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Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa

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Abstract

This work was designed to study how this ability is affected by different sperm treatments routinely used for *in vitro* fertilization (IVF) assay. In this study, boar sperm samples from epididymal or ejaculated origin were processed by three different methods: left unwashed (NW group), washed in Dulbecco's phosphate-buffered saline supplemented with 0.1% BSA (BSA group), and washed on a Percoll® gradient (PERCOLL group). After preparation of semen samples, changes in motility patterns were studied by CASA, calcium uptake by spectrofluorimetry, and ROS generation, spontaneous acrosome reaction, and lipid disorder by means of flow cytometry. Finally IVF assays were also performed with the different semen samples and penetrability results evaluated at 2 and 4 h post insemination (hpi). Independently of the sperm treatment, epididymal spermatozoa showed higher values of progressive motility, percentage of live cells with low lipid disorder, and penetration ability at 4 hpi than the corresponding ejaculated spermatozoa. Ejaculated spermatozoa showed higher levels of calcium uptake, ROS generation and percentage of spontaneous acrosome reaction than epididymal sperm. Regarding sperm treatments, PERCOLL group showed the highest values for some motility parameters (linearity of the curvilinear trajectory, straightness, and average path velocity/curvilinear velocity), ROS generation and penetration ability at 2 and 4 hpi; however this same group showed the lowest values for sperm curvilinear velocity and lateral head displacement. From all experimental groups, ejaculated-PERCOLL-treated spermatozoa showed the highest fertilization ability after 2 hpi. Results suggest that capacitation pathways can be regulated by suitable treatments making the ejaculated sperm able to reach capacitation and fertilize oocytes in similar levels than epididymal spermatozoa, although most of the studied capacitation-associated changes do not correlate with this ability.

Keywords: Ejaculated spermatozoa; Epididymal spermatozoa; Capacitation; *In vitro* penetration

1. Introduction

Capacitation is a vital phenomenon that a spermatozoon must undergo before it can fertilize an oocyte. It is a lengthy process in which early changes take place as rapidly as 1 min whereas full capacitation is accomplished within hours (depending on the mammalian species of interest). Capacitation confers upon the spermatozoon an ability to gain hyperactive motility, interact with oocyte zona pellucida (ZP), undergo acrosome reaction (AR), and initiate oocyte plasma membrane fusion [1]. Whereas this concept can still be considered valid, many studies have been carried out trying to characterize the specific changes in the sperm cell through the capacitation process. Among them, motility patterns [2–6], alterations in the sperm plasma membrane architecture [7–9], ROS generation [10], calcium uptake [11–13], or spontaneous AR [14] have been

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proposed as indicators of capacitation. However, none of the designed assays discriminate non-responding from responding cells at different time intervals of the specific process [15] and their relation to full ability for further oocyte penetration is unknown.

In vitro, sperm capacitation can readily be accomplished when provided culture conditions facilitate and support membrane changes and signal transduction pathway activation similar to those occurring *in vivo*. We have previously demonstrated that the methods of processing pig spermatozoa further affect different *in vitro* fertilization (IVF) parameters [16]. However, there is no detailed characterization of changes that sperm suffer after these different sperm treatments. Additionally, a wide difference exists among boar ejaculates and use of epididymal spermatozoa has become a more consistent option for pig IVF [17]. It has been reported that, *in vitro*, epididymal spermatozoa can reach capacitation and fertilize oocytes much easier than ejaculated spermatozoa [1], but this ability could be affected by capacitation treatments. *In vitro*, boar spermatozoa pre-incubated with seminal plasma (SP) display a lower fertilizing ability and it has been suggested that SP has sperm-coating components that firmly stick to the sperm surface inhibiting their fertilizing ability [18]. During epididymal maturation and ejaculation many glycoproteins and peptides secreted by the accessory glands bind to the sperm surface with varying affinities [19,20]; therefore, it is anticipated that they will have a significant effect on membrane function (glycodelines, [21], AWN-1, [22]). Equally, their differential removal during washing procedure in artificial media as a prelude to IVF may reverse SP or epididymal secretion effects, influencing capacitation and shortening sperm survival. Thus, we hypothesize that ejaculated spermatozoa are able to reach a capacitation status and to fertilize oocytes in similar levels than the epididymal spermatozoa as far as the treatments to remove seminal plasma can induce the suitable membrane alterations and signal transduction pathways necessary to bind and penetrate the oocytes. Additionally, we propose that some of the so-called "capacitation-associated changes", such as calcium increase, lipid disorder, and ROS production, might not correlate with *in vitro* fertilization ability thus not being useful to predict the penetration ability of a sperm sample.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain) unless otherwise indicated.

2.1. Sperm collection and handling

2.1.1. Ejaculated spermatozoa

Semen was routinely collected from mature fertile boars using the manual method. The sperm-rich fraction was collected in a pre-warmed thermo flask and the gel-fraction was held on a gauze tissue covering the thermo opening. The semen was then extended 1:2 with isothermal Beltsville Thawing Solution (BTS, [23]) and sperm concentration, motility, acrosome integrity, and normal morphology were microscopically evaluated by standard laboratory techniques. BTS consisted of 37.0 g glucose, 1.25 g EDTA, 6.0 g sodium citrate, 1.25 g sodium bicarbonate, and 0.75 g potassium chloride in 1 L distilled water with a final pH of 7.2. After dilution in the extender, a pool of semen from 4 different boars was used for all the experiments to avoid individual boar effect on the results.

2.1.2. Epididymal spermatozoa

Within 30 min of slaughter, porcine cauda epididymal sperm were collected by perfusion of the *ductus epididymidis* by washing with 2 mL BTS. These spermatozoa were evaluated as the ejaculated ones and used under the same criteria. A pool of samples from epididymis from four different boars was used for all the experiments.

2.2. Preparation of spermatozoa

The spermatozoa (ejaculated and epididymal) samples were: i) left unwashed (NW group), ii) washed in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.1% BSA (BSA group), or iii) washed on a Percoll® (Pharmacia, Uppsala) gradient (Percoll® group). After treatment, sperm samples were diluted in TALP medium [24] consisting of 114.06 mM NaCl, 3.2 mM KCl, 8 mM calcium lactate•$5H_2O$, 0.5 mM $MgCl_2$•$6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mL sodium lactate, 1.1 mM Na pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg BSA mL (fraction V, A-9647), 1 mg/mL PVA and 0.17 mM kanamycin sulphate, previously pre-equilibrated overnight at 38.5 °C in 5% CO_2 in 100% humidified air and with a final pH of 7.4.

Preparation of sperm samples for the three sperm treatments was as follows: for the NW-group, the semen samples (both epididymal and ejaculated) were diluted in TALP medium and sperm concentration adjusted according to experiment. The sperm concentration was assessed by a photometer (Spermacue, Minitüb, Germany). For BSA-group, samples (epididymal and ejaculated) were washed with DPBS supplemented with 0.1% BSA (fraction V, A-9647) by centrifugation (900 × *g*, 10 min, three

times). At the end of the washing procedure, the pellet was resuspended in TALP medium and sperm concentration adjusted according to experiment. Finally, Percoll-group involved layering a 0.5 mL aliquot of spermatozoa on a discontinuous 45 and 90% (v/v) Percoll® gradient for ejaculated spermatozoa and 45 and 75% (v/v) for epididymal spermatozoa [25]. The rationale to make two different Percoll® gradients depending on the spermatozoa origin is that it has been demonstrated that boar epididymal sperm do not separate well with the 45/90 gradient [25]. Both types of samples were then centrifuged (700 × *g*, 30 min) and pellet washed in TALP medium by centrifugation (700 × *g*, 10 min). Supernatant was discarded, pellet resuspended in TALP medium and sperm concentration adjusted according to experiment.

2.3. Motility assays

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, Microptic, Barcelona, Spain), previously validated in our laboratory [26]. The studied CASA-derived motility characteristics were the percentage of motile spermatozoa (%motil), percentage of motile progressive spermatozoa (%motil prog), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, μm), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), and beat cross-frequency (BCF, Hz).

A 7 μl drop of the sample both before (just ejaculated) and after sperm treatment, was placed on a warmed (37 °C) slide and covered with a 24 × 24 mm cover slip. The setting parameters were: 25 frames in which spermatozoa had to be present in at least 15 in order to be counted. Images were obtained at × 200 magnification in a contrast phase microscope and spermatozoa with a VAP <20 μm/s were considered immobile. A minimum of 5 fields per sample were evaluated, counting a minimum of 200 spermatozoa per sub-sample.

2.4. Flow cytometry assays

Evaluation of plasma membrane lipid packing disorder, ROS generation, and AR were made by flow cytometric on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, Florida, USA). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10000 events per sample were collected in list mode, and 4 measures per sample were recorded. Flow cytometric data were analyzed by the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude from the analysis the eventual remaining debris and aggregates.

2.4.1. Plasma membrane lipid packing disorder

To detect increase in plasma membrane lipid packing disorder, after the three different treatments and dilution in TALP, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 [27]. Merocyanine 540 is a hydrophobic dye that stains cell membranes more intensely if their lipid components are in a higher state of disorder, as is the case in capacitated spermatozoa. Stock solutions of M540 (1 mM) and Yo-Pro 1 (25 μM, Molecular Probes, Eugene, OR) were prepared in DMSO. For each sperm treatment, 1 mL of TALP diluted semen sample containing 5–10 × 10^6 cells was added to a mix of 2.7 μl M540 stock solution (2.7 μM final concentration) and 1 μl of Yo-Pro (25 nM final concentration). M540 fluorescence was recorded with a FL2 sensor using a 575 nm band-pass filter and Yo-Pro 1 with a FL1 sensor using a 525 nm band-pass filter. Cells were classified into three categories: low merocyanine fluorescence (viable, uncapacitated), high merocyanine fluorescence (viable, capacitated) or Yo-Pro-1 positive (dead).

2.4.2. Generation of reactive oxygen species (ROS)

Production of ROS was measured by incubating the samples in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (0.5 μM) [26] This fluorogenic probe is commonly used to detect cellular ROS production. H_2DCFDA is a stable cell-permeable non-fluorescent dye that is de-esterified intracellularly thus turning to highly fluorescent 2′,7′-dichlorofluorescin upon oxidation. Green fluorescence was recorded with a FL1 sensor using a 525 nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1) and used as an index of ROS generation. After the three different treatments and dilution in TALP, 1 mL sperm sample (10 × 10^6 cels/mL) was incubated for 15 min with 5 μL from H_2DCFDA stock solution (final concentration 0.5 μM). After incubation time, a subsample was taken to be measured by flow cytometry.

2.4.3. Acrosome reaction

After sperm treatment, seminal samples (1 mL of semen containing 5–10 × 10^6 cells) were incubated with 2 μL of fluorescein labeled lectin from the peanut plant *Arachis hypogaea* (FITC-PNA, 100 mg/ mL) and

5 μL of propidium iodide (PI) stock solution (500 mg/mL), at room temperature for 10 min [26]. Fluorescence was measured using a FL-1 sensor using a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor with a 575 nm band-pass filter to detect PI. Three sperm sub-populations were detected: live acrosome intact, live acrosome reacted and dead spermatozoa (with and without intact acrosome).

2.4.4. Determination of intracellular calcium activity

To measure intracellular free Ca^{2+} [28] spermatozoa treated by the three described methods were incubated with 2.5 μM fura-2/AM in a buffer medium consisting of 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose, and 1 mM pyruvate [29] for 45 min at 37 °C. The extracellular unloaded fura-2 was removed by centrifugation (700 × *g*, 5 min). Washed sperm were resuspended in the same buffer to a concentration of 3×10^8 cells/mL and incubated at 37 °C for 15 min in dark. Then, sperm were centrifuged (700 × *g*, 5 min) and resuspended in TALP medium. Fluorescence was monitored using an Aminco-Bowman 2 Spectronic Unicam fluorescence spectrofluorometer equipped with an AB Luminiscence Spectrometer (5.31 software) for a further 60 min [28]. Excitation wavelengths alternated between 340 and 380 nm with emission held at 510 nm. At the end of the experiments, sperm were lysed with Triton X-100 (0.5%), and then calcium was depleted by addition of 25mM EGTA (Fig. 1). Intracellular Ca^{2+} concentration $[Ca^{2+}]i$ was calculated as previously described by Grynkiewicz et al [30]. Equation used for calculation was as follows: $[Ca^{2+}]i = Kd \times (R\text{-}Rmin/Rmax\text{-}R) \times Sf/Sb$. Where R indicates the fluorescence ratio 340/380 nm λ; Rmin indicates the minimum values of fluorescence; Rmax indicates the maximal fluorescence values; Sf indicates the fluorescence intensity in the Ca^{2+}-free medium (excitation 380 nm λ); Sb: indicates the fluorescence intensity under Ca^{2+}saturation conditions and Kd (224 nmol) is the Ca^{2+} constant dissociation for Fura-2AM.

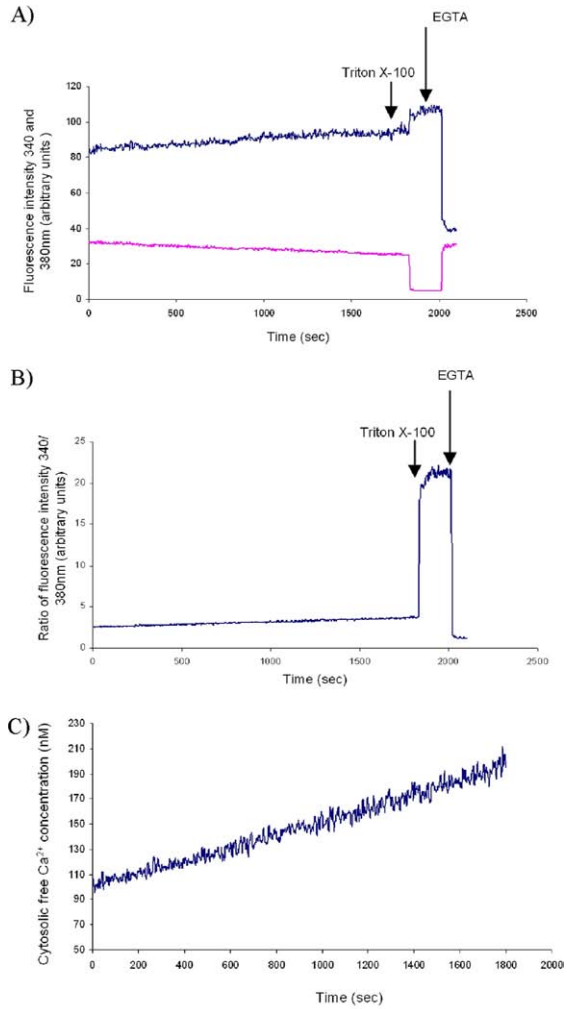

Fig. 1. Representative traces illustrating the intracellular calcium concentration in capacitated boar spermatozoa. A) Triton X-100 and EGTA addition to the samples is indicated by arrows. B) Ratio between measurements at 340/380 nm is shown. C) Final calcium concentration in the samples.

2.5. In vitro maturation and fertilization

2.5.1. Oocyte collection and in vitro maturation

Within 30 min of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline solution (0.9% NaCl, w/v) containing 100 μg/mL kanamycin sulphate at 37 °C, washed once in 0.04% (w/v) cetrimide solution and then twice in saline. Oocyte–cumulus cell complexes were collected from non-atretic follicles (3–6 mm diameter), washed twice in DPBS supplemented with 4 mg/mL polyvinyl alcohol (PVA), and twice more in maturation medium previously equilibrated for at least 3h at 38.5 °C under 5% CO_2 in 100% humidified air. Only oocytes harvested within 2 h of slaughter [31] with a complete and dense cumulus oophorus were matured. The medium used for oocyte maturation was NCSU-37 [32] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5μg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotrophin (Foligon, Intervet International BV, Boxmeer), 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, Barcelona, Spain), and 10% (v/v) pig follicular fluid. Groups

of 50 cumulus-oocyte complexes were cultured in 500 μL maturation medium for 20–22 h at 38.5 °C under 5% CO_2 in air. Once cultured, the oocytes were washed twice, transferred to fresh maturation medium without hormones nor dibutyryl cAMP, and cultured for a additional 20–22 h.

2.5.2. In vitro fertilization

Basic medium for IVF was TALP medium as describe above. After maturation oocytes were mechanically stripped of cumulus by gentle aspiration with a pipette. Denuded oocytes were washed three times in TALP medium and transferred in groups of 25–30 oocytes to a 4-well plate (Nunc, Roskilde, Denmark) containing 250 μL TALP medium previously equilibrated overnight at 38.5 °C under 5% CO_2. Sperm suspensions (250 μl) from each treatment group were added to each fertilization well to obtain a final concentration of 5×10^4 cells/mL. Two and four hours after insemination, oocytes were fixed, stained with 1% (w/v) lacmoid and examined at ×400 magnification under a phase-contrast microscope. The variables analyzed were percentage of penetrated oocytes (Penetration rate), mean number of spermatozoa per penetrated oocyte (S/O), percentage of oocytes reassuming meiosis (activated oocytes at Anaphase II, Telophase II or with female pronucleus) and percentage of penetrated oocytes with swollen heads or male pronuclei in the ooplasma (activated sperm) [16].

2.6. Statistical analysis

Data are expressed as mean ± SEM and were analyzed using two-way analysis of variance (ANOVA) where the source of spermatozoa (epididymal and ejaculated) and treatment applied (BSA, NW, Percoll®) were considered main variables. In those parameters measured during an incubation time (lipid membrane disorder, calcium uptake, ROS generation), this time variable was included in the ANOVA model as a covariate.

When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at $P \leq 0.05$.

2.7. Experimental design

2.7.1. Experiment 1. Motility parameters in ejaculated and epididymal spermatozoa after different treatments

Ejaculated and epididymal spermatozoa were prepared under the three different methods described above (BSA, NW and Percoll®) and resuspended in TALP medium. After 5 min of incubation (under 5% CO_2, 39 °C and humidified atmosphere), motility of sperm samples from BSA-group, NW-group and Percoll® group were studied by CASA. In the same day each replicate was run with ejaculated and epididymal spermatozoa. This experiment was carried out in 5 replicates.

2.7.2. Experiment 2. Plasma membrane lipid packing disorder in ejaculated and epididymal spermatozoa after different treatments

Ejaculated and epididymal sperm samples treated by the three different methods (BSA, NW and Percoll®), resuspended in TALP medium and kept in 35 mm diameter Petri dishes at 5% CO_2, 38.5 °C and humidified atmosphere. Just after TALP addition (time 0) a subsample for each group was withdrawn, resuspended with M540 and Yo-Pro 1 and assessed by flow cytometer as previously described. This experiment was carried out in 5 replicates with ejaculated and epididymal spermatozoa at the same time.

2.7.3. Experiment 3. Generation of reactive oxygen species (ROS) in ejaculated and epididymal spermatozoa after different treatments

Capacitated sperm by the different methods were incubated (5% CO_2, 38.5 °C and humidified atmosphere) for 15 min with H_2DCFDA. Then a subsample was taken and analyzed by flow cytometer (time 0) and following subsamples were analyzed every 15 min of incubation up to 135 min. This experiment was carried out in 5 replicates with ejaculated and epididymal spermatozoa at the same time.

2.7.4. Experiment 4. Kinetics of Ca^{2+} uptake in ejaculated and epididymal spermatozoa after different treatments

To determine the kinetics of Ca^{2+} uptake inside the sperm cells, ejaculated and epididymal sperm samples were prepared by the three methods (NW, BSA, and Percoll®). Then, spermatozoa were incubated with 2.5 μM fura-2/AM as described previously. Samples were centrifuged to discard unloaded Fura, resuspended in TALP medium and transferred into spectrofluorimeter. Then, a subsample for each group was measured from time 0 (just after transferring the samples) up to 60 min. This experiment was carried out in 7 replicates with ejaculated and epididymal spermatozoa at the same time.

Table 1
Motility parameters measured by CASA in epididymal and ejaculated boar spermatozoa just after collection and before sperm treatments.

Source	Progressive motility	% motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Epididymal	11.4 ± 3.2	32.9 ± 4.2	50.1 ± 2.2	14.6 ± 1.9	22.6 ± 1.2	24.3 ± 1.3	52.8 ± 1.7	43.9 ± 1.9	1.7 ± 0.2	2.8 ± 0.4
Ejaculated	44.6 ± 4.5	73.5 ± 2.3	57.3 ± 2.0	20.9 ± 1.1	35.5 ± 1.9	35.8 ± 1.3	58.3 ± 1.3	61.2 ± 1.93	2.0 ± 0.1	5.9 ± 0.2
P-value	<0.01	<0.01	0.02	0.01	<0.01	<0.01	0.01	<0.01	0.21	<0.01

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory; STR: straightness; ALH: amplitude of lateral head displacement; WOB: wobble (VAP/VCL); BCF: beat cross-frequency.

2.7.5. Experiment 5. Acrosome reaction in ejaculated and epididymal spermatozoa after different treatments

After preparation of ejaculated and epididymal sperm samples by NW, BSA and Percoll® methods, samples were resuspended in TALP medium and kept in 35 mm diameter Petri dishes at 5% CO_2, 38.5 °C in humidified atmosphere. After 30 min of TALP addition a subsample for each group was withdrawn, resuspended with PNA-PI and assessed by flow cytometer as previously described. This experiment was carried out in 5 replicates with ejaculated and epididymal spermatozoa at the same time.

2.7.6. Experiment 6. In vitro fertilization of in vitro matured oocytes with ejaculated and epididymal spermatozoa after different treatments

A total of 1282 *in vitro* matured oocytes were evaluated after insemination with epididymal and ejaculated spermatozoa prepared by the three treatments (NW, BSA, and Percoll®). Oocytes were assessed at 2 and 4 h post insemination. This experiment was carried out in 5 replicates with ejaculated and epididymal spermatozoa at the same time.

3. Results

In vitro treatments for spermatozoa try to enhance their ability to penetrate the oocytes. The first treatment (NW) consisted of the direct dilution of the sperm sample in TALP medium. It is supposed that capacitation process is not triggered by this treatment, due to presence of seminal plasma, and it will only take place in the IVF medium. The second treatment (BSA) consisted of three washes with the subsequent dilution of the pellet in PBS with BSA. This could mean an initial step in the capacitation process by contact of sperm with a cholesterol acceptor as BSA, enhancing lipid disorder and fusogenic potential. The third treatment, the Percoll® one, involved a differential centrifugation by a gradient, thus removing membrane vesicles and accelerating the capacitation process.

3.1. Experiment 1. Motility parameters in ejaculated and epididymal spermatozoa after different treatments

The motion ability of mammalian spermatozoa is acquired during their epididymal transit but observed only upon dilution with seminal plasma at time of ejaculation. For this reason, our first experiment tried to answer the following question: are the motility parameters affected by the source and the treatment of the sperm? The motility parameters were assessed both before (control) and after sperm treatment. Results before any sperm treatment showed that, as expected, epididymal spermatozoa have lower motility parameters than ejaculated ones (Table 1). Epididymal spermatozoa had significantly ($P < 0.05$) lower progressive motility, lower percentage of cells moving and lower values for VCL, VSL, VAP, LIN, STR, WOB, and BCF parameters. Once spermatozoa were processed,

Table 2
Motility parameters measured by CASA in epididymal and ejaculated boar spermatozoa after treatments and dilution in TALP medium.

Source	Progressive motility	% motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Epididymal	37.0 ± 1.0	70.8 ± 1.3	81.3 ± 1.6	38.3 ± 1.2	54.1 ± 1.5	44.6 ± 0.8	67.8 ± 0.7	64.0 ± 0.7	3.0 ± 0.1	5.5 ± 0.1
Ejaculated	29.2 ± 1.5	63.3 ± 1.3	69.8 ± 1.5	34.6 ± 1.2	48.1 ± 1.4	47.9 ± 1.1	69.8 ± 0.8	66.5 ± 0.8	2.5 ± 0.1	5.8 ± 0.1
P-value	<0.01	<0.01	<0.01	0.04	<0.01	0.02	0.08	0.03	<0.01	0.03

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory; STR: straightness; ALH: amplitude of lateral head displacement; WOB: wobble (VAP/VCL); BCF: beat cross-frequency.

Table 3
Motility parameters measured by CASA in epididymal boar spermatozoa prepared by three different treatments (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) and diluted in TALP medium.

Treatment	Progressive motility	% motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
NW	36.0 ± 1.9[a]	68.9 ± 2.4[a]	83.1 ± 3.2[a]	38.8 ± 2.7[a]	55.6 ± 3.1[a]	43.8 ± 1.7	66.8 ± 1.4	63.3 ± 1.4	3.1 ± 0.1[a]	5.3 ± 0.1[a]
BSA	40.3 ± 1.8[b]	73.4 ± 1.9[b]	84.2 ± 2.6[a]	39.5 ± 2.1[a]	55.4 ± 2.3[a]	44.2 ± 1.4	67.3 ± 1.2	63.8 ± 1.1	3.2 ± 0.1[a]	5.7 ± 0.1[b]
PERCOLL	34.7 ± 1.8[a]	70.1 ± 2.3[a,b]	76.7 ± 2.6[b]	36.6 ± 1.8[b]	51.5 ± 2.3[b]	45.7 ± 1.3	69.3 ± 0.9	64.9 ± 1.1	2.8 ± 0.1[b]	5.5 ± 0.1[a,b]
P-value	<0.01	0.05	<0.01	0.01	<0.01	0.92	0.66	0.63	<0.01	0.04

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory; STR: straightness; ALH: amplitude of lateral head displacement, WOB: wobble (VAP/VCL), BCF: beat cross-frequency.
[a,b,c]Different superscripts in the same column indicate significant differences ($P < 0.05$).

while all the three treatments produced a significant increase in progressive motility of epididymal spermatozoa compared to control (11.4 vs. 37.0), this positive effect on motility was not observed in ejaculated spermatozoa. In general terms, after treatments the different motion parameters were significantly higher (progressive motility, percentage of motility, VCL, VSL, VAP, ALH) or similar (STR) for epididymal spermatozoa (Table 2). Only LIN, WOB, and BCF were higher in ejaculated groups.

When data are analyzed by the different sperm treatments a different effect of any of them on motility patterns is observed. Therefore Percoll® treated spermatozoa tend to show lower velocity than BSA or NW treated groups, as can be observed for VCL values in both epididymal (Table 3) and ejaculated spermatozoa (Table 4). Similarly, higher linearity and straightness for ejaculated spermatozoa, lower curvilinear and average path velocities for epididymal ones, and lower lateral head displacement for both epididymal and ejaculated spermatozoa in Percoll® group was observed (Tables 3 and 4). These results suggest that sperm motility patterns depend both on the source (epididymal, ejaculated) and the treatment (NW, BSA, Percoll®) what might be crucial for their further penetration ability.

3.2. Experiment 2. Plasma membrane lipid packing disorder in ejaculated and epididymal spermatozoa after different treatments

The diffusion of lipids in the plasma membrane of ejaculated is influenced by seminal plasma proteins and the composition of the extender medium. It is believed that the membrane fluidity changes detected by M540 precede the calcium influx, making M540 a method for evaluating the early events of capacitation. Merocyanine 540 (M540) is a hydrophobic dye that has been shown to stain cell membranes more intensely if their lipid components are in a higher state of disorder, as is the case with capacitated spermatozoa.

The lipid membrane disorder was not affected by the sperm treatment, although it was different in epididymal vs. ejaculated spermatozoa (Table 5). After treatment, epididymal spermatozoa significantly showed higher percentages of live cells with low lipid disorder than the ejaculated spermatozoa. Percentages of dead cells were similar in all the groups, except for BSA-ejaculated spermatozoa group which showed a higher value. Thus, it seems the seminal plasma might play an important role to maintain the viability of the spermatozoa with high lipid disorder and its removal by BSA washes leads to an increase of cell death.

Table 4
Motility parameters measured by CASA in ejaculated boar spermatozoa prepared by three different procedures (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) and diluted in TALP medium.

Treatment	Progressive motility	% motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
NW	31.2 ± 3.0	67.4 ± 2.1[a]	72.7 ± 2.6[a]	35.0 ± 2.3	50.1 ± 2.6	46.2 ± 2.0[a]	67.5 ± 1.5[a]	66.0 ± 1.6[a,b]	2.6 ± 0.0[a]	5.7 ± 0.2
BSA	28.0 ± 2.7	60.3 ± 2.4[b]	71.8 ± 2.7[a,b]	33.3 ± 2.0	47.9 ± 2.5	45.3 ± 1.8[a]	67.3 ± 1.5[a]	64.9 ± 1.5[a]	2.6 ± 0.0[a]	5.9 ± 0.1
PERCOLL	28.4 ± 2.0	62.4 ± 2.2[a,b]	64.5 ± 2.5[b]	35.6 ± 2.0	46.3 ± 2.2	52.6 ± 1.7[b]	75.0 ± 1.2[b]	68.8 ± 1.4[b]	2.3 ± 0.0[b]	5.9 ± 0.1
P-value	0.80	0.01	0.03	0.14	0.58	<0.01	<0.01	<0.01	0.01	0.62

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory; STR: straightness; ALH: amplitude of lateral head displacement; WOB: wobble (VAP/VCL), BCF: beat cross-frequency.
[a,b] Different superscripts in the same column indicate significant differences ($P < 0.05$).

Table 5
Membrane lipid packing disorder in epididymal and ejaculated boar spermatozoa after the three different treatments (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) and dilution in TALP medium. Sperm samples were stained with Merocyanine 540 (M540) and Yo-Pro 1 and immediately evaluated by flow cytometry.

Source	Treatment	Low disorder and viable	High disorder and viable	Dead
Epididymal	NW	37.0 ± 9.5[a]	55.8 ± 9.3[a]	6.9 ± 0.5[a]
	BSA	24.5 ± 3.9[a]	62.0 ± 4.0[a,b]	12.8 ± 0.8[a]
	PERCOLL	28.6 ± 7.5[a]	58.9 ± 6.3[a,b]	12.0 ± 1.5[a]
Ejaculated	NW	10.0 ± 1.2[b]	79.2 ± 1.6[b]	10.4 ± 0.8[a]
	BSA	9.7 ± 0.7[b]	70.6 ± 3.5[a,b]	19.5 ± 3.3[b]
	PERCOLL	12.7 ± 2.1[b]	76.2 ± 2.2[a,b]	10.5 ± 0.5[a]

[a,b] Different superscripts in the same column indicate significant differences ($P < 0.05$).

3.3. Experiment 3. Generation of reactive oxygen species (ROS) in ejaculated and epididymal spermatozoa after different treatments

Capacitating spermatozoa produce controlled amounts of ROS that regulate the acquisition of hyperactivated motility, AR, zona pellucida binding and oocyte penetration, but also ROS are recognized for their deleterious effects on almost all tissues and cells. These features conducted us to study ROS generation in spermatozoa (epididymal, ejaculated) under different treatments (NW, BSA, Percoll®). The results showed that ROS generation increased during the incubation time and was affected by both sperm source ($P < 0.001$) and treatment ($P < 0.001$) (Fig. 2) but there was not a significant interaction between the two factors ($P = 0.25$). Ejaculated spermatozoa generated more ROS than epididymal during the 135 min of incubation. Spermatozoa treated in a Percoll® gradient generated more ROS than spermatozoa from the BSA group and both more than spermatozoa processed in the NW group.

3.4. Experiment 4. Kinetics of Ca^{2+} uptake in ejaculated and epididymal spermatozoa after different treatments

Since intracellular Ca^{2+} influx is one of the crucial biochemical events occurring during capacitation, this experiment was performed to describe changes in Ca^{2+} uptake between sperm groups and to know whether calcium levels could explain differences in oocyte penetration and activation. The calcium intake by the sperm was increasing along the incubation time (60 min) and was significantly affected by sperm source and treatment ($P < 0.001$) (Fig. 3). In general terms, ejaculated spermatozoa uptake a higher mean concentration of Ca^{2+} than epididymal ones ($P < 0.001$).

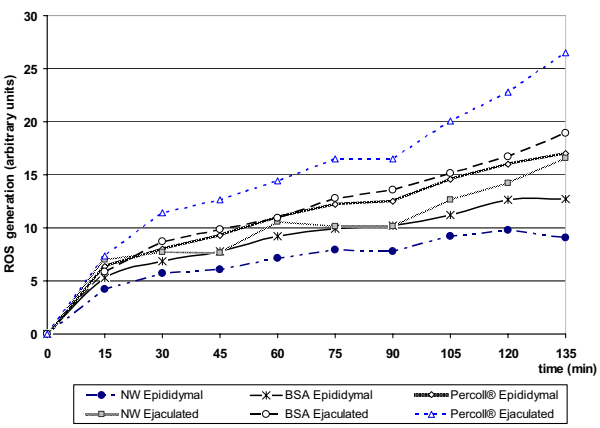

Fig. 2. ROS generation (mean channel of fluorescence) in epididymal and ejaculated boar spermatozoa prepared by three different treatments (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) and diluted in TALP medium. Sperm were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and measured flow cytometry for 135 min.

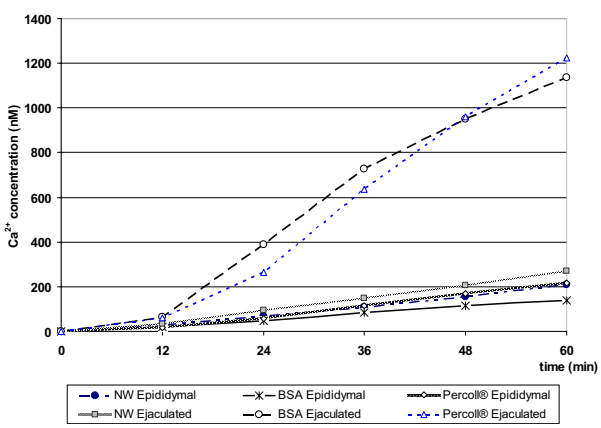

Fig. 3. Calcium concentration (nM) in epididymal and ejaculated boar spermatozoa prepared by three different procedures (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) and diluted in TALP medium. Fluorescence was measured by Fura-2 and monitored using spectrofluorimeter for 60 min.

Table 6
Acrosome reaction of epididymal and ejaculated boar spermatozoa prepared by three different procedures (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL). Sub-populations of spermatozoa after staining with FITC-PNA and PI examined by flow cytometry.

Source	Treatment	Live and intact acrosome	Live and acrosome reacted	Dead (intact and reacted)
Epididymal	NW	88.7 ± 1.2[a]	0.2 ± 0.1[a]	10.9 ± 1.2[a]
	BSA	47.5 ± 4.5[c]	0.5 ± 0.1[a,b,c]	51.8 ± 4.5[c]
	PERCOLL	54.6 ± 6.1[b,c]	0.9 ± 0.1[b,c]	44.4 ± 6.0[b,c]
Ejaculated	NW	72.1 ± 5.3[b]	1.0 ± 0.1[c]	26.7 ± 5.3[a,b]
	BSA	42.2 ± 5.3[c,d]	2.3 ± 0.2[d]	55.4 ± 5.5[c,d]
	PERCOLL	25.7 ± 2.7[d]	0.3 ± 0.1[a,b]	73.8 ± 2.7[d]

[a–d] Different superscripts in the same column indicate significant differences ($P < 0.05$).

Within the three groups of ejaculated sperm, the NW treatment induced a lower calcium uptake than BSA which also induced a lower uptake than Percoll® treatment (256.9 ± 59.1, 1049.6 ± 106.0, and 1324.3 ± 78.1, respectively). For epididymal sperm data were 236.5 ± 40.9, 137.3 ± 24.0, and 238.7 ± 49.8, respectively for NW, BSA, and Percoll groups (Fig. 3). However, the relation among factors was not lineal and there was a significant interaction between sperm source and treatment ($P < 0.001$).

3.5. Experiment 5. Acrosome reaction in ejaculated and epididymal spermatozoa after different treatments

Capacitation and the subsequent acrosome reaction (AR) are important events in the process of fertilization and they are essential for sperm to achieve the abilities to bind to and to penetrate the ZP and to subsequently fuse with the oocyte plasma membrane. The AR is an irreversible exocytotic process leading to the release of hydrolytic enzymes and enabling the sperm to penetrate the ZP. This experiment was designed to determine whether the sperm source or the treatment affect the percentage of spermatozoa undergoing spontaneous (premature) AR, since this percentage use to be very low in absence of any specific stimuli.

The results showed that the AR was significantly affected both by the source and sperm treatment ($P < 0.05$, Table 6). The percentage of live spermatozoa with an intact acrosome was higher in epididymal than in ejaculated spermatozoa. Among treatments, the NW-group presented the highest acrosomal stability. Also, from the data in Table 6 it could be inferred that a faster AR in the Percoll® ejaculated spermatozoa was induced, delivering the sperm to a quick death.

3.6. Experiment 6. In vitro penetrability of matured oocytes by ejaculated and epididymal spermatozoa after different treatments

It is generally accepted that capacitation and ability to undergo the AR induced by the zona pellucida are required steps for the sperm penetration into the oocyte. Thus, the sperm penetration ability was considered the tool of election to assess the capacitation process in our experimental groups. Two hours post insemination (hpi), the oocyte penetration rate was affected by treatments ($P < 0.05$) but not by the sperm source (Table 7), with a significantly higher penetration in Percoll® group from ejaculated origin compared to any other group.

At 4 hpi, the penetration percentages were different for epididymal and ejaculated spermatozoa, being higher for

Table 7
In vitro penetration ability of epididymal and ejaculated boar spermatozoa prepared by three different treatments (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) after 2 hours of coculture. N (number of inseminated oocytes).

Source	Treatment	N	Penetration rate	S/O	Activated oocyte	Activated sperm
Epididymal	NW	95	4.2 ± 2.0[a,b]	1.2 ± 0.2	75.0 ± 25.0	50.0 ± 28.8
	BSA	100	16.0 ± 3.6[b]	1.9 ± 0.3	87.5 ± 8.5	50.0 ± 12.9
	PERCOLL	96	14.5 ± 3.6[b]	1.5 ± 0.1	64.2 ± 13.2	42.8 ± 13.7
Ejaculated	NW	94	1.0 ± 1.0[b]	1.0	100	0
	BSA	92	6.5 ± 2.5[a,b]	1.0	83.3 ± 16.6	0
	PERCOLL	89	38.2 ± 5.1[c]	3.7 ± 0.6	85.2 ± 6.1	41.1 ± 8.5

Penetration rate: percentage of penetrated oocytes; S/O: mean number of spermatozoa per penetrated oocyte; Activated oocyte: percentage of oocytes reassuming meiosis (activated oocytes at Anaphase II, Telophase II or with female pronucleus); Activated sperm: percentage of penetrated oocytes with swollen heads or male pronuclei in the ooplasma.
[a–c] Different superscripts in the same column indicate significant differences ($P < 0.05$).

Table 8
In vitro penetration ability of epididymal and ejaculated boar spermatozoa prepared by three different treatments (Unwashed, NW; washed in PBS-BSA, BSA; and washed in Percoll, PERCOLL) after 4 hours of coculture. N (number of inseminated oocytes).

Source	Treatment	N	Penetration rate	S/O	Activated oocyte	Activated sperm
Epididymal	NW	129	84.5 ± 3.2[a]	9.2 ± 0.8[a]	88.9 ± 3.0	76.1 ± 4.1[a,b]
	BSA	142	84.5 ± 3.0[a]	16.8 ± 1.6[b,c]	82.5 ± 3.4	80.8 ± 3.6[a,b]
	PERCOLL	152	90.1 ± 2.4[a]	11.1 ± 0.8[a,b]	80.2 ± 3.4	78.1 ± 3.5[a,b]
Ejaculated	NW	84	27.3 ± 4.8[b]	1.7 ± 0.1[d]	78.2 ± 8.7	60.8 ± 10.4[b,c]
	BSA	93	30.1 ± 4.7[b]	1.7 ± 0.2[d]	92.8 ± 4.9	42.8 ± 9.5[c]
	PERCOLL	116	78.4 ± 3.8[c]	18.1 ± 1.0[c]	85.6 ± 2.1	89.0 ± 3.3[a]

Penetration rate: percentage of penetrated oocytes; S/O: mean number of spermatozoa per penetrated oocyte; Activated oocyte: percentage of oocytes reassuming meiosis (activated oocytes at Anaphase II, Telophase II or with female pronucleus); Activated sperm: percentage of penetrated oocytes with swollen heads or male pronuclei in the ooplasma.
[a,b,c]Different superscripts in the same column indicate significant differences ($P < 0.05$).

the former (86.38 vs. 45.31; Table 8). Regarding treatments, the highest values were obtained again for Percoll® group. The same pattern was obtained for the mean number of sperm per oocyte (S/O) with higher values for epididymal and Percoll® groups (Table 8).

The percentage of activated oocytes (oocytes reassuming meiosis, at Anaphase II, Telophase II or with female pronucleus) ranged from 78.2 to 92.8% and it was not affected by sperm source or treatment. However, the percentage of penetrated oocytes with activated spermatozoa inside (swollen heads or pronuclei) was affected by both factors and the values were higher for epididymal than for ejaculated groups and for Percoll® than for NW or BSA treatments.

All together, the results showed that Percoll® treated spermatozoa from ejaculated origin displayed the highest ability to quickly penetrate the oocyte and activate it 2 hpi. At 4 hpi this ability was similarly reached by the epididymal spermatozoa undergoing any of the three treatments, but remained lower for the ejaculated spermatozoa undergoing NW or BSA treatments.

4. Discussion

4.1. Source and treatment affect the ability of the spermatozoa to penetrate the oocyte

Epididymal and seminal fluids contain proteins and other macromolecules that adsorb to the sperm surface. These so-called decapacitating factors may either occlude key sites on sperm, suppress sperm functional activity, or even reverse capacitation [33]. A number of decapacitating factors, though found on ejaculated sperm or in seminal plasma, are of epididymal origin [34]. The desorption of these factors correlates temporally with the acquisition of fertilizing ability and conditions such as hypertonic incubation, that accelerate desorption, also accelerate capacitation [35].

Seminal plasma, however, also contains factors that enhance capacitation, as proteins isolated from bull seminal plasma (BSP) that facilitate a cholesterol efflux from the plasma membrane [36]. Other factors from seminal plasma, such as the fertility promoting peptide (FPP) and related compounds [37] are able to stimulate capacitation, while they inhibit the spontaneous AR. Hence, these factors seem to stimulate capacitation without necessarily decreasing membrane stability. From this information, it is not clear whether the so called capacitating or decapacitating factors either in the epididymal or seminal fluids are actually involved or related to the ability of the sperm to quickly penetrate the oocyte.

The results from the present work seem to indicate that some commonly used parameters to assess capacitation are related to fast sperm penetration ability, whereas some others are not. Epididymal and ejaculated spermatozoa responded differently to the *in vitro* capacitation treatments, and that response was subsequently reflected in the IVF results. In general terms, epididymal spermatozoa seemed to be more "stable" (= more decapacitated?) than ejaculated ones, since they were less affected by the treatments. Lipid disorder, ROS generation, calcium uptake and spontaneous AR were significantly lower in epididymal spermatozoa than in ejaculated ones undergoing the same treatments. However, motility and ability to penetrate the oocyte were higher in epididymal spermatozoa than in ejaculated ones, with the exception of the ejaculated-Percoll® group, which responded similarly to the epididymal ones. A first question, which we will try to answer below, arises from these results: are motility, lipid disorder, ROS generation, calcium uptake, and

spontaneous AR good indicators of *in vitro* ability to penetrate the oocyte and, consequently, of capacitation? Alternatively, a second interesting question, focusing on the problem from the opposite side, would be: are the NW and BSA treatments optimal enough to quickly overpass the decapacitating effect of seminal plasma?

4.2. Motility parameters and spontaneous acrosome reaction are related to the ability of the sperm to penetrate the oocytes in vitro

One of the most important modifications that mammalian sperm undergoes during the capacitation process is the change in the mean motility pattern of capacitated cells. *In vitro* incubation of sperm from fresh ejaculates in a capacitating medium has been shown to induce a significant increase in several sperm motility parameters such as VCL, VSL, VAP, LIN, STR, and WOB [6]. Moreover, it has been reported that stimulation of motility was characterized by an increase in the linearity (LIN) and progressive velocity [38], as we have been able to corroborate, not only for ejaculated spermatozoa but also for epididymal ones. TALP medium contains bicarbonate and it is known that boar spermatozoa are highly sensitive to the presence of bicarbonate, responding with a rapid motility activation [38] as it could be the case in the present work.

A relationship between these changes in the motility patterns and the different IVF parameters can be found in the present results. Epididymal spermatozoa showed a higher motility than ejaculated sperm after the treatments and all the motion parameters related with velocities were higher. Epididymal spermatozoa showed also a faster ability to penetrate and activate the oocyte than the corresponding ejaculated cells for each treatment. An exception can be observed in the ejaculated-Percoll® group, where the penetration and activation rates were significantly higher than those in any other group, independently of the source of the spermatozoa at 2 hpi, and similar to those for epididymal spermatozoa at 4 hpi. But, interestingly, the same exception can be found after a careful analysis of the motility results, where crucial parameters as LIN or STR where higher for this group compared to the others. These results support our initial hypothesis that, under certain conditions (Percoll® treatment in our case), ejaculated sperm can penetrate the oocyte in similar (even faster, in the present results) levels than the epididymal spermatozoa. It is possible that Percoll® treatment selects a sperm subpopulation that respond faster and more efficiently to the incubation medium. Ramió et al [6] determined four motile-sperm subpopulations in boar ejaculates subjected to "*in vitro*" capacitation and the subpopulation 4 was characterized by high values of velocity and linearity. It has been described that boar sperm from separate subpopulations have different sensitivities in reacting against the addition of activators like caffeine and bicarbonate to the medium [39]. In the same sense, it has been described that separation of human semen through a Percoll® gradient renders separate sperm subpopulations with different abilities to undergo capacitation depending on their overall protein tyrosine phosphorylation status [40]. All together, these observations would support our hypothesis and partially explain the results in the present work.

Further support could be found in the results from the AR assay, where lower proportions of spermatozoa undergoing spontaneous AR in the IVF medium were found for epididymal (in all the treatments) and ejaculated-Percoll® group cells. Because an intact acrosome is required for sperm binding to the zona pellucida, it was expected that higher spontaneous AR in the IVF medium, not induced by the contact of the sperm with the oocyte's zona pellucida, decreases the ability of the sperm to penetrate the oocytes, as it occurred. Holt et al [39] also showed that the incidence of spontaneous, ionophore- and zona-induced acrosome reactions were significantly higher in ejaculates with further low litter size. On the other side, it is worthy to mention that the proportion of live intact spermatozoa in the ejaculated-Percoll® group was significantly lower than in the other groups, suggesting the presence of increased proportions of capacitated cells ready to bind ZP and undergo the AR at every precise time. This observation contribute to explain the higher ability of this group of spermatozoa to fastly penetrate the oocyte or, if the oocyte is not accessible, to die.

4.3. Membrane lipid disorder, ROS generation and calcium intake do not explain the differences in penetrability among spermatozoa from different sources or treatments

Changes in the lipids of membrane are very fast and continuous along the whole capacitation process [41]. In general terms, the observed lipid disorder when spermatozoa were diluted in TALP medium can be explained because bicarbonate, in presence of albumin, affects the lipid composition and cholesterol levels in complete sperm suspension. Similarly, one possible explanation for the lower lipid disorder observed in epididymal spermatozoa compared to the ejaculated ones can be found in the fact that epididymal sperm has

less cholesterol (the phospholipids/cholesterol rate is higher than in ejaculated sperm) since they have not been in contact to the seminal plasma and, consequently, the efflux is lower.

Our results show that lipid disorder in ejaculated spermatozoa is not affected by sperm treatment but the kinetic of sperm penetration is faster when Percoll treatment is applied. From these observations we hypothesize that lipid disorder does not seem to be related to the velocity of sperm penetration. Similarly, the higher "stability" of the epididymal spermatozoa, showing lower values of lipid disorder, did not correspond with higher velocities of penetration than those found in the ejaculated-Percoll® group. Although Harrison and Gadella [42] have evidence for a relationship between phospholipids scrambling in response to bicarbonate and fertility (as might be expected), it is clear that the scrambling response itself cannot be used as a parameter for the fully capacitated state [43] and, following the same reasoning, as a parameter to predict the velocity and the ability of penetration.

It has been proposed that ROS are key modulators of the early signal transduction mechanisms leading to capacitation (for a review see [44]) and spermatozoa themselves are long known to be producers of ROS [45]. On the other side, redox activity is suppressed in epididymal spermatozoa by powerful antioxidants including glutathione peroxidase, SOD, and catalase (present both in epididymal plasma and intracellular sperm) that are loosely absorbed onto the sperm surface and released by discontinuous gradient centrifugation [46,47]. This observation fits the results from the present work, where epididymal spermatozoa produced less ROS than ejaculated ones. Similarly, it was expected that treatment affected ROS since the centrifugation process generates ROS and then, the highest level of ROS in Percoll® groups could be explained by the longer centrifugation time this treatment needs. However, if the level of ROS generation was assumed as an indicator of capacitation, the immediate conclusion would lead us to state that capacitation is then not related to the ability of penetration, because a relationship between ROS generation and penetration rate could not be found in our results.

A similar rationale can be used for the Ca^{2+} uptake results, where no further relationship with the sperm ability to penetrate the oocyte was found. Changes in intracellular ion concentrations are associated with different aspects of sperm function such as hyperactivation [13]. With the methodological approach used in the present work, neither the steady increase in the Ca^{2+} uptake along the 60 min of the assay for the six different experimental groups nor the mean values at the end of the assay let us correlate this parameter with the differences found in the motility, lipid disorder, AR, or IVF results. However, a similar tendency was observed for the Ca^{2+} uptake and the ROS generation in the different experimental groups.

In conclusion, ejaculated spermatozoa washed by a Percoll® gradient penetrate and activate the oocyte *in vitro* faster than those washed with BSA or kept unwashed, and at 2 hpi they penetrate the oocyte even faster than epididymal spermatozoa under any treatment. Higher linear motility and lower percentage of spontaneous AR could be related to this ability. It is proposed that Percoll® treatment of ejaculated spermatozoa can induce capacitation pathways making the sperm able to reach fertilization ability at similar levels to those found in the epididymal spermatozoa.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2010.06.002.

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