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Fertility of cryopreserved ovine semen is determined by sperm velocity

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ABSTRACT

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

1. Introduction

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

Thus, the objective in the present study was to explore laboratory techniques that would allow to quickly and effectively evaluate the potential fertility of a sperm sample. The present study follows the previous study by García-Alvarez et al. (2009a), which showed that heterologous *in vitro* fertilization assays were related to ram sperm fertility. In the present study, it was decided to study the behavior of sperm samples after incubation in the same medium used for *in vitro* fertilization, to determine if after

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"physiological-like" incubation the relation of sperm quality tests with fertility results would be improved.

To perform the present study, we have focused in flow cytometry as a tool with great statistical power because of its ability to analyze thousands of cells in a few seconds, improving existing analyses of fluorescence microscopy and allowing for new multi-parametric analyses (Martínez-Pastor et al., 2010). There have been many attempts to correlate flow cytometry results and fertility (Gillan et al., 2003). For instance, mitochondrial function and membrane integrity with fertility after artificial insemination with ram (Soler et al., 2008) or bull (Gualtieri et al., 2005) semen. However, García-Alvarez et al. (2009a) did not find any relation to fertility with the following sperm parameters: viability (PI membrane exclusion), membrane stability (YOPRO1), membrane phospholipid disorder (M540), and mitochondrial membrane potential (Mitotracker Deep Red), all assessed by flow cytometry. This study is a further step, testing additional sperm parameters in a modified experimental setting, to clarify the relation of several sperm variables to fertility.

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

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

To fully address this topic, sperm motility was evaluated using a computer-assisted semen analyzer (CASA) which provides precise and accurate information on sperm kinematic parameters (Gravance and Davis, 1995), allowing a more accurate prediction of fertility than the parameters assessed by the routine microscopic semen evaluation (Farrell et al., 1998; Malo et al., 2005). The objective assessment of sperm function could increase the chances of predicting the fertilizing capacity of a frozen-thawed semen sample or diagnosing infertility problems.

2. Materials and methods

2.1. Reagents and media

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The remaining of the chemicals (Reagent grade or higher) and propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes were purchased from Invitrogen (Barcelona, Spain). Stock solutions of the fluorescence probes were: 7.5 mM PI in water; 50 μm YOPRO1 in DMSO; 100 μg/mL FITC-PNA in water; 0.7 mM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) in DMSO; 5 μM fluo-3 in DMSO.

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

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

2.2. Animals and semen collection

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

2.3. Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. The extender used was prepared as described by Fiser et al. (1987). Fraction 1 was added 3:2 to semen and the sample was slowly cooled from 30 °C to 5 °C for 2 h. Then, the samples were further diluted (3:1) with Fraction 2 at 5 °C, reaching a final concentration of 200×10^6 sperm/mL, and held at this temperature for equilibration for 2 h (total refrigeration time at 5 °C was 4 h). At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-ml plastic straws and frozen. The straws were frozen in a programmable biofreezer (Planner) at −20 °C/min to −100 °C, and at −10 °C/min from −100 °C to −140 °C and then plunged into liquid nitrogen. Thawing was performed by putting the straws in a water bath with saline at 37 °C for 30 s, and the contents were transferred into a glass tube.

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2.4. Artificial insemination trials

Thawed sperm samples from the six males were used to inseminate 551 ewes in eight farms. Sperm samples from each male were used to inseminate between 11 and 262 females. The ewes were synchronized using progestagen pessaries (30 mg fluorogestone acetate, FGA; Chronogest, Intervet, The Netherlands) for 13 d followed by 500 IU equine chorionic gonadotrophin (eCG) at pessary removal. Ewes were inseminated intrauterine by laparoscopy at 55–58 h after pessary removal. Two technicians performed all intrauterine inseminations in different dates.

A male was considered to have contributed to a successful fertilization when the female lambed. Fertility rate for each male was calculated as follows: number of lambed ewes/number of ewes inseminated × 100. This rate was called male fertility.

2.5. Assessment of frozen-thawed sperm

Thawed samples were incubated for 2 h (37 °C) without dilution (*i.e.*, in the freezing extender) or after dilution 1:25 in SOF medium, at 5% CO_2 (38 °C). Samples were analyzed after this incubation time by CASA and flow cytometry. Sperm motility (subjective) was assessed for each sample after thawing. Percentage of individual motile sperm (motility) was noted.

2.6. Sperm motility assessed by CASA

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

2.7. Flow cytometry analyses

Sperm samples were analyzed using a Cytometer, Cytomics FC500 (Beckman Coulter, Brea, CA, USA). Excitation was provided by a 488 nm Argon-Ion laser. The FSC (forward – scattered light) and SSC (side-scattered light) signals were used to gate out debris (non-sperm events). FL1 photodetector (530/28BP filter) was used for YOPRO1, FITC-PNA, and JC-1; FL2 (575/26BP filter) for JC-1; FL3 (620SP filter) for PI events. The acquisition was controlled using the MXP software. All the parameters were read using logarithmic amplification. About 5000 sperm cells were acquired from each sample.

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

2.7.1. Sperm viability and apoptosis-like changes

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

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

The lipophilic cationic probe JC-1 was used to assess the mitochondrial status of the sperm. According to the manufacturer (Molecular Probes, Invitrogen Life Sciences, Fullerton, CA, USA.) and as described Robles and Martínez-Pastor (2013), JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when the mitochondrial membrane potential is great. Sperm samples were diluted with SOF-HEPES to a concentration of 5 × 10^6 sperm/mL, 300 mL of each sample were transferred to a polypropylene tube, and 1.2 mL of JC-1 stock solution (0.7 mM JC-1 in DMSO) was added. The tubes were incubated at 37 °C for 30 min in the dark. The stained sperm samples were then analyzed by flow cytometry, which identified cells with great mitochondrial membrane potential (hMMP; orange-stained cells).

2.7.3. Assessment of acrosomal integrity

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

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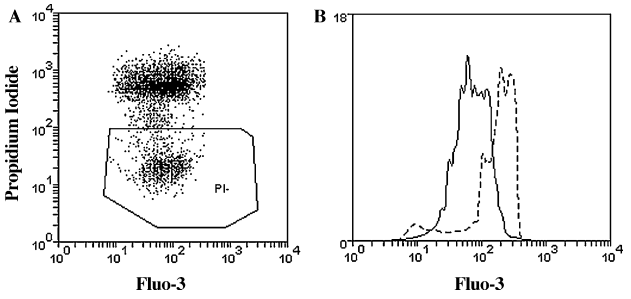

Fig. 1. Representative cytogram and histogram obtained by flow cytometry analysis of a ram sample after loading the sperm with Fluo-3, a specific stain for intracytoplasmatic calcium, and counterstained with propidium iodide (PI), a non-permeable membrane stain, for assessing viability. (A): Fluo-3/PI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

acrosomes and PI−/PNA+ as live cells with damaged acrosomes.

2.7.4. Detection of intracelular calcium concentration

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

2.8. Statistical analysis

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

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

3. Results

3.1. Semen evaluation

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

Table 2 presents the effect of dilution and incubation during 2 h in the freezing extender (37 °C) and in the SOF (38 °C, 5% CO_2) on flow cytometry variables. Results showed an overall decrease in sperm quality as assessed by flow cytometry after incubation, whereas no significant differences were observed between the two media ($P > 0.05$).

3.2. Correlations between sperm parameters and in vivo fertility

The sperm samples used in this study were selected based on heterogeneity regarding its *in vivo* fertility. Three males were selected with an average fertility above 50% and three males with an average fertility below 50%. Male fertility by intrauterine artificial insemination ranged from 22%

Table 1
Sperm motility parameters assessed by the CASA system Sperm Class Analyzer (SCA®). Values are expressed as Mean ± S.E.M. Sperm analyses were 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 SOF (38 °C, 5% CO_2).

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}	4.69 ± 0.3^{a}
2	Freezing extender	43.8 ± 11.9^{b}	9.2 ± 2.6^{b}	37.0 ± 7.4^{*b}	49.9 ± 7.3^{*b}	25.6 ± 5.7^{*b}	44.2 ± 3.6^{a}	2.1 ± 0.1^{b}	4.1 ± 0.6^{***b}
	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}

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* ($P < 0.001$).

Table 2
Results of sperm parameters evaluated by flow cytometry and its relation to *in vivo* fertility assessed by artificial insemination. Values are expressed as (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 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]

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 ($P < 0.05$).

to 83%, with a mean value of 44%. There were differences in fertility among males ($P = 0.003$).

The possible relation between kinematic and flow cytometry variables with *in vivo* fertility was studied using a lineal regression analysis. There were relationships between some kinematic parameters and field fertility only after 2 h of incubation in the freezing extender. In this regard, average-path velocity (VAP), the curvilinear velocity (VCL) and the head beat-cross frequency (BCF), showed a high positive correlation with fertility ($P = 0.044$ ($R^2 = 0.678$), $P = 0.027$ ($R^2 = 0.745$) and $P = 0.006$ ($R^2 = 0.852$), respectively; Fig. 2; Table 1).

4. Discussion

Recently, García-Alvarez et al. (2009a) showed that heterologous *in vitro* fertilization was a good procedure to predict the fertility of ram semen, unlike sperm evaluation by flow cytometry. However, methods based in IVF are costly in time needed to conduct the procedure and from a financial perspective. Therefore, in the present study two objectives were proposed. First, the objective was to study the quality of cryopreserved sperm using an incubation model in freezing extender or IVF media to determine if these stressful conditions could improve the relation between sperm quality variables (as assessed after incubation) with field fertility. Therefore, it could be concluded from these results whether these incubation models were practical, as a standard method for improving sperm quality assessment. Complementary with the first objective, the second objective aimed to identify laboratory techniques that would be most appropriate for, in the experimental approach defined by the first objective, to quickly and effectively evaluate the fertility potential of a sperm sample.

Motility has been considered one of the most important characteristics associated with the fertilizing ability of sperm (Saacke and White, 1972). In the present study, a decrease in motility variables was detected after the incubation, reflecting the stressful situation that sperm incur in these conditions. Interestingly, a positive relationship was detected among several kinematic variables (VCL, VAP, VSL, BCF) measured after the incubation in the extender and field fertility. To our knowledge, this is the first time a relationship was demonstrated between motility assessed by CASA and fertility of cryopreserved ram sperm. The difference in the present study with previous studies is the inclusion of a post-thawing incubation prior to the assessment. Thus, O'Meara et al. (2008) and García-Alvarez et al. (2010) did not detect a significant relationship between fertility and sperm quality (functional parameters or CASA, respectively) in thawed ram sperm. Results of the present study show the importance of pre-treating sperm before assessing the quality (in this case, submitting them to incubation at 37 °C). Malo et al. (2005) found differences in fertility between red deer stags related strongly to sperm swimming velocity parameters (VCL, VSL and VAP), and results of the present study support the hypothesis that sperm velocity is one of the key features in the process of fertilization, which has been also demonstrated in a large number of taxa (Gage et al., 2004; Holt et al., 1989). Greater motility is a result of a physiologically functional sperm, and sperm with decreasing motility are indicative of decreased sperm metabolism or failing organelles. This might indicate not only a decreased ability to reach the oocyte (of lesser importance in laparoscopic insemination), but reflects a lesser ability to undergo capacitation, the production of an excess of free radicals or to execute key steps in egg fertilization (Aitken et al., 2012; Martínez-Pastor et al., 2009). Results of the present study agree with studies in other species (bull: Amann et al., 2000; Farrell et al., 1998; Kathiravan et al., 2008; goat: Fernandez-Santos et al., 2011), which show that kinematic parameters as VCL, VSL and VAP are related to fertility. Other researchers have found positive correlations between different velocity parameters and fertility in human sperm (Fetterolf and Rogers, 1990).

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

It must be pointed out that these studies did not make use of a pre-treatment of thawed sperm prior to the study

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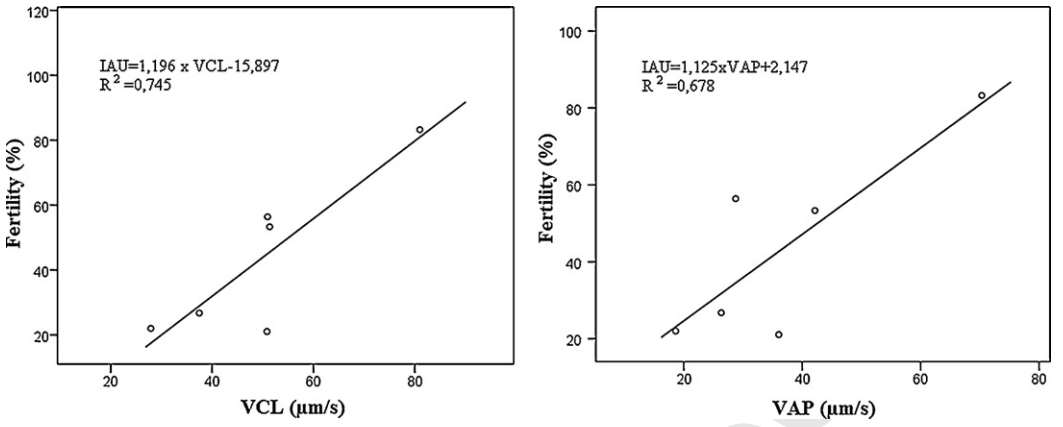

Fig. 2. Relationship between *in vivo* male fertility and VCL (velocity according to the actual path) and VAP (velocity according to the smoothed path). Regression lines and their equations are showed ($P < 0.05$). Data were obtained using a computer-assisted sperm analysis (CASA) system (Sperm Class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

of sperm quality. The incubation of sperm at physiological of above physiological temperatures is a challenge that may help to uncover the authentic fertility potential of the sample. This kind of treatment can be helpful for other kind of experiments. For instance, in a recent study the post-thawing quality of red deer sperm cryopreserved with different antioxidants were not very differentfrom the control, but after incubating the samples at 39 °C many differences were detected (Anel-López et al., 2012). Because in the present study it was found that there was a direct relationship between motion parameters and fertility, and mitochondria have been considered a fundamental organelle to sperm physiology (Mukai and Okuno, 2004; Peña et al., 2009), it would be logical to find a relationship between mitochondrial status and fertility. There, however, was not any relation between mitochondrial activity as measured using JC-1, and fertility in the present study. In this regard, Volpe et al. (2009) and Cheuquemán et al. (2011) indicated that JC-1 is suitable for detection of inner mitochondrial membrane potential changes in canine sperm, but it should always be associated with an objective motility analysis to avoid an incorrect evaluation of potential sperm fertility. García-Alvarez et al. (2009a) also showed that mitochondrial membrane potential of ram sperm, analyzed with MitoTracker deep red, was unrelated to fertility. Indeed, it has been considered that the main role of the sperm mitochondria was the production of energy for sperm motility, but this concept is under assessment and the roles of mitochondria will likely be broadened as more research assessing these roles is conducted (Marin et al., 2003; Miki et al., 2004; Mukai and Okuno, 2004). Mitochondria also have a crucial role in diverse cellular functions apart from energy production, such as modulation of the redox balance, osmotic regulation and Ca^{++} homeostasis (Peña et al., 2009). Therefore, the assessment of mitochondrial status could eventually offer important information relative to the sperm fertilizing ability, but this might require other experimental approaches.

Different researchers have demonstrated that intracytoplasmatic calcium efflux is a necessary component for capacitacion (Gualtieri et al., 2005). Although in the present study greater calcium concentrations were detected after incubation, there was not any relation between fertility and the relative intra-cellular calcium concentration with any treatment. Thus, the concentration of Ca^{2+} in the sperm after thawing or after incubation did not reflect the fertility of the sperm samples. That does not mean that Ca^{2+} concentrations are irrelevant. Measurement of the Ca^{2+} profile after treating sperm with progesterone or other physiological signals could offer relevant information related to sperm fertility (Arienti et al., 2010). The primary conclusion from the present research is that thawed semen samples with a greater sperm velocity is related to a greater field fertility, but only when measurements were conducted after 2 h of incubation in the freezing extender. Interestingly, incubation in SOF in conditions seeking to mimic the female oviductal environment did not yield any significant relationship with fertility. To our knowledge, no other researchers have found this relationship between sperm motility parameters and *in vivo* fertility of rams following intrauterine artificial insemination of ewes with frozen-thawed semen. Results cannot be generalized with conclusions of the present study, because of the limited number of males involved. Nevertheless, the experimental approach in the present study evaluating the semen samples after a 2 h incubation at 37 °C in the freezing medium merits further research, and it could be the basis of protocols for predicting the *in vivo* fertility of frozen-thawed ram sperm samples.

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