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Toward an integrative and predictive sperm quality analysis in *Bos taurus*

J.L. Yániz[a,*], C. Soler[b], C. Alquézar-Baeta[c], P. Santolaria[a]

[a] TECNOGAM Research Group, Environmental Sciences Institute (IUCA), Department of Animal Production and Food Sciences, University of Zaragoza, Huesca, Spain
[b] Department of Cellular Biology, Functional Biology and Physical Anthropology, University of Valencia, Valencia, Spain
[c] Department of Biochemistry and Molecular and Cellular Biology, Environmental Sciences Institute (IUCA), University of Zaragoza, Zaragoza, Spain

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ABSTRACT

There is a need to develop more integrative sperm quality analysis methods, enabling researchers to evaluate different parameters simultaneously cell by cell. In this work, we present a new multi-parametric fluorescent test able to discriminate different sperm subpopulations based on their labeling pattern and motility characteristics. Cryopreserved semen samples from 20 Holstein bulls were used in the study. Analyses of sperm motility using computer-assisted sperm analysis (CASA-mot), membrane integrity by acridine orange-propidium iodide combination and multi-parametric by the ISAS®3Fun kit, were performed. The new method allows a clear discrimination of sperm subpopulations based on membrane and acrosomal integrity, motility and morphology. It was also possible to observe live spermatozoa showing signs of capacitation such as hyperactivated motility and changes in acrosomal structure. Sperm subpopulation with intact plasma membrane and acrosome showed a higher proportion of motile sperm than those with damaged acrosome or increased fluorescence intensity. Spermatozoa with intact plasmalemma and damaged acrosome were static or exhibit weak movement. Significant correlations among the different sperm quality parameters evaluated were also described. We concluded that the ISAS®3Fun is an integrated method that represents an advance in sperm quality analysis with the potential to improve fertility predictions.

1. Introduction

Sperm evaluation techniques have greatly evolved in recent decades, especially with the incorporation of new methods of fluorescent staining, flow cytometry and computer-assisted sperm analysis (CASA) systems. Fluorescent markers and flow cytometry allow the assessment of numerous structural and functional characteristics of spermatozoa in large populations (Martinez-Pastor et al., 2010 Robles and Martinez-Pastor, 2013). CASA systems allow studying the motility and morphometry of spermatozoa in a much more detailed and objective way (Verstegen et al., 2002; Lu et al., 2014; Yániz et al., 2015b). All this makes it possible to analyze the type and extent of sperm damage more precisely.

In spite of these advances, the predictive capacity of the in vitro analysis on potential fertility of semen is still limited (Rodriguez-Martinez, 2003; Santolaria et al., 2015), although it may be improved using combined statistical analyses of various sperm quality parameters (Sellem et al., 2015; Utt, 2016). In consequence, the complexity of the sperm analysis has progressively been increased

* Corresponding author at: Departamento de Producción Animal y Ciencia de los Alimentos, Escuela Politécnica Superior de Huesca, Universidad de Zaragoza, Ctra. Cuarte S/N 22071 Huesca, Spain.
E-mail address: jyaniz@unizar.es (J.L. Yániz).

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with the hope of improving fertility predictions (Fraser et al., 2001 Yániz et al., 2008a). However, some of these quality parameters are highly correlated, with merely increasing the number of analytical tests does not always improve the predictive ability of the spermiogram (Brito et al., 2003; Utt, 2016).

A limitation of in vitro quality testing is that different parameters are usually assessed separately on the sample, losing the power of variables integration. There is a need to develop more integrative sperm quality analysis methods, enabling us to evaluate different parameters simultaneously cell by cell. With this aim, different fluorescent probes have been combined to obtain multi-parametric determinations of sperm quality. Fluorescent conjugates have been prepared to simultaneously assess sperm membrane and acrosome integrity (propidium iodide/FITIC-PSA or FITC-PNA; Peña et al., 1999; Nagy et al., 2003). Other combinations, such as IP/FITC-PSA/JC-1 (de Andrade et al., 2007) or IP/bis-benzamide/Mitotracker Green-MF/SBTI (Bussalleu et al., 2005), also allow to determine the mitochondrial activity. Despite achieving multi-parameter determinations, these treatments may cause alterations in spermatozoa, such as loss of motility. In this work, we present a new multi-parametric fluorescent test with the potential for improving fertility predictions that is able to discriminate different sperm subpopulations based on their membrane and acrosome integrity, functionality and motility characteristics.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all chemicals products were purchased from Sigma Chemical Co. (Madrid, Spain), and Milli-Q water (Millipore Ibérica S.A., Barcelona, Spain) was used for the solutions preparation.

2.2. Semen samples

Animal Care and Use Committee approval was not obtained for this study because no animals were used. Cryopreserved semen samples from 20 commercial Holstein bulls were used in the analyses. Straws with 0.25 mL of frozen semen were thawed for 1 min at 37 °C in a water bath and processed for sperm quality assessment.

2.3. Sperm quality assessment

2.3.1. Assessment of sperm motility by computer-assisted sperm analysis (CASA-Mot)

Computer-assisted sperm analyzer (ISAS®, Version 1.1, PROISER, Valencia, Spain) was used to assess sperm motility (Palacín et al., 2013). Two consecutive drops and at least 500 sperm cells were analyzed by CASA-Mot (Soler et al., 2016) for each sample.

2.3.2. Evaluation of sperm plasmalemma

Sperm viability (membrane integrity, SV) was determined using acridine orange (AO) and propidium iodide (PI) (Yániz et al., 2013b,c) using the DUO-VITAL kit (Halotech, Madrid, Spain). At least 200 sperm cells were examined per sample.

2.3.3. Multi-parametric assessment with the ISAS® 3Fun kit

Samples were labeled with the ISAS®3Fun kit (Proiser, Paterna, Spain) developed by the TECNOGAM research group. Briefly, 40 µl sample aliquot was pipetted into 0.6 ml Eppendorf centrifuge tubes, 4 µl of the fluorochrome combination provided by the kit was added and samples were incubated for 5 min at 37 °C in a water bath. Sample aliquots were directly placed in a prewarmed slide, covered, and assessed with fluorescence microscopy to evaluate the motility of fluorescent sperm subpopulations.

Digital images of the fluorescence-labelled sperm were obtained using an epifluorescence microscope (DM4500B Leica, Wetzlar, Germany) equipped with warmed stage and a triple-band bandpass filter (B/G/R triple-band filter cube, Leica, Wetzlar, Germany). A JenOptik ProgRes CF CCD (JenOptik AG, Jena, Germany) coupled with Jenoptik Progress Capture Pro image acquisition software was used for the evaluation of motility using time-lapse. Detection of the different fluorescent subpopulations and evaluation of their motility characteristics was performed with the ISAS® software (ISAS®, Version 1.1, PROISER, Valencia, Spain). The motility variables measured included the sperm cell motility percentage (MS, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), sperm linearity (LIN, as a measure of a curvilinear path, VSL/VCL), straightness (STR, as the linearity of the average path, VSL/VAP), wobble (WOB, oscillation measure of the actual path about the average path, VAP/VCL), and amplitude of lateral sperm head displacement (ALH, µm).

For a more detailed study of the morphology of sperm fluorescent subpopulations, digital images were obtained from the samples immobilized with formaldehyde, using the same microscope as described above equipped with a 63X plan apochromatic objective, and photographed with a Canon Eos 400D digital camera (Canon Inc., Tokyo, Japan). The camera was controlled by a computer by using DSLR Remote Pro software (Breeze Systems, Camberley, UK).

2.4. Statistical analyses

Statistical analyses were performed using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). Normality distributions and variance homogeneity of the median value score for each set were checked by the Kolmogorov–Smirnov and Levene tests, respectively. As data of sperm motility in the fluorescent stained samples were non-normally distributed, the Kruskal–Wallis test was