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Routine assessment of motility of ejaculated stallion spermatozoa using a novel computer-assisted motility analyzer (Qualisperm™)

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Abstract

Visual motility analysis is the basis for routine quality evaluation of stallion semen, although its prognostic value for fertilizing ability is considered low. The present study evaluated the ability of a novel computer-assisted motility analyzer (QualiSperm™) to determine the motility and velocity of ejaculated, extended stallion spermatozoa (collected from 10 stallions, 3 ejaculates/stallion) and following two different colloidal centrifugation methods (one- or two-layer), compared to visual evaluation by two independent operators. The Qualisperm™ instrument was able to retrieve and analyze ~10 times more spermatozoa per sample compared to routine visual estimation on the same time frame (~1,100 vs ~100 spermatozoa). The proportion of motile spermatozoa increased after the colloid-separation, compared to the extended ejaculates ($P < 0.05$) in some stallions. However, owing to the large variation seen among ejaculates and stallions, both for extended ejaculate ($P < 0.05$) as well as for the colloid centrifugations ($P < 0.01$), the differences were lost when the entire population was examined statistically. Interestingly, significant differences were seen for individual stallions between the measurements of Qualisperm™ and observers, as well as between observers ($P < 0.05$). Apart from the significantly higher number of spermatozoa analyzed at one time, the Qualisperm™ system provided a parameter that could simply not be estimated by visual assessment; mean sperm velocity (in µm/sec). Sperm velocity, upon which every computer assisted instrumentation base their evaluations, varied among stallions (and ejaculates within stallions, $P < 0.05$), with a tendency to increase after colloid-separation, thus suggesting the Qualisperm™ system might be able to differentiate sperm sub-populations. Due to its higher accuracy (in terms of sperm numbers examined) and speed, the Qualisperm™ system appears to be a suitable instrument for routine evaluation of equine semen.

Keywords: motility, Qualisperm™, subjective assessment, spermatozoa, stallion.

Introduction

Sperm motility is the parameter most frequently used to assess stallion semen quality, to measure sperm viability in the ejaculate and during/after any handling and preservation procedure, including refrigeration and even cryopreservation. Traditionally, sperm motility in stallion ejaculates is assessed by subjective estimation of the proportion of spermatozoa depicting progressive, rectilinear motility, using a light microscope. Problems inherent with this method include the wide variation between observers and between laboratories and its low predictive value in assessing the potential fertility of a semen sample (Watson, 1979). The use of Computer Assisted Semen Analysis (CASA), which allows objective measurement of several parameters of sperm motility, offers a more reliable, unbiased and repeatable means of assessing sperm motility than examination by eye (Colenbrander *et al*., 2003). Several CASA systems are available commercially, which vary in their mode of functioning and in their ability to detect and measure the motility of spermatozoa of different species. The majority of CASA systems (such as ISAS™ by Proiser, Hobson Sperm Tracker by Sound and Vision or CEROS™ by Hamilton Thorne), record the path and type of movement of a group of spermatozoa in a wet preparation under a cover slip using a video camera. The signal picked up by the camera is digitized and the information is processed by a computer which reconstructs each individual sperm path trajectory for a certain number of frames. Subsequently, these trajectories are mathematically processed, permitting them to be defined in a numerical form (Quintero-Moreno *et al*., 2003). The CASA system is, thereafter, able to determine a series of variables, including the number of moving spermatozoa, curvilinear velocity (VCL), linear velocity (VSL), linear coefficient (LIN), straightness coefficient (STR), frequency of head displacement (BCF), etc. The kinematic variables obtained from CASA, which cannot be determined by the human eye, are useful for research purposes, enabling, for example, the identification of sperm subpopulations coexisting in a stallion ejaculate (Quintero-Moreno *et al*., 2003).

Importantly, some of the above mentioned

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variables are considered absolute, such as the speeds of translation (VCL, VSL, VAP), while others are derived, or recalculated (proportions of moving spermatozoa, patterns of motility within the subpopulation of moving spermatozoa, wobble, dance, linearity, etc). In some instances, this recalculation might confound the results, especially in the case of the latter output variables (Katkov and Lulat, 2000). Moreover, they are not commonly found on studs because of their cost which ranges between 17,000 and 35,000 €. Although CASA does provide standardized and objective analysis, the apparatus tends to provide a surfeit of information which, albeit relevant for research purposes, it is not always relevant for routine use at a stud (Owen and Katz, 1993; Verstegen *et al*., 2002).

Recently, a new system for the analysis of sperm motility has been established. This system is called "QualiSperm™" (Biophos AG, Pfäffikon, Switzerland, http://www.biophos.com) and works with absolute values and, perhaps most important, it appears to be a suitable alternative for routine use. Qualisperm™ determines the motility and velocity of the spermatozoa, i.e. probably the most relevant absolute values for an AI station to use when assessing stallion semen samples, % motility (and its subclasses) and their mean speed (Rodriguez-Martinez, 2006). The system, which was recently validated for animal spermatozoa (Tejerina *et al*., 2008), uses newly-designed software which, instead of being based on trajectory identification as for conventional CASA instruments, it is based on statistical analysis of fluctuation, as is the case for Fluorescence Correlation Spectroscopy (FCS). The system measures the total number of spermatozoa in movement within a large field of view, through the frames captured by a high-resolution digital video camera (1280 x 1024 pixel, uEye UI-5640, IDS Imaging Development Systems GmbH, Obersulm, Germany) working on a range between 25 and 265 frames per second (e.g., from 1280 x 1024 to 320 x 240 pixels, respectively). The area of the wet preparation to be captured is large, and owing to the high resolution applied, the number of spermatozoa captured is high, between 10-20 times higher than for visual estimation. Thus, the precision of the motility analysis is potentially improved compared to conventional subjective assessment (Verstegen *et al*., 2002).

Contrary to conventional CASA instruments, the QualiSperm™ only analyzes two motility parameters: % of motility (disclosed in classes depending on their velocity) and the mean speed of the spermatozoa. These are the two most relevant variables for an AI station to use when assessing stallion semen samples, owing to their relation to fertility *in vitro* and *in vivo* (Holt *et al*., 1997, 2007; Hirano *et al*., 2001).

The purpose of the present study was to evaluate the QualiSperm™ assessment of sperm motility (compared to visual evaluation provided by two independent operators) and sperm velocity, for stallion ejaculated and colloid-separated spermatozoa using a density gradient (DGC) or a single layer (SLC). The formulations of the colloids used for DGC or SLC, containing glycidoxypropyl-trimethoxysilane-coated silica, were designed especially to separate spermatozoa from a native ejaculate (Thys *et al*., 2009 including stallion spermatozoa (Androcoll-E™; patent pending; Morrell *et al*., 2009).

Materials and Methods

Animals and husbandry

Ten warmblood stallions, ages ranging from 7 to 23 years, were housed under standard husbandry conditions at Flyinge AB, Flyinge, Sweden. Semen was collected up to three times a week using an artificial vagina according to standard methods. The trial took place during June, within the normal equine breeding season in Sweden (April to August). The experimental protocol had previously been reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Sperm handling

The concentration of spermatozoa in the original ejaculate was measured using a Spermacue photometer before extending the ejaculate 1:1 with either Kenney´s medium (4.9 g glucose, 2.4 g of skimmed milk powder, 0.15 g of dihydrostreptomycin, 0.15 g of penicillin, in 1,000 mL of water) or Nørlunds medium (purchased from Nørlunds Equine Hospital, Rue de Lund, Denmark) at 37°C. Aliquots (6 mL) of extended ejaculate were made available for the experiments. Kenney´s extender was used for stallions F, Y, T, A, R, K and Q; Nørlunds medium was used for stallions H, W and Z. The sperm concentration of the extended semen (1:1, see above) was determined using a Bürker counting chamber.

Colloidal centrifugation

Aliquots of stallion extended semen (containing up to 100 million spermatozoa per mL) were subjected to centrifugation (300 x *g* for 20 min) through one- or two-layer colloid columns (single-layer or gradient, respectively; Morrell *et al*., 2008) in order to separate spermatozoa from seminal plasma and recover those with normal morphology and viability. The formulations of the colloids used for the density gradient and single layer, containing glycidoxypropyl-trimethoxysilane-coated silica, were designed especially for stallion spermatozoa (Androcoll-E™; patent applied

for). The method of Density Gradient Centrifugation (DGC) has been described previously (Morrell and Geraghty, 2006). Briefly, a density gradient was prepared by pipetting 2 mL of the higher density layer into a centrifuge tube and carefully layering 2 mL of the lower density layer on top; an aliquot (1.5 mL) of extended semen containing up to $100x10^6$/mL was pipetted on top of the upper layer. The gradient was centrifuged at 300 x *g* for 20 min, after which the supernatant and most of the gradient material was discarded. The sperm pellet was transferred to a clean centrifuge tube containing 5 mL Kenney´s extender and was washed by centrifuging for 10 min at 500 x *g*, after which the sperm pellet was re-suspended in Kenney´s extender (1 mL). The method of Single Layer Centrifugation (SLC; Morrell *et al*., 2008, 2009) was similar to that for DGC with the exception that 4 mL of the higher density material was used instead of two layers of different densities (2 mL of each density). The sperm pellet was placed in a clean centrifuge tube containing 5 mL of Kenney´s medium and was washed by centrifugation for 10 min at 500 x *g*. Following washing, the sperm pellet was re-suspended in fresh Kenney´s medium (1 mL) for assessment of sperm concentration (Bürker chamber) and sperm motility.

Estimation of motility

Aliquots of the extended ejaculate and the colloid-separated sperm suspensions were split and assessed by visual estimation (two operators) and using the QualiSperm™ equipment (up to one hour after colloidal centrifugation).

Visual estimation. Aliquots (5 µL) of the sperm suspensions were placed on a pre-warmed glass slide (38°C) and covered with a cover slip. The evaluation was independently conducted by the two operators, three times each, using a microscope (Nikon Optiphot 2. Tokyo, Japan) equipped with phase-contrast optics and a thermal plate (38°C) at 100x magnification.

Computer-assisted estimation. Aliquots (5 µL) from the extended ejaculate or the colloid-separated sperm preparations were each loaded on a Makler chamber (Sefi-Medical, Haifa, Israel), which was transferred to a heated microscope stage at 38ºC. Sperm motility was observed using a positive phase-contrast system in a Nikon E200 microscope (Tokyo, Japan) at 100x magnification and recorded in one field at a rate of 50 frames/sec (200 frames in total) using a MV-D640-48-U2-10 Photon focus camera (Photon focus, AG, Lachen Switzerland). Sperm motility (%) and the speed (µm/s) were retrieved as parameters from the QualiSperm™ analysis. The analysis was performed twice by an independent operator.

Statistical analyses

The statistical analyses were made using SAS (SAS version 9.1, Cary, NC, USA). ANOVA was used to investigate differences between stallions and ejaculates. Means and standard deviations were calculated for the QualiSperm™ parameters and subjective motility estimations. The normality of the data was checked by Shapiro-Wilks test. If the data did not follow a normal distribution, the data were subjected to a Wilcoxon test to estimate the differences between means, whereas if the data followed a normal distribution, a Student´s t-test was performed to check differences between groups of data. Correlations between methods of motility estimation and between technicians were calculated using the Spearman's rank correlation coefficient. For all analyses, a significance level of $P < 0.05$ was used.

Results

The number of spermatozoa analyzed per method differed, e.g. the number of spermatozoa examined by the operators was estimated to ~100 while the QualiSperm™ retrieved ~1,100 spermatozoa per sample analyzed (a significantly higher proportion; $P < 0.01$).

The mean percentages of motile spermatozoa determined by the QualiSperm™ analysis and the visual assessment (two operators) of both the ejaculates and the spermatozoa collected after the colloid separations are shown in Table 1. The mean sperm motility, measured by QualiSperm™ in the extended ejaculate was similar to that measured after the colloid-separations. The visual estimates (by either observer) were higher than that recorded by the QualiSperm™ analysis both before and after colloid centrifugations, but remained non-significant. Observer 1 registered lower sperm motility after colloid centrifugations than Observer 2, but with a larger variation among stallions, leading to non-significant differences. There was a large variation between ejaculates and stallions (data not shown), both for extended ejaculate ($P < 0.05$) as well as for the colloid centrifugations ($P < 0.01$) with marked differences ($P < 0.05$) between the measurements of Qualisperm™ and observers, as well as between observers ($P < 0.05$) for some stallions. Also, for some stallions significant differences were seen between the motility measured in the extended ejaculate and after the colloid centrifugations for both assessment methods (QualiSperm™, $P < 0.05$; visual estimation, $P < 0.05$). Interestingly, the variation between ejaculates and stallions was lower for the QualiSperm™ instrument, probably owing to its wider limits compared to the visual evaluations.

Table 1. Means (± SD) of sperm motility (%) measurements by the QualiSperm™ system and the estimates by two observers (1 and 2) of spermatozoa in extended ejaculates and following one- (single layer centrifugation, SLC) or two-layer (density gradient centrifugation, DGC) colloid separation (n.s., n = 30 ejaculates from 10 stallions).

Assessed by	Extended sperm suspension	Colloid-separated spermatozoa	
		Single layer (SLC)	Gradient (GSC)
QualiSperm™	56.9 ± 13.4	69.7 ± 9.8	56.6 ± 18.3
Observer 1 (Visual)	69.7 ± 9.8	74.3 ± 19.8	74.2 ± 13.4
Observer 2 (Visual)	67.5 ± 10.3	85.7 ± 5.0	84.3 ± 5.2

Mean sperm velocities, discriminated per sperm source (e.g., extended semen vs single- or double-layer colloid-separation) are shown in Table 2. Variation existed among stallions and ejaculates within stallions ($P < 0.05$). Considering all ejaculates/stallions, mean sperm velocity increased in those spermatozoa separated by the colloid-preparations (76.5 ± 5.8 and 77.1 ± 5.0 µm/sec, respectively) compared to the mean speed recorded in the extended ejaculates (73.2 ± 3.0 µm/sec), indicating that the QualiSperm™ system seems able to differentiate between sperm populations.

Table 2. Sperm velocity (µm/sec, means ± SD) registered in extended-semen and after colloid centrifugation (SLC or DGC) using the QualiSperm™ system (Means ± SD, n = 30 ejaculates from 10 stallions).

Stallion	Extended sperm suspension	Colloid-separated spermatozoa	
		Single layer (SLC)	Gradient (GSC)
A	76.3 ± 7.3	80.7 ± 4.6	77.7 ± 6.5
F	74.5 ± 2.6[a]	76.3 ± 5.0**[b]	85.3 ± 2.1**[c]
H	69.0 ± 0.9	72.0 ± 19.2	77.7 ± 5.5
K	73.7 ± 0.6[a]	72.0 ± 3.0	74.7 ± 5.0
Q	70.5 ± 3.8	79.0 ± 4.0	76.7 ± 1.5
R	72.7 ± 2.0[a]	79.7 ± 1.2*	80.3 ± 4.2*
T	74.0 ± 4.4	80.7 ± 1.2	78.3 ± 1.1
W	78.8 ± 3.7[a]	75.7 ± 2.1	64.0 ± 19.2
Y	74.8 ± 3.2[a]	84.0 ± 3.6	75.0 ± 6.6
Z	68.0 ± 2.0[a]	71.3 ± 6.1	75.0 ± 6.2
Overall	73.2 ± 3.0	76.5 ± 5.8	77.1 ± 5.0

[a]Within column, superscript denotes significant differences between stallions ($P < 0.05$).
[bc]Denotes significant difference between single layer centrifugation (SLC) and density gradient centrifugation (DGC) ($P < 0.05$).
*, **Between columns, denotes significant difference from extended semen (*$P < 0.05$, ** $P < 0.001$).

Discussion

In the horse, as in other species of domestic animals, estimation of the fertility potential of males is an important step in animal breeding. The most reliable method is the retrospective analysis of the outcome of hundreds of matings or of artificial inseminations (AIs), procedures which are highly expensive and time-consuming (Colenbrander *et al*., 2003). In practice, the only feasible means of assessment is *in vitro* and, consequently, many laboratory methods are being developed to predict fertility without directly using AI (Colenbrander *et al*., 2003; Rodriguez-Martinez *et al*., 2006). With modern advances, in addition to the general characteristics of the spermatozoa (such as morphology, motility, integrity of the membrane and organelles, etc), it is possible to examine the spermatozoa-oocyte interaction and interaction with the female tract (Holt *et al*., 1997, 2007; Hirano *et al*., 2001). However, disadvantages of these methods include the need for skilled operators and specialized equipment to carry out some of these tests, as well as the time needed to obtain the result. Generally, AI stations need tests to be easy, cheap and fast.

There is an urgent need for objective means of assessing semen quality since stallions are selected for breeding, primarily on the basis of their pedigree and their athletic prowess or other phenotypic characteristics rather than on semen quality (Colenbrander *et al*., 2003). Currently, assessing ejaculate quality in AI-stations (as for other domestic livestock species) is mostly performed by measuring the concentration of spermatozoa (as a reflection of the number of spermatozoa produced by the male) and making a subjective assessment of the percentage of progressively motile spermatozoa. However, using such subjective motility analysis, variations of 30-60% have been reported in motility estimates of the same ejaculate (Verstegen *et al*., 2002).

The results of the present preliminary study confirm the inherent variation in sperm motility found among stallions. The variation between stallions was

detectable both from visual estimation and the QualiSperm™ instrumentation. Analyses could be done quickly by either method, but the limits set by the observers and by the computer-assisted instrument were clearly different, being wider for the latter. This difference would explain why the mean results were consistently lower for the QualiSperm™ compared to the visual observations, since its SDs were much larger. Whether this reflects the ability of the system to pick up differences not seen by the human eye should be studied in detail with a larger sample size.

The QualiSperm™ bases its performance on an absolute parameter, the speed of translation of the spermatozoa, a variable that cannot be estimated subjectively when visual screening is performed. Moreover, the number of assessed spermatozoa per single sample is much larger in the QualiSperm™ compared to visual assessment, since it is difficult for the human eye to cope with a quick observation of more than 20-25 spermatozoa in a microscopic field. The more spermatozoa present, the higher the over-estimation of motility given. Although the number of fields examined by each operator was at least 3 per sample, the number of spermatozoa was about ten times fewer than in the one field retrieved by the QualiSperm™. Similar differences were reported in another experiment using QualiSperm™ to examine boar semen (Tejerina *et al*., 2008).

Differences were also observed in the two observers´ estimates, particularly for the spermatozoa obtained after colloid-separation procedures. Moreover, when comparing separation procedures (single-layer column vs density gradient; Morrell *et al*., 2008), the visual outputs also differed; observer 1 found differences only between the percentage of motility in fresh semen and samples after single layer preparation, whereas observer 2 found differences for the two treatments in comparison with fresh semen, but not between treatments. Several reasons could account for these different levels of motility, particularly the fact that experience varied between observers. Since this is a problem that could influence the interpretation of the effect of the treatments, there is an obvious need for an "objective" instrument. However, there were significant differences for all the measures between the QualiSperm™ analysis and the estimations of the two observers, which is a matter of concern. Several explanations for these differences exist, mainly that the mean value of motile spermatozoa per sample is too imprecise a measure to be of much use and perhaps too difficult to compare when the number of assessed spermatozoa is as different as in the present study (10-fold difference).

The value of the QualiSperm™ system lies in its ability to assess velocity (an absolute value from which the software recalculates the proportions of sperm classes) as well as the proportions of motile spermatozoa, since velocity cannot be assessed objectively by eye. The low variability (as SDs) among ejaculates within stallion was noteworthy, while differences in sperm velocities were noticeable between stallions. This result suggests that the mean sperm velocity, if consistent within stallion, could be characteristic of each sire and may be correlated with, for example, fertility. Regrettably, the number of mares inseminated with processed, extended semen from the ejaculates evaluated in the present study was too small and inhomogeneous to provide any statistically significant relation with the outcome of the Qualisperm™. Further studies, with a larger mare population involved, are needed to solve this question.

There was a tendency for mean sperm velocity to increase when comparing the extended ejaculate and the spermatozoa retrieved after the colloid-separations, with a large variation among stallions. For some stallions the differences were significant, albeit without denoting differences between colloid-separation procedures. These results were not unexpected since the process would separate viable spermatozoa from those with less vitality, and hence motility. Such differences in motility were clear for the extended ejaculates. Moreover, previous studies with marmoset spermatozoa have shown that spermatozoa which have not been exposed to seminal plasma have a higher velocity than ejaculated spermatozoa (Morrell, 1997).

In conclusion, the QualiSperm™ is an interesting instrument for routine use, owing to its speed of evaluation, its low cost, the high number of spermatozoa assessed, and the provision of absolute parameters (speed of velocity and proportion of motile/immotile spermatozoa). For these reasons, and provided proper species-specific settings are installed, it may be an alternative to the customary visual evaluation. However, the output of parameters is restricted in comparison with other CASA instruments, which could be a disadvantage of this instrument for research purposes.

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