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Full Length Research Paper

Assessment of viability, chromatin structure stability, mitochondrial function and motility of stallion fresh sperm by using objective methodologies

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Methodologies, such as flow cytometry and computer assisted sperm analysis (CASA), provide objective, reproducible, rapid and multi-parametric evaluation of semen quality. In this study, semen samples collected from six stallions were analysed for viability (by propidium iodide), chromatin stability by sperm chromatin structure assay (SCSA) and mitochondrial membrane potential by JC-1 using flow cytometry. Total and progressive motility, average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL) were determined by CASA system. The cytofluorimetric analysis provided results with low intra-assay variability respect to motility analysis by CASA system. The data on viability and mitochondrial assessment were rather uniform between stallions. The SCSA was able to distinguish potential fertility levels between stallions. In fact statistical differences were found between stallions especially for %- DFI and SD-DFI parameter. The %-DFI parameter was negatively correlated with VCL parameter. The higher repeatability of %-DFI parameter respect to those of other SCSA parameters confirms the importance of this parameter notoriously related to fertility. In conclusion, the simultaneous assessment of different functional sperm parameters, by flow cytometry and CASA, may be allow to obtain detailed and repeatable evaluations of sperm quality in the stallion, usually not considered in breeding selection programs.

Key words: Flow cytometry, stallion, sperm quality.

INTRODUCTION

Pregnancy and foaling rates are considered true indexes of fertility in the horse. However, both of them are retrospective and dramatically influenced by factors extrinsic to the stallion, such as mare's reproductive capacity and breeding management. In many circum-stances, a prospective test is desired so that, likely, subfertility can be identified before a stallion embarks on his breeding career (Colenbrander et al., 2003). Constraints in horse breeding – small number of fertilized mares per ejaculate/ stallion and tremendous variations in mare management and insemination – did not allow carrying out trials similar to bovine species concerning artificial insemination techniques. For these reasons, the fertility trials of the horse are of little value and laboratory tests could help in accurately predicting the fertility of commercially produced stallion semen (Colenbrander et al., 2003). Thus, considerable effort has been invested in identifying

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Abbreviations: PI; Propidium Iodide, **AO;** Acridine Orange, **m;** Inner Mitochondrial Membrane Potential, **CASA;** Computer Assisted Sperm Analysis, **VAP;** Average Path Velocity, **VSL;** Straight-line Velocity, **VCL;** Curvilinear Velocity.

markers for functional sperm capacity that can more accurately predict the fertility of a stallion, such as ubiquitin and seminal plasma proteins ubiquitination (Sutovsky et al., 2003; Barrier-Battut et al., 2005). Given the limitations of the standard Breeding Soundness Examination in predicting fertility or even in identifying all seriously subfertile stallions, many different approaches have been investigated in the hope of finding a relatively straight-forward and inexpensive test closely correlated with fertility (Neild et al., 2005). The limitation of this approach is that most of tests only assess a limited number of attributes that a sperm must possess to fertilize oocyte. For this reason, the combined use of different tests could offer more promise for reliable assessment of functional attributes of sperm quality (Colenbrander et al., 2003; Rodriguez-Martinez, 2003; Gillan et al., 2005; Mocé and Graham, 2008).

The primary goal of all semen analyses is to determine the fertilizing potential of a semen sample, using rapid, automated and objective procedures although there is no laboratory assay that can be considered reliably correlated with fertility (Mocé and Graham, 2008). Most of laboratory assays are not objective and some of them, such as hypo-osmotic swelling test, morphology and motility assessed by microscopic observation, require also long procedures (Graham, 2001; Kuisma et al., 2006). Flow cytometry (FC) allows to simultaneously measure multiple sperm attributes which can be combined in a predictive equation (Graham, 2001; Kuisma et al., 2006).

Indeed, the flow cytometric analysis of cells is objective since it measures the amount of fluorescent stain in an unbiased manner and evaluates tens of thousands of cells in about a minute (Graham, 2001). The common cytofluorimetric parameters considered for sperm quality assessment are viability, chromatin structure stability and mitochondrial function (Graham, 2001; Gillan et al., 2005).

Membrane integrity is a fundamental requisite for sperm viability and for the success of fertilization. Viable spermatozoa are defined as cells that possess an intact plasma membrane. Different viability assays assess the integrity of different plasma membrane compartments (Mocé and Graham, 2008) that can be evaluated by microscopic or cytofluorimetric approach after cell staining (Garner et al., 1986; Magistrini et al., 1997; Merkies et al., 2000; Love et al., 2003). Many techniques, based on the use of viable (fluorescein diacetate and SYBR-14) and non-viable (propidium iodide – PI - and eosin-nigrosin) dyes allow to detect sperm viability (Martinez- Pastor et al., 2004). Sperm viability assessments by using nucleic acid stains (such as SYBR-14 and PI) are considered to be less variable than enzyme-based stains (such as carboxyfluorescein diacetate – CFDA- and fluorescein diacetate) and sperm DNA is believed to be a more appropriate cellular target due to its sustainability and staining uniformity (Gillan et al., 2005).

The sperm chromatin structure assay (SCSA), a procedure originally designed by Evenson et al. (1994), is based on the metachromatic properties of the fluorescent probe acridine orange (AO) in relation to the DNA structure. Acridine orange emits green or red fluorescence when intercalates to double-stranded DNA (native) or single-stranded DNA (damaged DNA structure), respectively. After acid treatment, the increased susceptibility of sperm cells to *in situ* DNA denaturation can be evaluated by FC. Sperm chromatin stability is related to the content of P1 and P2 protamines (Corzett et al., 2002). In the horse, the occurrence of both protamines (Andrabi, 2007) induces lower chromatin stability compared with species containing only P1 (Evenson et al., 2002). Additional factors, such as seminal plasma volume, storage time and extender type, could influence the intrinsic susceptibility of equine chromatin structure (Love, 2005). The integrity and structural stability of equine sperm DNA is associated with fertility (Love and Kenney, 1998).

Mitochondrial status plays an important role in determining sperm cell competence because of its relationship with the energetic status of the cell and motility and has been related to fertility (Martinez-Pastor et al., 2004). Rhodamine 123 and MitoTracker fluorochromes have been used to evaluate mitochondrial function of spermatozoa, but no distinction can be made between spermatozoa exhibiting different respiratory rates (Gillan et al., 2005). On the contrary, the potentiometric dyes, such as JC-1 probe, permit a distinction between spermatozoa with poorly and highly functional mitochondria by the determination of the inner mitochondrial membrane potential (m) since this molecule can exist in two different states (aggregates or monomers), each with a different emission spectra. If the JC-1 molecule remains in the monomeric form, it will fluoresce in green (that is, m^{low}) after passage through mitochondrial membrane. If it converts into the JC-1 aggregate form, it will fluoresce in orange (that is, m^{high}) (Reers et al., 1991; Mocé and Graham, 2008). The reliability and the sensitivity of JC-1 staining on stallion spermatozoa were assessed by FC by Gravance et al. (2000) and Mari et al. (2008).

Sperm motility is a very important parameter for sperm quality evaluation and it can be assessed by using Computer Assisted Sperm Analysis (CASA). The CASA system is based on the capture of successive microscopic images which are then digitized. Motile spermatozoa are identified in successive images, thus allowing to follow their trajectories. Finally obtained trajectories are processed, allowing the establishment of several kinetic characteristics. Sperm motility assessment by CASA is more objective and rapid than the microscopic evaluation although the inter- assay and intra-assay variability were similar for these two assay methods (Mocé and Graham, 2008). The CASA system was used for the evaluation of equine spermatozoal motility by several authors (Jasko et al., 1991; Varner et

al., 1991; Ball et al., 2001).

The aim of this study was to assess fresh sperm quality in the stallion by means of several, objective and rapid laboratory assays and to correlate in order to select the parameter useful for a future prediction of fertilizing potential of semen sample. In particular viability, SCSA and mitochondrial potential were assessed by FC and motility by CASA system.

MATERIALS AND METHODS

Materials

MitoProbe JC-1 assay kit (Molecular Probes). Propidium iodide (Sigma). Acridine orange (Sigma). Semen extender INRA 96 (IMV Technologies).

Semen collection

In this study, during a breeding season (from 14 February to 14 July), six mature stallions, routinely used in artificial insemination programs and located in Equine Reproduction Centre ("Pegasus" Bari, Italy) were used. During the experiments, horses were kept in boxes on straw and received two times a day two Kg of oat and five Kg of hay. Water was *ad libitum*. All experiments were carried out in June and July 2007. The management of stallions and the collection of semen samples were performed in accordance with health and welfare regulations in force.

Semen was collected by using an artificial vagina (Missouri model) with an in-line gel filter. Gel-free semen volume was measured with a graduate cylinder, spermatozoa concentration was assessed photometrically and motility parameters were estimated by using CASA system. Subsequently, semen was diluted with INRA 96 at 37°C, to a final concentration of about 20 – 25 x 10^6 spermatozoa/ml (Gravance et al., 2000). The extended semen was chilled for 1 h in commercial portable containers, to maintain viability during transport to the flow cytometer laboratory. From each stallion two semen samples were collected and analysed in two independent days, once a month.

Apparatus

Flow cytometric analyses were carried out by using a FACS Calibur (BD, Milan, Italy) equipped with 15 mW air-cooled Argon laser. After acquisition, data were evaluated by using Cell Quest v. 3.3 (BD, Milan, Italy) and Win List v. 5.0 (Verity Software House Inc., Topsham, ME, USA) software.

Sperm viability

Sperm viability was assessed by using PI that can enter the cell and stain the nucleus when the plasma membrane is damaged. The protocol of Papaioannou et al. (1997) with some modifications was used. Aliquots of extended semen, at the final concentration of 2 x 10^6 spermatozoa/ml, were transferred into 5 ml culture tubes (352052 BD Falcon, Becton Dickinson, Milan, Italy) and supplemented with 1 ml of PI solution (2 µg/ml). The tubes were gently mixed and further incubated for 30 min in the dark at room temperature. Each sample was analyzed by FC after acquisition of 20,000 total events at a flow rate of ~200 cells/s. An analysis gate was applied in the FCS/SSC dot-plot to restrict the evaluation to sperm cells and to eliminate small debris and other particles from analyses. Propidium iodide, excited at 488 nm, was read with 650/13 nm bandpass emission filter (FL-3) by using a logarithmic histogram. Viable spermatozoa with intact plasmalemma (PI-negative) were observed in the second decade while membrane-damaged cells (PI-positive) were visible in the fourth decade. Cell viability was expressed as percentage of PI-negative cells. For each stallion, sperm viability was analyzed on two different ejaculates.

Sperm chromatin structure assay (SCSA)

The SCSA protocol used in this study was described by Benzoni et al. (2008) . Fresh semen was diluted into 1 ml of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA and pH 7.4) at a final concentration of 2 x 10^6 spermatozoa/ml. In crushed ice, aliquots of semen (0.2 ml) were mixed with 0.4 ml of a low pH detergent solution (0.15 M NaCl, 0.1% Triton X-100, 0.08 N HCl and pH 1.4) for a partial *in situ* DNA denaturation. After 30 s, cells were stained by adding 1.2 ml of AO work solution (6 µg/ml in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0). After 3 min, cytometric analysis was carried out at a flow rate of ~200 cells/s and total acquisition of 20,000 events for each sample. Acridine orange was excited at 488 nm and its red fluorescence was read at 650/13 nm bandpass emission filter (FL3), while its green one was read at 530/30 nm bandpass emission filter (FL1). In order to standardize all analyses, FC was calibrated with semen collected from a stallion control with regular reproductive function and known SCSA parameters. The FC was adjusted such that the mean of FL1 and FL3 fluorescences were set on histograms with 1024 channel resolution, at 439 and 110 channels, respectively. Flow cytometer calibration was checked at each start-up of the FC and after every 5 samples. The instability of sperm structure chromatin was assessed by using three different parameters of DNA Fragmentation Index (DFI), as described by Benzoni et al. (2008): the mean ($\overline{X}$) DFI, calculated selecting those cells to the right of the main population on a dot-plot of red (FL3) versus green (FL1) fluorescence; the percentage (%) and the standard deviation (SD) DFI, calculated by using t parameter (which expresses the ratio between FL3/FL3+FL1) obtained by WinList 5.0 software. For each stallion, four determinations were carried out on each ejaculate.

Mitochondrial function assessment

A 200 M JC-1 stock solution in DMSO was prepared prior to use. For each sample, 1 x 10^6 sperm cells were suspended in 1 ml of warm PBS and JC-1 solution (2 M final concentration) was added. Cells were incubated for 30 min at room temperature. For each sample, a total of 20,000 events were analyzed at a flow rate of ~200 cells/s. As suggested by the protocol, in order to confirm the JC-1 sensitivity to changes in membrane potential, carbonyl cyanide 3-chlorophenylhydrazone (CCCP = 50 µM final concentration) was used as membrane potential disruptor. Emission filters of 535 and 595 nm were used to quantify the population of spermatozoa with green (m^{low} "inactive mitochondria") and orange (m^{high} "active mitochondria") fluorescence, respectively. Dot-plot for FL-1 (green) and FL-2 (orange) fluorescence was used. In order to determine the population of cells with m^{high}, the percentage of cells which concentrate JC-I into aggregates was considered. Because of technical problems, a single ejaculate for each stallion was assessed.

Motility determination

The CASA analysis was performed by using HTM-IVOS Sperm Analyzer v. 12.3 (Hamilton Thorne, Beverly, MA, USA). Briefly, the analysis was performed in a 10 µm Leja chamber at 37°C. Setting for cell detection (capture of 45 frames; 60 Hz) were: minimum

Table 1. Viability, sperm chromatin structure assay (SCSA) parameters, mitochondrial function and motility parameters of fresh spermatozoa collected from six stallions.

Parameters	Stallion 1	Stallion 2	Stallion 3	Stallion 4	Stallion 5	Stallion 6
Sperm viability (%)	93.5± 1.85	96.5 ±1.85	99 ± 1.85	97 ± 1.85	99.75 ± 2.77	98.75 ± 2.77
SCSA						
$\overline{X}$ -DFI	128 ± 9.63	121 ±9.63	134 ± 9.63	135.5 ± 9.63	141 ± 14.45	143 ± 14.45
%-DFI	16.5 ± 0.86a	17 ± 0.86b	29.5 ± 0.86c	32 ± 0.86c	4.25 ± 1.29d	8.25 ± 1.29e
SD-DFI	64 ± 2.59a	49.5 ± 2.59b	76.5 ± 2.59c	54 ± 2.59ab	83.25 ± 3.89ce	79.25 ± 3.89cf
Mitochondrial potential m high (%)	63	52	58	54	70	74
Motility						
Total motility (%)	87.5 ± 8.05a	86.5 ± 8.05a	63 ± 8.05a	65.5 ± 8.05a	88.12 ±12.08a	95.12 ± 12.08a
Progressive motility (%)	30 ± 8.12a	26.5 ± 8.12a	22.5 ± 8.12a	28.5 ± 8.12a	39.37 ±12.19a	18.37 ± 12.19a
VAP (µm/s)	125.5 ± 8.59a	118 ± 8.59a	114.5 ± 8.59a	94.5 ± 8.59a	104.87 ± 12.89a	128.87 ±12.89a
VSL (µm/s)	77 ± 9-6a	72.5 ± 9-6a	69 ± 9.6a	66.5 ± 9.6a	69.5 ± 14.4a	61.5 ± 14.4a
VCL (µm/s)	229 ± 13.67a	227 ± 13.67a	187.5 ±13.67a	189 ±13.67ab	209.87 ± 20.51a	263.87±20.51ac

LS means ± SD. a,b,c,d,e,f, LSmeans in the same row without common subscript differ (P < 0.05).

contrast 70, minimum cell size 4 pixel, path velocity (VAP) cut-off =50 µm/s and straightness (STR) threshold = 75%. The determination was carried out on 3000 spermatozoa (about 400 cells for each field with a total of 7 fields). Sperm kinetic assessment was based on the determination of the percentage of total motile cells, progressively motile cells, average path velocity (VAP-m/s), straight -line velocity (VSL– m/s) and curvilinear velocity (VCL– m/s) of motile cells. For each stallion, two ejaculates were collected with an interval of one month and analyzed.

Statistical analysis

Data were analysed by using the Statistical Analysis System package (SAS, 1998). General linear model (GLM) procedure was undertaken to assess the effect of age, breed and collection date on the semen quality parameters: motility, viability, SCSA para-meters and mitochondrial function assessment. Student's t test was used for comparison between least square means (LSmeans). Partial Correlation Coefficient among the variables were calculated on the residuals of the GLM analysis.

Repeatability of SCSA parameters was computed from the components of variance obtained by MIXED procedure on 6 stallions from which two or more measurements were performed. In the model collection date and breed are considered as fixed effect, the animal as random effect.

RESULTS

Parameters of sperm quality for each examined stallion are shown in Table 1. In Table 2, the correlations between parameters are reported, in Table 3 repeatabilities of SCSA parameters.

Viability assessment

The total mean viability was of 96.9 ± 2.64 (mean ± SD) and ranged from 92 to 99%. No statistical correlation between viability and other sperm parameters was found.

SCSA

The mean values of SCSA parameters are shown in Table 1. The total $\overline{X}$–DFI parameter ranged between 115 to 146 with a mean of 131.8 ± 10.63. The %-DFI parameter ranged between 7 to 35 with a mean of 20.84 ± 9.54. The total SD-DFI parameter ranged between 44 to 86 with a mean of 65.6 ± 14.2. Statistical differences between stallions were observed for %-DFI and SD-DFI parameters. Concerning %-DFI parameter, stallion 5 and 6 showed values significant lower than those of the other stallions. Concerning SD-DFI parameter, stallion 2 had the lowest values. Significant negative correlations was seen between %-DFI and VCL ($r = -0.98$; $p = 0.01$).

Repeatability values of %-DFI was high (0.91) showing high time-consistency of this trait. Repeatabilities for the other SCSA parameters were moderate to low: 0.47 and 0.11 for $\overline{X}$–DFI and SD-DFI respectively.

Mitochondrial function assessment

As observed in Figure 1a, the FL1-FL2 dot-plot, obtained after gating spermatozoa in FSC/SSC dot-plot, shows two cell populations: one with high green fluorescence and low orange fluorescence, (which is considered as the population with m^{low} "inactive mitochondria"), and another one with high green fluorescence and high orange fluorescence, (which is considered as the population with m^{high} "active mitochondria").

After the addition of a m disrupter CCCP, a decrease on m was recorded as only one population with high green fluorescence and low orange florescence

Table 2. Partial correlation coefficients from the residuals of General Linear Model (r -value) between viability, sperm chromatin structure assay (SCSA) parameters, mitochondrial membrane function and motility parameters in fresh equine spermatozoa.

Parameters		Mitochondial potential	SCSA			Motility (CASA)				
		m^{high} (%)	$\overline{X}$ -DFI	DFI %	SD-DFI	Tot (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
Sperm viability (%)		0.4	0.10	0.4	-0.24	0.45	0.66	0.38	0.55	-0.04
		p = 0.7	p = 0.8	p = 0.3	p = 0.8	p = 0.5	p = 0.3	p = 0.6	p = 0.4	p=0.9
Mitochondrial		/	0.8	0.9	0.4	1	0.8	-0.3	-0.8	-0.9
potential m^{high} (%)			p = 0.3	p = 0.08	p = 0.75	p = 0.08	p = 0.3	p = 0.7	p = 0.3	p=0.07
SCSA	$\overline{X}$ -	/	/	0.76	0.93	0.93	0.63	-0.13	0.25	-0.67
	DFI			p = 0.2	p = 0.06	p = 0.06	p = 0.3	p = 0.8	p = 0.7	p=0.3
	%-DFI	/	/	/	0.81	0.65	0.13	-0.71	-0.36	-0.98
					p = 0.18	p = 0.3	p = 0.8	p = 0.2	p = 0.6	p = 0.01
	SD-DFI	/	/	/	/	0.74	0.33	-0.36	-0.03	-0.71
						p = 0.25	p = 0.6	p = 0.6	p = 0.9	p = 0.3
Motility (CASA)	Tot (%)	/	/	/	/	/	0.83	0.07	0.47	-0.57
							p = 0.1	p = 0.9	p = 0.5	p = 0.4
	Prog (%)	/	/	/	/	/	/	0.59	0.87	-0.05
								p = 0.4	p = 0.1	p = 0.9
	VAP (µm/s)	/	/	/	/	/	/	/	0.91	0.77
									p = 0.08	p = 0.2
	VSL (µm/s)	/	/	/	/	/	/	/	/	0.44
										p = 0.5

Table 3. Repeatabilities of sperm chromatin structure assay (SCSA) parameters.

		Repeatability
SCSA	$\overline{X}$ -DFI	0.11
	%-DFI	0.91
	SD-DFI	0.47

was found (Figure 1b). The mean of m^{high}, derived from the analysis of all stallions, was of 61.83 ± 8.81 (range 52 - 74). No correlation between m^{high} and other sperm parameters was found.

Motility determination

Mean values for total motility was 77.9% ± 13.84 (range 54 - 94), 26.8% ± 8.97 (range 15 - 42) for progressive motility, 113.7 m/s ± 14.1 (range 83 - 128) for VAP, 70 m/s ± 9.27 (range 52 - 81) for VSL and 213 m/s ± 28 (range 168 - 264) for VCL (Table 1). As already discussed, a high (r = -0.98) negative correlation between VCL and %-DFI was found (Table 2).

DISCUSSION

Viability is the most common parameter evaluated for sperm quality by FC. The determination of sperm viability by using FC after PI staining produced similar results between stallions. Our sperm viability results, obtained by a single dye (PI), were higher than results reported in literature by dual DNA staining (SYBR-14/PI) (Merkies et al., 2000; Aziz et al., 2005). The PI staining can not omit from flow cytometric analyses (that is, gated out) particles without nuclei into viable cell (PI negative) percentage (Mocé and Graham, 2008), causing an overestimation of sperm viability. However, there was low correlation between viability and other spermatic functions, also considering low variation of sperm viability found among the six examined stallions. The lack of correlation between viability and mitochondrial function is not in agreement with the observations by Papaioannou et al. (1997) and could be probably related to the different probe (Rhodamine 123) used for mitochondria activity assessment.

Concerning the assessment of sperm chromatin quality, although significant differences on SCSA para-meters were found between stallions, the discrimination between fertile and subfertile stallions should be made by the following SCSA parameters described by Love et al. (2002): $\overline{X}$-DFI (199 ± 10 vs 228 ± 13), %-DFI (14 ± 4.4 vs 35 ± 8) and SD-DFI (45 ± 6.2 vs 60 ± 13). The %-DFI parameter is strictly associated with stallion fertility and it is able to discern fertility levels in the horse, as described by Love and Kenney (1998). In fact, ejaculates of stallions 1, 2, 5 and 6 could be considered excellent or

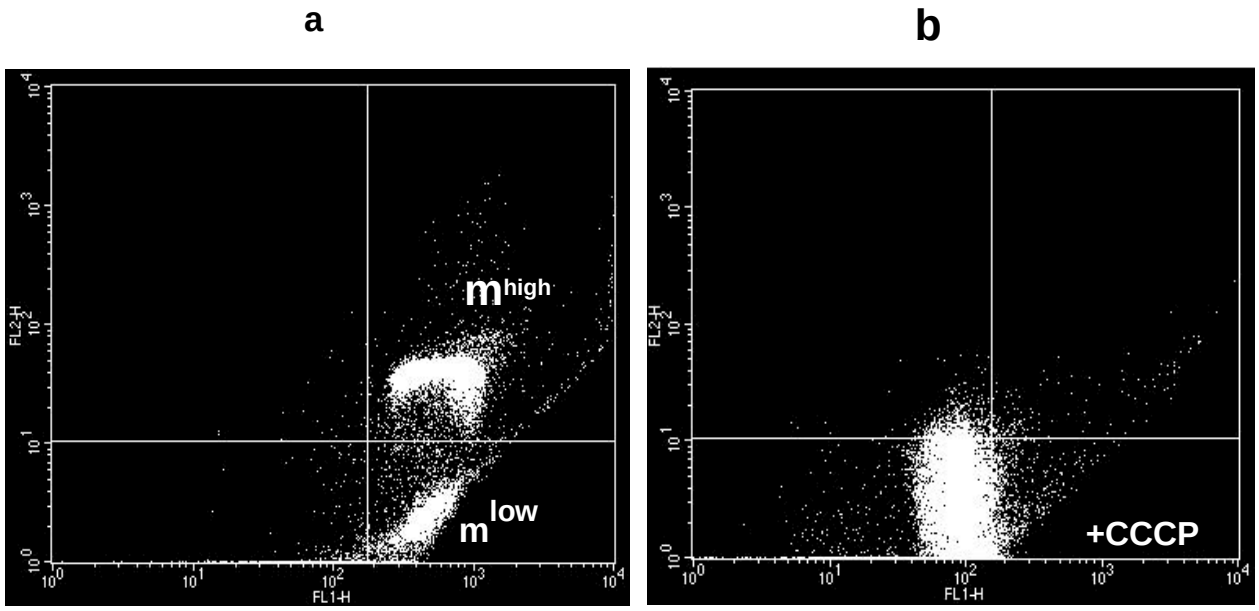

Figure 1. Flow cytometric dot plots of inner mitochondrial membrane potential (m) by using JC-1 probe in equine spermatozoa. a) The sperm population with m^{high} and m^{low} is indicated in the upper and lower right quadrant, respectively. b) A single population with m^{low} after addition of m disrupter CCCP.

good. While $\bar{X}$ –DFI is the least sensitive indicator of fertility because a higher degree of denaturability must occur in the whole spermatozoal population before this variable changes (Love and Kenney, 1998). In our study all ejaculates tested showed values of $\bar{X}$ –DFI parameter included in fertile range defined by Love et al. (2002). As reported by Love and Kenney (1998), the SD-DFI parameter, which reflects the degree of denaturability, is not highly correlated with fertility parameters in the stallions. Our SD-DFI values, which were higher in all stallions, could not be considered as indicator of subfertility.

In our study, the overall fertility rate (defined as per-centage of pregnant mares at 14 - 21 days after AI) for the whole season of stallions used was comprised between 78 and 89%. The integrity of sperm chromatin is important not only for fertilization but also normal embryonic development (D'Occhio et al., 2007). It has been proposed that alterations in chromatin structure may affect the rate of decondensation, a prerequisite for male pronucleus formation during fertilization and there-by, disrupt embryo development. Indeed, in humans, a reduced sperm chromatin stability has been related to recurrent abortion and it is possible that a similar mechanism explains why, while fertilization rates in horses appear to be very high, the early pregnancy loss rate is high (Colenbrander et al., 2003).

The negative correlation found between some SCSA and motility parameters in stallions is in agreement with related results reported by Giwercman et al. (2003) in human field. Moreover, the contemporaneous acquisition of motility and DNA reorganization around protamine molecules in the same organ (epididymis), could explain the negative correlation, found in our study, between SCSA and some motility parameters, as supposed in human field by Giwercman et al. (2003).

Mitochondrial membrane potential is a sensitive indicator of the functional status of mitochondria and it plays an important role because of its relationship with the energetic status and motility of spermatozoa (Martinez-Pastor et al., 2004). The lipophilic fluorescent probe, JC-1, has been validated in the assessment of bull and stallion spermatozoa using FC (Garner et al., 1997; Gravance et al., 2000) and provides a more rigorous estimate of metabolic function than rhodamine 123 or MitoTracker (Gravance et al., 2000). The percentages of spermatozoa with active mitochondria (m^{high}), found in our study, were homogeneous among the six examined stallions and in accordance with results reported by Mari et al. (2008) . The lack of relation between mitochondrial function and kinetic parameters, found in our study, was in agreement with data reported also in other species and could be explained by the involvement of many factors in sperm motility, not only mitochondrial status (Martinez-Pastor et al., 2004; Mari et al., 2008; Volpe et al., 2008).

The CASA system is one of the simplest and most reliable methods for studying sperm motility. The results of this processing are reflected in a series of parameters which precisely define the exact movement of each individual spermatozoon (Quintero Moreno et al., 2003). Conflicting opinions concerning relationship between motility and fertility in stallion were reported by Jasko et al. (1991) and Kuisma et al. (2006) although the spermatozoa motility and the quality of motility are still

the most reliable estimates in practice. The total motility and the VSL parameter found in our six stallions were in agreement with data reported by Jasko et al. (1991), Varner et al. (1991), Aurich et al. (2007) and Mari et al. (2008). Concerning VCL parameter, our samples are in agreement with Love et al. (2005) . The VAP values found in our study were similar to data reported by Mari et al. (2008) although this velocity parameter is not considered a reliable and repeatable parameter to analyze sperm quality, because of its high inter-assay variability (Kirk et al., 2005). The progressive motility values found in our study were similar to results found by Mari et al. (2008).

Other authors report, concerning this parameter, discordant results (Jasko et al., 1991; Varner et al., 1991; Albrizio et al., 2005). These discordant data could be related to different factors, such as stallion, ejaculation frequency, season, extender used for semen dilution, CASA parameters setting (threshold setting, specimen concentration, video digitalisation rate), as reported by several authors (Jasko et al., 1991; Davis and Katz, 1992; Brinsko et al., 2000; Ball et al., 2001; Sieme et al., 2004).

In conclusion, our study reported simultaneous evaluation of different functional sperm parameters by using objective methodologies, useful tools for complete sperm quality assessment in order to improve stallion's fertility. In general, fertility in stallions used in breeding programs is lower and more variable than in other farm animal species, primarily because current selection procedures are based on pedigree, looks and/or athletic performances, with little consideration of fertility or fertility potential (Neild et al., 2005). The modest consideration of stallion fertility could be greatly improved by the introduction of objective methodologies such as FC and CASA system.

As reported by Kirk et al. (2005), the flow cytometric data exhibited less intra-assay variability than did motility assays and the high repeatability of %-DFI parameter found in this study by using FC is in agreement with this statement. The sperm motility and chromatin integrity are significantly associated. Spermatozoa with excellent chromatin structure but without motility could be infertile or motile and normal spermatozoa with damaged DNA and chromatin could have an impaired functions, as reported by Giwercman et al. (2003) on human field. It is suggested that both parameters, obtained by objective methods (such as FC and CASA) can complement each other.

Further studies need to be carried out in order to establish the degree of reliability and variability of sperm functional parameters in relation to some factors, such as breed, seasonal influence, geographic location etc.

REFERENCES

Albrizio M, Guaricci AC, Maritato F, Sciorci RL, Mari G, Calamita G, Lacalandra GM, Aiudi GG, Minoia R, Dell'Aquila ME, Minoia P(2005). Expression and subcellular localization of the -opioid receptor in equine spermatozoa: evidence for its functional role. Reproduction 129:39-49.

Andrabi SMH (2007). Mammalian sperm chromatin structure and assessment of DNA fragmentation. J. Assist. Reprod. Genet. 24: 561-569.

Aurich C, Seeber P, Müller-Schlösser F (2007). Comparison on different extenders with defined protein composition for storage of stallion spermatozoa at 5°C. Reprod. Dom. Anim. 42:445-448.

Aziz DM, Ahlswede L, Enberges H (2005). Application of MTT reduction assay to evaluate equine sperm viability. . 64: 1350-1356.

Ball BA, Medina V, Gravance CG, Baumber J (2001). Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. Theriogenoly. 56: 577-589.

Barrier-Battut I, Dacheux JL, Gatti JL, Rouviere P, Stanciu C, Dacheux F, Vidament M (2005). Seminal plasma proteins and semen characteristics in relation with fertility in the stallion. Anim. Reprod. Sci. 89: 255-258.

Benzoni E, Minervini F, Giannoccaro A, Fornelli F, Vigo D, Visconti A (2008). Influence of in vitro exposure to mycotoxin zearalenone and its derivatives on swine sperm quality. Reprod. Toxicol. 25: 461-467.

Brinsko SP, Rowan KR, Varner DD, Blanchard TL (2000). Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. Theriogenoly. 53:1641-1655.

Colenbrander B, Gadella BM, Stout TAE (2003). The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Domest. Anim. 38: 305-311.

Corzett M, Mazrimas J, Balhorn R (2002). Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol. Reprod. Dev. 61:519-527.

Davis RO, Katz DF (1992). Standardization and comparability of CASA instruments. J. Androl. 13: 81-86.

D'Occhio MJ, Hengstberger KJ, Johnston SD (2007). Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim. Reprod. Sci. 101: 1-17.

Evenson DP, Larson KL, Jost LK (2002). Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J. Androl. 23:25-43.

Evenson DP, Thompson L, Jost L (1994). Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenoly. 41: 637-651.

Garner DL, Pinkel D, Johnson LA, Pace MM (1986). Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod. 34:127-138.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE (1997). Fluorometric assessment of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 57: 1401-1406.

Gillan L, Evans G, Maxwell WMC (2005). Flow cytometric evaluation of sperm parameters in relation to fertility potential period, Theriogenology 63: 445-457.

Giwercman A, Richthoff J, Hjøllund H, Bonde JP, Jepson K, Frohm B, Spanò M (2003). Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil. Steril. 80: 1404-1412.

Graham JK (2001). Assessment of sperm quality: a flow cytometric approach. Anim. Reprod. Sci. 68:239-247.

Gravance CG, Garner DL, Baumber J, Ball BA (2000). Assessment of equine sperm mitochondrial function using JC-1. Theriogenoyly. 53: 1691-1703.

Januskauskas A, Johannisson A, Rodriguez-Martinez H (2003). Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenoly 60: 743-758.

Jasko DJ, Lein DH, Foote RH (1991). The repeatability and effect of season on seminal characteristics and Computer-Aided Sperm Analysis in the stallion. Theriogenoly 35: 317-327.

Kirk ES, Squires EL, Graham JK (2005). Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenoly 64: 1422-1439.

Kuisma P, Andersson M, Koskinen E, Katila T (2006). Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta. Vet. Scand. 48: 14-22.

Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD (2005). Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenoly 63: 1584-91.

Love CC, Kenney RM (1998). The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenoly 50: 955-972.

Love CC (2005). The sperm chromatin structure assays: a review of clinical applications. Anim. Reprod. Sci. 89: 39-45.

Love CC, Thompson JA, Brisko SP, Rigby SL, Blanchard TL, Lowry VK, Varner DD (2003). Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenoly 60: 1127-1138.

Love CC, Thompson JA, Lowry VK, Varner DD (2002). Effect of storage time and temperature on stallion sperm DNA and fertility. Theriogenoly 57: 1135-1142.

Magistrini M, Guitton E, Levern Y, Nicolle JCI, Vidament M, Kerboeuf D, Palmer E (1997). New staining methods for sperm evaluation estimated by microscopy and flow cytometry. Theriogenoly 48: 1229-1235.

Mari G, Rizzato G, Merlo B, Iacono E, Seren E, Tamanini C, Galeati G, Spinaci M (2008). Quality and fertilizing ability *in vivo* of sex-sorted stallion spermatozoa. Reprod. Dom. Anim. DOI: 10.1111/j.1439-0531.2008.01314.x.

Martinez-Pastor F, Johannisson A, Gil J, Kaabi M, Anel L, Paz P, Rodriguez-Martinez H (2004). Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate a frozen-thawed ram semen. Anim. Reprod. Sci. 84:121-133.

Merkies K, Chenier T, Plante C, Buhr MM (2000). Assessment of stallion spermatozoa viability by flow cytometry and light microscopic analysis. Theriogenoly. 54:1215-1224.

Mocé E, Graham JK (2008). *In vitro* evaluation of sperm quality. Anim. Reprod. Sci. 105:104-118.

Neild DN, Gadella BM, Aguero A, Stout TAE, Colenbrander B (2005). Capacitation, acrosome function and chromatin structure in stallion sperm. Anim. Reprod. Sci. 89:47-56.

Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, Roche JF (1997). Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenoly 48: 299-312.

Quintero-Moreno A, Miró J, Teresa Rigau A, Rodríguez-Gil JE (2003). Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenoly 59:1973-1990.

Reers M, Smith TW, Chen LB (1991). J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochem. 7: 4480-4486.

Rodriguez-Martines H (2003). Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Domest. Anim. 38: 312-318.

Sieme H, Katila T, Klug E (2004). Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenoly 61: 769-784.

Sutovsky P, Turner RM, Hameed S, Sutovsky M (2003). Differential ubiquitination of stallion sperm proteins: possible implications for infertility and reproductive seasonality. Biol. Reprod. 68: 688-698.

Varner DD, Vaughan SD, Johnson L (1991). Use of a computerized system for evaluation of equine spermatozoal motility. Am. J. Vet. Res. 52: 224-230.

Volpe S, Leoci R, Aiudi G, Lacalandra MG (2008). Relationship between motility and mitochondrial functional status in canine spermatozoa. Proceedings of 6th ISCFR (Eds England G, Concannon P, Schäfer-Somi G,) Vienna (Austria), pp. 287-288.