Cool-stored and frozen-thawed stallion semen: thoughts on collection, evaluation, processing, insemination, and fertility

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Abstract

Semen evaluation is a fundamental part of breeding soundness examination of males, including stallions. New technologies have been implemented in both the research arena and clinical practice for analysis of stallion sperm; therefore, theriogenologists and general equine practitioners should become familiar with principles, technical advantages, and limitations of each of these assays. More importantly is the interpretation of results and its translation to clinical situations. Common methods used for storage of stallion sperm (e.g. cooled storage or freezing/thawing) can affect tests that should be performed when establishing quality and potential fertility of a semen sample. The present document describes routine semen analysis assays performed at the author's laboratory when determining sperm quality and potential fertility of stallions.

Keywords: Stallion, breeding soundness, testes, semen quality, fertility

Introduction

There are 3 main ways stallion semen is presented for artificial breeding after collection in an artificial vagina (AV): fresh semen, cool-stored (CS) semen (extended and packaged in a container that cools the semen as it is transported), and frozen-thawed (FT) semen (processed in a freezing extender and frozen for later use).

Cool-stored stallion sperm was introduced to the Warmblood industry in the 1980s to provide wider distribution of stallion sperm throughout North America.[1,2] Subsequently, the application was accepted in many breed registries, including Standardbred and Quarter Horse breeds. This technique introduced the concept of preserving stallion sperm in a liquid-cooled form for a short-term (24 - 72 hours), thus facilitating widespread distribution without the necessity to move either the stallion or mare. This allowed mares and stallions to remain on their home farm while reducing potential disease exposure and travel stress.

As with the introduction of any new technique, concerns arose regarding the maintenance of sperm quality and how best to maximize fertility. Initially, semen was extended using traditional dilution ratios for fresh semen (i.e. 1:1 or 1:2, semen:extender). Although this is effective in many cases, there are 'poor cooler' stallions. An initial cause of this phenomenon was related to a combination of high seminal plasma concentrations (50% for 1:1 or 33% for 1:2 dilution ratio) and inadequate extender components for the number of sperm in the sample. To address this problem, the concept of diluting semen to a final sperm concentration (i.e. 30 x 10^6/ml of final volume) was introduced.[3] This approach usually reduced effects of seminal plasma. Nevertheless, there are 2 challenges regarding cool-stored semen extension; providing enough metabolic nutrients to maintain longevity of sperm quality (motility, viability, and DNA quality) and having an amount of seminal plasma that is not detrimental to sperm longevity.

Evaluation of stallion sperm quality is a fundamental part of the breeding soundness examination[4] and includes the determination of sperm motility, viability, concentration, total sperm in the ejaculate, and sperm morphologic features. Routinely, the evaluation of sperm quality is limited to sperm motility using a technique such as light or phase-contrast microscopy. Additionally, sperm number is determined either by direct (e.g. hemocytometry that relies on identification of sperm) or indirect (e.g. spectrophotometry that uses light transmittance) methods.[5] Combining sperm concentration and motility information allow the practitioner to calculate a sperm 'dose' and the number of mares that can be bred from an individual ejaculate. In addition, these sperm parameters are used as part of a breeding soundness examination to determine the suitability of a stallion for breeding or aid in the diagnosis of subfertility.[4]

Although these methodologies (motility and concentration determination) have their limitations, they were sufficient when the horse industry relied exclusively on fresh semen. However, as the introduction of cool-stored and frozen-thawed semen

became popular and widespread, the horse industry was faced with more instances of judging stallions as 'subfertile' due to a change in the breeding method. This necessitated a more thorough understanding of sperm quality features in various processing techniques and how the results would be interpreted.

One area of particular importance became the evaluation of semen handling technique (e.g. AV preparation, handling, and cleaning; extender-type preparation and use) and its effects on sperm quality. The evaluation of sperm motility alone became insufficient and the introduction of additional sperm quality features became relevant.[5]

Semen collection

Semen collection procedures for cooled and frozen are similar to those employed for fresh semen. These procedures include correct maintenance, storage, and cleaning of the AV; proper extender handling and usage; and proper handling of the semen during and following collection. It is likely that what appear to be relatively minor unnoticed breaks in these procedures have limited noticeable impact when fresh semen is inseminated can have a substantial impact when semen is stored for an extended interval.

Semen processing

Processing semen after ejaculation should be a planned, efficient activity, realizing that any break in the system can result in a long-term decline in sperm quality. There are many commercial semen extenders, providing the practitioner with a variety of choices. In general, these extenders provide a similar level of protection to the sperm and there is little difference in sperm quality when they are compared. Occasionally, an individual extender will impart a more beneficial effect on sperm quality than another. Initially, after collection, a raw sample should immediately be evaluated using a phase-contrast microscope, to provide a general idea of sperm motility and sperm morphology. Although this is not an ideal method to evaluate sperm motility because of the inconsistent appearance of sperm concentration, it is nevertheless important to be aware of any potential concerns regarding sperm quality as processing proceeds. If sperm motility is only evaluated following extension, the clinician will be unable to differentiate poor sperm quality due to an inherent stallion problem or collection procedure, or because of a potentially toxic semen extender.

Sperm in fresh semen do not face the same challenges as those in CS or FT semen. The primary difference is the potential reduction in sperm quality of the inseminate of CT or FT compared to fresh semen due to the storage conditions. Although there are many instances when the fertility of CS and FT are good and may approach fresh semen, the variation in sperm quality within those methods is broader than with fresh. In effect, what may be very good when evaluated in the fresh state can be very different when cooled or frozen. This wide variation is a challenge to the practitioner because evaluation of sperm motility alone is often insufficient to render an adequate diagnosis and management plan. Other differences among fresh, CS, and FT include the inability to determine sperm concentration of CS and FT semen using spectrophotometric techniques, due to interference by nonsperm components (e.g. milk products and egg yolk). This limits the practitioners' ability to determine total sperm in a CS or FT sample received for breeding unless they use hemocytometry or a more expensive alternative (e.g. NucleoCounter®SP-100TM [Chemometec, Allerød, Denmark]) that can determine sperm concentration in extended samples.[6]

Sperm motility and viability

Sperm motility can be determined microscopically or using a computer-assisted motility analysis (CASA) system.[7] Although microscopy is more subjective, primarily due to the broad range in variation among readers, CASA systems, while more objective, are not without their own limitations. The limitations of both techniques become more relevant when CS and FT sperm are evaluated since sperm quality and sperm numbers for insemination are often limited compared to fresh semen. Recognizing a limitation in sperm quality becomes more important since 'suitability' for cooling and freezing is performed following a 'test' cool or freeze.

Addition of sperm viability (i.e. plasma membrane intactness) test has been an important adjunctive to evaluation of stallion sperm, especially for CS sperm. Even very low sperm motility may not be diagnostic or predictive of poor fertility, especially when cooled sperm are evaluated. Low sperm motility can occur in the presence of either high or low sperm viability.[8] The former (low motility/high viability) may result in normal fertility, whereas the latter is associated with low fertility. It is unclear why the low motility/high viability circumstance occurs, but elevated seminal plasma concentrations (> 5 - 10%) in the stored sample may be a factor. Seminal plasma is not a generic component of the semen and differs among stallions with respect to its ability to confer a positive or negative effect on the sperm quality of stored sperm.

Therefore, if the clinician is presented with a 'poor' cooler, an effort should be made to determine the viability to clarify the prognosis. The simplest method is the eosin-nigrosin stain commonly used for morphologic evaluation. This stain also 'doubles' as a viability stain rendering nonviable sperm a pink hue compared to white, viable sperm. In addition, the NucleoCounter®SP-100TM can also determine sperm viability as well as concentration.

A note on sperm motility parameters

Sperm motility, regardless of the method used, has long relied on total and progressive motility, with progressive motility considered the most 'sensitive' measure. As we move forward, we should consider that progressive motility is an archaic, outdated

term that has no meaning or relevance to clinical theriogenology. Why? The inability to clearly define this endpoint, either by definition or objective CASA evaluation, is a limitation. Nevertheless, even if there was consensus regarding a definition, what is the relevance of the value? For background, the initial intent of the term was to infer a population of sperm in a sample that had better motility because they were progressive rather than not. The lack of progressivity also suggested pathology was associated with those sperm. We now know that this is largely incorrect. The quality of sperm motion varies considerably, with the motility of some stallions characterized by a high percent of nonprogressive sperm; however, these individuals have other sperm characteristics (viability, DNA quality, and morphology) that are associated with fertile stallions, and they themselves are very fertile. This should not be interpreted only as an academic point. Rendering an incorrect interpretation of a stallion's sperm quality based solely on a low percent of progressive motility may have substantial economic impact on a stallion's value as a potential breeding prospect. The concept of 'doing no harm' is relevant!

Sperm concentration

This sperm quality measure is often overlooked and furthermore substantial misinterpretations are possible due to the method of evaluation. As mentioned above, these methods include a hemocytometer, spectrophotometric-type machines, as well as cytometry, including the NucleoCounter®SP-100TM and flow cytometry. Hemocytometry is the only true direct method, in which sperm are visually identified, making it a readily available specific test. The limitation of hemocytometry is that only a limited number of sperm are counted (100's) that may not represent an ejaculate with billions of sperm. An additional concern is reader variation in preparation and evaluation of the sample. These factors can have a substantial role and may result in inaccurate values. Spectrophotometric methods provide an efficient technique to determine sperm concentration but are limited by a lack of sperm specificity, especially when the sample is diluted, in which case the opacity of the seminal plasma itself may cause an artificial increase of 10 - 20 x 10^6 sperm/ml in the final concentration. Semen contaminants such as nonsperm debris (smegma) may also cause spurious values. A false high-value results in the perception that sperm numbers are adequate, when in fact subthreshold sperm numbers are inseminated.

The practitioner should also be concerned about the concentration of the sperm sample they have received (either cooled or frozen). Evaluation of sperm motility alone is an insufficient measure of sperm quality. Excellent sperm motility associated with low sperm numbers can result in low fertility.

Morphologic features

Evaluation of stallion sperm morphologic features differentiates normal and abnormal sperm forms.[9-11] Various techniques to prepare sperm include a dry mount in which the sperm is combined with a background stain (e.g. eosin-nigrosin [EN]) and allowed to dry on the slide. A wet-mount technique can be used in which the semen sample is fixed in a buffered-formol saline solution. The EN technique has the advantage of also evaluating the sperm viability and being a quicker and simpler technique requiring only a light microscope and low magnification. However, the EN technique can induce artifactual morphologic changes (e.g. bent tails and detached heads) due to composition changes in the stain or preparation error. In addition, the EN technique, due to the lesser quality of the image, may result in the clinician missing subtle, but fertility-limiting abnormalities such as abnormal midpieces. The BFS technique is usually performed at a magnification of 1000 x using either phase-contrast or differential interference microscopy, techniques that provide enhanced resolution compared to light microscopy.

Evaluation of sperm morphology is the only test that reflects the stallion's intrinsic sperm quality. Our laboratory has maintained a similar classification system as the stallion breeding soundness evaluation manual. In addition to percent normal sperm, the types of abnormalities include: abnormal acrosomes, heads, and midpieces; proximal and distal droplets; detached normal heads; bent midpieces and tails; coiled tails, and premature germ cells. Identifying specific abnormalities rather than combining abnormalities into large categories such as primary/secondary or major/minor classification systems is preferred, since individual sperm morphogenetic features can aid in the diagnosis, prognosis, and treatment of certain conditions. Some abnormalities are associated with transient conditions such as sperm accumulation (e.g. detached abnormal heads, hairpin tail, distal droplets) whereas others are associated with testicular dysfunction (abnormal heads and midpieces, coiled tails, and premature germ cells). Additionally, certain abnormalities, when they occur together in sufficient numbers, tend to be associated with a reduction in fertility.

Sperm DNA quality

Sperm DNA quality can be determined by the sperm chromatin structure assay (SCSA), a technique that uses a metachromatic dye, acridine orange, and a flow cytometer.[12] This technique measures the susceptibility of sperm DNA to be denatured after treatment with an acid-detergent solution. Upon exposure of the stained sperm to a laser, the acridine orange signal will shift from green fluorescence (double-stranded DNA), to red fluorescence (single-stranded DNA), if damage to the DNA backbone has been sustained.

Studies using SCSA have determined the relationship between sperm DNA integrity and stallion fertility.[13] In stallions with poor sperm quality as consequence of testicular dysfunction, a higher extent of DNA-susceptibility to denaturation is observed.[14] Such changes are similar to those observed in stallions with low fertility.[13] Furthermore, methods used for stallion sperm storage can have also a detrimental effect on sperm DNA integrity. For

example, studies conducted in our laboratory suggested that increased concentrations of seminal plasma (10 - 20%, v:v) in cooled stored semen[15] or storage time and temperature[16] increased the rate of sperm DNA damage. These findings have profound clinical implications, particularly when very diluted ejaculates are processed by simple dilution prior to shipping. Since the sperm concentration in the ejaculate is low, in theory less semen extender should be added to respect the 'minimal' sperm concentration that should be accommodated in a dose of cooled semen. Also, since some stallions are considered 'poor-coolers', some practitioners ship semen doses at 15 - 25°C. We have observed that both scenarios have reduced sperm quality and potential fertility of samples sent to our laboratory for sperm quality analysis. Our experience with the SCSA have led us to consider this assay, in conjunction with sperm morphology, as fundamental semen quality tests, making them valuable to draw conclusions regarding the intrinsic sperm quality of stallions.

Semen centrifugation and processing

It is in the best interest of the clinician to attempt to provide processing conditions that maintain sperm motility for the storage period of interest. Semen centrifugation using a cushion media has become a common technique to ensure that sperm quality is maximized. Semen centrifugation, like all techniques, must be performed correctly or complications can result in the 'treatment being worse than the disease.' Centrifugation has several advantages including standardization of seminal plasma concentration (< 10%), facilitate storage of sperm at a higher sperm concentration (250 x 10^6 sperm/ml); use of a deep-horn insemination technique, i.e. insemination of small-volume, high-concentrate sperm deposited at the tip of the uterine horn ipsilateral to the site of anticipated ovulation.

Cushion centrifugation is not restricted to fresh semen that is to be cool-stored but can also be exploited with shipped semen that is received, especially in a large volume sample with sperm of average to below-average quality. The process will 'freshen' the sample by removing the seminal plasma/extender supernatant and replacing it with a fresh extender, in a small volume (5 - 10 ml), enabling deep-horn insemination. Inseminating a smaller volume provides assurance that less semen volume will be refluxed out the vagina and more retained close to the site of ovulation, thus assuring that more sperm will colonize the oviduct. This effect may be particularly relevant for stallions with 'marginal' sperm quality. We have observed that centrifugation of stored semen can increase the longevity of stored sperm in circumstances when sperm motility is low, due to high concentrations of seminal plasma, while sperm viability remains high.[17]

A small volume of the extended sample should be retained for evaluation after 24 hours to assure sperm quality and to address any concerns the recipient of the sample may have after they receive the sample. It is also recommended that the sperm quality of the stallion be evaluated before the anticipated start of the breeding season to address potential concerns and modifications to routine semen processing.

Sperm quality evaluation

A thorough semen quality evaluation can be performed before the breeding season, with particular attention to the ability of the stallion's sperm to tolerate the cool-storage process. This evaluation should include total sperm motility, viability, and ensuring that an accurate determination of sperm concentration can be performed. Seminal plasma can have multiple effects on the longevity of cool-stored sperm quality. Historically, it was assumed that if a decline in sperm motility occurred after storage it was solely caused by a pathologic (i.e. toxic) effect of seminal plasma. When sperm viability was introduced as a recommended adjunct to the evaluation of sperm quality, it was recognized that sperm motility could decline dramatically after 24 hours of storage, yet sperm viability remained high.[8,17] This contrasted with stored samples in which both motility and viability were low after storage. We also became aware that those samples in which viability was retained also resulted in good fertility compared to samples that had poor viability. The relevance to the clinician is that 'poor' sperm motility after storage should not be assumed to be 'subfertile;' rather, those samples should be also evaluated for viability.

Evaluation of sperm morphological features should be considered an essential component of a routine evaluation of cool-stored sperm. 'Poor' cool-stored sperm quality commonly originates from features identified in a stallion's morphology. For instance, sperm storage (sperm 'accumulators' or spermiostasis) can render low sperm motility in cool-stored samples even when initial motility appears good. Sperm accumulation has a variety of morphological manifestations including detached heads, distal droplets, and distal midpiece reflexes (hairpin tails) in varying frequencies. The clinician should be aware that the presence of a limited number of these defects may signal a 'subclinical' manifestation of an accumulator, which may result in a stored sample of poor quality. Although sperm are motile initially, they are 'aged', and may be more 'fragile' to cooling and freezing.

Interpretation (epicrisis) of sperm quality

The evaluation of stallion sperm quality intends to render an opinion/interpretation of the results as they relate to the patient from which the sample was derived. The interpretation will consider the history of the stallion. Additionally, history of the semen sample (e.g. farm of origin; who collected and processed the sample; conditions under which the sample was exposed prior to your evaluation; fertility history; the difference in fertility between on-farm breedings with fresh versus cool-shipped sample) must also be considered in instances where the clinician is only presented with the semen and does not have first-hand contact with the stallion. Ideally, the clinician would like to know the initial sperm quality (motility, viability, and DNA quality) so that a comparison can be made between sperm

quality at the time of collection and the cool-stored or frozen sample. If the quality of the stored sample is inadequate, then the question becomes whether the sperm quality is inherent to that stallion (i.e. it came out of the stallion in that condition) or whether it was acquired during storage. Prognosis will tend to be worse if the condition is inherent to the stallion, although the possibility exists that short-term stress may have resulted in a transient decline in sperm quality (e.g. elevated ambient temperature during summer). In contrast, a precipitous decline in sperm quality due to storage may be 'treated' by a change in the conditions (e.g. collection technique, AV preparation, semen extender composition, centrifugation, etc.) prior to storage. Evaluation of the sperm morphologic features is helpful to determine the cause of poor sperm quality due to inherent versus acquired causes. A high percent of morphologically normal sperm should result in stored samples that exhibit good sperm motility and viability, whereas low motility and especially viability suggest sperm conditions prior to evaluation have been suboptimal.

Interpretation of sperm quality in our clinical practice is based on data in Tables 1 - 6. These tables represent studies that have attempted to provide perspective regarding the relationship between sperm quality features and fertility. Data regarding the evaluation of cool-stored stallion sperm and embryo recovery rate are in Table 1.[18] This study measured sperm quality features and the number of sperm that were received in the shipped sample. Threshold values give the practitioner perspective as to

Table 1. Sperm parameter, threshold value, the embryo recovery rate between 2 fertility groups (average and high) and sperm quality values between 2 fertility groups (Mean ± SD (range) for cool-stored stallion sperm

		Embryo recovery rate[2]		Sperm quality values[3]	
Sperm parameter	Threshold[1]	Average	High	Average	High
Total sperm motility (%)	≥65	93/174 (53)	153/230 (67)	50±11 (5 - 60)	69±5 (65 - 85)
Progressively sperm motility (%)	≥45	57/107 (53)	189/287 (64)	36±11 (0 - 45)	59±6 (50 - 75)
Viable sperm (%)	≥71	70/138 (51)	178/269 (66)	56±18 (0 - 71)	80±5 (71 - 92)
Morphologically normal (%)	≥47	46/97 (47)	170/269 (63)	35±10 (5 - 46)	64±10 (47 - 88)
Total sperm number (x 10^9)	≥1.14	64/129 (50)	181/272 (67)	0.78±0.22 (0.20 - 1.12)	1.91±0.86 (1.14 - 6.67)
Total motile sperm (x 10^9)	<0.60	53/114 (46)	189/283 (67)	0.4±0.1 (0.03 - 0.6)	1.2±0.6 (0.6 - 5.3)
Total morph. normal (x 10^9)	<0.94	117/222 (53)	84/112 (75)	0.57±0.23(0.06 - 0.94)	1.4±0.63 (0.94 - 4.20)
Total progressively motile sperm (x 10^9)	<0.55	61/129 (47)	181/268 (68)	0.4±0.2 (0.01 - 0.5)	1.1±0.5 (0.5 - 4.7)
Sperm concentration (x 10^6/mL)	≥31.6	72/141 (51)	182/277 (66)	22±6 (4 - 32)	60±48 (32 - 482)
Total viable sperm (x 10^6)	<0.74	59/120 (49)	186/280 (66)	0.5±0.2 (0.07 - 0.7)	1.4±0.7 (0.8 - 5.6)
Total PMVS (x 10^9)	<0.32	39/95 (41)	203/301 (67)	0.2±0.1 (0 - 0.3)	0.8±0.4 (0.3 - 3.8)
Total PMMS (x 10^9)	≤0.37	65/121 (54)	134/210 (64)	0.2±0.1 (.01 - 0.4)	0.7±0.4 (0.4 - 3.4)
% $COMP_{at}$	≥26.8	14/33 (42)	147/236 (62)	33±13 (27 - 72)	13±5 (4 - 27)
$Mean_{at}$	≤253	39/85 (46)	122/184 (66)	273±22 (254 - 374)	233±14 (186 - 254)
$Mode_{at}$	>233	44/93 (47)	117/175 (67)	244±16 (233 - 374)	216±13 (165 - 232)

1- Threshold value that separates the average and high fertility groups
2- Embryos recovered/total attempts (% recovery rate). Between columns, all rates are different ($p < 0.05$)
3- Represents Mean ± SD (range) sperm quality value for the average and high fertility groups. Between columns, all rates are different.
PMVS = Progressively motile, viable sperm
PMMS = Progressively motile, morphologically normal sperm
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what separates high from average fertility samples. As expected, samples with higher numbers of higher quality also result in an increased sperm recovery rate.

Information from a study that evaluated sperm motility, morphology, and DNA quality, based on fresh sperm quality, are in Tables 2-6.[11,13] These data evaluated relationships among seasonal pregnancy rates, per cycle pregnancy rates, and sperm quality. Stallions included in the study had no history of reduced fertility; therefore, they are assumed to represent differences in fertility among several levels of fertility. These tables are not intended to provide cutoffs or pass/fail conclusions, but rather to provide some perspective regarding interpretation of sperm quality. The reader can easily see that there is considerable variation within fertility levels. This should not be surprising, as mare and management factors have a large role in fertility outcomes.[19] 'Fertile' stallions can be 'subfertile' due to poor mare quality and/or poor semen handling/processing, etc. In contrast, 'subfertile' stallions can be 'treated' by breeding to mares of high reproductive quality and using enhanced breeding management and become 'fertile'. These tables also highlight the ability of per cycle pregnancy rates to identify differences in fertility compared to seasonal pregnancy. Seasonal pregnancy rate is an accumulation of breedings, whereas per cycle pregnancy rate determines the efficiency by which a stallion renders mares pregnant. Two stallions can have the same seasonal pregnancy rate, with 1 being much more inefficient (i.e. less fertile) than the other. Therefore, seasonal pregnancy rate is not as good at separating stallions of different fertility levels. This has led to the incorrect interpretations of sperm quality features, that stallions with different levels of sperm quality have the same fertility (due to similar seasonal pregnancy rates); thus, sperm quality is of little use in identifying those individuals of lesser fertility.

Table 2. The mean (+ SD) and (range) for stallion sperm motility variables for the seasonal pregnancy rate from mares bred with fresh semen

	1 (n = 22)	2 (n = 11)
Seasonal pregnancy rate (%)	90 ± 6 (79 - 100)	58 ± 21 (12 - 77)
Pregnant/cycle (%)	62 ± 16 (36 - 100)	41 ± 17 (8 - 66)
Pregnant/first cycle (%)	62 ± 22 (0 - 100)	48 ± 17 (14 - 80)
Total motility (%)[1]	78 ± 12 (44 - 92)	68 ± 20 (18 - 88)
Progressive motility (%)	72 ± 12 (43 - 88)	63 ± 20 (16 - 84)
Rapid (%)	69 ± 12 (41 - 87)	60 ± 20 (13 - 84)
Moderate (%)	3 ± 2 (1 - 8)	3 ± 1 (2 - 6)
Slow (%)	5 ± 2 (1 - 11)	4 ± 1 (2 - 6)
Anticrit	0.81 ± 0.44 (0 - 2)	0.73 ± 0.7 (0 - 2.3)
Critical value	88 ± 5 (74 - 95)	87 ± 6 (73 - 94)
Linearity	77 ± 4 (69 - 84)	76 ± 5 (67 - 82)
Path velocity (μ/s)	193 ± 23 (157 - 241)	178 ± 27 (104 - 199)
Progressive velocity (μ/s)[2]	173 ± 19 (143 - 208)	157 ± 25 (88 - 183)

Seasonal pregnancy rate groups- Group 1- ≥ 78% and ≤ 100%; Group 2- ≥ 0% and < 78%
n = number of stallions
1- p = 0.08
2- p = 0.06
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Table 3. Mean (± SD) and (range) for stallion sperm morphology variables for the seasonal pregnancy rate from mares

Morphology variables	1 (n = 56)	2 (n = 19)
Normal	51 ± 18 (11 - 85)	43 ± 23 (4 - 75)
Abnormal heads	12 ± 10 (0 - 45)	14 ± 10 (1 - 43)
Detached heads	2 ± 2 (0 - 10)	4 ± 10 (1 - 44)
Proximal droplets	19 ± 15 (2 - 60)	23 ± 17 (8 - 70)
Distal droplets	7 ± 6 (0 - 28)	5 ± 5 (0 - 17)
Bent midpieces	0.23 ± 0.62 (0 - 4)	1 ± 4 (0 - 17)
General midpiece abnormality	7 ± 6 (1 - 29)	8 ± 6 (3 - 26)
Hairpin tail	4 ± 4 (0 - 16)	5 ± 6 (0 - 20)
Coiled tail[1]	2 ± 2 (0 - 11)	4 ± 4 (0 - 14)
Premature germ cell	1.5 ± 1.7 (0 - 8)	1.3 ± 2.1 (0 - 7)

Seasonal pregnancy rate groups - Group 1- ≥ 78% and ≤ 100%; Group 2- ≥ 0% and < 78%
n = number of stallions
1-p = 0.02
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Table 4. The mean (± SD) and (range) for stallion sperm motility variables for the percent mares pregnant/cycle

	1 (n = 8)	2 (n = 20)	3 (n = 4)
Seasonal pregnancy rate (%)	97 ± 4 (90 - 100)	86 ± 10 (50 - 100)	61 ± 29 (12 -100)
Pregnant / cycle (%)	91 ± 10 (75 - 100)	56 ± 6 (45 - 74)	32 ± 13 (8 - 45)
Pregnant / first cycle (%)	91 ± 10 (75 - 100)	58 ± 16 (0 - 88)	34 ± 14 (0 - 50)
Total motility (%)[1]	83 ± 5 (76 - 91)[a]	76 ± 12 (44 - 92)[a]	48 ± 21 (18 - 66)[b]
Progressive motility (%)[2]	77 ± 6 (64 - 85)[a]	71 ± 12 (43 - 88)[a]	44 ± 20 (16 - 63)[b]
Rapid (%)[3]	74 ± 9 (57 - 83)[a]	68 ± 12 (41 - 88)[a]	42 ± 20 (13 - 60)[b]
Critical value[4]	87 ± 7 (74 - 95)a	89 ± 4 (81 - 95)[a]	83 ± 7 (73 - 90)[b]
Linearity	77 ± 3 (74 - 84)	77 ± 4 (67 - 82)	74 ± 4 (69 - 78)
Path velocity (μ/s)[5]	196 ± 26 (163 - 230)[a]	190 ± 19 (157 - 214)[a]	162 ± 41 (104 - 194)[b]
Progressive velocity (μ/s)[6]	174 ± 20 (146 - 206)[a]	171 ± 17 (143 - 208)[a]	139 ± 34 (88 - 166)[b]

Percent pregnant / cycle groups – Group 1- ≥ 76% and ≤ 100%; Group 2- ≥ 46% and < 76%; Group 3- ≥ 0% and < 46%.
n = number of stallions
1-p < 0.0001; 2-p < 0.001; 3-p < 0.0002; 4-p < 0.04; 5-p < 0.04; 6-p < 0.008
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Table 5. The mean (± SD) and range () for stallion sperm morphology variables for the percent mares pregnant/cycle

Morphology variables	1 (n = 11)	2 (n = 49)	3 (n = 13)
Normal[1]	67 ± 8 (50 - 76)[a]	48 ± 15 (17 - 83)[b]	41 ± 27 (7 - 85)[b]
Abnormal heads	9 ± 6 (1 - 22)	12 ± 9 (0 - 45)	17 ± 13 (1 - 43)
Detached heads[2]	2 ± 2 (1 - 8)[a]	2 ± 2 (1 - 10)[a]	6 ± 11 (0 - 44)[b]
Proximal droplets[3]	8 ± 3 (5 - 14)[a]	20 ± 14 (2 - 51)[b]	25 ± 18 (4 - 60)[b]
Distal droplets[4]	6 ± 4 (2 - 16)[a]	8 ± 7 (0 - 28)[a]	2 ± 2 (1 - 7)[b]
Bent midpieces	1 ± 1 (0 - 4)	0.2 ± 0.4 (0 - 1)	1 ± 5 (0 -17)
General midpiece abnormality[5]	6 ± 4 (1 - 14)[a]	7 ± 6 (1 - 29)[ab]	10 ± 6 (2 - 26)[b]
Hairpin tail	4 ± 3 (0 - 9)	4 ± 4 (0 - 16)	5 ± 6 (0 - 20)
Coiled tail[6]	1 ± 1 (0 - 3)[a]	2 ± 2 (0 - 7)[a]	5 ± 5 (0 - 14)[b]
Premature germ cell	1 ± 2 (0 - 6)	1 ± 2 (0 - 8)	2 ± 2 (0 - 7)

Percent pregnant/cycle groups – Group 1- ≥ 76% and ≤ 100%; Group 2- ≥ 46% and < 76%; Group 3- ≥ 0% and < 46%
n = number of stallions
1-p < 0.002
2-p < 0.03
3-p < 0.0008
4-p < 0.03
5-p < 0.03
6-p < 0.04
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Table 6. Means ± SD and range () for Sperm Chromatin Structure Assay (SCSA) values for each fertility group within each fertility parameter (SPR, FCP and PC). Means ± SD and range () for SPR, FCP and PC are given in the same row as the heading

Fertility parameter	SCSA variable	1	2	3
SPR		90 ± 6 (79 - 100)	58 ± 21 (12 - 77)	NA
	$Mean_{\alpha t}$[5]	232 ± 16 (203 -297)	241 ± 23 (222 - 296)	NA
	$SD_{\alpha t}$	85 ± 22 (50 - 149)	90 ± 26 (55-171)	NA
	$COMP_{\alpha t}$[3]	16 ± 8 (4 - 38)[a]	23 ± 13 (9 - 52)[b]	NA
FCP		89 ± 9 (78 - 100)	58 ± 7 (46 - 75)	25± 18 (0 - 45)
	$Mean_{\alpha t}$	227 ± 9 (213 - 243)	233 ± 16 (208 - 297)	237± 21 (203 - 281)
	$SD_{\alpha t}$[4]	73 ± 11 (54 - 96)	89 ± 21 (50 -149)	94 ± 30 (55 - 171)
	$COMP_{\alpha t}$[2]	12± 5 (4 - 28)[a]	17 ± 7 (6 - 38)[b]	25 ± 13 (5 - 52)[c]
PC		91± 10 (75 - 100)	56 ± 6 (45 - 74)	32± 13 (8 - 45)
	$SD_{\alpha t}$[4]	229 ± 11 (213 - 243)[a]	233 ± 15 (206 - 297)[a]	248± 27 (203 - 296)[b]
	$SD_{\alpha t}$	76 ± 12 (62 - 96)	87 ± 22 (50 - 149)	97 ± 30 (55 - 171)
	$COMP_{\alpha t}$[1]	13± 6 (7 - 28)[a]	17 ± 7 (4 - 38)[a]	27 ± 14 (5 - 52)[b]

Fertility groups - Seasonal pregnancy rate (SPR), group 1-SPR > 80%, group 2-SPR < 80%; first cycle pregnancy rate (FCP), group 1- FCP > 75%, group 2-FCP < 75% and
> 45%, group 3-FCP < 45%; overall cycles per pregnancy (PC), group 1- PC > 75%, group 2-PC < 75% and > 45%, group 3- PC <45%. Letters within rows are different, based on the following significance levels: 1- p < 0.0001; 2- p < 0.001; 3- p < 0.004; 4-p < 0.01; 5- p < 0.06.
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Conflict of interest

None

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