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3 # Use of chromatin stability assay, mitochondrial stain
4 JC-1, and fluorometric assessment of plasma
5 membrane to evaluate frozen-thawed ram semen

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16 **Abstract**

17 Cryopreservation of semen imposes deleterious effects on spermatozoa, either killing a certain
18 proportion of cells or causing subtle damages on sperm function in the surviving population, changes
19 not easily revealed by conventional assays. We have tested three functional assessment techniques
20 in frozen-thawed ram semen from six adult rams, cryopreserved following eight different protocols
21 (four extenders, and glycerol being added at two temperatures). Semen samples were thawed and
22 the following analyses were carried out: motility (CASA), membrane integrity (Hoescht 33258
23 and fluorometry), chromatin status (chromatin stability test and fluorescence-assisted cell sort-
24 ing, FACS) and mitochondrial activity (JC-1 and FACS). Fluorometry outcome did not correlate
25 with the other parameters and showed large variation, albeit discriminating among cryopreserva-
26 tion techniques ($P < 0.01$). Mitochondrial activity correlated, but with low values, with total and
27 progressive motility. However, good sperm motility and high velocity values were associated to
28 high mitochondrial membrane potential. The chromatin stability assay was also successfully car-
29 ried out, and had a good relationship with male factor (%COMP(α_t) and S.D.(α_t) parameters). In
30 conclusion, fluorometric assessment of membrane integrity albeit rendering poor results, merits im-
31 provement, being a low-cost and handy technique, especially for work in the field. On the other hand,
32 both assessments of chromatin stability and mitochondrial status (JC-1 staining), combined with

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FACS, are reliable techniques that can be used for the functional assessment of frozen-thawed ram semen.
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Keywords: Semen; Fluorometer; Plasma membrane integrity; Mitochondria; JC-1; Chromatin stability; Sheep-male reproduction

1. Introduction

Functional sperm analyses have been gaining importance during the last decades, since conventional techniques have not demonstrated to be able to accurately and repeatedly estimate the fertility of a semen sample (Correa et al., 1997). Thus, the development of techniques that pursue to evaluate the functional status of sperm organelles (acrosome, mitochondria) or the integrity of many cellular components (membranes, chromatin) (Graham, 2001), allows a different approach to the problem. In this work we have focused on membrane integrity, chromatin integrity and mitochondrial status.

Membrane integrity is a fundamental requisite for sperm viability and the success of fertilization. There are many techniques based in the use of permeating and non-permeating dyes, so membrane damaged cells can be detected. The combination of fluorescent probes and fluorescence microscopy or flow cytometry has proved to be objective and accurate (Harrison and Vickers, 1990; Garner et al., 1994), but the methods are slow (microscopy) or costly and unpractical in the field (flow cytometry). Instead, the use of an automated fluorometer is a low-cost and rapid approach that can be readily used on a routine basis (Gravance et al., 2000; Alm et al., 2001; Januskauskas et al., 2001).

Mitochondrial status plays an important role because of its relationship with the energetic status of the cell and motility, and has been related to fertility (Casey et al., 1993; Kasai et al., 2002). In this case, the fluorescent dye JC-1 has successfully been used to estimate mitochondrial membrane potential in sperm, both by fluorescence microscopy and flow cytometry (Garner et al., 1997; Papaioannou et al., 1997; Thomas et al., 1998; Troiano et al., 1998; Gravance et al., 2000).

Another important factor for sperm functionality is the integrity of the nuclear chromatin. This can be evaluated using a test where DNA denaturation in situ is performed (Evenson et al., 1980), which is a well-established technique that has proved its utility in many species (Dobrinski et al., 1994; Evenson et al., 1994; Sivashanmugam and Rajalakshmi, 1997; Muratori et al., 2000; Spano et al., 2000; Blottner et al., 2001). Defects in the structure or packaging of the chromatin can severely impair fertilization or embryo development (Spano et al., 2000; De Jonge, 2002). Thus the test has been used for fertility estimation, as well as detection of problems during spermatogenesis (Dobrinski et al., 1994; Evenson et al., 1994; Januskauskas et al., 2001; Blottner et al., 2001).

Information about the use of these techniques in ram semen is scarce. We have analyzed frozen-thawed ram semen samples, that were cryopreserved using eight different protocols. Our objective was to test fluorometry, JC-1, and chromatin stability assay with frozen-thawed ram semen, not only to assess their usability with this species, but also to look for differences between the cryopreservation protocols.

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74 ## 2. Materials and methods

75 ### 2.1. Source of cryopreserved semen

76 The semen assayed in the present study was part of a previous study in our laboratory
77 (Gil et al., 2003). Semen had been collected via artificial vagina from six crossbred sexually
78 mature rams and processed for deep-freezing in 0.25 ml plastic straws building up a series
79 of split-samples frozen in a programmable freezing chamber following eight different pro-
80 tocols (four extenders, 5, 10, 15 or 20% yolk, and glycerol being added at two temperatures,
81 5 or 15 °C) as described by Gil et al. (2003). Semen samples were thawed by immersion in
82 a 37 °C water bath for a minimum of 15 s. Straws were wiped dry and opened cut, and their
83 content collected in plastic tubes for analyses.

84 ### 2.2. Sperm motility assessment by CASA

85 A pre-warmed Makler chamber (10 μm depth; Sefi-Medical Instruments, Haifa, Israel)
86 was loaded with 5 μl of sample and observed under a phase-contrast microscope (400×), on
87 a warming stage (38 °C) (Nikon, Tokyo, Japan). At least six fields (minimum of 200 sper-
88 matozoa) were captured and analyzed by a CASA system (Strömberg-Mika Cell Motion
89 Analyzer; SM-CMA, MTM Medical Technologies, Montreux, Switzerland). Recorded pa-
90 rameters were: total motile spermatozoa (MS, %), progressively motile spermatozoa (PS,
91 %), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average-path
92 velocity (VAP, μm/s), and lateral head displacement (ALH, μm).

93 ### 2.3. Assessment of sperm plasma membrane integrity

94 Integrity of the sperm plasma membrane was assessed using the fluorescent dye bisben-
95 zimide (Hoescht 33258, H258, Riedel-de Haën). This fluorophore stains the DNA of cells
96 with damaged plasma membrane (impermeant dye).
97 A stock solution of H258 was prepared at 1 mg/ml concentration in distilled water and
98 filtered through a 0.22 μm filter. The stock solution was stored at −4 °C protected from
99 light. A working solution (10 μg/ml final concentration of H258) was prepared diluting
100 1 ml of the stock solution in 24 ml of Tris buffer (0.25 M Tris, 88 mM citric acid, 70 mM
101 fructose; Sigma–Aldrich, Tyresö, Sweden). It was stored at −20 °C in 3 ml aliquots.
102 From each frozen-thawed semen sample, 300 μl were taken and divided into two equal
103 splits. One split was kept on a warming plate (37 °C), and the other was quickly frozen in
104 LN_2 and re-warmed to room temperature three times, in order to kill all cells in the sample,
105 to be used as control for background fluorescence.
106 Aliquots of non-killed and killed splits (50 μl each one, all splits analyzed in triplicates)
107 were arranged into a well plate (Costar Black Opaque, No. 13300030, Corning Incorporated
108 Coming, NY, USA). There was a blank well for each sample, with 50 μl of extender.
109 Thereafter, 150 μl of H285 solution were added to each well. The well plate was in-
110 troduced in a plate reader (Bio-Tek Instruments, FL600 fluorescent plate reader, with a
111 360 nm/40 nm bandwidth excitation filter, and a 460 nm/40 nm bandwidth emission filter).
112 The data were collected from the top using static sampling with a 0.35 s delay, 10 reads per

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well and sensitivity settings at 60; instrument temperature was set at 38 °C. Before reading, the plates were pre-incubated for 5 min at 37 °C.

2.4. Assessment of sperm mitochondrial status

The lipophilic cation JC-1 was used to assess the mitochondrial status of spermatozoa (spz). According to the manufacturer (Molecular Probes, Eugene, OR, USA), JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when mitochondrial membrane potential is high. Sperm samples were diluted with Tris buffer down to 30×10^6 spz/ml, and 500 μl were transferred to a polypropylene tube. 0.5 μl of JC-1 stock solution (3 mM JC-1 in DMSO) were added. The tubes were kept in a water bath at 38 °C during 40 min.

Samples were analyzed on a FacsStar Plus flow cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an Ar ion laser (Innova 90, Coherent; Santa Clara, CA, USA), tuned at 488 μm, and running at 200 mW. Calibration was carried out daily using standard beads (Fluoresbrite plain YG 1.0 μM, Polysciences Inc., Warrington, PA, USA). Per sample, 50,000 events with a flow rate of ∼ 1500 cells/s. Percentage of orange stained cells was recorded, being considered as a population of cells with high mitochondrial membrane potential (hMMP).

2.5. Assessment of sperm chromatin stability

Chromatin stability was assessed by metachromatic staining with Acridine Orange (AO), based in the susceptibility of the sperm DNA to acid-induced denaturation in situ. The metacromatic fluorescent dye acridine orange shift from green (ds DNA) to red (ss DNA) fluorescence depending on the degree of DNA denaturation (Darzynkiewicz et al., 1975; Evenson and Jost, 2000). Samples were diluted with TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) into polypropylene tubes at a final sperm concentration of approximately 2×10^6 cells/ml. Aliquots (0.2 ml, two aliquots per sample) were dropped in LN_2 and then allowed to thaw at room temperature. This process was repeated two times. Thereafter, the samples were frozen again and stored in an ultra-cold freezer (−80 °C) until needed. For analysis, samples were thawed on crushed ice. Acid-induced denaturation of DNA in situ was attained adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl; pH 1.4). After 30 s, the cells were stained by adding 1.2 ml of a solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl; pH 6.0) containing 6 μg/ml AO. The stained samples were analyzed just 3 min after AO staining.

Flow cytometry analysis was carried out acquiring 10,000 events with a flow rate of ∼200 cells/s on the same FACS detailed above. Data corresponding to the red and green fluorescence of acquired particles (debris was electronically discarded) were recorded.

2.6. Data processing and statistical analysis

Fluorometry data were obtained as arbitrary fluorescence units. Viability was estimated calculating the ratio between non-treated and frozen aliquots, correcting for the blanks,

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152 using the following formula on the mean of triplicates:

$$\text{viability (\%)} = \left(1 - \frac{\text{sample} - \text{blank}}{\text{frozen sample} - \text{blank}}\right) \times 100 \quad 153$$

154 Results of the DNA denaturation test depend on function α_t, that express the shift from green
155 to red fluorescence and is expressed as the ratio of red cells to total (red + green) cells.
156 High values of α_t indicates high levels of chromatin abnormalities. Flow cytometry data
157 was retrieved with FCS Assistant (Ray Hicks, Cambridge, UK) software, and mean ($x(\alpha_t)$)
158 and standard deviation (S.D.(α_t)) of α_t were calculated for each sample. The percentage
159 of cells with high α_t values, called %COMP(α_t) (cells outside the main population), was
160 obtained directly from the red/green dot plot provided by the acquisition software (Cell
161 Quest Version 3, Becton Dickinson).
162 Statistical analyses were performed with the SAS^{TM}Version 8 package (SAS Institute
163 Inc., Cary, NC, USA). Data was tested for normality (Shapiro–Wilk test), and arc-sine
164 transformation was applied when needed.
165 The CORR procedure was used to obtain Pearson correlation coefficients between sperm
166 parameters. In order to further analyze the relationship between motility parameters and
167 hMMP, which were not quite evident in the correlation analysis, we also carried out an
168 one-way ANOVA with hMMP as factor, after transforming it to a categoric variable (dividing
169 it into "low", "medium" and "high" groups, of equal n). Group comparison was carried out
170 with the Student–Newman–Keuls (SNK) test ($P < 0.05$).
171 The effects of male (M), extender (E) and temperature of addition of glycerol (T) on sperm
172 quality (viability, chromatin and mitochondrial status) were analyzed with ANOVA, using
173 the general linear model (GLM) procedure. The initial model was proposed as follows:

$$Y_{ijkl} = \mu + \mathrm{M}_i + \mathrm{E}_j + \mathrm{T}_k + \mathrm{E}_j \times \mathrm{T}_k + e_{ijkl} \quad 174$$

175 where Y is the studied parameter (viability, hMMP, $x(\alpha_t)$, S.D.(α_t) and %COMP(α_t)). Group
176 comparison was carried out with the Student–Newman–Keuls (SNK) test ($P < 0.05$).

177 3. Results

178 We configured the flow cytometer for both the chromatin stability test and the mitochon-
179 drial status tests, according to the results of previous experiments in other species (Evenson
180 et al., 1994; Thomas et al., 1998; Troiano et al., 1998; Spano et al., 1999). For JC-1 assess-
181 ment, the particles corresponding to sperm cells had first to be identified and selected for
182 further analysis in an FSC/SSC (forward/side scatter of the laser light) plot. JC-1 plots (or-
183 ange/green fluorescence intensity) generally showed two populations, one of them with high
184 green fluorescence and low orange fluorescence (which was considered as the population
185 with low mitochondrial membrane potential—"inactive" mitochondria), and another with
186 high or medium green fluorescence and high orange fluorescence (which was considered
187 as the population with high mitochondrial membrane potential—"active" mitochondria).
188 Fig. 1 shows two JC-1 dot plots with different percentages of "green" and "orange" cells.
189 In general, samples with higher motility yielded larger " orange" populations.

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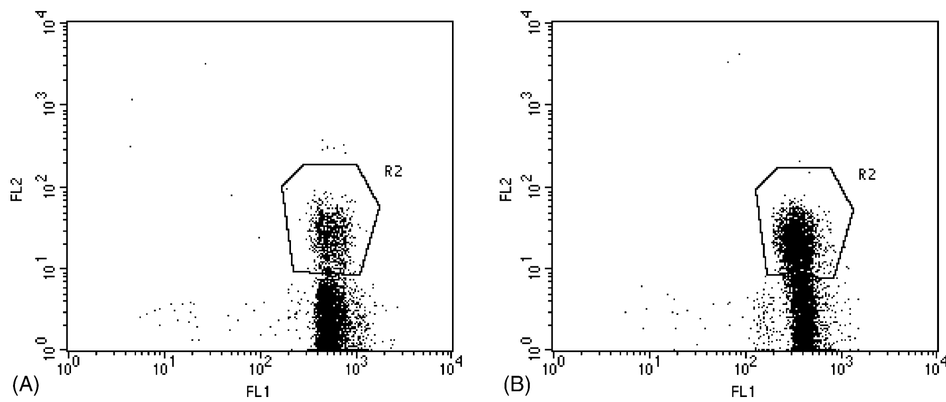

Fig. 1. Example of two flow cytometry dot plots for JC-1 staining. Units indicate fluorescence intensity (FL1 and FL2 stand for green and orange fluorescence, respectively). Region R2 encloses the high mitochondrial membrane potential (hMMP) cell population. Plot A belongs to a sample with hMMP = 16.6% and low motility (MS = 24.7%, PS = 15.3%, VAP = 130.9 μm/s), whereas plot B belongs to a sample with hMMP = 33.6% and better motility (MS = 51.9%, PS = 37.3%, VAP = 141.5 μm/s).

190 In the case of the chromatin stability assay, only a single dot plot was necessary (Fig. 2).
191 Particles were plotted depending on red and green fluorescence intensity. As debris is
192 considered to have very low fluorescence values, the region of the plot corresponding to the
193 lowest fluorescence levels was selected to exclude the particles in that region. Most of the
194 rest of the events (fluorescent sperm cells) grouped in a diagonal line close to the y-axis
195 (medium to high green fluorescence and low red fluorescence). Those particles appearing
196 in the zone on the right of that line (medium to high green fluorescence and medium to

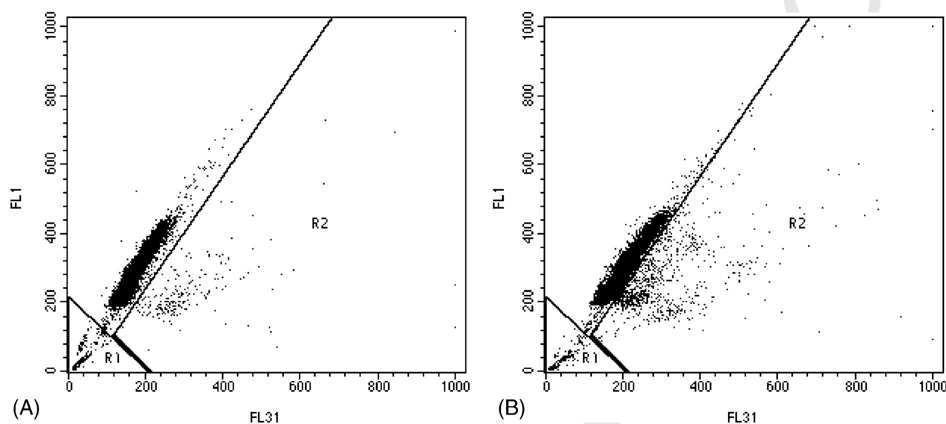

Fig. 2. Flow cytometry dot plots for the chromatin stability assay. Units indicate fluorescence intensity (FL1 and FL31 stand for green and red fluorescence, respectively). Debris (lower left corner, inside R1) were discarded. The elongated cloud on the left is the sperm population with good chromatin integrity. Dots inside R2 are cells out of the main population (COMP), bearing loose chromatin. The sperm sample in plot A has higher quality (regarding chromatin status) than the sample in plot B (0.33%, 0.02% and 2.46% vs. 0.38%, 0.04% and 6.37%, for $x(\alpha_t)$, S.D.(α_t) and %COMP(α_t), respectively).

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Table 1
Summary of collected data for each ram (mean ± S.D.)

Ram	Viability	hMMP	$x(\alpha_t)$	S.D.(α_t)	%COMP(α_t)
1	60.2 ± 4.0	18.3 ± 7.7	0.35 ± 0.01	0.023 ± 0.001	3.0 ± 0.7
2	52.2 ± 3.8	26.0 ± 4.3	0.36 ± 0.01	0.030 ± 0.003	4.1 ± 0.7
3	55.2 ± 3.0	25.3 ± 9.1	0.38 ± 0.02	0.036 ± 0.002	5.9 ± 0.5
4	57.0 ± 6.8	31.4 ± 3.3	0.37 ± 0.02	0.035 ± 0.004	5.7 ± 0.8
5	53.9 ± 9.2	21.4 ± 11.6	0.37 ± 0.01	0.038 ± 0.002	5.8 ± 0.4
6	47.9 ± 11.5	17.4 ± 5.9	0.36 ± 0.02	0.028 ± 0.006	2.9 ± 2.0

197 high red fluorescence) were considered as having loose chromatin (cells outside the main
198 population, %COMP(α_t)).
199 On the other hand, for the membrane status assessment by fluorometry, it was noted that
200 the CV's of the triplicates were quite high in many cases (>5% in about half of the cases, and
201 >10% in some of them, either for non-treated or killed aliquots). The relationship between
202 the CV's of the triplicates and the proportion of yolk of the correspondent extender was
203 checked, but not significance was found.
204 Table 1 shows a summary of the results. In general, we obtained low numbers for most
205 of the parameters. This must be interpreted differently depending on the parameter: low
206 values in chromatin stability parameters indicate good chromatin status (related to good
207 sperm quality), but low values in viability and hMMP are related to bad sperm quality.
208 Table 2 shows Pearson correlations between motility parameters and functional param-
209 eters (viability, hMMP, $x(\alpha_t)$, S.D.(α_t) and %COMP(α_t)). Viability does not significantly
210 correlate with any motility parameter. hMMP correlates with MS and PS, but not with ve-
211 locities, or ALH. Interestingly, chromatin stability parameters (S.D.(α_t) and %COMP(α_t))
212 correlate with many motility parameters. Nevertheless, these correlations were low.
213 Correlations between functional parameters were only significant between chromatin
214 stability parameters ($P < 0.001$). Correlation was specially high between S.D.(α_t) and
215 %COMP(α_t) ($r = 0.92$) (Table 3).
216 Group comparison for motility parameters, using hMMP as factor of variation, showed
217 that the "high hMMP" group had higher values for all motility parameters. There were no
218 significant differences between the "medium" and "low" hMMP groups (Table 4).
219 Results of GLM analysis are showed in Table 5. The significant model for viability
220 included both extender and temperature factors, whether the male factor was excluded from

Table 2
Correlation coefficients between functional parameters and motility

	MS	PS	VSL	VCL	VAP	ALH
Viability	0.29	0.13	−0.08	−0.04	−0.05	0.03
hMMP	0.33*	0.40**	0.19	0.18	0.12	0.11
$x(\alpha_t)$	0.04	0.11	0.23	0.34*	0.33*	0.13
S.D.(α_t)	0.32*	0.28	0.16	0.40**	0.31*	0.47**
%COMP(α_t)	0.31*	0.26	0.15	0.41**	0.28	0.53***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

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Table 3
Correlation coefficients between functional parameters

	hMMP	$x(\alpha_t)$	S.D.(α_t)	%COMP(α_t)
Viability	0.20	−0.24	−0.20	−0.10
hMMP		0.06	0.02	0.08
$x(\alpha_t)$			0.70***	0.60***
S.D.(α_t)				0.92***

*** $P < 0.001$.

Table 4
Comparison between low, medium and high hMMP groups regarding to motility parameters (mean ± SEM)

	hMMP		
Parameter	Low	Medium	High
MS	31.9 ± 2.6 a	44.4 ± 2.9 ab	41.5 ± 1.8 b
MP	19.3 ± 1.9 a	24.9 ± 2.2 a	27.4 ± 1.9 b
VSL	113.1 ± 3.7 a	106.1 ± 5.5 a	123.5 ± 3.6 b
VCL	159.5 ± 4.7 a	154.6 ± 5.1 a	171.3 ± 4.5 b
VAP	127.3 ± 3.9 a	121.2 ± 5.2 a	137.2 ± 3.7 b
ALH	3.4 ± 0.2 a	3.6 ± 0.1 a	3.7 ± 0.1 b

Different letters within rows indicate significant differences.

Table 5
Results of the ANOVA in the analysis of viability, hMMP and chromatin stability models (male, extender, temperature and E × T interaction were included as factors of variation; non-significant factors were excluded in the final models)

Factor	Viability	hMMP	$x(\alpha_t)$	S.D.(α_t)	%COMP(α_t)
Male	N.S.	*	**	***	***
Extender	**	N.S.	N.S.	N.S.	N.S.
Temperature	*	N.S.	N.S.	N.S.	N.S.
E × T	N.S.	N.S.	N.S.	N.S.	N.S.
R^2	0.3894	0.3877	0.3964	0.7820	0.7157

N.S.: non-significant.
* $P < 0.01$.
** $P < 0.05$.
*** $P < 0.001$.

221 the model. On the other hand, only male factor was significant in the models explaining
222 hMMP and chromatin stability. The interaction between extender and temperature was
223 not significant in any case. Group comparisons are showed in Figs. 3 and 4. In the case of
224 viability, the best extender was the one with 5% yolk, and the best temperature of addition of
225 glycerol was 5 °C. Male comparison for hMMP and chromatin stability parameters showed
226 statistical differences between many males. Regarding the chromatin stability parameters,
227 males 3, 4 and 5 seemed to form an homogeneous group (worse chromatin status).

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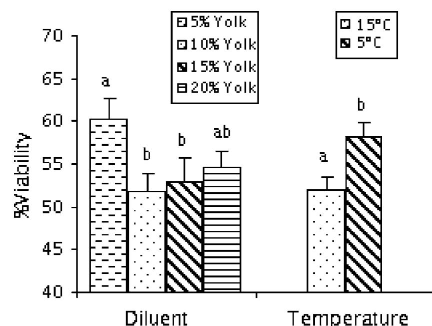

Fig. 3. Group comparison between different extenders and temperatures of addition of glycerol, regarding to viability assessment. Different letters indicate significant differences between groups.

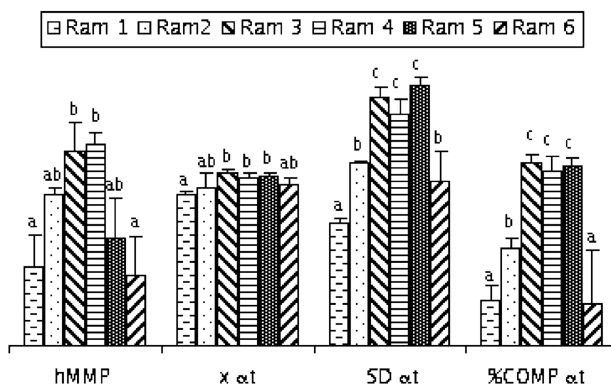

Fig. 4. Comparison between rams when male was a significant factor of variation for the correspondent parameter. Scales are different for each parameter (see Table 1). Different letters indicate significant differences.

228 ## 4. Discussion

229 We have carried out a laboratory trial to assess the usability and effectiveness of some
230 recently developed techniques in frozen-thawed ram sperm analysis. Our results show that
231 these techniques can be applied to frozen-thawed ram semen, but fluorometry needs further
232 improvement.

233 Plasma membrane integrity is undoubtedly a requirement for the success of fertilization.
234 The assessment of this characteristic has undergone a major improvement during the last
235 years, because of the use of new fluorescent dyes and flow cytometry (Graham et al.,
236 1990; Harrison and Vickers, 1990; Garner and Johnson, 1995; Garner et al., 1997; Paulenz
237 et al., 2002). Nevertheless, flow cytometry implies a high cost and it is not suitable for the
238 work in the field. Fluorescent microscopy can be used instead, but is a time-consuming
239 and less repeatable technique. A fluorometer is a relatively cheap device and can be easily
240 carried wherever it is needed. Many experiences have been carried out using an automated
241 fluorometer and fluorescent probes, in order to assess concentration, membrane integrity or

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mitochondrial status (Halangk and Bohnensack, 1982; Juonala et al., 1999; Gravance et al., 2000; Alm et al., 2001; Januskauskas et al., 2001). These experiences have been performed with avian, equine and bovine semen, with good results in general.

In this work, we have applied this technique to frozen-thawed ram semen, with a dubious outcome. Fluorescence reading is not so precise as other techniques, thus the use of triplicates for each sample. However, we have found an excessively high CV% among triplicates, which indicates the presence of an important experimental error. This explains the lack of correlation of the fluorometry with the other assessments, that contradicts the observations of other authors (Juonala et al., 1999; Januskauskas et al., 2001), who, in the other hand, found lower CV%. We did not carried out sperm washing after thawing, and, although we have not found a relationship between the CV% of the triplicates and the proportion of yolk in the extender, a suitable explanation could be that the extender interfered with the technique (either directly or because of its opacity). In an early work (Januskauskas et al., 2001), the rather low correlation between fluorescence output of permeabilized spermatozoa and its actual concentration was found to be due to the presence of the extender.

Regarding the cryopreservation protocols comparison, the extender with 5% yolk stands out as the best to preserve sperm viability, and the addition of the glycerolated extender at 5 °C is better than at 15 °C. A deeper study (Gil et al., 2003), found that there were not only an interaction between both factors, that was not noted in this study, but also that 10% yolk was statistically superior to 5 or 20% yolk. However, adding the glycerolated fraction at 5 °C improved viability over 15 °C, as we have found here. We have to consider that these authors used a double staining with fluorescent probes (SYBR-14/PI) and fluorescent microscopy, which is a more reliable method that the one described here.

Differences between our results and other works imply that the fluorometry method used to assess sperm viability may need extra improvement in order to fully adapt it to ram semen. Minor modifications, as washing the samples to remove extender, could improve the reliability of this technique. We have to take into account that some authors (Juonala et al., 1999; Alm et al., 2001), using fluorometry to assess sperm viability, have found significant correlations between this parameter and fertility, and other (Januskauskas et al., 2001) not only reported correlations, but also included viability as a significant factor in a multivariate model predicting non-return rates.

Mitochondrial membrane potential could be assessed in a quick manner using JC-1 staining and flow cytometry. This fluorescent probe has been used to assess sperm from many species (Garner et al., 1997; Thomas et al., 1998; Troiano et al., 1998; Gravance et al., 2000). Mitochondrial status has been related to motility and cell viability (Garner et al., 1997; Papaioannou et al., 1997; Thomas et al., 1998; Troiano et al., 1998), and even fertility (Kasai et al., 2002). Our results show some relationship between JC-1 staining and motility. Correlations were low, because sperm motility depends on many factors, not only mitochondrial status, and we could not consider different subpopulations regarding motility. However, group comparison of motility parameters, using hMMP as factor of variation, indicates that in fact high values of hMMP are related to high motility values. JC-1 staining performed as expected, considering the descriptions of other authors, and therefore it could be used for frozen-thawed ram sperm evaluation. However, it must be subjected to deeper analysis, in order to establish its actual relationship with sample quality (fertility).

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287 The DNA denaturation test is a reliable method to assess chromatin stability. It has been
288 utilized in many species to detect impaired spermatogenesis and provides good parameters
289 to predict sperm fertility (Dobrinski et al., 1994; Evenson et al., 1994; Angelopoulos et al.,
290 1998; Troiano et al., 1998; Spano et al., 2000; Acebedo, 2001; Januskauskas et al., 2001;
291 De Jonge, 2002). In this work we could reproduce the technique described by these authors,
292 using frozen-thawed ram sperm. The status of sperm chromatin occurs often as an individual
293 trait, very related to male factor rather than to other factors. In our study, male factor
294 explained very well the variation of S.D.(α_t) and %COMP(α_t) parameters (Table 5). These
295 two parameters have been preferably used in other studies. However, some authors have
296 chosen one of them instead of the other, because they often render different results in terms
297 of relationship with sperm quality and fertility (Ballachey et al., 1987; Evenson et al., 1994;
298 Spano et al., 2000; Januskauskas et al., 2001). We have found a good correlation and a
299 similar behavior between both parameters (Table 3, and Fig. 4). In our opinion, future
300 research should consider both of them when including more extensive data and fertility
301 parameters.

302 In our study, we found that S.D.(α_t) and %COMP(α_t) showed positive correlations with
303 many motility parameters, whereas $x(\alpha_t)$ correlated only with VCL and VAP. These results
304 do not match those of other authors, that detected negative correlations between chromatin
305 damage and motility parameters (Angelopoulos et al., 1998; Muratori et al., 2000). Positive
306 correlations may be due to individual differences between males, rather than to an actual
307 relationship between motility and chromatin status.

308 In conclusion, we have found that the assessment of plasma membrane integrity by means
309 of an automated fluorometer is an easy and attractive technique for its use in the field, but
310 the protocol used here needs to be revised for use with frozen-thawed ram semen, possibly
311 adding a previous washing step. On the other hand, JC-1 staining and chromatin stability
312 assay, which have been showed as good indicators of sperm quality in previous works, can
313 be successfully used in this species. However, more extensive studies are needed, especially
314 to determine their relationship with fertility.

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