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# Fibronectin stimulates human sperm capacitation through the cyclic AMP/protein kinase A pathway

**E. Martínez-León[1], C. Osycka-Salut[2], J. Signorelli[1], P. Pozo[1], B. Pérez[1], M. Kong[1], P. Morales[1,3], S. Pérez-Martínez[2], and E.S. Díaz[1,*]**

[1]Laboratory of Biology of Reproduction, Department of Biomedicine, Faculty of Health Sciences, University of Antofagasta, 1240000 Antofagasta, Chile [2]Laboratory of Biology of Reproduction in Mammals, Center for Pharmacological and Botanicals Studies (National Council of Scientific and Techniques Research), University of Buenos Aires, 1121 Buenos Aires, Argentina [3]Antofagasta Institute, University of Antofagasta, 1240000 Antofagasta, Chile

*Correspondence address. E-mail: emilce.diaz@uantof.cl

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**STUDY QUESTION:** Does fibronectin (Fn) stimulate the sperm capacitation process in humans?

**SUMMARY ANSWER:** Fibronectin stimulates human sperm capacitation.

**WHAT IS KNOWN ALREADY:** Capacitation is a process that occurs in the oviduct. It has been suggested that some molecules present in the oviductal fluid and cells as well as proteins present in the cumulus oophorus could be involved in the modulation of sperm function and their acquisition of fertilizing capacity. Fibronectin is a glycoprotein that is present in the fluid and the oviduct epithelium, and its receptor (alpha 5 beta 1 integrin) is present in human sperm. When alpha 5 beta 1 ($\alpha_5\beta_1$) integrin binds to fibronectin, intracellular signals similar to the process of sperm capacitation are activated.

**STUDY DESIGN, SIZE, DURATION:** Human sperm were selected via a percoll gradient and were then incubated in non-capacitated medium (NCM) or reconstituted capacitated medium (RCM), in the presence or absence of fibronectin for different time periods. A total of 39 donors were used during the study, which lasted 3 years.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Freshly ejaculated sperm from healthy volunteers were obtained by masturbation. All semen samples were normal according to the World Health Organization parameters. Six approaches were used to determine the effects of fibronectin on sperm capacitation: chlortetracycline (CTC) assay, heterologous co-culture of human sperm with bovine oviductal epithelial cells (BOEC), measurement of cyclic (c) AMP levels, activity of protein kinase A (PKA), phosphorylation of proteins in tyrosine (Tyr) residues, and induction of acrosome reaction with progesterone.

**MAIN RESULTS AND THE ROLE OF CHANCE:** When sperm were incubated in RCM in the presence of Fn, we observed differences with respect to sperm incubated in RCM without Fn (control): (i) a 10% increase in the percentage of sperm with the B pattern (capacitated sperm) of CTC fluorescence from the beginning of capacitation ($P < 0.001$); (ii) an effect on both the concentration of cAMP ($P < 0.05$) and PKA activity ($P < 0.05$) during early capacitation; (iii) an increase in the degree of phosphorylation of proteins on tyrosine residues after 60 min of capacitation ($P < 0.01$); (iv) an increase in the percentage of acrosome-reacted sperm in response to progesterone ($P < 0.05$); and (v) a decrease in the percentage of sperm attached to BOEC ($P < 0.05$). Moreover, we noted that the effect of Fn was specific and mediated by alpha 5 beta 1 integrin ($P < 0.001$). Fn by itself had no effect on sperm capacitation.

**LIMITATIONS, REASONS FOR CAUTION:** This study was carried out with sperm from young adult men. Men with abnormal semen samples were excluded. The results cannot be directly extrapolated to other mammalian species.

**WIDER IMPLICATIONS OF THE FINDINGS:** Currently, male subfertility has become a huge public health problem, which makes it imperative to develop new treatments. This is a novel discovery that extends our current knowledge concerning normal and pathological sperm physiology as well as events that regulate the process of fertilization.

**STUDY FUNDING/COMPETING INTEREST(S):** This study was supported by grants from FONDECYT (1130341, E.S.D. and 1120056, P.M.) and FONCYT (PIP 2011-0496, S.P.-M). The authors have no conflicts of interest.

**Key words:** human sperm / capacitation / fibronectin / cyclic AMP / protein kinase A

# Introduction

It is widely accepted that freshly ejaculated mammalian sperm are unable to fertilize an oocyte (Yanagimachi, 1994). For successful fertilization, the sperm must undergo a cascade of biochemical and physiological changes that allow binding and penetration of sperm into the oocyte. These events are time-dependent and have been termed 'capacitation' (Chang, 1984). This process normally occurs in the female genital tract; however, molecular understanding was developed based on the possibility of achieving this process *in vitro* by incubating freshly ejaculated sperm in a defined culture medium. Capacitation of sperm is associated with changes in the plasma membrane, metabolic events and post-translational modifications. This process includes cholesterol loss from the plasma membrane; changes in intracellular ion concentration; increase in intracellular pH and hyperpolarization of the plasma membrane; activation of flagellum movement, which occurs as soon as the sperm leave the epididymis; changes in the pattern of movement (hyperactivation); phosphorylation of proteins in tyrosine (Tyr) residues (de Lamirande *et al.*, 1997; Visconti *et al.*, 2011); and the ability to carry out the acrosome reaction (AR) stimulated by a physiological agonist. Interestingly, all of these events are regulated through the cyclic AMP (cAMP)/protein kinase A (PKA) system.

Cyclic AMP has been reported to be essential for events occurring during capacitation. In vertebrate sperm, cAMP is synthesized by two types of adenylyl cyclase (AC): a common family of transmembrane ACs (tmACs) and a soluble AC (Adcy10, also known as SACY or sAC) (Buck *et al.*, 1999; Jaiswal and Conti, 2003; Geng *et al.*, 2005). It is well known that the tmAC are regulated by G-protein and forskolin, whereas the SACY is activated by calcium ($Ca^{2+}$) and bicarbonate ($HCO_3^-$) (Forte *et al.*, 1983; Leclerc *et al.*, 1996; Tresguerres *et al.*, 2011). Sperm cells incubated in a capacitating medium show a rapid ($\leq$1 min) increase in PKA activity; then, the levels decline in the next 15 min, to increase to a peak at 30 min of incubation (Moseley *et al.*, 2005). In human sperm, an increase in the phosphorylation of PKA substrates (80 and 105 kDa) during capacitation has been described (O'Flaherty *et al.*, 2004; Bedu-Addo *et al.*, 2005). This increase was observed at 1 min of incubation, reaching a peak of phosphorylation 15 and 30 min later. After this time, there was a decrease in the phosphorylation of PKA substrates (Moseley *et al.*, 2005). This phosphorylation pattern correlates with the PKA activity in human sperm. Furthermore, post-translational modifications that occur during mammalian sperm capacitation have revealed a dramatic increase both in the number and level of tyrosine phosphorylated proteins in response to PKA activation by cAMP protein (Visconti *et al.*, 1995; Baker *et al.*, 2004, 2009; Bennetts *et al.*, 2004).

Capacitation occurs in the oviduct, where the sperm interact with molecules present in the fluid and/or oviductal epithelium that modulate sperm capacitation through a mechanism not yet defined (Quintero *et al.*, 2005; Suarez, 2008; Brewis and Gadella, 2010).

In mammals, the oviduct is a functional sperm reservoir, which provides an environment that allows maintenance and competence for successful fertilization of the oocyte (Harper, 1994). In different species, the sperm remain in the proximal region of the oviduct (isthmus) for a period of time and bind to the epithelial cells (Smith and Yanagimachi, 1990). Then, sperm capacitation induces the release of the sperm adhering to the oviduct epithelium, either by a rapid remodelling of the sperm plasma membrane, which involves adhesion molecules directed towards the oviductal epithelium, or by inducing sperm hyperactivation, which provides an additional force sufficient to release the sperm from their cellular contacts (Smith and Yanagimachi, 1991; Demott and Suarez, 1992; Lefebvre and Suarez, 1996). All these events extends sperm viability, delaying capacitation until signals associated with ovulation, such as progesterone or the presence of the cumulus-oocyte complex, induce sperm release allowing sperm to continue their transit to the upper region of the oviduct (ampulla) (Harper, 1994; Suarez, 2008; Kajanova *et al.*, 2012).

As capacitation is a crucial step in the acquisition of sperm fertilizing ability, it is likely that it is induced and controlled by several molecules and redundant mechanisms, and that cross talk between different pathways occurs during this process (Leclerc *et al.*, 1996; de Lamirande *et al.*, 1997; Zumoffen *et al.*, 2010). It has been suggested that some molecules present in the oviductal fluid, oviductal cells, and cumulus oophorus could be involved in the modulation of sperm function and their acquisition of fertilization capacity (Gadella and Harrison, 2000; Quintero *et al.*, 2005). These molecules include glycodelin-A (Chiu *et al.*, 2007), anandamide (Gervasi *et al.*, 2011; Osycka-Salut *et al.*, 2012) and fibronectin (Fn) (Diaz *et al.*, 2007).

Fn is a glycoprotein consisting of two similar subunits of 250 kDa, linked by disulphide bonds near the carboxyl terminal region (Kornblihtt *et al.*, 1996; Wierzbicka-Patynowski and Schwarzbauer, 2003). It is mainly found as a dimer in a soluble form and as an insoluble multimer of high molecular weight, maintained by covalent bonds and arranged as a fibrillar extracellular matrix component (Limper and Roman, 1992; Lucerna *et al.*, 2007). Fn has multiple domains through which it can bind to surface receptors and different molecules, such as collagen, enabling it to mediate a broad variety of biological functions (Wennemuth *et al.*, 2001), such as the adhesive and migratory behaviour of cells during embryogenesis, malignancy, haemostasis, wound healing, host defence, and maintenance of tissue integrity (Fusi and Bronson, 1992; Kornblihtt *et al.*, 1996; Midwood *et al.*, 2004).

Fn is present in the follicular and oviductal fluid and oviductal epithelium (Tsuiki *et al.*, 1988; Hung *et al.*, 1989; Honda *et al.*, 2004; Relucenti *et al.*, 2005; Makrigiannakis *et al.*, 2009). Makrigiannakis *et al.* (2009) found that the Fn immunostaining is localized to the luminal surface of ciliated cells, with particularly intense staining on the apex of the cilia. There are no differences in immunostaining throughout the menstrual cycle. Again, this distinct pattern is observed along the entire fallopian tube (isthmic, ampulla and fimbriae).

The binding of Fn to the cell surface is mediated by integrins; specifically, Fn binds to the $\alpha_5\beta_1$ integrin through the RGD (Arg-Gly-Asp) domain, and this integrin is present in human sperm (Glander and Schaller, 1993; Fusi *et al.*, 1996). Binding of integrins to their ligands activates different signal transduction pathways, such as an increase of intracellular $Ca^{2+}$, or activation of kinases, such as PKA, inositol

triphosphate/protein kinase C and Src, which are involved in the regulation of sperm function (Fusi and Bronson, 1992; Glander and Schaller, 1993; Diaz *et al.*, 2007; Lim *et al.*, 2008; Suh and Han, 2013). More specifically, many of these signalling pathways are involved in the capacitation process (Visconti *et al.*, 1998; Lefievre *et al.*, 2002; Signorelli *et al.*, 2012; Buffone *et al.*, 2014).

As Fn is present in the oviductal cells and its receptor ($\alpha_5\beta_1$ integrin) is present in the sperm (Glander and Schaller, 1993; Fusi *et al.*, 1996), we propose that the Fn-$\alpha_5\beta_1$ integrin complex could participate in the capacitation process. The aim of this study was to determine the effect of Fn on the capacitation process in human sperm. For this purpose, we evaluated the main events described during capacitation: mobilization of calcium ions via a chlortetracycline (CTC) assay, cAMP levels, PKA activity, Tyr phosphorylation pattern, and the ability of sperm to undergo the AR. Finally, we evaluated the effect of Fn during capacitation using a heterologous co-culture system (human sperm-bovine oviductal epithelial cells (BOEC)). Our results suggest that a subpopulation of human sperm is able to respond to the stimulation of Fn and undergoes capacitation. This is the first report that demonstrates that Fn stimulates human sperm capacitation in the presence of bovine serum albumin (BSA) and $HCO_3^-$ *in vitro*.

# Materials and Methods

## Chemicals and reagents

The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO, USA): Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK); CTC; BSA (A7030); HEPES; Ponceau Red; progesterone; ethylenediaminetetraacetic acid (EDTA); Hoechst 33258 (H258); Hoechst 33342 (H342); 1,4-Diazabicyclo[2.2.2] octane solution (DABCO); $Na_3VO_4$; NaF; sodium desoxycholate; Igepal CA-630 (NP-40); phenylmethylsulfonyl fluoride (PMSF); leupeptin; bestatin A and aprotinin. *Pisum sativum* agglutinin (PSA)-fluorescein isothiocyanate (FITC) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Fn, the chemiluminescence detection system and Immobilon P transfer membrane were purchased from Millipore Corporation (Bedford, MA, USA). The DC-protein method was obtained from Bio-Rad Laboratories Inc. (Hercules, CA, USA). M199 medium, gentamicin, fungizone and fetal calf serum (FCS) were obtained from Gibco (Invitrogen).

The following primary antibodies were used: monoclonal antibody against $\alpha_5$ integrin subunit (clone SAM-1) and polyclonal anti human immunoglobulin (Ig) G, purchased from Chemicon (Temecula, CA, USA); anti-phosphotyrosine (clone 4G10), obtained from Upstate Biotechnology (Lake Placid, NY, USA); and anti β-tubulin, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The deionized water used in these experiments was purified to 18 MV-cm with an EASY-pure UV/UF ion-exchange system (Barnstead/Thermolyne, Dubuque, IA, USA). All other chemicals were of analytical grade and obtained from standard sources.

## Media cultures

M199 medium supplemented with 50 μg/ml gentamicin, 1 μg/ml fungizone and 10% FCS (v/v) was used. FCS was used for oviductal incubations and for the development of monolayer cultures (Gualtieri and Talevi, 2000). Sperm handling and co-culture experiments were performed in modified Tyrode's medium with (reconstituted capacitating medium, RCM) or without 2.6% BSA and 25 mM $HCO_3^-$ (non-capacitating medium, NCM), as described previously by us (Signorelli *et al.*, 2013). Sperm viability and motility were not affected by the absence of $HCO_3^-$ and BSA (data not shown).

## Solubilization and handling of Fn

Fn was reconstituted in cold phosphate-buffered saline (PBS) (137 mM NaCl, 1.5 mM $KH_2PO_4$, 4.3 mM $Na_2HPO_4$, 2.7 KCl mM, pH 7.4) to a final stock concentration of 1000 μg/ml. The working concentration used in all experiments was 100 μg/ml.

## Semen collection and analysis

The research presented in this manuscript was approved by the Ethics Committee on Scientific Research at the University of Antofagasta. The institutional review board approved the use of all human semen samples described in this study. All donors signed a consent form for the use of their sperm cells for research purposes. Freshly ejaculated sperm from healthy volunteers were obtained by masturbation after 2–3 days of sexual abstinence. Semen samples were allowed to liquefy for 30–60 min at 37°C in a slide warmer. All semen samples were processed by the same person using the same equipment, and analyses of volume, pH, sperm concentration, and percentages of motility and viability of spermatozoa were performed. The mean values for semen parameters are summarized in Supplementary Table SI.

Ejaculate volume was measured with graduated pipettes and the pH was measured with pH paper. Sperm concentration, after an appropriate dilution, was assessed by a haemocytometer. Subjective sperm motility was assessed and we evaluate progressive motility (PR), non-progressive motility (NP), immotile sperm and total motility (PR + NP). All measurements were performed at room temperature or at 37°C. Viability was evaluated using the eosin–nigrosin staining method. All semen samples were normal according to the World Health Organization criteria (World Health Organization, 2010).

## Sperm suspension preparation

Motile sperm were separated using a double percoll gradient (40/80%), as described previously (Morales and Cross, 1989). Briefly, aliquots of semen were layered over the upper layer of the percoll gradient and centrifuged for 20 min at 300 g. The pellet was resuspended in 10 ml of NCM and then centrifuged at 300 g for 10 min. Finally, the sperm cells were resuspended in the appropriate medium at the required concentration.

## *In vitro* sperm capacitation

Approximately $5 \times 10^6$ sperm cells/ml were incubated for different times (1, 60, 180, 300 and 1080 min) at 37°C and 5% $CO_2$ in air, in RCM or NCM in the presence or absence of 100 μg/ml Fn.

## CTC assay

The sperm capacitation status was assessed using the CTC fluorescence assay method, as described previously (Ward and Storey, 1984; Kong *et al.*, 2009).

The CTC solution was prepared on the day of use and contained 750 mM CTC in a buffer of 130 mM NaCl, 5 mM cysteine and 20 mM Tris–HCl, with pH adjusted to 7.8. This solution was kept in the dark (wrapped in foil) at 4°C until use. To a 10 μl aliquot of capacitated sperm suspension (conditions indicated in *in vitro* sperm capacitation), 10 μl of CTC stock solution was rapidly added and then treated with 10 mg/ml H258 and incubated for 30 s, followed after 30 s by the addition of 2 μl of 2% glutaraldehyde in 1 M Tris buffer (pH 7.8). Twenty microlitres of this suspension was placed on a slide: once dry, a drop of DABCO mounting medium was carefully mixed in to retard the fading of the fluorescence and a cover slip was placed on top. Cells were assessed for their living/dead state using H258, as described previously (Cross *et al.*, 1986). In each sample, 200 live cells were assessed for CTC staining patterns, and in all cases the proportion of dead cells was very low. Three main patterns of CTC fluorescence could be identified: the F pattern, with uniform fluorescence over the entire head, was characteristic

of non-capacitated, acrosome intact cells; the B pattern, with a fluorescence-free band in the post-acrosomal region, which was characteristic of capacitated, acrosome-intact cells; and the AR pattern, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells (Lee *et al.*, 1987).

To test whether the effect of Fn was mediated by its receptor, $\alpha_5\beta_1$ integrin, we conducted an immunoneutralization test using an antibody against the $\alpha_5$ subunit of the receptor. Briefly, sperm capacitated in different conditions were pre-incubated at 37°C for 30 min with the antibody (1:100 dilution), and then the CTC assay was carried out as described above.

## Induction of the AR and evaluation of acrosomal status

To induce the AR, motile sperm were capacitated for different times, as mentioned above. Then, the cells were incubated with 7 μM progesterone for 15 min. At the end of this time, sperm viability and acrosomal status were evaluated using the supravital dye H258 and PSA-FITC, respectively, as described previously (Cross *et al.*, 1986).

To test whether the effect of Fn was mediated by $\alpha_5\beta_1$ integrin, we conducted an immunoneutralization test as described in CTC assay. Additionally, then exposed to progesterone (7 μM) for 15 min. After that, we determine acrosomal integrity, as described (Diaz *et al.*, 2007).

## Preparation of sperm extracts

After sperm capacitation, 25 × $10^6$ sperm/ml were washed twice in cold PBS 1× and centrifuged at 21 000 g for 30 s. The resulting sperm pellet was resuspended in homogenization buffer (50 mM HEPES, 10% glycerol, pH 7.4) and centrifuged for 30 s at 21 000 g in a Beckman microfuge. The pellet was resuspended in 70 μl of radioimmunoprecipitation (RIPA) lysis buffer (containing 150 mM NaCl, 50 mM Tris, 1% sodium dodecyl sulfate (SDS), 2 mM $Na_3VO_4$, 50 mM NaF, 2 mM EDTA, 1% sodium desoxycholate, 1% Nonidet P-40, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml bestatin A, 10 mg/ml aprotinin and pH 7.4). The sperm suspension was sonicated (Virsonic, Gardiner, NY) with six 60-W bursts for 20 s each, followed by centrifugation for 30 s at 14 000 g to remove nuclear and flagellar material. The supernatant was used for SDS-polyacrylamide gel electrophoresis (PAGE) and western blot protocols. All of these procedures were performed at 4°C. The protein concentration was determined by the DC-protein assay using BSA as protein standard.

## SDS–PAGE and western blotting

To carry out SDS–PAGE, aliquots of sperm extracts containing 10 μg of proteins were boiled for 5 min with sample buffer (500 mM Tris–HCl, 10% SDS, 30% glycerol, 1 M dithiothreitol and 0.01% bromophenol blue, pH 6.8) and then immediately settled on ice. Samples were resolved using 10% SDS–PAGE (10% acrylamide/bisacrylamide for the resolving gel and 5% acrylamide/bisacrylamide for the stacking gel) in a Mini Protein Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After SDS–PAGE, the gels were equilibrated in transfer buffer for 15 min and electrotransferred at 60 V for 150 min onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P) using a mini trans-blot cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The transfer was observed via Ponceau red stain. Blots probed with a mouse antibody against phosphotyrosine, clone 4G10 (1:1000) were blocked with PBS-Tween 20 (0.1%, v/v) and 3% non-fat dry milk for 60 min. Then, the blots were washed and incubated with an appropriate second biotinylated antibody. The reaction was enhanced with streptoavidinperoxidase conjugates, and a chemiluminescence kit was used to detect the horseradish-peroxidase-labelled protein according to the manufacturer's instructions. Pre-stained protein standards with a molecular mass range of approximately 250–10 kDa were used. Immunoblots were recorded as digital images (Gel Logic 2200PRO team). Finally, the image analysis system 'Image Processing and Analysis in Java' (ImageJ 1.42q, Center for Information Technology, NIH, Bethesda, MD, USA) was used for densitometric analysis of bands.

## Stripping the PVDF membranes

To confirm an equal loading of protein, blots that had been probed for phosphotyrosine proteins were stripped and reprobed with an antibody against β-tubulin. For this procedure, ~30 ml of stripping buffer, consisting of 2% (w/v) SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, was added to the membrane for 1 h with constant shaking at 60°C. The membrane was then washed three times for 10 min in Tris buffered saline, blocked and probed with the primary antibody, as described above.

## Bovine oviductal cell cultures

Bovine oviductal cell culture was assessed as described previously by Gervasi *et al.* (2009). Oviducts from bovines were collected at the time of slaughter, transported at 4°C, cleaned of the surrounding tissues and washed twice in sterile PBS at 4°C. Then, they were cut, flushed with sterile PBS and squeezed by pressure with tweezers. A monolayer of bovine oviduct epithelial cells (BOEC) was recovered from different animals, selected on the basis of ciliary beating, and pooled. BOEC were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium at 39°C in a 5% $CO_2$ atmosphere. Incubations were performed in six-well tissue culture dishes with 12-mm round cover slips on the bottom of the well. After 48 h, BOEC were washed by centrifugation (1500 g for 5 min) and seeded again in tissue culture dishes. The M199 medium was changed every 48 h. Once cell confluence was attained, the oviductal monolayers from the same pool of animals were washed twice in NCM and left in this medium for 60 min until aliquots of motile sperm were added.

## Release experiment and quantification of bound sperm

The release experiment was assessed as described by Gervasi *et al.* (2009), which we use as a method for evaluating spermatic capacitation. In addition, heterologous co-cultures of human sperm-BOEC were assessed as described by Ellington *et al.* (1998a,b, 1999).

Motile sperm selected in NCM were incubated for 2 min with Hoesch 33 342 (1 μg/ml). Then, the sperm were washed with NCM and centrifuged at 300 g for 5 min. After that, 14 × $10^6$ cells/ml were incubated with BOEC at 37°C with 5% $CO_2$ for 2.5 h. This duration of incubation was determined to be necessary for sperm binding. After this time, the medium was removed and washed three times with NCM to discard the sperm that were not attached to the epithelial cells. Subsequently, the co-cultures were incubated for different times with different treatments at 37°C with 5% $CO_2$. After co-culture, the oviductal monolayers with adhering sperm were fixed in glutaraldehyde (2.5% v/v) for 60 min at room temperature and then washed and mounted on a glass slide. The number of bound sperm was determined by analysing 20 fields of 0,11 $mm^2$/cover slip under a fluorescent microscope (400×). The results are expressed as number of sperm bound to BOEC.

## Total cAMP concentration

The total cAMP concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit from Arbor Assay (Cyclic AMP Direct EIA Kit) according to the manufacturer's instructions. Briefly, 30 × $10^6$ cells/ml were incubated for different times. After incubation, the sperm were lysed in the presence of 25 mg/ml IBMX (a phosphodiesterase inhibitor) to continue with the protocol indicated by the commercial kit.

### Specific enzymatic activity of PKA

The enzymatic activity of PKA was determined using a commercial ELISA manufactured by Arbor Assay (Protein Kinase A Activity Non-Radioactive Assay Kit) according to the manufacturer's instructions. To perform this determination, aliquots of 20 × $10^6$ spermatozoa/ml obtained in the different experimental protocols were lysed and the protocol performed as indicated by the commercial kit.

### Immunofluorescence of $\alpha_5$ integrin

To determine the presence of cells expressing $\alpha_5$ integrin, aliquots of sperm cells capacitated for 1080 min (5 × $10^6$ cells/ml) were fixed with 2% paraformaldehyde in PBS for 15 min, followed by three washes with PBS. Then, the samples were blocked with 0.1% BSA, 0.01% Tween-20 in PBS for 1 h at room temperature, washed and incubated at 4°C overnight with anti-FITC conjugated $\alpha_5$ integrin (SAM-1 clone, Chemicon). Aliquots were washed with PBS, gently smeared into a microscope slide and allowed to air dry (Signorelli *et al.*, 2013). Slides were mounted with DAKO fluorescence mounting medium (CA, USA). Slides were assessed by epifluorescence microscopy with oil objective lens magnification to determine the localization of detected $\alpha_5$ integrin (Supplementary Fig. S1).

### Statistical analyses

Data were analysed using one-way analysis of variance and Tukey's multiple comparison test for unequal replicates using the Instat program (3.01, GraphPad Software, Inc., La Jolla, CA, USA). A difference between groups of $P \leq 0.05$ was considered significant. All data are presented as the mean ± SEM.

## Results

### Presence of the human sperm capacitation

In order to verify the presence of $\alpha_5$ integrin in our sperm samples and to ensure the Fn acts through this receptor, we evaluated by immunofluorescence the presence of the receptor. As expected, we corroborated the presence of $\alpha_5$ integrin in human sperm (Supplementary Fig. S1) and observed that it is distributed mainly in the sperm head. This result is in agreement with those reported by other groups (Fusi *et al.*, 1996; Trübner *et al.*, 1997; Glander *et al.*, 1998).

### Fn stimulates human sperm capacitation in the B pattern

The CTC analysis provides a useful method for evaluating the intracellular calcium mobilization and to correlate this with the capacitation state of human spermatozoa (DasGupta *et al.*, 1993).

Sperm incubated in NCM exhibited a basal percentage of cells with the B pattern (average 10 ± 1.4%), which did not vary significantly in the different incubation times (Fig. 1). Similarly, sperm incubated in NCM + Fn showed no significant differences in the B pattern throughout the incubation times. When the spermatozoa were incubated in the presence of BSA and $HCO_3^-$ (RCM), we observed a rapid increase (≤1 min) in the percentage of capacitated sperm depicting the B pattern (22 ± 1.6%). Then, the percentage of sperm with the B pattern increased, achieving a maximum of 40 ± 1.6% at the end of the incubation. However, when Fn was added (RCM + Fn), the percentage of sperm with the B pattern rapidly and significantly increased at 1 min (10%) compared with RCM alone. This increase remained constant throughout the incubation period (60, 180 and 300 min) but at 1080 min there was no significant difference between RCM and RCM + Fn (Fig. 1). To confirm that Fn mediated this effect, we incubated the spermatozoa with an antibody that recognizes the $\alpha_5$ subunit of the integrin before adding Fn, and then we proceeded with the incubation. The results indicated that the effect exerted by Fn on sperm capacitation was completely prevented (Supplementary Fig. S2). These results suggest that Fn is not a capacitating molecule by itself, but when it is present in a capacitating medium (medium with BSA and $HCO_3^-$), it is able to increase the percentage of the sperm exhibiting the B pattern, as measured with the CTC assay.

### Fn stimulates the release of sperm from BOEC

To confirm the effect of Fn on sperm capacitation, we performed a heterologous co-culture of BOEC and human sperm cells as described in the materials and methods and evaluated the role of Fn in the release of the spermatozoa from oviductal epithelia (Fig. 2).

The results indicate that the incubation of co-cultures with NCM or NCM + Fn did not produce any effect on sperm binding or their release from BOEC (Fig. 2). However, when BSA and $HCO_3^-$ were added to the medium (RCM), there was a rapid decrease (≤1 min) in the number of sperm bound to BOEC. This effect was observed at all incubation times as shown in Fig. 2. Furthermore, when Fn was added to the co-cultures (RCM + Fn), we observed a rapid decrease (1 min) in the number of sperm bound to BOEC (42% compared with RCM alone). In this case, the decrease in the number of bound sperm with RCM + Fn was also observed at all times of incubation.

To confirm the specificity of the effect of Fn, the spermatozoa were incubated with an antibody that recognizes the $\alpha_5$ subunit of the integrin. In this case, the effect exerted by Fn on sperm attached to BOEC was completely abolished at 1 and 60 min (Supplementary Fig. S3).

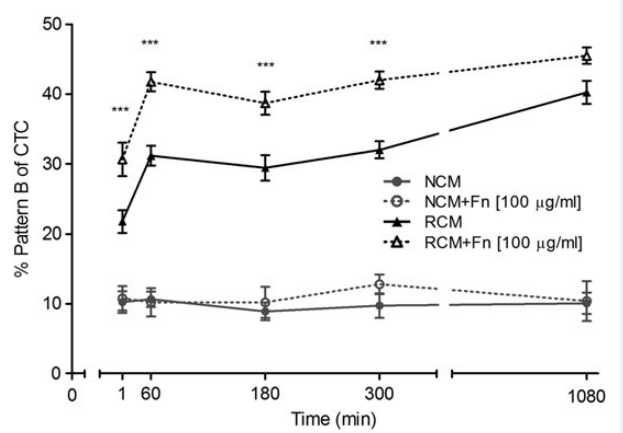


**Figure 1** Effect of fibronectin (Fn) on capacitation B pattern in human sperm. Human sperm were incubated for different times (1, 60, 180, 300 and 1080 min) in non-capacitated medium (NCM, grey circles) or reconstituted capacitated medium (RCM, black triangles) and in presence (dotted line) or absence of Fn (solid line). The capacitation state was evaluated using the chlortetracycline (CTC) assay, as described in the Materials and Methods. The results were obtained from 12 different donors and are expressed as the mean ± SEM of the percentage of cells with B pattern. ***$P \leq 0.001$ RCM + Fn versus RCM (ANOVA, Tukey's multiple comparison test).

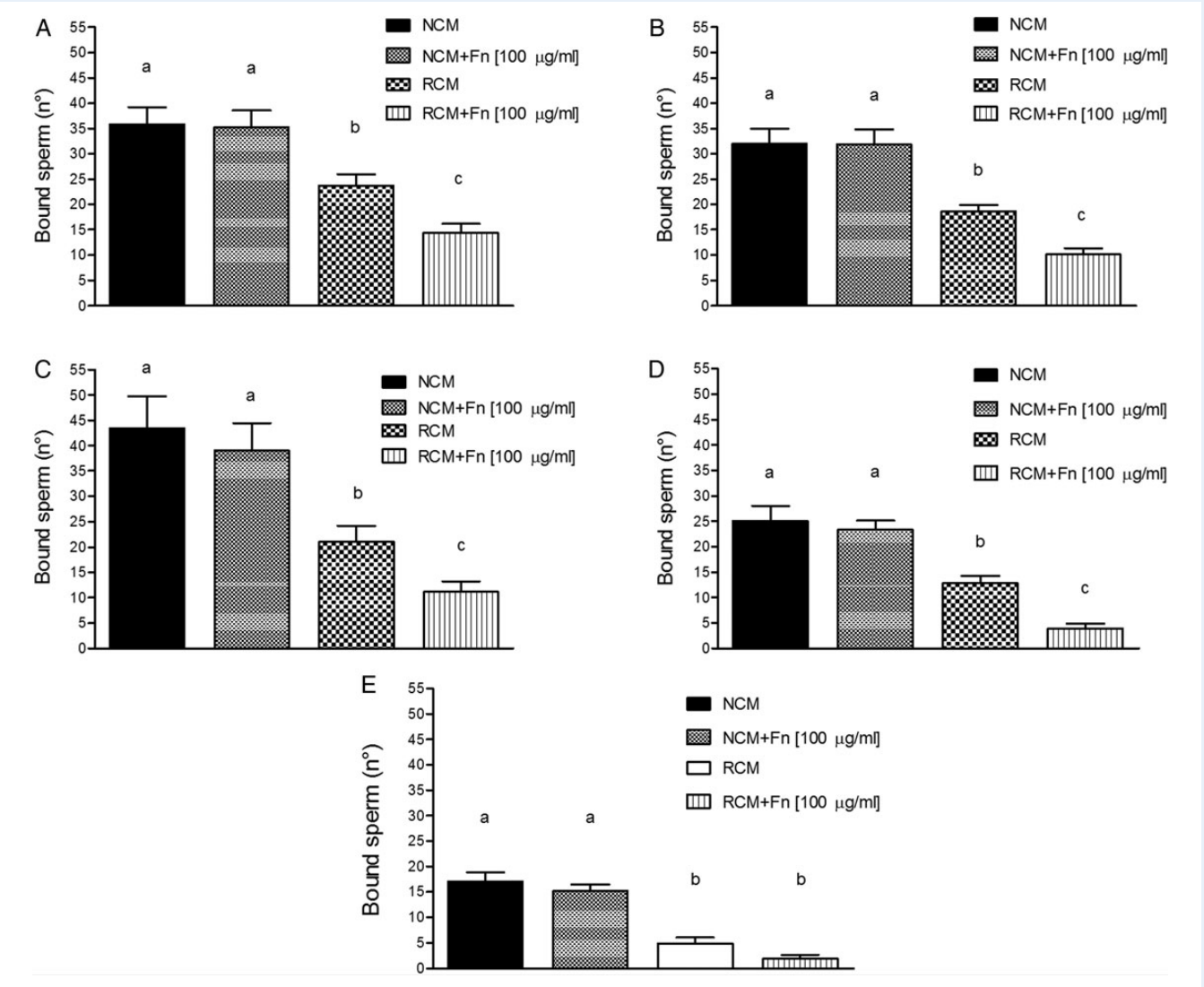


**Figure 2** Effect of Fn on human sperm release from bovine oviductal epithelial cell (BOEC) monolayers. 12 × $10^6$ sperm/ml and a monolayer BOEC were co-cultured for 2.5 h and then incubated for (**A**) 1, (**B**) 60, (**C**) 180, (**D**) 300 and (**E**) 1080 min in NCM or RCM with or without Fn (100 μg/ml). Bars represent the number of spermatozoa that remained attached to the monolayers of BOEC and were expressed as the mean ± SEM of six independent experiments. a ≠ b ≠ c $P \leq 0.05$ (ANOVA, Tukey's multiple comparison test).

In summary, incubation with Fn decreased the number of spermatozoa bound to BOEC, suggesting an increase in the number of capacitated sperm.

Spermatozoa were alive and motile during all incubation times (data not shown).

## Effect of Fn on cAMP concentration during human sperm capacitation

In the previous experiment, we observed that in RCM, Fn induces a further increase in the percentage of capacitated sperm. To determine whether this effect is mediated through cAMP/PKA pathways, we evaluated the effect of Fn on the sperm cAMP levels (Fig. 3). Spermatozoa incubated in NCM present a cAMP concentration of 16.5 ± 6.1 fmol/min/$10^6$ cells (mean ± SEM) that did not change during incubation. The concentration of cAMP in the spermatozoa incubated in RCM rapidly and significantly increased by ~455% (to 111.2 ± 9 fmol/min/$10^6$ cells) at 1 min of incubation compared with NCM. Interestingly, under these conditions, the cAMP levels markedly declined within the first hour and remained at this level for the rest of the capacitation period. Sperm incubated in the presence of Fn (RCM + Fn) increased the cAMP levels at the beginning (39%) and at the end (93%) of capacitation compared with sperm in RCM, achieving a cAMP concentration of 154.4 ± 8.7 fmol/min/$10^6$ sperm at 1 min of incubation and 53.6 ± 7.6 fmol/min/$10^6$ sperm at 1080 min. At 60 min of incubation, the sperm incubated in RCM + Fn showed a decreased cAMP concentration (by 47%, to 27.5 ± 7 fmol/min/$10^6$ sperm), and this decrease was sustained until 300 min of incubation. These results suggest that Fn modulates cAMP levels during capacitation.

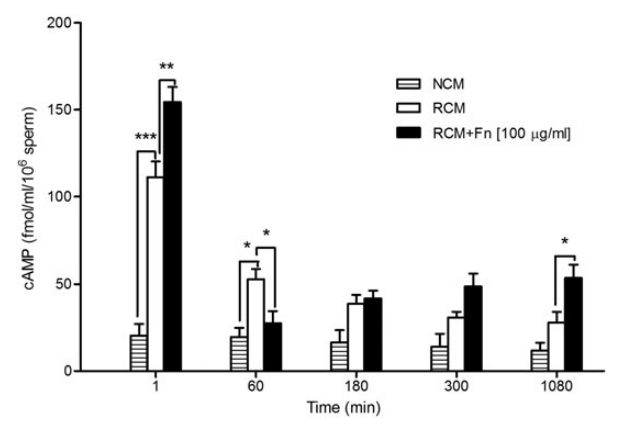


**Figure 3** Effect of Fn on cycle AMP (cAMP) levels during human sperm capacitation. Human sperm were incubated in NCM, RCM and RCM + Fn [100 μg/ml] at 1, 60, 180, 300 and 1080 min. Bars represent total cAMP concentration by ELISA commercial kit as detailed in Materials and Methods section. The results were obtained from eight different donors and were expressed as the mean ± SEM. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. (ANOVA, Tukey's multiple comparison test).

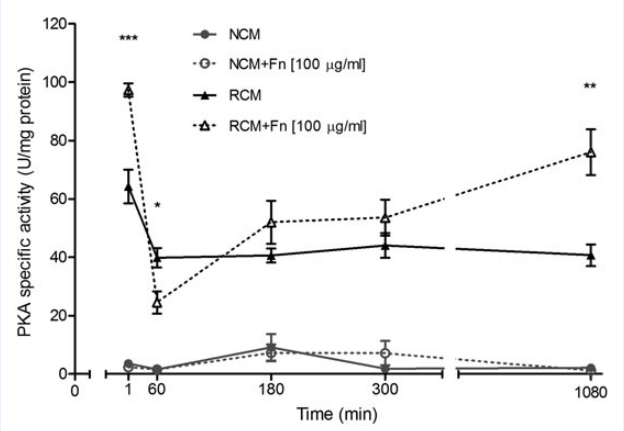


**Figure 4** Effect of Fn on protein kinase A (PKA) activity during human sperm capacitation. Sperm were incubated in NCM, RCM and RCM + Fn [100 μg/ml] at 1, 60, 180, 300 and 1080 min. PKA activity was measured in sperm extracts as indicated in Materials and Methods. The results were obtained from five different donors and were expressed as the mean ± SEM. $^{*}P \leq 0.05$ RCM + Fn versus RCM; $^{***}P \leq 0.001$ RCM + Fn versus RCM (ANOVA, Tukey's multiple comparison test).

## Effect of Fn on PKA activity during human sperm capacitation

In the previous experiments, we observed that Fn was able to modulated cAMP levels during capacitation. Thus, we evaluated whether Fn may regulate PKA activity (Fig. 4). The results indicate that the PKA enzymatic activity of sperm incubated in NCM and in NCM + Fn did not vary significantly during incubation (average 3.6 ± 1.7 and 3.8 ± 2.0 U/mg protein, respectively). Sperm incubated for 1 min in RCM increased their PKA activity rapidly and significantly to 64.3 ± 5.8 U/mg protein and then decreased it to 39.8 ± 3.4 U/mg protein after 60 min of incubation, which was maintained until the end of the capacitation period. However, when the sperm were incubated in the presence of Fn (RCM + Fn), the PKA activity increased rapidly and significantly by 53% at 1 min, and decreased significantly after 60 min by 36% relative to RCM without Fn. Additionally, at 180 min of incubation, the PKA activity increased again to reach a maximum at 1080 min of capacitation, which was higher than the maximum observed in sperm incubated in RCM alone (76 ± 7.8 U/mg of protein). It is noteworthy that the modulating effect of Fn on PKA activity correlates with that obtained on cAMP levels.

## Protein phosphorylation on Tyr residues (p-Tyr) during sperm capacitation

Activation of PKA results in increased phosphorylation of proteins on p-Tyr, which is essential for sperm to control their cellular processes, as they are transcriptionally inactive cells. In the next experiments, we evaluated the phosphorylation pattern on Tyr residues during human sperm capacitation (Fig. 5). Sperm were incubated in NCM or RCM with or without Fn for different times and then analysed by western blot. The non-capacitated sperm exhibited bands of p-Tyr in the presence or absence of Fn. At the beginning of capacitation (1 min of incubation), there were no significant changes in p-Tyr by incubating sperm in RCM with or without Fn. However, as confirmed by densitometric analysis (Fig. 5), an increase in the degree of phosphorylation on Tyr residues in high molecular mass bands (130-70 kDa) was observed in spermatozoa incubated in RCM with Fn from 60 min to the end of the capacitation time (180, 300 and 1080 min). We noted a global increase in tyrosine phosphorylation of proteins ranging in molecular mass between 12 and 150 kDa, as we previously described (Diaz *et al.*, 2007). We did not detect new proteins being phosphorylated by Fn. These results suggest that the presence of Fn in the RCM induces an increase in sperm p-Tyr after 60 min of capacitation.

## Effect of Fn on sperm capacitation through induction of the AR by progesterone

The AR is considered to be the end-point of the capacitation process. To further confirm that Fn stimulates a subpopulation of sperm to undergo capacitation, we evaluated the effect of Fn on the progesterone-induced AR, a physiological inducer of this process. When the sperm were incubated in RCM, we observed an increase in the percentage of reacted sperm, which did not exceed 10%, considered as spontaneous AR. Furthermore, when sperm were (Fig. 6A) stimulated with progesterone, an increase in the percentage of reacted sperm was observed, reaching 30 ± 2.9%. When the sperm were incubated in RCM plus Fn (RCM + Fn) throughout the capacitation process, we observed a similar effect of RCM. However, when the sperm were incubated in the presence of Fn and were stimulated with progesterone ((RCM + Fn) + progesterone), the percentage of reacted sperm significantly increased at 300 min (12%) and 1080 min (11%) of incubation with respect to sperm incubated in RCM alone (RCM + progesterone). The maximum percentage of acrosome-reacted sperm was observed at 1080 min and was 41 ± 3.4% of sperm incubated in ((RCM + Fn) +

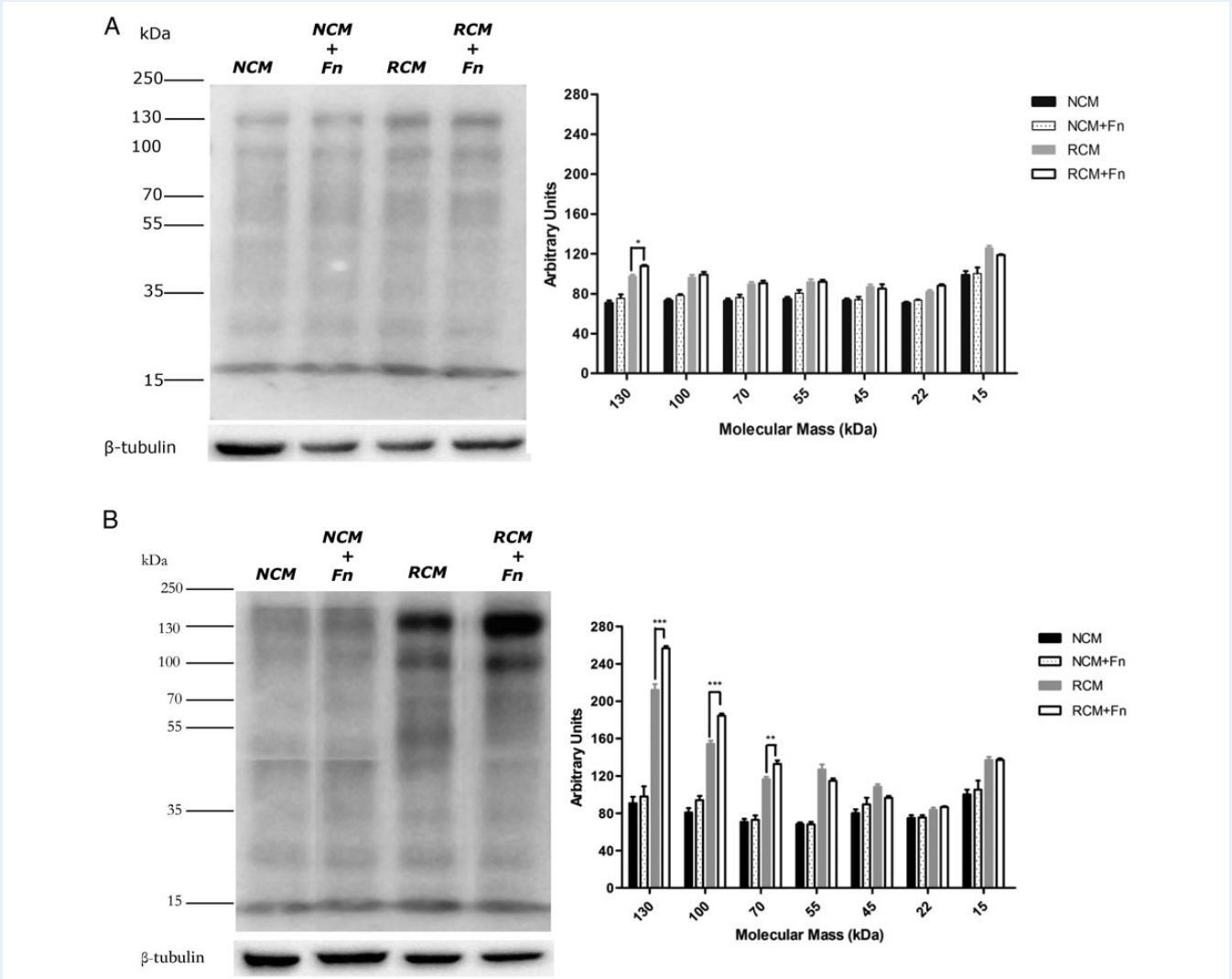


**Figure 5** Tyrosine (Try) phosphorylation pattern during human sperm capacitation induced by Fn. Ten microgram of sperm extracts incubated in NCM, RCM and RCM + Fn [100 μg/ml] at different times of capacitation (1 min (**A**), 60 min. (**B**), 180 min (**C**), 300 min (**D**) and 1080 min (**E**)) were subjected to SDS–PAGE and then transferred onto a PVDF membrane. The membrane was probed using a monoclonal anti-phosphotyrosine antibody (clone 4G10), as described in Materials and Methods. β-Tubulin was used as loading control. Molecular mass standards are indicated at the left. On the right side, the densitometric analysis of the bands is shown (mean ± SEM) of three experiment) *P, 0.01 RCM versus (RCM + Fn). A representative gel of three is shown (ANOVA, Tukey's multiple comparison test).

progesterone) versus 30 ± 2.9% of sperm incubated in RCM + progesterone (Fig. 6A). The addition of the antibody against the $\alpha_5$ subunit of the Fn receptor to the suspension of capacitated sperm in the presence of Fn prevented the stimulating effect of Fn (Fig. 6B), indicating that the effect of fibronectin is mediated by $\alpha_5\beta_1$ integrin. By contrast, the neutralization of the integrin with antibody against the $\alpha_5$ subunit of the Fn receptor, was unable to affect the progesterone-induced acrosome reaction (Fig. 6B). Finally, the incubation of capacitated sperm with an anti-human IgG antibody (control antibody) did not prevent the ability of Fn to induce the acrosome reaction (data not shown). These results demonstrate the specificity of antibody against the $\alpha_5$ subunit.

Overall these results demonstrate that Fn enhances human sperm capacitation in capacitating conditions and that its effect is specifically mediated by $\alpha_5$ integrin.

# Discussion

In this study, we provide evidence that Fn enhances human sperm capacitation. The evidence for this is as follows: (i) treatment with Fn increased the B pattern of capacitated sperm; (ii) treatment with Fn reduced the number of sperm bound to BOEC; (iii) both of these effects were blocked when the sperm were incubated with an antibody against the $\alpha_5$ subunit of integrin, indicating that the effect was specifically due to Fn; (iv) treatment with Fn increased the cAMP concentration and activity of PKA; (v) treatment with Fn induced an increase in the phosphorylation of proteins on Tyr residues; (vi) and finally, treatment with Fn induced an increase in the percentage of reacted sperm induced by progesterone. Furthermore, our results confirm the presence of $\alpha_5\beta_1$ integrin in human sperm and that the effect induced by Fn is mediated by

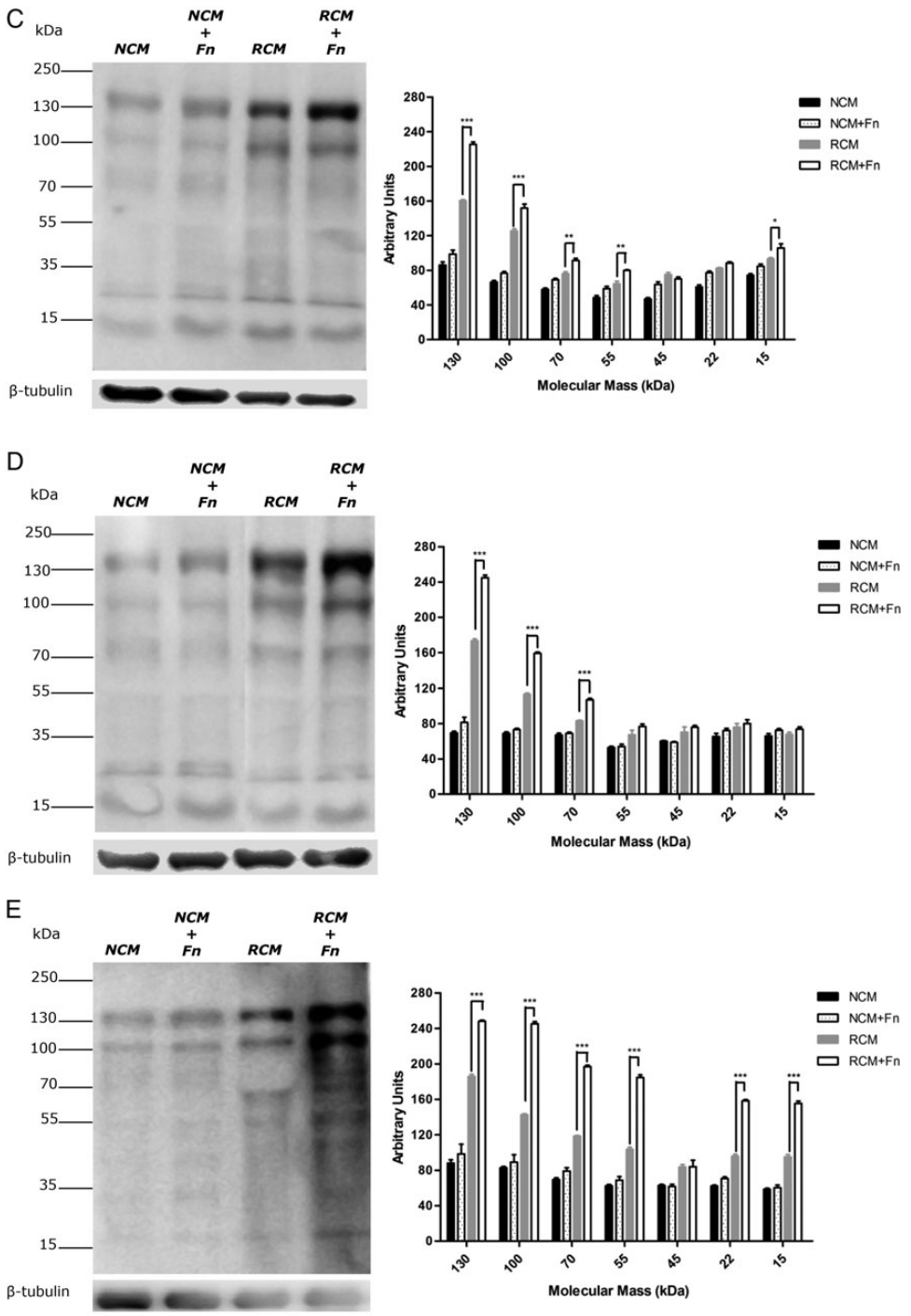


**Figure 5** (Continued).

this receptor. When evaluating the state of capacitation using CTC fluorescence, as expected, the percentage of sperm with the B pattern did not change over time in sperm incubated in NCM or NCM + Fn. This suggests that Fn by itself has no effect on capacitation. This could be because in non-capacitated sperm, the Fn receptor (alpha 5 beta 1 integrin) is inactive. When NCM was reconstituted with BSA and $HCO_3^-$ (RCM), a rapid increase in the number of capacitated sperm was observed from the start of the incubation time, showing a very similar behaviour to that described by Furuya *et al.* (1993) and Signorelli *et al.* (2013). Although, Fn has no capacitating effect by itself, the interesting and novel effect is that when sperm were incubated in RCM in the presence of Fn, the percentage of capacitated sperm increased by ~10% from the beginning of capacitation (1 min), which lasted until 300 min of incubation. Because we can find 20–300 × $10^6$ sperm/ml in a

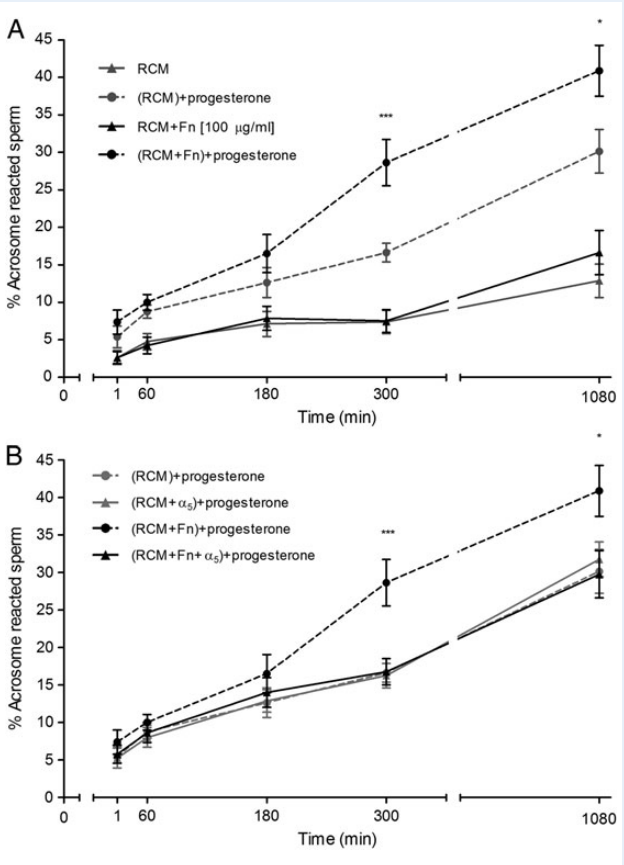


**Figure 6** Evaluation of sperm capacitation through induction of acrosome reaction (AR) by progesterone. (**A**) Sperm cells were incubated in RCM or RCM + Fn [100 μg/ml] at 1, 60, 180, 300 and 1080 min. In the specified condition progesterone was added for the last 15 min of incubation. Dotted line corresponds to cells incubated in presence of progesterone (7 μM) and solid line corresponds to sperm incubated in absence of progesterone. (**B**) Sperm cells were pre-incubated with the antibody (1:100 dilution), and then, were incubated in RCM or RCM + Fn [100 μg/ml] at 1, 60, 180, 300 and 1080 min. In the specified condition progesterone was added for the last 15 min of incubation. The state of the acrosome was evaluated using the PSA-FITC assay. Bars show the percentage of acrosome-reacted spermatozoa. Data are expressed as the mean ± SEM ($n = 8$). *$P \leq 0.05$ (RCM + Fn) + progesterone versus (RCM) + progesterone; ***$P \leq 0.001$ (RCM + Fn) + P versus (RCM) + progesterone (ANOVA, Tukey's multiple comparison test).

normal ejaculate (World Health Organization, 2010) and because usually only one sperm is able to penetrate the oocyte, a 10% increase in the amount of capacitated sperm induced by Fn is of great importance. Preliminary results from our laboratory support the CTC assay results shown above, because we have evidence that at the beginning of capacitation, ~7% of the sperm population in a normal ejaculate have an active $\alpha_5\beta_1$ integrin, suggesting that the increase of $Ca^{2+}$ and $HCO_3^-$ (early signs of the capacitation process) induces an increase in activation of the Fn receptor. It is noteworthy that at 1080 min no significant differences were observed between capacitated sperm in RCM with or without Fn. This is probably because although the effect of Fn on capacitation remains constant over time, RCM continues capacitation by itself, reaching a plateau in both cases (RCM alone and RCM with Fn).

Studies of sperm–oviduct interaction in different species showed the benefit of using co-cultures *in vitro* to evaluate the physiology of sperm function during exposure to oviductal epithelial cell (Dobrinski *et al.*, 1996; Suarez *et al.*, 1997). However, this system has been difficult to adapt for human tissues due to the difficulties in obtaining Fallopian tube epithelium from normal donors. Previous reports indicate that the co-cultures of human sperm with oviductal epithelial cell from macaques or bovines maintain human sperm function, and this system may be useful for the study of human sperm–oviduct interactions (Ellington *et al.*, 1998b). In accordance with this background and CTC measurements, we observed that Fn stimulates the release of human sperm incubated in RCM from BOEC at all times studied, suggesting an increase in the number of capacitated sperm. It was noteworthy that at 18 h (1080 min) of incubation, the number of human sperm attached decreased, probably because the incubated BOEC times were too long and because the co-incubation was not performed in a sterile environment.

Note that the Fn effect observed in the CTC assay and the co-culture techniques was specific because when the sperm were pre-incubated in the presence of an antibody against $\alpha_5\beta_1$ the effect of Fn was prevented.

It is known that during mammalian sperm capacitation, increased concentrations of $Ca^{2+}$ and $HCO_3^-$ result in the activation of SACY enzyme (Visconti, 2009) and that this activation makes a significant contribution to the capacitation process (Buffone *et al.*, 2014) by increasing the intra- (Visconti *et al.*, 1998; Battistone *et al.*, 2013) and extracellular cAMP levels (Osycka-Salut *et al.*, 2014). To determine whether Fn regulates the levels of cAMP, we measured cAMP concentration during capacitation. When the sperm were incubated in NCM, the cAMP levels were almost undetectable throughout the incubation, whereas when sperm were incubated in RCM a significant increase in cAMP levels was observed at 1 min, which significantly decreased at 60 min of incubation and then remained almost undetectable throughout the capacitation time. Determination of cAMP levels during the process of capacitation, both in NCM and RCM, has been assessed in other studies in human and bovine spermatozoa, and describe a cAMP production profile similar to that described here (Lefievre *et al.*, 2002; Battistone *et al.*, 2013; Osycka-Salut *et al.*, 2014).

The effect exerted by Fn on cAMP levels could be explained by modulation of intracellular $Ca^{2+}$ levels, as described by Diaz *et al.* (2007). Furthermore, Abrams *et al.* (1991) found that the concentration of intracellular $Ca^{2+}$ dually modulates the activity of certain ACs. AC types 1, 2, 3, 4 and 8 were found in mammalian sperm (Baxendale and Fraser, 2003). Of those, AC types 1, 3 and 8 can be regulated by $Ca^{2+}$ (Sunahara and Taussig, 2002). These antecedents support our results and lead us to postulate that the binding of Fn to its integrin activates $Ca^{2+}$ channels at the beginning of capacitation, increasing the intracellular $Ca^{2+}$ concentration, thereby activating some ACs and increasing levels of cAMP. However, as described by Abrams *et al.* (1991), high concentrations of $Ca^{2+}$ would produce a negative regulation of ACs. This could be overcome by some phosphodiesterases, whose activation is dependent on $Ca^{2+}$ (Dimitriadis *et al.*, 2008), and would contribute to the decrease in the concentration of cAMP, taking it to lower levels than in RCM alone, as we observed after 60 min of incubation. Likewise, the second messengers, as described previously, are transient, regulating the concentration of $Ca^{2+}$ in the sperm over time, explaining the

gradual increase in cAMP levels towards the end of the capacitation. Furthermore, we have observed that as capacitation progresses the amount of active integrin increases (manuscript in preparation), leading to the recovery of cAMP levels, as observed at the end of incubation. Notably, further studies are needed to investigate the exact mechanisms by which Fn is able to modulate the levels of cAMP.

During the capacitation process, the immediate consequence of increased cAMP is the activation of PKA. We observe that the PKA activity increased rapidly in capacitated sperm incubated in RCM alone and then decreased at 60 min, staying almost undetectable until 1080 min of capacitation. These results are consistent with those of Moseley *et al.* (2005), who analysed the PKA activity up to 90 min of incubation. In the presence of Fn, we observed similar behaviour, although more pronounced. At the end of the incubation (1080 min), we observed an increase in the PKA activity, which may be because the sperm underwent the AR and intracellular signals associated with this event stimulate PKA activity (De Jonge *et al.*, 1991; Lefievre *et al.*, 2002). Likewise, it should be noted that the effect of Fn on PKA activity correlates with cAMP levels; to date there are no studies monitoring PKA activity during 18 h of incubation, and the effect of Fn on the activity of this kinase in sperm has not been evaluated previously.

Mature sperm are transcriptionally inactive cells, so post-transcriptional modifications are vital for their normal operation. This makes protein phosphorylation the primary method for controlling its cellular processes. Protein phosphorylation is considered a late event in the capacitation process (Visconti, 2009). However, the time at which the increase in p-Tyr residues occurs is controversial, especially in short capacitation times. Our results show that no substantial change in p-Tyr throughout the capacitation period was observed in NCM, which agrees with the results described in the literature (Carrera *et al.*, 1996; Aitken *et al.*, 1998; Moseley *et al.*, 2005; Battistone *et al.*, 2013). With regard to the effect of sperm incubated in RCM on p-Tyr, there is broad consensus that phosphorylation increases during capacitation (Visconti *et al.*, 1995; Leclerc *et al.*, 1996; Visconti and Kopf, 1998; Ficarro *et al.*, 2003; Naz and Rajesh, 2004; Battistone *et al.*, 2014) and our results are in agreement with these data. Consistent with the results described by Carrera *et al.* (1996), our data indicate that from 60 min of capacitation, human sperm incubated in RCM begin to experience phosphorylation of proteins on p-Tyr. Moseley *et al.* (2005) indicated that human sperm change the pattern of p-Tyr phosphorylation from the beginning of capacitation, which increases to 90 min and then decreases. On the other hand, Battistone *et al.* (2013) evaluated the p-Tyr pattern through the capacitation process and found that protein phosphorylation on tyrosine residues begins to appear from 6 h of capacitation, a fact that disagrees with our results. To the best of our knowledge, this is most likely due to differences in implementation of the western blot technique. In the present study, we used a biotin-conjugated secondary antibody, which amplifies the signal, making our method more sensitive to detect phosphorylated proteins.

In sperm incubated in RCM in presence of Fn, we observed that there was an increase in the level of p-Tyr from 60 min incubation onwards. Diaz *et al.* (2007) found that by incubating human sperm in the presence of Fn, there is an increase in the degree of phosphorylation of p-Tyr from the beginning of capacitation, increasing more intensely from 5 h onwards. What is interesting is the inescapable relationship between our results described above for the CTC technique, heterologous co-cultures, measuring cAMP levels and the specific activity of PKA.

Finally, to demonstrate the capacitating effect of Fn on human sperm, we evaluated the final event that shows the status of sperm capacitation, namely the AR. Our results indicated that the addition of progesterone in a RCM medium induced an increase of the AR. These results are consistent with those previously described in the literature by many authors (Diaz *et al.*, 2007; Tapia *et al.*, 2011; Battistone *et al.*, 2013; Sagare-Patil *et al.*, 2013; Wertheimer *et al.*, 2013). Furthermore, our results indicated that the addition of Fn to RCM increased the ability of sperm to react with progesterone, supporting the fact that this glycoprotein increases the number of capacitated sperm compared with that obtained in a conventional capacitating medium containing BSA and $HCO_3^-$. Furthermore, the results demonstrate that the effect of Fn is specific and mediated by $\alpha_5$ integrin.

As mentioned above, the capacitation process is divided into early and late events, and the AR is considered one of the final events in capacitation. This makes sense because we observe a significant difference in the percentage of reacted sperm from 300 min of capacitation for sperm incubated in the presence of Fn. At the same time, it is widely accepted that a capacitated sperm must not necessarily undergo the AR. This is probably because: (i) the human sperm need time to be prepared for the AR; (ii) the capacitation and AR processes are activated by different intracellular signals and (iii) capacitation and AR are temporally different and independent processes. For example, Sagare-Patil *et al.* (2013) observed that human sperm capacitated for 2 h and then incubated in the presence of progesterone activate the PI3K-AKT pathway, which regulates sperm motility and hyperactivation but not the AR. Furthermore, if sperm are capacitated for longer times, progesterone is capable of triggering the AR, activating other signalling pathways, such as protein kinase C and the extracellular regulated kinase (ERK) family (O'Toole *et al.*, 1996; Luconi *et al.*, 1998). This shows that intracellular pathways of capacitation and the AR process are different although they are stimulated by the same molecule, in our case Fn.

With respect to the moment when a sperm is able to trigger the AR, there is substantial controversy. Reports describe that the human sperm is able to trigger the AR at 3.5 h (de Lamirande and Gagnon, 2002), 4 h (Leclerc *et al.*, 1996), 5 h (Diaz *et al.*, 2007) and 6 h (Battistone *et al.*, 2013) after capacitation onset. The AR is considered the final event of the capacitation process, and the molecular machinery must necessarily differ from the initial step of capacitation. Moreover, motility and hyperactivation are prerequisites for capacitation. This shows that the intracellular pathways of the capacitation process and AR are different, regardless of their shared stimuli.

*The in vivo* effect of Fn on sperm physiology is difficult to know because the physiological concentrations and fluctuation in the oviductal fluid during the female cycle are unknown. Moreover, the existence of hormonal regulation of proteins present in the oviductal fluid has been reported in many species, such as frog (Xiang *et al.*, 2004), hamster (Hall *et al.*, 1977), and cow (Killian *et al.*, 1989; Gervasi *et al.*, 2013). Additionally, hormonal regulation of several glycoproteins of the extracellular matrix in the human fallopian tube has also been reported (Diaz *et al.*, 2012). It is possible that Fn is regulated hormonally and that this regulation is related to time for sperm capacitation.

The present study is pioneering in demonstrating the stimulatory effects of Fn on the capacitation process in human sperm. These effects have been validated by evaluating 6 events associated with sperm capacitation: the movement of $Ca^{2+}$ bound to membrane phospholipids using a CTC assay, cell interactions in heterologous co-culture,

cAMP levels, the specific activity of PKA, tyrosine phosphorylation and induction of the AR.

## Supplementary data

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

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## Authors' roles

E.M.-L. contributed substantially to the acquisition and analysis of the data and the drafting of the manuscript. C.O.-S. contributed to the acquisition of the data and the final approval of the manuscript version. E.M.-L., J.S., P.P., B.P., M.K., P.M., S.P.-M. and E.S.D. substantially contributed to the study design, the critical revision of the manuscript for intellectual content and the final approval of the version to be published. E.S.D. (with assistance from S.P.-M.) managed the project.

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## Conflict of interest

None declared.

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