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Authors: E. Del Olmo, A. Bisbal, A. Maroto-Morales, O García-Alvarez, M. Ramon, P Jimenez-Rabadan, F. Martínez-Pastor, A.J. Soler, J.J. Garde, M.R. Fernandez-Santos

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1 # Fertility of cryopreserved ovine semen is determined by sperm velocity

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3 E. Del Olmo[a], A. Bisbal[a], A. Maroto-Morales[a], O García-Alvarez[a], M. Ramon[b], P

4 Jimenez-Rabadan[b], F. Martínez-Pastor[c], A.J. Soler[a], J.J. Garde[a], M.R. Fernandez-Santos[a*]

5

6 [a]*Biology of Reproduction Group, National Wildlife Research Institute (IREC) UCLM-CSIC-*

7 *JCCM, 02071 Albacete, Spain*

8 [b]*Regional Center of Animal Selection and Reproduction (CERSYRA) JCCM, 13300*

9 *Valdepeñas, Spain*

10 [c]*ITRA-ULE, INDEGSAL, University of León, León, Spain.*

11

12

13 *Corresponding author: María del Rocío Fernandez Santos; ETSIA. Campus

14 Universitario s/n, 02071-Albacete (España);Phone: +34 967 599 200+2581; Fax: +34 967 599

15 238; e-mail: MRocio.Fernandez@uclm.es

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18 **ABSTRACT**

19 The present study aims to examine the predictive value of some sperm parameters on
20 male fertility. Semen samples from six Manchega rams were collected and cryopreserved.
21 Sperm quality was assessed after thawing and after 2 h of incubation, either in the freezing
22 extender (37 °C) or after dilution in Synthetic Oviductal Fluid (SOF) (38 °C, 5% CO_2),
23 attempting to mimic the physiological conditions of the female reproductive tract. The
24 following sperm parameters were evaluated: motility and kinetic parameters by computer-
25 assisted semen analyzer (CASA), and sperm viability (propidium iodide), mitochondrial
26 membrane potential (JC-1), apoptotic-like membrane changes (YO-PRO-1), acrosomal status
27 (PNA-FITC), and intracellular calcium (fluo-3) by flow cytometry. Results showed no
28 significant differences between incubation media neither after thawing nor after incubation.
29 There were no significant correlations between fertility and sperm parameters assessed by
30 flow cytometry. However, after incubation in the freezing extender, sperm samples from
31 males with poor fertility yielded less linearity and velocity ($P < 0.05$) as indicated by motility
32 parameters analysed by CASA. These results indicate that kinematic sperm motility
33 parameters evaluation by CASA might be useful to identify samples with poor fertility.

34

35 *Keywords:* Fertility, Cryopreserved semen, Ram, Flow cytometry, CASA

36 **1. Introduction**

37 The assessment of the fertility potential of a semen sample has been the paramount
38 objective of semen analysis, to predict the outcome of a future artificial insemination. Many
39 studies have aimed at uncovering this relationship between sperm quality parameters and *in*
40 *vivo* fertility, with different outcomes (Papadopoulos et al 2005, Rodríguez-Martínez 2003,
41 Schneider et al. 1999, Zhang et al. 1998). Conventional semen assessment using light
42 microscopy has been increasingly replaced by fluorescent staining techniques, flow cytometry
43 and computer-assisted sperm analysis (CASA) (Hallap et al. 2006). Moreover, an increasing
44 number of techniques for *in vitro* semen evaluation have aimed at evaluating more precisely
45 characteristics of the sperm that are essential for fertility. However, any study has yielded a
46 conclusive link among sperm quality and fertility.

47 Thus, the objective in the present study was to explore laboratory techniques that
48 would allow to quickly and effectively evaluate the potential fertility of a sperm sample. The
49 present study follows the previous study by García-Alvarez et al. (2009a), which showed that
50 heterologous *in vitro* fertilization assays were related to ram sperm fertility. In the present
51 study, it was decided to study the behavior of sperm samples after incubation in the same
52 medium used for *in vitro* fertilization, to determine if after "physiological-like" incubation the
53 relation of sperm quality tests with fertility results would be improved.

54 To perform the present study, we have focused in flow cytometry as a tool with great
55 statistical power because of its ability to analyze thousands of cells in a few seconds,
56 improving existing analyses of fluorescence microscopy and allowing for new multi-
57 parametric analyses (Martínez-Pastor et al. 2010). There have been many attempts to
58 correlate flow cytometry results and fertility (Gillan et al. 2003). For instance, mitochondrial
59 function and membrane integrity with fertility after artificial insemination with ram (Soler et

60 al. 2008) or bull (Gualteri et al. 2005) semen. However, García-Alvarez et al. (2009a) did not
61 find any relation to fertility with the following sperm parameters: viability (PI membrane
62 exclusion), membrane stability (YOPRO1), membrane phospholipid disorder (M540), and
63 mitochondrial membrane potential (Mitotracker Deep Red), all assessed by flow cytometry.
64 This study is a further step, testing additional sperm parameters in a modified experimental
65 setting, to clarify the relation of several sperm variables to fertility.

66 Thus, with the present study it was decided to evaluate different sperm parameters and
67 their relation to *in vivo* fertility, to study whether these assays have the attributes to determine
68 the potential fertility of a sperm sample. Acrosomal status (peanut agglutinin - PNA -
69 conjugated with fluorescein), viability and apoptosis (using the fluorochromes propidium
70 iodide (PI) and YOPRO1, respectively) were evaluated because the activation of apoptotic
71 pathways could be responsible for poor fertility resulting from use of a sperm sample for
72 artificial insemination. Moreover, the presence of active mitochondria is important because
73 they participate in many regulatory and maintenance processes, and could also be linked to
74 sperm death (Aitken et al. 2007), so mitochondrial activity (JC-1) was evaluated.

75 Calcium is an intracellular messenger that has a key role in sperm capacitation.
76 Recently, Marquez and Suarez (2007) established the relationship among capacitation status
77 and intracellular calcium concentration in frozen-thawed sperm. In this regard, intracellular
78 calcium concentrations were analyzed as a factor related to sperm capacitation (fluo-3)
79 (Maxwell and Watson 1996).

80 To fully address this topic, sperm motility was evaluated using a computer-assisted
81 semen analyzer (CASA) which provides precise and accurate information on sperm kinematic
82 parameters (Gravance and Davis 1995), allowing a more accurate prediction of fertility than
83 the parameters assessed by the routine microscopic semen evaluation (Farrel et al. 1998, Malo

84 et al. 2005). The objective assessment of sperm function could increase the chances of
85 predicting the fertilizing capacity of a frozen-thawed semen sample or
86 diagnosing infertility problems.

87

88 **2. Materials and methods**

89 *2.1. Reagents and media*

90 Flow cytometry equipment, software and consumables were purchased from Beckman
91 Coulter (Fullerton, CA, USA). The remaining of the chemicals (Reagent grade or higher) and
92 propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes
93 were purchased from Invitrogen (Barcelona, Spain). Stock solutions of the fluorescence
94 probes were: 7.5 mM PI in water; 50 µmYOPRO1 in DMSO; 100 µg/mL FITC-PNA in
95 water; 0.7 mM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine
96 iodide) in DMSO; 5 µM fluo-3 in DMSO.

97 All fluorescent stocks were kept at -20 °C, in the dark until needed. The freezing
98 extender was prepared using reagent-grade chemicals purchased from Panreac Química S.A.
99 (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA). The fluorochrome
100 acridine orange was of electrophoretic grade and purchased from Polysciences Inc.
101 (Warrington, PA, USA).

102 Syntetic Oviductal Fluid (SOF) was composed of: NaCl 107 mM, KCl 7.17 mM,
103 KH_2PO_4 1.19 mM, $Ca_2Cl•2H_2O$ 1.71 mM, $MgCl_2•6H_2O$ 0.49 mM, $NaHCO_3$ 25.07 mM, Na
104 lactate 3.3 mM, Na pyruvate 0.3 mM and glutamine 200 mM.

105

106 *2.2. Animals and semen collection*

107 All animal procedures were performed in accordance with Spanish Animal Protection
108 Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. Adult
109 males belong to the Regional Center for Animal Selection and Reproduction in Valdepeñas
110 (CERSYRA). Six males of white Manchega sheep breed (age>3 years) were used. Males
111 were selected based on average fertility by artificial insemination. Thus, three males were
112 selected with an average fertility over 50 % and three males with an average fertility under
113 50%. After males were chosen, semen collection, and the intrauterine insemination and the
114 assessment of sperm quality was performed. Semen collection was performed with an
115 artificial vagina. The volume, concentration, mass motility (0: no movement to 5: strong
116 movement) and motility (%) immediately after collection were evaluated. Only the ejaculates
117 with mass movement greater than 4 and individual motility greater than 80% after 10 minutes
118 in a warm bath at 37 °C were used in the present study.

119

120 *2.3 Cryopreservation of semen*

121 After initial semen evaluation, each ejaculate was diluted with the freezing extender.
122 The extender used was prepared as described by Fiser et al. (1987). Fraction 1 was added 3:2
123 to semen and the sample was slowly cooled from 30 °C to 5 °C for 2 h. Then, the samples
124 were further diluted (3:1) with Fraction 2 at 5 °C, reaching a final concentration of $200x10^6$
125 sperm/mL, and held at this temperature for equilibration for 2 h (total refrigeration time at
126 5 °C was 4 h). At the end of the cooling and equilibration period, the extended semen was
127 loaded into 0.25-ml plastic straws and frozen. The straws were frozen in a programmable
128 biofreezer (Planner) at -20 °C/min to -100 °C, and at -10 °C/min from -100 °C to -140 °C and

129 then plunged into liquid nitrogen. Thawing was performed by putting the straws in a water
130 bath with saline at 37 °C for 30 s, and the contents were transferred into a glass tube.
131
132 *2.4. Artificial insemination trials*
133 Thawed sperm samples from the six males were used to inseminate 551 ewes in eight
134 farms. Sperm samples from each male were used to inseminate between 11 and 262 females.
135 The ewes were synchronized using progestagen pessaries (30 mg fluorogestone acetate, FGA;
136 Chronogest, Intervet, The Netherlands) for 13 d followed by 500 IU equine chorionic
137 gonadotrophin (eCG) at pessary removal. Ewes were inseminated intrauterine by laparoscopy
138 at 55 to 58 h after pessary removal. Two technicians performed all intrauterine inseminations
139 in different dates.
140 A male was considered to have contributed to a successful fertilization when the
141 female lambed. Fertility rate for each male was calculated as follows: number of lambed
142 ewes/number of ewes inseminated ×100. This rate was called male fertility.
143
144 *2.5. Assessment of frozen-thawed sperm*
145 Thawed samples were incubated for 2 h (37 ℃) without dilution (i.e., in the freezing
146 extender) or after dilution 1:25 in SOF medium, at 5 % CO_2 (38 ℃). Samples were analyzed
147 after this incubation time by CASA and flow cytometry. Sperm motility (subjective) was
148 assessed for each sample after thawing. Percentage of individual motile sperm (motility) was
149 noted.
150

151 *2.6. Sperm motility assessed by CASA*

152 Semen were diluted down to 10–20×10^6 sperm/mL and loaded into a Makler counting
153 chamber (10 µm depth) at 37 °C. The CASA system consisted of a triocular optical phase
154 contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan), equipped with a warming
155 stage at 37 °C and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg,
156 Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were
157 captured and analyzed using the Sperm Class Analyzer (SCA2002) software (Microptic S.L.;
158 Barcelona, Spain). Sampling was conducted using a ×10 negative phase contrast objective (no
159 intermediate magnification). Image sequences were saved and analyzed afterwards. Software
160 settings were adjusted to ram sperm. The standard parameter settings were as follows: 25
161 frames/s; 20 to 90 µm^2 for head area; VCL > 10 µm/s to classify a spermatozoon as motile.
162 For each sperm, the software rendered the percentage of motile sperm (TM), the percentage of
163 progressive motile sperm (PM) three velocity parameters (VCL: velocity according to the
164 actual path (µm/s); VSL: velocity according to the straight path (µm/s); VAP: velocity
165 according to the smoothed path (µm/s), LIN: linearity (%); ALH: amplitude of the lateral
166 displacement of the sperm head (µm); and BCF: head beat-cross frequency, (Hz). These
167 parameters have been defined elsewhere (Mortimer 1997).

168 *2.7. Flow cytometry analyses*

169 Sperm samples were analyzed using a Cytometer, Cytomics FC500 (Beckman Coulter,
170 Brea, CA, USA). Excitation was provided by a 488 nm Argon-Ion laser. The FSC (forward –
171 scattered light) and SSC (side-scattered light) signals were used to gate out debris (non-sperm
172 events). FL1 photodetector (530/28BP filter) was used for YOPRO1, FITC-PNA, and JC-1;
173 FL2 (575/26BP filter) for JC-1; FL3 (620SP filter) for PI events. The acquisition was

174 controlled using the MXP software. All the parameters were read using logarithmic
175 amplification. About 5000 sperm cells were acquired from each sample.

176 Staining solutions were prepared using SOF-HEPES (10 mL of SOF medium
177 supplemented with 23.5 mg of HEPES). Sperm were diluted in 0.5 mL of the different
178 staining solutions in polypropylene tubes for flow cytometry (final concentration $5x10^6$
179 sperm/mL).

180

181 *2.7.1 . Sperm Viability and apoptosis-like changes*

182 State of plasma membrane (apoptosis-like changes) with YOPRO1 and the viability
183 with propidium iodide (PI) were assessed (García-Alvarez et al. 2009b). A staining solution
184 using SOF-HEPES was prepared by adding 50 nM YOPRO1 , and 15 µM PI. Amounts of 20
185 µL of sample were diluted in 0.5 mL of staining solution in polypropylene tubes for flow
186 cytometry. The tubes were allowed to equilibrate for 15 min in the dark and then analyzed by
187 low cytometry. The PI stains the nucleus of sperm with damaged plasma membranes. YO-
188 PRO1 stains the nucleus when the membrane permeability increases, a phenomenon
189 associated to apoptosis in other cell types (Martínez-Pastor et al. 2009). YOPRO1-/PI- were
190 considered viable sperm (indicating live sperm with intact plasmalemma), whereas YOPRO-
191 1+/PI- were considered as sperm with continuous plasmalemma, but with apoptotic-like
192 disorders.

193 *2.7.2. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)*

194 The lipophilic cationic probe JC-1 was used to assess the mitochondrial status of the
195 sperm. According to the manufacturer (Molecular Probes, Invitrogen Life Sciences, Fullerton,
196 CA, USA.) and as described Robles and Martínez-Pastor (2013), JC-1 changes reversibly its
197 fluorescence from green (monomeric status) to orange (multimeric status) when the

198 mitochondrial membrane potential is great. Sperm samples were diluted with SOF-HEPES to
199 a concentration of 5×10^6 sperm/mL, 300 mL of each sample were transferred to a
200 polypropylene tube, and 1.2 mL of JC-1 stock solution (0.7 mM JC-1 in DMSO) was added.
201 The tubes were incubated at 37 °C for 30 min in the dark. The stained sperm samples were
202 then analyzed by flow cytometry, which identified cells with great mitochondrial membrane
203 potential (hMMP; orange-stained cells).

204

205 *2.7.3. Assessment of acrosomal integrity*

206 Acrosomal status was assessed in a 12 μM PI and FITC-PNA 1 μg/mL staining
207 solution. The PNA (peanut agluttinin) binds specifically to the internal side of the external
208 membrane of the acrosome, labelling acrosome-damaged sperm. The fluorescent technique
209 allows distinguishing among four sperm populations: PI-/PNA- were considered as living
210 cells with intact acrosomes, PI+/PNA- as dead cells with intact acrosomes, PI+/PNA+ as dead
211 cells with damaged acrosomes and PI-/PNA+ as live cells with damaged acrosomes.

212

213 *2.7.4. Detection of intracelular calcium concentration*

214 To assess the amount of intracellular calcium existing in the cytoplasm and reserves
215 held by the sperm, samples were stained in a 5 μM fluo-3 and 12 μM PI in SWB (Sucrose
216 Wash Buffer) as described by Harrison et al. (1993). The fluo-3 has affinity for Ca^{2+} and to a
217 lesser extent by Mg^{2+}; when it binds to these cations, it emits green fluorescence. Mean of
218 green fluorescence of living cells (PI-) was evaluated (Fig. 1). A replicate was performed
219 adding to a second tube 1 μM of calcium ionophore (A23187) and incubating 10 min. The
220 average calcium content of viable sperm in each sample was estimated by the ratio of
221 untreated and ionophore-treated tubes.

222 *2. 8. Statistical analysis*

223 All statistical analysis was performed using SPSS for Windows version 17.0 (SPSS
224 Inc., Chicago, IL, USA). All variables that were not normal were transformed using the arc
225 sine (percentages) or decimal logarithm. Statistical signification was considered when $P <$
226 0.05.

227 A preliminary study was conducted to estimate the effects of some environmental
228 factors on fertility outcomes. These factors were: year and season of insemination, farm in
229 which females were managed, technicians who perform the inseminations and the number of
230 ewes inseminated per male. All factors showed a significant effect with the exception of
231 number of ewes inseminated per male. Therefore, prior to examining the relationships among
232 male fertility and sperm traits, fertility outcome was corrected by all these significant factors
233 as a way to reduce the variability due to other factors than the sperm characteristics. The study
234 of the relationships among male fertility by intrauterine insemination and sperm features were
235 performed by using a uni-variate linear regression.

236

237 **3. Results**

238 *3.1. Semen evaluation*

239 The effects of sperm incubation either in the freezing extender or in the SOF medium
240 are summarized in Table 1 and 2. Table 1 shows several motility variables as yielded by the
241 CASA system. No significant differences ($P > 0.05$) were detected between the two media
242 after thawing and after 2 h of incubation. Motility parameters PM, VAP, VCL, VSL and ALH
243 decreased during incubation, regardless of the medium used for incubation. Total motility,
244 however, decreased ($P < 0.05$) only in those samples diluted with the freezing extender, while
245 no significant differences were observed when a SOF medium (38 °C, 5% CO_2) was used.

246 Table 2 presents the effect of dilution and incubation during 2 h in the freezing
247 extender (37 °C) and in the SOF (38 °C, 5% CO_2) on flow cytometry variables. Results
248 showed an overall decrease in sperm quality as assessed by flow cytometry after incubation,
249 whereas no significant differences were observed between the two media ($P > 0.05$).
250
251 *3.2. Correlations between sperm parameters and in vivo fertility*
252 The sperm samples used in this study were selected based on heterogeneity regarding
253 its *in vivo* fertility. Three males were selected with an average fertility above 50% and three
254 males with an average fertility below 50%. Male fertility by intrauterine artificial
255 insemination ranged from 22% to 83%, with a mean value of 44%. There were differences in
256 fertility among males ($P = 0.003$).
257 The possible relation between kinematic and flow cytometry variables with *in vivo*
258 fertility was studied using a lineal regression analysis. There were relationships between some
259 kinematic parameters and field fertility only after 2 h of incubation in the freezing extender.
260 In this regard, average-path velocity (VAP), the curvilinear velocity (VCL) and the head beat-
261 cross frequency (BCF), showed a high positive correlation with fertility ($P = 0.044$ (R^2=
262 0.678), $P = 0.027$ (R^2= 0.745) and $P = 0.006$ ($R^2 = 0.852$), respectively; Fig. 2; Table.1).
263
264 **4. Discussion**
265 Recently, García-Alvarez et al. (2009a) showed that heterologous *in vitro* fertilization
266 was a good procedure to predict the fertility of ram semen, unlike sperm evaluation by flow
267 cytometry. However, methods based in IVF are costly in time needed to conduct the
268 procedure and from a financial perspective. Therefore, in the present study two objectives
269 were proposed. First, the objective was to study the quality of cryopreserved sperm using an
270 incubation model in freezing extender or IVF media to determine if these stressful conditions

271 could improve the relation between sperm quality variables (as assessed after incubation) with
272 field fertility. Therefore, it could be concluded from these results whether these incubation
273 models were practical, as a standard method for improving sperm quality assessment.
274 Complementary with the first objective, the second objective aimed to identify laboratory
275 techniques that would be most appropriate for, in the experimental approach defined by the
276 first objective, to quickly and effectively evaluate the fertility potential of a sperm sample.

277 Motility has been considered one of the most important characteristics associated with
278 the fertilizing ability of sperm (Saacke and White 1972). In the present study, a decrease in
279 motility variables was detected after the incubation, reflecting the stressful situation that
280 sperm incur in these conditions. Interestingly, a positive relationship was detected among
281 several kinematic variables (VCL, VAP, VSL, BCF) measured after the incubation in the
282 extender and field fertility. To our knowledge, this is the first time a relationship was
283 demonstrated between motility assessed by CASA and fertility of cryopreserved ram sperm.
284 The difference in the present study with previous studies is the inclusion of a post-thawing
285 incubation prior to the assessment. Thus, O'Meara et al. (2008) and García-Alvarez et al.
286 (2010) did not detect a significant relationship between fertility and sperm quality (functional
287 parameters or CASA, respectively) in thawed ram sperm. Results of the present study show
288 the importance of pre-treating sperm before assessing the quality (in this case, submitting
289 them to incubation at 37 °C). Malo et al. (2005) found differences in fertility between red deer
290 stags related strongly to sperm swimming velocity parameters (VCL, VSL and VAP), and
291 results of the present study support the hypothesis that sperm velocity is one of the key
292 features in the process of fertilization, which has been also demonstrated in a large number of
293 taxa (Gage 2004, Holt et al. 1989). Greater motility is a result of a physiologically functional
294 sperm, and sperm with decreasing motility are indicative of decreased sperm metabolism or

295 failing organelles. This might indicate not only a decreased ability to reach the oocyte (of
296 lesser importance in laparoscopic insemination), but reflects a lesser ability to undergo
297 capacitation, the production of an excess of free radicals or to execute key steps in egg
298 fertilization (Aitken et al. 2012, Martínez-Pastor et al. 2009). Results of the present study
299 agree with studies in other species (bull: Amann et al. 2000, Farrel et al. 1998, Kathiravan et
300 al. 2008; goat: Fernandez-Santos et al. 2011), which show that kinematic parameters as VCL,
301 VSL and VAP are related to fertility. Other researchers have found positive correlations
302 between different velocity parameters and fertility in human sperm (Fetterolf and Rogers
303 1990).

304 Flow cytometry has been successful as a tool for the study of several physiological
305 features of sperm (Petrunkina et al. 2007), and many tests have been related with *in vivo*
306 fertility (Gillan 2003, Januskauskas et al. 2000, Wilhelm et al. 1996). However, the study of
307 ram sperm by flow cytometry has not been related to field fertility. For instance, O'Meara et
308 al. (2008) did not found that viability or acrosomal status of ram sperm studied by flow
309 cytometry were related to *in vivo* fertility. In 2007, Rodríguez-Martínez and Barth indicated
310 that both modern sperm evaluation techniques and conventional techniques have been related
311 to fertility, but these relationships are modest and quite variable between laboratories.

312 It must be pointed out that these studies did not make use of a pre-treatment of thawed
313 sperm prior to the study of sperm quality. The incubation of sperm at physiological of above
314 physiological temperatures is a challenge that may help to uncover the authentic fertility
315 potential of the sample. This kind of treatment can be helpful for other kind of experiments.
316 For instance, in a recent study the post-thawing quality of red deer sperm cryopreserved with
317 different antioxidants were not very differentfrom the control, but after incubating the
318 samples at 39 °C many differences were detected (Anel-López et al. 2012). Because in the

319 present study it was found that there was a direct relationship between motion parameters and
320 fertility, and mitochondria have been considered a fundamental organelle to sperm physiology
321 (Mukai and Okuno 2004, Peña et al. 2009), it would be logical to find a relationship
322 between mitochondrial status and fertility. There, however, was not any relation between
323 mitochondrial activity as measured using JC-1, and fertility in the present study. In this
324 regard, Volpe et al. (2009) and Cheuquemán et al. (2011) indicated that JC-1 is suitable for
325 detection of inner mitochondrial membrane potential changes in canine sperm, but it should
326 always be associated with an objective motility analysis to avoid an incorrect evaluation of
327 potential sperm fertility. García-Alvarez et al. (2009a) also showed
328 that mitochondrial membrane potential of ram sperm, analyzed with MitoTracker deep
329 red, was unrelated to fertility. Indeed, it has been considered that the main role of the sperm
330 mitochondria was the production of energy for sperm motility, but this concept is under
331 assessment and the roles of mitochondria will likely be broadened as more research assessing
332 these roles is conducted (Marin et al. 2003, Miki et al. 2004, Mukai and Okuno 2004).
333 Mitochondria also have a crucial role in diverse cellular functions apart from energy
334 production, such as modulation of the redox balance, osmotic regulation and Ca^{++}
335 homeostasis (Peña et al. 2009). Therefore, the assessment of mitochondrial status could
336 eventually offer important information relative to the sperm fertilizing ability, but this might
337 require other experimental approaches.

338 Different researchers have demonstrated that intra-cytoplasmatic calcium efflux is a
339 necessary component for capacitacion (Gualteri et al. 2005). Although in the present study
340 greater calcium concentrations were detected after incubation, there was not any relation
341 between fertility and the relative intra-cellular calcium concentration with any treatment.
342 Thus, the concentration of Ca^{2+} in the sperm after thawing or after incubation did not reflect
343 the fertility of the sperm samples. That does not mean that Ca^{2+} concentrations are irrelevant.

344 Measurement of the Ca^{2+} profile after treating sperm with progesterone or other physiological
345 signals could offer relevant information related to sperm fertility (Arienti et al. 2010). The
346 primary conclusion from the present research is that thawed semen samples with a greater
347 sperm velocity is related to a greater field fertility, but only when measurements were
348 conducted after 2 hours of incubation in the freezing extender. Interestingly, incubation in
349 SOF in conditions seeking to mimic the female oviductal environment did not yield any
350 significant relationship with fertility. To our knowledge, no other researchers have found this
351 relationship between sperm motility parameters and *in vivo* fertility of rams following
352 intrauterine artificial insemination of ewes with frozen-thawed semen. Results cannot be
353 generalized with conclusions of the present study, because of the limited number of males
354 involved. Nevertheless, the experimental approach in the present study evaluating the semen
355 samples after a 2 h incubation at 37 °C in the freezing medium merits further research, and it
356 could be the basis of protocols for predicting the *in vivo* fertility of frozen-thawed ram sperm
357 samples.
358
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366
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513 Table 1

514 Sperm motility parameters assessed by the CASA system Sperm Class Analyzer (SCA®). Values are expressed as Mean ± S.E.M.. Sperm analyses were

515 conducted immediately after thawing or after dilution in Synthetic Oviductal Fluid (SOF), and after 2 h of incubation in the freezing medium (37 °C) or in

516 SOF (38 °C, 5% CO_2).

517

Time (h)	Treatment	TM	PM	VAP	VCL	VSL	LIN	ALH	BCF
0	Freezing Extender	83.8 ± 3.8^a	24.8 ± 4.2^a	70.1 ± 3.9^a	89.5 ± 2.5^a	49.7 ± 6.5^a	47.0 ± 3.9^a	2.9 ± 0.2^a	4.9 ± 0.2^a
	SOF	79.8 ± 4.0^a	21.6 ± 3.9^a	64.8 ± 7.0^a	83.5 ± 6.0^a	43.9 ± 6.7^a	45.4 ± 3.2^a	2.8 ± 0.2^a	4.6 ± 0.3
2	Freezing Extender	43.8 ± 11.9^b	9.2 ± 2.6^b	$37.0 \pm 7.4^{*b}$	$49.9 \pm 7.3^{*b}$	$25.6 \pm 5.7^{*b}$	44.2 ± 3.6^a	2.1 ± 0.1^b	$4.1 \pm 0.6^{**}$
	SOF	63.1 ± 10.0^a	8.8 ± 1.2^b	29.4 ± 2.3^b	43.1 ± 1.9^b	19.3 ± 2.12^b	38.4 ± 1.6^a	2.1 ± 0.1^b	3.3 ± 0.2^b

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Motility parameters: TM: % total motile sperm, PM: % sperm with a progressive movement; VAP: velocity according to the smoothed path (µm/s), VCL: velocity according to the actual path (µm/s); VSL: velocity according to the straight path; LIN: linearity (%); ALH: amplitude of the lateral displacement of the sperm head;µm); the BCF (head beat-cross frequency, Hz).[ab]Different letters indicate differences between incubation times ($P < 0.05$)* correlation with fertility *in vivo* ($P<0.05$). *** correlation with fertility *in vivo* ($P<0.001$).

540 Table 2

541 Results of sperm parameters evaluated by flow cytometry and its relation to *in vivo* fertility assessed by artificial insemination. Values are expressed as

542 (Mean ± S.E.M.) provided by cytometry and their relation to fertility. Sperm analyses were conducted immediately after thawing or dilution in SOF and after

543 2 h of incubation, either in the freezing medium (37 °C) or after dilution in Synthetic Oviductal Fluid (SOF) medium (38 °C, 5% CO_2).

Time (h)	Treatment	Viability (%)	hMMP (%)	Apoptotic-like membrane changes (%)	Acrosomal integrity (%)	Intracellular calcium rate (%) (viable cells)
0	Freezing Extender	26.5 ± 4.7^{a}	29.5±4.3^{a}	3,9±0,4^{a}	19.4±3.0^{a}	46,0±9.0^{a}
	SOF	32.7 ± 3.3^{a}	29.8±5.1^{a}	5,7±0,8^{a}	22.7±2.8^{a}	39,7±9.0^{a}
2	Freezing Extender	15.3±2.4^{a}	14.6±2.7^{b}	5,0±1,1^{a}	9.8±1.8^{a}	55,9±9.0^{b}
	SOF	24.1±3.4^{a}	16.3±3.8^{a}	6,7±1,6^{a}	12.4±3.2^{a}	54,4±9.0^{a}

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Cytometry parameters: Positive sign (+) indicates cell staining, negative sign (-) indicate the lack of staining for each flourochrome. PI: propidium iodide.

Viability: % of YO-PRO-/PI- (membrane intact) sperm; hMMP: % of sperm with high mitochondrial membrane potential (JC-1 +); Apoptotic-like

membrane changes: % of YO-PRO+/PI- sperm; acrosomal integrity: % of PNA+/PI- sperm; Intracellular calcium rate (viable cells): result of dividing the average fluo-3 mean fluorescence by the average fluo-3 mean fluorescence after incubating with 1 µM calcium ionophore, as percentage.[a,b] Different letters indicate differences between incubation times ($P < 0.05$).

552 **Figure Captions**

553 **Fig. 1.** Representative cytogram and histogram obtained by flow cytometry analysis of a ram
554 sample after loading the sperm with Fluo-3, a specific stain for intracytoplasmatic calcium,
555 and counterstained with propidium iodide (PI), a non-permeable membrane stain, for
556 assessing viability. (A): Fluo-3 /PI dot plot, showing a gate to discard PI positive sperm
557 (membrane damaged). (B): Fluo-3 /PI negative (Non-damaged membrane) histogram,
558 showing only fluorescence from viable sperm (gated). The histogram shows fluorescence
559 results from an untreated sample (solid line) and after incubating with 1 μM of calcium
560 ionophore (dashed line). The mean fluorescence was obtained from each histogram, and a rate
561 was obtained by dividing the non-treated mean by the ionophore-incubated mean, resulting in
562 an estimation of the intracellular calcium concentration. Data were obtained from Cytomics
563 FC500 Cytometer (Beckman Coulter, Brea, CA, USA).

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566 **Fig. 2.** Relationship between *in vivo* male fertility and VCL (velocity according to the actual
567 path) and VAP (velocity according to the smoothed path). Regression lines and their
568 equations are showed ($P<0.05$). Data were obtained using a computer-assisted sperm analysis
569 (CASA) system (Sperm Class Analyzer, SCA2002, Microptic S.L.: Barcelona, Spain).

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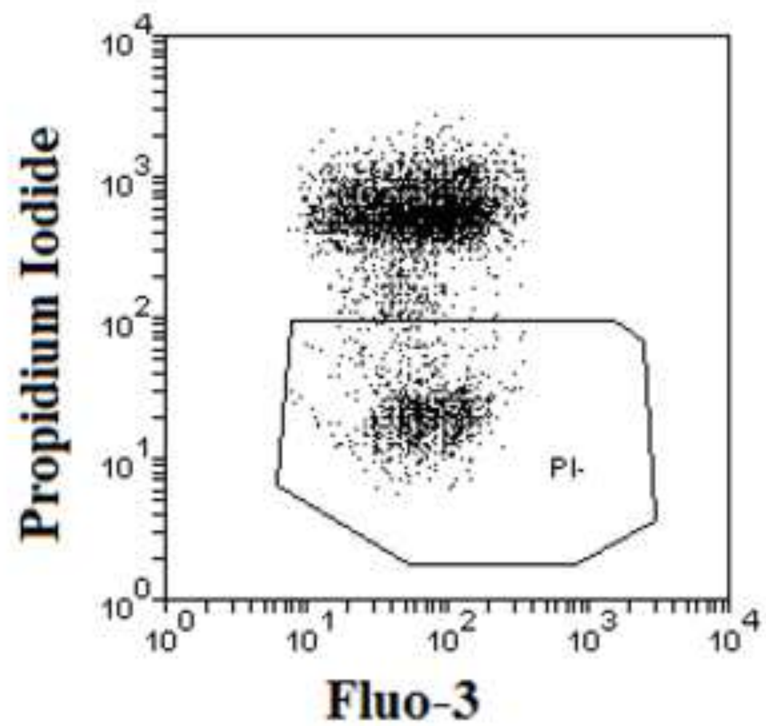
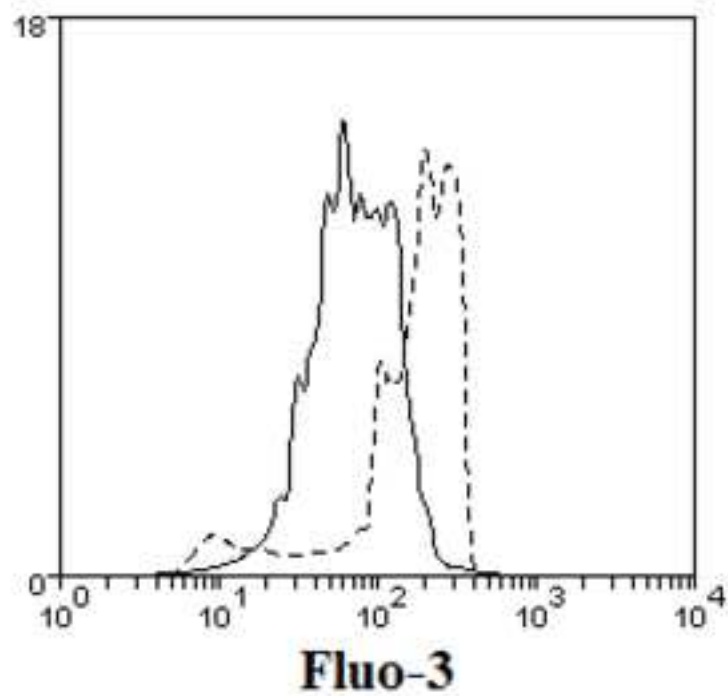
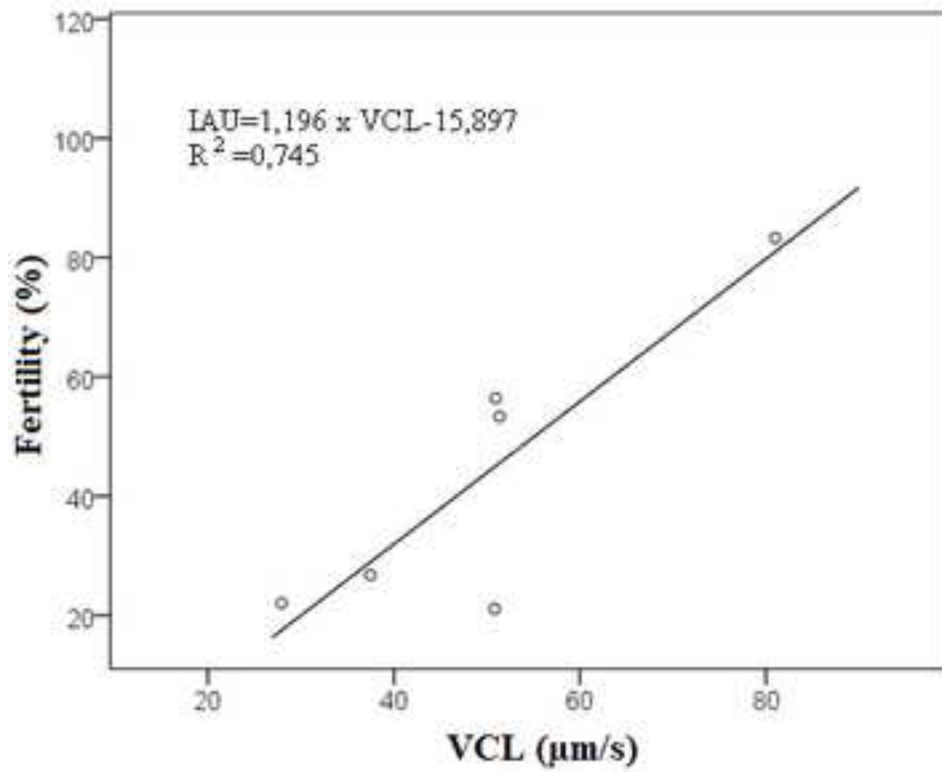
Figure

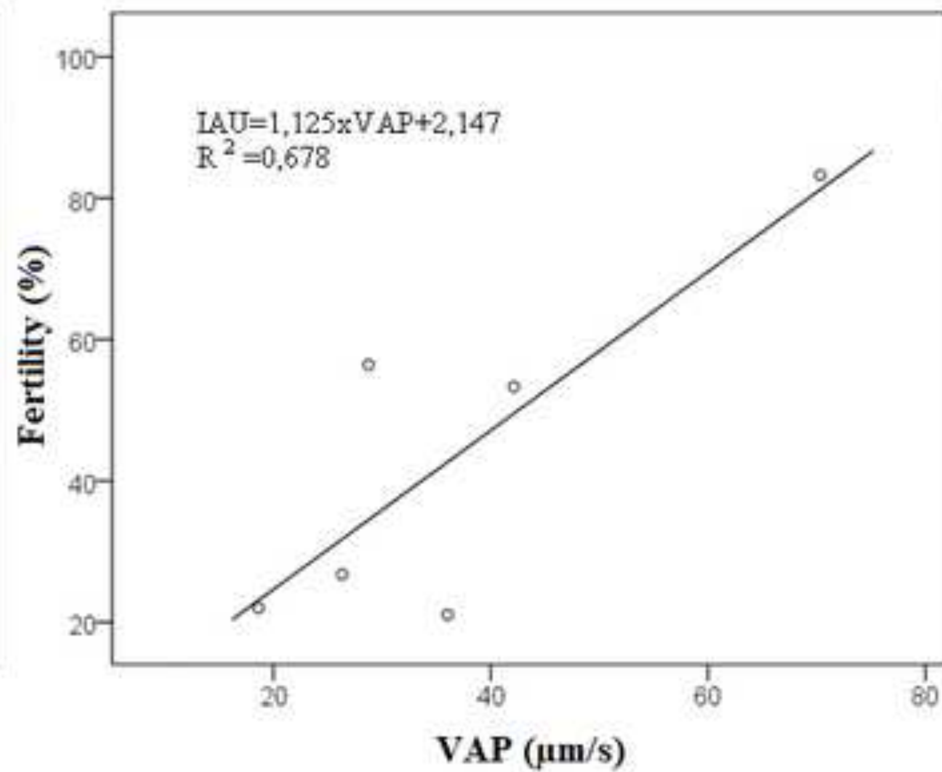
Manuscript





Figure





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