CSIRO PUBLISHING

Reproduction, Fertility and Development, 2018, **30**, 919–923
https://doi.org/10.1071/RD17441

Determining the relationship between bull sperm kinematic subpopulations and fluorescence groups using an integrated sperm quality analysis technique

J. L. Yániz[A,C], *I. Palacín*[A], *K. S. Caycho*[A], *C. Soler*[B], *M. A. Silvestre*[B] *and P. Santolaria*[A]

[A]TECNOGAM Research Group, Environmental Sciences Institute (IUCA), Department of Animal Production and Food Sciences, University of Zaragoza, 22071, Huesca, Spain.
[B]Department of Cellular Biology, Functional Biology and Physical Anthropology, University of Valencia, 46100, Burjassot, Valencia, Spain.
[C]Corresponding author. Email: jyaniz@unizar.es

Abstract. The aim of the present study was to determine whether there is an association between the kinematic sperm subpopulations and fluorescent groups in bulls using a new fluorescent staining method that allows classification of spermatozoa into groups depending on their acrosomal and membrane integrity, as well as functional status, without inhibiting sperm motility. Cryopreserved semen samples from 10 Holstein bulls were used in the study. A multiparametric analysis of results obtained by the ISAS 3Fun kit (Proiser) was performed. The different fluorescent groups were detected and their motility characteristics evaluated using ISAS software. Clustering procedures using the kinematic data resulted in the classification of spermatozoa into three kinematic sperm subpopulations. The distribution of kinematic sperm subpopulations was different between the fluorescent sperm groups ($P < 0.001$), although the correlation between them was low ($r = 0.113$; $P < 0.01$).

Additional keywords: *Bos taurus*, CASA-Mot, fluorescence labelling, sperm quality.

Received 22 October 2017, accepted 8 February 2018, published online 16 April 2018

Introduction

The spermatozoa of mammalian ejaculates are heterogeneous and can be classified into different subpopulations according to their motility, morphometry and functional characteristics. The distribution of these subpopulations varies greatly between males of a given species in bulls (Dorado *et al.* 2010) and rams (Yániz *et al.* 2015*b*), and even in the different splits of the same ejaculate (Santolaria *et al.* 2016). However, in certain studies the sperm subpopulation structure showed little variation (Muiño *et al.* 2009; Dorado *et al.* 2010), although cooling and cryopreservation processes may induce substantial changes in the distribution of sperm subpopulations, evidencing differences between males (Muiño *et al.* 2009). Some have suggested an association between the distribution of sperm subpopulations and male fertility (Ramón *et al.* 2013; Yániz *et al.* 2015*a*) or the ability to survive freeze–thawing procedures (Núñez-Martínez *et al.* 2006; Ortega-Ferrusola *et al.* 2009), although others have failed to find any association with field fertility after AI (Santolaria *et al.* 2015).

The classification of spermatozoa into different subpopulations is usually performed using cluster statistical procedures on detailed motility or morphometry data provided by computer-aided sperm analysis (CASA) systems. This process of analysis is time consuming, and consequently the determination of sperm subpopulations has been limited to research studies.

Investigation of sperm motility is considered a main element in any analysis of seminal quality (Yániz *et al.* 2018). However, motility is not the only requirement for fertilisation, and the simultaneous investigation of different sperm quality parameters improves the ability of spermiogram to predict male fertility (Sellem *et al.* 2015; Gliozzi *et al.* 2017). There is a need for more integrative methods able to simultaneously evaluate different aspects of sperm integrity and functionality.

Another way of approaching the problem is to assess several variables in a single test. The combination of fluorescent probes has led to multiparametric sperm quality determinations. For example, fluorescent conjugates have been prepared to simultaneously assess plasma and acrosomal membrane integrity (Peña *et al.* 1999; Nagy *et al.* 2003), but not motility. In a recent study, we described a new fluorescent staining technique in bulls that allows classification of the spermatozoa into groups depending on their acrosomal and membrane integrity, as well as functional status, without inhibiting motility (Yániz *et al.* 2017). Using this method, we observed differences in motility patterns between the fluorescence sperm subpopulations. The aim of the present study was to determine whether there is an association between

the kinematic and fluorescent sperm subpopulations in bulls using a new fluorescent staining method. The existence of such an association may simplify the investigation of sperm quality subpopulations.

Materials and methods

Semen collection and processing

Cryopreserved semen samples from 10 commercial Holstein bulls were used in the analyses. Semen was collected using an artificial vagina, and the ejaculates were immediately transported to the laboratory. The percentage of motile spermatozoa was measured using the ISAS-Mot (Proiser) CASA-Mot system, and the criterion for acceptability was 70% motile spermatozoa. The ejaculate was then extended in BullXcell (IMV Technologies) to a final concentration of 23×10^6 spermatozoa per 0.25-mL semen straw (IMV Technologies) and samples were cryopreserved according to the manufacturer's instructions. Briefly, straws were cooled to 4°C over 3 h and then frozen in a programmable freezer (IMV Technologies) to −140°C as follows: -5°C min^{-1} from +4°C to −10°C, -40°C min^{-1} from −10°C to −100°C and thereafter -20°C min^{-1} from −100°C to −140°C. After straws had been frozen, they were submerged and stored in liquid nitrogen at −196°C until use. Before use, straws were thawed for 1 min at 37°C in a water bath and the processed for the study of sperm subpopulations and fluorescent groups.

Multiparametric assessment

Samples were labelled with the ISAS 3Fun kit (Proiser) as described previously (Yániz *et al.* 2017). The labelling mix includes fluorochromes able to simultaneously evaluate plasma membrane and acrosomal integrity, enzyme activity and sperm motility. Briefly, a 40-μL aliquot of the samples was pipetted into 0.6-mL Eppendorf tubes, 4 μL fluorochrome mix provided in the kit was added and the tubes were incubated for 5 min at 37°C. Then, samples were placed on a prewarmed slide, covered and assessed under fluorescence microscopy to evaluate the motility of fluorescent sperm groups.

Digital images were obtained using an epifluorescence microscope (DM4500B; Leica) equipped with warmed stage and a standard Blue/Green/Reed filter set (excitation: 420-430, 495-615, 570-620 nm). Motility was evaluated in time-lapse sequences using a JenOptik ProgRes CF microscope camera and a Jenoptik Progress Capture Pro image acquisition software. The different fluorescent groups were detected and their sperm motility characteristics evaluated using ISAS-Mot Version 1.1 (Proiser).

This method allows for a clear discrimination of sperm fluorescent groups based on membrane and acrosomal integrity, motility and cellular functional state. Three groups of motile spermatozoa were described (Fig. 1): (1) those with an intact membrane and acrosome (MNAN; normal); (2) those with an intact membrane and damaged acrosome (MNAD); and (3) those with increased fluorescence intensity in the head and flagellum (IFI).

Images were taken at 1-s intervals, with a velocity of image capturing of one photograph every 50 ms. Minimum sperm curvilinear velocity (VCL) of 10 μm s^{-1} was used to classify a spermatozoon as motile. The motility variables measured included VCL, straight line velocity (VSL; μm s^{-1}), average path velocity (VAP; μm s^{-1}), sperm linearity (LIN; as a measure of a curvilinear path, calculated as VSL/VCL), straightness (STR; as the linearity of the average path, calculated as VSL/VAP), wobble (WOB; oscillation measure of the actual path about the average path, calculated as VAP/VCL) and the amplitude of lateral sperm head displacement (ALH; μm). Two consecutive drops and at least 100 sperm cells were analysed by CASA-Mot for each sample.

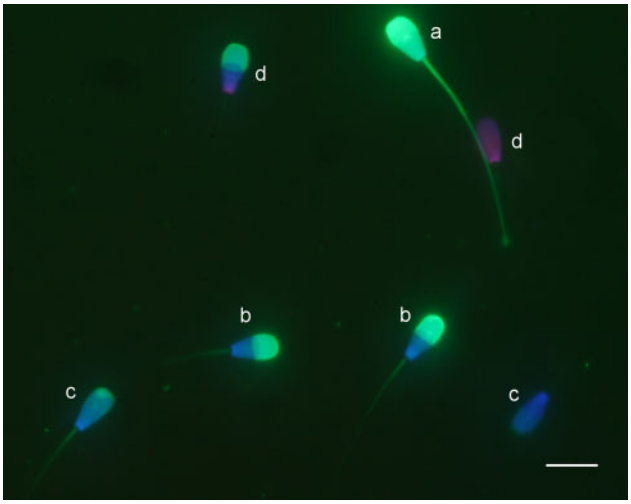

Fig. 1. Multiparametric assessment of sperm quality in bulls showing the presence of different fluorescent sperm groups: a, increased fluorescence intensity; b, intact plasma membrane and acrosome; c, intact plasma membrane and damaged acrosome; d, damaged plasma membrane. Scale bar = 10 μm.

Statistical analyses

Statistical analyses were performed using SPSS version 24.0 (SPSS Inc.). Normality of distribution and homogeneity of variance were checked using the Kolmogorov–Smirnov and Levene tests respectively. Clustering procedures were performed to identify sperm subpopulations from the CASA-Mot data (Santolaria *et al.* 2016). The first step was to perform a principal component analysis (PCA) of the motility data. The purpose of the PCA is to derive a small number of linear combinations (principal components) from a set of variables that retain as much of the information in the original variables as possible. This allows many variables to be summarised in a few, jointly uncorrelated, principal components. A preferred result is when there are few principal components accounting for a large proportion of the total variance. To determine the number of principal components that should be used in the next step of the analysis, the criterion was set to use only those components with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion). The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. This analysis allowed for the identification of sperm subpopulations and the detection of outliers. Chi-squared tests were used to investigate the

Table 1. Differences in sperm motility parameters between the three fluorescent sperm subpopulations detected with the ISAS 3Fun kit (Proiser) that exhibit motility

Data are the mean ± s.d. Within rows, different superscript letters indicate significant differences ($P < 0.001$). MNAN, spermatozoa with an intact membrane and acrosome; MNAD, spermatozoa with an intact membrane and damaged acrosome; IFI, spermatozoa with increased fluorescence intensity in the head and flagellum; VCL, curvilinear velocity, VSL straight line velocity; VAP average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency

Sperm subpopulation	Motility parameters						
	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})	LIN (%)	STR (%)	WOB (%)	ALH (μm)
MNAN	108.09 ± 42.88^{a}	65.24 ± 38.29^{a}	86.77 ± 38.14^{a}	60.10 ± 26.90^{a}	73.05 ± 28.06^{a}	76.67 ± 20.06^{a}	5.50 ± 2.75^{a}
IFI	198.76 ± 89.76^{b}	160.99 ± 80.96^{b}	183.18 ± 89.65^{b}	80.49 ± 14.75^{b}	88.16 ± 10.45^{b}	91.02 ± 12.19^{b}	9.98 ± 4.87^{b}
MNAD	46.90 ± 22.14^{c}	28.74 ± 21.59^{c}	32.90 ± 24.54^{c}	49.34 ± 28.48^{c}	71.14 ± 24.57^{a}	55.82 ± 29.30^{c}	1.99 ± 1.39^{c}

Table 2. Results of the principal component analysis of kinematic parameters, performed on the CASA-Mot data from 10 bulls

PC, principal component; CASA, computer-aided sperm analysis; VCL, curvilinear velocity, VSL straight line velocity; VAP average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency

CASA parameters	PC1	PC2
VCL	0.879	−0.447
VSL	0.981	−0.056
VAP	0.951	−0.280
LIN	0.599	0.788
STR	0.393	0.771
WOB	0.591	0.534
ALH	0.829	−0.483
BCF	−0.162	−0.148

distribution of sperm kinematic subpopulations between the fluorescent groups. Because the data were not normally distributed, non-parametric Spearman's correlation coefficient was used to assess the correlations between sperm kinematic subpopulations and fluorescent groups. In all cases, the level of significance was set at $P \leq 0.05$ (two-tailed).

Results

In the analysis of kinematic variables, spermatozoa in the IFI group showed strong sperm motility compared with MNAN spermatozoa, whereas MNAD spermatozoa were slow and had weak movement (Table 1).

From the two-step cluster analysis, PCA rendered two principal components with Eigenvalues >1 (PC1 and PC2; Table 2), which accounted for more than 78% of the cumulative variance. PC1 was related to rapid movement, whereas PC2 was related to high LIN, STR and WOB, including slow VCL and narrow ALH.

The second clustering analysis, with the two principal components as variables, revealed the presence of three sperm subpopulations (Table 3). Subpopulation 1 ($SP1_{mot}$) had high negative values for PC1, so this cluster includes slow spermatozoa. Subpopulation 2 ($SP2_{mot}$) had high positive values for PC1, so this cluster includes fast spermatozoa. Subpopulation 3 ($SP3_{mot}$) had positive values for PC2 and slightly negative values for PC1, so this cluster included spermatozoa with intermediate speed and straight trajectories (high LIN, STR). Of all spermatozoa, 21.3%, 18.5% and 60.2% were included in $SP1_{mot}$, $SP2_{mot}$ and $SP3_{mot}$ respectively.

Table 3. Results of the two-step cluster procedure in bulls with the kinematic indices (principal component (PC) 1, PC2) as variables

Data are given as the mean ± s.d. SP_{mot}, motility subpopulation

Cluster	PC1	PC2
$SP1_{mot}$	−0.923 ± 0.536	1.363 ± 0.818
$SP2_{mot}$	1.680 ± 0.778	−0.458 ± 0.622
$SP3_{mot}$	−0.191 ± 0.410	0.623 ± 0.444

Table 4. Percentage distribution of kinematic sperm subpopulations in the different fluorescent subpopulations determined using the ISAS 3Fun kit (Proiser)

The distribution of kinematic sperm subpopulations differed significantly among the fluorescence sperm groups ($P < 0.001$). MNAN, spermatozoa with an intact membrane and acrosome; MNAD, spermatozoa with an intact membrane and damaged acrosome; IFI, spermatozoa with increased fluorescence intensity in the head and flagellum; SP_{mot}, motility subpopulation

Sperm subpopulation	Sperm subpopulation		
	$SP1_{mot}$	$SP2_{mot}$	$SP3_{mot}$
MNAN	26.34	6.10	67.56
IFI	1.45	60.14	38.41
MNAD	40.00	0.00	60.00

The distribution of kinematic sperm subpopulations was different between fluorescent sperm groups (Table 4). The greatest percentage (67.7%) of MNAN spermatozoa were classified in $SP3_{mot}$, whereas IFI spermatozoa were predominantly (60.1%) classified in $SP2_{mot}$. MNAD spermatozoa had a higher percentage of spermatozoa classified in $SP1_{mot}$ (40%) than the other two fluorescence groups, although 60% of MNAD spermatozoa were grouped in $SP3_{mot}$. The correlation between kinematic subpopulations and fluorescent groups was low ($r = 0.113$; $P < 0.01$).

Discussion

Fertility is a complex issue that depends upon numerous factors associated with both males and females. Sperm quality analysis may help reduce the effects of males on reproductive success after AI. However, traditional sperm quality tests are only able to measure one or just some independent parameters (or attributes) involved in the fertilisation process (Mocé and Graham 2008), and numerous studies have demonstrated that the combination of different sperm traits in multiple regression models shows a stronger correlation with fertility than single traits (Zhang *et al.* 1998; Januskauskas *et al.* 2003; Gillan *et al.* 2008; Sellem *et al.* 2015; Gliozzi *et al.* 2017). In any case, the use of complex statistical methods is unpractical, and there is a need to develop more integrative sperm quality analysis combining the determination of complementary sperm traits in a few tests.

With the aim of understanding basic and applied aspects of sperm function, we developed the ISAS 3Fun, which combines different parameters of sperm quality, including sperm motility, plasma and acrosomal membrane integrity and functional status, in a single test (Yániz *et al.* 2017). We observed that ISAS 3Fun-derived fluorescence sperm groups have different motility characteristics, suggesting a logical association with kinematic sperm subpopulations.

Results from the present study demonstrate that the distribution of motile sperm subpopulations is different between fluorescence sperm groups, although the correlation between them is low. This low correlation may be attributed to the fact that the study of sperm subpopulations was performed using the overall sperm population from 10 bulls. We observed substantial between-bull variability in motility characteristics and in the distribution of kinematic sperm subpopulations, in agreement with previous reports in different species (Muiño *et al.* 2009; Dorado *et al.* 2010; Soler *et al.* 2014). As a consequence, the overall study of the entire population of spermatozoa of different males to obtain the motility subpopulations may have contributed to the low correlations (Valverde *et al.* 2016). In this respect, when spermatozoa are classified within males, according to VCL and fluorescent pattern, in 72.2% of cases IFI spermatozoa are faster and in 90.5% of cases MNAD spermatozoa are slower.

The use of cryopreserved bull semen samples in the present study may have also affected the distribution of sperm subpopulations. Using fresh semen samples, Muiño *et al.* (2009) observed a similar distribution of motile sperm subpopulations between the 10 bulls included in the study. However, after the freeze–thawing process the distribution of sperm subpopulations was modified, yielding significant differences between bulls.

The ISAS 3Fun test allows observation of live spermatozoa at different stages, although there is a gradual transition between them (Yániz *et al.* 2017). It is possible that the fluorescent modifications are not completely synchronised with the changes in sperm motility. Furthermore, the establishment of sperm fluorescent groups is based primarily on head fluorescence patterns, whereas sperm kinematic subpopulations depend on flagellar motility characteristics. It is also believed that the head and tail are independent compartments in mammalian spermatozoa, separated by the posterior ring (Eddy and O'Brien 1994). In fact, a genetic defect in bulls has been described that causes the detachment of the flagella from the head in most spermatozoa (Eddy and O'Brien 1994), and the resulting detached flagella are metabolically active, remain motile and are able to penetrate cervical mucus. These aspects may have also played a role in the low correlations obtained between the motility subpopulations and fluorescent sperm groups in the present study.

Conclusion

The sperm fluorescent groups defined using the ISAS 3Fun kit show different patterns of sperm motility, although there is no clear correspondence between these groups and the sperm kinematic subpopulations obtained using cluster procedures.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Spanish Ministerio de Economía, Industria y Competitividad (MINECO; Grants AGL2014-52775-P and AGL2017-85030-R), and the Diputación General de Aragón-Fondo Social Europeo (DGA-FSE; Grant A40).

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