Steroid hormone receptors and direct effects of steroid hormones on ram spermatozoa

S Gimeno-Martos, M González-Arto, A Casao, M Gallego, J A Cebrián-Pérez, T Muiño-Blanco and R Pérez-Pé

Department of Biochemistry and Molecular and Cell Biology – Environmental Sciences Institute of Aragón (IUCA), Faculty of Veterinary Medicine, University of Zaragoza, Spain

Correspondence should be addressed to R Pérez-Pé; Email: rosper@unizar.es

Abstract

This study was based on the assumption that steroid hormones present in the female genital tract may have a rapid effect on ram spermatozoa by interaction with specific surface receptors. We demonstrate the presence of progesterone (PR) and estrogen (ER) receptors in ram spermatozoa, their localization changes during *in vitro* capacitation and the actions of progesterone (P4) and 17 β -estradiol (E2) on ram sperm functionality. Immunolocalization assays revealed the presence of PR mainly at the equatorial region of ram spermatozoa. Western blot analyses showed three bands in ram sperm protein extracts of 40–45 kDa, compatible with those reported for PR in the human sperm membrane, and both classical estrogen receptors (66 kDa, ER α and 55 kDa, ER β). ER α was located in the postacrosomal region of all the spermatozoa and ER β on the apical region of 63.7% of the cells. The presence of ER β was correlated with the percentage of non-capacitated spermatozoa evaluated by chlortetracycline staining ($R=0.848$, $P<0.001$). This significantly decreased after *in vitro* capacitation and nearly disappeared when acrosome reaction was induced. The addition of P4 and E2 before *in vitro* capacitation resulted in a higher ($P<0.001$) acrosome-reacted sperm rate compared with the control (13.0%), noticeably greater after 3 h and when added to a high-cAMP medium (37.3% and 47.0% with E2 and P4, respectively). In conclusion, the results of this study demonstrate for the first time that ovine spermatozoa have progesterone and estrogen receptors and that both steroid hormones are related with the induction of the acrosome reaction.

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Introduction

Steroid hormones, such as estrogens and progesterone, play a crucial role in the regulation of reproductive events in mammals. It is well established that these hormones regulate gene expression in the hypothalamic–hypophyseal gonadal axis through nuclear receptors (Beato *et al.* 1996). Apart from their genomic action, steroid hormones exert rapid effects on several types of cells by binding to receptors in the plasma membrane, generally affecting signal transduction responses (see review in Bishop & Stormshak 2008).

Progesterone (P4) and 17- β estradiol (E2) are present in the female genital tract. The concentrations of these hormones in the follicular fluid have been estimated in the nanomolar range (Carson *et al.* 1981), and part of this fluid is released into the oviduct together with the oocyte at the moment of ovulation. Furthermore, after ovulation, the cumulus cells surrounding the oocyte secrete P4 and E2 (Vanderhyden & Tonary 1995, Chian *et al.* 1999), which could reach micromolar levels (Frederick *et al.* 1991) and diffuse to form a molecular gradient toward the edge of the cumulus matrix and beyond (Teves *et al.* 2006). Thus, it is very difficult to estimate the steroid

hormone concentration to which the spermatozoa are exposed, even more so when considering other factors such as the moment of the oestrus cycle, the number of cells constituting the cumulus matrix, the proximity to the oocyte or differences between mammal species.

A large number of studies can be found in the literature concerning the non-genomic actions of P4 and E2 on spermatozoa, especially in humans (reviewed in Bishop & Stormshak 2008 and Baldi *et al.* 2009). P4 may stimulate the hyperactivation (Uhler *et al.* 1992), chemotaxis (Jaiswal *et al.* 1999, Oren-Benaroya *et al.* 2008), *in vitro* capacitation (de Lamirande *et al.* 1998, Yamano *et al.* 2004) and the acrosome reaction in human spermatozoa (Osman *et al.* 1989, Kay *et al.* 1994). In other mammalian species, P4 has mainly been related with the induction of the acrosome reaction in mouse (Roldan *et al.* 1994), pig (Melendrez *et al.* 1994), goat (Somanath *et al.* 2000) and stallion (Meyers *et al.* 1995), and with chemotaxis in rabbit (Guidobaldi *et al.* 2008). On the other hand, the findings relating to the effects of E2 on spermatozoa are contradictory. While for some authors the main role of this hormone may be to modulate the progesterone effects on hyperactivation (Fujinoki 2010,

Fujinoki *et al.* 2016), capacitation (Sebkova *et al.* 2012) or the acrosome reaction (Vigil *et al.* 2008), others suggest a direct stimulating effect on sperm functionality (Adeoya-Osiguwa *et al.* 2003, Ded *et al.* 2013). There are scarcely any studies about the non-genomic effects of steroid hormones on spermatozoa in ovine.

The way in which steroid hormones exert rapid non-genomic actions might either involve the nuclear steroid receptors acting on different cellular signalling or be mediated by membrane receptors, which could be either the classical receptors targeted to the plasma membrane or totally different ones (Luconi *et al.* 2004). In human spermatozoa, several studies have shown evidence of the presence of functionally active novel membrane receptors for progesterone (PR), excluding the existence of the classical nuclear receptors (Castilla *et al.* 1995, Luconi *et al.* 1998). However, the expression of the conventional isoforms PRA and PRB has also been reported (De Amicis *et al.* 2011), suggesting the presence of different types of PRs. Numerous authors have attributed the rapid effects of E2 on spermatozoa to its binding to the classical estrogen receptors (ER α and ER β) (Aquila *et al.* 2004), although the G protein-coupled estrogen receptor (GPER) has recently been identified in humans (Rago *et al.* 2014). Likewise, putative steroid receptors mediating fast effects on sperm functionality have been described in other species such as dog, goat, pig or stallion (Cheng *et al.* 1998, Sirivaidyapong *et al.* 1999, Somanath *et al.* 2000, De Amicis *et al.* 2011, Arkoun *et al.* 2014), but there is no information concerning ovine spermatozoa.

Given the above information, we can hypothesize that steroid receptors may mediate the fast effects of steroid hormones in ram spermatozoa. To prove this hypothesis, the specific aims of this study were (1) to evidence the presence of progesterone and estrogen receptors in ram spermatozoa; (2) to determine whether *in vitro* capacitation has any effects on their localization and (3) to study the effects of both steroid hormones on ram sperm functionality.

Material and methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

Semen collection and processing

Semen was collected from nine 2- to 5 year-old Rasa Aragonesa rams using an artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding (ANGRA), were kept at the Experimental Farm of the Veterinary School of the University of Zaragoza under uniform nutritional conditions, with an abstinence period of two days. Second ejaculates were pooled and used for each assay, to avoid individual differences (Ollero *et al.* 1996).

All experimental procedures were performed under Project License PI34/11 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza.

A seminal plasma-free sperm population was obtained using a dextran swim-up procedure as described previously (Garcia-Lopez *et al.* 1996) performed in a medium with the following composition: 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM MgSO₄, 0.3 mM sodium pyruvate, 0.3 mM K₂HPO₄, 5 mg/mL bovine serum albumin (BSA), 30 mg/mL dextran, 1.5 IU/mL penicillin and 1.5 mg/mL streptomycin (pH 6.5).

In vitro capacitation and acrosome reaction induction

For the induction of *in vitro* capacitation, aliquots of swim-up-selected spermatozoa (1.6×10^8 cells/mL) were incubated for 3 h at 39°C in a humidified incubator with 5% CO₂ in air. Incubations were performed in a TALP medium (Parrish *et al.* 1988) composed of 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.3 mM NaH₂PO₄, 21.6 mM Na lactate, 3 mM CaCl₂, 0.4 mM MgCl₂, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose and 5 mg/mL BSA (pH 7.3 adjusted with NaOH) or in a high-cAMP medium (cocktail, cAMP-PKA pathway), already successfully demonstrated for capacitating ram spermatozoa (Grasa *et al.* 2006 Colas *et al.* 2008), composed of dibutyryl-cAMP (db-cAMP, Sigma Chemical Co.; 1 mM), caffeine and theophylline (both inhibitors of phosphodiesterases, Sigma Chemical Co.; 1 mM each), okadaic acid (OA, a broad spectrum phosphatase inhibitor, Sigma Chemical Co.; 0.2 μ M) and methyl- β -cyclodextrin (M- β -CD, Sigma Chemical Co.; 2.5 mM).

The acrosome reaction was induced by the addition of lysophosphatidylcholine (LPC) to the high-cAMP-capacitated samples (Parrish *et al.* 1988). To this end, 5 μ L LPC (300 μ g/mL) was added to 95 μ L capacitated samples and incubated at 39°C, 5% CO₂ and 100% humidity for 20 min (Gomez *et al.* 1997).

Control samples without the addition of the cocktail or LPC were maintained under the same conditions in both the treatments: *in vitro* capacitation and acrosome reaction.

In order to evaluate the effects of 17- β estradiol (E2) and progesterone (P4) on sperm functionality, hormones were added to aliquots of swim-up-selected spermatozoa (1.6×10^8 cells/mL) diluted in a TALP or a high-cAMP (cocktail) medium. Both hormones were diluted separately in DMSO and PBS and added to the sperm samples to yield final concentrations of 1 μ M, 10 nM or 100 pM of each. The final concentration of DMSO in all the samples was 0.1%. A control group containing the same DMSO concentration was included.

Sperm motility analysis

Motility kinematic parameters underwent computer-assisted measurement using a CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain) with a video camera (Basler A312f, Basler Vision Components, Exton, PA, USA) mounted on a microscope (Nikon eclipse 50i, Nikon) equipped with a 10x

negative-phase contrast lens and a 10× projection ocular. Samples (6 µL) were placed between pre-warmed slides and cover slips and maintained at 37°C during analysis by a heated slide holder. From a single field, 25 consecutive digitalized images were analysed. The percentages of total motile (TM) and progressive motile (PM) spermatozoa were evaluated. The kinematic parameters recorded for each spermatozoon were curvilinear velocity (VCL, µm/s: the average path velocity of the sperm head along its actual trajectory); straight line velocity (VSL, µm/s: the average path velocity of the sperm head along a straight line from its first to its last position); average path velocity (VAP, µm/s: the average velocity of the sperm head along its average trajectory); percentage of linearity (LIN, %: the ratio between VSL and VCL); percentage of straightness (STR, %: the ratio between VSL and VAP); wobble coefficient (WOB, %: the ratio between VAP and VCL); mean amplitude of lateral head displacement (ALH, µm: the average value of the extreme side-to-side movement of the sperm head in each beat cycle) and beat cross-frequency (BCF, Hz: the frequency with which the actual sperm trajectory crosses the average path trajectory).

Sperm viability

Two microlitres of carboxyfluorescein diacetate (CFDA, 1 mM) and propidium iodide (PI, 0.75 mM) were added to 200 µL of sperm samples (6×10^6 cells/mL) based on a modification of the procedure described by Harrison and Vickers (Harrison & Vickers 1990). Samples were incubated at RT in darkness for 15 min and analysed by flow cytometry. Measurements were performed on a Beckman Coulter FC 500 (Beckman Coulter Inc., Brea, CA, USA) with CXP software, equipped with two lasers of excitation (Argon ion laser 488 nm and solid state laser 633 nm) and 5 filters of absorbance (FL1-525, FL2-575, FL3-610, FL4-675 and FL5-755, ± 5 nm each band pass filter). A minimum of 20,000 events was counted in all the experiments. The sperm population was gated for further analysis on the basis of its specific forward (FS) and side scatter (SS) properties; other non-sperm events were excluded. A flow rate stabilized at 200–300 cells/s was used. The argon laser and filters of 525 and 675 nm were used to avoid overlapping. The monitored parameters were FL1 (CFDA) and FL4 (PI).

Assessment of capacitation status by CTC staining

CTC is a fluorescent antibiotic that binds to membrane proteins of sperm cells. The fluorescence of bound CTC is enhanced by intracellular calcium, and the capacitation-induced changes in the labelling patterns showed by Ward and Storey (Ward & Storey 1984) are widely considered to reflect the sperm capacitation state, although the molecular basis of CTC staining to sperm cells is not still understood. A CTC solution (750 µM) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 µM cysteine (pH 7.8) and passed through a 0.22-µm filter (Merck Millipore). Thereafter, 20 µL CTC solution and 5 µL of 12.2% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.8) were added to a 18 µL sperm sample and incubated at 37°C in the dark for 10 min. At room temperature,

a 4-µL aliquot of the stained sample was placed on a glass slide and mixed with 2 µL of 0.22 M triethylenediamine (DABCO) in glycerol:PBS (9:1). Samples were covered with 24×48 mm coverslips, sealed with colourless enamel and stored in the dark at 4°C. For the evaluation of CTC patterns, samples were examined using a Nikon Eclipse E-400 microscope (Kanagawa, Japan) under epifluorescence illumination using a V-2A filter. All samples were processed in duplicate and at least 150 spermatozoa were scored per slide. Three sperm types were identified (Gillan *et al.* 1997): (1) non-capacitated (NC), showing an even distribution of yellow fluorescence over the head, with or without a bright equatorial band; (2) capacitated (C), with fluorescence on the anterior portion of the head and (3) acrosome-reacted cells, showing no fluorescence on the head, with or without a bright equatorial band.

Avidin-biotin peroxidase assays

Aliquots of 4×10^6 spermatozoa were fixed with 3.7% formaldehyde (v/v) in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.76 KH_2PO_4 , pH 7.2) for 20 min at room temperature. Then, the cells were then centrifuged at 900×g for 5 min and the pellet was resuspended in PBS. After fixation, 40 µL of cell suspension was smeared onto poly-L-lysine-coated slides and maintained at room temperature for 3 h to ensure good adhesion onto the slide.

Slides were rehydrated in PBS, and endogenous peroxidase was inactivated with 1.70% hydrogen peroxide in 100% ethanol for 30 min. The slides were then washed in PBS and incubated in horse serum as a blocking reagent (supplied by Vector, Los Angeles, CA, USA) for 45 min, followed by incubation with the specific antibody at a 1:50 dilution overnight. The chosen antibody to detect the progesterone receptor was PR (C-19) (Santa Cruz Biotechnology; Cat# sc-538, RRID:AB_632263), an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of PR of human origin, but also recommended for detection of progesterone receptors (PRA and PRB) in additional species such as equine, canine, bovine, porcine and avian. Antibodies used for the detection of estradiol receptors were rabbit polyclonal antibodies against ERα and ERβ, respectively (ERα Santa Cruz Biotechnology; Cat# sc-7207, RRID:AB_640249, and ERβ Santa Cruz Biotechnology; Cat# sc-8974, RRID:AB_2102246).

Subsequently, the slides were incubated with a biotinylated anti-rabbit antiserum for 40 min. An avidin-biotin-peroxidase complex (from Vector) was then applied for 45 min. The binding sites of the primary antibodies were visualized by diaminobenzidine (DAB) and hydrogen peroxide solution (20 mg DAB in 100 mL of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.005% H_2O_2) for 5 min. Counter-staining with Carazzi's haematoxylin was followed by dehydration and mounting. As negative controls, all samples were incubated with normal serum instead of the primary antibody, with the remaining procedure being the same.

The cells were examined under a Nikon Eclipse E-400 microscope (Kanagawa, Japan) under bright field illumination at 1000× magnification. Microscopic images were captured and processed with Nikon image software.

Indirect immunofluorescence assays

Slides prepared as described previously were washed three times with PBS, and non-specific binding sites were blocked with 5% BSA (w/v) in PBS for 4 h at room temperature in a humidity chamber. After three washes in PBS, spermatozoa were incubated overnight at 4°C with the primary antibody (anti-ER α (RRID:AB_640249), anti-ER β (RRID:AB_2102246) and anti-PR (RRID:AB_632263) rabbit polyclonal antibody, respectively; Santa Cruz Biotechnology), diluted 1:50 (v/v) in PBS containing 1% BSA (w/v). After three washes in PBS, the cells were incubated with the secondary antibody (Alexa Fluor 488 chicken anti-rabbit; Thermo Fisher Scientific; Cat# A-21441, RRID:AB_2535859), diluted 1:800 (v/v), for 1.5 h at room temperature in a humidity chamber. The slides were then washed three times with PBS before the addition of 5 μ L of 0.22 M triethylenediamine (DABCO) in glycerol:PBS (9:1 v/v) to enhance and preserve cell fluorescence. Finally, the preparations were covered with coverslips, sealed with colourless enamel and visualized using a Nikon Eclipse E-400 microscope under epifluorescent illumination. All samples were processed in duplicate and at least 150 spermatozoa were scored per slide.

Western blotting

For PR and ER detection, sperm proteins were extracted from raw semen by diluting samples in PBS (10^8 cells/mL) and centrifuging in a microfuge at 900 $\times$ g for 6 min at room temperature. The supernatant was discarded, and the pellet was resuspended with 100 μ L of extraction buffer (125 mM Tris-HCl (pH 6.8), 2% SDS, 10% β -mercaptoethanol, 20% glycerol and 0.02% bromophenol blue). For the detection of phosphorylated proteins on tyrosine residues, aliquots of 3.2×10^7 cells of TALP- or cocktail-incubated samples were directly suspended in 100 μ L of extraction buffer. In both cases, after incubation at 100°C in a sand bath for 5 min, the samples were centrifuged again at 7500 $\times$ g for 5 min at 4°C. The supernatant was recovered and, after adding 10% of a protease inhibitor cocktail, was stored at -20°C.

For SDS-PAGE, 5×10^6 cells were loaded on 10% (w/v) SDS-PAGE gels, separated by standard SDS-PAGE and transferred onto a PVDF membrane using a transfer unit (Trans-Blot Turbo Transfer System, Bio-Rad). After the blocking of non-specific sites with 5% BSA in PBS for 4 h, the proteins were detected by incubating overnight at 4°C with a primary antibody diluted 1:1000 in 0.1 Tween20 PBS containing 1% BSA. For the detection of steroid hormone receptors, the above-mentioned anti-ER α , -ER β and -PR rabbit polyclonal antibodies were used. After extensive washing, the membranes were incubated for 1 h and 15 min at room temperature with a secondary donkey anti-rabbit antibody (Donkey Anti-Rabbit IgG, IRDye 800CW Conjugated antibody; LI-COR Biosciences, Lincoln, NE, USA; Cat# 926-32213, RRID:AB_621848 LI-COR Biosciences,) diluted in 0.1 Tween20 PBS containing 1% BSA (dilution 1: 35000 and 1:15000 for PR and ER, respectively). As positive controls, we used the commercial cell lysates specifically recommended and supplied by the antibody manufacturer (Santa Cruz Biotechnology). MCF7 whole-cell lysate, specifically recommended as the positive control

for PR and ER α , is derived from the MCF7 cell line (human breast adenocarcinoma). F9 whole-cell lysate, specifically recommended as the positive control for ER β , is derived from the F9 cell line (mouse testicular teratoma). Liver protein extract was also used as positive controls for all the above-mentioned antibodies, on the basis of several studies reporting the presence of PR and ER in hepatic tissue (Xu *et al.* 2004, Varas & Jahn 2005, Dressing *et al.* 2011). Liver protein extracts were obtained from ovine liver following the same protocol as sperm protein extracts. We carried out the Western blotting protocol using the corresponding antibody in each case.

For the detection of phosphorylated proteins on tyrosine residues, the procedure was the same as described above but using a mouse monoclonal anti-phosphotyrosine antibody (Monoclonal Antibody, clone 4G10; Millipore; Cat# 05-321, RRID:AB_309678) as a primary antibody (dilution 1:1000) and a Donkey Anti-Mouse IgG, IRDye 800CW Conjugated antibody (LI-COR Biosciences; Cat# 926-32212, RRID:AB_621847) as a secondary antibody (dilution 1:15000). Anti-actin antibody (Sigma-Aldrich; Cat# A2066, RRID:AB_476693) produced in rabbit was used as a loading control (1:500 in 0.1 Tween20 PBS containing 1% BSA), followed by incubation with a secondary Donkey Anti-Rabbit IgG, IRDye 680RD Conjugated antibody (LI-COR Biosciences; Cat# 926-32223, RRID:AB_621845) diluted 1:15000.

Finally, the membranes were scanned after washing using the Odyssey Clx Infrared Imaging System (LI-COR Biosciences). To prove that the signal was specific, Western blotting omitting either primary or secondary antibodies was performed (data not shown).

Western blot images were quantified using Odyssey Clx Infrared Imaging System software (LI-COR Biosciences) to determine the relative intensity of the tyrosine phosphorylated protein bands. The total intensity signal of each lane was evaluated as the sum of the peak intensity of all bands in the lane and normalized to the actin loading control.

Statistical analysis

Differences between groups in motility, viability, CTC staining and receptor distribution were analysed by means of the chi-square test. Differences in protein tyrosine phosphorylation levels were analysed by ANOVA followed by the Bonferroni *post hoc* test after evaluation of the data distribution by the Kolmogorov-Smirnov test. The correlation between the capacitation state and ER β immunolocalization of the spermatozoa was analysed by Pearson's correlation test, after evaluation of the data distribution by the Kolmogorov-Smirnov test. All statistical analyses were performed using SPSS, version 14.0 (SPSS).

Results

Identification and immunolocalization of progesterone and estrogen receptors in ram spermatozoa

The avidin-biotin peroxidase complex assays revealed that progesterone receptors (PR) were located at the equatorial region of all the spermatozoa and also

Figure 1 Immunocytochemical localization of progesterone receptor (A), estrogen receptor α (B) and β (C) in ram spermatozoa. Magnification 1000x. Negative controls are shown (D).

appeared faintly on the midpiece of the tail (Fig. 1A). Both estrogen receptors, ER α and ER β , are also present in ram spermatozoa. The immunocytochemistry assays revealed that ER α was located at the postacrosomal region of all the cells although the labelling was always weaker than those with the other antibodies used (Fig. 1B). Two sperm subpopulations regarding ER β signalling were found: one showing no antibody labelling (ER β -, sperm 1 in Fig. 1C) and another showing intense reactivity on the apical region of the acrosome (ER β +, 63.7 $\pm$ 4.6%, sperm 2 in Fig. 1C). Curiously, some of the ER β + cells appeared to be losing the stained region (sperm 3 in Fig. 1C). In all the assays, the omission of the primary antibody resulted in no staining, which rules out the possibility of non-specific binding of the secondary antibodies (Fig. 1D).

Western blot analyses for PR in ram sperm protein extracts identified three protein bands corresponding to a molecular weight ranging between 40 and 45 kDa.

Figure 2 Western blot analysis of the presence of progesterone receptor (A), estrogen receptor α (B) and β (C) in ram sperm protein extracts (SP, lane 1 in A, B and C). Positive controls: MCF7 cell extracts (MCF7, lane 2, A and B), F9 cell extracts (F9, lane 2, C) and ovine liver protein extracts (L, lane 3, A, B and C).

The molecular weights of these bands coincide with those found in the positive controls (Fig. 2A), but not with the expected values for either the classical or the nuclear PR described by the commercial supplier of the antibody (81 kDa and 116 kDa for PRA and PRB, respectively). Regarding estrogen receptors (ER), Western blot analyses revealed a band of approximately 65 kDa for ER α (SP, lane 1 of Fig. 2B) and another of 55 kDa for ER β (SP, lane 1 of Fig. 2C), which correspond to the molecular weight of the amino acid sequence recognized by these antibodies (66 and 56 kDa for ER α and ER β respectively), according to the manufacturer. The bands observed were also found in the positive control recommended by the manufacturer (MCF7 and F9 cell lysates for ER α and ER β , respectively, lane 2 of Fig. 2B and C respectively) and in the ovine liver protein extracts (L, lane 3 of Fig. 2B and C, respectively).

Changes in the immunolocalization of progesterone and estrogen receptors according to the sperm capacitation state

In order to evaluate a possible variation in the localization of steroid hormone receptors associated to sperm capacitation, we investigated the presence and cellular distribution of PR, ER α and ER β in control, capacitated and acrosome-reacted samples by indirect immunofluorescence. As expected, the assessment of the capacitation state by CTC staining revealed a significant increase ($P < 0.05$) in the capacitated (C) and acrosome-reacted (AR) sperm patterns after the induction of both *in vitro* capacitation with the cocktail (high-cAMP concentration) and the acrosome reaction with LPC, compared with the control group (Table 1). However, no change was observed in PR and ER α localization after both processes (data not shown). In contrast, significant differences in the percentage of spermatozoa showing ER β labelling (ER β + cells) were evidenced (Table 1). The high ER β + sperm rate in control samples (65.2 $\pm$ 3.7%) significantly decreased after incubation with either the cocktail (24.6 $\pm$ 1.2%; $P < 0.05$) or LPC (6.3 $\pm$ 1.6%; $P < 0.05$). On the basis of these results, we investigated whether there was any correlation between the ER β labelling and the different capacitation patterns determined by the CTC staining (Fig. 3). Statistical analysis showed a highly significant positive correlation between the presence of ER β and the NC sperm rate ($r = 0.848$, $P < 0.001$; Fig. 4) and a highly negative correlation with both the C ($r = -0.524$, $P < 0.01$) and AR ($r = -0.811$, $P < 0.001$) sperm rate.

Effects of different concentrations of progesterone (P4) and 17 β -estradiol (E2) on sperm functionality

Once the presence of progesterone and ERs in ram spermatozoa was demonstrated, we assessed the effects of steroid hormones on several sperm functionality

Table 1 Effect of *in vitro* induction of capacitation and acrosome reaction on the estrogen receptor β labelling in ram spermatozoa.

Sperm sample	ER β + cells (%)	Chlortetracycline (CTC) staining patterns		
		NC (%)	C (%)	R (%)
Control	65.2 $\pm$ 3.7 ^a	72.1 $\pm$ 1.3 ^a	18.4 $\pm$ 1.5 ^a	9.2 $\pm$ 1.0 ^a
<i>In vitro</i> capacitated	24.6 $\pm$ 1.2 ^b	51.5 $\pm$ 3.2 ^b	30.9 $\pm$ 3.1 ^b	18.1 $\pm$ 0.7 ^b
AR	6.3 $\pm$ 1.6 ^c	26.7 $\pm$ 1.8 ^c	27.9 $\pm$ 0.9 ^b	45.4 $\pm$ 1.3 ^c

Percentage of spermatozoa with estrogen receptor β labelling (ER β +) assessed by indirect immunofluorescence (IIF), and capacitation status (NC: non-capacitated; C: capacitated; R: acrosome reacted) assessed by chlortetracycline (CTC) staining, in control, *in vitro* capacitated and acrosome reacted samples. Results are shown as mean $\pm$ S.E.M., $n = 10$. Different letters (a, b, c) mean statistical differences of $P < 0.05$.

markers. After 3 h of incubation in capacitating conditions in high-cAMP (cocktail) medium, a significant decrease in viability (sperm membrane integrity), motility (total and progressive) and velocity (VCL, VSL and VAP) was found ($P < 0.001$, compared to control at 0 h). P4 and E2 at 100 pM induced a deeper decrease in TM sperm ($65.2 \pm 4.5\%$, $68.2 \pm 2.3\%$ vs $75.0 \pm 2.1\%$ for cocktail samples with 100 pM P4 and E2, and without hormones, respectively). Progressive motility, kinematic parameters and sperm viability were not affected by the presence of hormones in incubation media (Table 2).

Regarding the capacitation status (CTC staining), no change was found after 1 h of incubation with both hormones in TALP-incubated samples (Fig. 5A). However, after 3 h of incubation, the effect of both hormones was significant, leading to a decrease in the NC sperm rate with both low and high concentrations of E2 and P4, concomitant with a higher percentage of AR spermatozoa compared with the control samples with no hormone ($25.5 \pm 8.3\%$ and $28.3 \pm 9.8\%$ for 100 pM of both hormones vs $13.0 \pm 6.4\%$ in control samples, $P < 0.001$) (Fig. 5A). Furthermore, 1 μ M of E2 led to a significant increment in the capacitated sperm rate ($P < 0.01$). However, incubation with 10 nM of both hormones did not significantly affect the physiological sperm status (Fig. 5A). Despite the considerable differences observed in CTC staining, statistical analysis of the densitometric evaluation of the protein tyrosine phosphorylation did not reveal any significant change (Fig. 5C and E).

When steroid hormones were added to the cocktail-incubated samples, differences in the percentages of different CTC staining subtypes could already be detected after the first hour of incubation (Fig. 5B). As expected, the addition of a cocktail that maintains high intracellular cAMP levels led to a highly significant increment in the percentage of capacitated sperm relative to the control (TALP-incubated sample) ($53.8 \pm 6.2\%$ vs $30.5 \pm 6.2\%$, $P < 0.001$). Inclusion of P4 in the cocktail medium did not significantly modify this percentage relative to cocktail samples without the hormone, but gave rise to a significant increment in the percentage of AR spermatozoa (from $8.2 \pm 1.3\%$ up to $23.5 \pm 4.7\%$ with 1 μ M P4, $P < 0.001$). After 3 h of incubation, the proportion of capacitated cells was increased to $69.7 \pm 4.3\%$ in cocktail samples without P4, while in the presence of this hormone between 41 and 45% of capacitated spermatozoa remained in all the samples, and this difference was highly significant ($P < 0.001$). Furthermore, the percentage of AR spermatozoa, which barely changed in cocktail samples without P4 ($13.0 \pm 2.0\%$), reached more than 40% in samples with the hormone (up to 47.0 ± 3.7 with 100 pM), this difference being highly significant in all cases ($P < 0.001$).

The inclusion of E2 in the cocktail medium did not account for any significant change in the proportion of capacitated cells after 1 h of incubation in capacitating conditions, similar to that observed in the presence of P4 (Fig. 5B). However, the percentage of AR cells was significantly increased to $23.5 \pm 9.0\%$ with the highest

Figure 3 Representative images of immunocytochemical localization of ER β (bright field and epifluorescence, A, B and C) and capacitation status assessed by chlortetracycline (CTC) staining (epifluorescence, D, E and F) in control (A and D), *in vitro* capacitated (B and E) and acrosome-reacted (C and F) ram spermatozoa (magnification 1000 $\times$).

Figure 4 Graphic representation of the correlation found in ram spermatozoa between the presence of estrogen receptor β (ER β), localized by indirect immunofluorescence (IFF), and the non-capacitated sperm rate assessed by chlortetracycline (CTC) staining. Experiments were replicated 12 times with control, *in vitro* capacitated and acrosome-reacted sperm samples in each experiment ($n=36$).

E2 concentration ($P<0.001$). After 3 h of incubation, the proportion of capacitated cells increased in all the cocktail-containing samples. The addition of E2 accounted for a significant decrease in the capacitated sperm pattern from $69.7 \pm 4.3\%$ in samples with no E2 to 45.7 ± 3.18 and $54.7 \pm 5.3\%$ with 10 nM and 1 μM E2 respectively. In contrast, the percentage of AR cells was significantly higher in samples incubated with E2 (up to $37.3 \pm 8.6\%$ with 10 nM E2 vs $13.0 \pm 2.0\%$ in the control cocktail samples, $P<0.001$). Densitometric evaluation of protein tyrosine phosphorylation showed an increase in the total band intensity in the cocktail samples in the presence of P4, which was significant with 100 pM ($P<0.05$) and 10 nM ($P<0.01$) relative to the control at 3 h, while the inclusion of E2 did not reveal any significant change (Fig. 5D and F).

Discussion

Progesterone and 17 β -estradiol are present in the female genital tract, and several studies have shown their ability to exercise direct effects on spermatozoa (reviewed in Bishop & Stormshak 2008 and Baldi *et al.* 2009) presumably by interaction with a specific receptor on the sperm surface (Luconi *et al.* 2004). In this study, we have revealed the presence of PR at the equatorial region of ram spermatozoa, with also a faint signal at the midpiece. This localization is in concordance with that described in human spermatozoa (Sabeur *et al.* 1996), but not in other species such as pig and goat where PR has mainly been detected on the postacrosomal and apical regions (Somanath & Gandhi 2002, De Amicis *et al.* 2012). Our results showed the presence of both ER on

the head of ram spermatozoa, ER α at the postacrosomal part and ER β at the apical region. Localization of both ERs partially coincides with that described in stallion (Arkoun *et al.* 2014), mouse (Sebkova *et al.* 2012) and human (Solakidi *et al.* 2005) spermatozoa, although other studies have shown a prevalent labelling at the flagellum of human and pig spermatozoa (Rago *et al.* 2007, Guido *et al.* 2011), where we did not detect any signalling at all. These differences could be due to either a specie-specific expression pattern or the use of different antibodies in the immunodetection assays. Additionally, differences between the localization of both ER suggest different roles in sperm physiology. Although ER α was evidenced at the postacrosomal region of all the spermatozoa, ER β was found at the apical region of only about 63% of the cells, and some of them appeared to be losing their stained region. Therefore, we can hypothesize that this phenomenon may be attributed to an ER β redistribution related to capacitation, and the data obtained support this idea. We found a strong significant correlation ($R=0.848$, $P<0.001$) between the presence of ER β and the percentage of NC spermatozoa. Moreover, the percentage of labelled cells (ER β +) significantly decreased after *in vitro* capacitation, and nearly disappeared when the acrosome reaction was induced by LPC. All these data indicate that ER β is involved in capacitation and the acrosome reaction of ram spermatozoa. In contrast, the expression and localization of ER α and PR did not change during capacitation or the acrosome reaction. Despite that we could assume that steroid hormone receptors described in the present study are associated to the plasma membrane as no permeabilisation was performed in the immunofluorescence assays, we cannot exclude the putative presence of intracellular receptors mediating rapid effects.

Classical nuclear receptors for progesterone, PR (A and B), are generated from an alternative splicing of the same gene (Conneely *et al.* 1989, Kastner *et al.* 1990) and have a molecular weight of 94 (PRA) and 120 kDa (PRB) in human spermatozoa (De Amicis *et al.* 2011). In the present study, Western blot analyses for PR in ram sperm protein extracts identified three protein bands corresponding to a molecular weight between 40 and 45 kDa, which are different from those reported for A and B isoforms of the classical or nuclear P4 receptor of human spermatozoa, or those described by the commercial supplier of the antibody (81 kDa and 116 kDa for PRA and PRB, respectively). A functionally active novel membrane PR has been reported in human spermatozoa, with two proteins bands of similar molecular masses (46–48 and 50–52 kDa, (Sabeur *et al.* 1996) or 54 and 57 kDa (Luconi *et al.* 1998). Other putative sperm membrane PRs have also been described in dog (Cheng *et al.* 2005), goat (Somanath & Gandhi 2002) or boar (Wu *et al.* 2006) spermatozoa. We can speculate that the proteins detected in the present study

Table 2 Effect of steroid hormones on viability and motility of ram spermatozoa.

	Progesterone (3 h)				17β-Estradiol (3 h)			
	Control (0 h)	Control (3 h)	100pM	10nM	1 μM	100pM	10nM	1 μM
TALP-incubated samples								
Viability (%)	65.6 ± 3.1	58.9 ± 3.0 ^a	58.2 ± 3.5 ^a	63.0 ± 4.7	58.4 ± 4.0 ^a	62.0 ± 4.1	57.9 ± 3.7 ^a	60.2 ± 5.3
Total motility (%)	85.5 ± 3.0	80.3 ± 4.2 ^a	82.8 ± 2.4	82.5 ± 4.6	80.7 ± 2.2 ^a	81.7 ± 3.4	75.7 ± 4.7 ^a	82.7 ± 2.4
Progr. motility (%)	31.0 ± 2.4	30.0 ± 2.3	30.2 ± 1.8	30.3 ± 1.5	33.8 ± 1.4	27.8 ± 3.1	30.3 ± 2.8	31.8 ± 1.1
VCL (μm/s)	108.8 ± 7.2	88.4 ± 5.6	86.9 ± 4.6 ^a	89.7 ± 4.3	90 ± 3.2	93.6 ± 3.8	95.8 ± 2.7	90.0 ± 2.9
VSL (μm/s)	57.6 ± 3.5	44.4 ± 3.8	46.4 ± 4.3	47.9 ± 1.8	49.7 ± 4.6	49.9 ± 2.6	53.9 ± 2.8	49.7 ± 2.9
VAP (μm/s)	82.8 ± 5.6	63.9 ± 5 ^a	63.3 ± 4.3 ^a	66.6 ± 1.9	67.2 ± 4.5	69.6 ± 2.4	72.9 ± 2.2	67.3 ± 3.1
LIN (%)	53.2 ± 2.0	50.0 ± 3.0	53.1 ± 3.6	54.2 ± 3.6	55.3 ± 4.0	53.9 ± 4.3	56.4 ± 3.1	55.3 ± 3.0
STR (%)	69.7 ± 1.2	69.3 ± 2.1	72.6 ± 2.2	72.2 ± 2.7	73.7 ± 2.5	71.8 ± 3.4	73.8 ± 2.4	73.7 ± 2.1
WOB (%)	76.3 ± 2.0	71.9 ± 2.4	72.8 ± 2.9	74.6 ± 2.3	74.8 ± 3.0	74.6 ± 2.4	76.2 ± 1.9	74.7 ± 2.2
ALH (μm)	2.9 ± 0.2	3.1 ± 0.1	3.1 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.2
BCF (Hz)	8.5 ± 0.2	8.1 ± 0.1	8.3 ± 0.1	8.3 ± 0.1	8.5 ± 0.1	8.4 ± 0.2	8.5 ± 0.1	8.5 ± 0.2
Cocktail-incubated samples								
Viability (%)	73.3 ± 3.3	54.7 ± 3.9 ^a	54.0 ± 3.8 ^a	52.2 ± 3.7 ^a	55.2 ± 3.2 ^a	52.8 ± 3.5 ^a	57.5 ± 3.6 ^a	55.7 ± 3.5 ^a
Total motility (%)	88.5 ± 1.4	75.0 ± 2.1 ^a	65.2 ± 4.5 ^{a,b}	71.8 ± 3.8 ^a	72.2 ± 4.9 ^a	68.2 ± 2.3 ^{a,b}	73.7 ± 2.9 ^a	74.3 ± 5.2 ^a
Progr. motility (%)	42.2 ± 3.8	23.8 ± 4.8 ^a	20.0 ± 3.9 ^a	24.3 ± 4.4 ^a	21.3 ± 5.0 ^a	20.5 ± 5.1 ^a	25.3 ± 2.8 ^a	22.0 ± 2.3 ^a
VCL (μm/s)	110.7 ± 7.4	80.3 ± 3.3 ^a	76.4 ± 2.4 ^a	83.2 ± 3.9 ^a	81.3 ± 2.6 ^a	75.1 ± 2.5 ^a	84.9 ± 3.5 ^a	79.6 ± 5.6 ^a
VSL (μm/s)	70.1 ± 4.9	42.5 ± 4.6 ^a	40.7 ± 2.9 ^a	44.5 ± 3.7 ^a	40.8 ± 2.9 ^a	37.8 ± 4.1 ^a	46.2 ± 2.9 ^a	42.1 ± 4.1 ^a
VAP (μm/s)	93.2 ± 6.7	60.8 ± 4.4 ^a	58.5 ± 2.9 ^a	64.8 ± 3.8 ^a	61.3 ± 2.3 ^a	54.5 ± 2.9 ^a	65.1 ± 3.0 ^a	60.9 ± 4.8 ^a
LIN (%)	63.4 ± 2.2	52.8 ± 4.6	53.5 ± 3.9	53.6 ± 4.1	50.1 ± 3.0	50.6 ± 5.5	54.8 ± 4.0	52.8 ± 2.8
STR (%)	75.3 ± 1.7	69.4 ± 3.1	69.3 ± 2.5	68.6 ± 3.1	66.3 ± 3.0	68.5 ± 4.1	70.9 ± 3.1	68.9 ± 1.8
WOB (%)	84.1 ± 1.3	75.4 ± 3.4	76.8 ± 3.3	77.8 ± 2.4	75.5 ± 1.5	72.8 ± 4.0	76.8 ± 2.6	76.4 ± 2.3
ALH (μm)	2.6 ± 0.1	2.6 ± 0.2	2.5 ± 0.1	2.5 ± 0.2	2.6 ± 0.1	2.7 ± 0.2	2.7 ± 0.1	2.6 ± 0.2
BCF (Hz)	8.5 ± 0.2	8.4 ± 0.2	8.5 ± 0.2	8.4 ± 0.2	8.4 ± 0.2	8.1 ± 0.2	8.3 ± 0.1	8.2 ± 0.1

Percentage of viable (PI-), total and progressive motile spermatozoa and kinematic parameters before treatment (0 h) and after 3 h of incubation in capacitating conditions (39°C, 5% CO₂, 100% humidity) in TALP and Cocktail media either with or without different progesterone and 17 β -estradiol concentrations. Mean values $\pm$ s.e.m. ($n=6$). Significant differences related to control 0 h (A) and to control 3 h (B) $P<0.001$.

Figure 5 Effect of incubation with different progesterone and 17 β -estradiol doses on the capacitation state of ram spermatozoa. Assessment of the capacitation status (CTC staining) of ram spermatozoa of control samples at 0 h, and after 1 h and 3 h of incubation at 39°C and 5% CO₂ with different progesterone and 17 β -estradiol doses in TALP medium (A) and in the cocktail medium (B). Significant differences related to control samples (TALP medium) at 3 h: ($^{**}P < 0.01$ and $^{***}P < 0.001$). Significant differences related to cocktail samples at 1 h and 3 h: ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). Mean values $\pm$ S.E.M. ($n = 4$). Protein tyrosine phosphorylation during *in vitro* capacitation analysed by Western blotting and quantified by densitometry: (C and E) control samples (TALP medium); (D and F) cocktail samples. Significant differences related to control samples at 3 h of the incubation: ($^{*}P < 0.05$ and $^{**}P < 0.01$). Mean values $\pm$ S.E.M. ($n = 4$).

might be either different isoforms or two different types of a specific ovine sperm plasma membrane PR. Our chosen anti-PR antibody was an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of PR of human origin, where the steroid-binding site is located. We may presume that this important domain may be highly conserved between the putative membrane and nuclear receptors, as has been hypothesized by other authors (Luconi *et al.* 2004), and probably between different species as well. Indeed, the antibody used here has also been recommended for the detection of PR in different species, not only mammalian such as equine, canine, bovine and porcine, but also avian. However, our findings do not allow us to confirm the existence of the classical nuclear PR in ram spermatozoa, as already described in human (De Amicis *et al.* 2011) or porcine (De Amicis *et al.* 2012) spermatozoa. Conversely, we have demonstrated the existence of the two types of classical nuclear estrogen receptors (ER α and ER β) in ram spermatozoa. A band of about 66 kDa and another of 55 kDa were evidenced when anti-ER α and anti-ER β antibodies were used, respectively, which were coincident with those described for conventional ER α and ER β found in human spermatozoa (Aquila *et al.* 2004) and in other cell types (Mauro *et al.* 2014, Rizza *et al.* 2014). The signal of the band corresponding to ER β was much more intense than that matching with ER α , in comparison with their respective positive controls. This is in concordance with results of other authors who have reported that ER β is the most highly expressed isoform in spermatozoa (Aquila *et al.* 2004). With the antibodies used in this study, directed against a region of N-terminus corresponding to amino acids 2–185 of ER α and 1–150 of ER β of human origin, we did not detect any other putative ER. However, we cannot ignore the possible existence of specific estrogen-binding proteins on the sperm surface as revealed by other authors using antibodies directed against the steroid-binding domain of the classical ER (Luconi *et al.* 1999). It is worth highlighting that this is the first time that steroid hormone receptors have been described and localized in ram spermatozoa.

Progesterone and 17 β -estradiol are able to exert rapid, non-genomic effects on spermatozoa and probably play an important role *in vivo* regulating several sperm functions involved in the fertilization process (reviewed in Bishop and Stormshak 2008 and Baldi *et al.* 2009). One necessary requisite to reach the oocyte is the sperm's ability to move properly through the female genital tract. In this regard, a positive effect of progesterone on the progressive motility of human spermatozoa has been shown (Contreras & Llanos 2001), although this effect was not confirmed by other authors (Wang *et al.* 2001). Studies about the effects of estradiol on motility are scarce and apparently contradictory. While some authors have reported that this hormone induces sperm motility (Guido *et al.* 2011), others have

showed that it decreases significantly the percentage of spermatozoa with progressive motility (Gautier *et al.* 2016). On the basis of our results, neither progesterone nor 17 β -estradiol seemed to exert any influence on motility when ram spermatozoa were incubated in TALP medium. Likewise, we did not observe any effect on membrane sperm integrity, in contrast to previous results obtained with pig spermatozoa (De Amicis *et al.* 2012). Most descriptions of the actions of steroid hormones on spermatozoa are related to capacitation and the acrosome reaction. In the present study, we could not observe any significant change in the percentage of spermatozoa displaying a capacitated pattern in samples incubated with progesterone in a TALP medium, which is a commonly used medium to induce capacitation in most species. Due to the special difficulty to induce *in vitro* capacitation in ram spermatozoa (Grasa *et al.* 2006, Colas *et al.* 2008), we repeated the experiments adding a cocktail of compounds that maintains high intracellular cAMP levels, already successfully demonstrated for capacitating ram spermatozoa after three hours of incubation (Grasa *et al.* 2006, Colas *et al.* 2008). We found that the presence of P4 resulted in a significant decrease in capacitated spermatozoa, concomitant with an increase in both the AR cells and protein tyrosine phosphorylation. Likewise, 100 pM P4 also led to a significant decrease in TM sperm. Although several reports suggest a positive effect of progesterone on the capacitation of human spermatozoa (reviewed in Baldi *et al.* 2009), such results were obtained with very high hormone concentrations, much higher than those supposed to be in the site of capacitation, before the spermatozoa reached the oocyte. However, there are no doubts about the role of P4 in the induction of the acrosome reaction in most species, as demonstrated in human (Osman *et al.* 1989), mouse (Roldan *et al.* 1994), pig (Melendrez *et al.* 1994), stallion (Meyers *et al.* 1995) and goat (Somanath *et al.* 2000). In this study, we show for the first time, to the best of our knowledge, the influence of P4 on the ram sperm acrosome reaction. The fact that changes in the chlortetracycline staining patterns were not accompanied by a decrease in membrane integrity could suggest that P4 would bring ram spermatozoa to an end-point situation, ready to react in response to physiological stimuli such as the zona pellucida signals. This could be defined as a priming effect as described by other authors (Roldan *et al.* 1994, Sumigama *et al.* 2015). Conflicting results have been reported concerning the effects of 17 β -estradiol on capacitation and the acrosome reaction. While some authors have described a direct stimulatory action (Adeoya-Osiguwa *et al.* 2003, Ded *et al.* 2013), others have reported a role in the modulation of the progesterone effects (Vigil *et al.* 2008, Sebkova *et al.* 2012). Our results have demonstrated the ability of 17 β -estradiol to induce changes associated to the acrosome reaction by itself, especially when ram spermatozoa are incubated in a medium that increases

the intracellular cAMP level. Furthermore, 100pM E2 resulted in a significant decrease in TM sperm, according to the results showed in equine (Gautier *et al.* 2016) and human (Guido *et al.* 2011) spermatozoa with E2 at μ M concentrations. These results could point to different effects of E2 on sperm motility depending on concentration in a species-specific manner. The increase in the percentage of AR spermatozoa obtained with E2 was lower than that induced by P4 and did not trigger any modification in the phosphotyrosine pattern. This difference of results between both capacitation markers, i.e. CTC pattern and protein P-tyrosine rate evaluation could be explained on the basis that capacitation is a sequential process and several phenomena are concomitant with the beginning of the acrosome reaction (Baldi *et al.* 2000, Guraya 2000). It is widely considered that CTC staining is able to reflect the sperm capacitation state, although the molecular basis of CTC staining has not been fully understood yet (Rathi *et al.* 2001). Furthermore, changes in the content and localization of proteins phosphorylated at tyrosine residues (apart from others at serine and threonine) during *in vitro* capacitation have been shown and could be detected by Western blotting (Visconti *et al.* 1995). Both assays are considered as capacitation markers but events that induce these changes do not have to be simultaneous, but they might likely bring about in different moments of the capacitation process, and some molecules may trigger one of the events while not the another.

Numerous steroid hormones have been found to rapidly influence the ion channel activity in several cellular types (review in Nemere *et al.* 2003). In human sperm, progesterone activates the principal Ca^{2+} channel, CatSper (Lishko *et al.* 2011). CatSper-mediated Ca^{2+} influx leads to Ca^{2+} elevation in the sperm head (Xia *et al.* 2007), and thus, it could contribute to the Ca^{2+} -dependent acrosome reaction. We could speculate that binding of progesterone to its specific receptors in sperm might activate CatSper via a signalling cascade and result in the acrosome reaction. 17 β -estradiol could also influence an ion channel since it induces a rapid and sustained increase in the intracellular calcium concentration as well (Luconi *et al.* 1999), but nevertheless until now there is no evidence for a link between 17 β -estradiol and CatSper.

In conclusion, our study demonstrates that ovine spermatozoa have progesterone and estrogen receptors and that the presence of ER β on the sperm surface decreases after capacitation and almost disappears after the acrosome reaction. Both steroid hormones are related with the induction of the acrosome reaction in ram spermatozoa.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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