Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar
http://dx.doi.org/10.5424/sjar/2013112-3494

Spanish Journal of Agricultural Research 2013 11(2), 362-365
ISSN: 1695-971-X
eISSN: 2171-9292

Short communication. Evaluation of a commercial kit based on acridine orange/propidium iodide to assess the plasma membrane integrity of ram sperm

J. L. Yániz[1]*, I. Palacín[1], S. Vicente-Fiel[1], J. Gosalvez[2], C. López-Fernández[2] and P. Santolaria[1]

[1] *Institute of Environmental Sciences (IUCA), Departamento de Producción Animal y Ciencia de los Alimentos, Escuela Politécnica Superior de Huesca. Universidad de Zaragoza, Ctra. Cuarte, s/n. 22071 Huesca, Spain*
[2] *Departamento de Biología, Unidad de Genética. Universidad Autónoma de Madrid. Madrid, Spain*

Abstract

This study was designed to develop a semiautomatic computer assisted methodology to evaluate the membrane integrity of ram spermatozoa using a commercial kit based on acridine orange/propidium iodide (AO/PI) labelling and ImageJ software. The study was divided into two experiments. In the first trial, the new computer-assisted method was validated by mixing fresh semen samples with different volumes of freeze killed spermatozoa to determine proportions of damaged spermatozoa in the final samples. The proportion of damaged spermatozoa in each sample determined by the automated procedure where highly correlated ($R^2 = 0.97$, $p < 0.001$) with the predicted theoretical values. In the second trial, the new method was compared with a previously validated method of membrane integrity assessment based on phase-contrast/propidium iodide (PH/PI) methodology. Measurements by AO/PI were, on average, 4.0% larger than measurements by PH/PI (SD = 7.02%) and 1.79% smaller than measurements of sperm motility determined by CASA (SD = 4.83). The AO/PI method was also more repeatable than the PH/PI. The double staining methodology coupled with the routine for image analysis allowing automatic determination of sperm membrane integrity means a reduction in processing time of 75% compared to the previously developed method using a single fluorochrome (3 *vs* 12 min on average if the incubation period was included). This facilitates its use when a large number of samples are analysed. Our results validate the new computer assisted method for assessing sperm membrane integrity in sheep. The new method developed, in addition to being a free tool, allows quick automatic determination of sperm viability, which facilitates its use in routine semen analysis.

Additional key words: fluorescence microscopy; *Ovis aries*; sperm quality; sperm viability.

Methods based on fluorochrome-labelling of sperm to assess membrane integrity by fluorescence microscopy, flow cytometry or fluorometry are progressively replacing those classical methodologies based upon bright microscopy (Garner & Johnson, 1995; Alm *et al*., 2001). This occurs because in addition to the more repeatable results obtained among different experimental trials, they are compatible with automation (Yaniz *et al*., 2012). In a previous study, we described a new methodology to evaluate the membrane integrity of ram spermatozoa based on fluorescence microscopy and image analysis (Yaniz *et al*., 2008). In this case, negative-phase contrast microscopy combined with one emitting fluorochrome (PI) under fluorescence microscopy was used to determine the number of total and membrane-damaged spermatozoa in a given field. The present study constitutes a step further, improving the rationale of the previous methodology and focused on decreasing the sperm incubation time to distinguish live/dead sperm using a dual fluorescent DNA probe (acridine orange AO/ PI) combined with free Image J software to automatically count live/dead ram sperm.

* Corresponding author: jyaniz@unizar.es
Received: 04-09-12. Accepted: 19-03-13

Abbreviations used: AO (acridine orange); CASA (computer-asssisted sperm analysis); d (day/s); PI (propidium iodide); SV (sperm viability); 2SD (2 × standard deviation)

Semen was collected with the aid of an artificial vagina from adult rams belonging to the Rasa Aragonesa breed. The semen of each ejaculate was diluted in INRA-96 diluent (IMV Technologies, L'Aigle, France) to $200 \cdot 10^6$ sperm mL^{-1}, kept in sterile glass tubes and stored in a refrigerator at 15°C. Within the first 8 h of storage, the semen samples were carefully mixed and diluted to $50 \cdot 10^6$ sperm mL^{-1} with a TRIS-based diluent (Yaniz *et al*., 2012), immediately before the assessment of sperm plasma membrane integrity and motility. The minimum criterion for acceptability was 70% sperm motility.

Computer assisted sperm analyser (ISAS®, Version 1.0, PROISER, Valencia, Spain) was used to assess sperm motility (Yániz *et al*., 2011). Briefly, two 5-µL-drops/sample were placed in a pre-warmed slide, covered, and five fields/drop and at least 500 spermatozoa/sample were analysed by computer-assisted sperm analysis (CASA). Sperm viability (SV) was assessed using the DUO-VITAL kit (Halotech, Madrid, Spain), and following manufacturer instructions. Analyses were performed in replicate slides, and 5 fields/slide were assessed. A minimum of 250 and 500 spermatozoa were evaluated per slide and method, respectively, for each sample. This procedure results in selective labelling of dead and live cells visualised in red and green colours, respectively. Non-viable sperm were considered when stained with any red fluorescence (PI +), indicating plasma membrane damage. Digital images of the fluorescence-labelled sperm were obtained using epifluorescence microscope (DM4500B Leica, Germany) under a 10× magnification objective, equipped with the appropriate filter sets and with a JenOptik ProgRes CF CCD (JenOptik AG, Jena, Germany). The total number of sperm and the percentages of membrane-intact and membrane-damaged sperm were determined using Image J processing open software (version 1.42, available online at http://rsbweb.nih.gov/ij/download.html), and two plug-ins created for this purpose (available online at http://dl.dropbox.com/u/59049260/Membrane_A.txt and http://dl.dropbox.com/u/59049260/Membrane_B.txt). At least 300 sperm were examined per sample.

The study was divided into two experiments. In the first trial, fresh semen samples were mixed with different concentrations of dead sperm (treated with three cycles of freezing to –20°C and thawing to room temperature) to determine the damaged spermatozoa proportions in the samples. Ram spermatozoa from 12 different ejaculates (6 rams, 2 ejaculates/ram) were diluted to $800 \cdot 10^6$ sperm mL^{-1} in INRA-96, and mixed with different proportions (0%, 25%, 50%, 75% and 100%) of killed spermatozoa from the same ejaculate. The mix was diluted to $50 \cdot 10^6$ sperm mL^{-1} in INRA-96 and in a TRIS-based medium (Yániz *et al*., 2012) and assessed for sperm viability and motility by CASA. Results were compared to the theoretical values predicted on the basis of the estimations made on fresh and frozen samples. The relation between theoretical and actual values of sperm viability in experiment 1 was evaluated by linear regression test using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). In the new method, one colour image was captured for a given microscopy field and, after processing, the number of membrane-intact and membrane-damaged sperm were automatically recorded (Fig. 1). The ImageJ software allowed automatic selection of all sperm heads for a given image with high precision. The proportions of damaged sperm cells in each sample determined by the automated procedure are highly correlated ($R^2 = 0.97$, $p < 0.001$) with the predicted theoretical values (Fig. 2).

In the second trial, the new method was compared with a previously validated method of membrane integrity assessment (PH/PI, Yániz *et al*., 2008). Ram spermatozoa from 18 different ejaculates (6 rams, 3 ejaculates/ram) were diluted in the INRA-96 extender to a concentration of $800 \cdot 10^6$ sperm mL^{-1} at room temperature, and stored in a refrigerator at 15°C until analysis. Aliquots from each sample were diluted to $50 \cdot 10^6$ sperm mL^{-1} in a TRIS-based medium (Yániz *et al*., 2012) and in INRA-96, to assess plasma membrane integrity, with the two methods, and motility by CASA, as explained above. Repeatability and method-agreement analyses were done with Microsoft Excel 2010 software (Microsoft, Redmond, WA), following the method described by Bland & Altman (1986) and Nagy *et al*. (2003). Briefly, we calculated differences between pairs of repeated measurements (for repeatability) or between the two methods of viability assessment and between viability and motility (for method-agreement). The mean of the differences between the paired measurements on the same samples (d) was calculated. The 95% limits of agreement were calculated as $d \pm 2SD$, where SD is the standard deviation of the differences between paired measurements. The average difference between repeated measurements of the double AO/PI staining

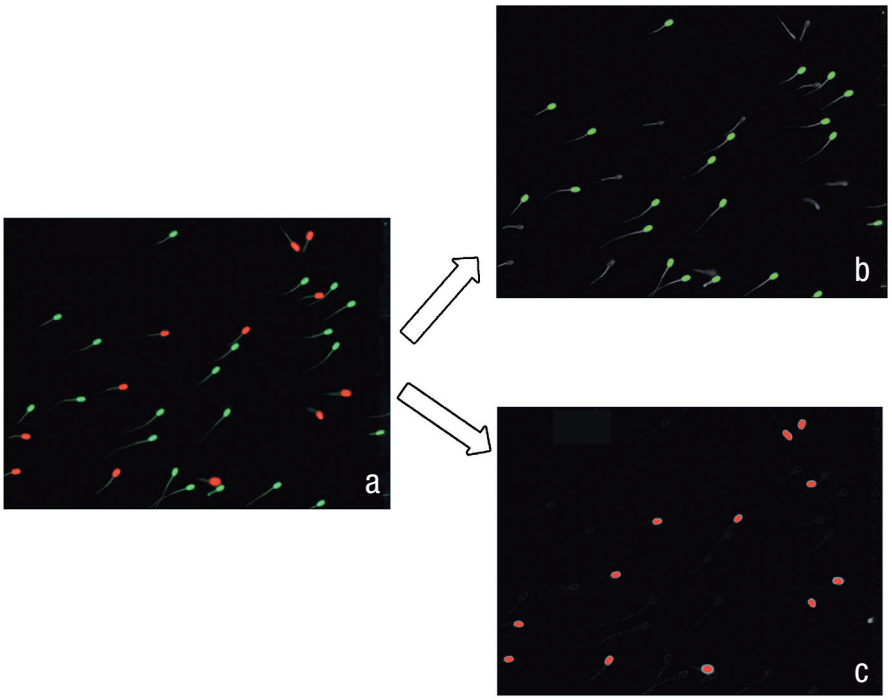

Figure 1. Microscopy field after staining the sample with acridine orange/propidium iodide (a). Live sperm (green) and dead sperm (red) are clearly distinguishable. After colour channel-splitting of the image with the aid of Image J software, live (b) and dead (c) sperm were automatically identified and counted.

was d = 0.44% (SD = 2.5%), with the British Standards Institution repeatability coefficient (2SD) being 5%. This repeatability was better than for the previously developed method (PH/PI, d = 0.93, 2SD = 8.2). As method-agreement analysis showed, measurements by AO/PI were, on average, 4.0% larger than measurements by PH/PI (SD = 7.02%) and 1.79% smaller than measurements of sperm motility determined by CASA (SD = 4.83). The double staining methodology coupled with the routine for image analysis allowing automatic determination of sperm membrane integrity means a reduction in processing time of 75% compared to the previously developed method using a single fluorochrome (3 *vs* 12 min on average if the incubation period was included). This facilitates its use when a large number of samples are analysed.

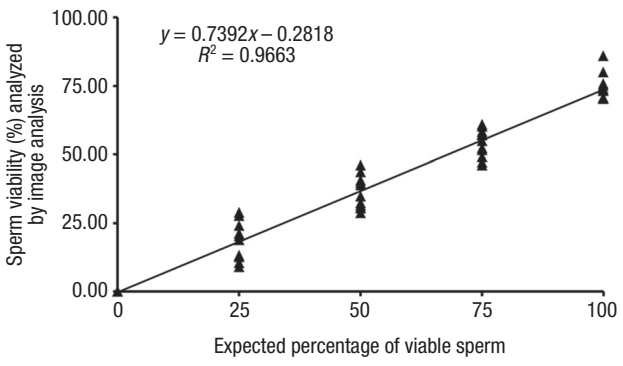

Figure 2. Regression plot for correlations between percentages of sperm analysed by the new computer-assisted method and the expected percentages.

The results of the present study suggest that our new method based on fluorescence microscopy and image analysis may be an efficient and low-cost alternative to the conventional way of assessing sperm membrane status, if a fluorescence microscope is available. The new method developed, in addition to being a free tool, allows automatic determination of sperm viability in a reduced time, which facilitates its use in routine semen analysis.

We had also performed a previous short experiment to compare the evolution of membrane-damaged sperm during incubation, but similar results were obtained at 0 and 10 min of incubation using the kit (data not shown). Likewise, similar results were obtained in the two slides analysed per sample, with the second slide being incubated for a longer time. Perhaps the

penetration of PI on damaged sperm may depend on the concentration of the dye, which seems to be high in the commercial kit we tested.

Incubation of the samples with fluorochromes is usually considered necessary prior to sperm membrane integrity assessment (Harrison & Vickers, 1990; Garner & Johnson, 1995; Pintado *et al*., 2000; Brito *et al*., 2003; Yániz *et al*., 2008). In the present study, incubation was avoided using the DUO-VITAL commercial kit, which combines acridine orange and propidium iodide fluorochromes, since penetration of the AO is immediate, as is the penetration of PI in damaged sperm. In these cells, PI produces a good level of replacement of the AO bound to DNA. The use of acridine orange as permeable fluorochrome causes swift immobilisation of spermatozoa when the microscope light illuminates a specific field. The use of formaldehyde to immobilise sperm is thus not necessary. In addition to cutting the time taken to get the final results, this may also help reduce the possible effects of iatrogenic damage that will increase the proportion of dead cells as incubation time increases, providing data which are closer to those presented by the ejaculate.

Acknowledgements

The authors thank Neil Macowan for assistance with the English translation and the CITA (DGA) for their help with procuring semen samples. This work was supported by the Spanish MINECO (grants IPT-010000-2010-33, BFU201016738/ BFI and AGL2011-30353-C02-01).

References

Alm K, Taponen J, Dahlbom M, Tuunainen E, Koskinen E, Andersson M, 2001. A novel automated fluorometric assay to evaluate sperm viability and fertility in dairy bulls. Theriogenology 56: 677-684.

Bland JM, Altman DG, 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1: 307-310.

Brito LFC, Barth AD, Bilodeau, GoeseelsS, Panich PL, Kastelic JP, 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 60: 1539-1551.

Garner DL, Johnson LA, 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 53: 276-284.

Harrison RAP, Vickers SE, 1990. Use of fluorescent-probes to assess membrane integrity in mammalian spermatozoa. J Reprod Fertil 88: 343-352.

Nagy S, Jansen J, Topper EK, Gadella BM, 2003. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 68: 1828-1835.

Pintado B, De La Fuente J, Roldan ERS, 2000. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin: accuracy in the assessment of cell viability. J Reprod Fertil 118: 145-152.

Yaniz JL, Santolaria P, Marco-Aguado MA, Lopez-Gatius F, 2008. Use of image analysis to assess the plasma membrane integrity of ram spermatozoa in different diluents. Theriogenology 70: 192-198.

Yaniz JL, Mateos JA, Santolaria P, 2011. Zwitterionic buffers preserve ram semen quality more efficiently than Tris during storage at 15 degrees C. Small Rumin Res 95: 54-60.

Yaniz JL, Mateos JA, Santolaria P, 2012. Tris buffer improves fluorescence yield of ram spermatozoa when evaluating membrane integrity. Microscop Res Techn 75: 520-523.