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ORIGINAL RESEARCH

Picomolar gradients of progesterone select functional human sperm even in subfertile samples

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ABSTRACT: More than 1 million infertility treatments are practiced around the world per year, but only 30% of the couples succeed in taking a baby home. Reproductive technology depends in part on sperm quality, which influences not only fertilization but also embryo development and implantation. In order to provide a better quality sperm subpopulation, innovative sperm selection techniques based on physiological sperm features are needed. Spermatozoa at an optimum state may be selected by following an increasing concentration gradient of picomolar progesterone, a steroid secreted by the cumulus cells at the time of ovulation. In this study we developed a method to recruit spermatozoa at the best functional state, based on sperm guidance toward progesterone. The sperm selection assay (SSA) consists of a device with two wells connected by a tube. One well was filled with the sperm suspension and the other with picomolar progesterone, which diffused inside the connecting tube as a gradient. The sperm quality after the SSA was analyzed in normal and subfertile semen samples. Several sperm parameters indicative of sperm physiological state were determined before and after the SSA: capacitation, DNA integrity and oxidative stress. After the SSA, the mean level of capacitated spermatozoa increased three times in normal and in subfertile samples. The level of sperm with intact DNA was significantly increased, while sperm oxidative stress was decreased after sperm selection. Interestingly, the exposure to a progesterone gradient stimulated the completion of capacitation in some spermatozoa that could not do it by themselves. Thus, the SSA supplies a sperm population enriched with spermatozoa at an optimum physiological state that may improve the assisted reproductive technology outcome.

Key words: sperm selection / sperm chemotaxis / progesterone

Introduction

Infertility is a disease that is increasing worldwide, affecting >70 million couples, with about 50% of the cases due to male inability to fertilize the female oocyte (Boivin *et al.*, 2007). In the last 40 years, several techniques have been developed to treat infertility (ART, Assisted Reproduction Technology). More than 1 million infertility treatments are practiced around the world per year, but only 30% of the couples succeed in taking a baby home. The remaining frustrated couples try again, sometimes several times, an expensive and emotionally disturbing treatment (Nygren *et al.*, 2011). Even though ART allows the birth of babies that would be impossible under natural circumstances, it is still necessary to improve the procedures to increase treatment efficiency.

The success of ART depends in part on sperm quality. Indeed, the influence of spermatozoa is notable even beyond fertilization, during embryo development and implantation (Barroso *et al.*, 2009). Sperm preparation prior to ART involves the removal of seminal plasma. However, for ICSI—the ART mainly used nowadays (Nygren *et al.*, 2011), which involves the injection of one spermatozoon into the oocyte—further sperm selection is needed. In general, the operator subjectively selects the sperm for its apparent good morphology and motility, but these parameters are poor indicators of sperm fertilizing potential (Lewis, 2007; Barratt *et al.*, 2011). In order to provide a better quality sperm subpopulation for ICSI, additional sperm selection techniques have been recently developed. These methods base sperm selection on surface charge, apoptosis markers, membrane maturity or

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ultramorphology. However, the controversial reports indicate a need to improve them or to develop new technologies (Said and Land, 2011).

An ideal method for sperm selection should be based on sperm physiological aptitudes that better reflect its competence to fertilize the oocyte. Capacitation is a physiological state that enables the spermatozoon to fertilize the oocyte (Eisenbach, 1999). Interestingly, capacitated spermatozoa may be oriented by following an increasing concentration gradient of an attractant molecule, a phenomenon called sperm chemotaxis. This is a guidance mechanism observed *in vitro* (Eisenbach and Giojalas, 2006), which may transport and retain spermatozoa at the fertilization site (Guidobaldi *et al*., 2012). Though several molecules have been suggested to attract human spermatozoa (Eisenbach and Giojalas, 2006), in the context of gamete interaction prior to fertilization, progesterone has biological importance (Guidobaldi *et al*., 2008; Oren-Benaroya *et al*., 2008) for several reasons. After ovulation, this hormone is secreted by the cumulus cells that surround the oocyte (Bar-Ami *et al*., 1989; Vanderhyden and Tonary, 1995; Chian *et al*., 1999; Yamashita *et al*., 2003; Guidobaldi *et al*., 2008), diffusing to form a molecular gradient toward the periphery of the cumulus and beyond (Teves *et al*., 2006). Notably, a gradient of very low concentrations (picomolar) of progesterone is sufficient to chemically attract capacitated human spermatozoa (Teves et al., 2006, 2009a, b; Blengini et al., 2011). The aim of the study was to develop a sperm selection assay (SSA) based on sperm exposure to picomolar gradients of progesterone, to provide a sperm population enriched with spermatozoa in an optimum functional state for fertilization.

Materials and Methods

Criteria for semen sample classification

Experiments were designed for human semen samples in accordance with the Declaration of Helsinki. The study was conducted with the approval from the Ethics Committee of the National Clinical Hospital (National University of Cordoba, Argentina; permit number: 061/10) and with written informed consent from the patients.

Sperm samples were collected by masturbation after 3–5 days of sexual abstinence and then classified according to the reference values suggested by the WHO (2010). Semen samples were considered normal ($n = 21$) on the basis of percentile 25 of population values for sperm concentration, motility and morphology (Cooper *et al*., 2010). Additionally, subfetile semen samples classified as terazoospermic ($n = 21$), asthenoteratozoospermic ($n = 26$), oligozoospermic ($n = 5$), and those with a history of unexplained sterility ($n = 6$) were included in the study. All samples were provided by the Laboratorio de Andrología y Reproducción (LAR, Córdoba, Argentina) with the exception of those of unexplained sterility, which were kindly provided by the Centro Integral de Ginecología, Obstetricia y Reproducción (CIGOR, Córdoba, Argentina).

Sperm preparation

The seminal plasma was removed by a discontinuous Percoll gradient (Aitken and Clarkson, 1988). The highly motile sperm population was adjusted to 7×10^6 cells/ml with HAM-F10 medium containing 25 mM HEPES and L-glutamine (Invitrogen, Argentina), supplemented with 1% human albumin (Laboratorio de Hemoderivados, Universidad Nacional de Córdoba, Argentina) and then incubated under capacitating conditions at 37°C in 5% CO_2 in air for 4 h, unless otherwise indicated.

Sperm selection assay

The device consists of two wells (each with a maximum capacity of 250 μl) connected by a tube of 2 mm length per 2.5 mm diameter. In addition, the device is provided with a hermetic closing system which includes screw caps over a rubber o-ring and holes to favor the skip of air while screwing the caps. One well (W1) is first filled with 130 μl of the sperm suspension and the other (W2) with 130 μl of the attractant molecule solution (Fig. 1A). Thus, in the SSA device, progesterone diffuses from W2 to W1 through the connecting tube according to Fick's law applied to one-dimension diffusion with a continuous supply of molecules (Supplementary data, Fig. S1). The experimental conditions to use the SSA were previously defined as shown in Supplementary data, Fig. S2. The cap was placed in W2 and the connecting tube was filled with the culture medium described above. Spermatozoa at a concentration of 6×10^6/ml were placed in W1, which was closed with a cap. Immediately after, the cap of W2 was removed and a 10 pM solution of progesterone (Sigma-Aldrich, St. Louis, USA) diluted in culture medium was added inside the well, closing it with the cap. The device was incubated at 37°C in 5% CO_2 in air for 20 min. The cap of W2 was taken off and the sperm suspension was removed from the well.

Sperm capacitation determined as the ability to phosphorylate proteins in tyrosine residues (PY)

For each sperm sample, the SSA was run in parallel with 10–20 devices with or without 10 pM progesterone. After the SSA, spermatozoa collected from W2 (from each set of devices) were pooled and fixed in 1% formaldehyde in PBS for 20 min at room temperature. After washing in PBS, the samples were resuspended in RIPA and Laemmli loading buffer and then boiled for 10 min. Proteins were loaded onto 8% SDS-PAGE gel, electrophoretically separated and transferred to nitrocellulose membrane (BioRad,Hercules, USA). Membranes were blocked for 2 h in 5% BSA-TTBS and then incubated overnight, first with antibody anti-phosphotyrosine (1:5,000; 4G10, Millipore, Billerica, USA) at 4°C, followed by anti-mouse IgG biotin-conjugated secondary antibody (1:10,000; Sigma-Aldrich, St. Louis, USA) and streptavidin-peroxidase (1:2,000; Sigma-Aldrich, St. Louis, USA) at room temperature. Total phosphorylated proteins were visualized by enhanced chemiluminescence and the whole lane was quantified for relative intensity with the ImageJ software (ver. 1.38; NIH, Bethesda, USA).

Sperm capacitation determined as the ability to undergo induced acrosome reaction

The percentage of spermatozoa that underwent the induced acrosome reaction was determined as an indirect indicator of the level of capacitated spermatozoa (Jaiswal *et al*., 1998, 1999) as previously described (Fabro *et al*., 2002), and staining the acrosome according to Larson and Miller (1999). Briefly, the sperm suspension was divided into two aliquots, one of which was stimulated with 8 μM A23187 (Sigma-Aldrich, St. Louis, USA) for 30 min at 37°C and the other kept as control. Then the cells were fixed in 1% formaldehyde in PBS for 20 min at room temperature. The sperm suspensions were washed by centrifugation in 100 mM ammonium acetate. The pellet was let dry over a slide and spermatozoa were stained with Coomassie Blue G250 (Sigma-Aldrich, St. Louis, USA) for 10 min. The state of the acrosome (intact or reacted; Supplementary data, Fig. S3) was observed at 1000× under light microscope (Olympus, Center Valley, USA) in 200 cells counted at random in each duplicated slide. The percentage of capacitated spermatozoa was determined as the difference in the percentage between induced and spontaneous acrosome-reacted spermatozoa.

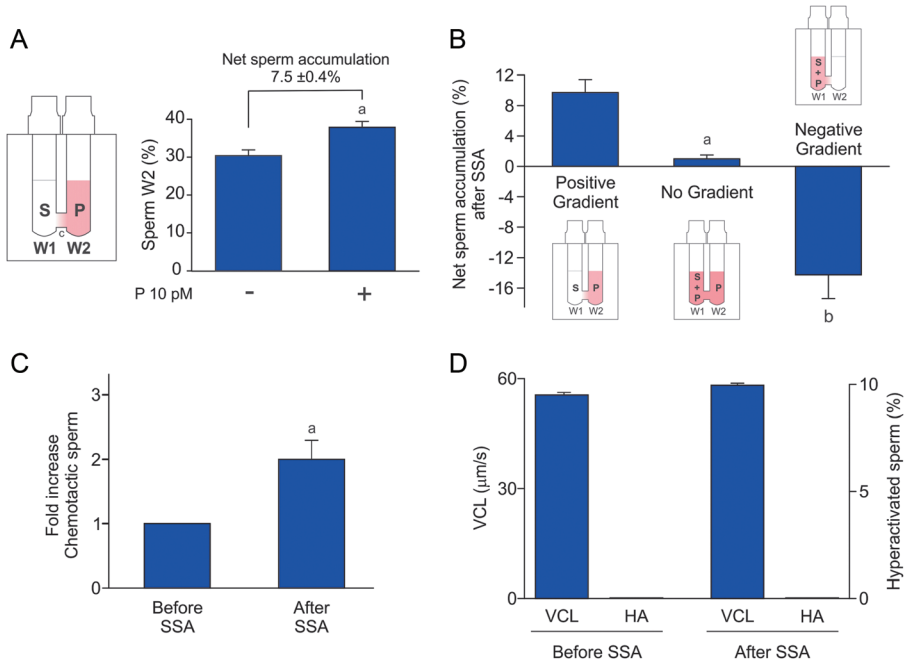

Figure 1 The SSA selects spermatozoa by chemotaxis. (**A**) a schematic representation of the SSA device is on the left, showing well 1 (W1) containing sperm (S) and well 2 (W2) progesterone (P), and inside the connecting tube (c) a positive gradient of progesterone is formed from W2 to W1. The percentage of spermatozoa accumulated in W2 after the SSA performed with or without progesterone and the corresponding difference expressed as the net sperm accumulation are shown on the right. Data are expressed as mean ± SE of 21 independent experiments performed with normal semen samples. [a]significant differences versus without progesterone ($P < 0.01$). (**B**) Inhibition of sperm chemotaxis by disrupting the progesterone gradient. The experimental conditions are shown in the corresponding drawings of the SSA device, indicating the wells with sperm (S) or progesterone (P) loaded in each case. Data are expressed as mean ± SE of three independent experiments performed with normal semen samples. Significant differences versus the positive gradient (control): [a]$P < 0.05$, [b]$P < 0.001$. (**C** and **D**) Fold increase in the percentage of chemotactic sperm, curvilinear velocity (VCL) and percentage of hyperactivated sperm (HA) determined before the SSA and in the sperm population recovered from W2 after the SSA. Data are expressed as mean ± SE of three independent experiments performed with normal semen samples. [a]significant differences versus before the SSA ($P < 0.05$).

Sperm DNA integrity

The level of DNA integrity was evaluated by means of the 'Comet Assay' kit (Trevigen, Gaithersburg, USA) according to the manufacturer's instructions. Briefly, the sperm suspension was mixed with low-fusion agarose solution and then spread over the slide. The samples were treated with the lysis solution for 30 min at 4°C. To allow a complete unfolding of DNA helix, the samples were treated with an alkaline solution for 20 min at room temperature in the dark, and then the electrophoresis was performed. Sperm chromatin was stained with SYBR-Green and observed in an inverted fluorescence Nikon Ti-S microscope (Nikon Instruments Inc., New York, USA) at 600×. The fluorescence digital images were taken with a Nikon DSQi1 and captured by means of the software NIS-elements Br ver. 3.06 (Nikon Instruments Inc., New York, USA). A hundred sperm nuclei per treatment were photographed at random and analyzed with the Comet Score software ver. 1.5 (TriTeck Corp, Virginia, USA). The percentage of spermatozoa with intact DNA was calculated as described by others (Hughes *et al.*, 1996), considering a DNA fragmented spermatozoon as when the head of the comet shows <80% of the fluorescence (Supplementary data, Fig. S4).

Sperm protein tyrosine phosphorylation (PY) chemotactic pattern

Spermatozoa were immunostained for PY as previously described (Teves *et al.*, 2009b). Briefly, the cells were fixed and permeabilized with 1% formaldehyde and 0.2% Triton X-100. Then the sperm were incubated with 3% H_2O_2 and washed with PBS. Slides were blocked with 5% BSA-PBS/0.2% Triton X-100 for 30 min at room temperature and then incubated with a mouse monoclonal anti-phosphotyrosine antibody (4G10; Millipore, Billerica, USA) diluted 1:50 in a solution of 1% BSA-PBS/0.2% Triton X-100, overnight at room temperature. The negative control was similarly treated with normal mouse serum. After washing several times with 1% BSA-PBS/0.2% Triton X-100, slides were incubated with anti-mouse IgG secondary antibody biotin-conjugated (Sigma-Aldrich, St. Louis, USA), diluted 1:100 in a solution of 1% BSA-PBS/0.2% Triton X-100 for 2 h. Then the samples were washed with PBS and treated with streptavidin-peroxidase (LAB-VISION, UK) for 30 min. Finally, cells were stained with the AEC kit (Sigma-Aldrich, St. Louis, USA) and observed under a light microscope (Olympus, Center Valley, USA). The percentage of sperm showing the PY chemotactic pattern (equatorial band and tail) was determined in 200 cells counted at random in each duplicated slide.

Sperm morphology

Spermatozoa were stained on a slide with the Papanicolaou technique (Cooper *et al.* 2010), and sperm morphology was evaluated under light microscope at 1000× magnification following Kruger's strict criteria (Kruger *et al.*, 1986). The percentage of spermatozoa with normal morphology was determined in 200 cells at random in duplicate slides. Sperm morphology was determined by an expert operator who routinely performs

this analysis in an andrology laboratory, which is subjected to regular internal control by calculating the coefficient of variation and to external quality control procedures performed by the University of Buenos Aires (Argentina).

Sperm oxidative stress

Sperm nitric oxide (NO) and superoxide anion (O_2^-) production were determined by fluorescence markers, as previously reported by others (Aitken *et al*., 2006; de Lamirande *et al*., 2009). Spermatozoa were incubated with either 10 μM DHE (Molecular Probes, Eugene, USA) for NO or DAF2-DA (Molecular Probes, Eugene, USA) for O_2^-, diluted in Ham F-10 without albumin for 20 or 60 min, respectively, at 37°C. Samples were then washed by centrifugation with cold PBS and then fixed with 1% formaldehyde for 20 min at room temperature. After washing in PBS, the pellet was smeared on a slide and let dry in the dark. Then the cells were washed once with distilled water. Once dried, the slide was mounted with antibleaching (SlowFade® Light Antifade Kit; Molecular Probes, Eugene, USA). Digitalized images were captured in an inverted fluorescence microscope at 1000× (Nikon Instruments Inc., New York, USA). The average fluorescence intensity (mean gray values) of the sperm head from 100 cells per treatment was determined with the ImageJ software (ver. 1.38, NIH, Bethesda, USA).

Sperm chemotaxis and other kinematic parameters

This parameter was determined in a chemotaxis chamber by videomicroscopy and image analysis as previously described (Fabro *et al*., 2002; Guidobaldi *et al*., 2008). Briefly, the chemotaxis chamber consists of two wells separated by a 2-mm wall, one filled with medium with or without 10 pM progesterone and the other one with spermatozoa. The chamber was sealed with a coverslip, thus forming a capillary space between both wells where a one-dimension attractant concentration gradient is formed in the direction of the well containing the spermatozoa. Fifteen minutes after sealing the chamber, the sperm movement was recorded in the middle of the separating wall with a digital camera connected to a phase contrast microscope (Olympus, Center Valley, USA). Recordings were performed at 6 Hz with the Virtualdub software (ver. 1.6.16, Avery Lee; http://www.virtualdub.org/). The sperm tracks were analyzed by image analysis with the ImageJ software (ver. 1.38, NIH, USA) and the MtrackJ plugin (ver. 1.1.0, Eric Meijering; http://www.imagescience.org/ meijering/software/mtrackj/). For each sperm track, the distance traveled along the gradient axes (DX) and the absence of gradient axes (DY) were calculated. Then, sperm directionality was determined by the quotient DX/|DY|. When the value was ≥1, the spermatozoon was considered oriented toward the attractant well. The chemotactic responding subpopulation was determined as the difference in the percentage of 'oriented spermatozoa' between the progesterone solution and the culture medium calculated in 150 spermatozoa per treatment with the SpermTrack software (ver. 4.0, Universidad Nacional de Córdoba, Argentina). In the same sperm tracks, sperm curvilinear velocity and hyperactivation was determined as previously described (Fabro *et al*., 2002). The percentage of motile spermatozoa was objectively determined in an observation chamber at 37°C by means of the videomicroscopy and image analysis system described above, in 200 sperm per treatment.

Sperm parameters calculated after the SSA

(i) *Net sperm accumulation in W2 after the SSA*. This parameter was calculated as the difference in the percentage of spermatozoa recovered from W2 with or without progesterone, which was determined by dividing the number of spermatozoa recovered from W2 after the SSA by the number of spermatozoa placed in W1 before the SSA, multiplying the result by 100. (ii) *Fold increase after the SSA for*: (a) *the percentage of spermatozoa* (capacitated, with intact DNA, with normal morphology, with chemotactic behavior or showing the PY chemotactic pattern), (b) *the absolute number of capacitated spermatozoa* (by multiplying the number of spermatozoa by the percentage of capacitated spermatozoa divided by 100) and (c) *the level of sperm oxidative stress* (as the fluorescence intensity emitted by the fluorescence probes for sperm NO and the superoxide anion O_2^- production). For each sperm parameter, the fold increase was calculated by dividing the corresponding value after the SSA by that before the SSA.

Statistical analysis

Significant differences between mean values were determined by means of the Student 't' test (with previous square root arccosine transformation of data) or a non-parametric Mann–Whitney by means of the software SigmaPlot ver. 11.0 (SPSS, Inc., Chicago, USA), where differences between treatments were considered statistically significant at $P \leq 0.05$.

Results

The sperm selection assay selects capacitated spermatozoa

We previously observed by analyzing single sperm track directionality that a 10 pM gradient of progesterone stimulates chemotaxis in capacitated sperm but not chemokinesis nor hyperactivation (Teves *et al*., 2006). Therefore, to apply the chemotaxis phenomenon to recruit capacitated spermatozoa, we designed a new device (Fig. 1A) preserving the physical properties and the experimental conditions of the chemotaxis chamber used in the past (Fabro *et al*., 2002; Teves *et al*. 2006, 2009a, b). Moreover, to keep the attractant gradient (exclusive requirement for chemotaxis), the SSA was provided with a hermetic closing system combined with a sequential procedure that prevents the mechanical displacement of fluids between wells. In addition, the selected sperm subpopulation can be easily recovered at the end of the assay. Next we experimentally defined the optimum conditions to run the SSA (Supplementary data, Fig. S2), which were similar to those used with the chemotaxis chamber. Then, we performed the SSA with normal semen samples running two devices with or without placing progesterone in W2. As expected, after the SSA, spermatozoa were significantly accumulated in the W2 containing progesterone (Fig. 1A). We next verified whether the observed sperm accumulation was indeed due to the recruitment of capacitated spermatozoa.

Initially, the SSA was designed to recruit capacitated spermatozoa as an inherent attribute of chemotaxis (Cohen-Dayag *et al*., 1995; Fabro *et al*., 2002); therefore, the occurrence of chemotaxis in the SSA was verified in different ways. Since chemotaxis is the only kinematic mechanism that is dependent on the attractant distribution as a concentration gradient, we next performed classic control procedures for chemotaxis. These kinds of assays involve the disruption of the attractant gradient and are recommended for accumulation assays to discard effects different from chemotaxis (Eisenbach, 1999; Eisenbach and Giojalas, 2006). Thus, the 'positive' gradient of progesterone from W2 to W1 was avoided in two ways. Firstly, when the same concentration of the hormone is loaded in both wells, no attractant gradient is generated; therefore, chemotactic cells are expected to move at random without preferably accumulating in W2. Under this experimental condition, sperm accumulation in W2 was significantly reduced in comparison with the value observed in the positive gradient control, suggesting that sperm were evenly distributed between wells (Fig. 1B, middle

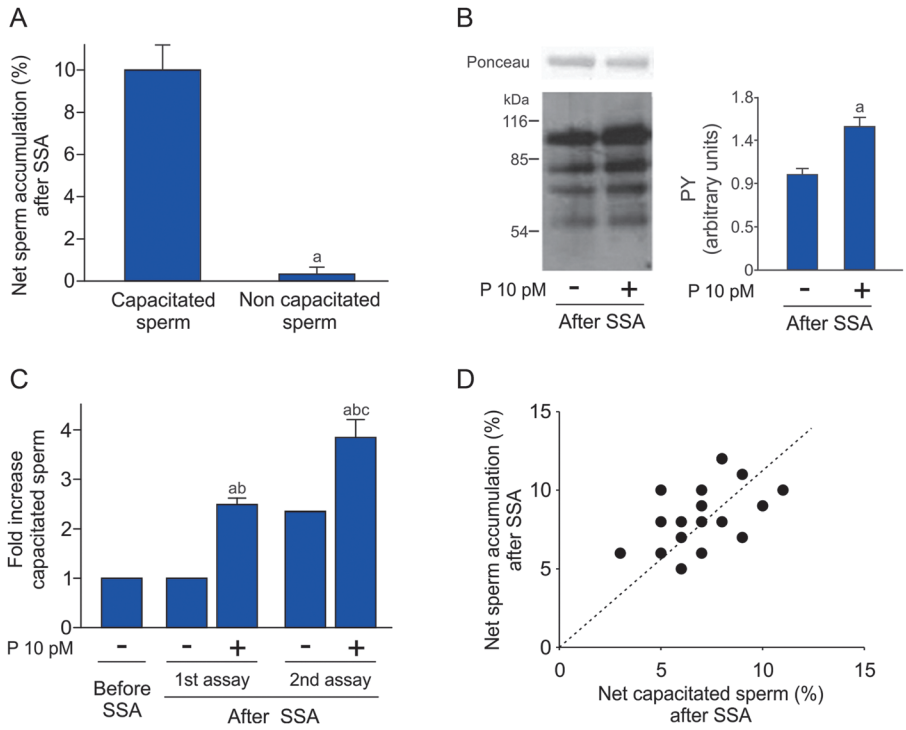

Figure 2 The SSA selects capacitated spermatozoa. (**A**) Net sperm accumulation after the SSA performed with or without previously incubating spermatozoa under capacitating conditions. Data are expressed as mean ± SE of three independent experiments performed with normal semen samples. [a]significant differences versus the control group performed with capacitated sperm ($P < 0.01$). (**B**) Representative Western Blot for PY after the SSA with or without progesterone and the corresponding quantification referred to as Ponceau labeling. Data are expressed as mean ± SE of four independent experiments performed with normal semen samples. [a]significant differences versus without progesterone ($P < 0.05$). (**C**) Fold increase in the level of capacitated spermatozoa before and after one or two consecutive SSAs, with or without progesterone. Data are expressed as mean ± SE of three independent experiments performed with normal semen samples. [a]significant differences versus before the SSA ($P < 0.05$), [b]significant differences versus without progesterone ($P < 0.05$), [c]significant differences versus after the first SSA with progesterone ($P < 0.05$). (**D**) Correlation of the net sperm accumulation obtained after the SSA with the net percentage of capacitated sperm recovered from W2. Correlation coefficients and statistical significance are mentioned in the text. Data represent results from 21 independent experiments with normal semen samples.

bar). Secondly, when progesterone is loaded together with spermatozoa, a 'negative' gradient of the hormone is formed from W1 to W2, in which some cells swim out by chance from W1. While navigating along the connecting tube, chemotactic sperm are expected to sense the negative gradient of progesterone, and as a consequence, they return to W1 (Blengini *et al.*, 2011). This experimental setting provoked a negative sperm accumulation in W2 in comparison with the positive attractant gradient run as the control, meaning that spermatozoa were retained in W1 (Fig. 1B, right bar).

Furthermore, we investigated the chemotactic response of spermatozoa recovered from W2 in a chemotaxis chamber by single-cell directionality analysis. If chemotaxis is taking place in the SSA, a significant increment in chemotactic sperm is expected in W2. After the SSA, the level of chemotactic sperm was around two times higher than the value observed before running the SSA (Fig. 1C). As expected for this steroid concentration (Teves *et al.*, 2006), neither chemokinesis nor hyperativation was observed in these spermatozoa (Fig. 1D).

In order to confirm whether capacitated sperm were indeed recruited in W2, several approaches were performed. Thus, sperm accumulation in W2 was only observed when spermatozoa were previously incubated under capacitating conditions (Fig. 2A). We next evaluated the recruitment of capacitated sperm by determining the increment in phosphorylated proteins in tyrosine residues. Consistently, the level in protein phosphorylation was significantly increased after running the SSA with progesterone (Fig. 2B). However, the latter technique does not provide information about the proportion of capacitated spermatozoa recruited in W2. Therefore, hereafter we determined the level of capacitated spermatozoa as the sperm's ability to undergo the induced acrosome reaction (Jaiswal *et al.*, 1998, 1999). After the SSA, capacitated spermatozoa increased >2-fold only in the presence of progesterone (Fig. 2C). Moreover, when the sperm population recovered from W2 was used to run a second SSA, the enrichment in capacitated cells was almost 4-fold in comparison with the level of capacitated sperm before the SSA (Fig. 2C), reinforcing the notion that the SSA recruits capacitated sperm. Furthermore, after the SSA the level of capacitated spermatozoa in W2 was highly correlated with the level of sperm accumulated in W2 ($r^2 = 0.94$, $P < 0.001$, $n = 21$; Fig. 2D). In other words, the higher the amount of recruited capacitated sperm the higher the number of accumulated sperm after the SSA. Taken together, these results confirm that the SSA selects and concentrates capacitated spermatozoa.

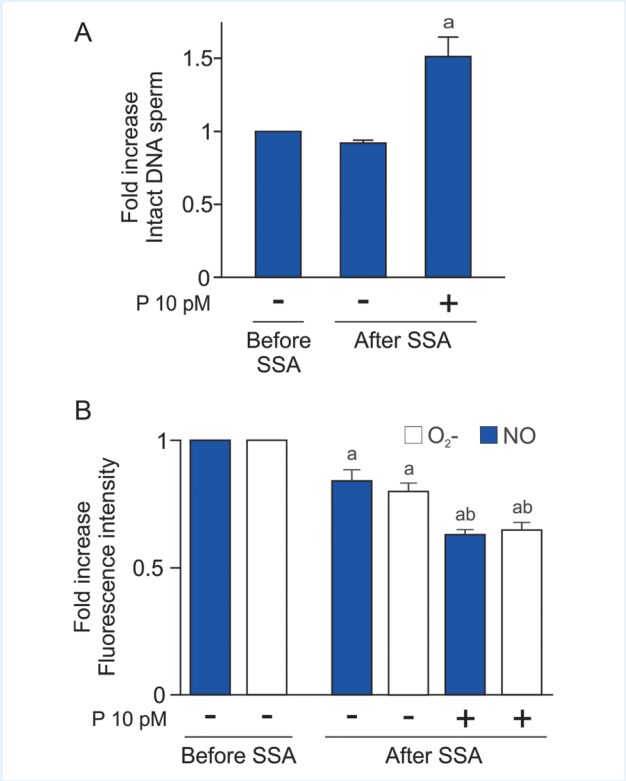

Figure 3 The selected sperm population is enriched with spermatozoa in the best functional state. (**A**) Fold increase in the level of sperm showing intact DNA before the SSA and after the SSA, with or without progesterone. Data are expressed as mean ± SE of four independent experiments performed with normal semen samples. [a]significant differences versus before the SSA and after the SSA without progesterone ($P < 0.05$). (**B**) Fold increase in the sperm NO and superoxide anion O_2^- content before the SSA and after the SSA with or without progesterone. Data are expressed as mean ± SE of four independent experiments performed with normal semen samples. [a]significant differences versus before the SSA ($P < 0.05$), [b]significant differences versus after the SSA without progesterone ($P < 0.05$).

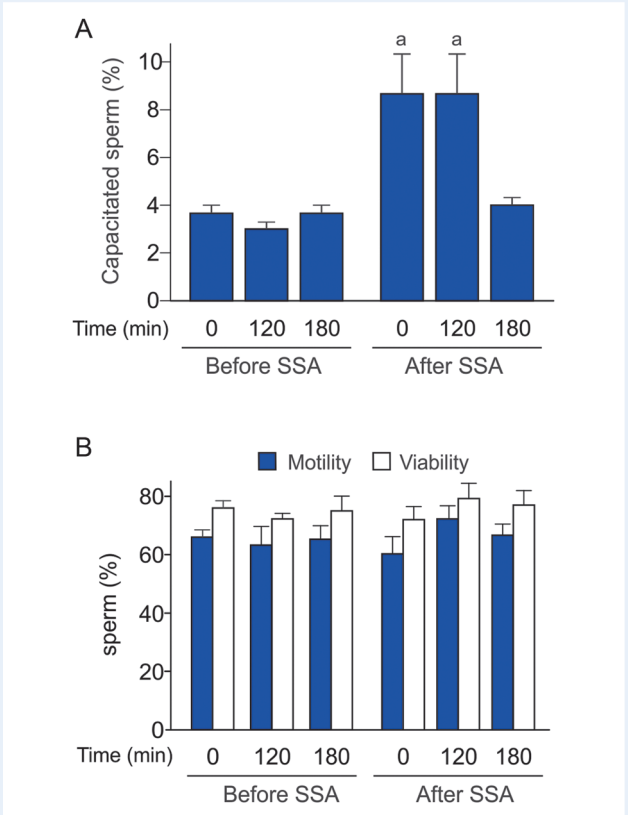

Figure 4 Kinetics of the sperm physiological state after the SSA. (**A**) capacitated sperm and (**B**) motile and viable sperm, before and after the SSA. Data are expressed as mean ± SE of three independent experiments performed with normal semen samples. [a]significant differences versus before SSA and after the SSA at 180 min ($P < 0.05$).

The selected sperm population is enriched with spermatozoa in the best functional state

We further examined the quality of the sperm population recovered from W2 in normal semen samples. The integrity of sperm DNA has been associated with the success of fertilization, embryo development and implantation (Barroso *et al.*, 2009), whereas DNA fragmentation increases during the conventional preparation of spermatozoa associated with oxidative stress (Aitken and De Iuliis, 2010). Similar to capacitation, the SSA containing a progesterone gradient favors the selection of a sperm population enriched with spermatozoa with intact DNA (Fig. 3A). Consistently, sperm oxidative stress, measured as the level of superoxide anion and NO in spermatozoa, was significantly decreased after the SSA, where the reduction was higher in the presence of progesterone (Fig. 3B).

Since the capacitation state is transitory (Cohen-Dayag *et al.*, 1995), we next verified the duration of sperm capacitation and its relation to sperm motility and viability after the SSA. The sperm population recovered from W2 after the SSA with progesterone were washed by centrifugation, then resuspended in culture medium and further incubated for different periods of time. Interestingly, the capacitation state of spermatozoa recovered from W2 lasted for 2 h (Fig. 4A) as observed by others (Cohen-Dayag *et al.*, 1995), while sperm motility and viability remained constant (Fig. 4B). These features provide a time window to further use the enriched sperm population selected by the SSA.

In summary, the sperm population selected by progesterone in the SSA is enriched not only with spermatozoa that are capacitated but also with intact DNA, low level of oxidative stress and high motility and viability.

Picomolar gradients of progesterone stimulate sperm capacitation

If the sole effect of the SSA was to recruit capacitated sperm in W2, the potency of the method should be the same for all the semen samples (around 2-fold increase as shown in Fig. 2C). However, the increase in capacitated cells in W2 was inversely proportional to the level of sperm capacitation the sample had before the SSA (Fig. 5A; $r^2 = -0.64$, $P < 0.002$, $n = 21$). Two groups were arbitrarily defined: samples where the potency of the SSA was relatively constant (group 1) and samples where the potency of the SSA was highly variable (group 2) and interestingly associated with a low incidence of

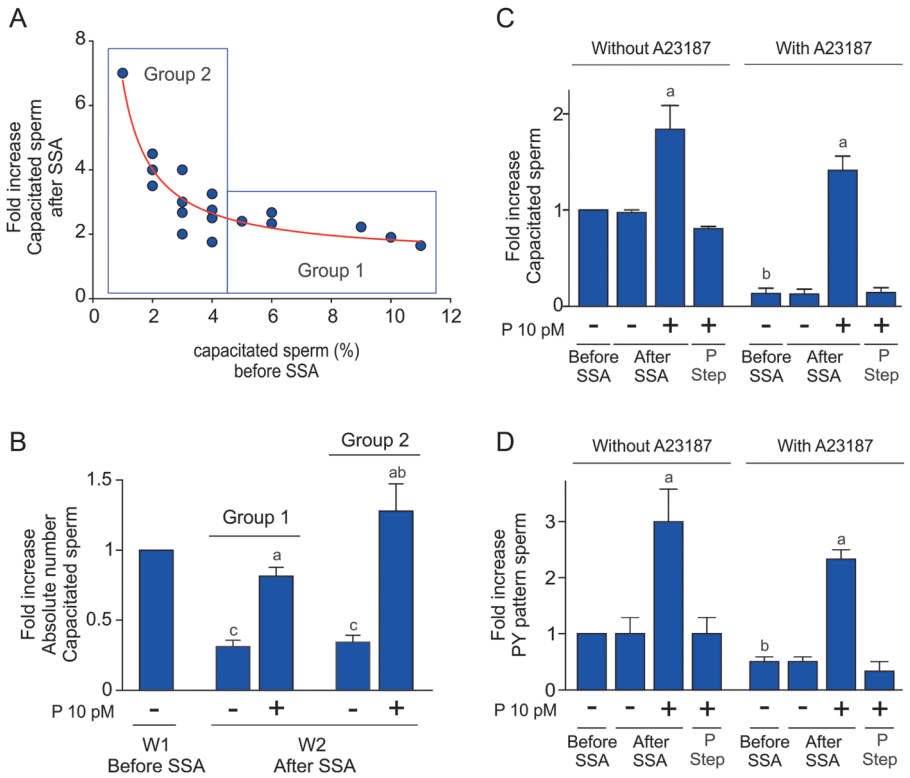

Figure 5 Picomolar gradients of progesterone stimulate sperm capacitation. (**A**) Correlation between the fold increase in the level of capacitated spermatozoa after the SSA and the percentage of capacitated sperm before the SSA. Two groups were arbitrarily defined: samples where the potency of the SSA was relatively constant (group 1) and samples where the potency of the SSA was highly variable (group 2) which have lower incidence of spontaneous capacitation. Correlation coefficients and statistical significance are noted in the text. (**B**) Fold increase in the absolute number of capacitated spermatozoa before and after the SSA, performed with or without progesterone in groups 1 and 2 described in A. Data are expressed as mean ± SE of independent experiments performed with 6 (group 1) and 15 (group 2) normal semen samples, [a]significant differences versus after the SSA without progesterone ($P < 0.01$). [b]significant differences versus before the SSA ($P < 0.01$), [c]significant differences versus before the SSA ($P < 0.001$). (**C**) Sperm samples were or were not treated with calcium ionophore (A23187), and after washing, the cells were loaded in W1 to perform the SSA with or without progesterone. The fold increase in capacitated spermatozoa was calculated before and after the SSA. Additionally, sperm were incubated with 10 pM progesterone homogenously distributed for 20 min (same period as the SSA) as a control (P step). Data are expressed as mean ± SE of five independent experiments performed with normal semen samples. [a]significant differences versus before and after the SSA without progesterone and the step application of the hormone ($P < 0.01$). [b]significant differences versus before the SSA without calcium ionophore pretreatment ($P < 0.001$). (**D**) Sperm samples were or were not induced to acrosome react with calcium ionophore (A23187), and after washing, the cells were loaded in W1 to perform the SSA with or without progesterone. The fold increase in sperm expressing the PY chemotactic pattern was calculated before and after the SSA. In addition, sperm were incubated with 10 pM progesterone homogenously distributed for 20 min (same period as the SSA) as a control (P step). Data are expressed as mean ± SE of 4 independent experiments performed with normal semen samples. [a]significant differences versus before and after the SSA without progesterone and the step application of the hormone ($P < 0.05$). [b]significant differences versus before the SSA without calcium ionophore pretreatment ($P < 0.01$).

spontaneous capacitation. This analysis led to the suspicion that in samples of group 2 more capacitated cells than those loaded in W1 were recruited in W2. Therefore, we calculated the absolute number of capacitated spermatozoa recovered from W2 which was seen to be different in both groups. Thus, in samples from group 1, most capacitated spermatozoa loaded in W1 were recovered from W2 (Fig. 5B). Conversely, in samples of group 2, there was a significantly higher absolute number of capacitated spermatozoa collected in W2 than that loaded in W1 (Fig. 5B). The enrichment in capacitated spermatozoa in W2 was observed only under a gradient of progesterone, since in both groups one third of the capacitated spermatozoa loaded in W1 swam randomly to W2. These results suggest that the exposure to gradients of pM progesterone not only recruits capacitated spermatozoa but may also promote sperm capacitation. We further explore the latter possibility.

When capacitated sperm are induced to acrosome react, they cannot further be oriented by chemotaxis toward follicular fluid (Cohen-Dayag *et al.*, 1995; Fabro *et al.*, 2002). Therefore, we designed experiments where capacitated spermatozoa were induced to acrosome react with calcium ionophore (Jaiswal *et al.*, 1998, 1999) and then treated and untreated spermatozoa were used to run the SSA in parallel, determining in each case the level of capacitated cells. Thus, in the case the exposure to progesterone gradient stimulates sperm capacitation, this effect would be observed in the remaining population of acrosome intact

sperm. When the sperm population treated with A23187 was exposed to a gradient of progesterone, a stimulation of sperm capacitation was observed. In contrast, a homogeneous distribution of 10 pM progesterone for the same incubation time did not promote the appearance of new capacitated cells (Fig. 5C, right set of four bars). These results matched those obtained without prior treatment with calcium ionophore, in which a similar increase in capacitated spermatozoa was observed only under the progesterone gradient (Fig. 5C, left set of four bars). Additionally, we previously described a protein tyrosine phosphorylation pattern (PY; sperm labeling in the equatorial band and tail, associated with an intact acrosome), which was considered representative of chemotactic cells (Teves *et al.*, 2009b). Considering that sperm chemotaxis is correlated with sperm capacitation (Cohen-Dayag *et al.*, 1995; Fabro *et al.*, 2002), we next treated the sperm population with calcium ionophore as mentioned above, and then evaluated the level of sperm expressing the PY pattern after the SSA. Immediately after the ionophore treatment, the level of sperm expressing the PY pattern was significantly reduced. However, this effect was reverted when the cells were exposed to a gradient of progesterone in which the level of PY sperm was significantly increased. As expected, a homogeneous distribution of 10 pM progesterone did not affect the level of spermatozoa with an acrosome intact PY pattern (Fig. 5D, right set of four bars). These results also matched those obtained without prior treatment with calcium ionophore (Fig. 5D, left set of four bars).

Taken together, these results suggest that exposure to a progesterone gradient stimulates and/or completes the capacitation process in some sperm that could not do so during standard incubation before running the SSA, effect that is particularly notable in samples with poor initial level of capacitation.

SSA provides an enriched sperm population even in subfertile semen samples

We next investigated the efficiency of the SSA in subfertile semen samples. Independently of the type of semen pathology, the sperm samples behaved similar to the normal samples, showing an ~ 3-fold increase in the level of capacitated sperm after the SSA (Fig. 6A). Sperm morphology is a classical sperm parameter used for evaluating semen quality and also to choose a spermatozoon to inject inside the oocyte by ICSI (Franken and Oehninger, 2012). Therefore, we evaluated whether the SSA improved the selection of sperm with normal morphology in teratozoospermic samples which are characterized by a poor normal morphology. The level of sperm with normal morphology was not enhanced after the SSA in teratozoospermic samples (Fig. 6B). Besides, neither did the SSA improve the level of good morphology sperm in normozoospermic samples. However, the exposure to a progesterone gradient selects sperm with intact DNA in teratozoospermic samples at a similar extent than that observed in normal ones (Figs 3A and 6C). We next investigated whether the potency of the SSA was also dependent on the initial level of capacitated sperm. Two groups were also observed in the subfertile samples, in which the potency of the SSA was inversely correlated with the level of capacitation the sample had before running the SSA ($r^2 = -0.81$, $P < 0.000002$, $n = 58$; Fig. 7A and B). Supplementary data, Table SI summarizes the percentage of capacitated sperm before and after the SSA for group 1 and 2 in all semen sample types. Hence, similar to normal samples, the SSA apparently stimulates

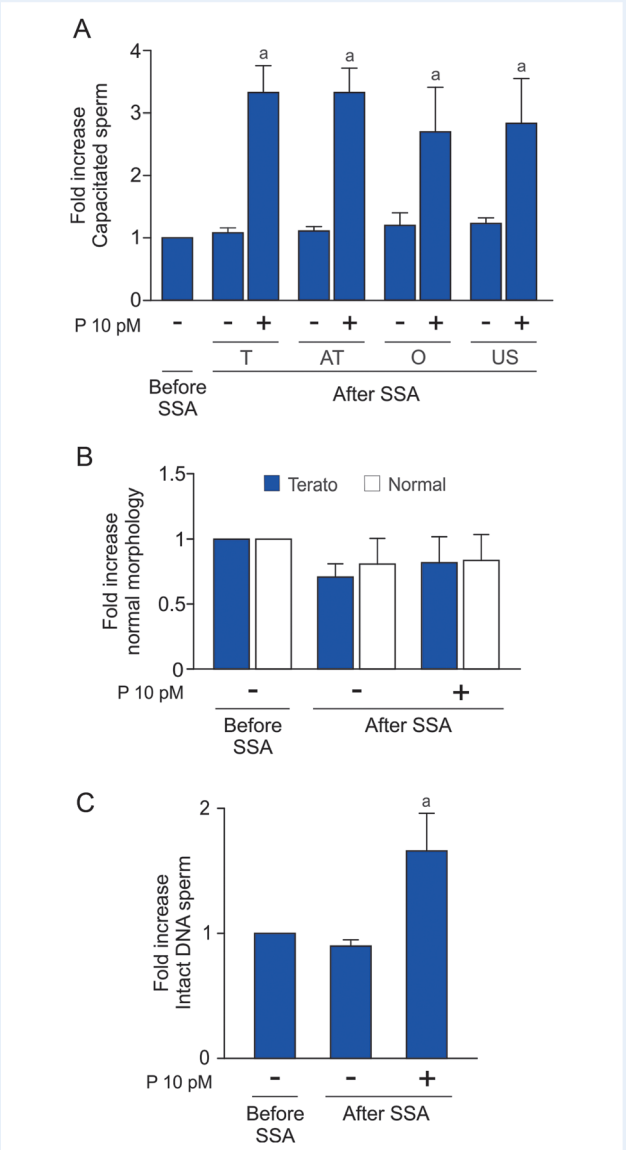

Figure 6 The SSA provides an enriched sperm population even in subfertile semen samples. (**A**) Fold increase in the level of capacitated spermatozoa before and after the SSA, with or without progesterone, for several subfertile sperm samples. Data are expressed as mean ± SE of independent experiments performed with samples from 21 teratozoospermic (T), 26 asthenoteratozoospermic (AT), 5 oligozoospermic (O) and 6 unexplained sterility (US). [a]significant differences versus before the SSA or after the SSA without progesterone ($P < 0.01$). (**B**) Fold increase in the level of sperm showing normal morphology before the SSA and after the SSA with or without progesterone in teratozoospermic and normal samples. Data are expressed as mean ± SE of four independent experiments. (**C**) Fold increase in the level of sperm showing intact DNA before the SSA and after the SSA with or without progesterone. Data are expressed as mean ± SE of four independent experiments performed with teratozoospermic semen samples. [a]significant differences versus before the SSA and after the SSA without progesterone ($P < 0.05$).

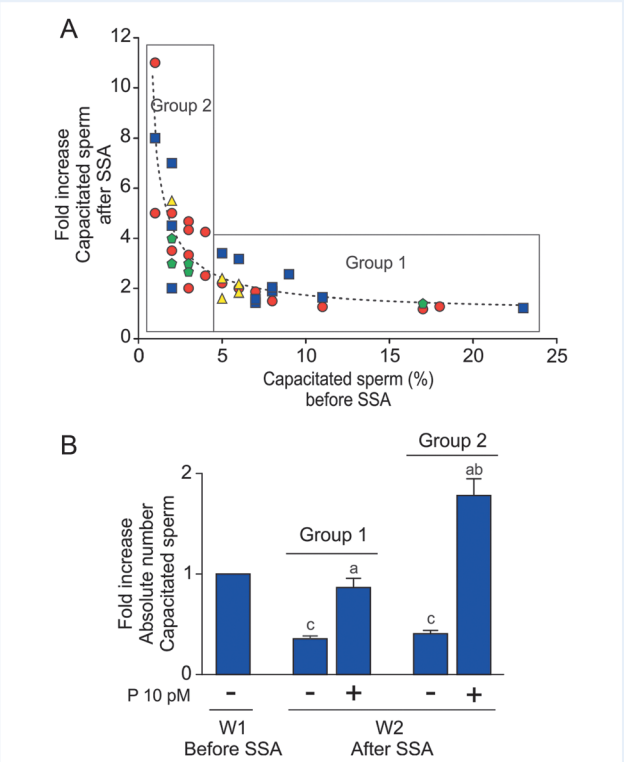

Figure 7 Picomolar gradients of progesterone stimulate sperm capacitation even in subfertile samples. (**A**) Correlation between the fold increase in the level of capacitated spermatozoa after the SSA and the percentage of capacitated sperm before the SSA. Two groups were arbitrarily defined: samples where the potency of the SSA was relatively constant (group 1) and samples where the potency of the SSA was highly variable (group 2) which have lower incidence of spontaneous capacitation. Correlation coefficients and statistical significance are noted in the text. (blue square: teratozoospermic; red circle: asthenoteratozoospermic; yellow triangle: oligozoospermic; green pentagon: unexplained sterility). (**B**) Fold increase in the absolute number of capacitated spermatozoa before and after the SSA performed with or without progesterone for groups 1 and 2 described in (A). Data are expressed as mean ± SE of independent experiments with 26 (group 1) and 32 (group 2) subfertile semen samples. [a]significant differences versus after the SSA without progesterone ($P < 0.001$); [b]significant differences versus before the SSA ($P < 0.01$), [c]significant differences versus before the SSA ($P < 0.001$).

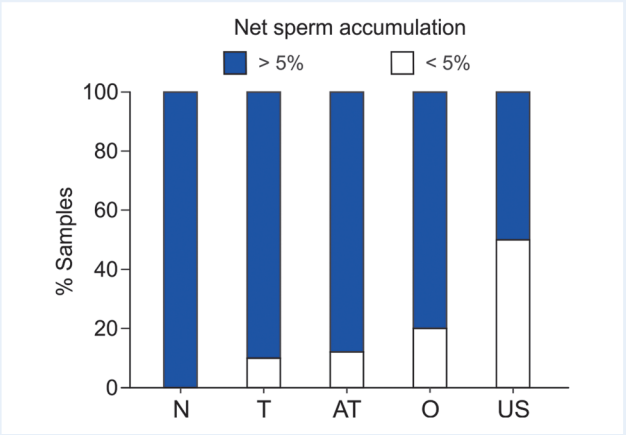

Figure 8 The SSA for diagnosis. Percentage of samples showing a net sperm accumulation after the SSA of ≥5% (black bar) or <5% (white bar) determined for each semen sample type (N, 21 normal; T, 21 teratozoospermic; AT, 26 asthenoteratozoospermic; O, 5 oligozoospermic; US, 6 unexplained sterility).

sperm capacitation preferentially in those samples that have a poor initial level of capacitation.

Collectively, the results of this work show that the SSA provides a population enriched with spermatozoa in the best functional state both in normal and subfertile semen samples. Considering that the SSA is based on the selection of sperm according to their physiological state, we next evaluated the aptitude of the SSA to diagnose a semen sample. Since in normal samples the lowest value observed in net sperm accumulation after the SSA was 5%, the percentage of subfertile samples with a value lower than 5% was calculated (Fig. 8). Most pathological samples behaved physiologically similar to normal samples, while a minority did not optimally respond to the SSA. Notably, only half of unexplained sterility samples behaved as normal ones. These results suggest that the physiological state of the semen sample should be considered for diagnosis.

Discussion

The efficiency of current infertility treatments is still low and the use of the best spermatozoa is essential for a successful procedure (Said and Land, 2011). Preferably, sperm selection should be based on the physiological aptitude of the sperm for fertilization. A recognized sperm feature is capacitation, by which spermatozoa become ready to fertilize the oocyte (Eisenbach, 1999). Capacitated spermatozoa can be recruited by chemotaxis (Eisenbach and Giojalas, 2006), a mechanism that contributes to the transport and retention of spermatozoa to the fertilization site (Guidobaldi *et al*., 2012). Specifically, capacitated spermatozoa can be attracted by very low quantities of progesterone (Teves *et al*., 2006). Therefore, we developed a method, the SSA, to select functional spermatozoa on the basis of sperm chemotaxis toward progesterone, a steroid that interacts with sperm by the time of ovulation. Although the rationale behind the SSA design should guarantee the recruitment of capacitated sperm, it was experimentally verified by different approaches. Here, we provide evidence that the application of the SSA with a gradient of picomolar amounts of progesterone not only selects spermatozoa that are capacitated, with intact DNA, reduced oxidative stress, good viability and motility, but most importantly, may also promote sperm capacitation.

The ability of sperm chemotaxis to recruit capacitated spermatozoa was already reported (Cohen-Dayag *et al*., 1995; Fabro *et al*., 2002); however, we observed that the magnitude of the enrichment depends on the initial level of capacitated cells the sample has. Thus, there is an additional benefit of the SSA for those poorly capacitated samples, in which the level of good spermatozoa may be increased up to 11-fold after the SSA. Interestingly, this observation was independent of the semen sample type as classified according to conventional criteria, where even normal samples benefitted by the SSA. Initially, we expected that the maximum efficiency of the SSA would be to recruit most of the

capacitated cells loaded in W1. However, in samples with a low level of spontaneous capacitation, the absolute number of capacitated cells recruited after the SSA was significantly higher than that loaded in W1. Indeed, when capacitated spermatozoa were induced to acrosome react before the SSA, a short exposure of 20 min to a gradient of progesterone induced and/or completed capacitation in some spermatozoa that could not do so during the sperm preparation performed prior to the SSA. This effect was exclusive of the influence of a progesterone gradient, since a step administration of the hormone did not stimulate sperm capacitation. It was suggested in some previous reports that progesterone may promote sperm capacitation (Kay *et al*., 1994; de Lamirande *et al*., 1998; Thundathil *et al*., 2002; Yamano *et al*., 2004). However, high concentrations of this steroid have been applied homogeneously distributed to spermatozoa, conditions dissimilar to the gradual distribution the spermatozoa may find *in vivo* (Teves *et al*., 2006).

On the other hand, the DNA of spermatozoa is susceptible to being damaged during sample preparation for ART procedures (Tamburrino *et al*., 2012) and when this happens, fertilization, embryo development and implantation may be affected (Aitken *et al*., 2009). After running the SSA in the presence of progesterone, a selection of spermatozoa with intact DNA was observed even in teratozoospermic samples, a common sperm pathology. The enrichment in intact DNA spermatozoa is consistent with the reduction in reactive oxygen species observed after the SSA, since the oxidative stress is known to cause DNA damage (Aitken and De Iuliis, 2010). The decrease in the oxidative stress was also observed (though to a lower extent) after the SSA without progesterone, suggesting that it may be associated with sperm motility, since only motile sperm swim to W2. Besides, the level of sperm with normal morphology was not improved in teratozoospermic samples either, which have a poor sperm morphology. This observation is in line with previous reports suggesting that sperm morphology does not necessarily reflect the physiological state or the DNA integrity of the sperm (Lewis, 2007; Avendaño and Oehninger, 2011). Moreover, data from Supplementary data, Table SI show that in normal samples and those that are normal but with a history of unexplained sterility, most have a low initial level of capacitation. This suggests that the traditional classification of semen samples on the basis of sperm morphology, motility or number does not necessary reflect the physiological state of the semen sample, which should be seriously considered for semen diagnosis. Although the SSA could be used to diagnose the physiological state of a semen sample, the corresponding reference values need to be defined.

Since the SSA not only provides a subpopulation enriched with spermatozoa in the best functional state but also prepares additional sperm for fertilization, it may have several medical applications. Although today it is not possible to identify the spermatozoon in the best functional state (capacitated, with intact DNA and a low level of oxidative stress), the use of an enriched sperm population selected by a natural procedure would increase the chance to pick out the optimum spermatozoon for ICSI. Besides, the use of such a selected sperm subpopulation may be useful even for IVF, since only capacitated spermatozoa may pass through the oocyte vestment to fertilize it (Eisenbach, 1999). It is worth mentioning that a semen sample usually has a subpopulation of optimal spermatozoa, and the benefit of the SSA is to improve this initial value. Thus, after the SSA, poor capacitating samples (group 2) had an optimal average of capacitated sperm (~10%; Supplementary data, Table SI), meaning that the SSA improves the level of capacitated cells to normal values (Cohen-Dayag *et al*., 1995). Interestingly, good capacitating samples (group 1) also enhance the capacitation level after the SSA to a 15% average value (Supplementary data, Table SI), meaning that even good capacitating samples also benefit from the SSA. Although the efficiency of the SSA is notable, it does not provide a pure population of spermatozoa at the best functional state. However, it should be considered that healthy sperm selected by the SSA may produce good-quality embryos which are, at the end, the ones to be transferred. Nevertheless, further studies are needed to verify the efficacy of the SSA, if any, to successfully treat human infertility.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

L.V.G. and M.M.M. performed experiments; H.A.G. and M.E.T. designed the device and procedure of use; D.R.U. and A.I.M. performed the DNA fragmentation assays; R.I.M. analyzed sperm morphology; L.C.G. designed the study, obtained financial support, designed the device and procedure of use and wrote the manuscript.

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Conflict of interest

The Consejo Nacional de Investigaciones Científicas y Técnicas is the owner of the device patent application and L.C.G., H.A.G., L.V.G., M.E.T., M.M.M. and D.R.U. are the inventors.

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