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Dynamics of sperm subpopulations based on motility and plasma membrane status in thawed ram spermatozoa incubated under conditions that support *in vitro* capacitation and fertilisation

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Abstract. The present study evaluated modifications occurring in thawed ram spermatozoa during incubation in different media that supported *in vitro* capacitation and fertilisation, and examines how these changes relate to IVF. Thawed sperm samples were incubated under capacitating (Cap) and non-capacitating (non-Cap) conditions for 0, 1 and 2 h and used in an IVF test. During incubation, changes related to membrane status and the motility pattern of spermatozoa were assessed, the latter being used to characterise sperm subpopulations. A significantly greater increase ($P \leq 0.05$) in the percentage of spermatozoa with higher membrane fluidity was observed in samples incubated with Cap medium from the beginning of incubation. In addition, changes over time in the distribution of the motile subpopulation were particularly evident when spermatozoa were incubated with Cap medium, with a noted increase in spermatozoa classified as 'hyperactivated like', with major changes occurring after 1 h incubation. Both characteristics (i.e. membrane fluidity and the percentage of the hyperactivated-like subpopulation) were significantly related with *in vitro* fertility, and only sperm samples incubated with the Cap medium were capable of fertilising oocytes. These results support the idea that changes in sperm membrane fluidity and motility pattern (i.e. an increase in hyperactivated spermatozoa) are needed for fertilisation to take place.

Additional keywords: hyperactivation, membrane fluidity.

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Introduction

Prior to the occurrence of fertilisation, spermatozoa undergo a complex physiological process that involves biochemical, physical and metabolic modifications known as capacitation (Yanagimachi 1994). These changes in the spermatozoa include changes in the composition and distribution of membrane phospholipids, cholesterol levels, protein tyrosine phosphorylation, intracellular ion concentrations and hyperpolarisation of the sperm plasma membrane, which will enable the spermatozoa to develop the acrosome reaction (AR; Visconti *et al.* 2002) and zona pellucida binding (Buffone *et al.* 2009). Likewise, capacitation includes changes in the motility pattern of spermatozoa, allowing them to express a movement termed 'hyperactivation' (Topper *et al.* 1999; Ho and Suarez 2001).

Depending on the species, *in vitro* capacitation may be modulated by chemically defined media. In the ram, oestrous sheep serum is considered to be necessary for *in vitro* capacitation and fertilisation (Cognié *et al.* 2003). This substance induces cholesterol efflux from the sperm plasma membrane (Huneau *et al.* 1994), increasing membrane fluidity. Other physiological events documented in ram capacitated spermatozoa are increased tyrosine phosphorylation (Pérez-Pé *et al.* 2002; Sidhu *et al.* 2004) and changes in the motility pattern (Vulcano *et al.* 1998). However, fertilisation represents the benchmark endpoint of capacitated spermatozoa (Yanagimachi 1994; Visconti and Kopf 1998).

It is recognised that ejaculates are heterogeneous in many different aspects and sperm populations with different structural and functional grades exist within a sperm sample (Buffone *et al.* 2004). Many authors have studied variations in the subpopulation structure through the capacitation process based on several sperm traits. For example, Harrison (1996) reported that spermatozoa incubated under capacitation conditions showed a rapid increase in the population of spermatozoa with

high membrane fluidity. Ramió *et al.* (2008), in the pig, identified four sperm subpopulations based on sperm motility in semen samples incubated in capacitating and non-capacitating media, describing changes in the percentages of each subpopulation throughout the capacitation period. Goodson *et al.* (2011) also found changes in the distribution of motile subpopulations of mouse spermatozoa subjected to capacitation conditions. These authors identified spermatozoa with a hyperactivated pattern during capacitation.

More important than the presence of structural and functional changes during capacitation is the fact that sperm populations may react differently to capacitation, which would eventually affect the fertility results (Holt and Van Look 2004).

Against this background, the aim of the present study was to evaluate changes that occur in thawed ram spermatozoa based on plasma membrane status and motility pattern after incubation under conditions that support *in vitro* capacitation and fertilisation, and to determine how these changes may be related to fertility.

Materials and methods

Animals and reagents

Animals were handled in accordance with Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2003/65. Four rams of the Manchega ovine breed (>3 years of age) were used. Males were maintained and managed at the Regional Center of Animal Selection and Reproduction (Valdepeñas, Spain).

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The remaining chemicals (reagent grade or higher) and the fluorescence probes propidium iodide (PI), peanut agglutinin (PNA)–fluorescein isothiocyanate (FITC) and merocyanine 540 were obtained from Sigma (Madrid, Spain). YO-PRO-1 was purchased from Invitrogen (Barcelona, Spain).

Semen collection and cryopreservation

Semen were collected using an artificial vagina. Volume, concentration, wave motion and subjective sperm motility were assessed shortly after collection. The volume of ejaculates was measured in a conical graduated tube and sperm concentration was calculated using a spectrophotometer. The wave motion was assessed from fresh non-diluted semen and was scored over a scale of 0–5, with 0 indicating no movement and 5 indicating strong wave movement.

Because computer-assisted sperm analysis (CASA) and flow cytometry analysis of spermatozoa had to be performed at a later stage, spermatozoa were stored by cryopreservation. Only ejaculates with wave motion and sperm motility (subjective) values ≥4% and ≥80%, respectively, were frozen with the freezing extender Biladyl (20% egg yolk and 7% glycerol; Minitüb, Tiefenbach, Germany), as described by García-Álvarez *et al.* (2009).

Sperm evaluation

Straws were thawed by placing them in a water bath at 37°C for 20 s. Then, sperm selection was performed using a discontinuous density gradient on Percoll (1 mL of 45% Percoll over 1 mL of 90% Percoll; Sigma) at room temperature (20–22°C). The thawed semen samples were layered on top of the two layers of Percoll and centrifuged at 700*g* for 10 min (O'Meara *et al.* 2005; Papadopoulos *et al.* 2005; García-Álvarez *et al.* 2010). After centrifugation, the supernatant was carefully discarded and the sperm pellet was divided into two parts.

One part of the sperm pellet was diluted with the medium frequently used for *in vitro* capacitation and fertilisation (Cap; synthetic oviducal fluid (SOF) Takahashi and First 1992) supplemented with 2% oestrous sheep serum,(1 μL mL^{-1} heparin and 1 μL mL^{-1} hypotaurine (Berlinguer *et al.* 2009). The other part of the sperm pellet was incubated in SOF medium supplemented only with 0.1% polyvinyl alcohol (PVA; non-Cap medium). The PVA was added to inhibit the formation of sperm aggregates and prevent sticking of the gametes to various surfaces (Visconti *et al.* 1999). The final sperm concentration after dilution was 10×10^6 spermatozoa mL^{-1} and the diluted spermatozoa were incubated for 2 h at 38.5°C under 5% CO_2. The sperm parameters assessed in samples incubated in both media for 0 (corresponding to 10 min of incubation), 1 and 2 h, are described below.

Sperm motility was assessed by CASA, as described by Tamayo-Canul *et al.* (2011). A prewarmed (37°C) Makler counting chamber (10 μm depth; Sefi-Medical Instruments, Haifa, Israel) was loaded with 5 μL sample. The CASA system consisted of a triocular optical phase contrast microscope (Eclipse 80i; Nikon, Tokyo, Japan) 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 analysed using Sperm Class Analyzer (SCA2002) software (Microptic, Barcelona, Spain). Samples were examined at ×10 magnification (negative phase contrast) using a microscope with a warmed stage (37°C). Software settings were adjusted to ram spermatozoa and at least five fields or 200 spermatozoa were saved for later analysis. For each sperm sample analysed, SCA2002 yielded the following data: percentage of motile spermatozoa (SM), curvilinear velocity (VCL; μm s^{-1}), straight line velocity (VSL; μm s^{-1}), average path velocity (VAP; μm s^{-1}), linearity index (LIN; %), straightness (STR; %), wobble (WOB; %), amplitude of lateral head displacement (ALH; μm) and beat cross frequency (BCF; Hz).

Flow cytometry analyses were also performed on sperm samples incubated with Cap and non-Cap media. Membrane stability was assessed by YO-PRO-1, whereas sperm viability was determined by PI staining (Martínez-Pastor *et al.* 2008). Acrosomal status was assessed by PNA-FITC (Martinez-Pastor *et al.* 2006; García-Álvarez *et al.* 2009), and membrane fluidity was evaluated by using the hydrophobic dye merocyanine 540 (Harrison 1996). Sperm samples were diluted to a concentration of 1×10^6 spermatozoa mL^{-1} in each of two staining solutions (see below)using bovine gamete medium (BGM-3). One of the staining solutions was prepared by combining 15 μM PI (stock solution 7.5 mM in Milli-Q water), 50 nM YO-PRO-1 (stock solution 1 mM in dimethylsulfoxide (DMSO)) and 1 μM merocyanine 540 (stock solution 3.4 mM in DMSO). The second staining solution was prepared by adding the same concentration of PI to 10 μg mL^{-1} PNA-FITC (stock solution 0.2 mg mL^{-1}) in polypropylene tubes for flow cytometry analysis. The tubes

were kept in the dark for 15 min and then sperm samples were run through a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA). A 488 nm argon ion laser of the cytometer was used to excite the different fluorochromes, namely YO-PRO-1, merocyanine 540, PI and PNA-FITC. Forward scatter (FSC) and side scatter (SSC) signals plus the fluorescence light for each fluorochrome were acquired using three photodetectors: FL1 for YO-PRO-1 and PNA-FITC (530/28BP filter); FL2 for merocyanine 540 (575/26BP filter); and FL3 for PI (670 LP filter). Acquisition was controlled using MXP software (Beckman Coulter, Brea, CA, USA). All parameters were read using logarithmic amplification. An acquisition template was set up in the software that allowed us to first discriminate spermatozoa from debris within the events acquired. The filtered events were displayed in a dot plot showing either FL1/FL3 (YO-PRO-1 vs PI and PNA-FITC vs PI) and FL2/FL1 (merocyanine 540 vs YO-PRO-1). A total of 5000 events was acquired from each sample, saving the data in flow cytometry standard (FCS) version 2 files. The YO-PRO-1^-/PI^- sperm subpopulation was considered to indicate viable spermatozoa with intact membranes (sperm viability), whereas the PNA-$FITC^-$/PI^- sperm subpopulation was considered to indicate viable spermatozoa with an intact acrosome. The merocyanine 540^+/YO-PRO-1^- subpopulation was considered to indicate viable spermatozoa with high membrane fluidity. We thought it appropriate to consider only the YO-PRO-1^- subpopulation to study spermatozoa with high membrane fluidity (merocyanine 540^+), preventing the confounding effect of an 'apoptotic' sperm subpopulation. Thus, the proportion of merocyanine 540^+ spermatozoa within the population of viable spermatozoa was calculated (VIABmerocyanine$^+$).

Experiments were replicated three times.

IVF test

Sperm samples incubated with Cap and non-Cap media at 0 h, corresponding to 10 min incubation, were used in an IVF test to assess the fertilisation ability of the spermatozoa. The IVF test was replicated three times per incubation treatment (Cap and non-Cap) and 20 oocytes were used per well and replicate ($n = 120$).

The IVF protocol was similar to that described by García-Álvarez *et al.* (2011). Briefly, ovaries were collected at an abattoir from sheep at least 4 years of age. The ovaries were transported to our laboratory in saline solution (25–30°C) within 1–2 h after removal. Ovaries were sliced using a microblade and the follicle content was released in TCM-199 medium supplemented with HEPES (2.38 mg mL^{-1}), heparin (2 μL mL^{-1}) and gentamycin (40 μg mL^{-1}). Cumulus–oocyte complexes (COC) were washed in TCM-199–gentamycin (40 μg mL^{-1}), and those with dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected and randomly placed in four-well plates containing 500 μL TCM-199 supplemented with cysteamine (100 μM), FSH and LH (10 μg mL^{-1} each), fetal calf serum (FCS; 10%) and gentamycin (40 μg mL^{-1}), before being matured at 38.5°C in 5% CO_2. After 24 h, half the COC were washed with non-Cap medium and the other half were washed with Cap medium. Cumulus cells were removed by gentle pipetting. Oocytes were transferred to four-well plates containing 450 μL non-Cap or Cap medium under mineral oil. Finally, spermatozoa from each incubation treatment (non-Cap and Cap) were coincubated with the oocytes in the corresponding medium at a final concentration of 1×10^6 spermatozoa mL^{-1} for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Presumptive zygotes were cultured in SOF enriched with amino acids and bovine serum albumin (Gardner *et al.* 1994) at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2.

Fertility was assessed as the cleavage rate 24 h post insemination (h.p.i.; two blastomeres) by phase contrast microscopy.

Statistical analysis

Statistical analyses were performed using the R statistical package (R Development Core Team 2012). Prior to statistical analysis, the normal distribution of data was evaluated by graphical methods and the Kolmogorov-Smirnov normality test. When necessary, percentages were arcsine transformed. Differences were considered significant when $P \leq 0.05$, unless indicated otherwise.

An analysis to identify sperm subpopulations based on motility data was performed for each treatment and incubation time point. The cluster procedure was performed following the multistep process described by Martinez-Pastor *et al.* (2005). Briefly, data from CASA were merged into a single dataset. In all, 8930 motile spermatozoa were captured, each one being defined by eight kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF). A variable group analysis and correlation analysis were used to select which of these eight parameters could be used as initial classifiers. This analysis has been used by other authors (Maroto-Morales *et al.* 2012) for studying different sperm characteristics. Based on the results of this analysis, VCL, LIN and ALH were finally selected as initial classifiers for clustering. Cluster analysis was divided in two parts: (1) a non-hierarchical cluster analysis using VCL, LIN and ALH as classifiers; and (2) a hierarchical cluster analysis performed on the clusters obtained from the previous analysis. Non-hierarchical methods are often used with large datasets before trying a hierarchical analysis to reduce the data to a few initial clusters and then passing them on to the hierarchical procedure. Three different subpopulations were identifying in all treatments and incubation times.

We examined changes in sperm viability, acrosomal and plasma membrane status and in subpopulation distribution based on sperm motility within treatments (Cap and non-Cap) for each incubation time point (0 (10 min), 1 and 2 h) and within incubation time for each treatment. These analyses were performed using a multivariate mixed effects model that considered the two media and the three times as fixed factor and the variability among replicates as random effect.

In addition, for both incubation media (Cap vs non-Cap), we examined whether each of the sperm parameters evaluated (sperm viability, proportion of spermatozoa with an intact acrosome, proportion of merocyanine$^+$ spermatozoa within the population of viable spermatozoa (VIABmerocyanine$^+$) and subpopulations (SP) 1, SP2 and SP3) was related to differences in fertility based on the IVF test. Those sperm features that were related to fertility (VIABmerocyanine$^+$ and the hyperactivated sperm subpopulation (SP3)) were included as

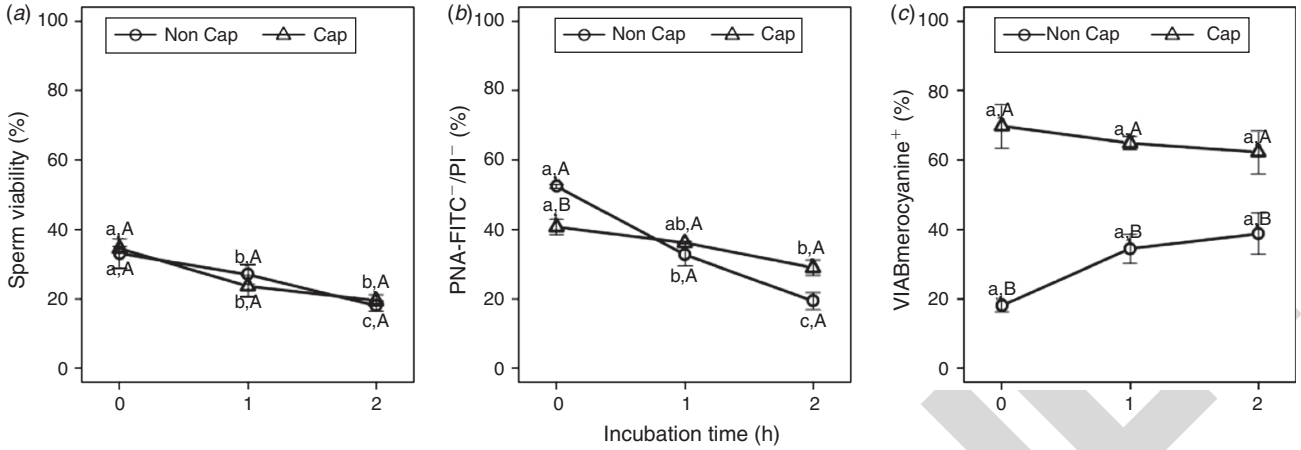

Fig. 1. (*a*) Sperm viability, (*b*) peanut agglutinin (PNA)–fluorescein isothiocyanate (FITC)- negative and propidium iodide (PI)-negative spermatozoa (percentage of viable spermatozoa with an intact acrosome) and (*c*) the proportion of merocyanine 540-positive spermatozoa within the viable sperm (YO-PRO-1 negative) population (VIABmerocyanine$^+$) for sperm samples incubated in the presence of non-capacitating (non-Cap) or capacitating (Cap) media for different periods of time (0 (≡10 min incubation), 1 and 2 h). Data are the mean ± s.e.m. Symbols with different uppercase letters (A, B) indicate significant differences between treatments (non-Cap vs Cap; $P \leq 0.05$); symbols with different lowercase letters (a, b, c) indicate significant differences between incubation times points (0, 1 and 2 h; $P \leq 0.05$).

independent variables in a multivariate regression analysis considering the *in vitro* fertility measure (cleavage rates at 24 h.p.i.) as a dependent variable.

Results

Characteristics of thawed sperm samples incubated with Cap and non-Cap media over time

Sperm viability decreased over time for both incubation conditions, with no significant differences between treatment groups ($P > 0.05$; Fig. 1*a*).

The proportion of viable spermatozoa with intact acrosomes (PNA-FITC$^-$/PI$^-$) decreased significantly ($P \leq 0.05$) in sperm samples incubated in non-Cap medium over 2 h incubation, whereas the proportion remained constant for spermatozoa incubated in Cap medium (Fig. 1*b*). However, there were no significant differences between the treatments for the duration of incubation, with the exception of at 0 h (10 min incubation), at which time the percentage of PNA-FITC$^-$/PI$^-$ spermatozoa was significantly higher ($P \leq 0.05$) in sperm samples incubated with non-Cap compared with Cap medium (52.41 ± 0.54% vs 40.71 ± 2.41%, respectively).

In the present study, the VIABmerocyanine$^+$ sperm population remained stable for the duration of incubation for both treatments (Fig. 1*c*), but was significantly greater ($P \leq 0.05$) in samples incubated with Cap compared with non-Cap medium (69.74 ± 6.33% vs 18.13 ± 1.78%, respectively, at 0 h (10 min incubation); 64.86 ± 1.84% vs 34.48 ± 4.19%, respectively, at 1 h; and 62.34 ± 6.14% vs 38.73 ± 14.71%, respectively, at 2 h).

Characterisation of sperm subpopulations based on motility parameters

Three sperm subpopulations were identified and well defined based on the motility parameters VCL, LIN and ALH (Table 1): (1) SP1 consisted of slow, non-linear spermatozoa with low ALH; (2) SP2 was characterised by fast, linear spermatozoa with medium ALH; and (3) SP3 consisted of rapid, non-linear spermatozoa with high ALH. The latter subpopulation (i.e. SP3) could represent the hyperactivated spermatozoa described by others (Mortimer and Mortimer 1990).

Table 1. Motility characteristics for different sperm subpopulations in thawed ram sperm samples

Data are the mean ± s.e.m. Within a column, values with different superscript letters differ significantly ($P < 0.001$). SP1, slow, non-linear spermatozoa with low lateral head displacement (ALH); SP2, fast, linear spermatozoa with medium ALH; SP3, rapid, non-linear spermatozoa with high ALH; VCL, curvilinear velocity; LIN: linearity index

	VCL (μm s^{-1})	LIN (%)	ALH (μm)
SP1	23.74 ± 1.39^A	33.17 ± 2.44^A	1.38 ± 0.05^A
SP2	124.30 ± 6.60^B	85.79 ± 1.46^B	2.01 ± 0.10^B
SP3	187.90 ± 7.55^C	52.07 ± 3.19^C	4.98 ± 0.25^C

Evolution of sperm subpopulations based on motility parameters in ram thawed sperm samples during incubation with Cap and non-Cap media

The three sperm subpopulations were observed in sperm samples incubated with Cap and non-Cap media (Fig. 2). The evolution of the sperm subpopulation classified as SP1 throughout incubation was similar for sperm samples incubated with non-Cap and Cap media, with higher values at 0 h (10 min incubation) that decreased after 1 and 2 h incubation (Fig. 2*a*). For SP2, there were no significant changes in this subpopulation throughout incubation for sperm samples incubated in non-Cap sperm medium (Fig. 2*b*). However, there was a decrease in the SP2 population throughout incubation for samples incubated in Cap medium, with lower values

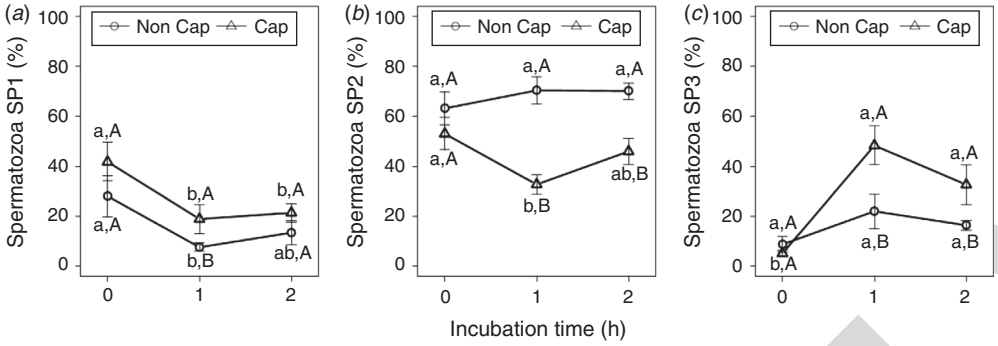

Fig. 2. Motile sperm subpopulations for samples incubated in the presence of non-capacitating (non-Cap) or capacitating (Cap) media and changes in these populations throughout incubation (0 (≡10 min incubation), 1 and 2 h). (*a*) Subpopulation (SP) 1 (slow, non-linear spermatozoa with low lateral head displacement (ALH)), (*b*) SP2 (fast, linear spermatozoa with medium ALH) and (*c*) SP3 (rapid, non-linear spermatozoa with high ALH). Data are the mean ± s.e.m. Symbols with different uppercase letters (A, B) indicate significant differences between treatments (non-Cap vs Cap; $P \leq 0.05$); symbols with different lowercase letters (a, b, c) indicate significant differences between incubation times points (0, 1 and 2 h; $P \leq 0.05$).

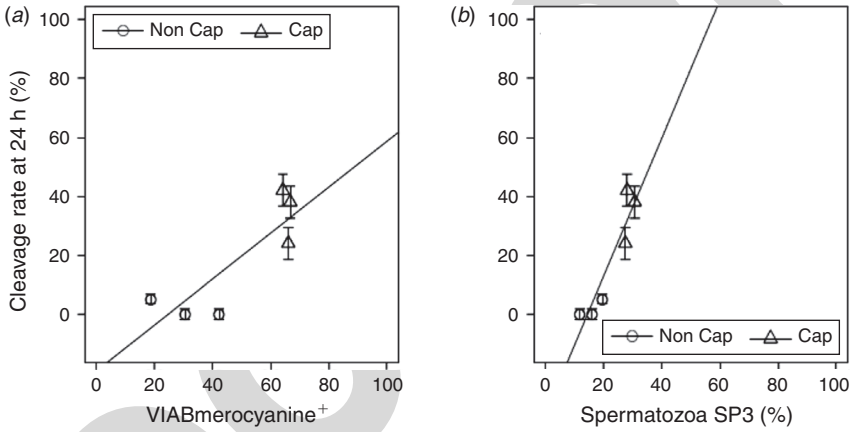

Fig. 3. Linear regression analysis of the relationship between *in vitro* fertility (cleavage rate at 24 h post insemination) and (*a*) the proportion of merocyanine 540-positive spermatozoa within the viable sperm (YO-PRO-1 negative) population (VIABmerocyanine$^+$) (proportion of merocyanine+ calculated within viable spermatozoa population (YO-PRO-1-)) and (*b*) motile sperm subpopulation 3 (SP3) for sperm samples incubated in the presence of non-capacitating (non-Cap) or capacitating (Cap) media.

compared with samples incubated in non-Cap medium (Fig. 2*b*). Finally, there were marked changes in the SP3 subpopulation for samples incubated in Cap medium for 1 and 2 h compared with 0 h (10 min incubation; Fig. 2*c*). However, there were no changes in the SP3 subpopulation for samples incubated with non-Cap medium throughout incubation. In addition, there was a higher percentage of spermatozoa classified as SP3 in samples incubated in Cap versus non-Cap medium at 1 and 2 h (Fig. 2*c*).

IVF for sperm samples incubated with Cap and non-Cap media

The effect of incubation medium on the ability of the spermatozoa to subsequently fertilise oocytes was evaluated. Sperm samples incubated in non-Cap medium exhibited a significant reduction in their ability to fertilise oocytes compared with spermatozoa incubated with Cap medium (1.66 ± 1.66% vs 34.66 ± 5.45%, respectively).

Relationships between characteristics of thawed sperm samples incubated with Cap and non-Cap media and in vitro *fertility*

A relationship was found for the VIABmerocyanine$^+$ and SP3 sperm population and IVF ($R^2 = 0.580$; $P = 0.013$). Sperm samples incubated with Cap medium had significantly higher values for these two parameters and also greater IVF rates (Fig. 3*a*, *b*).

Discussion

The present study examined differences in the response of ram spermatozoa incubated in two different media, one of which is

typically used for IVF and the other with a similar composition but without capacitation agent. The results showed that under capacitation conditions, spermatozoa undergo different structural and functional changes that lead to successful fertilisation. Moreover, such changes result in an increase in membrane fluidity and changes in the movement pattern towards a fast, non-linear movement. These changes did not occur in all spermatozoa, indicating a heterogeneous response. Thus, the percentage of spermatozoa that showed the changes was related to the fertilising capacity of the sample.

In vitro capacitation, in the case of the ram, can be induced by oestrous sheep serum (Cognié *et al.* 2003). This non-defined substance induces cholesterol efflux from the plasma membrane (Huneau *et al.* 1994), which results in higher membrane fluidity. Conversely, the cryopreservation process causes changes to the plasma membrane, resulting in alterations in phospholipids and increased membrane fluidity (Watson 1995). The increase in sperm plasma membrane fluidity, triggered by phospholipid disorder, can be detected as an increase in merocyanine 540 staining (Harrison 1996; Rathi *et al.* 2001; Gadella and Van Gestel 2004; Harrison and Gadella 2005). In the present study, sperm samples incubated in both Cap and non-Cap media contained a subpopulation of spermatozoa with high membrane fluidity, both initially and throughout the incubation process. This could be due to an effect of sperm cryopreservation. Many researchers (Bailey *et al.* 2000; Watson 2000) have shown changes similar to capacitation after cryopreservation, although it is thought that the expression of these changes follows a different pathway to that of true capacitation (Green and Watson 2001). In the present study, samples incubated in Cap medium contained a significantly higher proportion of viable spermatozoa with high membrane fluidity (VIABmerocyanine^{+}) from the beginning of incubation compared with samples incubated in non-Cap medium. This could be due to cryopreservation sensitising the spermatozoa to capacitating agents (Parrish 1991; Watson 1995; Pons-Rejraji *et al.* 2009). Furthermore, confirmation that changes in plasma membrane phospholipids are important for the acquisition of fertilising ability (and thus evidence that spermatozoa have been capacitated) was the significant relationship found between VIABmerocyanine^{+} spermatozoa and fertility.

Capacitation is important to prepare the spermatozoa for the acrosome reaction (Tsai *et al.* 2010), an event that occurs later in the capacitation process (Gadella 2013). In the present study, at the beginning of incubation (after 10 min), a small sperm subpopulation of the sperm samples incubated in Cap medium seemed to undergo a spontaneous (premature) acrosomal loss, possibly because the action of the capacitating agent on those spermatozoa deteriorated to a greater extent after the cryopreservation process. It is known that cryopreservation affects Ca^{2+} regulation in surviving spermatozoa (Bailey and Buhr 1993). Thus, the immediate response of a group of spermatozoa samples incubated in Cap medium regarding acrosomal loss could be the consequence of a lack of Ca^{2+} regulation after cryopreservation. In addition, spermatozoa incubated in non-Cap medium exhibited a more marked decrease in the proportion of PNA-FITC^{-}/PI^{-} spermatozoa, whereas this acrosome loss was slower in samples incubated in Cap medium. Our results agree with those reported by Robertson *et al.* (1988), who found that the proportion of spermatozoa with an intact acrosome was maintained in samples incubated under capacitation conditions for a period of 3 h.

Another feature that takes place during the capacitation process and has been linked to fertility is the change that occurs in the motility pattern of spermatozoa (Ho and Suarez 2001). Hyperactivated spermatozoa show a change in the pattern of movement, from a progressive path to a figure-of-eight pattern (Yanagimachi 1970), and many authors have characterised this movement after incubating spermatozoa under capacitating conditions (Mortimer and Maxwell 1999; Marquez and Suarez 2007; Suarez 2008; McPartlin *et al.* 2009; Goodson *et al.* 2011). In the present study, we evaluated these changes at sperm subpopulation levels as a way to better examine how motility patterns change throughout the incubation period. A total of three sperm subpopulations was identified. The SP3 subpopulation may correspond to hyperactivated spermatozoa, with sperm motility characteristics (VCL, LIN and ALH) similar to that described by others for characterising hyperactivation movement (Mortimer and Mortimer 1990). The SP2 subpopulation may represent those spermatozoa with progressive-intermediate movement and they could be the spermatozoa that subsequently show hyperactivation movement (Goodson *et al.* 2011). Finally, the SP1 subpopulation may represent those slow and weakly motile spermatozoa that do not respond to the capacitating stimulus. This sperm classification is in agreement with that reported by Mortimer and Mortimer (1990), who classified human spermatozoa into three distinct groups: (1) spermatozoa with progressive and not hyperactivated motility; (2) spermatozoa with hyperactivated motility; and (3) spermatozoa with a transitional motility between these two groups.

We also investigated the changes that occurred in the subpopulation structure throughout incubation for samples incubated in Cap and non-Cap media. For sperm samples incubated in non-Cap medium, the proportion of spermatozoa with progressive and linear motility (SP2) remained constant throughout incubation, being also the majority subpopulation. In contrast, sperm samples incubated in Cap medium exhibited a significant decrease in the percentage of SP2 spermatozoa after 1 h incubation, which was accompanied by an increase in the proportion of hyperactivated spermatozoa (SP3) at this time. The increase in SP3 spermatozoa could be due to changes in the spermatozoa belonging to the SP2 group, which respond to capacitating conditions. The transition of spermatozoa from one subpopulation to another is a dynamic process and may be influenced by several factors, such as capacitation conditions. Ramió *et al.* (2008) have found that the proportion of spermatozoa classified within a subpopulation based on sperm motility varies with incubation time under capacitation conditions, and that spermatozoa can pass from one subpopulation to another during this period. We have also evaluated the relationships between each of the sperm motile subpopulations with fertility. Spermatozoa in the SP3 subpopulation, which exhibited the greatest and significant change throughout incubation in Cap medium, was the only subpopulation related to *in vitro* fertility. The proportion of the SP3 subpopulation was higher in samples incubated in Cap medium, but this population was also present

in samples incubated in non-Cap medium, albeit at a lower level. Because Cap samples were able to fertilise oocytes, it seems necessary for a certain level of hyperactivated-like spermatozoa to be present for fertilisation to take place.

In conclusion, incubation of sperm samples in medium that support *in vitro* capacitation and fertilisation leads to changes that are consistent with an increase in the proportion of viable spermatozoa with high membrane fluidity, as well as an increase in the sperm subpopulation characterised by a hyperactivated-like motility pattern; these features are related to fertility.

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