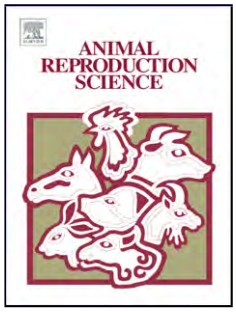

Underlying molecular mechanism in the modulation of the ram sperm acrosome reaction by progesterone and 17β-estradiol

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Underlying molecular mechanism in the modulation of the ram sperm acrosome reaction by progesterone and 17β−estradiol

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Highlights

- P4 and E2 induce changes in P-Tyr location in ram sperm.
- P4, but not E2, increments ROS and Ca^{2+} levels in ram sperm.
- P4 and E2 did not induce hyperactivation in ram sperm.
- Neither P4 nor E2 seem to modulate AR in ram sperm by the cAMP-PKA pathway.

ABSTRACT

Steroid hormones progesterone (P4) and 17β−estradiol (E2) not only have important functions in regulation of reproductive processes in mammals but also have direct effects on spermatozoa. There can be induction of the acrosome reaction in ram spermatozoa by P4 and E2 and, in the present study, there was further investigation of mechanisms underlying this effect. In a medium containing agents that increase cAMP, the presence of both P4 and E2 led to changes in the localization of proteins phosphorylated in tyrosine residues evaluated by indirect immunofluorescence. The inclusion of P4 at 1 μM in the media induced an increase in $Ca^{2+}{}_i$ and mobilization in the area of the acrosome (Fluo-4 and Rhod-5 staining, respectively), an increase in ROS (H_2DCFDA staining) and a substantial disruption of the acrosome (evaluated using RCA), while E2 did not

have these effects. There were no effects on cAMP concentrations or PKA activity with inclusion of these hormones in the media. The inclusion of P4 at 100 pM in the media led to changes in values for sperm kinematic variables which could indicate there was an inhibition of the hyperactivation caused by agents that induce an increase in cAMP concentrations. In conclusion, results from the present study indicate that P4 and E2 promote mechanisms regulating the acrosome reaction in ram spermatozoa, however, these effects on mechanisms are different for the two hormones, and for E2, require further clarification.

Keywords: Spermatozoa; Acrosome reaction; Steroid hormones; Ca^{2+} mobilization; Tyrosine-phosphorylation; ROS.

1. Introduction

To acquire fertilizing capacity, ejaculated spermatozoa must undergo a complex process, capacitation, during the transit of these cells through the female reproductive tract. Capacitation, which enables the sperm to undergo the acrosome reaction (AR) (Yanagimachi 1994), involves changes in the sperm plasma membrane (Gadella et al. 2008), Ca^{2+} transport (Lishko et al. 2011), an increase in concentrations of reactive oxygen species (ROS) (de Lamirande and Lamothe 2009) and the activation of a signal transduction cascade that promotes sperm protein tyrosine phosphorylation and motility changes related to the induction of hyperactivated motility (Visconti et al. 2002).

During sperm transit through the female reproductive tract, the milieu surrounding the spermatozoa contains different concentrations of physiological substances such as steroid hormones [see review in (Frederick et al. 1991; Baldi et al. 2009)]. Apart from the genomic actions of these hormones, progesterone (P4) and 17β−estradiol (E2) have rapid direct effects on spermatozoa, mediated,

at least in part, by binding to the receptors in the plasma membrane of these cells [reviewed by (Bishop and Stormshak 2008)]. In previous studies, it was determined that there were P4 and estrogen receptors in ram spermatozoa. It was also ascertained that steroid hormones are related to the induction of the acrosome reaction based on results from chlortetracycline (CTC) staining, and that P4 induces tyrosine phosphorylation of sperm proteins (Gimeno-Martos et al. 2017). In relation with the molecular mechanisms underlying sperm capacitation and the subsequent acrosome reaction, results from several studies indicate that P4 induces Ca^{2+} oscillations due to its release from intracellular Ca^{2+} stores (Harper et al. 2004) or extracellular influx because of the CatSper activation (Lishko et al. 2011; Strunker et al. 2011). Furthermore, it has been suggested that P4 increases cyclic adenosine monophosphate (cAMP) (Parinaud and Milhet 1996) and cyclic guanosine monophosphate (cGMP) concentrations and modulates the activity of protein kinase A (PKA) and C (PKC) (Sagare-Patil et al. 2012; Baron et al. 2016). The induction of sperm hyperactivated motility is also thought to be modulated by P4 (Noguchi et al. 2008; Sumigama et al. 2015). Results are inconsistent from previous studies that focused on the effect of E2 on capacitation or hyperactivation of sperm because findings in some studies indicated there was a direct stimulating function of E2 on spermatozoa (Ded et al. 2010; Bosakova et al. 2018), while other results lead to the postulation that there are only a modulatory actions of P4 on sperm capacitation (Vigil et al. 2008; Fujinoki 2010).

The main objective of the present study was to examine, in greater depth, the molecular mechanisms regulated by both P4 and E2 that modulate capacitation and the acrosome reaction in ram spermatozoa. There, therefore,

was evaluation of the effect of different P4 and E2 concentrations on acrosomal membrane integrity, capacitation status and changes in both the localization of tyrosine-phosphorylated proteins and intracellular Ca^{2+} stores of spermatozoa. Additionally, there was quantitation of the intracellular concentrations of Ca^{2+}, ROS, cAMP and PKA. Furthermore, changes in values for kinematic variables that are related to hyperactivation were assessed.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), including P4 (Reference number P8783) and E2 (E8875).

2.1. Sperm preparation

All experiments were performed using fresh ram spermatozoa. Semen was obtained from eight mature Rasa Aragonesa rams (2–4 years old), using an artificial vagina. All the rams belonged to the National Association of Rasa Aragonesa Sheep Breeders and were housed with uniform nutritional regimens occurring at the Experimental Farm of the University of Zaragoza (Spain). All experimental procedures were accomplished as described for the Project Licence PI19/17 approved by the Ethics Committee for Animal Experiments, University of Zaragoza (Spain).

Two ejaculates were collected every 2 days and a pool of second ejaculates was used for each assay, to avoid individual differences (Ollero et al. 1996). A seminal plasma-free sperm population was obtained using a dextran swim-up procedure (García-López et al. 1996) performed in a medium devoid of $NaHCO_3$ and $CaCl_2$. In all experiments, an aliquot of the swim-up sample was

evaluated to be sure that changes that occurred in capacitated samples were due to incubation in capacitating conditions and were not originally present in the non-capacitated swim-up samples. Evaluated variables were capacitation status (CTC staining), immunolocalization of tyrosine phosphorylated proteins, motility, plasma and acrosome membrane integrity, ROS and Ca^{2+} concentrations and Ca^{2+} localization in sperm cells.

2.2. In vitro capacitation

In vitro capacitation was performed by incubating aliquots of swim-up-selected spermatozoa (1.6×10^8 cells/mL) for 3 h at 39 °C in a humidified incubator with 5% CO_2 in air. Incubations were performed in Tyrode's albumin lactate pyruvate (TALP) medium (Parrish et al. 1988). Due to the difficulty of inducing *in vitro* capacitation in ram sperm, TALP medium was supplemented with several compounds, already proven to be effective for capacitating ram spermatozoa (Grasa et al. 2006; Colas et al. 2008). The assumption was that addition of a combination of db-cAMP (a cell-permeable cAMP analog) and phosphodiesterases inhibitors (without cAMP degradation actions) would lead to a greater concentrations of sperm intracellular cAMP. This modified TALP medium (i.e., medium containing agents that increase intracellular cAMP) is referred to as a "cocktail" medium. Also, a control sample, without the addition of the compounds to enhance concentrations of cAMP, was maintained under the same conditions as the capacitated samples (control).

To have a positive control for the acrosome reaction, lysophosphatidylcoline (LPC) was added to a "cocktail" sample after 3 h of incubation in capacitating conditions (Marti et al. 2000).

The P4 and E2 were solubilized separately in DMSO and PBS and added to “cocktail”-incubated sperm samples to final concentrations of 1 μM and 100 pM, respectively. The final concentration of DMSO in all samples was 0.1%. There were also additions so that there was the same DMSO concentration in all control samples.

Analysis of samples that are subsequently described in this manuscript were performed after 3 h of incubation in capacitating conditions, except for the cAMP and PKA assays, which were evaluated at 5 min after the beginning of *in vitro* capacitation using the previously reported procedures of Colás et al. (2010).

2.3. Assessment of capacitation status using CTC staining

The CTC is a fluorescent antibiotic that binds sperm membrane proteins. Fluorescence of bound CTC is enhanced by intracellular Ca^{2+}, and CTC staining patterns change in capacitated and acrosome-reacted spermatozoa (Ward and Storey 1984). Samples were stained and processed as described previously (Gimeno-Martos et al. 2017).

Spermatozoa were classified as having one of the following three staining patterns (Gillan et al. 1997): non-capacitated (even distribution of fluorescence in the head, with or without fluorescence of the equatorial band), capacitated (with fluorescence in the acrosome) and acrosome-reacted cells (having no fluorescence in the head, with or without fluorescence of the equatorial band). Samples were examined using a Nikon Eclipse E-400 microscope with epifluorescence illumination and with a V-2A filter. All samples were processed in duplicate and at least 150 spermatozoa were classified per slide.

2.4. Immunolocalization of tyrosine phosphorylated proteins (TyrPP) in spermatozoa

The extent of localization of tyrosine phosphorylated proteins (TyrPP) in spermatozoa was analyzed using indirect immunofluorescence (IIF) using the method previous described by Tardfi (Tardif et al. 2001) and as adapted by Matás (Matás et al. 2011). Samples were processed and slides were prepared using procedures that have been previously described (Gimeno-Martos et al. 2017). A monoclonal anti-phosphotyrosine antibody (4G10, Cat# 05-321, RRID:AB_309678, Millipore, CA, USA) and an Alexa Fluor 594 chicken anti-mouse (Cat#A-21201, RRID: AB_2535787; Thermo Fisher Scientific, Waltham, MA, USA) were used as primary and secondary antibodies, diluted 1:200 and 1:600 (v/v), respectively. Sperm were visualized using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) with epifluorescence illumination utilizing a B-2A filter. At least 200 cells per sample were evaluated. The following staining patterns were identified based on the regions of the sperm cell that were labeled: E: equatorial region; A: acrosome; AE: acrosome + equatorial region; AF: acrosome + flagellum; EF: equatorial region + flagellum; AEF: acrosome + equatorial region + flagellum; F: flagellum.

2.5. Sperm motility evaluation

Two drops of 2 µL of each sample, diluted to a final concentration of 3 × 10^7 cells/mL, were placed onto a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel), maintained at 37 °C in a heated slide holder. Spermatozoa were recorded using a video camera (Basler acA1920; Basler Vision Components) mounted on a microscope (Nikon Eclipse 50i) equipped with

a 10x negative-phase contrast lens.

The settings for video analysis were as follows. Frames per second: 60; number of frames: 120; resolution images: 800×600 pixels; minimum cell size: 10 μm^2; maximum cell size: 100 μm^2; progressive motility: straightness coefficient (STR)>80% and average path velocity (VAP)>90 μm/s; minimum curvilinear velocity (VCL): 10 μm/s; VCL lesser threshold: 100 μm/s; VCL upper threshold: 200 μm/s; minimum track length: 30 frames and maximum displacement between frames: 20 μm. Percentages of total motile (TM), progressive motile (PM) and all kinematic variables for spermatozoa included in the the OpenCASA motility module (Alquézar-Baeta et al. 2019) were evaluated. This software also included the determination of DANCE (μ^2/s), a measure of the pattern of sperm motion VCL x ALHmean, and "fractal dimension" (FD), an estimate of the extent to which a line fills a plane). An increment in both variables has been related to hyperactivated spermatozoa in reports resulting from several previous studies (Robertson et al. 1988; Mortimer 1990; Mortimer et al. 1996; Mortimer 1997).

2.6. Flow cytometry analyses

All measurements were performed using Beckman Coulter FC 500 with CXP software (Beckman Coulter Inc., Brea, CA, USA) equipped with two excitation lasers (argon-ion laser, 488 nm; and solid-state laser, 633 nm) and five absorbance filters (FL1–525, FL2–575, FL3–610, FL4–675 and FL5–755; ± 5 nm each bandpass filter). A flow rate stabilized at 200 to 300 cells/s was used. A minimum of 20,000 events were counted in all the experiments. The sperm population was identified for further analysis by the specific forward (FS) and side

scatter (SS) properties; thus, a FS area compared with SS area density plot was acquired using adjusted gate settings and was used to exclude non-sperm particles from the analysis. Data are reported using a logarithmic scale.

2.6.1. Evaluation of sperm plasma membrane integrity

To determine cell viability (membrane integrity), a modification of the procedure described by Harrison and Vickers (Harrison and Vickers 1990) was used (Gimeno-Martos et al. 2019). For the gated sperm cells, percentages of viable spermatozoa (CFDA+/PI) were evaluated.

2.6.2. Evaluation of acrosomal membrane integrity

To determine cell membrane acrosome integrity, 5 µL of 20 µg/ml *Ricinus communis* agglutinin (FITC-RCA, Vector Laboratories, Burlingame, CA, USA) and PI (1.5 mM), and 3 µL of formaldehyde (0.005% final concentration) were added to 300 µL of sperm samples (final concentration 5 x 10^6 cells/mL). This protocol was originally described by Martí et al. (Marti et al. 2000) and adapted for conducting flow cytometry. The samples were incubated at 37 °C in darkness for 15 min. For the gated sperm cells, percentages of viable spermatozoa with damaged acrosomes were determined (PI-/ RCA+).

2.6.3. Intracellular content of ROS

Production of peroxides was quantified by incubating spermatozoa in the presence of the dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). The fluorescent probe was combined with PI to exclude the non-viable sperm population from the analysis (Guthrie and Welch 2006). The assay was conducted using procedures previously described (Gimeno-Martos et al. 2019).

For the gated sperm cells, percentages of live sperm with greater than average concentrations of ROS (PI-/DCF+) were determined using flow cytometry.

2.6.4. Intracellular Ca^{2+} concentrations

To detect intracellular Ca^{2+} (Gee et al. 2000), the procedure described in Colás et al was used (Colas et al. 2010). For the gated sperm cells, viable spermatozoa with relatively greater concentrations of intracellular Ca^{2+} (PI^-/ Fluo4-AM^+) were considered.

2.7. Ca^{2+}distribution into cell compartments

Although Fluo-4 staining is a suitable method for analyzing Ca^{2+} concentrations using flow cytometry, Rhod-5N results are more useful for assessing calcium localization in cell compartments of ram spermatozoa using microscopy (Miguel-Jimenez et al. 2018). The procedure described in Yeste et al (Yeste et al. 2015) was used. Samples were examined using a Nikon Eclipse E-400 microscope with epifluorescence illumination utilizing a V-2A filter. At least 200 cells per sample were evaluated.

2.8. Cyclic AMP assays

The intracellular cAMP content was quantified using a Direct cAMP enzyme immunoassay system (Sigma Aldrich, St. Louis, MO, USA) utilizing the manufacturer's instructions. As previously described (Colas et al. 2010), because of the very small concentrations of intracellular cAMP in sperm samples, the acetylated option for conducting the procedure was used, and samples were

processed after 5 min of incubation in capacitating conditions. The optical density was read at 405 nm to quantify concentrations of cAMP.

2.9. PKA activity analysis

The PKA enzymatic activity was determined using a commercial ELISA kit (PKA Activity Kit K027-H1, Arbor Assay, MI, USA). Sperm samples were processed after 5 min of incubation in capacitating conditions and aliquots of 8×10^7 spermatozoa were processed utilizing the manufacturer's instructions. The optical density was read at 450 nm to quantify the activity of PKA.

2.10. Statistical analysis

Normality and homoscedasticity of the continuous variables were analyzed using the Kolmogorov-Smirnov test and Levene test, respectively. When there was determination there was normality and homoscedasticity of data (PKA concentrations and values for kinematic variables except for WOB), mean differences between experimental groups were evaluated using an ANOVA, followed by utilization of the Tukey *post-hoc* test. When there was not normality and homoscedasticity of data (cAMP concentration and WOB values), the Kruskal-Wallis test was conducted with subsequent use of the Dunn's post-test. Mean differences between groups for categorical variables (viability, acrosome integrity, concentrations of intracellular ROS using H_2DCFDA, concentrations of intracellular Ca^{2+} by FLUO 4-AM, Rhod5-N subpopulations using microscopy, CTC staining and immunolocalization of P-Tyr proteins) were compared using means of Chi-square independence test followed by Fisher's exact test (Shan

and Gerstenberger 2017). Correlation between the capacitation state and P-Tyr immunolocalization of spermatozoa were determined using the Pearson´s correlation test. All statistical analyses were performed using GraphPad InStat software (Version 3.01) and SPSS statistic software (v. 21). Data are expressed as mean ± standard error of the mean (S.E.M.) of five experiments.

3. Results

3.1. *Effect of steroid hormones on the localization of tyrosine-phosphorylated proteins during in vitro ram sperm capacitation*

In swim-up samples, the most abundant immunotype had simultaneous labelling at both the acrosome and the equatorial region (AE, 58.0 ± 2.6%). The second most abundant subpopulation had only equatorial (E, 29.2 ± 4.2%) or only acrosomal labelling (A, 12.8 ± 2.3%; Fig. 1). In general, only spermatozoa with labelling of the head were observed, while there were almost none of the sperm cells with a stained flagellum. Incubation in capacitating conditions had very little effect on modification of the distribution of TyrPP immunotypes when these cells were incubating in TALP medium (control samples, Fig. 1). The addition of agents to increase cAMP concentrations (“cocktail”) resulted in there being TyrPP in the sperm tail, and with the most frequent immunotype there was simultaneous labelled of the acrosome, equatorial region and flagellum (AEF, 44.5 ± 6.2%). The addition of LPC to “cocktail”-containing samples led to changes in TyrPP location, with there being decreased tail labelling and increased labelling at the equatorial and acrosomal regions as a result of the LPC addition (E and A, $P< 0.001$, Fig. 1).

When there was P4 at 1 μM in the “cocktail”-medium, there was a decrease in the AEF subpopulation (32.8 ± 1.4%; $P< 0.001$), concomitant with an

increase in sperm labelling at the equatorial region and flagellum (EF; $P< 0.05$). In addition, the percentage of spermatozoa with labelling only at the acrosomal region was also greater ($P <0.01$). Unlike P4, the addition of E2 (1 μM) to "cocktail"-containing samples did not result in any change in the AEF subpopulation (Fig. 1).

At the relatively lesser concentrations (100 pM), the addition of both steroid hormones induced an increase in the subpopulation of sperm labelled only at the equatorial segment (E; $P <0.001$; Fig. 1). This effect was not observed when there were larger concentrations of both hormones added to the media.

On the basis of these results, there was investigation of whether there was any correlation between TyrPP immunotypes and the different capacitation patterns determined using CTC staining (Supplementary figure 1). Results from conducting the statistical analyses indicated that was a marked positive correlation between percentages of sperm of immunotype AE and that were non-capacitated (NC) ($r = 0.783$, $P<0.0001$), and AEF and capacitated (C) ($r = 0.571$, $P<0.0001$) sperm. The correlation between EF and acrosome-reacted (AR) spermatozoa was less but also significant ($r = 0.295$, $P<0.05$; Table 1).

3.2. Effect of steroid hormones on acrosomal membrane integrity of ram spermatozoa

The initial percentage of viable spermatozoa with a damaged acrosome (PI-/RCA+) in swim-up samples was greater ($P<0.001$) after 3 h of incubation in capacitating conditions, with differences ($P<0.001$) between control and samples containing relatively greater concentrations of cAMP ("cocktail"; Fig. 2a).

When there was only P4 at the relatively greater concentration was there induction of an increase in the percentage of viable spermatozoa with a damaged acrosome compared with those sperm cells incubated in the “cocktail” media without hormones (P< 0.001). Even so, the effect of LPC was much more pronounced (P<0.0001, Fig. 2a). Results from plasma membrane integrity assays (CFDA/PI) indicated that addition of steroid hormones to the media did not alter sperm viability during *in vitro* capacitation (Table 2).

3.3. Effect of steroid hormones on ram sperm ROS concentrations

The addition of agents to increase the concentrations of intracellular cAMP (“cocktail”) led to an increase of viable spermatozoa with relatively greater ROS concentrations (PI-/DCF+) than control samples (P< 0.01, Fig 2b). The inclusion of P4 in media at 1 μM led to an increased percentage of sperm in this subpopulation to an even greater extent, with the percentage being greater than in samples incubated in the “cocktail” media (P<0.05). The inclusion of E2 in the media at both concentrations resulted in there being similar percentages of sperm in this sub-population as in the control samples (Fig. 2b).

3.4. Effect of steroid hormones on concentrations and location of intracellular Ca^{2+} *in ram spermatozoa*

Percentages of viable sperm with relatively greater intracellular Ca^{2+} concentration (PI^-/ $Fluo4\text{-}AM^+$) were greater when there was incubation in the “cocktail” media after 3 h of incubation in capacitating conditions (P< 0.001, Fig. 2c). Among the assayed hormones, only when there was inclusion of P4 at 1 μM

in the “cocktail” media was there an increment in this subpopulation compared with the “cocktail” media without hormones being added (P <0.05, Fig. 2c).

When the Ca^{2+} localization within intracellular stores was analyzed using fluorescence microscopy utilizing Rhod-5-AM, there were five sperm subpopulations ascertained based on the staining characteristics: I) spermatozoa with a very intense fluorescent spot at the end of the midpiece; II) with fluorescence in the neck and midpiece; III) with fluorescence in the neck, acrosome and midpiece; IV) with fluorescence in the entire head and midpiece; and V) with fluorescence in the head and “dotted” staining in the midpiece (Fig. 3a). In swim-up samples, there was a greater percentage of sperm with a fluorescent spot at the midpiece (subtype I), which was markedly decreased after 3 h incubation in capacitating conditions, especially when there was incubation with the “cocktail” (P <0.001, Fig. 3b). In “cocktail”-containing samples, there was a larger subpopulation II of sperm compared with the control (P <0.001) and inclusion of P4 in the media at 1 μM (P< 0.05) and E2 at both concentrations (P<0.001) resulted in a marked decrease in this subpopulation. When there was inclusion of P4 in the media, there was also an association with a mobilization of Ca^{2+} in the area of the acrosome (increment of subtype III) compared with samples incubated with the “cocktail” without hormone (P< 0.001). Furthermore, the inclusion of E2 in the media at 100 pM also led to an increase in the percentage of sperm with fluorescence in the head and dotted staining in the midpiece (Subtype V).

3.5. Effect of steroid hormones on the cAMP-PKA pathway

As expected, incubation in capacitating conditions with the “cocktail” that leads to an increase in intracellular cAMP concentrations induced an increase in this second messenger (P <0.05 when compared “cocktail” and control samples, Fig. 4a). Incubation with the steroid hormones, however, did not result in any changes, although there was a slight increase when there was inclusion of the largest concentration of both hormones in the media.

Because cAMP activates the protein kinase A (PKA), there was quantitation of the concentrations of this enzyme to elucidate whether a slight increase in concentrations of this second messenger due to the presence of steroid hormones could lead to changes in PKA activity. Neither the addition of P4 nor that of E2 resulted in changes related to “cocktail”-containing samples, in which there was a substantial increase in PKA concentrations (P <0.001, Fig. 4b). When the experiments were repeated in TALP medium, there were no differences as a result of the addition of steroid hormones to the media (Supplementary Figure 2).

3.6. Effect of steroid hormones on sperm motility characteristics

The results from the study of sperm kinematic variables of samples recorded at 60 fps indicated there were differences due to the addition of agents that resulted in an increase in cAMP. In “cocktail”-incubated samples, therefore, there was a lesser progressive motility and VSL, LIN, WOB and STR relative to control samples (P<0.001, Table 2). Furthermore, in these “cocktail” incubated samples there was a greater VCL, ALH mean and maximum, DANCE and fractal dimension (P< 0.001).

The addition of P4 at 100 pM to the media resulted in greater percentages of progressively motile spermatozoa and LIN, and lesser total motility relative in the samples incubated with “cocktail” without addition of hormones (Table 2). In regard to E2, there was only a greater percentage of progressively motile spermatozoa relative to incubation in the “cocktail” media when there was addition of E2 at the largest concentration (1 μM).

4. Discussion

In a previous study, there were progesterone and estrogen receptors detected in ram spermatozoa and there was an association of both hormones, P4 and E2, with the induction of acrosome reaction (Gimeno-Martos et al. 2017). In the present study, there was investigation of the mechanism underlying this effect.

The capacity of P4 to induce the acrosome reaction has been studied in many males of different species, such as the stallion (Cheng et al. 1998), bull (Therien and Manjunath 2003), mouse (Roldan et al. 1994), boar (Melendrez et al. 1994; Wu et al. 2006), hamster (Llanos and Anabalón 1996) and human (Sabeur et al. 1996). Results from a previous study (Gimeno-Martos et al. 2017) indicated that the inclusion of P4 in media resulted in a marked increase in both acrosome-reacted spermatozoa and protein tyrosine phosphorylation. During capacitation, not only has an increase in tyrosine-phosphorylated proteins (TyrPP) been reported, but also there are changes in localization of these proteins throughout the sperm plasma membrane (Visconti et al. 1995; Petrunkina et al. 2003). Indirect immunofluorescence assays have been conducted to elucidate whether the increase in TyrPP concentrations in the

presence of P4 was accompanied by changes in localization of TyrPP. Incubation with agents that increase cAMP ("cocktail") is necessary for successful *in vitro* ram sperm capacitation (Grasa et al. 2006; Colas et al. 2008). When there was inclusion of the "cocktail" in the media, there was an increase in TyrPP labelling in the sperm flagellum. The inclusion of P4 in media resulted in a marked decrease in the most frequent subpopulation in samples incubated with the "cocktail", which was correlated with percentages of capacitated sperm (according to CTC staining) and which occurred concomitant with a marked increase in the immunotype of acrosome-reacted spermatozoa. The induction of the acrosome reaction by P4, therefore, induced the disappearance of acrosome labelling in capacitated spermatozoa. This occurred in concordance with the incremental increase in percentage of viable sperm with a damaged or altered acrosome based on findings when a lectin-based assay was conducted.

Optimal concentrations of ROS are essential for capacitation and induction of an acrosome reaction, among other aspects of sperm functionality (Amaral et al. 2013). In the present study, there was a marked increase in peroxide concentrations in "cocktail"-containing samples consistent with results from a previous study (Gimeno-Martos et al. 2019). The addition of P4 at the largest concentration resulted in a further increase in ROS concentrations. These results are consistent with those from previous studies where there was an effect of P4 on ROS concentrations during human sperm capacitation (de Lamirande et al. 1998; Ghanbari et al. 2018).

Furthermore, the addition of P4 to the media at the largest concentration also induced an increase in intracellular Ca^{2+} concentrations in treated samples. This observed increment could be due to either a Ca^{2+} influx from the extracellular

medium, as occurs in other species (Yeste et al. 2015; Romarowski et al. 2016), or an efflux from the intracellular stores, such as the acrosome (Lucchesi et al. 2016). Results from the present study indicate the addition of P4 to the media during *in vitro* capacitation led to a change of Ca^{2+} localization, and there was an increase in the sperm subpopulation with Ca^{2+} storage in the acrosome, neck and midpiece. It could be inferred that P4 functions to induce Ca^{2+} mobilization from the flagellum to the acrosome, maintaining the cation location at the neck and midpiece, which was the predominant position when there was incubation with the "cocktail".

Findings from the evaluation of E2 effects in the present study, indicated the percentage of AR spermatozoa (evaluated by CTC staining) was less than that induced by P4 and LPC, but greater compared to when there was incubation with the "cocktail" without hormones being added. These results were consistent with previous results evaluating the effects of E2 (Gimeno-Martos et al. 2017). With inclusion of E2 in the media, there were no differences in the percentage of viable sperm with a damaged acrosome when there was evaluation using RCA staining. Inconsistent results on the effect of estrogens on sperm capacitation and acrosome reaction have been reported in other species. A direct stimulating function of E2 during *in vitro* capacitation has been described for the boar and mouse (Ded et al. 2010; Bosakova et al. 2018), whereas for humans and hamsters there is only a modulatory action of the P4 (Vigil et al. 2008; Fujinoki 2014). Differences in results among studies may be due to the use of hormones from different sources or may be species-specific differences.

Although results from a previous study did not indicate there were modifications in total concentrations of TyrPP by E2 in ram spermatozoa

(Gimeno-Martos et al. 2017), the results from the present study indicate there are marked changes in localization, with differences depending on the hormone concentration, as previously reported for mice (Sebkova et al. 2012). At the relatively smaller E2 concentration, there was an increase in the subpopulations with TyrPP-labelling that was restricted to specific areas of the head (acrosome or equatorial region). At the largest concentration of E2, the staining extended towards the flagellum (acrosome and flagellum, AF) where there was the greatest abundance of staining. Unlike P4, the addition of E2 did not result in changes in the subpopulation of capacitated sperm. Furthermore, the addition of E2 did not induce an increase in intracellular ROS or Ca^{2+}concentrations, processes that were promoted when there was incubation with the largest concentration of P4. Interestingly, inclusion of E2 in the media led to changes in the localization of Ca^{2+}, resulting in a decrease in the subpopulation of sperm with uniform labelling in the neck and midpiece and an increase in the subpopulation with fluorescence in the head and irregular labelling in the midpiece. This indicates Ca^{2+} is gradually lost from these regions. The Ca^{2+} mobilization towards the acrosome, however, was not observed with addition of E2 to the media, unlike what occurred with the addition of P4.

Neither addition of P4 nor E2 to the media induced an increase in cAMP concentrations, probably because these concentrations were already increased in the sperm incubated in a medium with agents that increased the concentrations of cAMP. Furthermore, the presence of steroid hormones did not lead to any change in PKA activity. To ensure that this lack of effect was not due to a saturation of PKA with cAMP induced by the medium used, the assay was repeated for the quantitation of PKA concentrations following incubation in TALP

medium. The fact that there were no changes due to the presence of steroid hormones in these conditions indicates that the cAMP-PKA pathway is not involved in the effects on ram spermatozoa. Results from other studies indicated P4 induces an increase in cAMP concentrations in human spermatozoa and, therefore, cAMP signalling is not directly involved in the Ca^{2+} response to P4 in this species (Strunker et al. 2011). The possibility that the observed effects of steroid hormones on ram spermatozoa are modulated by a different mechanism, such as PI3K/AKT and MAPK pathways, as occurs in human spermatozoa (Aquila et al. 2004; Sagare-Patil et al. 2013), cannot be discounted, and may be explored in further studies.

There were attempts to determine the effects of steroid hormones on hyperactivated sperm motility and kinematic variables in the present study using an open-source software that was recently developed (Alquézar-Baeta et al. 2019). Because there have been many reports based on results from studies that indicate there is a large amount of variability in results when there is use of different CASA systems (Holt et al. 1994; Boryshpolets et al. 2013; Lu et al. 2014), there was not a comparison numerically with other studies. There, therefore, was analyses of the differences between samples collected for evaluation of those variables for which an increase or decrease was related to hyperactivation in ram spermatozoa (Vulcano et al. 1998; Mortimer and Maxwell 1999; García-Álvarez et al. 2014). For spermatozoa incubated in capacitating conditions with agents that induced an increase in cAMP concentrations, there was an incremental increase in VCL, ALH, DANCE and fractal dimension, concomitant with a decrease in VSL, STR, WOB, LIN and progressive motility, all changes related to hyperactivation. The incubation with P4 at 100 pM induced an

increase in the percentages of progressive motility and LIN and a decrease in total motility, which could indicate there was an inhibition of the hyperactivation caused by agents that induce an increase in cAMP concentrations. Incubation with E2 at the largest concentration also led to an increase in sperm progressive motility. These results are not consistent with studies in other species, mice and humans, in which steroid hormones induced an increase in the percentage of hyperactivated spermatozoa (Sagare-Patil et al. 2012; Perez-Cerezales et al. 2016; Bosakova et al. 2018). The results from the present study also differed from those described in a previous study (Gimeno-Martos et al. 2017) in which there were no effects of P4 or E2 on progressive motility or values for kinematic variables when there was use of a different CASA system, recording conditions and instrument settings.

5. Conclusions

In conclusion, results from the present study indicate that although P4 and E2 promote changes conducive to the acrosome reaction in ram spermatozoa, the mechanisms of both hormones are different. While the addition of P4 to the medium results in an increase in Ca^{2+} concentrations and a mobilization of this ion and transport into the acrosomal area, an increase in ROS and a significant disruption of the acrosome, E2 does not induce these changes. The cAMP-PKA pathway does not appear to be involved in the effects of P4 on ram sperm because neither P4 nor E2 addition to the medium resulted in an increase in hyperactivated ram sperm incubated in a medium containing large concentrations of cAMP. It, however, has to be taken into account that the present study was

conducted in *in vitro* capacitation conditions and that perhaps the mechanisms in the female reproductive tract are different.

Credit to author statement

Silvia Gimeno-Martos: Methodology, Validation, Investigation, Formal analysis, Writing-Original draft.

Sara Miguel-Jiménez: Validation, Investigation.

Adriana Casao**:** Conceptualization, Formal analysis, Supervision, Writing-Reviewing.

Jose Alvaro Cebrián-Pérez: Conceptualization, Supervision.

Teresa Muiño-Blanco: Conceptualization, Supervision, Writing-Reviewing. Rosaura Pérez-Pe: Conceptualization, Resources, Supervision, Writing-Reviewing, Project administration and Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

The authors declare no conflicts of interest to report.

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Figure legends

Fig. 1. (a) Representative microscopy images (magnification x1000) of the location of tyrosine-phosphorylated proteins, evaluated by indirect immunofluorescence (IIF) in swim-up selected spermatozoa (control 0 h) and after 3 hours of incubation in capacitating conditions (39 °C, 5% CO_2 and 100% humidity) in TALP (control) or TALP with agents increasing cAMPconcentrations ("cocktail") without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2), and after the induction of the acrosome reaction by lysophosphatidylcoline (LPC) and **(b)** percentage of the seven immunotypes identified by IIF; The images are simply to illustrate the patterns observed: E: equatorial region; A: acrosome; AE: acrosome + equatorial region; AF: acrosome + flagellum; EF: equatorial region + flagellum; AEF: acrosome + equatorial region + flagellum; F: flagellum; **P*< 0.05, ** *P*< 0.01 and ****P*< 0.001 indicate differences relative to "cocktail" sample; Values are shown as mean values ± S.E.M.; (*n* = 5)

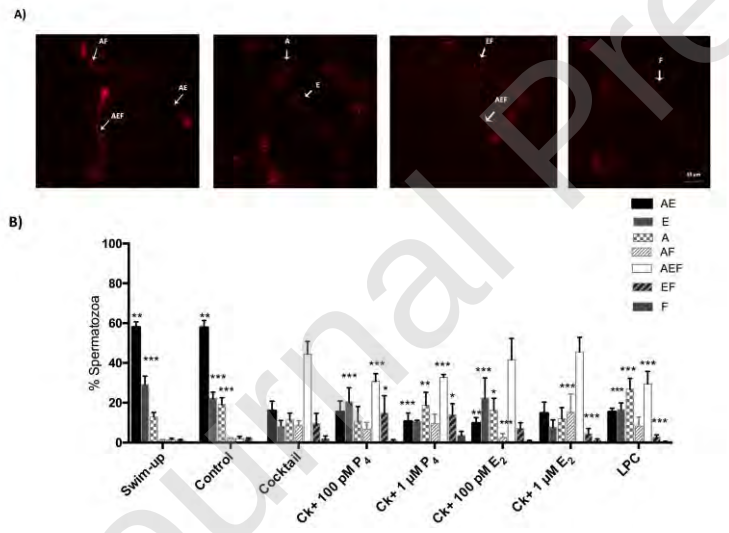

Fig. 2. (a) Percentage of viable spermatozoa with damaged acrosome membrane (RCA+/PI-) evaluated by *Ricinus communis* agglutinin swim-up selected spermatozoa and after 3 hours of incubation in capacitating conditions (39 °C, 5% CO_2 and 100% humidity) in TALP (control) or TALP with agent that increased cAMP concentrations ("cocktail") without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2), and after the induction of the acrosome reaction by lysophosphatidylcoline (LPC) Values are shown as mean

± S.E.M. (*n* = 5); **P*< 0.05, ** *P*< 0.01 and ****P*< 0.001 indicate differences compared with the "cocktail" sample; **(b)** Percentage of viable spermatozoa with greater ROS concentrations (PI-/DCF+) in swim-up selected spermatozoa and after 3 hours of incubation in capacitating conditions (39 °C, 5% CO_2 and 100% humidity) in TALP (control) or TALP with agents increasing cAMP concentrations ("cocktail") without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2); Values are expressed as mean ± S.E.M. (*n* = 5); ## *P*< 0.01 and ### *P*<0.001 indicate differences relative to control sample and **P*< 0.05 to "cocktail" sample **(c)** Percentage of viable spermatozoa with relatively greater intracellular calcium evaluated using Fluo4-AM stain (Fluo4-AM+/PI-) in swim-up selected spermatozoa and after 3 hours of incubation in capacitating conditions (39 °C, 5% CO_2 and 100% humidity) in TALP (control) or TALP with agent increasing cAMP concentrations ("cocktail") without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2); Results are expressed as mean ± S.E.M.; *n* = 5; ### *P*< 0.001 indicates differences compared with control and * *P*< 0.05 and *** *P*< 0.001 with "cocktail" sample

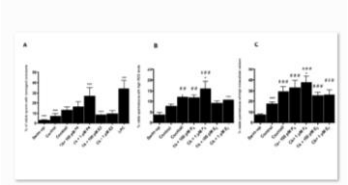

Fig. 3 (a) Representative microscopy images (magnification x1000) of the calcium location into the cell compartments, evaluated using Rhod-5N-AM and **(b)** percentage of the different localization patterns in swim-up selected ram spermatozoa (control 0 h) and after 3 hours of incubation in capacitating conditions (39 °C, 5% CO2 and 100% humidity) in TALP (control) or TALP with agent increasing cAMP concentrations ("cocktail") without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2) ;The following staining patterns were identified: I) spot at the end of the midpiece; II) staining of the neck and midpiece; III) staining of the neck, acrosome and midpiece; IV) staining of the entire head and midpiece, and V) staining of the entire head and dotted midpiece; **P*< 0.05, ** *P*< 0.01 and ****P*< 0.001 indicate

differences relative to “cocktail” sample; Values are shown as mean ± S.E.M.; (*n* = 5)

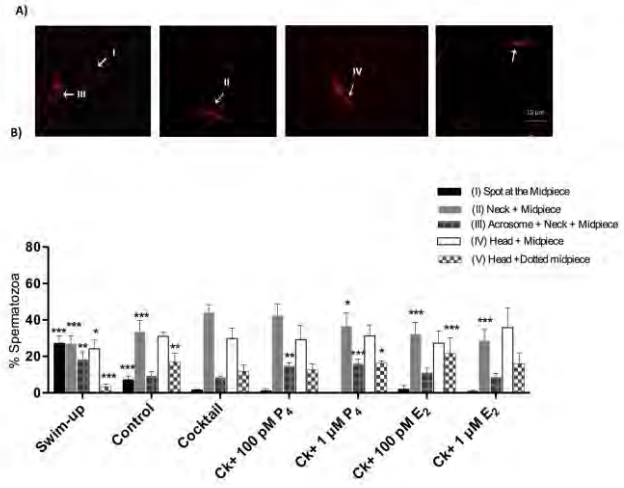

Fig. 4. Assessment of intracellular cAMP content **(a)** and protein kinase A (PKA) activity **(b)** in swim-up selected ram spermatozoa after 5 minutes of incubation in capacitating conditions (39 °C, 5% CO_2 and 100% humidity) in TALP (control) or TALP with agent increasing cAMP concentrations (“cocktail”) without or with two concentrations (100 pM or 1 µM) of progesterone (P4) or 17β-estradiol (E2); Data are expressed as pmol cAMP/10^7 cells (a) or as arbitrary units (U) /10^7 cells (b) (mean values ± S.E.M.; *n* = 5); # *P*< 0.05, ## *P*< 0.01 and ### *P*< 0.001 indicate differences relative to control sample

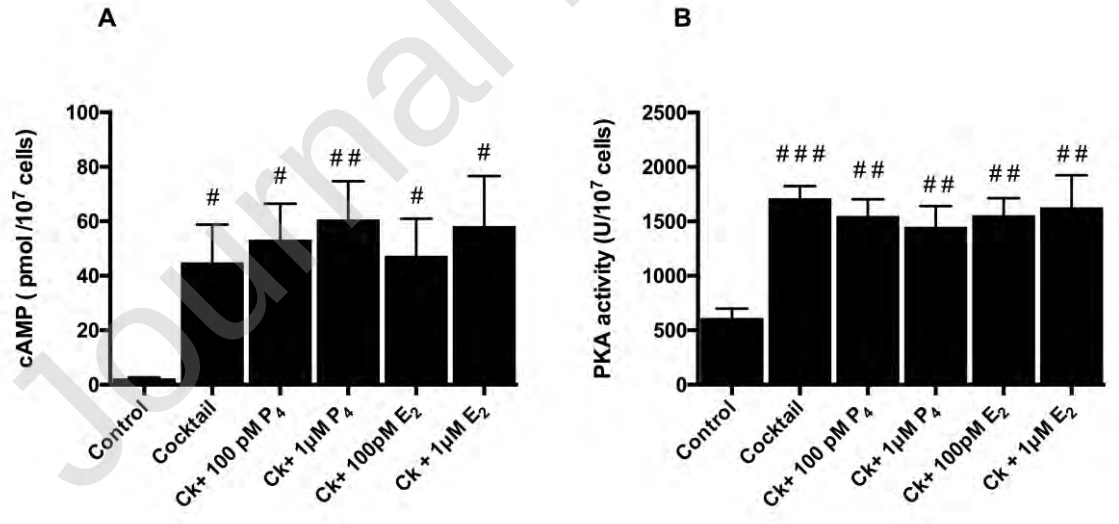

Table 1

Correlation between the localization of tyrosine-phosphorylated proteins (evaluated by indirect immunofluorescence (IIF)) and capacitation status (evaluated by chlortetracycline staining (CTC)) in ram spermatozoa; Experiments were replicated six times with the three treatment groups (control, *in vitro* capacitated and LPC-induced acrosome reacted) in each experiment; *$P<$ 0.05, ** $P<$ 0.01 and ***$P<$ 0.001 indicate differences

Sperm immunotype	CTC staining pattern		
	Non-capacitated	Capacitated	Acrosome Reacted
AE (acrosome and equatorial region)	*r* = 0.7835, ***	*r* = -0.5278, ***	*r* = -0.5731, ***
A (acrosome region)	*r* = -0.1302, *	*r* = -0.1717, *	*r* = 0.1483, *
E (equatorial region)	*r* = 0.2962, *	*r* = -0.2690, ns	*r* = -0.09394, ns
AEF (acrosome, equatorial and flagellum)	*r* = -0.5828, ***	*r* = 0.5714, ***	*r* = 0.2942, *
AF (acrosome and flagellum)	*r* = -0.2952, *	*r* = 0.1762, ns	*r* = 0.1750, ns
EF (equatorial and flagellum)	*r* = -0.2901, *	*r* = -0.0635, ns	*r* = 0.2956, *
F (flagellum)	*r* = -0.2346, ns	*r* = -0.1534, ns	*r* = 0.2301, ns

Table 2
Effect of progestrone and estradiol on viability and motility of ram spermatozoa

Percentage of viable (6-CFDA+/PI-), total and progressive motile spermatozoa and kinematic variables recording at 60 frames/s before capacitation (swim-up) and after 3 h of incubation in capacitating conditions (39 °C , 5% CO2 and 100% humidity) with cAMP agents ("cocktail"), "cocktail" with different progesterone and 17β-estradiol or without this compound (control); Values are mean ± SEM; (*n*=5); Differences letters indicate differences between treatment groups

			"Cocktail"-incubated samples (3 h)				
Variable	Swim-up	Control	"Cocktail"	+ 100 pM P4	+ 1µM P4	+ 100 pM E2	+ 1µM E2
Viability(%)	76 ± 1.67^{a}	52.7 ± 2.33^{b}	60.5 ± 2.91^{c}	63.3 ± 4.41^{c}	57.2 ± 3.85^{bc}	59 ± 2.15^{c}	61.1 ± 0.8^{c}
VSL (µm/s)	88.1 ± 6.61^{a}	87.6 ± 4.26^{a}	50.6 ± 8.18^{b}	60.8 ± 6.11^{b}	49.8 ± 7.21^{b}	56.8 ± 7.93^{b}	53.7 ± 8.7^{b}
VCL (µm/s)	171 ± 6.6^{a}	178 ± 5.17^{a}	211 ± 7.34^{b}	199 ± 15.5^{ab}	202 ± 9.11^{ab}	221 ± 7.93^{b}	201 ± 13.4^{ab}
VAP (µm/s)	119 ± 6.52	113 ± 4.98	116 ± 3.88	116 ± 3.19	112 ± 3.57	123 ± 4.1	114 ± 9.2
LIN (%)	49.5 ± 2.24^{a}	46.4 ± 2.49^{a}	25.7 ± 2.78^{b}	32.9 ± 5.81^{c}	26.8 ± 4.04^{b}	27.3 ± 2.85^{b}	29.1 ± 2.58^{b}
WOB (%)	68.4 ± 1.78^{a}	61.9 ± 2.53^{a,b}	53.8 ± 0.627^{b}	58.4 ± 3.98^{a,b}	54.1 ± 1.14^{a,b}	54.1 ± 0.78^{a,b}	55.4 ± 1.22^{a,b}
STR (%)	71 ± 2.19^{a}	72.8 ± 2.62^{a}	48.2 ± 4.1^{b}	54.2 ± 5.67^{b}	49.3 ± 6.61^{b}	50.5 ± 4.35^{b}	53.9 ± 5.53^{b}
ALH_{mean}(µm)	2.13 ± 0.08^{a}	2.26 ± 0.13^{a}	2.91 ± 0.09^{b}	2.81 ± 0.23^{b}	2.93 ± 0.25^{b}	3.06 ± 0.14^{b}	2.80 ± 0.11^{b}
ALH_{max} (µm)	4.34 ± 0.1^{a}	4.38 ± 0.22^{a}	5.77 ± 0.14^{b}	5.65 ± 0.48^{b}	5.87 ± 0.42^{b}	6.24 ± 0.32^{b}	5.38 ± 0.22^{b}
BCF (Hz)	39.4 ± 1.03^{a}	35.3 ± 1.33^{b}	34.7 ± 1.61^{b}	34.3 ± 0.72^{b}	33.9 ± 0.55^{b}	35.5 ± 1.08^{b}	34.3 ± 2.01^{b}
DANCE($\mu m^2/s$)	382 ± 24.6^{a}	494 ± 52.1^{a}	738 ± 40.9^{b}	650 ± 115^{ab}	717 ± 93.7^{b}	807 ± 65.6^{b}	675 ± 48.2^{ab}

MAD(°)	184 ± 2.83	182 ± 3.97	187 ± 2.65	183 ± 3.9	182 ± 3.77	178 ± 5.08	176 ± 2.25
Fractal dimension (FD)	1.24 ± 0.02[a]	1.3 ± 0.04[a]	1.7 ± 0.08[b]	1.57 ± 0.08[b]	1.64 ± 0.09[b]	1.67 ± 0.07[b]	1.63 ± 0.09[b]
Progressive motility (%)	40.3 ± 4.48[a]	44.9 ± 3.29[a]	15.3 ± 5.71[b]	26.1 ± 8.56[c]	14 ± 3.43[b]	20 ± 4.78[b]	21.3 ± 5.94[c]
Total motility(%)	85.1 ± 3.2[a]	69.6 ± 7.58[b]	67 ± 4.93[b]	58.7 ± 7.4[c]	66.4 ± 4.89[b]	68.3 ± 4.99[b]	63.9 ± 7.6[b]