

## Technology Report

# Influence of different cellular concentrations of boar sperm suspensions on the induction of capacitation and acrosome reaction

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**Abstract.** We aimed to analyze the influence of different cellular concentrations of boar sperm suspensions on the induction of capacitation and acrosome reaction. When spermatozoa were incubated at 100 or 200 mill/ml, significant increases in protein tyrosine phosphorylation in the p32 protein were observed, compared to those at 50 mill/ml. In addition, sperm concentration-dependent increases were observed in plasma membrane lipid disorganization (50 mill/ml vs. 200 mill/ml), induction of the acrosome reaction (50 mill/ml vs. 100 mill/ml and 200 mill/ml), and sperm viability (50 mill/ml vs. 100 mill/ml and 200 mill/ml). Our data indicate that an increase in sperm concentration stimulates the induction of capacitation and acrosome reaction in boars.

**Key words:** Acrosome reaction, Boar spermatozoa, Capacitation

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Currently, assisted reproductive techniques (ARTs) are widely implemented for domestic species. In recent years, significant advances in ARTs have facilitated the development and improvement of *in vitro* fertilization (IVF) protocols, resulting in the commercialization of *in vitro*-produced embryos. To this end, great efforts have been put into the design of fertilization media depending on the requirements of the species. Generally, fertilization media are supplemented with different substances, such as heparin in cattle [1] or caffeine in pigs [2], to induce sperm capacitation. Capacitation of spermatozoa occurs in the female reproductive tract, and consists of a series of events that render spermatozoa capable of fertilizing an oocyte [3, 4]. Early capacitation-related events include an increase in the intracellular levels of bicarbonate and calcium, which induce cholesterol efflux from the plasmalemma, phospholipid scrambling, and an increase in membrane fluidity [5]. These early events induce the activation of adenylyl cyclases, resulting in increased intracellular cyclic adenosine monophosphate (cAMP) levels. Increase in cAMP activates protein kinase A (PKA), which leads to an increase in overall protein tyrosine phosphorylation (PY) [6, 7], and specifically, in the p32 protein in boar spermatozoa [8]. Finally, capacitated spermatozoa must undergo the acrosome reaction, which is an exocytotic process required for successful fertilization [9]. To improve the composition of fertilization media, most studies so far have focused on determining the composition of natural reproductive fluids, such as follicular, uterine, or oviductal fluids, to create a physiological environment

more conducive to capacitation [10]. However, little attention has been given to mimicking the physiological conditions of the sperm reservoir where many spermatozoa accumulate, even though such phenomenon is believed to be important for successful fertilization [11, 12].

The objective of the present study was to analyze the influence of different cellular concentrations of boar sperm suspensions on the induction of capacitation and acrosome reaction *in vitro*. To achieve this objective, boar spermatozoa were incubated at different cellular concentrations (50, 100, and 200 mill/ml) in Tyrode's complete medium (TCM; a capacitating medium). After incubation (4 h at 38.5°C), capacitation-related events such as plasma membrane lipid disorganization, PY, phosphorylation of PKA substrates, and the induction of acrosome reaction, were analyzed. When boar spermatozoa were incubated at 100 or 200 mill/ml, a significant increase in PY in p32 was observed (Fig. 1A;  $P < 0.05$ ). This increase in PY was not accompanied by a significant rise in the levels of phosphorylated PKA substrates (Fig. 1B;  $P > 0.05$ ). In addition, sperm concentration-dependent increases were observed in the percentage of spermatozoa showing plasma membrane lipid disorganization ( $25.6 \pm 4.7$  [50 mill/ml] vs.  $44.9 \pm 5.8$  [200 mill/ml], mean %  $\pm$  SEM,  $P < 0.05$ ; Fig. 2A) and the percentage of live acrosome-reacted spermatozoa ( $6.6 \pm 0.6$  [50 mill/ml] vs.  $14.3 \pm 2.2$  [100 mill/ml] and  $33.6 \pm 4.1$  [200 mill/ml],  $P < 0.05$ ; Fig. 3A). Furthermore, a significant increase was observed in the percentage of live spermatozoa at 100 and 200 mill/ml, compared to 50 mill/ml ( $58.4 \pm 3.1$  [50 mill/ml] vs.  $66.6 \pm 2$  [100 mill/ml] and  $73.2 \pm 1.4$  [200 mill/ml],  $P < 0.05$ ; Fig. 3B).

In the present study, different approaches that have been previously validated were used to determine the induction of capacitation in boar spermatozoa, which are as follows: a) increased disorder in plasma membrane lipid packing using Merocyanine 540 [13], b) increased PY in p32 [8, 14], and c) increased phosphorylation of PKA substrates [15]. The results obtained in this work show that the 4 h incubation of boar spermatozoa at higher cellular concentrations

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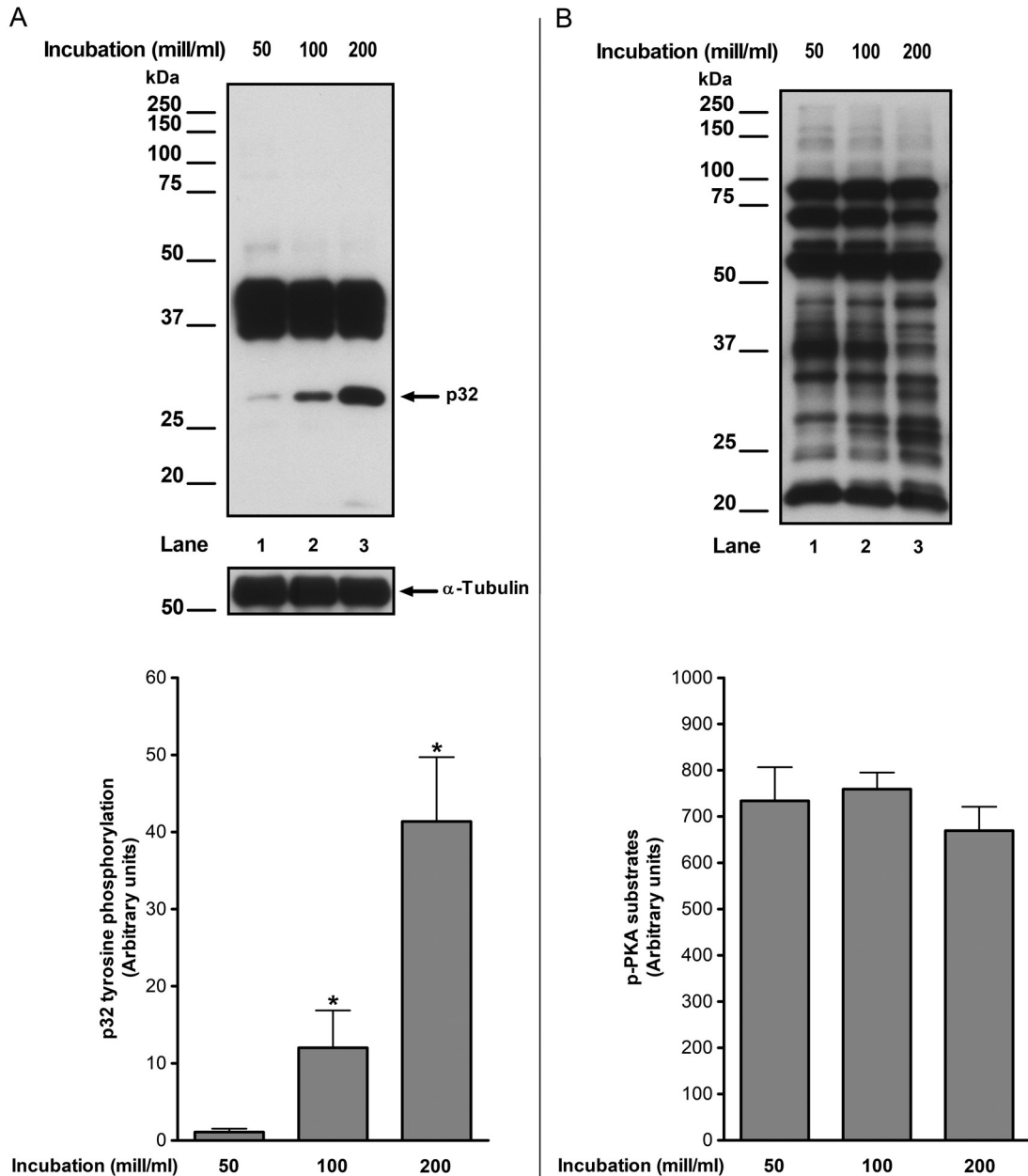
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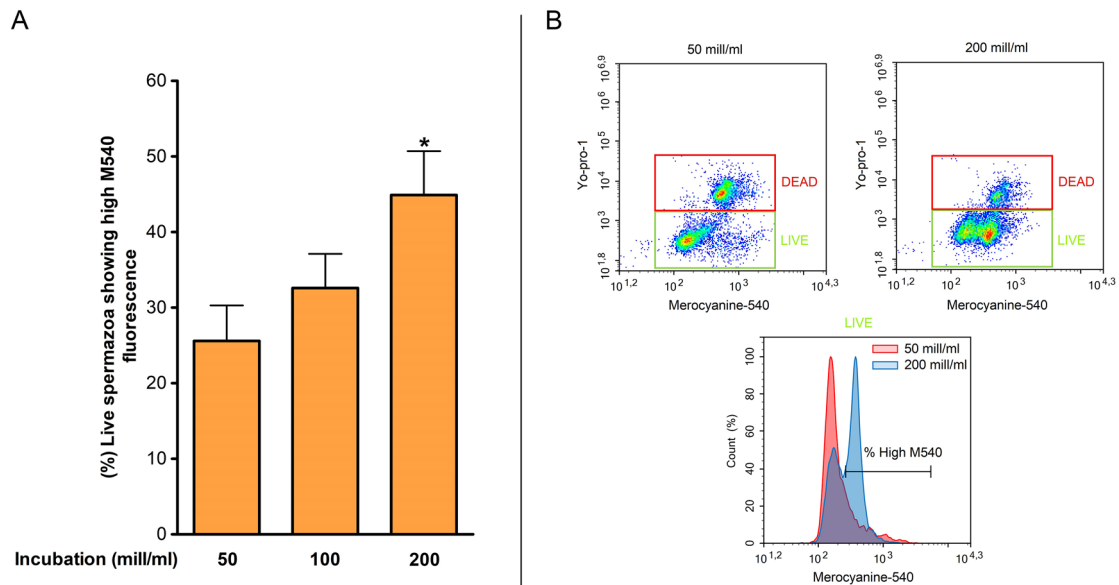


**Fig. 1.** Effect of sperm concentration during incubation on protein tyrosine phosphorylation (A) and phosphorylation of PKA substrates (B). Spermatozoa were incubated at concentrations of 50, 100 or 200 mill/ml in TCM for 4 h at 38.5°C. Subsequently, spermatozoa were lysed and western blotting was performed using an anti-phosphotyrosine antibody (A; upper panel) or an anti-phospho-PKA substrate antibody (B; upper panel). The immunoblots are representative of 8 independent experiments (n=8);  $\alpha$ -tubulin levels are shown as loading control. The bars represent the mean  $\pm$  SEM of the optical densities of p32 (A; lower panel) and phospho-PKA substrates (B; lower panel) in arbitrary units. Bars marked with \* differ from the 50 mill/ml concentration significantly ( $P < 0.05$ ).

in the capacitating medium induced a significant increase in events related to sperm capacitation. The effect of sperm concentration on PY has previously been studied in stallion spermatozoa, and it was found that PY induction occurs when spermatozoa are incubated in the capacitating medium (containing calcium, bicarbonate, and bovine serum albumin [BSA]) at a low concentration (10 mill/ml), and not at a relatively higher concentration (30 mill/ml) [16]. Therefore, an opposite scenario is observed in pigs, as in our conditions, high sperm concentration induce an increase in PY of p32 protein, indicating species-specific differences.

Interestingly, a 4 h incubation of boar spermatozoa at the highest

cellular concentrations in a capacitating medium induced spontaneous acrosome reaction more effectively. In this regard, it must be mentioned that the classical theory, which argues that the spermatozoon has to maintain acrosome integrity and reach the oocyte's zona pellucida (ZP) to achieve fertilization has been deeply questioned. Instead, it has been postulated that spermatozoa lose their acrosome at the level of the oviductal ampulla, well before reaching the oocyte, and these acrosome-free spermatozoa are capable of crossing the ZP and fertilize the oocyte [17, 18]. Likewise, other studies on mouse spermatozoa have shown that, even in the presence of a PKA inhibitor (H-89), addition of calcium ionophore induces acrosome reaction, and these



**Fig. 2.** Effect of sperm concentration during incubation on plasma membrane lipid organization. Spermatozoa were incubated at 50, 100 or 200 mill/ml in TCM for 4 h at 38.5°C. Following this, plasma membrane lipid organization was assessed by flow cytometry using Merocyanine 540 (M540). Panel (A) shows the percentage of viable spermatozoa with high M540 fluorescence (mean  $\pm$  SEM;  $n = 6$ ). Bars marked with \* differ from the 50 mill/ml concentration significantly ( $P < 0.05$ ). Panel (B) shows representative plots acquired at 50 and 200 mill/ml.

spermatozoa are able to fertilize the oocyte. Hence, these data indicate that the activation of the PKA pathway is not mandatory to achieve fertilization and can be bypassed [19]. This theory is in agreement with our results, as after sperm incubation, we observed consistent capacitation-related events (induction of plasma membrane lipid disorganization and a rise in PY of p32 protein), and an increase in the live acrosome-reacted spermatozoa, which was not associated with an increase in phosphorylation of PKA substrates. The relationship between the appearance of the phosphorylated form of p32 and acrosome integrity has been previously studied in boar spermatozoa. Tabuchi *et al.* [20] showed an increase in PY in p32 and in the percentage of dead spermatozoa with damaged acrosomes subjected to rapid thawing cycles. However, our study showed that viability was not impaired in accordance with the findings reported by Harayama *et al.* [21], which showed an increase in PY in p32, and an increase in the percentage of live acrosome-reacted spermatozoa, after the activation of the cAMP signaling cascade. Therefore, the increase observed in the percentage of live acrosome-reacted spermatozoa at concentrations of 100 and 200 mill/ml could indicate that capacitation has occurred, and that they are ready to fertilize. Interestingly, sperm incubation at higher cellular concentrations exerted a protective effect on sperm plasmalemma, as more spermatozoa remained viable when incubated at 100 mill/ml and 200 mill/ml after 4 h, compared to the lower concentration (50 mill/ml). These results may in part reflect that, among other factors, high sperm concentrations in the sperm reservoir may contribute to maintaining high sperm viability prior to fertilization, as less than 20% of the spermatozoa retrieved at the sperm reservoir *in vivo* were classified as dead [22]. Hence, the reported incubation conditions at high cellular concentrations could mimic those found in the oviductal reservoir in boars; however, functional experiments such as IVF are needed to fully determine whether live acrosome-reacted spermatozoa have the capacity to fertilize an oocyte, as previously reported in mouse spermatozoa [17].

At present, the exact mechanism involved in the increase in capacitation-related events and acrosome reaction induced by high

sperm concentrations in boar spermatozoa remains to be elucidated. However, one possibility is that the increase in sperm concentration during incubation induces reactive oxygen species (ROS) production, associated with cellular metabolism. This increase in ROS production could induce sperm capacitation and the acrosome reaction, as previously reported [23, 24].

In conclusion, our study shows that increasing sperm concentration during incubation under capacitating conditions triggers capacitation-related events and spontaneous acrosome reaction in boars, while maintaining plasmalemma intactness. These findings may be useful for improving the IVF outcomes in pigs.

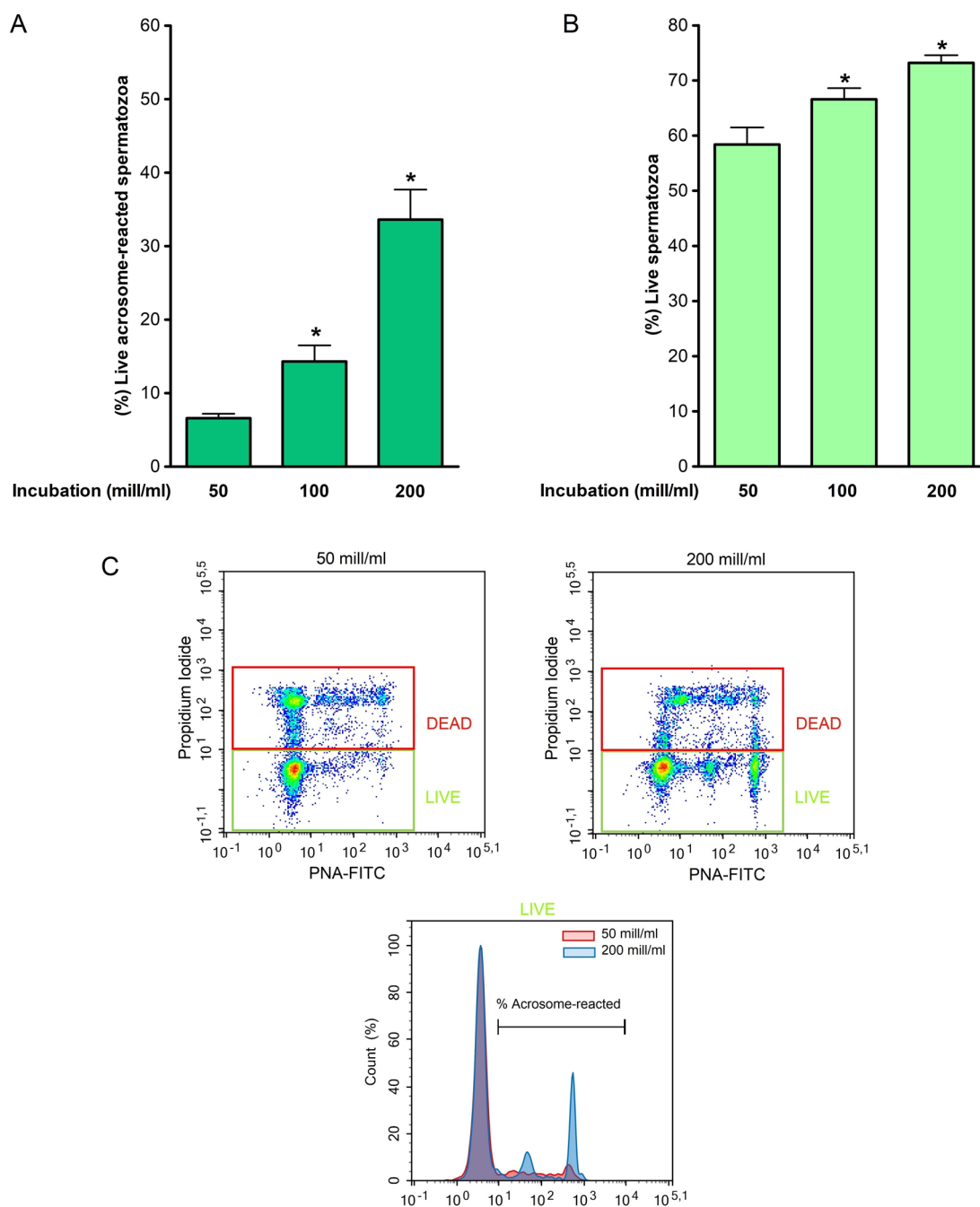
## Methods

### Medium

Tyrode's complete medium (TCM) consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM HEPES, 1 mM CaCl<sub>2</sub>, 15 mM NaHCO<sub>3</sub>, and 3 mg/ml BSA. TCM was prepared on the day of use, and its pH was adjusted to 7.45. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

### Semen collection and processing

Porcine seminal doses were purchased from a commercial boar station (Tecnogenext, S.L., Mérida, Spain). Duroc boars were maintained according to institutional and European regulations. In each experiment, seminal doses from three different males were pooled, centrifuged at 900  $\times$  g for 4 min at room temperature (RT; 22–25°C), and washed with phosphate-buffered saline (PBS). Finally, spermatozoa were diluted in TCM (500  $\mu$ l final volume) to a final concentration of 50, 100, or 200 mill/ml, in 5 ml round bottom plastic tubes from BD Falcon (San Jose, CA, USA), and incubated for 4 h in a water bath at 38.5°C in air.



**Fig. 3.** Effect of sperm concentration during incubation on acrosome reaction and viability. Spermatozoa were incubated at concentrations of 50, 100 or 200 mill/ml in TCM for 4 h at 38.5°C. Following this, acrosome reaction and viability were simultaneously assessed by flow cytometry using PNA-FITC and PI. Panel (A) shows the percentage of live acrosome-reacted spermatozoa, and panel (B) shows the percentage of viable spermatozoa (mean  $\pm$  SEM; n = 8). Bars marked with \* differ from the 50 mill/ml concentration significantly ( $P < 0.05$ ). Panel (C) shows representative plots acquired at 50 and 200 mill/ml.

### Western blotting

After incubation, spermatozoa were processed as described previously by González-Fernández *et al.* [25]. Briefly, spermatozoa were lysed in Laemmli buffer 2X (Bio-Rad, Hercules, CA, USA), and the protein content was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Lysates (15  $\mu$ g protein) were loaded and electrophoresed in 10% SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany). Membranes were incubated with an anti-

phosphotyrosine monoclonal antibody (clone 4G10) (#05-321) (Merck KGaA, Darmstadt, Germany) (diluted 1:5000, v/v, in Tris-buffered saline-Tween 20 solution [TBS-T] containing 3% BSA), or with anti-phospho-PKA substrate (100G7E) polyclonal antibody (#9624) from Cell Signaling Technology (Danvers, MA, USA) (diluted 1:1000, v/v, in TBS-T containing 3% BSA) overnight at 4°C. As a loading control,  $\alpha$ -tubulin levels were analyzed using an anti- $\alpha$ -tubulin antibody (#sc-8035) from Santa Cruz Biotechnology (Santa Cruz, CA, USA; diluted 1:5000, v/v, in TBS-T containing 3% BSA) overnight

at 4°C. The next day, the membranes were incubated for 45 min at RT (22–25°C) with a secondary anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (#sc-516102 or #sc-2357) from Santa Cruz Biotechnology (Santa Cruz, CA, USA; diluted 1:5000, v/v, in TBS-T containing 3% BSA). Following secondary antibody incubation, membranes were washed for 20 min in TBS-T, then incubated with SuperSignal™ West Pico Kit (Thermo Fisher Scientific, Waltham, MA, USA), and exposed to Hyperfilm™ ECL (Amersham, Arlington Heights, IL, USA). Densitometric analysis was performed using the Gel-Pro Analyzer™ software (ver. 4.0) (Media Cybernetics, Bethesda, MD, USA).

### Flow cytometry

Samples were analyzed using an ACEA NovoCyte™ flow cytometer (ACEA Biosciences, San Diego, CA, USA) equipped with blue and red lasers (488/640 nm). Flow cytometry experiments and data analysis were performed using the ACEA Novo Express™ software (ACEA Biosciences, San Diego, CA, USA). Forward scatter (FSC) and side scatter (SSC) were used to gate the sperm population and exclude debris. Spermatozoa were analyzed at a rate of 400–800 cells/sec, and data were collected for 10,000 cells in each treatment. After sperm incubation and before flow cytometry analysis, samples were mixed by quickly pipetting three times before dilution in PBS, to avoid agglutination as much as possible. To maintain the same concentration (1–3 mill/ml in PBS), different dilutions were performed (25 µl of sample in 475 µl of PBS for 50 mill/ml, 12 µl of sample in 488 µl of PBS for 100 mill/ml, and 6 µl of sample in 494 µl of PBS for 200 mill/ml).

### Evaluation of plasma membrane lipid organization by flow cytometry

Sperm plasma membrane lipid organization was assessed as described previously by staining with Merocyanine 540 (M540) and Yo-Pro-1 (Thermo Fisher Scientific, Waltham, MA, USA) [26]. Diluted samples were incubated with M540 (6 µM) and Yo-Pro-1 (75 nM) at 38.5°C for 15 min. After excitation at 488 nm, fluorescence was detected using a 572 ± 28 nm band pass filter for M540, and a 530 ± 30 nm band pass filter for Yo-Pro-1. Results are expressed as the percentage of spermatozoa with high M540 fluorescence, taking into account only viable cells (Yo-Pro-1 negative).

### Evaluation of the acrosome reaction and viability by flow cytometry

Acrosome reaction and viability were assessed after staining with peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC) and Propidium Iodide (PI) [27]. PNA-FITC and PI were added to diluted sample (final concentration of PNA-FITC was 0.4 µg/ml and that of PI was 4.8 µM), and were incubated at RT in the dark for 5 min. The fluorescence of PNA-FITC and PI were measured using 530 ± 30 nm and 675 ± 30 nm band pass filters, respectively. Live acrosome-reacted spermatozoa are expressed as the percentage of PNA-positive cells, taking into account only viable cells (PI-negative). Results of viable spermatozoa are expressed as the percentage of PI-negative cells (LIVE).

### Statistical analysis

An ANOVA followed by Dunnett's post-hoc test was used to compare the different treatments. All data are shown as the mean ± standard error of the mean (SEM). The level of significance was set at  $P < 0.05$ . Analyses were performed using the SigmaPlot software (ver. 12.0) for Windows (Systat Software, Chicago, IL, USA).

**Conflict of interests:** The authors declare no conflict of interest.

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