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The Acidic Probe LysoSensor™ is not Useful for Acrosome Evaluation of Cryopreserved Ram Spermatozoa

D Castro-González[1], M Álvarez[1], J Muro[1], MC Esteso[1,2], P de Paz[3], L Anel[1] and F Martínez-Pastor[2,4]

[1]Department of Animal Reproduction and Obstetrics, University of León, León; [2]Biology of Reproduction Group, National Wildlife Research Institute (IREC), UCLM-CSIC-JCCM, Albacete; [3]Department of Molecular Biology, University of León, León; [4]Institute of Regional Development (IDR), Section of Cynegetics and Cattle Resources, University of Castilla-La Mancha, Albacete, Spain

Contents

To try new acrosomal probes for assessing ram spermatozoa, we compared the LysoSensor™ probe, which labels acidic organelles, with the frequently used peanut agglutinin acrosomal probe (PNA-PE; phycoerythrin as fluorescent moiety). The previous microscopic observations showed a lack of relationship of LysoSensor™ with acrosomal status. Semen was obtained from five rams and frozen in four pools. Each pool was analysed carrying out a triple staining propidium ioide/PNA-PE/LysoSensor™ Green DND-189 to test acrosome labelling, and a double staining SYBR-14/PI, to assess sperm viability. Stained samples were analysed by flow cytometry. All measurements were replicated. Data were processed using agreement and repeatability tests. LysoSensor™ labelling did not agree with PNA (mean of differences: 30.8%; coefficient of agreement: 22.6%), confirming microscopic observations. Nevertheless, when LysoSensor™ was compared with SYBR-14/PI, the agreement was high (mean of differences: −0.05%; coefficient of agreement: 5.07%). Repeatability of both methods was high and similar. LysoSensor™ did not seem to specifically stain the acrosome, but it may accumulate in the cytoplasm and label viable spermatozoa. Therefore, LysoSensor™ might not be used as an acrosomal probe in ram spermatozoa, but it could be used in other kind of studies, taking advantage of its pH sensitivity.

Introduction

The identification of the intact acrosome has been the object of lively research and a lot of approaches have been proposed (Rijsselaere et al. 2005). Among many others, fluorescence techniques have played a central role. Lectin-based probes are based in a protein that bind sugar moieties, generally in the acrosomal matrix or external membrane, and have a fluorochrome attached. They have been widely used, but also criticized either for their lack of specificity or for being unable to label a damaged acrosome in some circumstances (D'Cruz and Hass 1992; Parinaud et al. 1993). Other probes use antibodies against antigens on the internal acrosomal membrane (Carver-Ward et al. 1996; Tao et al. 1993), but they are more complicated to obtain than lectins, might depend on species, and sometimes they are not able to label partially damaged or reacted acrosomes. Indeed, these probes must penetrate and bind to their respective targets, but this process can be hindered by many factors (loss of membrane, thick acrosomal matrix, incomplete membrane vesiculation, etc.) (Jaiswal et al. 1999). To compensate for these disadvantages, several studies have tested probes that are based in accumulating in acidic compartments rather than binding to specific targets. The LysoTracker™ probe was employed in bovine and murine spermatozoa (Codelia et al. 2005; Thomas et al. 1997). However, its use never spread, and other studies in human and ruminants found non-specific labelling and other problems (Harper et al. 2006; F. Martinez-Pastor, unpublished data).

LysoSensor™ is a similar probe, designed for labelling acidic organelles. Apparently, it was previously used only once on sperm analysis, for evaluating the acrosomes of *Xenophus laevis* spermatozoa (Ueda et al. 2002). Here, we report our experience with this probe. The experiments performed were done during the evaluation of cryopreserved ram semen (part of another ongoing study). Our initial hypothesis was that the probe would stain intact acrosomes, and reacted or damaged acrosomes would present reduced or absent fluorescence. However, in the preliminary observations with fluorescence microscopy, we observed that the fluorescence seemed not to inform about acrosomal status. Therefore, we combined our analysis with a viability stain, to test if LysoSensor™ labelling was related to the integrity of the plasmalemma. Our objective was to determine if LysoSensor™ labelling agreed either with acrosomal or with live/dead stains and, in any case, to define some parameters (concordance and repeatability) that would help to qualify it for future use.

Materials and Methods

Reagents

Live/Dead Sperm Viability Kit [propidium ioide (PI) and SYBR-14] and LysoSensor™ Green DND-189 were purchased from Invitrogen (Barcelona, Spain). PE-PNA, Peanut agglutinin conjugated with phycoerythrin (Phycoprobe R-PE-PNA) was purchased from Biomeda Corp. (Foster City, CA, USA). CellWash optimized phosphate-buffered solution (PBS) was purchased from Becton Dickinson (Madrid, Spain). Other reagents were purchased from Sigma (Madrid, Spain).

Sperm collection and cryopreservation

Spermatozoa were obtained using an artificial vagina from five adults during four consecutive weeks. Procedures followed the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences. Ejaculates were pooled and diluted 1 : 1 in Tes-Tris-Fructose extender (Anel et al. 2003), complemented

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with either 5–15% of LDL, or 20% egg yolk. LDL were purified in our laboratory from egg yolk (McBee and Cotterill 1971). After the initial dilution of the ram semen at 35°C, the semen was slowly cooled to 5°C over a period of 90 min, subsequently diluted at 100×10^6 spermatozoa/ml and packaged in 0.25-ml French-straws, and frozen in liquid nitrogen (KRYO 10 Series III Planer biofreezer) at −20°C/min down to −100°C. For semen analysis, straws were thawed for 6 s at 65°C in a waterbath.

Flow cytometry analysis

For flow cytometry analysis, samples were diluted in CellWash optimized PBS to 6×10^6 spermatozoa/ml in polystyrene tubes (Becton Dickinson). We carried out two different staining protocols, based on the one proposed by Nagy et al. (2003). The first one was designed to verify if the staining pattern of PE-PNA and LysoSensor™ were coincident. We added PI (24 μM), PE-PNA (2 μg/ml) and LysoSensor™ Green DND-189 (1 μM) to the sample. In another tube, we added PI (24 μM) and SYBR-14 (100 nM) to assess sperm viability. The samples were kept in the dark for 30 min, and then run through a Becton Dickinson FACScalibur flow cytometer. A 488-nm Ar-Ion laser was used for providing the excitation beam. Fluorescence from SYBR-14 and LysoSensor™ was read with the FL1 photodetector (530/28BP filter). Fluorescence from PI was read with the FL3 photodetector (670LP filter), and fluorescence from PE-PNA was read with the FL2 photodetector with the FL2 photodetector (585/42BP filter). The acquisition was controlled using the CELL QUEST PRO 3.1 software, using logarithmic amplification for all the parameters. We acquired 10 000 spermatozoa from each sample, saving the data in FCS v. 2 files. The analysis of the flow cytometry data was carried out using WINMDI v. 2.8 (The Scripps Research Institute, La Jolla, CA, USA). All the analyses were performed twice to calculate repeatability of each parameter.

Samples were also observed at ×400 with an epifluorescence microscope to interpret flow cytometry analyses. We used a Nikon Optiphot microscope (Nikon, Tokyo, Japan), equipped with a 450- to 490-nm excitation filter, a 510-nm dichroic-beam splitter and a 520-nm barrier filter.

Statistical analysis

Statistical analyses were carried out using the *R* statistical environment (R Development Core Team, 2007). To compare results from PE-PNA (acrosome labelling) and SYBR-14/PI (viability) with LysoSensor™, we carried out an agreement analysis following Bland and Altman (1986). This analysis consists in calculating the differences of the measurement methods to compare, obtaining the mean of the differences and the agreement coefficient (twice the standard deviation of the differences). Good agreement is defined by an average difference near zero and a small agreement coefficient. Furthermore, we calculated the repeatability of each parameter in the same manner as the agreement, but in this case we calculated the differences of the replicates, and estimated a repeatability coefficient. In all cases, we used the Wilcoxon signed rank test for checking if the differences differed significantly from 0.

LOW RESOLUTION COLOUR FIG

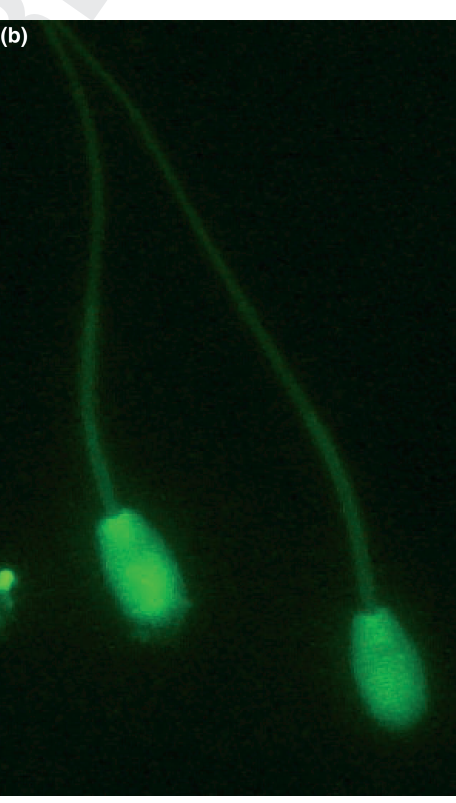

Fig. 1. Photomicrographies (×40) of spermatozoa stained with the LysoSensor™ probe. (a) Left: transmitted light (phase contrast); the spermatozoon on the left has a clearly damaged acrosome. (b) Right: epifluorescence; both spermatozoa have a similar bright and the fluorescence is present not only in the whole head, but also in the midpiece and in the principal piece of the flagellum. The acrosomal region is not brighter than the rest of the head

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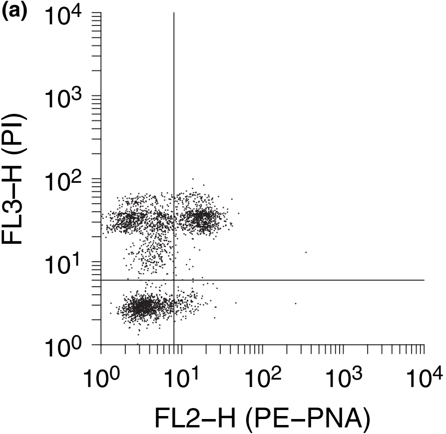
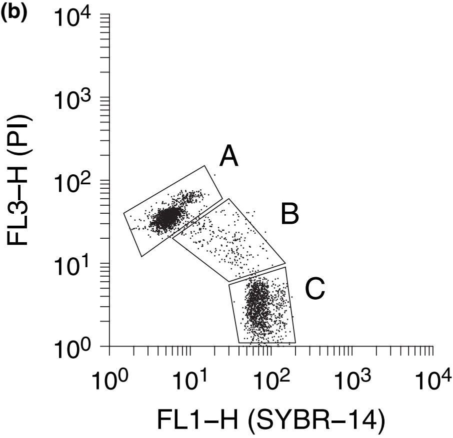
Results

A preliminary microscopic analysis showed that PE-PNA stained the acrosomal cap of acrosome-damaged spermatozoa (confirmed by evaluating the acrosomal ridge by phase contrast). However, LysoSensor™ (LS) labelled the whole spermatozoa, with no signs of compartmentalization of the fluorescence. Moreover, we confirmed that many acrosome-damaged spermatozoa (identified by phase contrast) retained the fluorescence in the whole head, whereas other spermatozoa, apparently with intact acrosomes, lacked LysoSensor™ fluorescence (Fig. 1). Interestingly, all LS–spermatozoa were stained by PI (which labels membrane-damaged spermatozoa). In the flow cytometry analysis, the PE-PNA/PI stain rendered four populations (Fig. 2a): PE-PNA−/PI−, viable spermatozoa with intact acrosomes (30.7 ± 1.1%); PE-PNA+/PI−, viable spermatozoa with damaged acrosomes (3.3 ± 0.4%); PE-PNA−/PI+, non-viable (membrane damaged) spermatozoa with intact acrosomes (33.4 ± 1.9%); and PE-PNA+/PI+: non-viable spermatozoa with damaged acrosomes (32.7 ± 1.6%). The combination LS/PI rendered three populations instead (Fig. 2c): LS−/PI + (69.7 ± 1%), LS+/PI + (8.0 ± 0.6%) and LS+/PI− (29.2 ± 1.0%). There was no agreement between the percentage of spermatozoa with intact acrosomes defined by each method (mean of differences: 30.8%; coefficient of agreement: 22.6%).

Following Garner et al. (1994), the SYBR-14/PI stain rendered three populations (Fig. 2b): PI + spermatozoa, membrane-damaged (59.1 ± 1.2%); SYBR-14 + spermatozoa, membrane-intact (29.1 ± 1.0%); SYBR-14+/PI + spermatozoa, 'moribund' (11.5 ± 1.0%). When comparing LS/PI results with SYBR-14/PI (considering LS+/PI− as viable), both methods had a good agreement. Regarding the percentage of viable spermatozoa (SYBR-14+/PI− and LS+/PI−), the mean of the differences was 0.05% (non-significantly different from 0) and the agreement coefficient was 5.07%. The agreement was poorer when evaluating the double-stained population ('moribund'), although the mean difference was still low (3.53%, $p < 0.001$), and the agreement coefficient was similar (6.02%). The repeatability coefficients of both methods were low (indicating high repeatability): 3.98% for SYBR-14/PI and 2.46% for LS/PI (percentage of viable spermatozoa in both cases).





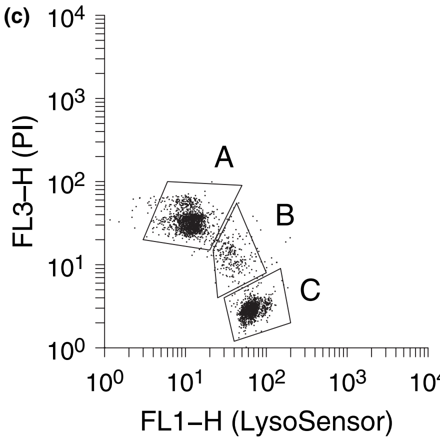


Fig. 2. Flow cytometry dot plots corresponding to the three stain combinations tested. PE-PNA/PI dot plots (a) could be divided into four populations: unstained (lower-left quadrant; live), orange fluorescence (lower-right; live, damaged acrosome), red fluorescence (upper-left; dead) and double fluorescence (upper-right; dead, damaged acrosome). SYBR-14/PI dot plots (b) presented three populations: red fluorescence (A, dead), double fluorescence (B, moribund) and green fluorescence (C, live). LysoSensor™/PI dot plots (c) resembled SYBR-14/PI dot plots, rather than PE-PNA/PI dot plots (the A, B and C populations may be homologous to SYBR-14/PI ones, but it could not be confirmed in this study). Therefore, LysoSensor™ fluorescence does not seem to inform about acrosomal status, like PE-PNA fluorescence does

Discussion

The number of fluorescent probes available for sperm evaluation was increasing considerably during the last 20 years. In many cases, the same fluorescent probe designed for somatic cells has successfully been used in spermatozoa after little or no modifications. Nevertheless, in other cases the results were not those expected. The sperm acrosome is an acidic vesicle originating from the Golgi apparatus (Moreno et al. 2000), and therefore the use of pH-sensitive probes, designed for staining lysosomes and other acidic organelles may be considered. During the acrosome reaction or if the acrosome is damaged, the acrosomal pH would increase (Harper et al. 2006), therefore allowing identifying altered acrosomes unequivocally. However, as we have showed here, it is not clear that these probes can be useful for this purpose. In our experiment, LysoSensor™ stained the

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whole spermatozoon, and the flow cytometry results were not compatible with acrosomal staining. Ueda et al. (2002) reported that *Xenopus laevis* spermatozoa could be stained with LysoSensor™ for discriminating between intact and reacted spermatozoa, thus it is possible that species differences exist. Nevertheless, in that study, LysoSensor™ stained the rest of the spermatozoon too, although the acrosomal cap remained brighter than the cytoplasm. The findings of our study might be related to the problems detected by Harper et al. (2006) using the LysoTracker™ probe in human semen. These authors reported that LysoTracker™ stained the spermatozoa heterogeneously. Thus, while some spermatozoa displayed a stained acrosome, others (apparently with intact acrosomes) were stained in the post-acrosomal region only, or showed fluorescence in the whole head. Furthermore, stimulation with the ionophore A23187 induced complex effects, generally resulting into changes in cytoplasmic fluorescence, but not because of changes on acrosome fluorescence.

Instead of being related to the PE-PNA labelling, we obtained a great coincidence with the results of the widespread used Live/Dead Sperm Viability Kit. The most suitable explanation is that LysoSensor™ accumulates in the whole cytoplasm and that cytoplasm pH is low enough to activate LysoSensor™ fluorescence. Thus, when plasmalemma damage occurs, intracellular pH might increase and the cell would lose the green fluorescence. We cannot confirm that LysoSensor™ is a suitable probe for sperm viability assessment, as our study was limited, and we cannot discard that LysoSensor™ would perform differently if tested in other species. However, more interesting is the possibility that this probe could be used for detecting cytoplasm pH changes after physiological stimuli or in toxicity trials. We also observed that the stain was very specific for spermatozoa (possibly because of its pH sensitivity), thus it could be used for discriminating between spermatozoa and debris, a use that was previously demonstrated for the SYBR-14/PI combination (Nagy et al. 2003). This kind of data could complement other studies on the dynamics of sperm death (Martinez-Pastor et al. 2008), but further work is necessary to assess its suitability.

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Author contributions

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Author's address (for correspondence): Felipe Martínez-Pastor, Grupo de Biología de la Reproducción, IREC, ETSIA, Campus Universitario s/n, 02071 Albacete, Spain. E-mail: felipe.martínez@uclm.es

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