability but unexplained (idiopathic) subfertility or infertility. It is important to highlight the recent increase in reports of fertility problems in stallions related to disorders of genetic origin.

Key words: *stallion-subfertility-idiopathic-Acrosome-FKBP6*

Introduction

In equine production systems, stallions are approximately 5% of the horse population, since selection pressure in registered purebreds is strongly biased towards males, with some exceptions, such as the Polo Argentino breed, in which the pressure is primarily directed towards females (Cunningham, 2000; Martinez, 2021). Unlike other domestic production species, in horses selection is based (at best) on individual performance; pedigree (close relatives -and sometimes very distant ones-); conformation and progeny, but very rarely on fertility. It´s estimated that at least 30-40% of stallions in commercial breeding programs are moderately fertile and at least 8-12% are subfertile. Within this last category, 0.5-3% suffers severe subfertility (less than 10% pregnancy rate/cycle). Two to 20% of the total reported cases of subfertility in stallions are considered idiopathic, meaning that the cause of the subfertility is unknown or was not established, which generates significant economic losses to the industry (Turner, 2018).

Fifteen percent of human couples have fertility problems, 45-50% of them related to the male factor and in 25-40% of cases it´s idiopathic. Surprisingly, 15-20% of infertile men have sperm counts considered "normal" or acceptable (Lefievre et al, 2007).

Semen analysis has been part of the clinical-andrological examination of the stallion for over 60 years to determine reproductive aptitude or estimate the "potential fertility". However, its systematic and more formal context was proposed by Kenney et al. in 1983 with the edition of "Clinical fertility evaluation of the stallion". In this text, methodology, semen standards and quantitative ranges for the tests were detailed; therefore, it established a landmark in the clinical andrology in horses. With the logical and expected changes of scientific advances in knowledge and technology, is maintained until today (Kenney et. al, 1983; Withesell et al., 2020).

In summary, this protocol, which clearly establishes that a fertility test in a stallion is much more than a sperm analysis, propose the following summarized steps:

- Physical exam-health status
- Sexual behavior assessment - libido
- Mounting capacity-penetration-ejaculation
- External and internal genitalia examination

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- Sampling for bacteriological cultures (sexually transmitted infections)
- Semen quality assessment
- Real fertility rates-Pregnancies (if the stallions have performed natural mating or artificial insemination)

In addition to providing an order of precise actions and quantitative standards (i.e: $1x10^9$ progressively motile morphologically normal spermatozoa/ejaculate; 75% pregnancy rate/40 mares by natural mating or 120 by artificial insemination; >180 cc testicular volume, etc.), Kenny´s manual established three categories in the fertility test according to the results obtained: 1) satisfactory; 2) questionable and 3) unsatisfactory breeder. This was known as an estimate of "potential fertility", something that is currently more appropriately called "current fertility" in reference to the specific moment in which the andrological examination is carried out.

Complementarily research enriched the structure of fertility evaluation in stallions with multiple and original contributions since the last 50 years. In particular, and only as an example, given the variety, quantity and quality of publications, we believe it´s pertinent to highlight the early work of the Institut du Cheval and INRA Nouzilly in France. In particular, the studies of Clement (1992, 1998) who raised and discussed the sensitivity of the Kenney and colleagues proposal as a fertility indicator on two groups of stallions: 1) fertile (n = 29) and 2) subfertile (n = 30) based on strict inclusion and exclusion criteria and real fertility tests (pregnancies) on experimental controlled groups of mares.

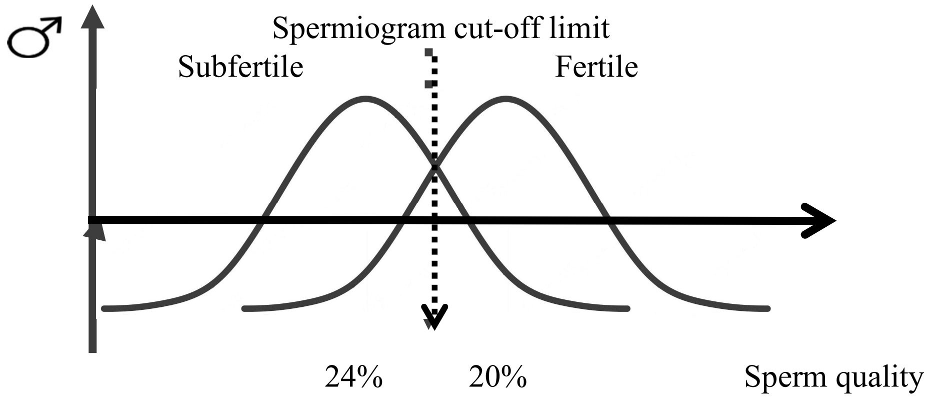

Figure 1. Distribution of stallions according to their sperm quality and real fertility. Adapted from Clement et al. Appreciation of the sexual function of l´etalon, Le Point Vet.v29,n191,p343-348; 1998.

According to their results, 24% of stallions with real acceptable fertility could be rejected due to their seminal characteristics and 20% of the subfertile ones could be accepted (in breeding or sales programs) given that they presented acceptable sperm analysis, which clearly emphasized the relative value, in some cases, of the spermiogram as an in vitro predictor test.

When there is categorical data from the standardized andrological examination (i.e. oligospermia, severe low libido, <15% progressive motility, etc.), presumptive diagnosis of subfertility is generally simple. However, with non-categorical data (i.e with just some acceptable andrological parameters but pregnancy rates per cycle of 30-50%, etc.), diagnosis is more complex. Therefore, it is convenient to have clear guidelines in reference to examination, diagnosis and clinical predictions when we face clients and pre-purchase fertility exams:

- Fertility can be estimated by multiple endpoints and/or combination of endpoints from actual fertility data and/or laboratory parameters (which are also variable).
- The majority of clearly subfertile stallions can be detected by single or combined tests.
- Fertility (actual or potential) of a stallion based on the andrological exam depends on the type and degree (sensitivity and specificity) of the tests, especially if it is not an extreme case of subfertility or infertility (i.e.azoospermia, ejaculatory failure, etc.).

Recently, due to the advances in molecular evaluation techniques, both for sperm (CASA, flow cytometry, sperm multiparametric approach) and seminal plasma (proteomics, metabolomics), the techniques and seminal values considered as standards for decades have been updated, for "field" tests and for highly complex laboratories (Turner, 2005; Ball et al. 2008; Love 2011, 2018; Sieme & Distl, 2012; Peña et al., 2018; Griffin et al., 2019).

The objective of this work is to present the concept of subfertile stallion based on the current state of knowledge and advanced molecular diagnostic technologies, in addition to emphasizing the cases of idiopathic subfertility in which there is a discrepancy between the semen analysis predictive data and the real fertility under field conditions.

Subfertile stallions

A subfertile stallion is one that does not exceed the limits of minimum-maximum cuts (human, variable, fallible) of a clinical physical examination and/or data from a spermiogram and/or stipulated or expected pregnancy rates according to a standard. However, we face certain questions such as which are the standards, methods, techniques and complexity necessary to determine the actual fertility. These questions have been the subject of debate for years, and still are, due to the clinical importance of trying to establish predictive values with high reliability using a combination of sensitive and specific methods and that, ideally, can be used in field conditions. For this, it is convenient to keep in mind that subfertility is not always related to semen quality, for example in stallions with copulatory failure or low libido, which is not always permanent, as in the case of sporadic or eventual urospermia / hemospermia, testicular trauma, fever etc., and that fertility is a dynamic, multifactorial and (most of the times) quantifiable attribute (Varner et al., 2014; Turner, 2018).

There are, at least, two well-differentiated scenarios regarding the prediction or estimation of fertility in stallions: 1) those with no real fertility data (have not been used for breeding nor AI, or there is no record of it) and 2) those with real fertility data (have bred mares and/or their semen has been used in AI programs). In commercial programs is not expected to have reliable and precise data, and analysis are difficult due to the number of variables (mares categories and fertility, management conditions, health, nutritional and environmental status, etc.), however, this information is helpful to build clinical indices. The most common parameters are: 1) Per season pregnancy rate (PSPR) = Pregnant mares/mares bred; 2) Cycles/pregnancy rate (CPP) = number of cycles bred /pregnant mares and 3) First cycle pregnancy rate (FCPR) = Pregnant in the first cycle/total pregnant mares. Of these, the most sensitive indicator of true fertility is the Cycles/pregnancy rate (Love, 2018; Whitesell, 2020).

In fertile stallions, these data are also useful to establish two subcategories: 1) high fertility, and 2) moderate fertility. Both acceptable, but clinically different (Table 1).

Table 1. Fertile stallions real fertility indicators. Adapted from (Withesell, 2020). PSPR = per season pregnancy rate; CPP = cycles per pregnancy; FCPR= first cycle pregnancy rate

	Fertility	
	High	**Moderate**
PSPR (%)	>75 (85-95)	> 60 < 75
CPP*	< 1.9	> 1.9
FCPR (%)	> 50	> 40 < 50

Considering only the sperm analysis, and leaving aside for a moment all the other factors of the andrological exam, we know that only 0.0007% of the ejaculated spermatozoa reach the ampulla (Cazales, 2020), in optimal fertilization conditions. Several questions arise from this statement: how many sperm and which attributes should they have to fertilize naturally? How many should we evaluate in a conventional spermiogram under field conditions? Which methods should we use to evaluate them? How should we interpret the results? By objectively analyzing as many attributes and cells as possible, the more acute/sensitive the test will be and better the prediction of current fertility (Table 2).

Table 2. Sperm structural and functional attributes. Adapted from Varner&Johnson, 2007.

Attribute	**Mechanisms / function**
Ability to move through the uterus to the oviduct	Energy production, mitochondrial function, mechanisms of sperm movement
Compacted chromatin	DNA integrity (SCSA, COMET; TUNEL)
Membrane integrity - Changes associated with maturation	Membrane and acrosome integrity, training
COC and ZP penetration	Intact and functional acrosome
Oocyte activation and embryonic development	Phospholipase C-zeta
Pronuclei formation in the zygote	DNA integrity

In order to answer these questions, standardized tests and procedures have been developed (BSE-Breeding Soundness Examination). They can be used in field conditions, require basic equipment and in general can be primary detectors in severe cases of subfertility/infertility. These tests are considered good predictors in stallions that have poor sperm quality and in which subfertility is due to factors related to semen, since it is a limiting factor in general fertility (Love, 2018). However, the fact that BSE is considered a good predictor of subfertility in these conditions does not mean the same for high or acceptable fertility, due to the multiple factors that influence it. For an update and a critical look at the scope of the conventional BSE (Table 3), we recommend the excellent works of Love (2011; 2018) and Whitesell (2020).

Table 3. Routine tests in andrological examinations and additional structural and functional tests to evaluate more attributes.

Routine tests	**Additional tests**
Concentration	Acrosome-RA Integrity
Motility	Membrane integrity (FL)
Morphology	Mitochondria (M potential)
Membrane integrity	Sperm capacitation
Sperm survival	Chromatin integrity
Testicular exam	Oxidative stress
Bacterial culture	Apoptosis

Computerized systems for sperm kinetics and other structural attributes analysis (CASA-Computer Assisted Sperm Analysis-), both laboratory and portable, and flow cytometry, which allows multiparametric analysis of sperm functional attributes, have evolved rapidly in the last decade. These are available for professionals, offering equipment of high-sensitivity, operational, small, economically accessible, which allow us to infer a new era of sperm analysis and consequently a greater understanding of cells and potentially a clinical improvement in the prediction of fertility in what it is already called “flow spermetry” (Peña et al., 2018; Hernandez Aviles et al., 2019).

One of the challenges we may face in equine assisted reproductive clinical practice is stallions with satisfactory BSE results, and real (“actual”) extremely low or negative fertility rates, ruling out factors such as mares and management. Although we have already mentioned that the relative frequency of subfertility in stallions is 8 to 12%, within this category, idiopathic subfertility can be as high as 20%; similar to what occurs in humans.

Faced with this clinical scenario, in general, the first actions usually taken by practitioners (or even managers) are: quantify other attributes in semen (DNA fragmentation; membrane integrity, mitochondrial function, apoptosis); get second opinions, methods and interpretations; treat stallions non-specifically (food supplements, probiotics, antioxidants, antibiotics, hormones); modify mares management (ovulation induction, post-ovulation uterine flushings); change mares categories (mares of proven fertility or maidens); selection of sperm subpopulations by colloids fractions separation, seminal plasma removal, different extenders.; pre and post ovulation AI, etc. In some cases, the change of so many variables simultaneously, without a diagnosis, may bring a solution to the clinical problem, but possibly, we will never know the cause or when or how it will be repeated. Nevertheless, when we face subfertility/infertility after a long time, work, expectations and money with no positive results, we realize that we are dealing with something more complex.

Table 4. Fluorochromes for a multiparametric approach to sperm quality in stallions. (Adapted from Hernandez-Aviles, 2019).

Organelle/ Function	Interpretation	Fluorochromes	Color
Plasma membrane integrity	Damaged Intact	PI / Hoescht 33258 SYBR-14 / 6CFDA	Red-Blue Green/Green
Acrosome integrity	Damaged Reacted	FITC-PSA FITC-PNA	Green Green
Mitochondrial integrity/function	Intact High/low membrane potential	MitoTracker Green MTG	Green PM Red-Uncolored
DNA Integrity	Intact Damaged	SCSA	Green Orange
ROS/oxidative stress	Superoxide anion prod. Lipid peroxidation	DHE/MitoSOX CII-BODIPY	Red Green
Sperm capacitation	Tyrosine Phosphorylation	FITC-AC monoclonal	Green
Post fertilization oocyte activation	Phospholipase C-Zeta	FITC-AC monoclonal	Green

Stallions with this clinical phenotype (acceptable andrological examinations, including spermiogram and low or negative fertility) have been initially described by Meyers (1995), who suspected that acrosomal reaction failures, and consequently fertilization ones, might be involved, although progressive motility and other sperm parameters remained within ranges considered normal for the species. Therefore, he decided to perform a progesterone-induced acrosomal reaction test in four subfertile stallions. The results are shown in Table 5. The acrosome reaction was verified using transmission electron microscopy, which was considered the gold standard at the time and, although it could not be taken as a diagnosis of certainty in terms of cause-effect relationship, it was shown that there was a significant difference between both groups.

Table 5. Results of acrosomal reaction induced by progesterone in fertile and subfertile stallions. Adapted from Meyers, 1995.

Stallions	Acrosomal Reaction	
	Induced (P4)	Control
Fertile (n=5)	19.7[a]	8.5
Subfertile (n=4)	5.8[b]	4.4

Some years later, Varner et al. (2001) described similar cases in Thoroughbred stallions and developed the acrosomal reaction test using the more potent compound A23187, obtaining similar results and concluding that acrosomal dysfunction could be the cause of subfertility/infertility in stallions with this clinical phenotype.

In 2007, Brinsko et al. described seven referred stallions cases with similar clinical history and andrological examinations and performed the acrosomal reaction test (Table 6) reinforcing the hypothesis of acrosomal dysfunction and infertility.

Table 6. Results of induced acrosome reaction in fertile and subfertile stallions. Adapted from Brinsko, 2007.

	Pregnancy rate/cycle (%)	Induced acrosomal reaction (%)
Fertile (n=6)	**>50**	**95.8**
Subfertile (n=7)	**<15**	**2.8**

Raudsepp et al. (2012), decided to study the cases of the seven stallions documented by Brinsko in 2007, genetically testing 89 Thoroughbred stallions (7 subfertile and 82 fertile). The 7 subfertile stallions, positive for acrosomal dysfunction in 2007, were positive (100%) for the combined AA/AA genotype (homozygous for the A allele) of the chr13 SNP in FKBP6 exon 5, a protein related to infertility and acrosomal reaction failure in other species, but only 9% of the fertile stallions tested positive for FKBP6. Based on these data, Castaneda et al (2021) decided to test a significant population of Thoroughbred stallions with accurate fertility records and determined the prevalence of the FBKP6 genotype in fertile and subfertile stallions (518 stallions from 7 countries). The rate of positive animals for FKBP6 was 4.1% (18/158), referring to the general population of stallions under study. However, 15 of the 18 (83.3%) FKBP6 positives had a history of low fertility. Out of a subpopulation of 150 stallions with detailed fertility histories, 13 (8.6%) had less than 46% pregnancy rate/cycle (low fertility) and of those 13, 4 of them (30.8%) were positive to FKBP6, with pregnancy rates/cycle between 2 and 36% and pregnancy rates per season lower than 31%, showing a significant association between low fertility and combined AA/AA FKBP6 genotype.

Finally, Hernandez Aviles et al. (2022) tested the clinical casuistry of stallions referred to the Texas A&M University clinic between 2003 and 2020. Only 21/1128 presented idiopathic subfertility (1.86%), but 8 of those 21 (38.1%), were positive for FKBP6 being the population prevalence of 0.7% (8/1128). As a synthesis of these cited studies and at least a part of the reports of acrosomal dysfunction and molecular genotyping, all the Thoroughbred stallions that had low rates of induced AR were positive to acrosomal dysfunction (FKPB6+). The prevalence of acrosomal dysfunction (FKBP6+) in stallions with idiopathic subfertility may be as high as 38%, but the frequency in the population (so far) is low (0.7- 4%). The AD can affect 97% of sperm, while the remaining 3% could fertilize and may be responsible for the very low, but possible, pregnancy rates.

We have recently reported three cases with similar clinical phenotypes, always in Thoroughbred stallions, young, with acceptable andrological tests and severe subfertility or infertility, all positive to the FKBP6 test (Losinno et al., 2023).

As a general synthesis of the clinical cases, the limitations of the andrological tests that can be carried out in field conditions, and the risk of false negatives, we could conclude that the evaluation of the acrosomal status is essential in cases of stallions with good motility, concentration, morphology and viability but unexplained (idiopathic) subfertility or infertility (Neild et al, 2005). It is important to highlight the increase in reports of fertility problems in stallions related to disorders of genetic origin (autosomal abnormalities in spermatozoa; CRISP3 protein in seminal plasma; FKPB6, etc.) (Hamann et al. 2007; Kjollestrom et al., 2016; Schrimpf et al, 2015, 2016; Castaneda el al, 2018; Sulliman et al, 2018; Hillburger et al, 2019; Kimble et al. 2019; Ruiz et al, 2019; Hernandez Aviles et al., 2023;

The standard clinical-andrological examination (BSE) is useful for stallions that do not pass the basic criteria (according to the cut-off limit) but very limited for some subfertile stallions remembering that the standard semen analysis only provides a moderate information on the functional competence of sperm (Blanchard et al., 2010; Losinno, 2018).

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