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Oligomycin A-induced inhibition of mitochondrial ATP-synthase activity suppresses boar sperm motility and *in vitro* capacitation achievement without modifying overall sperm energy levels

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Abstract. Incubation of boar spermatozoa in a capacitation medium with oligomycin A, a specific inhibitor of the F_0 component of the mitochondrial ATP synthase, induced an immediate and almost complete immobilisation of cells. Oligomycin A also inhibited the ability of spermatozoa to achieve feasible *in vitro* capacitation (IVC), as measured through IVC-compatible changes in motility patterns, tyrosine phosphorylation levels of the acrosomal p32 protein, membrane fluidity and the ability of spermatozoa to achieve subsequent, progesterone-induced *in vitro* acrosome exocytosis (IVAE). Both inhibitory effects were caused without changes in the rhythm of O_2 consumption, intracellular ATP levels or mitochondrial membrane potential (MMP). IVAE was accompanied by a fast and intense peak in O_2 consumption and ATP levels in control spermatozoa. Oligomycin A also inhibited progesterone-induced IVAE as well as the concomitant peaks of O_2 consumption and ATP levels. The effect of oligomycin on IVAE was also accompanied by concomitant alterations in the IVAE-induced changes on intracellular Ca^{2+} levels and MMP. Our results suggest that the oligomycin A-sensitive mitochondrial ATP-synthase activity is instrumental in the achievement of an adequate boar sperm motion pattern, IVC and IVAE. However, this effect seems not to be linked to changes in the overall maintenance of adequate energy levels in stages other than IVAE.

Additional keywords: acrosome exocytosis, ATP, chemiosmosis, O_2 consumption.

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Introduction

Sperm capacitation has been defined as the overall sum of changes that a spermatozoon undergoes after being ejaculated that allow it to fertilise the oocyte (for a classical definition of the process see Austin 1951; Chang 1951; Yanagimachi 1994). Capacitation results in altered plasma membrane architecture and permeability, which ultimately modulates flagellar activity and renders the sperm apical-head plasma membrane fusogenic (Rodríguez-Martínez 2007). Physiologically, spermatozoa acquire fertilisation competence in the female reproductive tract, but capacitation should also be achieved *in vitro* in a defined medium (Visconti and Kopf 1998). Furthermore, the *in vitro*-capacitated spermatozoa are prone to subsequent *in vitro* acrosome exocytosis (IVAE). IVAE can be induced by several systems; one that better mimics *in vivo* exocytosis is incubation with progesterone. Progesterone is a steroid hormone that induces calcium influx into spermatozoa (Blackmore *et al.* 1990; García and Meizel 1999; Publicover *et al.* 2007) and triggers multiple subsequent physiological responses essential for successful fertilisation, like sperm motility hyperactivation,

chemotaxis towards the egg and induced-acrosome exocytosis (Teves *et al.* 2006; Wu *et al.* 2006).

Several intracellular changes are known to occur during capacitation and subsequent acrosome exocytosis, including increases in membrane fluidity, cholesterol efflux, changes to intracellular Ca^{+2} and cAMP concentrations, protein tyrosine phosphorylation and changes in swimming patterns and chemotactic motility (Eisenbach and Giojalas 2006). Hyperactivated motility is one of the best-characterised phenomena associated with capacitated spermatozoa (Suárez 1996). All of these changes require a significant consumption rhythm of ATP (Chang 1951, 1984; Austin 1952; Yanagimachi 1989; Tulsiani *et al.* 2007). Despite this, little is known regarding the energy sources from which spermatozoa transform their entire physiology, including motility, to hyperactivation during capacitation. In fact, the source of ATP that supports not only sperm motility but also other processes, such as the achievement of capacitation, has long been debated in the field of gamete research (see, as examples, Ford 2006; Ruíz-Pesini *et al.* 2007).

In mammalian spermatozoa there are two main pathways for ATP production: glycolysis, which occurs along the entire length of the principal piece of the flagellum, and mitochondrial respiration, centred on mitochondria of the midpiece. Mitochondrial respiration is the most efficient source of ATP and, in this way, it would be inferred that, under normal conditions, the ATP required for sperm motility is mainly obtained through mitochondrial respiration. Hence, mitochondrial status has been related not only to sperm motility in species like man (Piomboni *et al.* 2012), bull (Garner and Thomas 1999), horse (Gravance *et al.* 2000*a*) and ram (Martínez-Pastor *et al.* 2004), but also to fertilisation ability in humans (Kasai *et al.* 2002) and the achievement of other important processes such as progesterone-induced acrosome exocytosis in *in vitro*-capacitated boar spermatozoa (Ramió-Lluch *et al.* 2011). However, several works strongly indicate that mitochondria are not the only energy source for sperm motility, at least in several species, and glycolysis has also been related to the maintenance of important sperm functions, such as motility. In this way, it has been described that mouse spermatozoa remain motile after the addition of agents able to inhibit mitochondrial oxidative phosphorylation (Travis *et al.* 2001; Mukai and Okuno 2004). Furthermore, the gene knockout of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) caused the appearance of non-motile mouse spermatozoa and a significant reduction in the ATP content (10% of the total) despite having no deficiency in oxygen consumption (Miki *et al.* 2004). These findings suggest that active glycolysis is required in mice spermatozoa to support motility. Accordingly, Marín *et al.* (2003) reported that glycolysis plays a significant role as an energy source in boar spermatozoa. This would be in accordance with the presence of active and specific glycolytic activity in mammalian spermatozoa that was complementary and even more important than mitochondrial-based energy production (Mukai and Okuno 2004). This is linked to the presence of a sperm-specific form of GAPDH, which is tightly bound to the fibrous sheath (Kamp *et al.* 2003). In addition, several studies have documented the relationship between glycolysis and capacitation-dependent cell signalling (Urner and Sakkas 2003). Following all of these results, Guthrie *et al.* (2008) propose glycolysis as a specialised and sperm-specific source of ATP to maintain motility in mammalian spermatozoa. However, the pre-eminence of glycolysis is not clear in all species. In this respect, it has been described in species like rat, ram and boar that the blocking of GAPDH does not impair motility when sperm cells were incubated in the presence of lactate and pyruvate (Ford and Harrison 1981, 1985). This leads to the questioning of glycolysis as the pre-eminent energy source for mammalian sperm motility (Ford 2006). In fact, the joint analysis of all of these data suggests that the maintenance of sperm function would be the result of the equilibrium between energy obtained from glycolysis and mitochondrial respiration. Moreover, after seeing the noticeable differences observed among species, the equilibrium between glycolysis and mitochondrial respiration would be different, depending on the specific functional and metabolic characteristics of each mammalian species (Ruíz-Pesini *et al.* 2007; Storey 2008; Piomboni *et al.* 2012).

Mitochondrial respiration is composed of two consecutive, separate steps. The first step is the citric acid cycle, or Krebs cycle. The main function of this step is the production of NADH. This NADH is needed for the net formation of ATP, which is produced in the second step of mitochondrial respiration. This second step is, in fact, composed of two parallel processes. One of these processes is the electron-transport chain, in which the NADH produced in the Krebs cycle is reduced after interaction with O_2, thus producing free protons. The other process is chemiosmosis, catalysed by the ATP-synthase complex, in which ATP is formed from ADP and inorganic phosphate (Grüber *et al.* 1994). This formation includes interaction with the free protons formed by the electron-transport chain through a proton gradient (Grüber *et al.* 1994). The optimal functioning of mitochondrial respiration, and hence the net formation of ATP from this source, is obtained after a close regulation of the rhythms of the Krebs cycle, electron-transport chain and chemiosmosis. Furthermore, mitochondrial respiration is also instrumental in the maintenance of a proper redox status of spermatozoa. It is noteworthy that mitochondrial respiration is a major site in the synthesis of free oxidative radicals (reactive oxygen species; ROS) through electron leakage via the electron-transport chain (Nohl *et al.* 2003), whereas reductive potential is also produced via NADH synthesis (Rich 2003). The equilibrium between the mitochondrial synthesis of both ROS and reductive potential is important, not only for the maintenance of proper basal sperm function, but also for the achievement of a sustainable capacitation status (see de Lamirande *et al.* 1997; O'Flaherty *et al.* 2006; Donà *et al.* 2011 as examples). Thus, mitochondrial respiration can modulate the achievement of capacitation both through the maintenance of energy levels and the achievement of the proper redox status and intracellular ROS levels. In eukaryotic cells, the rhythm of mitochondrial respiration and, thus, the concomitant rhythm of all of the associated processes mentioned before can be controlled by many separate systems. Changes in intracellular levels of ATP and ADP (Chance and Williams 1955) and intramitochondrial concentrations of Ca^{2+} (Brown 1992), among

others, are some of the most potent regulators of mitochondrial respiration.

Taking into account all of these data, the main aim of this work was to test the effect of the specific inhibition of mitochondrial ATP-synthase activity on both the maintenance of motility and the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis. This inhibition was performed by incubating boar spermatozoa in the presence of oligomycin A as a specific inhibitor of the oligomycin A-sensitive F_0 component of the mitochondrial ATP-synthase complex without any direct action on other components of mitochondrial respiration like the F_1 component of the ATP-synthase complex, the Krebs cycle, the electron transport chain (Chappell and Greville 1961; Grüber *et al.* 1994; Piomboni *et al.* 2012) or other ATPases such as dynein ATPase, which is directly related to the control of flagellar contractions (Belles-Isles *et al.* 1986; Bhattacharyya and Pakrashi 1993; Vívenes *et al.* 2009). Our experimental design was based on a simultaneous analysis of sperm mitochondrial activity and several molecular markers of motility and capacitation during the achievement of IVC and subsequent IVAE by incubating boar spermatozoa under two separate conditions: (1) standard IVC–IVAE process through the incubation in a standard capacitation medium (CM) specifically designed for boar, in which capacitation is induced in the absence of bicarbonate, but in the presence of bovine serum albumin (BSA; see Ramió *et al.* 2008; Ramió-Lluch *et al.* 2011, 2012*a*, 2012*b*) and (2) incubation of spermatozoa in CM containing oligomycin A. Furthermore, the addition of oligomycin A was carried out in two separate designs. In the first design, the inhibitor was added at time 0 h of the incubation of spermatozoa in CM, thus testing the effect of oligomycin A on both sperm motility and the achievement of IVC. The second design consisted of the addition of oligomycin A together with progesterone after 4 h of sperm incubation in CM in capacitating conditions. This design will test the effect of oligomycin A in the achievement of feasible IVAE. The analysed mitochondrial function and IVC markers included determination of the rhythm of O_2 consumption, intracellular ATP levels, mitochondrial membrane potential, objective motility, tyrosine phosphorylation status of the p32 acrosomal protein, fluidity and integrity of cell membranes and, as a final result of the achievement of IVC, progesterone-induced achievement of IVAE.

Materials and methods

Ethics

All procedures described within were approved by the Autonomous University of Barcelona Animal Care and Use Committee and were performed in accordance with the Animal Welfare Law issued by the Catalan Government (Generalitat de Catalunya, Spain).

Suppliers

All of the supplies were of analytical grade and came from Sigma, Boehringer-Mannheim (Mannheim, Germany) or Merck (Darmstadt, Germany), unless otherwise stated.

Boar semen collection

Commercial artificial insemination (AI) doses from boars of proven fertility were obtained from a commercial farm (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain). The spermatozoa were manually collected, maintained at 37°C in a water bath and diluted to 2×10^7 spermatozoa mL^{-1} in a commercial dose extender for refrigerated semen (MR-A Extender; Kubus, SA, Majadahonda, Spain) and distributed in 90-mL commercial doses. Six to seven of the 90-mL doses obtained from different ejaculates, chosen at random, were placed in a portable refrigerator at 16°C and immediately send to the laboratory. The approximate transporting time from the commercial farm to the laboratory was 45 min, as previously reported (Ramió-Lluch *et al.* 2011).

In vitro *capacitation and acrosome exocytosis procedures*

Fifty mL of each AI dose were washed thrice by centrifugation at 600*g* for 5 min at 16°C. Each time, spermatozoa were re-diluted in a 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (NCM). The osmolarity was 304 ± 5 mOsm and pH was adjusted to 7.4. After the last wash, the spermatozoa were resuspended in capacitating medium (CM) to a final concentration of $50–70 \times 10^6$ spermatozoa mL^{-1}. The CM was composed of NCM with 5 mg mL^{-1} bovine serum albumin (BSA) added. Incubation in CM was maintained for 4 h at 38.5°C in a 5% CO_2 atmosphere, as described by Ramió-Lluch *et al.* (2011). Simultaneously, a parallel semen aliquot from the same ejaculate was also subjected to the same washing and centrifugation process. After the final wash, however, this aliquot was again resuspended in NCM and was subsequently incubated for 4 h at 38.5°C in a 5% CO_2 atmosphere. This aliquot was utilised as a negative control for the achievement of IVC.

The induction of IVAE was performed through the addition of progesterone to the sperm suspension in CM, as described previously (Jiménez *et al.* 2003; Ramió *et al.* 2008). For this purpose, progesterone was added to a final concentration of 10 μg mL^{-1} to boar spermatozoa that had been incubated in CM for 4 h at 38.5°C in a 5% CO_2 atmosphere. After thoroughly mixing, the spermatozoa were further incubated for an additional 1 h at 38.5°C in a 5% CO_2 atmosphere. The same progesterone incubation protocol was carried out in the aliquots incubated in NCM. These aliquots were then utilised as negative controls for the achievement of progesterone-induced IVAE.

Sperm aliquots of 1.5 mL were taken at 0, 2 and 4 h of incubation in CM and 5, 15, 30 and 60 min after the addition of progesterone. When stated, spermatozoa were incubated in CM with 2.4 μM oligomycin A added (final concentration). As indicated above, the inhibitor was added in one of two separate experimental designs. The first design was based on the addition of oligomycin A to the CM at the same time as spermatozoa (Time 0 h). This design evaluated the effect of oligomycin A on the achievement of IVC status. The second design involved the addition of 2.4 μM oligomycin A together with 10 μg mL^{-1} progesterone after 4 h of incubation in CM in order to evaluate

the effect of oligomycin A on capacitated spermatozoa subjected to IVAE.

Evaluation of the achievement of capacitation and progesterone-induced acrosome exocytosis status

The evaluation of both IVC and IVAE was performed through the analysis of several well-known and previously described IVC- and IVAE-linked parameters (Ramió *et al.* 2008; Ramió-Lluch *et al.* 2011). These parameters were the percentage of viable spermatozoa subjected to progesterone-induced acrosome exocytosis (true acrosome exocytosis), the mean values of several motility parameters after a computer-assisted sperm-analysis system (CASA) and the increase in tyrosine phosphorylation levels of the acrosomal p32 protein. Furthermore, the analysis performed through flux cytometry on changes in cell-membrane lipid permeability changes is also a feasible parameter that contributes to the evaluation of the achievement of both IVC and IVAE. However, cytometric analyses will be detailed as a whole in another part of this section, since the specific particularities of this type of analysis made a global description of all of the parameters evaluated by using this system easier. In summary, the achievement of capacitation was evaluated after the overall evaluation of capacitation-compatible changes in sperm motility, p32 tyrosine phosphorylation, cell-membrane lipid permeability and especially the ability to respond to the induction of acrosome exocytosis after progesterone stimulus.

The percentage of true acrosome exocytosis was analysed through the simultaneous estimation of both the percentages of viable and altered acrosomes by using the bis-benzamidine–propidium iodide-Mitotracker Green FM-Alexa Fluor 488-conjugated lectin trypsin-inhibitor from soybean (SBTI) staining, as described in Bussalleu *et al.* (2005). This technique started with the incubation of an aliquot of sperm suspension in a solution of 15 mM bis-benzamidine (proportion 1 : 1000, v/v) for 10 min at 37°C. After this, a 2 mM propidium iodide solution was added (proportion 6 : 1000, v/v) and the spermatozoa were further incubated for 10 min at 37°C. Afterwards, the sperm suspension was centrifuged at 1500*g* for 10 min at 15°C and the supernatant discarded. The sperm pellet thus obtained was resuspended and subsequently incubated for 20 min at 37°C in 1 mL of a solution of 100 nM Mitotracker Green FM and FM-Alexa Fluor 488-conjugated SBTI in NCM. After this incubation, samples were immediately centrifuged at 1500*g* for 12 min at 15°C. The resultant sperm pellet was again resuspended in 100 mL of NCM at 37°C. Finally, the sperm suspension obtained was spread onto slides and fluorescence was immediately determined in a Zeiss Axioskop-40 fluorescence microscope (Karl Zeiss GmbH, Jena, Germany) with the appropriate filters. Viable and altered acrosome percentages were determined after counting 200–300 spermatozoa per slide at a magnification of 1000×. Unaltered acrosomes were considered to be those that showed a faint-to-moderate and uniform STBI lectin stain. On the contrary, altered acrosomes showed a very faint and non-uniform stain. Spermatozoa subjected to true acrosome exocytosis were considered to be those that showed, after the stimulation of IVAE, positive viability (blue stain of the sperm head) and an intense and non-uniform STBI lectin stain. Finally, non-viable spermatozoa showed an intense red stain of the head.

The CASA analysis was performed by using a commercial system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). The procedure started with a previous warming of semen samples at 37°C for 5 min in a water bath. Afterwards, 5-mL aliquots of these samples were placed onto a warmed (37°C) slide and covered with a 22 × 22 mm coverslip. Our CASA system was based upon the analysis of 25 consecutive, digitalised photographic images obtained from a single field at a magnification of 100× in a positive phase-contrast field. The velocity of image capturing was one photograph every 40 ms. Three separate fields were taken for each sample. The sperm motility descriptors obtained were described following Ramió *et al.* (2008). Settings programmed for all of the utilised motility parameters were the following:

Range of area particles: 10–80 μm^2.
Connectivity: a minimum of 11 images for all parameters, but a minimum of 10 images only for the mean amplitude of lateral head displacement (ALH).
Parameter ranges:
Mean velocity (VAP): the mean velocity of the sperm head along its average trajectory ($\mu m\ s^{-1}$).
Linear velocity (VSL): the straight trajectory of the spermatozoon per unit of time ($\mu m\ s^{-1}$).
Straightness coefficient (STR): (VSL/VAP) × 100 (%).
Mean amplitude of lateral head displacement (ALH): the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (μm).
Frequency of head displacement (BCF): the frequency with which the actual sperm trajectory crosses the average path trajectory (Hz).
Total motility was defined as the percentage of spermatozoa that showed a VAP above 10 $\mu m\ s^{-1}$.

Oxygen consumption measurement

Sperm oxygen consumption was estimated with a Clark oxygen electrode (Oxytherm; Hansatech Instruments Ltd, Norfolk, UK) linked to recorder system software (Oxygraph; Hansatech Instruments), as published in Ramió-Lluch *et al.* (2011). Temperature was maintained at 38.5°C by using a circulating water system throughout the DW1 oxygen electrode chamber. Furthermore, constant stirring by a magnetic flea ensured homogeneous distribution of O_2. The zero point was set by adding ~50 mM (estimated concentration) $Na_2S_2O_4$ to the distilled water-filled chamber (total volume 700 μL). Measurements were made by adding 900 μL of CM heated to 38.5°C followed by 100 μL of the sperm solution. The plunger was inserted to expel air, and O_2 consumption was monitored for 3 min. The mean sperm concentration in the DW1 chamber was 8×10^6 spermatozoa mL^{-1}. Data are presented as nmol O_2 consumed per viable spermatozoon.

Analysis of intracellular ATP levels

To determine the intracellular content of ATP, 250-μL aliquots of the sperm suspensions were taken and immediately centrifuged at 1000*g* for 1 min at 15°C. Supernatants were discarded

and the resultant cellular pellets were immediately frozen in liquid N_2. Samples were stored at −80°C until analysis. In all cases, a separate 10-μL aliquot was also collected for analysis of the total protein. To determine the concentrations of ATP, the frozen pellets were homogenised by sonication in 200 μL of ice-cold 10% (v/v) $HClO_4$. Afterwards, homogenates were centrifuged at 10 000*g* for 15 min at 4°C, and the supernatants obtained were neutralised with 5M K_2CO_3 before analysis. The ATP of samples was determined through the enzymatic technique described in Lambrecht and Transtschold (1974). Total protein content of the corresponding aliquot samples was determined by the Bradford method (Bradford 1976) using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of tyrosine phosphorylation levels of the acrosomal p32 protein

This analysis was performed from 1.5-mL aliquots of boar sperm suspensions. Cells were first centrifuged at 10 000*g* for 30 s at 15°C and the resultant pellet was immediately frozen at −196°C in liquid N_2 and stored at −80°C before use. When stated, sperm pellets were homogenised by sonication in 200 μL of ice-cold 50-mM Tris-HC buffer (pH 7.4) containing 1 mM EDTA, 10 mM EGTA, 25 mM DTT, 1.5% (w/v) Triton X-100, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM benzamidin, 10 μg mL^{-1} leupeptin and 1 mM Na_2VO_4, this latter added to avoid changes in the overall phosphorylation of the homogenates. Samples were then centrifuged at 13 000*g* for 15 min at 4°C. Supernatants were recovered and total protein content of the samples was determined through the Bradford method (Bradford 1976) by using a commercial kit (Bio-Rad Laboratories). Supernatants were immediately stored at −80°C until use. The sperm protein samples were boiled for 1 min before being transferred to the sodium dodecyl sulfate (SDS) gel, and polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was carried out following the standard protocol established in Laemmli (1970). The total amount of protein loaded in each lane was 15 μg.

Western blot analysis was carried out following the standard protocol of transferring the SDS–PAGE to nitrocellulose membranes (Burnette 1981). Transference was tested through the staining of membranes with red Poinceau stain (Bannur *et al.* 1999), which also allowed for the determination that the presence of BSA in the medium did not interfere with the position of p32 (data not shown). Transferred samples were tested by applying an anti-pTyr antibody (PY-20; Chemicon International, Temecula, CA, USA) as in Medrano *et al.* (2006). The final dilution of the primary antibody was of 1 : 1000 (v/v). Immunoreactive proteins were detected by using peroxidase-conjugated anti-mouse secondary antibody (Amersham, Buckinghamshire, UK). The reaction was developed with an ECL-Plus detection system (Amersham). Previous works showing the specificity of the anti-pTyr antibody used have already been published (Ramió-Lluch *et al.* 2011; Ramió-Lluch *et al.* 2012*b*), indicating the suitability of this specific antibody.

Flow cytometric analyses

Flow cytometry analyses were performed as in Yeste *et al.* (2013). Analyses were carried out in accordance with the recommendations of the International Society for Advancement of Cytometry (ISAC; see Lee *et al.* 2008). The sperm concentration of all of the analysed samples was adjusted to 1×10^6 spermatozoa mL^{-1} in a final volume of 500 μL. Spermatozoa were then stained with the appropriate combination of fluorochromes, following the protocols described below. Samples were evaluated through a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, California, USA). This instrument, whose original configuration provided by the manufacturer had not been altered, was equipped with two light sources: an arch-discharge lamp and an argon–ion laser (488 nm) set at a power of 22 mW. In our case, only the single-line visible light (488 nm) from the argon laser was used. Cell diameter and volume were directly measured with the Cell Laboratory Quanta SC cytometer employing the Coulter principle for volume assessment. This system has the forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size of the bead in channel 200 on the volume scale.

Optical filters were the following:

(1) FL1 (green fluorescence): Dichroic/Splitter; DRLP, 550 nm; BP filter, 525 nm; detection width, 505–545 nm.
(2) FL2 (orange fluorescence): DRLP, 600 nm; BP filter, 575 nm; detection width, 560–590 nm.
(3) FL3 (red fluorescence): LP filter, 670 nm; detection width, 655–685 nm.

Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (YO-PRO-1 and JC-1), while FL2 was used to detect JC-1_{agg} and FL3 was used to detect merocyanine-540 (M-540).

The sheath flow-rate was set at 4.17 μL min^{-1} in all analyses. Both EV and side scatter (SS) were recorded in a linear mode (in EE vs SS dot plots) for a minimum of 10 000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter <7 μm) and cell aggregates (particle diameter >12 μm). In this manner, the sperm-specific events were positively gated on the basis of EV and SS distributions. When stated, compensation was used to minimise spill-over of green fluorescence into the red channel.

Information on the events was collected as List-mode Data files (.LMD). The files generated were then analysed using the Cell Laboratory QuantaSC MPL analysis software (Version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs FL2 and FL1 vs FL3) and to analyse the cytometric histograms. Data obtained from flow-cytometry experiments were corrected according to the procedures described in Petrunkina and Harrison (2010) and Petrunkina *et al.* (2010). Each assessment for each sample and parameter was repeated three times in independent tubes in order to calculate the corresponding mean ± standard error of the mean (s.e.m.).

Unless otherwise stated, all fluorochromes used for these analyses were purchased from Molecular Probes (Invitrogen, Eugene, Oregon, USA) and were diluted with dimethyl sulfoxide (DMSO; Sigma). Flow-cytometry data were corrected

following the procedure described in Petrunkina and Harrison (2010), as stated at the end of this section. The cytometric analyses performed were those specified in the following paragraphs.

Analysis of membrane lipid changes (M540/YO-PRO-1)

Membrane lipid changes were assessed using the co-staining protocol for M-540 and YO-PRO-1 described by Harrison *et al.* (1996). In this procedure, sperm cells stained with the M-540 stain (M-540+ spermatozoa) presented an increase of their cell-membrane permeability that allowed the stain to enter the cell. Thus, these cells have been described as 'highly permeable cells'. Concomitantly, positivity to the YO-PRO-1 stain indicates an apoptotic-like type of membrane alteration, completely separated from that expressed by the M-540 stain (Idziorek *et al.* 1995; Harrison *et al.* 1996). Cells that showed this type of alteration have been described as 'YO-PRO-1+ cells', since we have not performed additional tests that confirm the instigation of a definite apoptotic process in these cells. This procedure allowed us to detect simultaneous changes in cell-membrane permeability (M-540 stain) and apoptotic-like changes (YO-PRO-1 stain). Cells that combined both types of membrane changes have been described as 'YO-PRO-1+/M-540+ cells'. Sperm samples were incubated for 10 min at 38°C with M-540 and YO-PRO-1 at a final concentration of 400 μM and 40 μM, respectively. The fluorescence of M-540 was detected through FL-2, while that of YO-PRO-1 was detected through FL-1.

Analysis of mitochondrial membrane potential

The potential of the mitochondrial membrane (MMP) was determined following the protocol described in Garner and Johnson (1995). Following this procedure, samples were incubated with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide) at a final concentration of 0.3 μM at 38°C for 30 min in the dark. Two different emission filters (FL-1 and FL-2) were used to differentiate two sperm populations:

(1) Spermatozoa with high MMP (JC-1 aggregates): these cells corresponded to the orange-stained spermatozoa, which appeared in the upper half of the diagram in FL1 vs FL2 dot-plots.
(2) Spermatozoa with low MMP (JC-1 monomers): these cells corresponded to the green-stained spermatozoa, which appeared in the lower half of the diagram in FL1 vs FL2 dot-plots.

These results allowed calculation of the percentage of spermatozoa with high MMP. Finally, data were not compensated in any way.

Correction of cytometric data: identification of non-DNA containing particles

In some flow-cytometry assessments there is the danger to perform an overestimation of the analysed sperm particles through contamination with non-DNA-containing particles. These particles, such as cytoplasmic droplets or cell debris will often show EV/FS and SS characteristics similar to those of spermatozoa, and thus cannot be excluded via light scatter (Petrunkina and Harrison 2010). Taking this into account, the percentage of non-DNA-containing particles was determined as described in Yeste *et al.* (2013). For this purpose, 5 μL of each sperm sample coming from each of the five treatments at each relevant time-point were diluted with 895 μL of milliQ-distilled water. Samples were then stained with PI at a final concentration of 10 μM and incubated at 38°C for 3 min, according to the procedure described in Harrison *et al.* (1996). Percentages of non-DNA-containing particles (f) were utilised to correct the percentages of non-stained spermatozoa (q_1) in each sample in a dual-staining analysis according to the following formula:

$$q_1' = \frac{q_1 - f}{100 - f} \times 100$$

where q_1' is the percentage of non-stained spermatozoa after correction.

General statistics

Statistical analyses were performed using the SPSS 15.0 for Windows statistical package (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± s.e.m. The data obtained from the analysis of all sperm parameters were tested for normality and homoscedasticity using both the Shapiro–Wilk and Levene tests. When needed, data were transformed using arcsine square root (arcsin $\sqrt{x}$) before a generalised lineal model (GLZM) was performed for repeated measures. In our model, the inter-subject factor was the treatment and the intra-subject factor was the time after thawing (i.e. 30 min or 240 min). In all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were calculated using Sidak's test.

Non-parametric procedures were used in parameters in which no transformation remedied the normality. The Friedman's test was performed as the non-parametric alternative to GLZM. Afterwards, the Wilcoxon matched-pairs test was utilised to evaluate differences between treatments as well as the effects of post-thawing time. In all statistical analyses, the minimal level of significance was set at $P < 0.05$.

Results

Effects of oligomycin A on sperm viability and true acrosome exocytosis

Incubation of boar spermatozoa in the standard CM induced a progressive decrease in the percentage of viability, which dropped from 86.0% ± 2.2% at 0 h to 62.9% ± 4.5% after 4 h of incubation (mean ± s.e.m.; Table 1). The subsequent addition of progesterone was followed by a further decrease in viability, which reached values of 54.6% ± 4.5% 60 min after the addition (Table 1). A much more intense drop in viability was observed in cells incubated in NCM throughout all of the determined incubation time (Table 1). Surprisingly, the incubation of cells in a CM medium supplemented with oligomycin A partially counteracted the observed decrease in viability. Thus, viability values of spermatozoa incubated in the presence of the inhibitor were 71.0% ± 1.0% after 4 h of incubation and 67.0% ± 4.5% 60 min after the addition of progesterone. These values were

Table 1. Mean values of viability, true acrosome exocytosis and several chosen motion parameters of boar spermatozoa subjected to *in vitro* capacitation and further progesterone-induced *in vitro* acrosome exocytosis in the presence or absence of oligomycin

Viability, true acrosome exocytosis, VAP, STR, ALH and BCF have been defined in the Materials and Methods section. Control, cells were subjected to a standard incubation in the capacitation medium; Oligomycin A IVC, cells were incubated in the presence of 2.4 μM oligomycin A from the start of the experiment; Oligomycin A IVAE, cells were incubated with 2.4 μM oligomycin A added together with 10 μg mL^{-1} progesterone after 4 h incubation in the capacitation medium; Non-capacitated, cells were incubated at the indicated times in a medium similar to that of the capacitation medium but without BSA (these cells were utilised as negative controls of capacitation, as indicated by the results obtained in percentages of viability and true acrosome exocytosis (constant and intense drop in viability together a total lack of progesterone-induced acrosome exocytosis)). Results are expressed as mean ± s.e.m. for seven separate experiments. Different superscripts in a row indicate significant ($P < 0.05$) differences between groups. Asterisks indicate significant ($P < 0.05$) differences, when compared with the respective Control values. Statistical analyses were performed as described in the Materials and Methods section

Parameter	Treatment	IVC			IVAE		
		0 h	2 h	4 h	5 min	30 min	60 min
Viability (%)	Control	86.0 ± 2.2^{a}	64.7 ± 4.2^{b}	62.9 ± 4.5^{b}	61.0 ± 2.0^{b}	55.3 ± 4.7^{b}	54.6 ± 4.6^{b}
	Oligomycin A IVC	84.2 ± 3.8^{a}	$73.4 \pm 3.6^{b*}$	$71.0 \pm 1.0^{b*}$	$68.9 \pm 2.0^{b*}$	$66.7 \pm 2.8^{b*}$	$67.0 \pm 4.5^{b*}$
	Oligomycin A IVAE	86.8 ± 1.3^{a}	63.4 ± 3.3^{b}	60.2 ± 1.1^{b}	$53.7 \pm 2.1^{c*}$	49.0 ± 3.3^{c}	60.8 ± 1.2^{d}
	Non-capacitated	85.9 ± 2.5^{a}	$56.4 \pm 3.0^{b*}$	$47.7 \pm 1.3^{c*}$	$48.1 \pm 1.5^{c*}$	$40.3 \pm 2.7^{d*}$	$31.6 \pm 1.1^{e*}$
True acrosome exocytosis (%)	Control	5.1 ± 3.3^{a}	4.6 ± 3.5^{a}	4.6 ± 3.9^{a}	20.7 ± 4.2^{b}	68.4 ± 6.2^{c}	72.3 ± 6.2^{c}
	Oligomycin A IVC	4.9 ± 2.3^{a}	6.3 ± 1.9^{a}	5.4 ± 2.0^{a}	19.2 ± 2.2^{b}	$19.9 \pm 1.4^{b*}$	$21.5 \pm 1.2^{b*}$
	Oligomycin A IVAE	5.4 ± 3.0^{a}	5.1 ± 2.0^{a}	4.9 ± 1.0^{a}	17.1 ± 2.2^{b}	$20.1 \pm 1.4^{b*}$	$22.2 \pm 2.2^{b*}$
	Non-capacitated	4.8 ± 2.5^{a}	4.1 ± 2.1^{a}	3.4 ± 1.7^{a}	$3.0 \pm 1.6^{a*}$	$3.3 \pm 2.2^{a*}$	$3.0 \pm 2.8^{a*}$
VAP (μm s^{-1})	Control	48.0 ± 0.9^{a}	57.1 ± 0.8^{b}	65.3 ± 1.4^{c}	63.2 ± 1.7^{c}	63.8 ± 1.3^{c}	59.6 ± 1.1^{bc}
	Oligomycin A IVC	$37.3 \pm 1.2^{a*}$	$36.9 \pm 1.2^{a*}$	$36.5 \pm 0.9^{a*}$	$34.3 \pm 1.5^{a*}$	$36.7 \pm 1.5^{a*}$	$36.7 \pm 2.1^{a*}$
	Oligomycin A IVAE	49.9 ± 1.2^{a}	59.0 ± 1.3^{b}	67.2 ± 1.3^{c}	$37.6 \pm 1.2^{d*}$	$35.4 \pm 1.1^{d*}$	$35.8 \pm 1.2^{d*}$
STR (%)	Control	63.9 ± 1.0^{a}	75.6 ± 1.3^{b}	75.9 ± 1.4^{b}	74.5 ± 1.7^{b}	78.6 ± 1.1^{bc}	79.8 ± 1.3^{c}
	Oligomycin A IVC	$58.2 \pm 1.6^{a*}$	$60.7 \pm 1.4^{a*}$	$66.6 \pm 2.2^{b*}$	$57.6 \pm 2.4^{a*}$	$60.2 \pm 1.0^{a*}$	$65.1 \pm 2.6^{b*}$
	Oligomycin A IVAE	64.2 ± 0.7^{a}	72.9 ± 3.1^{b}	77.6 ± 0.5^{c}	$61.0 \pm 1.0^{a*}$	$64.4 \pm 1.2^{a*}$	$53.0 \pm 3.1^{d*}$
ALH (μm)	Control	4.12 ± 0.20^{a}	3.82 ± 0.27^{a}	4.14 ± 0.27^{a}	4.50 ± 0.22^{b}	4.42 ± 0.26^{ab}	4.30 ± 0.20^{ab}
	Oligomycin A IVC	$3.68 \pm 0.17^{a*}$	$2.49 \pm 0.14^{b*}$	$2.87 \pm 0.14^{c*}$	$2.66 \pm 0.14^{c*}$	$2.84 \pm 0.14^{c*}$	$2.88 \pm 0.12^{c*}$
	Oligomycin A IVAE	4.11 ± 0.11^{a}	3.83 ± 0.20^{a}	4.04 ± 0.11^{a}	$3.05 \pm 0.11^{b*}$	$2.81 \pm 0.13^{b*}$	$2.90 \pm 0.14^{b*}$
BCF (Hz)	Control	6.3 ± 0.2^{a}	6.2 ± 0.1^{a}	6.3 ± 0.1^{a}	6.4 ± 0.1^{b}	6.4 ± 0.2^{a}	6.3 ± 0.2^{a}
	Oligomycin A IVC	$4.9 \pm 0.1^{a*}$	$4.1 \pm 0.4^{a*}$	$4.6 \pm 0.1^{a*}$	$3.3 \pm 0.1^{b*}$	$3.1 \pm 0.2^{b*}$	$3.3 \pm 0.2^{b*}$
	Oligomycin A IVAE	6.1 ± 0.3^{a}	6.4 ± 0.2^{a}	6.4 ± 0.2^{a}	$3.7 \pm 0.2^{b*}$	$4.2 \pm 0.2^{b*}$	$3.7 \pm 0.2^{b*}$

significantly higher ($P < 0.05$) than those observed in cells incubated in the standard CM medium. On the other hand, the addition of oligomycin A in a simultaneous manner as progesterone did not modify viability at any of the tested points (Table 1).

As expected, the addition of progesterone to cells incubated for 4 h in a standard CM medium induced a progressive increase in the percentage of acrosome exocytosis, which reached maximal values 1 h after progesterone addition (Table 1). As expected, no increase was observed in cells incubated in NCM medium (Table 1). Remarkably, the addition of oligomycin A prevented this increase (Table 1). A similar lack of acrosome exocytosis stimulation was observed when oligomycin A was added together with progesterone 4 h after incubation in a standard CM medium (Table 1).

Effects of oligomycin A on sperm motility

Total motility suffered a slight, progressive decrease after incubation in a standard CM for 4 h. Thus, this parameter went from 82.8% ± 4.1% at 0 h of incubation to 70.2% ± 3.5% after 4 h of incubation. Subsequent progesterone addition was related to a more intense decrease of this parameter, which reached values of 57.4% ± 2.2% 60 min after progesterone addition (Fig. 1). As in the case of viability, a much more intense drop in total motility was observed in cells incubated in NCM throughout all of the determined incubation time (Table 1).

The addition of oligomycin A to the CM induced an immediate and almost complete inhibition of sperm motility, which had values of 9.8% ± 1.4% at 0 h of incubation and of 5.6% ± 1.2% after 4 h of incubation in the CM medium followed by another 60 min of incubation in the presence of progesterone (Fig. 1). Likewise, the addition of oligomycin A together with progesterone after 4 h incubation in the CM medium also induced a rapid and almost complete inhibition of sperm motility, reaching total motility values of 7.0% ± 1.9% 60 min after the incubation (Fig. 1).

Regarding motion parameters, as expected, the incubation in a standard CM for 4 h induced a progressive increase of VAP and STR (Table 1). These results were concordant with those previously published by our laboratory (Ramió *et al.* 2008; Ramió-Lluch *et al.* 2011) and other authors (García Herreros *et al.* 2005), which were indicative of the achievement of IVC in boar, despite that in other species motility parameters suffered other type of changes in the achievement of IVC. Subsequent progesterone addition had little effect on these parameters, although an overall, slight decrease was observed after 60 min of incubation (Table 1).

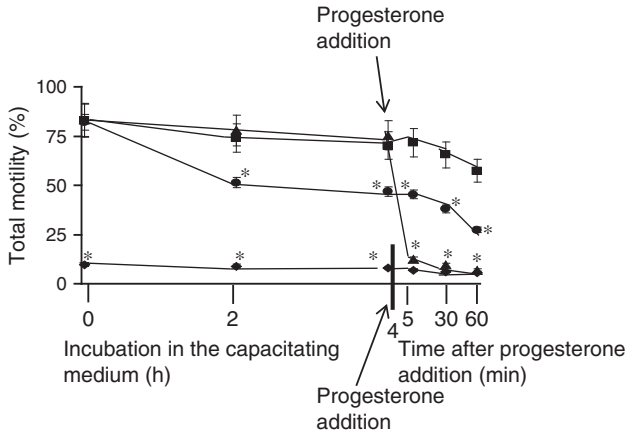

Fig. 1. Percentages of total motility of boar spermatozoa subjected to *in vitro* capacitation and subsequent *in vitro* acrosome reaction in the presence or absence of oligomycin A. Boar spermatozoa were incubated for 4 h and then had 10 μg mL^{-1} progesterone added and were then subjected to further incubation for 60 min, as described in the Materials and Methods section. Likewise, the separate experimental groups have also been defined in the appropriate Materials and Methods section. Total motility has been defined in the Materials and Methods section. ■ Spermatozoa incubated in standard capacitation medium. ◆ Spermatozoa incubated in capacitation medium with 2.4 μM oligomycin A added from the beginning of the incubation. ▲ Spermatozoa incubated in capacitation medium for 4 h and subsequently with 10 μg mL^{-1} progesterone and 2.4 μM oligomycin A added together. ● Spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These cells were utilised as negative controls of capacitation, as indicated by the results shown here (constant and intense drop in total motility when compared with spermatozoa incubated in the standard capacitation medium). Results are expressed as mean ± s.e.m. for seven separate experiments. Asterisks indicate significant ($P < 0.05$) differences, when compared with the respective values obtained in cells incubated in capacitation medium.

The addition of oligomycin A to the CM induced an overall decrease in almost all of the analysed motion parameters in the very few detected motile spermatozoa. These effects were observed immediately after the addition of oligomycin A and were maintained for all of the analysed incubation times (Table 1). A similar effect was obtained when oligomycin A was added together with progesterone after 4 h of incubation in the standard CM (Table 1).

Effects of oligomycin A on tyrosine phosphorylation levels of the acrosomal p32 protein

As expected, tyrosine phosphorylation levels of the acrosomal p32 protein were faint or even nonexistent in boar spermatozoa taken at the 0 h time-point of incubation in the CM (Fig. 2). The subsequent incubation in CM induced a progressive increase in tyrosine phosphorylation levels of the acrosomal p32 protein, which was, in all cases, evident after 4 h of incubation (Fig. 2*a*, *c*). On the contrary, no tyrosine phosphorylated p32 protein was observed in any case when spermatozoa were incubated for 4 h in NCM (Fig. 2*d*). This is the result of the failure to achieve a feasible IVC of spermatozoa incubated in NCM. Tyrosine phosphorylation of p32 was maintained or, in some cases, further increased following the addition of

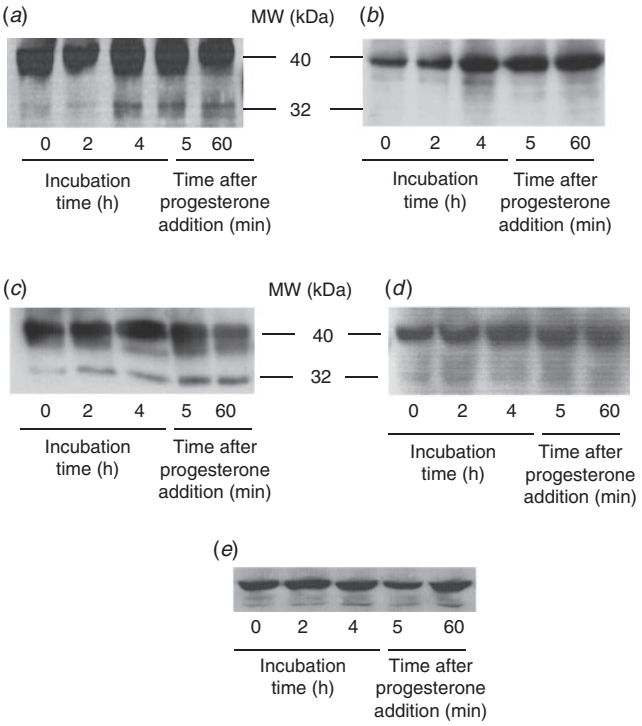

Fig. 2. Tyrosine phosphorylation levels of the p32 protein after the induction of *in vitro* capacitation and subsequent *in vitro* acrosome reaction in the presence or absence of oligomycin A. Boar spermatozoa were incubated for 4 h and then had 10 μg mL^{-1} progesterone added and were then subjected to further incubation for 60 min, as described in the Materials and Methods section. (*a*) Spermatozoa incubated in capacitation medium. (*b*) Spermatozoa incubated in the capacitation medium with 2.4 μM oligomycin A added at Time 0 h. (*c*) Spermatozoa incubated in capacitation medium for 4 h and subsequently with 10 μg mL^{-1} progesterone and 2.4 μM oligomycin A added together. (*d*) Spermatozoa incubated in a medium similar to the capacitation medium but without BSA. These were utilised as a negative control of the achievement of capacitation, as compared with the capacitation medium, as illustrated by the lack of increase in tyrosine phosphorylation of the p32 protein. (*e*) Western blot against β-tubulin of spermatozoa incubated in capacitation medium. This is shown as a sample of the utilisation of β-tubulin as an internal control for the total amount of protein loaded in each lane. Data shown are representative images for seven separate experiments.

progesterone after 4 h of incubation in CM. The p32 tyrosine phosphorylation levels were maintained 60 min after progesterone addition (Fig. 2*a*).

The addition of oligomycin A at the 0 h time-point of incubation prevented, in all cases, the increase of tyrosine phosphorylation levels. In fact, there was no tyrosine phosphorylated p32 protein in any of the experiments carried out after 4 h of incubation in CM (Fig. 2*b*). The addition of oligomycin together with progesterone after 4 h of incubation in CM, however, did not have any clear effect on the observed tyrosine phosphorylation levels of p32 (Fig. 2*c*).

Effects of oligomycin A on sperm membrane fluidity and stability

Sperm cells incubated in CM showed a steady increase in membrane fluidity, as the M-540 test indicated. The percentage

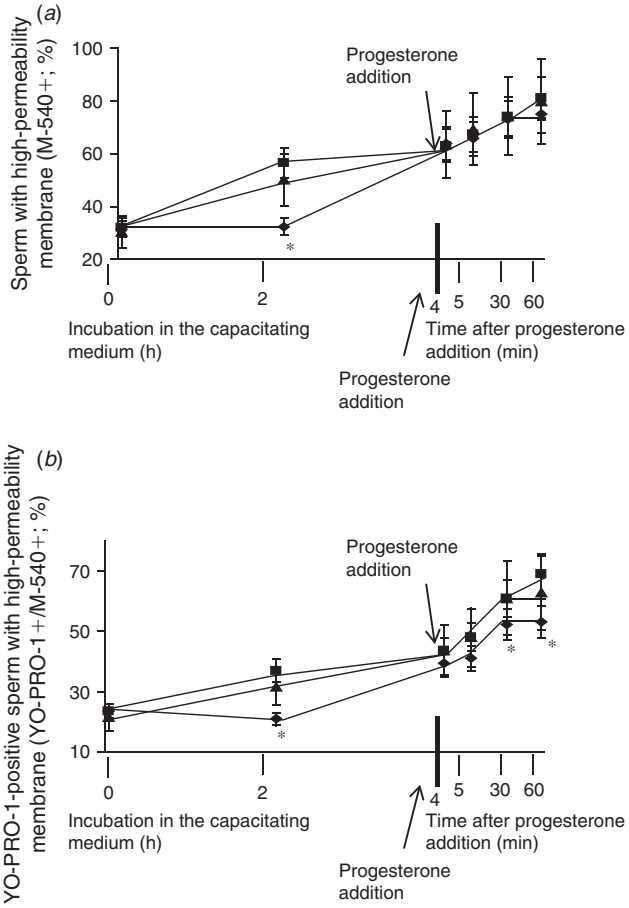

Fig. 3. Percentages of both boar spermatozoa with high-permeability cell membranes and boar spermatozoa with positive YO-PRO-1 stain simultaneously with concomitant high-permeability cell membrane following merocyanine-540 stain after the induction of *in vitro* capacitation and subsequent *in vitro* acrosome reaction in the presence or absence of oligomycin A. Boar spermatozoa were incubated for 4 h and then had 10 μg mL^{-1} progesterone added and were then subjected to further incubation for 60 min, as described in the Materials and Methods section. Likewise, the separate experimental groups have also been defined in the appropriate Materials and Methods section. The analysed parameters have been defined in the Materials and Methods section. (*a*) Percentages of cells with high-permeability cell membranes. (*b*) Percentages of spermatozoa with positive YO-PRO-1 stain simultaneously with concomitant high-permeability cell membranes. ■ Spermatozoa incubated in capacitation medium. ◆ Spermatozoa incubated in capacitation medium with 2.4 μM oligomycin A added from the beginning of the incubation. ▲ Spermatozoa incubated in a standard capacitation medium for 4 h and subsequently with 10 μg mL^{-1} progesterone and 2.4 μM oligomycin A added together. Results are expressed as mean ± s.e.m. for seven separate experiments. Asterisks indicate significant ($P < 0.05$) differences, when compared with the respective control values.

of total cells that presented high cell-membrane-fluidity characteristics increased from 32.7% ± 5.8% at 0 h to 63.4 ± 7.3% after 4 h of incubation (Fig. 3*a*). The subsequent progesterone addition did not modify this tendency, and the percentage of total cells with highly fluid membranes reached values of 81.5% ± 7.9% 60 min after progesterone addition (Fig. 3*a*). The addition of oligomycin A in the CM at Time 0 h only had a significant effect after 2 h of incubation with the percentage of cells with high-fluidity membranes significantly lower ($P < 0.05$) than that observed in control samples (Fig. 3*a*). The simultaneous addition of progesterone and oligomycin A to cells incubated for 4 h in CM did not modify this parameter (Fig. 3*a*).

Similar results were observed when membrane fluidity was simultaneously analysed with YO-PRO staining. The percentage of cells which had high-fluidity membranes and were concomitantly positive for the YO-PRO stain steadily increased after incubation in CM. Again, this percentage was further increased after the addition of progesterone, reaching values of 69.6% ± 6.9% 60 min after progesterone addition (Fig. 3*b*). The incubation of sperm cells in CM with oligomycin A added had a lowering effect, although it was only statistically significant ($P < 0.05$) at 2 h of incubation. The addition of progesterone to the oligomycin A-complemented CM after 4 h of incubation partially counteracted the observed increase in the percentage of YO-PRO-positive, high-fluidity-membrane cells. The simultaneous addition of progesterone and oligomycin A to cells incubated for 4 h in CM did not significantly modify the percentage of YO-PRO-positive, high-fluidity-membrane spermatozoa (Fig. 3*b*).

Effects of oligomycin A on O_2 consumption and intracellular ATP levels

Boar sperm cells showed a low level of O_2 consumption rhythm at 0 h of incubation in a standard CM of 1.8 ± 0.4 nmol 10^{-7} viable cells (Fig. 4*a*). This rhythm was not significantly changed during incubation after 4 h in the standard CM (Fig. 4*a*). Addition of progesterone after 4 h of incubation in the CM induced a rapid, intense and transient peak in O_2 consumption, which reached values of 3.3 ± 0.7 nmol 10^{-7} viable cells 5 min after the addition of the hormone (Fig. 4*a*). Afterwards, O_2 consumption decreased, reaching minimal values 60 min after the progesterone addition (Fig. 4*a*).

The addition of oligomycin A to the CM, both at Time 0 h and concomitantly with progesterone addition, in all cases inhibited the progesterone-induced peak of O_2 consumption observed after 4 h of incubation in CM (Fig. 4*a*). However, oligomycin A did not cause any other change in O_2 consumption throughout all of the experimental procedure, with no differences being observed between cells incubated in the CM and those incubated in the presence of the inhibitor (Fig. 4*a*).

The dynamics of ATP intracellular levels followed a pattern similar to that observed in the rhythm of O_2 consumption. Thus, as shown in Fig. 4*b*, boar spermatozoa did not significantly modify ATP levels after 4 h of incubation in a standard CM, which went from 9.1 ± 1.8 nmol mg^{-1} protein at 0 h to 6.5 ± 1.3 nmol mg^{-1} protein. The addition of progesterone after 4 h of incubation in CM induced a rapid, significant ($P < 0.05$) and intense increase in ATP levels, which reached values of 14.2 ± 2.2 nmol mg^{-1} protein 5 min after progesterone addition (Fig. 4*b*). Subsequently, ATP levels suffered a progressive decrease, reaching values of 5.3 ± 0.4 nmol mg^{-1} protein 60 min after progesterone addition (Fig. 4*b*). The addition of oligomycin A to the CM did not significantly modify ATP levels

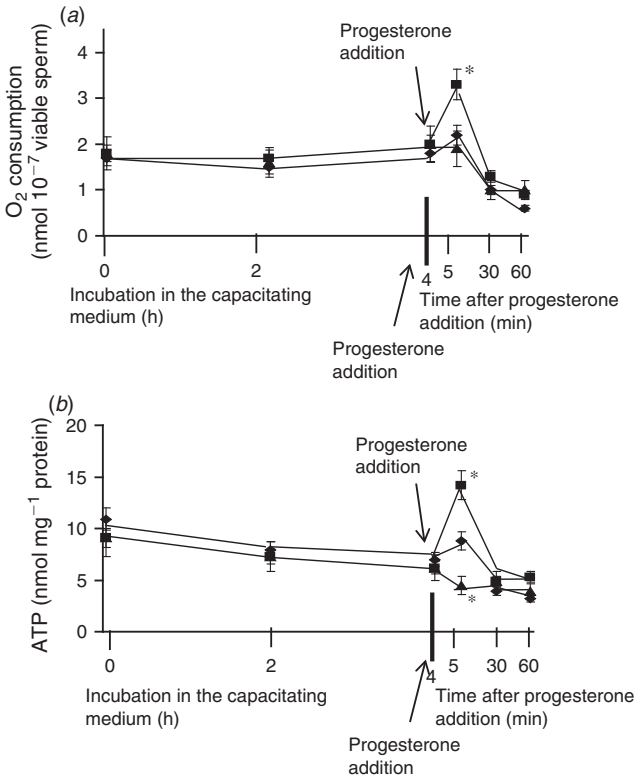

Fig. 4. Rhythm of O_2 consumption and intracellular ATP levels of boar spermatozoa subjected to *in vitro* capacitation and subsequent *in vitro* acrosome reaction in the presence or absence of oligomycin A. Boar spermatozoa were incubated for 4 h and then had 10 μg mL^{-1} progesterone added and were then subjected to further incubation for 60 min, as described in the Materials and Methods section. Likewise, the separate experimental groups have also been defined in the appropriate Materials and Methods section. Parameters have been defined in the Materials and Methods section. (*a*) Rhythm of O_2 consumption. (*b*) Intracellular ATP levels. ■ Spermatozoa incubated in capacitation medium. ◆ Spermatozoa incubated in capacitation medium with 2.4 μM oligomycin A added from the beginning of the incubation. ▲ Spermatozoa incubated in a standard capacitation medium for 4 h and subsequently with 10 μg mL^{-1} progesterone and 2.4 μM oligomycin A added together. Results are expressed as mean ± s.e.m. for seven separate experiments. Asterisks indicate significant ($P < 0.05$) differences, when compared with the respective control values.

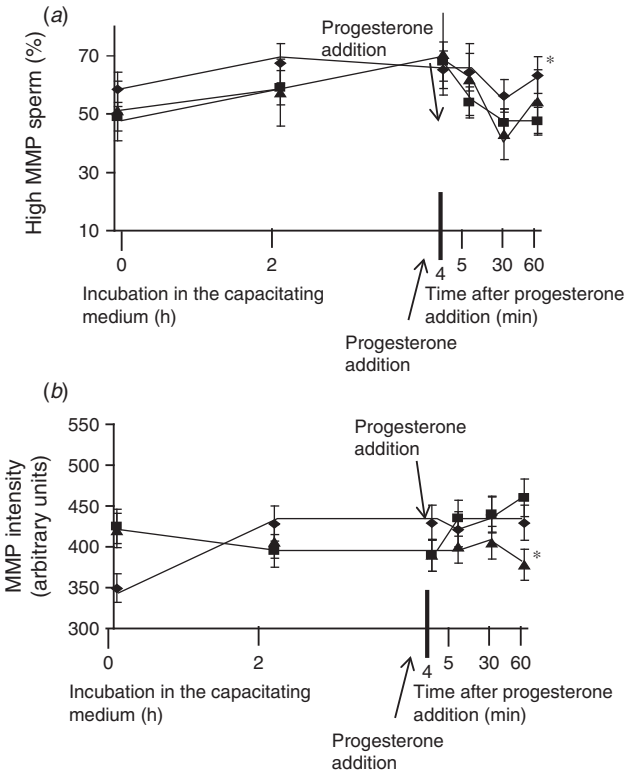

Fig. 5. Percentages of boar spermatozoa with high mitochondrial membrane potential and mean intensity levels of JC-1 stain of boar spermatozoa with high mitochondrial membrane potential following the JC-1 stain after the induction of *in vitro* capacitation and subsequent *in vitro* acrosome reaction in the presence or absence of oligomycin A. Boar spermatozoa were incubated for 4 h and then had 10 μg mL^{-1} progesterone added and were then subjected to further incubation for 60 min, as described in the Materials and Methods section. Likewise, the separate experimental groups have also been defined in the appropriate Materials and Methods section. Parameters have been defined in the Materials and Methods section. (*a*) Percentages of boar spermatozoa with high mitochondrial membrane potential. (*b*) Mean intensity levels of JC-1 stain of boar spermatozoa with high mitochondrial membrane potential. ■ Spermatozoa incubated in capacitation medium. ◆ Spermatozoa incubated in capacitation medium with 2.4 μM oligomycin A added from the beginning of the incubation. ▲ Spermatozoa incubated in a standard capacitation medium for 4 h and subsequently with 10 μg mL^{-1} progesterone and 2.4 μM oligomycin A added together. Results are expressed as mean ± s.e.m. for seven separate experiments. Asterisks indicate significant ($P < 0.05$) differences, when compared with the respective control values.

after 4 h of incubation. However, the progesterone-induced transient increase of ATP was abolished by oligomycin A, both when added at the start of the incubation and when added together with progesterone (Fig. 4*b*). There was no other effect on ATP levels of oligomycin A than that of the inhibition of the progesterone-induced ATP peak.

Effects of oligomycin A on mitochondrial membrane potential

The percentage of boar spermatozoa with high MMP progressively increased during incubation in CM. Thus, this value went from 49.5% ± 7.5% at 0 h to 68.4% ± 9.0% after 4 h of incubation (Fig. 5*a*). The subsequent addition of progesterone induced a decrease of this percentage, which reached values of 47.6% ± 7.0% 30 min after the addition of the hormone (Fig. 5*a*); this decrease was maintained 60 min after progesterone addition.

The incubation of boar spermatozoa in CM with oligomycin A added did not have any significant effect on the percentage of high-MMP boar spermatozoa after 4 h of incubation. However, the subsequent addition of progesterone did induce a decrease of this percentage that was smaller than that observed in control CM. In fact, this decrease was completely counteracted 60 min after progesterone addition (Fig. 5*a*). The combined addition of progesterone and oligomycin A to cells incubated for 4 h in CM

did not induce any significant separate effect, when compared with spermatozoa incubated in CM and with progesterone alone subsequently added (Fig. 5*a*).

Regarding the mean intensity values of MMP, this parameter did not significantly change during incubation of cells in CM for 4 h (Fig. 5*b*). The subsequent addition of progesterone induced a progressive increase of this intensity (Fig. 5*b*). Incubation of sperm cells in CM with oligomycin A added did not show any significant change in this pattern. However, when oligomycin A was added together with progesterone after 4 h of incubation, the progesterone-induced increase in MMP intensity was counteracted, reaching values of 378.2 ± 8.5 arbitrary units 60 min after the addition of the effectors (Fig. 5*b*).

Discussion

Our results clearly show that the maintenance of the activity of the F_0 component of the mitochondrial ATP-synthase complex is essential for the maintenance of boar sperm motility in basal conditions, defined as an incubation time in CM below 4 h, as well as for the achievement of both IVC and subsequent progesterone-induced IVAE. However, the specific importance of the F_0 component activity seems to be different in each case, depending on the exact functional status in which boar spermatozoa are analysed. This is highlighted when comparing the results of motility obtained with the addition of oligomycin A to the CM. Taking into account these results it is evident that the F_0 component-dependent production of ATP is needed to maintain motility. On the contrary, the maintenance of sperm viability does not essentially require the activity of this component, as the results involving viability and acrosome status during the first 4 h of incubation in the CM indicate. The specific necessity of an active oligomycin A-sensitive ATP synthase, and thus of a functional whole chemiosmosis step, to maintain motility is highlighted by the fact that the addition of oligomycin A inhibits motility without affecting the rhythm of O_2 production or the MMP during practically all of the incubation time in the CM. The lack of effect on MMP and O_2 consumption indicates that the effect of oligomycin A is exerted without any significant effect on the O_2-consuming production of reductive potential, a role which is played by the combined action of the Krebs cycle and the subsequent electronic chain, but not directly by chemiosmosis. A similar lack of a direct relationship between MMP and the electron transport chain and sperm motility has already been observed in human and dog (Nascimento *et al.* 2008) and bull (Krzyzosiak *et al.* 1999). Furthermore, the inhibition of the mitochondrial oligomycin A-sensitive ATP synthase suppresses tumour necrosis factor (TNF)-induced apoptosis in HeLa cells without affecting either MMP or overall intracellular ATP levels (Shchepina *et al.* 2002), showing thus an effect similar to that demonstrated here on sperm motility. Reinforcing the explanation of the effect induced by oligomycin A, Aitken *et al.* (2012) have recently indicated that the inhibition of sperm motility mediated by the presence of free oxidative radicals is carried out without significant changes in MMP in species like human, rat and horse. However, a positive correlation between sperm motility and MMP has been found in rat (Gravance *et al.* 2000*b*) and, in species such as human it has been proposed that the MMP ensures, when sufficiently high, a proper sperm motility (Espinoza *et al.* 2009). This highlights the great differences that can be found between species, making the interspecies comparison of results regarding mammalian sperm function thus very difficult.

Our results show that the control that oligomycin A-sensitive ATP synthase exerts on boar sperm motility seems not to be linked to the overall effect that this inhibition has on the overall sperm ATP intracellular levels. This can be deduced by the fact that the inhibition of the oligomycin A-sensitive ATP synthase did not have any significant effect on the overall intracellular ATP levels. This result is compatible with previous results that indicated that mitochondrial respiration of boar spermatozoa incubated in the presence of glucose contributes as much as 5% to the overall energy production of the cell, the other 95% being obtained from glycolysis (Marín *et al.* 2003). The fact that the relatively low levels of energy produced through the oligomycin A-sensitive ATP synthase by itself directly affects sperm motility despite the overall sperm energy levels not being essentially affected is striking, although a similar lack of relationship has been observed in the oligomycin A-induced inhibition of TNF-induced apoptosis in HeLa cells (Shchepina *et al.* 2002). We can only speculate on this point. In this respect, a link between the overall ATP content and motility has been observed in experiments involving mouse spermatozoa with a lack of lactate dehydrogenase C activity (Odet *et al.* 2013). This result is, of course, different from that found by our group in boar spermatozoa. These differences strongly suggest the existence of species-specific differences in the mechanisms by which mammalian spermatozoa modulate their ATP levels. All of these apparently contradictory and difficult-to-explain results clearly indicate the necessity of more in-depth studies focusing on this point.

The achievement of feasible IVC seems also to be dependent on a correct oligomycin A-sensitive ATP-synthase activity. This is evident when the effects of the addition of oligomycin A to the CM at the start of the IVC process are analysed. In this case, IVC-linked phenomena such as changes in membrane fluidity status, phosphorylation of the acrosomal p32 protein and intracellular Ca^{2+} levels are either abolished or altered. It is obvious that the majority of these IVC-linked processes are ATP-consuming, although the exact origin of this ATP, either from glycolysis or from mitochondrial respiration, is not known. In any case, the fact that the oligomycin A-induced inhibition of ATP-synthase activity was not related to a significant decrease in intracellular ATP levels seems to indicate that the overall failure in obtaining energy sources would not be the main reason for the oligomycin A-induced failure in the achievement of IVC in our conditions. In this respect, we must remember that in species such as mice spermatozoa can utilise ATP from either glycolysis or mitochondrial respiration indifferently to maintain their overall function (Pasupuleti 2007). This would indicate that the final source of the total energy needed to maintain overall sperm function would not be primordial, since this source can be modified in accordance with the environmental circumstances. If we consider this hypothesis as being correct, the maintenance of a basal activity of the oligomycin A-sensitive F0 component of the ATP synthase is important to

maintain essential sperm functions, like the ability to achieve feasible IVC. However, this activity would be important not only for its overall contribution to the total intracellular ATP levels, but also for the necessity of this minimal amount of ATP to start or maintain sperm functions like the IVC-linked changes in membrane fluidity and acrosomal p32 phosphorylation.

It is well known that all of the effectors included in the oligomycin family have a more or less intense inhibitory effect on the cell membrane ion channel Na^+/K^+-ATPase, regardless of other more specific effects caused by each compound of the family (Garrahan and Glynn 1967; Sachs 1980). In our case, the inhibitory effect of oligomycin A on boar sperm Na^+/K^+-ATPase is only partial, taking into account that the estimated K_i of oligomycin A for this enzyme is ~4.5 μM (Arato-Oshima *et al.* 1996), whereas the concentration of oligomycin A utilised in our experiments was 2.4 μM. At first glance, the partial inhibition of the Na^+/K^+-ATPase induced by oligomycin A would not have any significant effect on the achievement of IVC by boar spermatozoa. In fact, Na^+/K^+-ATPase activity in cells like spermatozoa would be mainly related to the adaptation of cells to environmental osmotic changes (Alberts *et al.* 1983; Rodríguez-Gil and Rigau 1996; Caiza de la Cueva *et al.* 1997; Peris *et al.* 2000). However, it has been described that the complete inhibition of Na^+/K^+-ATPase activity in boar spermatozoa by incubation with ouabain does not modify the ability of these cells to respond to sudden changes in osmolarity (Rodríguez-Gil and Rigau 1996). This seems to indicate that, in boar spermatozoa, Na^+/K^+-ATPase plays a secondary role as osmotic regulator. Nevertheless, it is possible that the partial inhibition of Na^+/K^+-ATPase could have some effects on other points, such as boar sperm cell-membrane stability. We must remember that this ATPase is a transmembrane protein (Shinoda *et al.* 2009), and the destabilisation of its function could putatively affect cell-membrane structure, yielding to the launching of apoptotic-like phenomena of membrane destabilisation. However, this hypothesis seems unlikely, since the results shown here regarding merocyanine-540, and especially YO-PRO-1, do not suggest the activation of apoptotic-like changes on cell-membrane structure caused by the oligomycin A incubation. Thus, we do not have any strong reason to suppose that any of the observed effects of oligomycin A on boar sperm motility and the achievement of IVC and IVAE were a direct consequence of the partial inhibition of the cell-membrane Na^+/K^+-ATPase activity.

Our results also indicate that the progesterone-induced IVAE of boar spermatozoa is another event that requires a correct functioning of the oligomycin A-sensitive ATP-synthase activity. Notwithstanding, IVAE requires something more than the mere maintenance of this activity. First of all, IVAE achievement is concomitant with an intense, fast and transient increase in not only O_2 consumption, as previously published (Ramió-Lluch *et al.* 2011), but also in intracellular ATP levels. The addition of oligomycin A both at the start of the IVC process and simultaneously with progesterone after 4 h of incubation concomitantly inhibits the progesterone-related O_2 consumption peak, the ATP level increase and the subsequent IVAE induction. These results would indicate that both the O_2 consumption peak and the concomitant increase in ATP are linked to a sudden and transitory activation of the F_0 modulated, oligomycin A-sensitive ATP synthase and, thus, of the chemiosmotic step in its entirety. This additional and transitory energy source would be needed in order to establish the molecular mechanisms involved in the achievement of progesterone-induced IVAE. Again, more studies would be needed in order to clarify this issue.

As final conclusions, our results clearly indicate that a proper functioning of the F_0 component, sensitive to oligomycin A, of the mitochondrial ATP-synthase complex is instrumental in the achievement not only of a proper boar sperm motion pattern, but also in that of feasible IVC and subsequent progesterone-induced IVAE. However, the exact manner in which the F_0 component of the ATP-synthase complex plays its role seems to vary depending on the exact functional status of the boar sperm cell.

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