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Silvia Gimeno Martos

Effect of hormones of the female reproductive tract on ram sperm functionality

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Tesis Doctoral

EFFECT OF HORMONES OF THE FEMALE REPRODUCTIVE TRACT ON RAM SPERM FUNCTIONALITY

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UNIVERSIDAD DE ZARAGOZA
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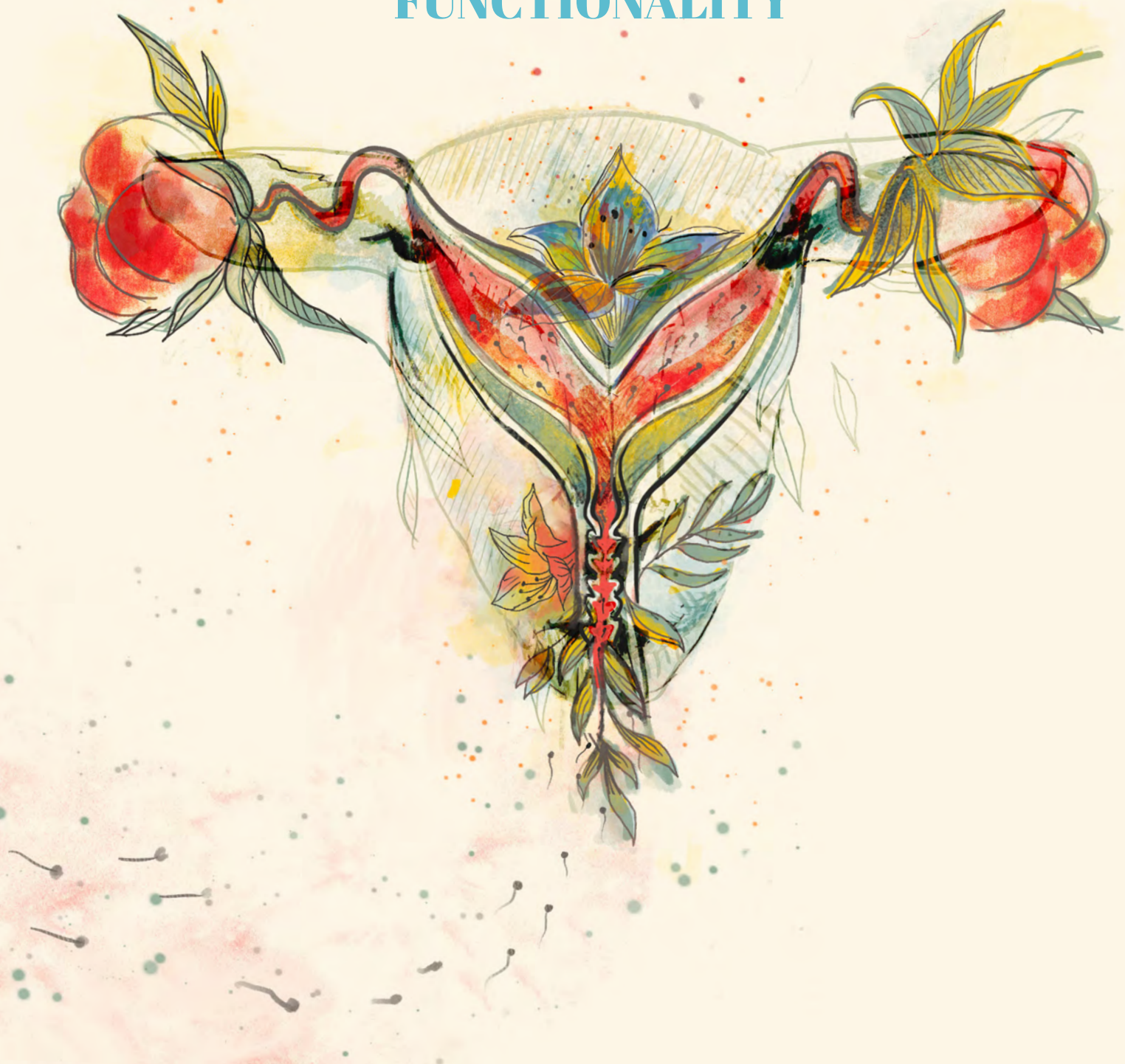
EFFECT OF HORMONES OF THE FEMALE REPRODUCTIVE TRACT ON RAM SPERM FUNCTIONALITY



SILVIA GIMENO MARTOS

TESIS DOCTORAL 2020

Universidad de Zaragoza

FACULTAD DE VETERINARIA

Departamento de Bioquímica y Biología Molecular y Celular

TESIS DOCTORAL

Effect of hormones of the female reproductive tract on ram sperm functionality

Memoria presentada por

SILVIA GIMENO MARTOS,

graduada en Biotecnología

para optar al título de Doctor con

Mención Internacional

Zaragoza, Septiembre 2020

Universidad Zaragoza
1542

Las Doctoras **Rosaura Pérez Pe** y **Adriana Casao Gascón**, Profesoras Titulares de Zoología del Departamento de Bioquímica y Biología Molecular y Celular de la Universidad de Zaragoza

INFORMAN:

Que el trabajo realizado por la graduada **Silvia Gimeno Martos** con título: **"**Effect of hormones of the female reproductive tract on ram sperm functionality" ha sido realizado bajo su dirección en la Facultad de Veterinaria de Zaragoza y reúne las condiciones exigidas para optar al grado de **Doctor con Mención Internacional**, por lo que consideran procedente su presentación.

Y para que conste a los efectos oportunos firman la presente en Zaragoza a 1 de septiembre de 2020.

Rosaura Pérez Pe **Adriana Casao Gascón**

Este trabajo se ha realizado en el Departamento de Bioquímica y Biología Molecular y Celular de la Universidad de Zaragoza, financiado por los proyectos AGL 2014-57863-R, AGL-2017-83799-R y A07-17R (BIOFITER), y la beca predoctoral del Ministerio de Economía y Competitividad BES-2015-072034

Escuela de Doctorado
Universidad Zaragoza
1542

INFORME VALORACIÓN TESIS DOCTORAL / PhD THESIS ASSESSMENT REPORT

Doctorando/a /Doctoral Student: …Silvia Gimeno Martos

Título de la Tesis / Title of the Thesis: "Effect of hormones of the female reproductive tract on ram sperm functionality"

D./Dª. Ana Patricia Grasa Molina …

☐ Como …… del Tribunal que ha juzgado la tesis doctoral / As …….. of the Doctoral Committee that has evaluated the PhD thesis

☐ **X** Como evaluador externo de la tesis / As external examiner of the PhD thesis

emite el informe que sigue /issues the following report.

En Oxford, a 17 de Julio de 2020

Firma/Sign.: Dr Patricia Grasa Molina
University of Oxford

Comente la originalidad del trabajo presentado, la relevancia del mismo dentro del dominio al que pertenece la tesis, la metodología y la calidad de la memoria presentada. Incluya, en su caso, los comentarios que desee hacer llegar al doctorando (adjunte las hojas que sean necesarias).
Discuss the originality and relevance of the work as well as the quality of the dissertation. You may include any comments that you want to send to the PhD student (please, attach as many pages as necessary).

There is no doubt that the candidate investigations on the effect of steroid hormones and melatonin on sperm functionality have opened up new research lines on the regulation of sperm fertilising ability. Moreover, a better understanding of the mechanisms of action of these hormones could help to improve cryopreservation protocols aimed to increase sperm survival and fertilising ability to improve fertility after artificial insemination.

The candidate has demonstrated that she has detailed knowledge of the field as her review of the literature references major works and places them in context. The introduction describes in detail the current knowledge about the cellular and molecular mechanisms regulating sperm capacitation and acrosome reaction as well as the role of female secreted hormones (steroids and melatonin) and their receptors on sperm functionality in different species of mammals.

The experimental design is well structured to achieve the main objectives, which are succinctly and clearly stated. The vast amount of work she has generated has fructified in four original research papers/manuscripts (2 already published in high-impact journals in the field of reproduction and 2 accepted for publication).

I hereby recommend that this thesis should be awarded with the degree of Doctor of Philosophy.

In accordance with the provisions of Regulation (EU) 2016/679 on Personal Data Protection, please be aware that your data will be added to the students' file, whose purpose is academic, research and administrative management, including the thesis. Your right to access, rectify and cancel data may be exercised in writing to the UZ Administrator adding a copy of your identity document. In case of disagreement with his answer you cancontact dpd@unizar.es or clamming to aepd.es
All the information about it can be consulted in: protecciondatos.unizar.es

Bogotá, 22 de julio de 2020

A quien interese

Apreciado Sr/Sra

Referencia: Reporte de la evaluación de la tesis doctoral de la señorita Silvia Gimeno Martos.

Les envío mi apreciación sobre la tesis de doctorado titulada "Effect of hormones of the female reproductive tract on ram sperm functionality", presentada por la señorita Silvia Gimeno Martos, en cumplimiento parcial de los requisitos para optar el título de doctor por la Universidad de Zaragoza, España, con Mención Internacional.

Después de leer con detenimiento e interés el documento, he quedado grata y favorablemente impresionado, con la gran cantidad de información generada de forma rigurosa, sobre los efectos que ejercen a nivel celular y molecular, hormonas del tracto reproductivo femenino en el funcionamiento del espermatozoide ovino.

El trabajo generó nuevo conocimiento sobre la acción de la Progesterona, Estradiol y Melatonina presentes en el tracto reproductor femenino, en la regulación del funcionamiento del espermatozoide. Se destaca el aporte al conocimiento de los mecanismos moleculares que rigen la acción de estas hormonas, así como el descubrimiento de la presencia de receptores para las hormonas esteroides, en el espermatozoide ovino. En este sentido, el trabajo no solo se aplica a la especie ovina, sino que mantiene una constante comparación con otras especies de mamíferos.

La secuencia en la investigación es de destacar, y deja ver que fue bien concebido y desarrollado. Su escrito se presenta de forma amena y de fácil comprensión, por lo que es mi sentir y parecer, que la candidata debe ser elogiada y su trabajo debe ser galardonado con un doctorado con mención internacional. Aprovecho este momento, para resaltar la labor de las dos orientadoras, ya que sin duda han contribuido enormemente a la alta calidad del trabajo presentado.

El escrito inicia con una introducción que permite establecer la secuencia seguida en el desarrollo del trabajo doctoral, en un inicio se establece la presencia por primera vez de los receptores para las hormonas esteroides (progesterona y estradiol) en el espermatozoide del

carnero; posteriormente se determina el papel que juegan estas hormonas en el funcionamiento del espermatozoide, para lo cual se tienen en cuenta aspectos moleculares y celulares. Establecido el rol que juegan estas hormonas, se procedió a determinar si los efectos que ejercían las hormonas eran mediados por sus receptores, para lo cual se usaron agonistas y antagonistas de las hormonas, determinándose que la acción es mediada por los receptores de estas.

La investigación con las hormonas esteroides se complementó con el desarrollo de trabajos *in vitro*, que profundizaron en el conocimiento de los mecanismos moleculares que acompañan al proceso de capacitación espermática y reacción del acrosoma. Se destacan aquí los trabajos que evalúan la fosforilación de las proteínas en residuos de tirosina y su distribución tras la capacitación del espermatozoide. Otros aspectos moleculares que se analizaron tuvieron que ver con el efecto de las hormonas en los niveles de calcio intracelular y de las especies reactivas de oxígeno. A nivel celular, se destacan los efectos sobre la estructura acrosomal y los parámetros cinéticos relacionados con la hiperactivación del espermatozoide, que tienen estas hormonas.

El trabajo con la melatonina se centró en establecer los mecanismos moleculares por los que esta, ejerce una doble acción (capacitante o descapacitante) sobre el espermatozoide ovino. Comprobándose que altas concentraciones de esta inhibe la capacitación y la reacción acrosómica del espermatozoide, mientras que a bajas concentraciones promueve la hiperactivación espermática.

Los resultados de este trabajo doctoral, abre caminos para adelantar nueva investigación, y la posibilidad del uso de estas hormonas en la formulación de diluyentes o de medios empleados en la Inseminación artificial, o en la preservación de dosis seminales, tal como se manifiesta en las conclusiones.

En cuanto a la metodología empleada, se utilizó una gran cantidad de procedimientos metodológicos, que dan idea de la gran preparación, dedicación y dominio de las técnicas por parte de la estudiante doctoral, y del inmenso trabajo que tuvo esta tesis doctoral. Metodologías como la inmunofluorescencia indirecta, la inmunocitoquímica y el western blot se emplearon para lograr encontrar los sitios de localización de los receptores para las hormonas esteroides en el espermatozoide ovino. Llama también la atención sobre el dominio alcanzado de las técnicas moleculares que permitieron establecer los efectos de las hormonas analizadas, así como también las técnicas para el análisis a nivel celular. Aspectos como la citometría de flujo, junto a las técnicas de tinción, son de resaltar.

Los resultados se presentan en cuatro artículos publicados en revistas de alto reconocimiento internacional, lo cual dice mucho del gran desarrollo, rigurosidad y aporte a la ciencia, que ha hecho este trabajo doctoral. La discusión es amplia, centrada y de gran profundidad, abarca todos los aspectos planteados en los resultados, todo esto demuestra que la candidata ha

alcanzado la madurez necesaria para afrontar los compromisos relacionados con el desarrollo de proyectos de investigación, seguramente podrá realizar discusiones críticas con gran claridad en el debate, y el gran número de herramientas metodológicas le permitirán realizar grandes aportes para el desarrollo de la ciencia, en especial en este mundo complejo de la reproducción.

Las conclusiones están de acuerdo con los objetivos planteados y definitivamente muestran un camino a seguir.

Por todo lo descrito, es mi pensamiento y decisión evaluar positivamente la presente Tesis doctoral de la señorita Silvia Gimeno Martos, y recomiendo a la Comisión de Doctorado de la Universidad de Zaragoza que la Tesis se apruebe en el panel de evaluación final, con las calificaciones del caso.

Solo me resta felicitar a la doctoranda, por tan magnífico trabajo.

Atentamente

Jaime Antonio Cardozo Cerquera
Investigador PhD asociado.

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Además de los artículos publicados que se recogen en el apartado “Resultados”,otros resultados fueron presentados en los siguientes congresos:

- **XXXVIII Congreso de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM).,**

AUTORES: S. Gimeno-Martos, A. Casao, T. Muino-Blanco J.A. Cebrían-Pérez and R. Pérez-Pé.
TÍTULO: “Melatonin affects calcium, protein tyrosine phosphorylation and ROS levels during in vitro ram sperm capacitation”
TIPO DE PARTICIPACIÓN: Presentación de póster
LUGAR DE CELEBRACIÓN: Valencia.
FECHA: 7-10 Septiembre, 2015.

- **X Biennual Meeting of the Association for Applied Animal Andrology (AAAA).**

AUTORES: S. Gimeno, L. Del Molino, A. Casao, J.A. Cebrián-Pérez, T. Muino-Blanco, R. Pérez-Pé.
TÍTULO: **“Effects of 17- estradiol and progesterone on ram sperm functionality”**
TIPO DE PARTICIPACIÓN: Presentación de póster
PUBLICACIÓN: P24
LUGAR DE CELEBRACIÓN: Tours (Francia).
FECHA: 24-26 Junio 2016.

- **XIII Congreso Internacional de la Sociedad Española de Reproducción Animal(AERA).**

AUTORES: T Muino-Blanco, J Peinado, P Nadal, S Gimeno-Martos, A Casao, JA Cebrián-Pérez, R Pérez-Pé.
TÍTULO: “An implemented dextran/swim- up technique with estradiol for the separation of a sperm subpopulation on enriched in non- capacitated with no phosphatidylserine exposure cell”
TIPO DE PARTICIPACIÓN: Presentación de póster
PUBLICACIÓN: *Reproductionin Domestic Animals.* Vol. 52-Issue S2; P168.
LUGAR DE CELEBRACIÓN: Lisboa.
FECHA: 27-29 Octubre, 2016.

- **XIII Congreso Internacional de la Sociedad Española de Reproducción Animal(AERA).**

AUTORES: R Perez-Pe, P Nadal, J Peinado, S Gimeno-Martos, A Casao, JA Cebrian-Perez, T Muiño-Blanco.
TÍTULO: “The inclusion of progesterone in the dextran/swim- up procedure increases therecovery of ram capacitated sperm rate”
TIPO DE PARTICIPACIÓN: Presentación de póster
PUBLICACIÓN: *Reproductionin Domestic Animals.* Vol. 52-Issue S2; P200
LUGAR DE CELEBRACIÓN: Lisboa.
FECHA: 27-29 Octubre, 2016.

- **XVII Jornadas sobre producción animal de la Asociación Interprofesional por el Desarrollo Animal (AIDA). Zaragoza, 30-31 Mayo de 2017.**

AUTORES: Gimeno, S., Alquezar-Baeta, C., Peinado, J., Casao, A., Cebrían-Pérez, JA., Muiño-Blanco, T., y Pérez-Pé.
TÍTULO: "Influencia de progesterona y estradiol en la orientación espermática ovina"
TIPO DE PARTICIPACIÓN: Comunicación oral
REVISTA/LIBRO: ITEA-AIDA. XVII Jornadas sobre Producción Animal ITEA.
VOLUMEN/PÁGINAS/EDITORIAL/AÑO: Tomo I: 395-397. (2017). Depósito legal: Z-769-2017.
LUGAR DE CELEBRACIÓN: Zaragoza.
FECHA: 30-31 Mayo de 2017.

- **XVII Jornadas sobre producción animal de la Asociación Interprofesional por el Desarrollo Animal (AIDA). Zaragoza, 30-31 Mayo de 2017.**

AUTORES: Alquézar-Baeta, C., Gimeno-Martos, S., Santorromán, M.J., Santolaria, P., Yániz, J., Casao, A., Cebrían-Pérez, J.A., Muino-Blanco, T. y Pérez-Pé, R.
TÍTULO: "OpenCASA: un proyecto abierto para el desarrollo de software libre orientado al análisis de espermatozoides"
TIPO DE PARTICIPACIÓN: Comunicación oral
REVISTA/LIBRO: ITEA-AIDA. XVII Jornadas sobre Producción Animal ITEA.
VOLUMEN/PÁGINAS/EDITORIAL/AÑO: Tomo I: 398-400. (2017). Depósito legal: Z-769-2017.
LUGAR DE CELEBRACIÓN: Zaragoza.
FECHA: 30-31 Mayo de 2017.

- **XVII Jornadas sobre producción animal de la Asociación Interprofesional por el Desarrollo Animal (AIDA). Zaragoza, 30-31 Mayo de 2017.**

AUTORES: Casao, A., Gimeno-Martos, S., Abecia, J.A., Cebrián-Pérez, J.A., Muiño-Blanco, T. y Pérez-Pé, R.
TÍTULO: **"17-β Estradiol and progesterone effect on ram sperm capacitation and fertilizing ability"**
TIPO DE PARTICIPACIÓN: Comunicación oral
REVISTA/LIBRO: ITEA-AIDA. XVII Jornadas sobre Producción Animal ITEA.
VOLUMEN/PÁGINAS/EDITORIAL/AÑO: Tomo I: 392-394. (2017). Depósito legal: Z-769-2017.
LUGAR DE CELEBRACIÓN: Zaragoza.
FECHA: 30-31 Mayo de 2017.

- **XXXIII Scientific Meeting of the Association of Embryo Technology in Europe (AETE)., Septiembre 2017.**

AUTORES: Santorromán, M.J., Gimeno-Martos, Casao, A.,Cebrián-Pérez, J.A., Muino-Blanco, T. and Pérez-Pé, R.
TÍTULO: "Progesterone-induced changes in the ram sperm acrosome reaction are inhibited by the antagonist mifepristone"
TIPO DE PARTICIPACIÓN: Presentación de póster
LUGAR DE CELEBRACIÓN: Bath, UK.
FECHA: 13-14 de Septiembre de 2017.

- **XXII Congreso Internacional de la Sociedad Europea de Reproduccón Animal (ESDAR).Córdoba, 27-29 Septiembre de 2018.**

AUTORES: S. Gimeno-Martos, S. Miguel, A. Casao, J.A. Cebrían-Pérez, T. Muiño-Blanco and R. Pérez-Pé.
TÍTULO: **"**Protein array-based strategies for identifying ram sperm capacitation-associated changes in apoptotic signaling molecules"
TIPO DE PARTICIPACIÓN: Presentación de póster
PUBLICACIÓN: *Reproductionin Domestic Animals.* Vol. 53-Suppl.2. 139-140
LUGAR DE CELEBRACIÓN: Córdoba.
FECHA: 27-29 Septiembre de 2018.

- **XVIII Jornadas sobre producción animal de la Asociación Interprofesional por el Desarrollo Animal (AIDA). Zaragoza, 7-8 Mayo de 2019.**

AUTORES: Gimeno-Martos, S., Miguel-Jimenez S., Casao A., Cebrián-Pérez, J.A., Muiño-Blanco, T. y Pérez-Pé, R.
TÍTULO: "Efecto de las hormonas esteroideas en la localización y los niveles de calcio intracelular durante la capacitación espermática ovina"
TIPO DE PARTICIPACIÓN: Comunicación oral
REVISTA/LIBRO: ITEA-AIDA. XVIII Jornadas sobre Producción Animal ITEA.
VOLUMEN/PÁGINAS/EDITORIAL/AÑO: Tomo I: 367-369. (2019). Depósito legal: Z-818-2019.
LUGAR DE CELEBRACIÓN: Zaragoza.
FECHA: 7-8 Mayo de 2019.

- **XXXV Scientific Meeting of the Association of Embryo Technology in Europe (AETE).**

AUTORES: **"** Gimeno-Martos, S., Pina-Beltrán B.., Casao A., Cebrián-Pérez, J.A., Muiño-Blanco, T. y Pérez-Pé,R.
TÍTULO: "Identification and localization of NADPH oxidase 5 in ram spermatozoa"
TIPO DE PARTICIPACIÓN: Presentación de póster
LUGAR DE CELEBRACIÓN: Murcia.
FECHA: 13-14 de Septiembre de 2019.

- **XV International Congress of the Spanish Association for Animal Reproduction (AERA).**

AUTORES: Gimeno-Martos, S.,Casao A., Cebrián-Pérez, J.A., Muiño-Blanco, T. y Pérez-Pé,R.
TÍTULO: **"**Melatonin modifies the cAMP level with no change on PKA activity on ram spermatozoa"
TIPO DE PARTICIPACIÓN: Presentación de póster
PUBLICACIÓN: *Reproductionin Domestic Animals.* Vol. 54-Issue S4; P70
LUGAR DE CELEBRACIÓN: Toledo.
FECHA: 7-9 Noviembre, 2019

A mis padres

Consejos de supervivencia para jóvenes sensibles

No te fíes de todos aquellos visionarios que te hablen de la vida sin caballos galopando en su mirada.

Ni escuches a quien no alce la vista de vez en cuando para mirarle las bragas a una estrella. No verás a ninguno de ellos llorando de emoción tras un orgasmo, o por una canasta sobre la bocina del eterno segundón. Sigue el ejemplo de los locos necesarios que se abrochan a la vida cuando quitan un botón, de los que encuentran a Dios al abrir tu cremallera.

Síguelos a ellos. A los que piensan que solo el amor puede hacer que lo imposible se vuelva a repetir. A los poetas que saben que quien tiene un lápiz lleva un paraíso en el bolsillo. Sigue solo a esos. A los que buscan la hermosura en la niebla de un poema. A aquellos que cuando tocan una piel comprenden todo.

Y huye. Huye de quien tenga tanta razón que nunca tenga nada. De aquellos que jamás dudan, porque estarán mintiendo. Huye de quien no crea que un Lunes tiene un callejón hacia el Nirvana. De todo aquel que no considere que no hay niños malcriados, sino adultos que malcrían. Y huye del hombre que no piense que quien aparta los ojos de la pobreza también se ha vuelto un cómplice al hacerlo. De quien te diga que la felicidad es un crucero con pulsera y no una muchacha con la risa floja y ojos hambrientos de infinitos.
No te fíes de quien defienda a esos
corruptos, que cuando sobra agua se inventan un modo de vender la sed.

Y ama. Ama aunque nunca tengas suelto. Recuerda que no hay peor amor que el que no se da por miedo a que te dañen. Que ningún amor no correspondido puede matarte, salvo aquel que no sientes por ti mismo. Conviene no olvidar que uno y uno suman uno entero cuando de quien te enamoras es de ti. Que cuando te caigas, nadie te convenza que la solución está en democratizar el suelo para todos, sino en encontrar la escalera de subida hacia ti mismo y para eso tendrás que preguntar a las baldosas sobre el golpe.

Evita los consejos, cualesquiera que sean.
Que no pongan tu corazón al frente, y olvida también estos consejos…
uno a uno, y sé feliz.

Marwan

Tras cinco intensos y bonitos años, ha llegado la hora de plasmar y resumir mi agradecimiento tras haber finalizado esta bonita etapa que me ha hecho crecer tanto profesional como personalmente en todos los sentidos. Aún recuerdo el día que cruce la puerta del Departamento y el grupo me dio la oportunidad de realizar el TFM con ellos, comenzando así una gratificante etapa en la ciudad de Zaragoza, que paso de ser una estancia de un año a seis queridos años y, convertirse en mi segunda casa, ¡quien lo iba a decir!

En primer lugar, agradecer a mis directoras de la Tesis por haberme dado la oportunidad y haber confiado en mí. Rosaura, por ser la templanza en persona, apoyo incondicional desde el primer momento, por transmitirme todo tu conocimiento, por todas las horas sentadas en tu despacho exprimiendo los resultados y sacándome las fuerzas y el optimismo en todo momento. Adri, gracias por tu dedicación desde el minuto uno, por transmitirme tu conocimiento tan sabio sobre la estadística, *photoshop* y miles de tips que siempre se salían de mi alcance, por encontrar siempre una solución a cualquier problema y hacer del mismo una nueva oportunidad de aprendizaje. Muchas gracias a las dos por todo, os estaré eternamente agradecida por ser mis mentoras en este ámbito.

Pepín y Tere, por ser el alma del grupo, y transmitirnos toda vuestra sabiduría, paciencia y amor por la ciencia, sin vosotros nada hubiera sido lo mismo. Pepín, por transmitirme la curiosidad por todo, por ayudar y estar dispuesto en todo momento a arreglar cualquier “cacharro” y salvar así los experimentos, y por cuidarnos tanto a todas. Tere, por tener siempre el despacho abierto a cualquier duda y consulta y estar dispuesta a echar una mano con tu conocimiento y cariño, por asesorarme siempre. Seguir disfrutando de esta preciosa etapa como abuelos.

Gracias a Marta y Carol, que estabais cuando llegue y me transmitisteis todo lo necesario para seguir la saga.

No sabría cómo agradecer todo lo vivido a mis dos compañeras de este gran viaje por todo lo sufrido y vivido juntas: Melissa y Sara. Melissa, por ayudarme en cada momento y transmitir tu energía para trabajar siempre con una sonrisa, por haber sido mi compañera no solo de trabajo sino de la vida, de miles de momentos tantos buenos como malos, de nuestro piso compartido y de nuestra crianza de gatos, te estaré eternamente agradecida. Sara, por ser ese gran apoyo en estos últimos años, ayudarme con tu sabiduría de Word y Power point que tanto me ha servido, por nuestros desayunos y charlas, nuestras horas

escuchando la Oreja de Van Gogh que nos salvaban los días eternos de experimentos y miles de cosas. Me llevo una gran familia para toda la vida.

Por supuesto, no puedo olvidarme del informático que apareció en mi primer año de doctorado y trajo un poquito de multidisciplinaridad al grupo, Carlos, que paso de ser un compañero de trabajo a convertirse en uno de mis grandes apoyos de esta tesis y en un buen amigo para toda la vida, por todas las horas en el laboratorio y sobre todo, por todas las horas en los bares y terrazas arreglando el mundo. A María, por aparecer y ser un cómplice más de esta tesis, gracias por ser mi otro apoyo incondicional en todo momento y ser mi compañera de muchas aventuras.

A todos los que vinieron de fuera y se llevaron un poquito de mí, a Ashley, nuestra americana favorita, nunca olvidaremos todo los momentos tan emocionantes en los que te acompañamos en esa etapa tan bonita, a Juliano, por llevarme un gran amigo y confidente argentino, a Luisa por aparecer en el último período de esta Tesis y dejarme conocer tu tierra preciosa. A todos los chicos que han pasado por el laboratorio y he crecido compartiendo horas en el laboratorio : Patri, Jorge, Adrián, Sofía, Paula, Virginia, Blanca y en especial a Vicky (espero que puedas seguir nuestro legado).

A Mariano Buffone y todas sus chicas (Ana, Pauli, Guille y Lis) por haberme abierto las puertas de su laboratorio en Buenos Aires y haberme enseñado tanto del mundo murino. A Violeta, por abrirme las puertas de su casa y haber sido una más de la familia, te estaré agradecida siempre.

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Si bien la lista dentro era extensa, esto no podría haber sido posible sin todo el apoyo de mis amigos y familia que tanto adoro.

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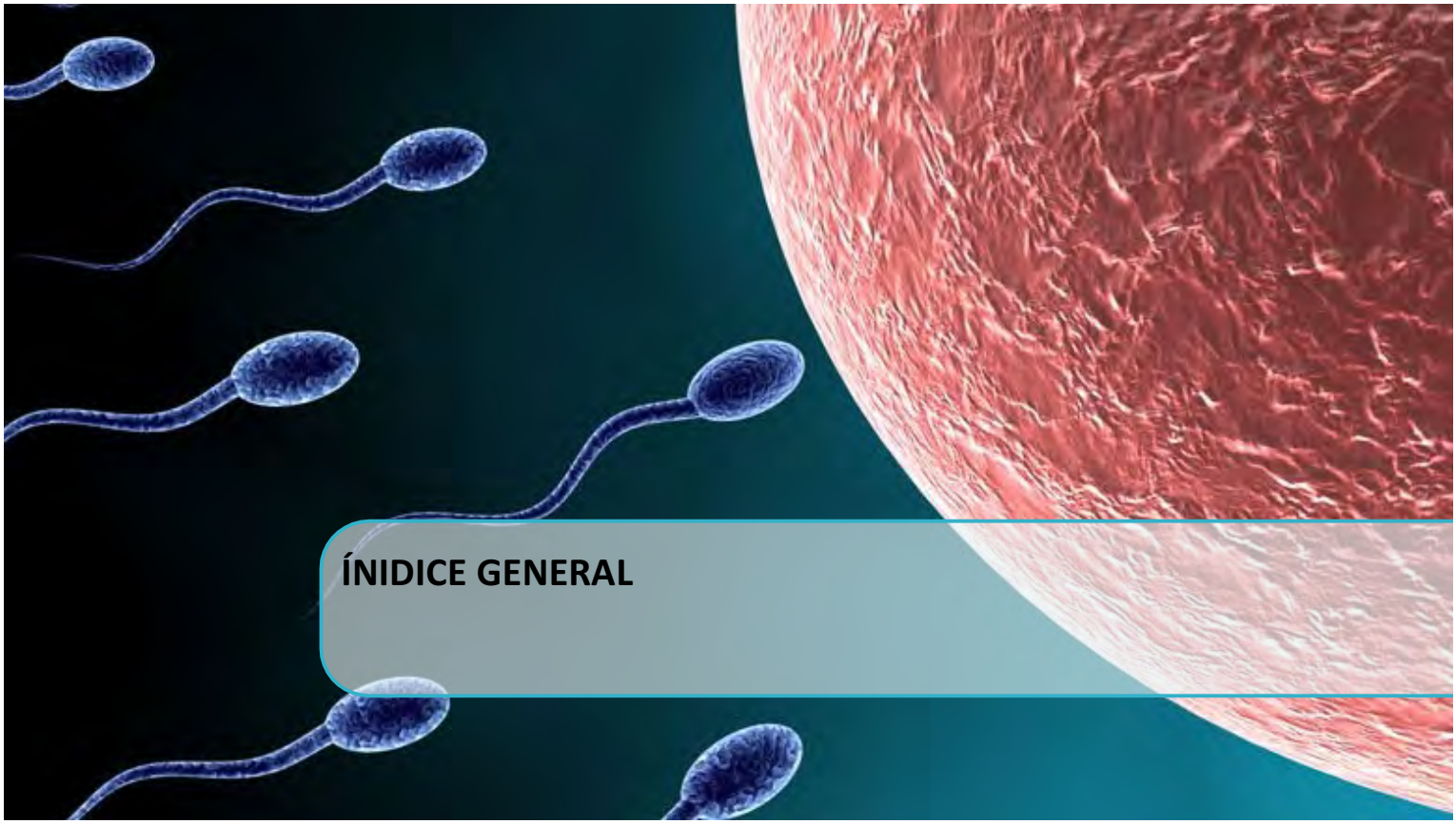

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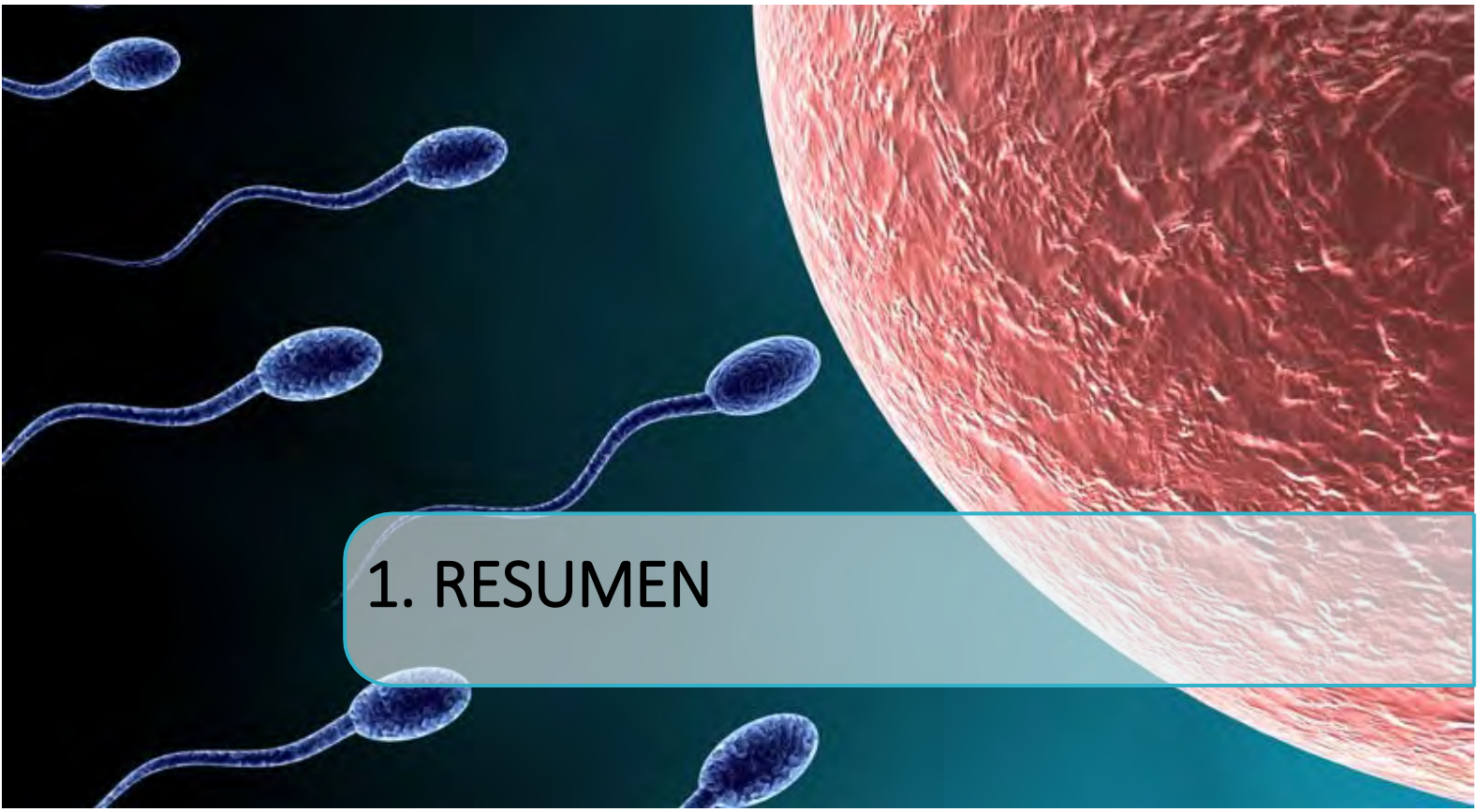


1. RESUMEN

La presencia de hormonas en el tracto reproductor femenino y su papel en la regulación de la reproducción ha sido tema de estudio en múltiples especies. Entre ellas se encuentran las hormonas esteroideas, como la progesterona y el estradiol, y otras como la melatonina.

Las hormonas esteroideas, ejercen su mecanismo de acción mediante la regulación de la expresión génica tras su unión a receptores nucleares, también llamados clásicos. Sin embargo, estas hormonas también pueden ejercer sus efectos de manera no genómica en diferentes tipos celulares, provocando respuestas rápidas a través de receptores presentes en la membrana o en el citoplasma. En espermatozoides de diferentes especies se han descrito y localizado este tipo de receptores para progesterona y 17-β estradiol, así como acciones no genómicas sobre procesos que tienen lugar en el tracto reproductor femenino como la capacitación, hiperactivación, quimiotaxis y la reacción acrosómica. Sin embargo, hasta el día de hoy, no han sido descritos los receptores para estas hormonas en espermatozoides ovinos, por lo que este hito constituyó el **primer objetivo de esta tesis**. Nuestros estudios mediante ensayos de inmunofluorescencia indirecta e inmunocitoquímica **pusieron en evidencia, por primera vez, la localización del receptor de progesterona (PR) en** en la zona ecuatorial y la pieza intermedia **del espermatozoide ovino**. Los análisis de *western-blot* de los extractos proteicos de los espermatozoides para el PR permitieron identificar un receptor con un peso molecular diferente al receptor clásico descrito en humano, por lo que se trataría de una isoforma del receptor clásico o de un receptor de membrana específico para la especie ovina. Así mismo, también se evidenció, **por primera vez, la presencia de los receptores de estrógenos, ERα y ER$_{\beta}$ en el espermatozoide ovino**, localizándose en la zona post-acrosomal y en la región apical respectivamente, y correspondiendo su peso molecular a los de los receptores nucleares clásicos. Además, nuestros resultados demostraron una correlación positiva entre la presencia del receptor ERβ y el porcentaje de espermatozoides no capacitados evaluados por la tinción con clorotetraciclina. El porcentaje de células marcadas (ERβ+) disminuyó significativamente tras la capacitación *in vitro*, y prácticamente desapareció tras la inducción de la reacción acrosómica con lisofosfatidilcolina (LPC). Sin embargo,

la localización de los receptores PR y ERα no cambió durante la capacitación ni tras la reacción acrosómica (Artículo 1).

Una vez demostrada la presencia de los receptores para progesterona y estrógenos en el espermatozoide de morueco, nos planteamos, como **segundo objetivo**, **determinar la implicación de ambas hormonas esteroideas, progesterona y 17-β estradiol, en la funcionalidad del espermatozoide ovino.** Se llevaron a cabo experimentos con diferentes concentraciones de hormonas en un medio con y sin sustancias elevadoras del cAMP (*cocktail*) que promueven la capacitación *in vitro* en la especie ovina. Ambas hormonas provocaron un aumento del porcentaje de espermatozoides reaccionados (AR) tras 3 horas de incubación, pero únicamente la progesterona a baja (100 pM) y media (10 nM) concentración en un medio *cocktail* provocó un aumento en la fosforilación de proteínas en residuos de tirosina (TyrPP). Ambas hormonas a baja concentración también provocaron una disminución de la motilidad total cuando se añadieron a los espermatozoides en un medio *cocktail*. Por lo tanto, **nuestros resultados ponen de manifiesto por primera vez el papel de la progesterona y 17-β estradiol sobre la funcionalidad espermática ovina y la capacidad de inducir la reacción acrosómica en el espermatozoide ovino (Artículo 1).**

Una vez determinados los efectos de la progesterona y 17-β estradiol en la funcionalidad del espermatozoide ovino, nos planteamos estudiar, **como tercer objetivo, si dichos efectos estaban mediados por su unión a los receptores, PR, ERα y ERβ, que habíamos evidenciado.** Para ello, incubamos espermatozoides ovinos en condiciones capacitantes, en presencia o ausencia de agonistas y antagonistas de PR y ER. La incubación con los agonistas provocaron un aumento del porcentaje de espermatozoides reaccionados (AR), en algún caso superior al inducido por la hormona, mientras que los antagonistas inhibieron el efecto inductor de la progesterona y el 17-β estradiol sobre la reacción acrosómica. Además, la incubación con los agonistas, tanto de PR como de ER, provocó un descenso de la motilidad progresiva mientras que la incubación con los antagonistas (previa a la adición de las hormonas) provocó un aumento significativo de este parámetro. **Estos resultados confirman que tanto la progesterona como el 17-β estradiol son capaces de inducir la**

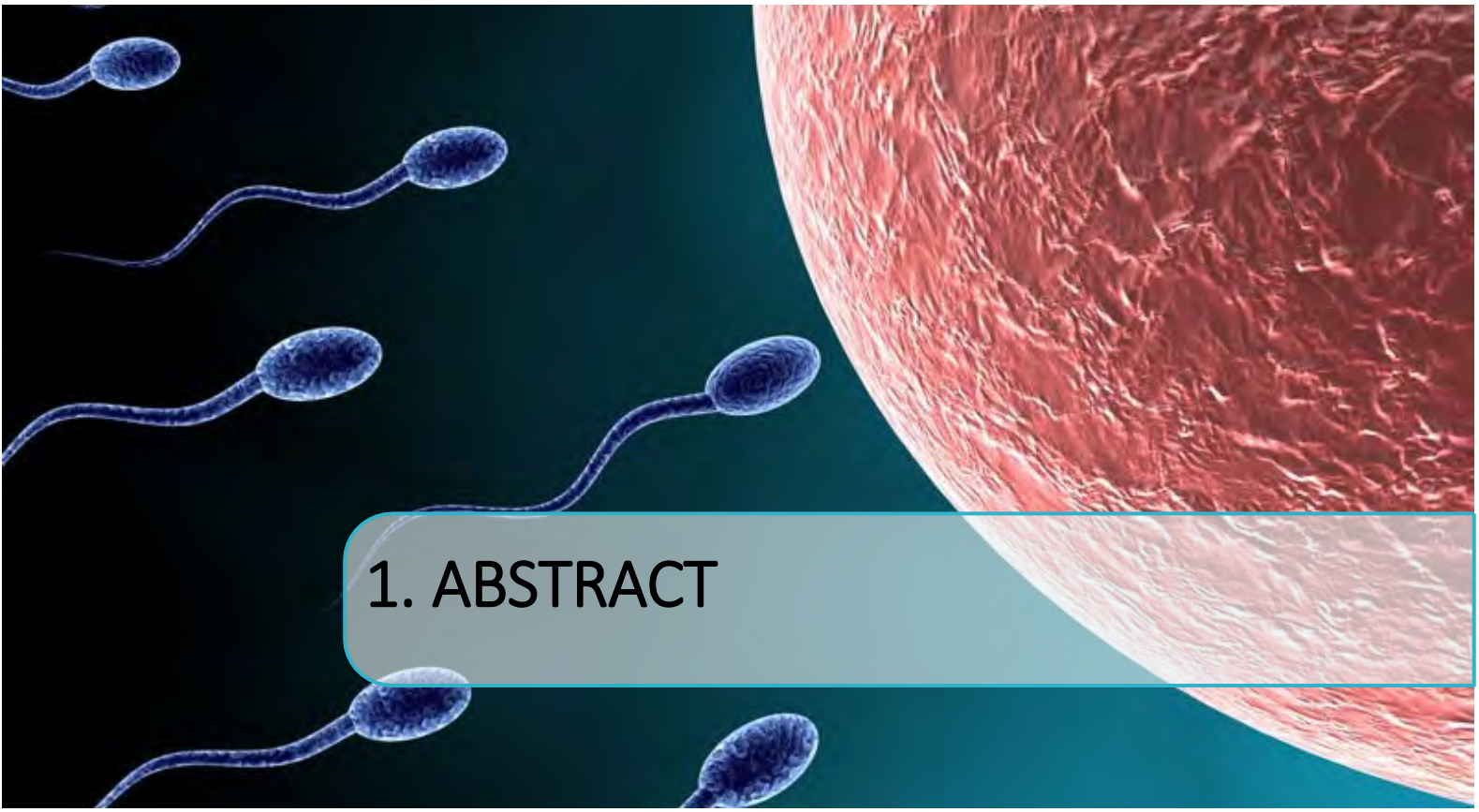
reacción acrosómica en espermatozoides ovinos, y que esta acción está mediada por su unión a sus receptores (Artículo 2).

El cuarto objetivo de esta tesis fue profundizar en el conocimiento del mecanismo de acción de la progesterona y el 17-β estradiol en el espermatozoide ovino, analizando los mecanismos moleculares subyacentes al proceso de capacitación y reacción acrosómica. Para ello, evaluamos la distribución de TyrPP tras la capacitación in vitro mediante IIF. Ambas hormonas provocaron cambios diferentes en la localización de las TyrPP en los espermatozoides. La progesterona dio lugar a la desaparición de TyrPP del acrosoma, provocando un descenso de la subpoblación con marcaje en acrosoma, zona ecuatorial y flagelo, que correlacionaba con espermatozoides capacitados, y un aumento de la subpoblación con marcaje en la zona ecuatorial y flagelo, correlacionada con los espermatozoides reaccionados, mientras que este efecto no se observó en los espermatozoides incubados con estradiol. Además, la progesterona provocó un aumento en los niveles de calcio intracelular y una movilización de este ion hacia el acrosoma, así como un aumento en los niveles de especies oxígeno reactivas (ROS) y una rotura significativa del acrosoma (evidenciada mediante el uso de lectinas). Sin embargo, el 17-β estradiol no dio lugar a ninguno de estos cambios. La vía cAMP-PKA no parece estar involucrada en los efectos de ninguna de estas hormonas esteroideas en el espermatozoide ovino. Finalmente, ni la progesterona ni el estradiol provocaron un aumento en los parámetros cinéticos relacionados con la hiperactivación espermática (Artículo 3). Así, nuestros resultados demostraron que, aunque la progesterona y el 17-β estradiol promueven mecanismos conducentes a la inducción de la reacción acrosómica en los espermatozoides ovinos, estos mecanismos son diferentes.

Por último, quisimos profundizar en el efecto de otra hormona presente en el tracto reproductor femenino, la melatonina, sobre la funcionalidad espermática ovina. Esta hormona es secretada por la hipófisis y controla la estacionalidad reproductiva a través también del eje hipotálamo-hipofisario-gonadal, pero también se secreta en otros tejidos y es capaz de ejercer efectos directos sobre las células, incluyendo los espermatozoides. En estudios previos, nuestro grupo identificó los receptores de

melatonina, MT_1 Y MT_2 , en la membrana plasmática del espermatozoide ovino y demostró que, entre otras acciones, esta hormona provocaba efectos opuestos en función de su concentración, sobre la capacitación espermática ovina. Por ello, **el último objetivo de esta tesis se centró en investigar los mecanismos moleculares por los que la melatonina ejerce su acción dual capacitante/descapacitante sobre el espermatozoide ovino.** Se comprobó que la melatonina a alta concentración (1 µM) daba lugar a la inhibición del proceso de capacitación y de reacción acrosómica mediante la disminución de los niveles de fosfotirosinas y de ROS. Por otro lado, tras un estudio de análisis de componentes principales (PCA), se pudo comprobar que la melatonina a concentraciones bajas (100 pM y 10 nM) promueve la hiperactivación. Sin embargo, no se pudo confirmar el papel de la vía cAMP-PKA en estos procesos, ya que aunque la melatonina a concentración alta fue capaz de modificar los niveles de cAMP, no provocó cambios en la actividad de la enzima PKA durante la capacitación espermática ovina (Artículo 4 + Anexo I).

En conclusión, los resultados de esta tesis doctoral demuestran el papel de las hormonas esteroideas, progesterona y 17-β estradiol, en la inducción de la reacción acrosómica, y el papel dual capacitante/descapacitante de la melatonina en el espermatozoide ovino, lo que abre nuevas posibilidades para el uso de las mismas, o bien agonistas o antagonistas de sus receptores, para la formulación de diluyentes o medios empleados en inseminación artificial o para el mantenimiento de las dosis seminales. Así mismo, también abre nuevas perspectivas en la investigación del mecanismo de acción de estas hormonas a través otras vías distintas a la vía cAMP-PKA y que podrían estar implicadas en la funcionalidad del espermatozoide ovino.



1. ABSTRACT

The hormones present in the female genital tract, such as estrogens, progesterone and others like melatonin, play a crucial role in the regulation of the reproductive events, and they have been studied in several species.

Steroid hormones regulate gene expression through nuclear, also called classical receptors. Apart from their genomic action, these hormones can exert rapid effects on several types of cells by binding to plasma membrane or cytoplasmic receptors . Several studies have revealed the presence of progesterone (PR) and estrogen receptors (ERα and ERβ) in spermatozoa from different species, and have shown direct non-genomic effects on sperm capacitation, hyperactivation, chemotaxis or acrosome reaction. However, to the date, there is no information concerning the presence of steroid hormones receptors in ovine spermatozoa. For this reason, this has been the first aim of this doctoral thesis. Our study, by means of inmunofluorescence indirect (IIF) and immunocytochemical staining, demonstrates, for the first time, that progesterone and estrogen receptors are present in ovine spermatozoa . PR is located at the equatorial region and midpiece of ram spermatozoa. Western blot analyses for PR in ram sperm protein extracts showed that the molecular weight of the ovine PR was different to the nuclear PR described in human spermatozoa, so it could be an isoform of the classic receptor or a specific membrane receptor for ovine sperm. Moreover, our results also showed the presence of both ER on ram spermatozoa, ERα being located at the postacrosomal region and ERβ at the apical region. The molecular weights of both estrogen receptors are coincident with those described as classical receptors. Also, we found a strong significant correlation between the presence of ERβ and the percentage of non-capacitated spermatozoa, evaluated by chlortetracycline (CTC). The percentage of labelled cells (ERβ+) significantly decreased after in vitro capacitation and nearly disappeared when the acrosome reaction was induced by lysophosphatidylcoline (LPC). In contrast, the localization of ERα and PR did not change during capacitation or the acrosome reaction (Article 1).

Once we demonstrated the presence of PR and ER, the second aim of this study was to determine the effects of both steroid hormones, progesterone and 17-β estradiol on ram sperm functionality. Ram spermatozoa were incubated in a medium

with different concentrations of hormones and without or with cAMP- elevating agents (*cocktail*) to induce in vitro capacitation. We found that the presence of both hormones increased the percentage of acrosome reacted (AR) cells after 3 hours of incubation in capacitating conditions, but only progesterone at low (100 pM) and medium concentration (10 nM) increased protein tyrosine phosphorylation when cAMP-elevating compounds were added. Likewise, both hormones at low concentration (100 pM) also led to a significant decrease in total motility sperm. Thus, our study demonstrates, for the first time, that steroid hormones can exert rapid effects on ram sperm functionality and that they are able to induce the acrosome reaction in ram spermatozoa (Article 1).

Once we identified the effect of progesterone and 17-β estradiol on ram sperm functionality, we decided, as a third aim of this doctoral thesis, to investigate whether that effects are receptor-mediated. For this purpose, we incubated ram spermatozoa with receptors agonists or antagonists in capacitating conditions. The incubation with PR or ER agonists increased the percentage of acrosome reacted spermatozoa, in some cases superior to that induced by the hormone. On the other hand, the incubation with receptors antagonists prevented the induction of the acrosome reaction by progesterone or 17-β estradiol. Moreover, a decrease in progressive motility was detected after the incubation with receptors agonists, and an increase with receptors antagonist. These results confirm that progesterone and 17-β estradiol can induce acrosome reaction in ram spermatozoa on their own, and that this effect is receptor-mediated (Article 2).

The four aim of this doctoral thesis, was to investigate the molecular mechanisms underlying the capacitation and acrosome reaction induced by of both hormones on ram spermatozoa. For this purpose, we evaluated, by IIF, the localization of phosphorylated proteins on tyrosine residues (TyrPP) after in vitro capacitation. Both hormones led to different changes in TyrPP. Progesterone significantly decreased sperm subpopulation immunolabeled in the acrosome, equatorial zone and flagellum, that correlated with capacitated spermatozoa, and increased the one that was labelled in the equatorial zone and flagellum and correlated with AR spermatozoa. However,

17-β estradiol did not lead those modifications. Furthermore, progesterone significantly increased the intracellular calcium levels together with a mobilization of this ion towards the acrosome, an increase in ROS levels and a substantial breakage of the acrosome (evaluated by lectins). Nonetheless, 17-β estradiol did not provoked these changes. The cAMP-PKA pathway did not seem to be involved in these effects and neither progesterone nor 17-β estradiol changed the kinetic parameters related to sperm hyperactivation.

In conclusion, this study shows that, although progesterone and 17-β estradiol promote mechanisms conducive to the acrosome reaction in ovine spermatozoa, these mechanisms are different (Article 3).

Finally, we wanted to delve into the effect of another hormone present in the female reproductive tract, melatonin, on sheep sperm functionality. This hormone is secreted by the pituitary and controls reproductive seasonality through the hypothalamic-pituitary-gonadal axis as well, but it is also secreted in other tissues and is capable of exerting direct effects on cells, including sperm. Previous studies of our research group demonstrated the presence of melatonin receptors (MT_1 Y MT_2) on ram spermatozoa and that melatonin modulates ram sperm capacitation depending on the concentration. Thus, the aim of the last study was to elucidate the mechanism through which melatonin regulates ram sperm capacitation. Melatonin at high concentration (1 µM) decreased both the percentage of capacitated and AR spermatozoa by decreasing protein tyrosine phosphorylation and ROS levels. Moreover, the PCA analyses revealed that the addition of melatonin at low (100 pM) and medium (10 nM) concentration increased the proportion of the hyperactived subpopulation. However, the present study did not confirm the involvement of the cAMP-PKA pathway on the melatonin effects in ram sperm capacitation, and although melatonin at high concentration was able to of the decrease of cAMP levels, it did not change PKA activity (Article 4 + Annex I).

Globally, the results of all these studies have demonstrated the role of steroid hormones, progesterone and 17-β estradiol, on the acrosome reaction in ram spermatozoa and the dual effect of melatonin in ram sperm capacitation. These

hormones, or the agonists or antagonists of their receptors, could be used in diluents for artificial insemination or for the conservation of the seminal doses. Additionally, this work opens new perspectives in the investigation of the mechanism of action of these hormones through different pathways than the cAMP-PKA pathway and that could be involved in the functionality of ram sperm.

2. REVISIÓN BIBLIOGRÁFICA

2. REVISIÓN BIBLIOGRÁFICA

2.1. TRÁNSITO DEL ESPERMATOZOIDE POR EL TRACTO REPRODUCTOR FEMENINO

Tras el coito, los espermatozoides, junto con el plasma seminal, son depositados en el tracto reproductor femenino (TRF). Sin embargo, estos espermatozoides eyaculados no tienen capacidad fecundante. Esta capacidad la adquieren durante su paso por el TRF, mediante el proceso de **capacitación**, que culmina con la reacción acrosómica (RA). La capacitación, como se explicará en detalle más adelante, incluye una serie secuencial de profundos cambios bioquímicos y biofísicos en el espermatozoide que le confieren la capacidad de fecundar al ovocito (Yanagimachi 1994).

Por tanto, los espermatozoides, que en su viaje por el TRF estarán expuestos a ambientes muy distintos, entrarán en contacto con gran variedad de fluidos y sufrirán modificaciones en su estado fisiológico.

A diferencia de otras especies como la equina, en las que los espermatozoides se depositan directamente en el útero (Rickard et al. 2019), el viaje de los espermatozoides ovinos comienza en la vagina y deben atravesar las criptas del cérvix, el útero y la unión útero-tubárica antes de llegar al oviducto (**Fig. 2.1).** El cérvix de la oveja, de entre 4 y 7 cm de longitud, está formado principalmente por tejido conectivo y posee una pared gruesa y un lumen compuesto por un conjunto de anillos o pliegues. Esta peculiar anatomía supone un inconveniente en el momento de utilizar un catéter para la inseminación artificial (IA) en esta especie, dando lugar a bajas tasas de fecundación (Fair et al. 2019).

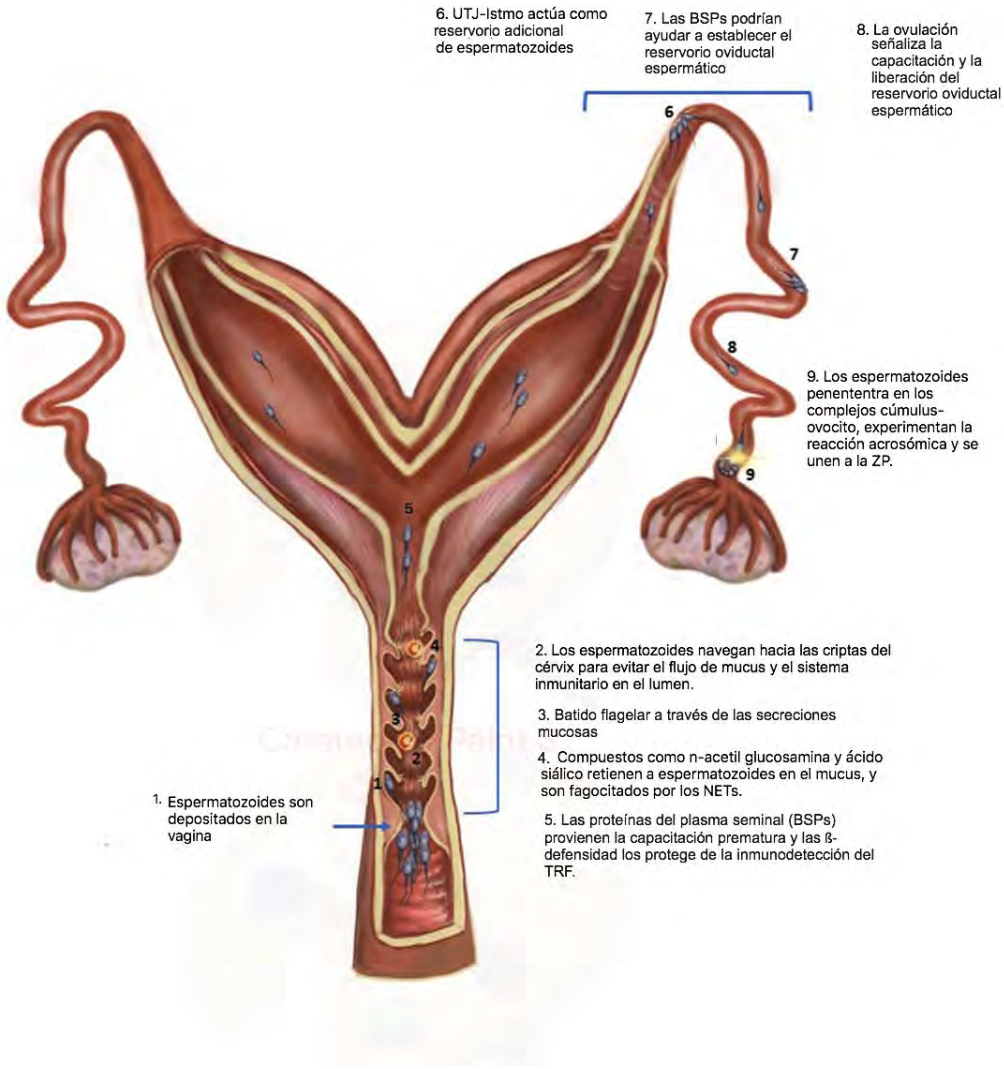


Fig. 2.1. *Trayecto de los espermatozoides ovinos por el tracto reproductor femenino. Modificada de (Rickard et al. 2019).*

El fluido existente en el lumen del cérvix se denomina mucus y procede de secreciones del propio cérvix. La producción de este mucus cervical es continua pero variable dependiendo de la fase del ciclo reproductivo (Maddison et al. 2016). En el cérvix los espermatozoides se enfrentan a obstáculos físicos, bioquímicos e inmunológicos como primera medida de selección espermática. En este ambiente, los espermatozoides mótiles son capaces de avanzar a través del mucus y formar pequeños reservorios en las criptas del cérvix (Rath et al. 2008). Asimismo, las glicoproteínas ß-defensinas, presentes en la superficie de los espermatozoides, los protegen del sistema inmune en el TRF (Hall et al. 2017). Por otro lado, las BSPs (del inglés, *binder sperm proteins*) presentes en el plasma seminal impiden una

capacitación prematura de los espermatozoides (Pini et al. 2018). Sin embargo, aquellos espermatozoides con motilidad baja y morfología anormal son atrapados y arrastrados por el flujo del mucus presente en el lumen gracias a la activación del sistema inmune. En este caso, los espermatozoides son eliminados por los neutrófilos, que, bien los fagocitan, o bien los atrapan, tras su extrusión, en las llamadas NETS (del acrónimo en inglés, *neutrophil extracelular traps*) (Alghamdi et al. 2009). De esta forma, el cérvix actúa como primera barrera en el TRF, seleccionando una población espermática con alta calidad, capaz de avanzar a zonas más próximas al ovocito, y eliminando los espermatozoides con baja calidad.

Una vez atravesado el cérvix, los espermatozoides se mueven por el útero hasta la unión utero-tubárica (UTJ, *utero-tubal juction*), y finalmente alcanzan el oviducto. En esta última zona, los espermatozoides permanecen retenidos en pequeños reservorios del istmo, mediante uniones al epitelio ciliado oviductal. Este fenómeno fue observado por primera vez en hámster y conejo (Yanagimachi and Chang 1963) y, posteriormente, en la especie ovina (Hunter and Nichol 1983), bovina (Hunter and Wilmut 1984) y murina (Suarez 1987). Los espermatozoides se van capacitando conforme se van liberando de estos reservorios y contactan con diferentes moléculas presentes en el fluido oviductal (Hunter 2012). De esta manera, el reservorio oviductal juega un papel muy importante en el mantenimiento de la capacidad fecundante de un eyaculado entre el estro y la ovulación de la hembra, ya que sincroniza la capacitación con la ovulación. Además controla la cantidad de espermatozoides que llegan a la ampolla, contribuyendo así a la prevención de la poliespermia durante la fecundación (revisado por Rickard (2019)).

Asimismo, la orientación espermática en el oviducto parece jugar un papel esencial para la llegada con éxito hasta el ovocito. Hasta la fecha, se han propuesto tres mecanismos diferentes: **termotaxis** (Bahat et al. 2003), **reotaxis** (Miki and Clapham 2013) y **quimiotaxis** (Armon and Eisenbach 2011), y cada uno de los cuales responde a un estímulo específico: gradiente de temperatura, flujo de fluido y gradiente de concentración, respectivamente. Además de la capacitación, y en relación con ella, los espermatozoides sufren un cambio en su patrón de movimiento denominado **hiperactivación flagelar**. Cuando entran en el TRF, los espermatozoides

se mueven avanzando en línea relativamente recta, pero en las inmediaciones del ovocito el patrón de movimiento se modifica, las oscilaciones del flagelo se vuelven asimétricas y las trayectorias de los espermatozoides adquieren un patrón en forma de ocho (Yanagimachi 1994). Asimismo, en aquellas especies en las que el istmo del oviducto constituye un reservorio espermático, como hemos comentado previamente, la hiperactivación es un proceso importante para la liberación de los mismos de las criptas del epitelio oviductal. Este movimiento hiperactivado permitirá a los espermatozoides atravesar tanto la capa de células del *cúmulus* como la zona pelúcida (ZP) que rodean al ovocito (Ho and Suarez 2001a). Estos dos procesos, capacitación e hiperactivación son complementarios y se desarrollan simultáneamente en condiciones fisiológicas. Sin embargo, también pueden producirse de manera independiente aun compartiendo vías o rutas de señalización (Ho and Suarez 2001a).

Por último, debe de llevarse a cabo la **reacción acrosómica** (RA), un proceso de exocitosis controlado que permite al espermatozoide atravesar la ZP del ovocito, y que es imprescindible para culminar la fecundación con éxito. Sólo aquellos espermatozoides previamente capacitados son capaces de experimentar la RA en el tracto reproductor femenino (Saling et al. 1979) (**Fig.2.2.).**

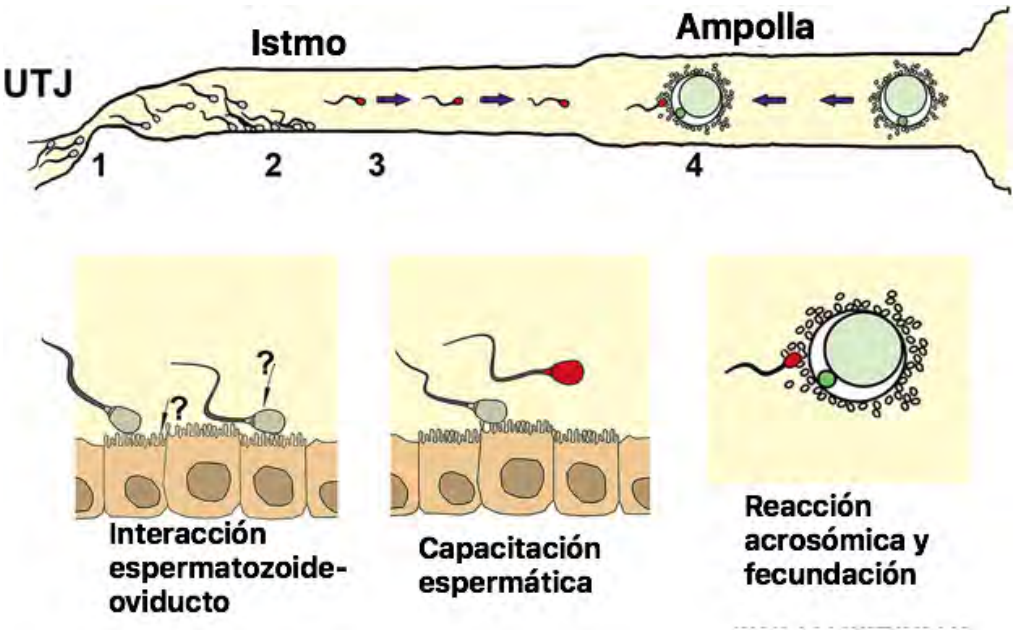


Fig.2.2.*Principales eventos durante el tránsito del espermatozoide por el tracto reproductor femenino: 1) Paso de los espermatozoides del útero al oviducto a través de la unión útero-tubárica (UTJ). 2) Formación del reservorio de espermatozoides en el istmo. 3) Liberación de los espermatozoides capacitados (representados con cabeza*

roja) del istmo a la ampolla. 4) Localización del complejos cúmulus-ovocito por parte de los espermatozoides. Modificada de Gervasi &Visconti (2016), a su vez adaptada de Coy (2012).

2.2. CAPACITACIÓN ESPERMÁTICA

Como ya hemos mencionado, la capacitación es un pre-requisito indispensable para que se produzca la fertilización e incluye una serie secuencial de profundos cambios bioquímicos y biofísicos en el espermatozoide. La capacitación espermática fue descrita por primera vez en 1951 por Min Chueh Chang y Colin Russell Austin en trabajos independientes (Austin 1951; Chang 1951). A día de hoy, a pesar de que han transcurrido casi 70 años desde estos primeros trabajos, no se han elucidado totalmente los mecanismos moleculares que regulan este proceso.

En la actualidad se sabe que durante la capacitación se produce la salida de parte del colesterol de la membrana plasmática, en concreto de los microdominios denominados *rafts lipídicos*. Estudios llevados a cabo en otras células relacionan la salida del colesterol de los *rafts* con la activación de vías de señalización celular en las que están implicadas las tirosina quinasas, las proteínas G y otras moléculas (Simons and Toomre 2000). De manera que, en el caso del espermatozoide, el colesterol regula la capacitación a través de su salida y/o reordenación en los *rafts* lipídicos, provocando la activación de vías moleculares entre las que destaca cAMP-PKA (Boerke et al. 2008). Además, esta salida de colesterol da lugar a un aumento de la permeabilidad de la membrana plasmática (Langlais and Roberts 1985), lo que provoca un influjo de Ca^{2+} y bicarbonato al interior del espermatozoide (Revisado por Leahy y Gadella (2015)). Estos eventos, además van acompañados de un aumento de los niveles de especies reactivas de oxígeno (ROS) (de Lamirande and Lamothe 2009) y de la fosforilación de las proteínas en residuos de tirosina (Revisado por Gadella y Luna (2014).

Por todo ello, cuando se pretende llevar a cabo una **capacitación espermática *in vitro***, es necesario, además de unas determinadas condiciones de temperatura y humedad, añadir una serie de compuestos que provoquen estos efectos descritos.

Mientras que en la capacitación *in vivo* las encargadas de secuestrar el colesterol son las lipoproteínas HDLs que se encuentran en el fluido uterino y oviductal, en los medios de capacitación *in vitro* el aceptor de colesterol más usual es la albúmina sérica bovina (BSA), que da lugar a un aumento de la fluidez de la membrana. También se utilizan las metil-β-ciclodextrinas (M-β-CD), que tienen una alta afinidad por los esteroles y la capacidad de inducir la capacitación *in vitro* en ratón, toro, humano (Osheroff et al. 1999; Visconti et al. 1999a) y ovino (Grasa et al. 2006). Además, se cree que el tiempo requerido para la capacitación *in vitro* en las distintas especies está relacionado con la velocidad de salida de colesterol de la membrana espermática (Yanagimachi 1994).

La capacitación también es un proceso dependiente del ión **HCO_3^-** (Visconti et al. 2002). Las bajas concentraciones de HCO_3^- en el epidídimo mantienen a los espermatozoides sin capacitar hasta que los altos niveles de este anión en el TRF disparan este proceso (Visconti et al. 1998). Por ello, numerosos estudios demuestran la necesidad de este ion en medios de capacitación *in vitro* en la mayoría de las especies como la murina (Lee and Storey 1986), bovina (Breininger et al. 2010), porcina (Gadella and Harrison 2000), humana (Battistone et al. 2013) y ovina (Grasa et al. 2006)

Otro ión necesario para provocar la capacitación *in vitro* de los espermatozoides es el Ca^{2+}. La concentración de Ca^{2+} intracelular aumenta debido a su entrada del exterior, o a su salida de los reservorios intracelulares, como son el acrosoma, la envoltura nuclear redundante (RNE, *redundant nuclear envelope*) y la pieza intermedia, donde se localizan las mitocondrias (Costello et al. 2009). Este aumento se produce gracias a la apertura de canales permeables al mismo, tanto en la membrana plasmática como en la membrana de orgánulos intracelulares, como son **Ca_v** (Voltaje-gated calcium channel) (Darszon et al. 1999), **CNG** (del acrónimo en inglés, *Cyclic Nucleotide-gated Channels*) (Weyand et al. 1994) **y CatSper (**del acrónimo en inglés, *Cation Channels of Sperm*) (Ren et al. 2001), el único específico del espermatozoide.

Como hemos mencionado previamente, la capacitación también va acompañada de la producción de especies reactivas de oxígeno (ROS). El espermatozoide tiene la capacidad de generar ROS de forma fisiológica (Agarwal et al. 2003), mayoritariamente **anión superóxido (O_2^-), peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (**OH^-**)**. Las ROS juegan un papel importante a concentraciones adecuadas en este y otros procesos como la hiperactivación, RA y en la fusión de membranas durante la fecundación (**Fig.2. 3**) (Kothari et al. 2010). En cambio, un exceso de ROS puede provocar daños, tanto en la membrana plasmática como en la membrana acrosomal y en el ADN (Aitken 2017), dando lugar a la activación de vías intrínsecas de la apoptosis celular (Aitken et al. 2012) y como consecuencia, la incapacidad del espermatozoide de interaccionar con la ZP y fecundar al ovocito (de Castro et al. 2016). En relación con las ROS, el plasma seminal juega un papel muy importante dado que contiene enzimas antioxidantes capaces de eliminarlas, así como diversas moléculas antioxidantes no enzimáticas como la melatonina, el glutatión y las vitaminas C y E que pueden proteger al espermatozoide de un estrés oxidativo (Awad et al. 2006; Casao et al. 2010a).

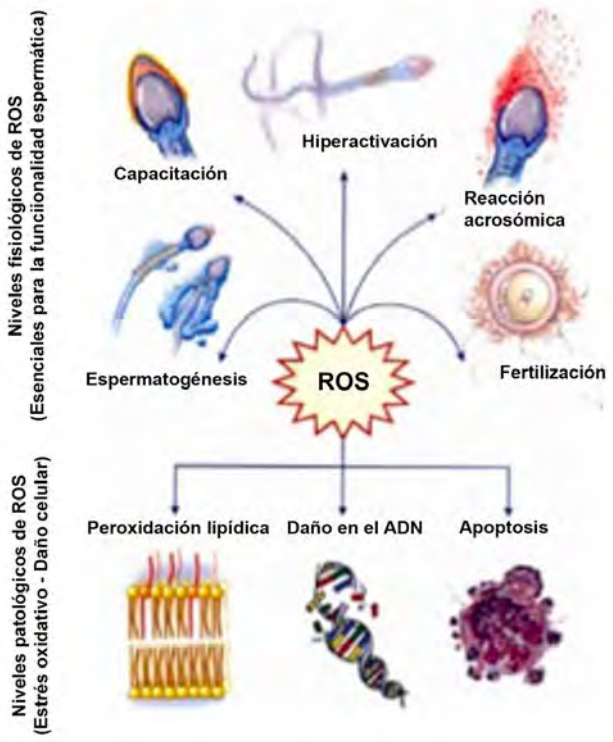


Fig.2. 3.*Efectos fisiológicos y dañinos de ROS en la funcionalidad espermática. Imagen modificada de Kothari (2010).*

En el proceso de capacitación, el cAMP (del inglés; *cyclic adenosine 3´-5´monophosphate*) desempeña un papel fundamental como segundo mensajero. Sus niveles intracelulares dependen de su formación mediante la enzima adenilato ciclasa (AC), y de su degradación por las fosfodiesterasas (PDEs; de las siglas en inglés *phosphodiesterases*). La activación de la AC está provocada por el aumento de Ca^{2+} intracelular así como del HCO_3^-, junto con ROS. En los medios de capacitación *in vitro* para espermatozoides de determinadas especies, se pueden añadir análogos del cAMP, e incluso inhibidores de las fosfodiesterasas para evitar su degradación (Colas et al. 2008).

Como se ha comentado, el rápido aumento del contenido de cAMP durante la capacitación (Harrison and Miller 2000) estimula inmediatamente la actividad de la enzima protein quinasa A (PKA) (Lefievre et al. 2002). PKA es una holoenzima tetramérica, y la unión de dos moléculas de cAMP a cada subunidad reguladora desencadena su disociación y la activación de las subunidades catalíticas, dando lugar a la fosforilación en residuos de Ser/Thr (Gronborg et al. 2002). Además, indirectamente, PKA también regula la fosforilación de proteínas en residuos de tirosina (Naz and Rajesh 2004). Todas estas fosforilaciones se llevan a cabo con la ayuda de las **proteínas de anclaje de Protein kinasa A (AKAPS;** de las siglas en inglés A *Kinase Anchoring Proteins*). AKAPs se encargan de dirigir las subunidades catalíticas a distintos compartimentos subcelulares para regular la fosforilación de proteínas a través de quinasas y fosfatasas (Beene and Scott 2007). Se ha averiguado que en el caso de los espermatozoides de mamíferos, PKA y AKAPs se encuentran presentes en el flagelo (Wertheimer et al. 2013).

Esta ruta de señalización descrita es la vía clásica cAMP-PKA, pero en el espermatozoide se han descrito otras, como la que implica a la familia de las **MAPKS** (del acrónimo en inglés, *Mitogen-actived protein kinases*). Esta familia de serina/treonina quinasas fosforilan múltiples tipos de proteínas (Raman et al. 2007) a través de su unión de ligandos extracelulares, o intracelularmente por medio de ROS. Dentro de esta familia se incluyen quinasas reguladas por señales extracelulares o **ERK**

(del acrónimo en inglés, *Extracelular Regulated Kinases*), quinasas c-Jun N-terminalo **JNKs** (del acrónimo en inglés, *c-Jun-N-terminal Kinase*), **MAPK p38 y MAPK ERK5.**

Varios estudios han asociado la activación de las MAPKs con el proceso de capacitación y la inducción de RA en varias especies (de Lamirande and Gagnon 2002; Almog et al. 2008; Awda and Buhr 2010; Nixon et al. 2010). En el caso de la especie ovina, nuestro grupo evidenció la implicación, además de la clásica ruta cAMP/PKA, de la ruta MAPK ERK1/2 en la capacitación, activada por la unión del factor de crecimiento epidérmico (EGF, del acrónimo en inglés *epidermal growth factor*) a su receptor (EGFR) (Luna et al. 2012). Recientemente, también hemos identificado las enzimas JNK y p38 en el espermatozoide ovino, y demostrado su participación tanto en la capacitación como en la apoptosis, sugiriendo que ambos procesos podrían compartir vías de señalización molecular (Luna et al. 2017a).

Como hemos mencionado anteriormente, durante la capacitación en el espermatozoide se incrementa la **fosforilación de las proteínas en residuos de tirosina** (Visconti et al. 1995). Las modificaciones post-traduccionales en el espermatozoide son muy frecuentes y de vital importancia, ya que se trata de una una célula terminalmente diferenciada, altamente compartimentalizada y con una actividad transcripcional y traduccional prácticamente residuales. Por ello, los mecanismos de fosforilación/defosforilación, y en concreto las fosforilaciones en residuos de tirosina, juegan un papel fundamental en los procesos como la capacitación e hiperactivación y son un requisito para la RA y que el espermatozoide fecunde al ovocito (Visconti et al. 1995; Flesch and Gadella 2000).

Esta modificación post-traduccional es dependiente de la presencia de BSA y HCO_3^- (Bedu-Addo et al. 2005) en los medios de incubación, así como del tiempo de incubación. Está directamente relacionada con la vía cAMP-PKA (**Fig.2.4**) (Visconti et al. 2002), ya que el uso de inhibidores de PKA, como H89, da lugar a una drástica disminución de la fosforilación de tirosinas en el espermatozoide durante la capacitación, mientras que la adición de análogos de cAMP promueve dicha

fosforilación (Thundathil et al. 2002; Grasa et al. 2006).

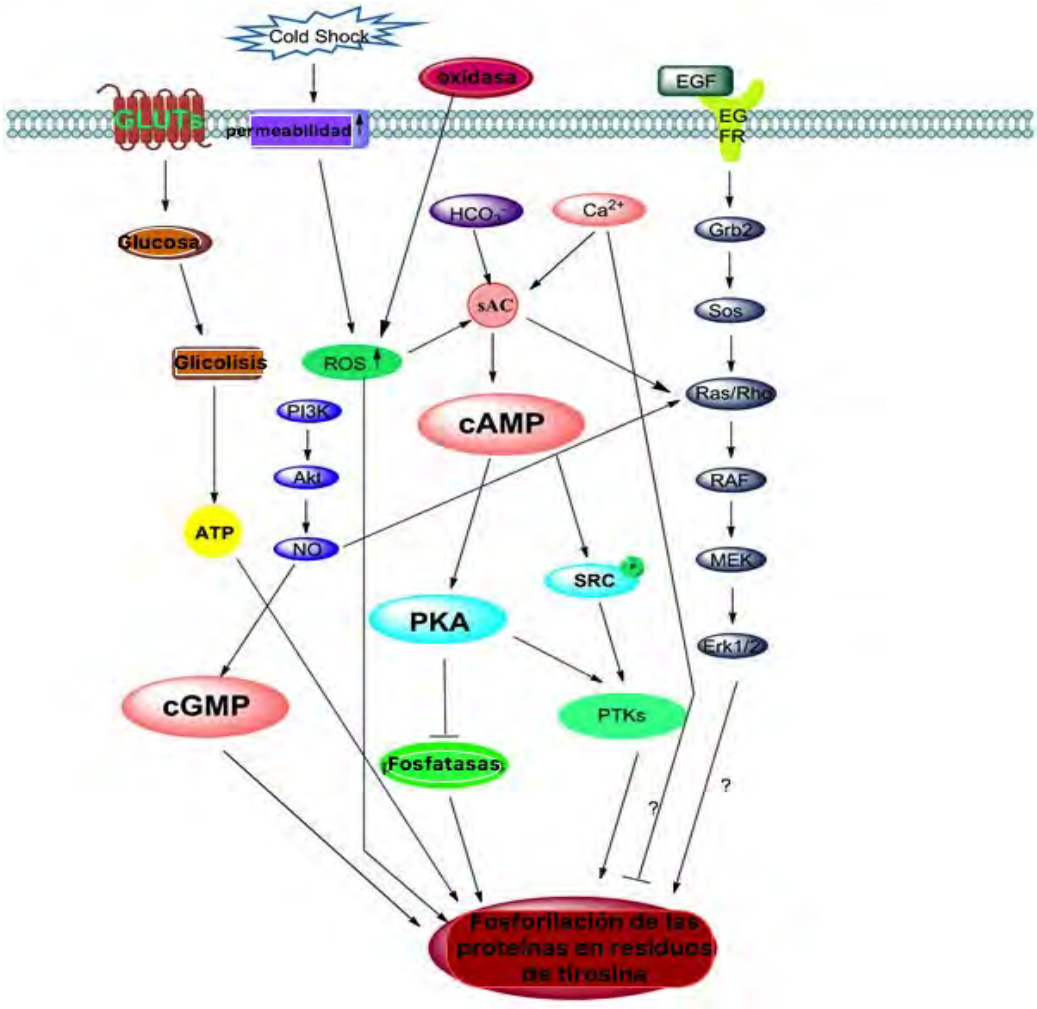


Fig.2.4.*Esquema de los mecanismos que regulan la fosforilación en residuos de tirosina durante la capacitación (Tomada de la revisión de Jin& Yang (2017)).*

A lo largo de los años, numerosos estudios han descrito la fosforilación en residuos de tirosina de proteínas de membrana fenómeno en espermatozoides humanos (Naz 1996), de toro (Galantino-Homer et al. 1997), ratón (Visconti et al. 1998), hámster (Visconti et al. 1999b), cerdo (Kalab et al. 1998), búfalo (Roy and Atreja 2008), caballo (Pommer et al. 2003) y morueco (Perez-Pe et al. 2002; Grasa et al. 2006). Este fenómeno se asocia con determinados eventos dependiendo de su localización. En el flagelo, donde es más abundante en la mayoría de las especies (Carrera et al. 1996; Mahony and Gwathmey 1999; Pommer et al. 2003; Urner and Sakkas 2003), se relaciona directamente con cambios en motilidad e hiperactivación

(Nassar et al. 1999; Kong et al. 2008) e indirectamente con la unión al ovocito y la fusión de gametos (Urner et al. 2001; Liu et al. 2006). Por otra parte, la fosforilación en tirosinas de proteínas localizadas en el cuello y la cabeza del espermatozoide parece estar relacionada con la preparación para la RA y su unión a la ZP (Naz et al. 1991; Asquith et al. 2004; Grasa et al. 2009; Sati et al. 2014).

2.3. REACCIÓN ACROSÓMICA

El acrosoma es una vesícula secretora que cubre la parte anterior de la cabeza del espermatozoide debajo de la membrana plasmática de la célula. Esta estructura deriva del aparato de Golgi y está delimitada por la membrana acrosomal externa (adyacente a la membrana plasmática) y por la membrana acrosomal interna (colindante a la envoltura nuclear). Fue descrita por primera en el espermatozoide de erizo por Dan y cols., en 1952 (Dan 1952) y en mamíferos por Austin y Bishop, en 1958 (Austin and Bishop 1958).

La reacción acrosómica (RA) es un proceso de exocitosis que permite al espermatozoide penetrar en la ZP del ovocito y es un requisito para realizar la fecundación con éxito. Sólo aquellos espermatozoides previamente capacitados son capaces de experimentar la RA (Saling et al. 1979). En mamíferos, el acrosoma se hincha (*swelling*), se fusionan la membrana acrosomal externa con la membrana plasmática y se forman poros entre ellas. Tras la fusión, los componentes presentes en la matriz acrosomal, mayoritariamente enzimas, se liberan y permiten al espermatozoide atravesar la ZP del ovocito (Kim et al. 2011).

Durante más de 30 años la comunidad científica aceptaba como dogma que únicamente el espermatozoide de mamíferos con el acrosoma intacto es capaz de unirse a la ZP del ovocito y producirse la RA (Breitbart and Shabtay 2018). Sin embargo, dos estudios recientes llevados a cabo de forma independiente y utilizando ratones transgénicos han demostrado que, por lo menos en esa especie, el espermatozoide puede experimentar la RA antes de unirse a la ZP del ovocito, en zonas ascendentes del istmo y la ampolla (La Spina et al. 2016; Muro et al. 2016). Otros estudios apuntan

a las células del *cúmulus,* y concretamente la proteína sindecan-1 de las mismas, como capaces de inducir la RA *in vivo* (Joshi et al. 2014). A pesar de estos hallazgos, es necesaria la interacción entre el espermatozoide y la ZP para que se produzca la fecundación (Avella et al. 2013). Alguna de las proteínas presentes en la membrana acrosomal del espermatozoide, como la denominada IZUMO1, sufren un cambio en su localización tras la RA, pasando a la región ecuatorial, y juegan un papel clave en la fusión con la membrana plasmática del ovocito (Inoue et al. 2005; Satouh et al. 2012).

Aunque tanto el aumento intracelular de Ca^{2+} como el pH_i intervienen en la inducción de la RA (Yanagimachi 1994; Aitken 1997), este evento también viene determinado por la unión de determinados ligandos a la membrana del espermatozoide. Los ligandos fisiológicos de la RA más relevantes son una de las glicoproteínas constitutivas de la ZP del ovocito, la ZP3, y la progesterona (Harper et al. 2006; Florman et al. 2008). Su unión a los receptores presentes en la membrana plasmática del espermatozoide da lugar a la activación de los mismos y a la apertura tanto de canales específicos de Ca^{2+} como el CatSper (Strunker et al. 2011) como de canales en la membrana acrosomal externa, provocando un aumento de Ca^{2+} en el citoplasma (**Fig.2.5.).**

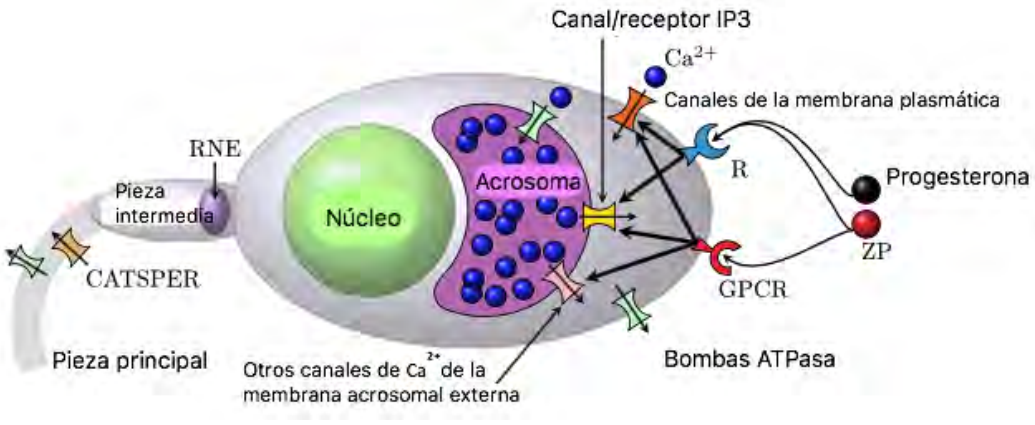


Fig.2.5.*Esquema de la reacción acrosómica en la cabeza del espermatozoide de mamíferos inducida por ZP o progesterona (Modificada de J. Simon y Fauci (2018)).*

La unión de los ligandos a los receptores provoca la activación tanto de la enzima AC como de la fosfolipasa C (PLC, *phospholipase C*) (**Fig. 2.6.).** En el caso de la AC, ésta

provoca el aumento de $Ca^{2+}{}_i$ a través de la PKA. Por otro lado, la PLC hidroliza el fosfatidilinositol bifosfato (PIP2) en diacilglicerol (DAG) e inositol trifosfato (IP3). Así, el IP3 se une a su receptor IP3R localizado en la membrana acrosomal externa (Walensky and Snyder 1995), en la envoltura nuclear redundante (RNE; *redundant nuclear envolture*) y en la región del cuello (Ho and Suarez 2001b; Ho and Suarez 2003). Este receptor controla la apertura de un canal dependiente de voltaje y provoca la salida de Ca^{2+} del espacio intraacrosomal al citoplasma (Revisado por Breitbart y Shabtay (2018). Además, el DAG activa la protein-kinasa C (PKC), que es capaz de abrir canales voltaje y dependientes de Ca^{2+}- presentes en la membrana plasmática, y dar lugar así a un aumento del $Ca^{2+}{}_i$ presente en el citosol (Breitbart et al. 1992; Spungin and Breitbart 1996).

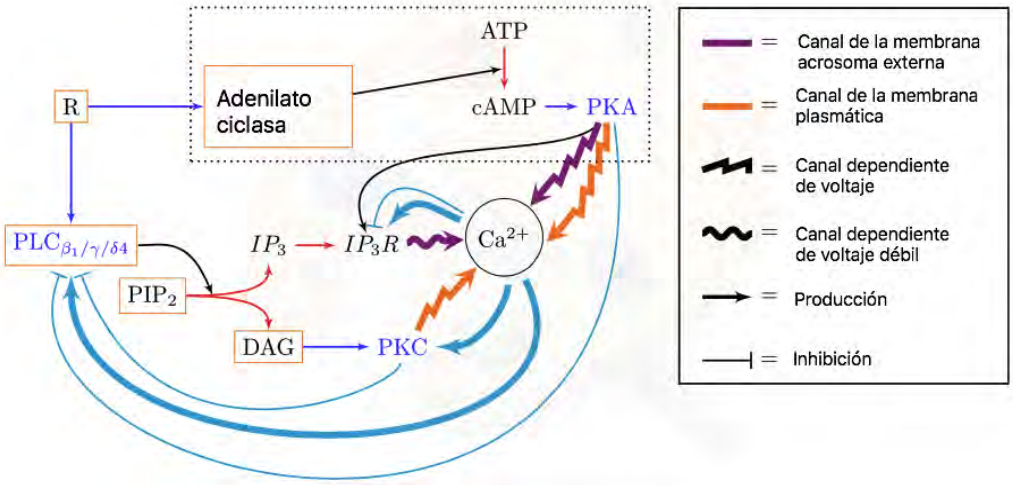


Fig. 2.6.*Diagrama representativo de los mecanismos moleculares de la RA iniciada por la glicoproteína ZP3 o la progesterona tras su unión a receptores acoplados a proteínas G (R).Imagen modificada de (Simons and Fauci 2018).*

Este aumento de calcio intracelular es necesario, pero no suficiente, para que se produzca la RA. Entre otros eventos necesarios se encuentran la despolimerización de la F-actina llevada a cabo por la proteína **gelsolina** (Finkelstein et al. 2010) y la activación de **Epac dependiente de cAMP.** Esta EPAC, a su vez, activa a las **proteínas SNARE** (*soluble SNF attachment protein*), que participan en la fusión de membranas durante la RA (De Blas et al. 2005).

En ocasiones, la RA ocurre prematuramente de manera espontánea y es conocida como **sAR** (*spontaneous Acrosome Reaction*), provocando una baja tasa de fecundación. Se ha propuesto que la polimerización de actina evitaría esta sAR durante la capacitación mediante dos vías diferentes: la vía Ca^{2+}/Calmodulina protein kinasa II (CaMKII) y la vía de PLD (del inglés *phospholipase D*) activadas por las enzimas PKA y IP3K (del inglés *phosohoinositide-3-kinase*) respectivamente (Ackermann et al. 2009; Shabtay and Breitbart 2016).

Durante años, el término "reacción acrosómica" ha sido usado como un proceso con dos únicos estados: espermatozoides con "acrosoma intacto" o con "acrosoma reaccionado". Pero algunos estudios revelan la existencia de estadios intermedios entre ellos, encontrando cuatros subtipos en la especie murina y hasta seis en la especie humana, gracias al uso de microscopios electrónicos de transmisión (Lee and Storey 1985; Yudin et al. 1988). Por ello, algunos autores han adaptado el término "exocitosis acrosomal" para referirse al conjunto de eventos que se producen como un proceso continuo y que se manifiesta en diferentes estadios del espermatozoide con respecto al acrosoma.

2.4 HORMONAS DEL TRACTO REPRODUCTOR FEMENINO

2.4. 1. HORMONAS ESTEROIDEAS: PROGESTERONA Y ESTRADIOL

2.4.1.1. ESTRUCTURA QUÍMICA Y BIOSÍNTESIS

La **progesterona**, o pregnen-4-en-3,20-diona (**P4,** $C_{21}H_{30}O_2$), pertenece a la familia de los progestágenos dentro del grupo de las hormonas esteroideas. Su estructura química consiste en cuatro hidrocarburos cíclicos interconectados con grupos funcionales oxigenados, de cetonas y dos sustituyentes metilos (**Fig. 2.7**)

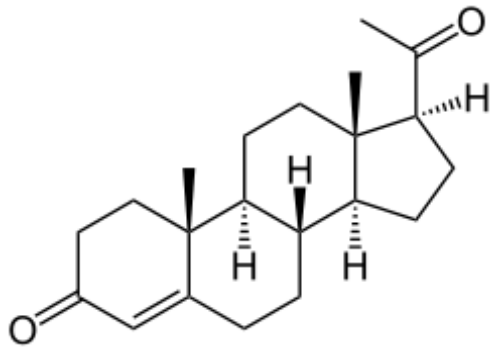


Fig. 2.7. *Estructura química de la progesterona.*

En mamíferos, la P4 se sintetiza a partir de la pregnenolona, que a su vez deriva del colesterol, en el cuerpo lúteo del ovario (Taraborrelli 2015), la placenta durante la gestación (Tuckey 2005)y las glándulas adrenales (Balfour et al. 1957; Tuckey 2005; Taraborrelli 2015). Es una hormona esencial en los ciclos reproductivos y el mantenimiento de la gestación (Taraborrelli 2015; Morel et al. 2016), aunque también actúa en el sistema nervioso, respiratorio y óseo (Bayliss and Millhorn 1992; Schumacher et al. 2014; Rossetti et al. 2016).

El **17β-estradiol, estradiol** o (17β)-estra-1,3,5(10)-triene-3,17-diol (**E2**, $C_{18}H_{24}O_2$) pertenece a la familia de los estrógenos, y se caracteriza por tener dos grupos hidroxilo en su estructura molecular (**Fig. 2. 8**).

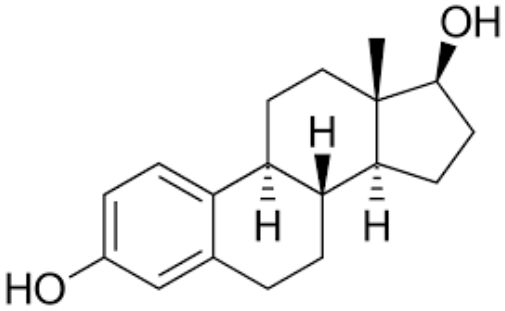


Fig. 2. 8. *Estructura química de 17β-estradiol.*

En mamíferos, el E2 se sintetiza principalmente en las células de la granulosa del folículo ovárico (Vanderhyden and Tonary 1995), pero también en las células de Leyding y Sertoli del testículo (Carreau et al. 2006), por lo que su principal función es reproductiva, aunque también se puede sintetizar en menor medida en el tejido

adiposo y la corteza suprarrenal (Barakat et al. 2016). Al igual que el resto de las hormonas esteroideas, es un derivado del colesterol, aunque en el caso de E2, el intermediario clave es la androstenediona, es convertida a testosterona, y que a su vez se convierte en estradiol gracias a la enzima aromatasa.

2.4.1.2. MECANISMO DE ACCIÓN DE LAS HORMONAS ESTEROIDEAS

Tanto la progesterona como el estradiol ejercen su acción clásica a través de los receptores citosólicos o nucleares, regulando la expresión génica de genes diana (Truss and Beato 1993). El receptor nuclear descrito para progesterona (PR) presenta dos isoformas (PRA, con un peso molecular de 94 kDa y PRB con 120 kDa), que están codificadas por el mismo gen pero transcriptas por diferentes promotores (Grimm et al. 2016).

Por su parte, se han identificado dos receptores de estrógenos funcionales, ERα y ERβ, que comparten un alto nivel de homología en su secuencia de aminoácidos primaria y son muy similares en su estructura terciaria (Nilsson et al. 2001), por lo que tienen una afinidad similar a agonistas y antagonistas (Barkhem et al. 1998).

Junto con la vía de acción genómica o clásica, numerosos estudios recientes han revelado que tanto la P4 como el E2 pueden ejercer su acción sobre distintos tipos celulares de manera no genómica. Estos efectos no genómicos de las hormonas esteroideas se caracterizan por ser demasiado rápidos (de segundos a pocos minutos) para ser compatibles con cambios en el RNAm, y pueden ser observados en células altamente especializadas, como los espermatozoides, que carecen de la capacidad de llevar a cabo la síntesis de proteínas (Revelli et al. 1998). Esta vía de acción no genómica provoca la activación de vías de transducción y de segundos mensajeros (Bishop and Stormshak 2008) y podría estar mediada por los receptores nucleares clásicos localizados en caveolas de la membrana plasmática, por otro tipo de receptores de membrana específicos o bien mediante una ruta independiente de receptores que implicaría modificaciones en la membrana plasmática (**Fig. 2. 9**) (Baldi et al. 2009).

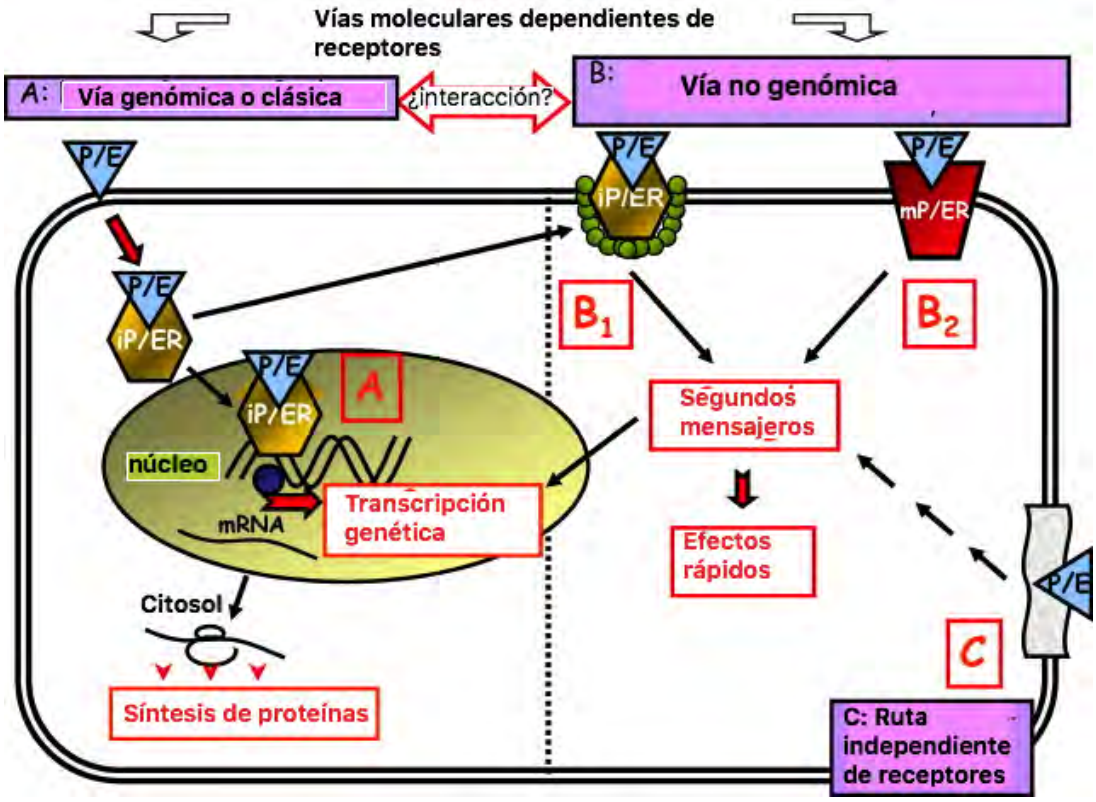


Fig. 2. 9. *Mecanismos de acción propuestos de las hormonas esteroideas en las células. A) Ruta genómica clásica. B) Ruta no genómica regulada por segundos mensajeros, mediada por receptores nucleares clásicos asociado a caveolas de la membrana (B1), mediada por receptores de membrana específicos (B2) o independiente de los receptores (C) Imagen modificada de Baldi (2009).*

2.4.1.3. EFECTO DE LAS HORMONAS ESTEROIDEAS SOBRE EL ESPERMATOZOIDE

Como se ha mencionado anteriormente, tanto la P4 como el E2 se sintetizan en el ovario de los mamíferos. Estas hormonas pueden alcanzar concentraciones en el rango nanomolar en el folículo ovulatorio durante la ovulación (Carson et al. 1981), y parte de este fluido pasa al oviducto junto con el ovocito tras la ovulación. Además, tras la ovulación y ya en el oviducto, las células del *cúmulus* que rodean al ovocito secretan P4 y E2 que pueden alcanzar concentraciones micromolares (Vanderhyden and Tonary 1995; Chian et al. 1999) dando lugar a un gradiente de concentración desde las proximidades del ovocito a zonas más alejadas del mismo (Teves et al. 2006; Sagare-Patil et al. 2012).

En las últimas décadas, numerosos estudios han demostrado la presencia de receptores para estrógenos y progesterona en espermatozoides de numerosas especies. Dentro de los receptores para progesterona, se han identificado tanto los nucleares clásicos, en las especies humana (De Amicis et al. 2011) y porcina (De Amicis et al. 2012), como los no genómicos en espermatozoides humanos (Sabeur et al. 1996; Luconi et al. 1998), equinos (Cheng et al. 1998), caprinos (Somanath and Gandhi 2002) y porcinos (Wu et al. 2006). De igual forma, se han identificado los receptores de estrógenos ERα y ERβ en espermatozoides humanos (Mosselman et al. 1996), equinos (Arkoun et al. 2014) o murinos (Sebkova et al. 2012). Por otro lado, también se han identificado receptores de estrógenos con pesos moleculares diferentes a los habitualmente descritos en la membrana de espermatozoides humanos (Luconi et al. 1999) o porcinos (Wu et al. 2006) y recientemente se ha descubierto un receptor de estrógenos ligado a proteína G (GPER) en espermatozoides porcinos (Rago et al. 2014) y equinos (Arkoun et al. 2014).

Además, también se han realizado numerosos estudios sobre el papel que las hormonas esteroideas ejercen en los diferentes procesos que tienen lugar de manera fisiológica en el tránsito del espermatozoide por el tracto reproductor de la hembra: **la capacitación, la quimiotaxis e hiperactivación y la reacción acrosómica.**

❖ EFECTO DE LAS HORMONAS ESTEROIDEAS SOBRE LA CAPACITACIÓN ESPERMÁTICA

El efecto positivo de la P4 sobre la capacitación espermática sólo se ha demostrado en el espermatozoide humano (Kay et al. 1994; Thundathil et al. 2002) y a concentraciones altas (1-30 µM), mientras que en otras especies sólo se ha demostrado de forma concluyente su acción sobre la RA. La inducción de la capacitación por la P4 parece estar relacionado con un aumento de la producción de ROS (de Lamirande et al. 1998) y la activación de las MAPK, pero no de la vía cAMP/PKA (Thundathil et al. 2002). Concentraciones crecientes de P4 inducen la fosforilación de proteínas de la familia de las MAPK, como la ERK1/2, p38MAPK o c-Jun

N-terminal kinasa 1 (JNK1) y promueve la fosforilación de proteínas en residuos de tirosina (Sagare-Patil et al. 2012).

Por otro lado, el E2 parece inducir la capacitación en espermatozoides de ratón (Adeoya-Osiguwa et al. 2003; Sebkova et al. 2012) y cerdo (Ded et al. 2010), mientras que en otras especies como la humana parece modular o inhibir los efectos de la progesterona (Luconi et al. 1999). En aquellas especies en las que E2 estimula la capacitación, esta hormona produce el aumento de la fosforilación en residuos de tirosinas de proteínas de la cabeza en espermatozoide (Sebkova et al. 2012; Bosakova et al. 2018) aunque también parece suprimir el aumento de fosfotirosinas provocado por la hormona P4 en el espermatozoide de hámster (Fujinoki 2010).

❖ EFECTO DE LAS HORMONAS ESTEROIDEAS EN LA HIPERACTIVACIÓN Y QUIMIOTAXIS

La incubación con P4 parece aumentar la motilidad progresiva (Contreras and Llanos 2001) y la motilidad hiperactivada en espermatozoide humano (Uhler et al. 1992; Jaiswal et al. 1999), de hámster (Noguchi et al. 2008) y de macaco (Sumigama et al. 2015). Sin embargo, otros autores no han podido confirmar este efecto de la progesterona (Wang et al. 2001)

El efecto de la P4 sobre la hiperactivación y la actividad flagelar se ha relacionado, en la especie humana, con un aumento de Ca^{2+}, provocado por su salida de los reservorios intracelulares (Harper et al. 2004), o por la entrada de Ca^{2+} del exterior mediante la activación indirecta de los canales Catsper (Carlson et al. 2003; Marquez and Suarez 2007) a través de la enzima serin-hidrolasa *α/β hydrolase domain–containing protein 2* (ABH2) (Mannowetz et al. 2017). Tras su estimulación por la P4, ABHD2 hidroliza los endocanabinoides 2-araquidonoglicerol (2AG) y 1-araquidonoglicerol (1AG), que actúan como inhibidores del canal CatSper. Así, esta hidrólisis, junto con la alcalinización intracelular, produce la apertura del canal CatSper y la hiperactivación espermática (Miller et al. 2016).

En el caso del espermatozoide de hámster, sin embargo, se ha descrito que la P4 estimularía el proceso de hiperactivación mediante dos vías diferentes (**Fig. 2.10**): la

unión de la P4 a su receptor activaría la fosfolipasa C (LPC), que aumentaría la producción de IP3 y diacilglicerol (DAG), que a su vez produciría la liberación de Ca^{2+} intracelular de los reservorios intracelulares y la activación de la PKC; o bien a través de la activación de la vía cAMP/PKA, la producción de cAMP y activación de la enzima PKA (Fujinoki et al. 2016).

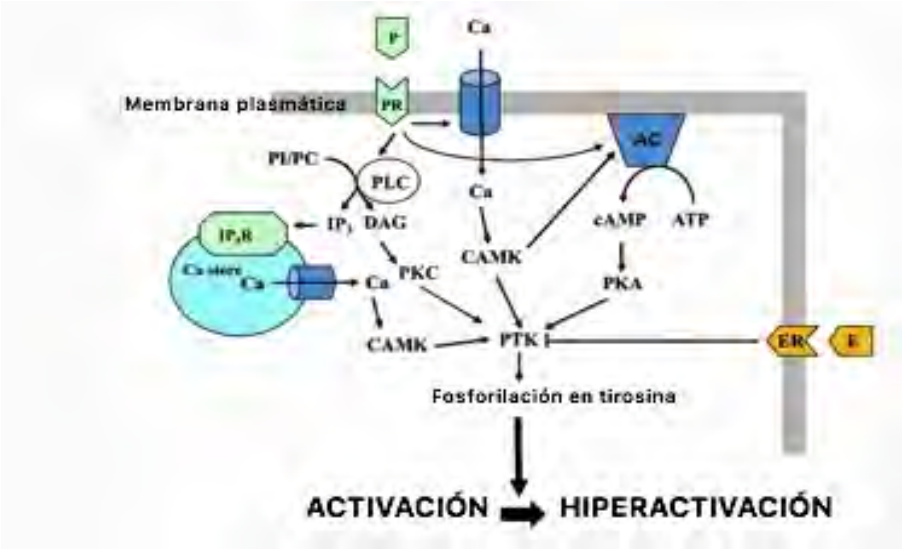


Fig. 2.10. *Representación gráfica del posible mecanismo de regulación de la hiperactivación por progesterona y estradiol en espermatozoide de hamster (Fujinoki et al. 2016).*

En cuanto al efecto del E2 sobre la **motilidad e hiperactivación,** esta hormona parece aumentar la motilidad en espermatozoide humano (Guido et al. 2011) y bovino (Çiftci and Zülkadir 2010) y la hiperactivación en ratón (Bosakova et al. 2018). Sin embargo, otros estudios no encontraron ningún efecto del E2 sobre la motilidad en espermatozoide humano (Wang et al. 2001) o incluso un efecto negativo en espermatozoide equino (Gautier et al. 2016). En otras especies, como en hámster, parece suprimir el proceso de hiperactivación provocado por la P4 (Fujinoki 2010).

Como se ha comentado, las hormonas esteroideas están presentes en el fluido folicular y son secretadas por las células del *cúmulus* (Vanderhyden and Tonary 1995), dando lugar a un gradiente de concentración desde las proximidades del ovocito a zonas más alejadas del mismo. Dado que la quimotaxis es el movimiento guiado en respuesta a un gradiente de una sustancia, se ha postulado un posible **papel**

quimiotáctico para estas hormonas. En el caso de la P4, sólo se ha demostrado este efecto sobre espermatozoides humanos, evaluando la acumulación celular (Jaiswal et al. 1999; Wang et al. 2001), o cambios en la orientación espermática (Teves et al. 2006). Sin embargo, no se tiene evidencia de un efecto quimioatrayente del E2 sobre espermatozoides humanos, que es la única especie en la que se ha estudiado (Wang et al. 2001).

❖ EFECTO DE LAS HORMONAS ESTEROIDEAS EN LA REACCIÓN ACROSÓMICA

La RA puede ser inducida por diferentes moléculas presentes en el tracto reproductor femenino como bien hemos mencionado anteriormente, siendo la P4 la más estudiada a día de hoy. En la especie humana, el efecto inductor de la RA por la P4 fue descrito por primera vez por Osman *et al.* (1989) y confirmado posteriormente por otros autores (Uhler et al. 1992; Sabeur et al. 1996). Este mismo efecto también se ha identificado en especies domesticas como el ratón (Roldan et al. 1994), cerdo (Melendrez et al. 1994), caballo (Meyers et al. 1995), cabra (Somanath et al. 2000) o el toro (Therien and Manjunath 2003).

Como hemos explicado anteriormente, la RA implica la apertura de canales de Ca^{2+} presentes tanto en la membrana plasmática como en la membrana acrosomal externa y RNE. En este contexto, la P4 es capaz de inducir la RA mediante diferentes vías a través del aumento del Ca^{2+} intracelular (Baldi et al. 1991; Romarowski et al. 2016) o también puede darse de manera rápida y dependiente del Ca^{2+} extracelular (Blackmore et al. 1991; Darszon et al. 2011) mediante el canal CatSper (Tamburrino et al. 2014; Rehfeld et al. 2016). Por otro lado, la RA mediada por P4 en espermatozoide humano también está estimulada mediante proteín-fosfatasas y quinasas como PP2B (del acrónimo en inglés serine /threonine-protein phosphatase 2B), PKC y PKA (Baron et al. 2016).Además, la P4 también puede activar la proteína Epac dependiente de cAMP, y provocar la liberación de Ca^{2+}desde el acrosoma vía Rap1/PLC/IP3 (Lucchesi et al. 2016) a través del receptor de IP3 (Li et al. 2019).

Mientras que el efecto inductor de la P4 sobre la RA ha sido ampliamente estudiado, hay muchos menos estudios sobre el papel del E2 en este proceso. Algunos indican que esta hormona no tiene ningún efecto sobre la RA (Francavilla et al. 2003), pero la mayoría apuntan al efecto inhibidor del E2 sobre la RA inducida por P4 (Luconi et al. 1999; Baldi et al. 2000b; Vigil et al. 2008), evitando el aumento de Ca^{2+} intracelular y la fosforilación de proteínas en tirosinas (Luconi et al. 1999; Sebkova et al. 2012). Sin embargo, también existen otros trabajos que concluyen que esta hormona también es capaz de provocar por si misma la inducción de la RA, al menos en el ratón(Bosakova et al. 2018).

2.4.2. MELATONINA

2.4.2.1. ESTRUCTURA Y BIOSÍNTESIS

La melatonina es una hormona descubierta en 1917 por *MacCord y Allen* (1917) y aislada por primera vez a finales de los años 50 por *Lerner* y col. en pinealocitos bovinos (Lerner et al. 1960). Se trata de una indolamina denominada **N-acetil-5-metoxitriptamina (Fig. 2. 11).**

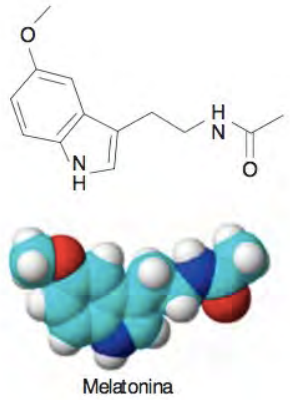


Fig. 2. 11. *Estructura química de la melatonina.*

La síntesis de melatonina comienza con la captación del aminoácido triptófano del torrente sanguíneo. Mediante un proceso de hidroxilación y decarboxilación, este aminoácido se convierte en serotonina que durante el periodo de oscuridad, es transformado por la enzima arilalquilamina N-acetil transferasa (ANNAT) en N-acetilserotonina. Este compuesto a su vez es metilado por la enzima N-acetilserotonina O-metiltransferasa (ASMT) dando lugar a la melatonina ((Boutin et al. 2005). Esta molécula es poco soluble en agua, pero soluble en etanol y otros solventes orgánicos como el dimetilsulfóxido (DMSO), por lo que tiene la facilidad de atravesar la membrana celular de forma pasiva (Yu et al. 2016).

Esta hormona se encuentra presente en todos los taxones filogenéticos incluyendo bacterias, eucariotas unicelulares, algas, plantas, invertebrados y animales vertebrados (Reiter et al. 2013b). En vertebrados la melatonina es secretada durante la noche por los pinealocitos de la glándula pineal de forma que se produce un pico máximo de concentración nocturno y unos niveles basales diurnos (Rollag and Niswender 1976). Por ello a la melatonina también se la conoce como la **hormona de la oscuridad**. Además, su secreción está regulada por ritmos circadianos y circanuales (Reiter 1991) de forma que su secreción varía en función de la duración de las noches a lo largo del año (**Fig. 2. 12**).

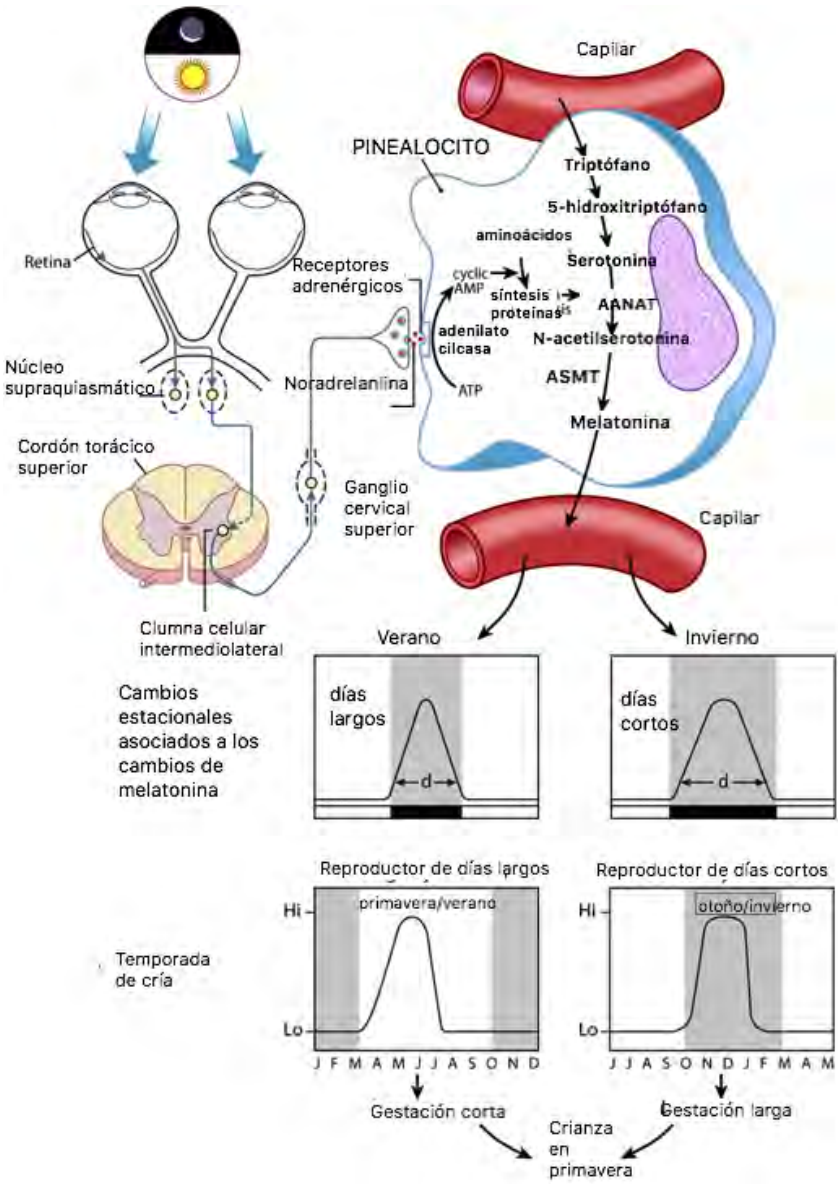


Fig. 2. 12. *Representación de la regulación luz/oscuridad de la producción de melatonina en mamíferos (Imagen de Reiter (2009)) .*

En los últimos años se ha demostrado la síntesis extrapineal de melatonina durante las horas diurnas en el tracto gastrointestinal (Huether 1993), retina (Reiter 1996; Tosini and Menaker 1996), células del sistema inmune (Carrillo-Vico et al. 2004), médula ósea (Tan et al. 1999), bazo, hígado, riñón y corazón (Sanchez-Hidalgo et al. 2009), piel (Slominski et al. 2008) e incluso en el folículo ovárico (Brzezinski et al. 1987). Sin embargo, esta melatonina extrapineal no se secreta al torrente sanguíneo ni parece seguir un ritmo circadiano, lo que sugiere que su función podría estar

relacionada con un papel antioxidante en el tejido en el cual se produce (Tan et al. 2003).

Además de su gran poder antioxidante, gracias a su capacidad de bloquear los efectos de los radicales libres de oxígenos (ROS) y de nitrógeno (RNS) (Reiter et al. 2009), la melatonina controla un gran número de funciones fisiológicas, entre las que destacan el metabolismo de azúcares y lípidos en adipocitos (Alonso-Vale et al. 2008), la regulación del sistema inmune (Carrillo-Vico et al. 2006), la regulación de procesos reproductivos mediante la modulación de la síntesis y secreción de las hormonas gonadotrópicas (Arendt 1998; Malpaux et al. 2001), además de un efecto quimiotáctico en células del epitelio pigmentario retinal (Shirakawa and Ogino 1987) y en leucocitos (Peña et al. 2007), e incluso un papel modulador del citoesqueleto (Benitez-King 2006).

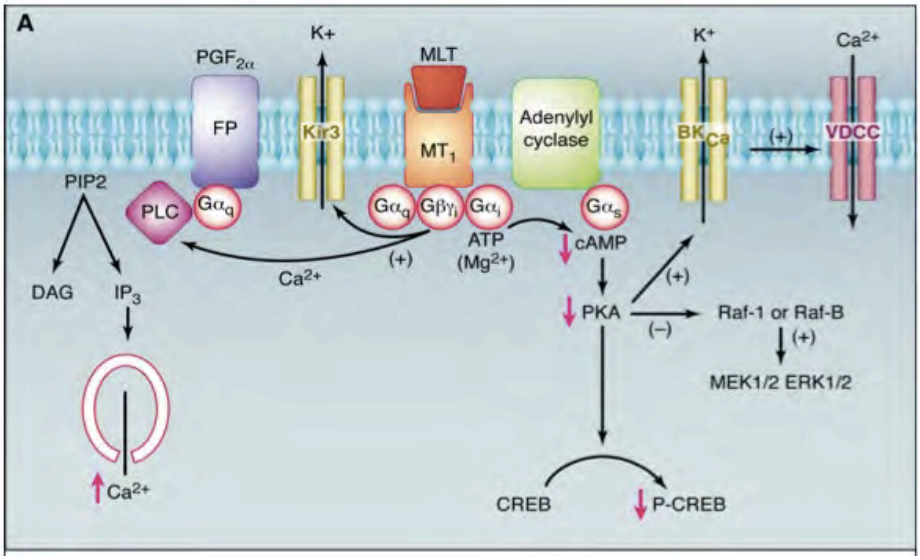
2.4.2.2. MECANISMO DE ACCIÓN DE LA MELATONINA

El mecanismo de acción de la melatonina es complejo y, a excepción de su papel antioxidante, sus acciones principales parecen estar mediadas por su unión receptores específicos de membrana. Los receptores de membrana para la melatonina descritos en mamíferos se denominan **MT_1 y MT_2**, y pertenecen a la familia de receptores acoplados a proteínas G (GPCR, *G protein-coupled receptors*), también conocidos como 7TM por estar constituidos por siete dominios transmembrana (Reppert 1997). Ambos receptores contienen una alta homología en su secuencia amino-terminal y, en células somáticas que expresan ambos receptores, estos tienen mayor tendencia a formar heterodímeros MT_1/MT_2 u homodímeros de MT_1 que homodímeros de MT_2 (Ayoub et al. 2002). Además, se ha descrito un tercer receptor de la melatonina, inicialmente denominado receptor MT_3, (Ebisawa et al. 1994) presente en aves, anfibios, peces y en algunos mamíferos como el hámster (Nosjean et al. 2000) y que se ha identificado como la quinona reductasa 2 (Boutin and Ferry 2019).

Los receptores MT_1 y MT_2 se encuentran presentes en el sistema nervioso central y en tejidos periféricos como vasos sanguíneos, medula ósea, riñón, páncreas y corteza adrenal (Dubocovich and Markowska 2005). En la hembra, estos receptores también

se han detectado en los complejos *cúmulus*-ovocitos, células de la granulosa y en el cuerpo lúteo del ovario y en la placenta(Reiter et al. 2013a). En el aparato reproductor masculino estos receptores se han identificado en el testículo (Frungieri et al. 2005; Izzo et al. 2010) y en células epiteliales de próstata (Gilad et al. 1996). En la especie ovina, nuestro grupo de investigación identificó recientemente ambos receptores en testículo, ampolla, vesícula seminal y conducto deferente (Gonzalez-Arto et al. 2017).

Las vías de transducción de señal desencadenadas por la unión de melatonina a sus receptores han sido ampliamente estudiadas en células somáticas (Jockers et al. 2008). Tras su unión a ambos receptores se produce una disminución de la producción de cAMP (Reppert et al. 1994; Browning et al. 2000) y de la actividad de la enzima PKA (Witt-Enderby et al. 2000), que a su vez da lugar a la disminución de la proteína CREB (*cAMP response element-binding*)**,** y, en el caso de MT1, a la fosforilación de las MEK1, MEK2 y ERK1/2 (Witt-Enderby et al. 2000). La unión de la melatonina a ambos receptores también produce la activación de la enzima LPC (Masana and Dubocovich 2001), que convierte PIP2 en DAG e IP3 **(Fig. 2.13),** provocando la salida de calcio de reservorios intracelulares, y aumentando la actividad de la PKC en el caso de MT_2 (Hunt et al. 2001). Finalmente, la unión de melatonina al receptor MT_2, inhibe la producción de cGMP (cyclic Guanosine monophosphate) (Petit et al. 1999) (**Fig. 2.13b).**



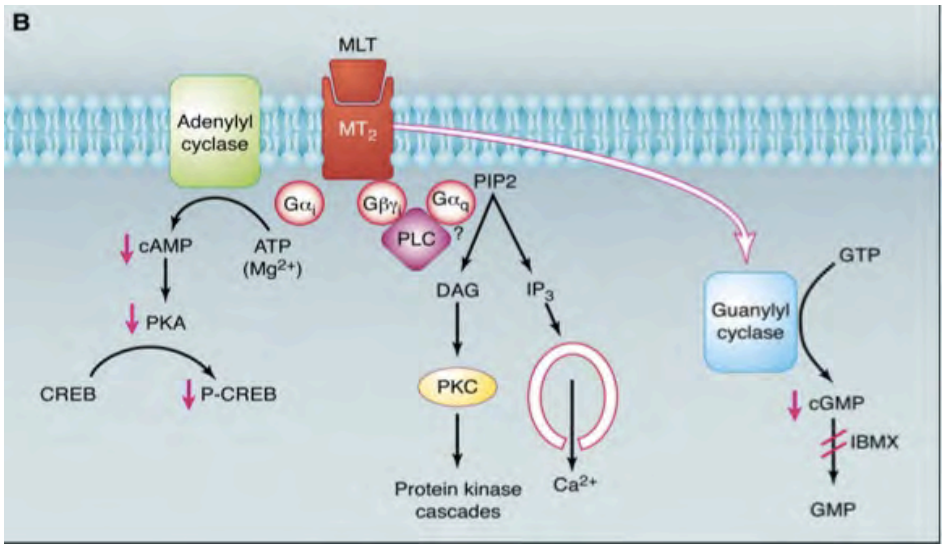


Fig. 2.13. *Representación de las principales vías de transducción de señales reguladas por el receptor MT_1* ***(A)*** *y por el receptor MT_2* ***(B)*** *(Masana and Dubocovich 2001).*

Por otro lado, gracias a su naturaleza anfipática, la melatonina también tiene la capacidad de atravesar la membrana plasmática fácilmente y unirse a diferentes proteínas de unión a Ca^{2+}, entre las que se encuentran la calreticulina, la cual se relaciona con cambios a nivel nuclear (Macias et al. 2003) y la calmodulina, relacionada con la regulación del citoesqueleto. En células somáticas, la unión de la melatonina a la calmodulina, inhibiría la formación del complejo calcio-calmodulina (Benitez-King 2006) y, por tanto, daría lugar a la inhibición de la actividad de enzimas como la calcio calmodulina kinasa II (CaMKII).

Todas estas vías de señalización activadas por la melatonina, también están implicadas en la fisiología espermática, en concreto en la capacitación espermática y en la RA (Breitbart 2002; Almog et al. 2008; Breitbart and Shabtay 2018), por lo que la melatonina podría jugar un papel regulador importante en estos fenómenos.

2.4.2.3. EFECTO MODULADOR DE LA MELATONINA SOBRE LA ESTACIONALIDAD REPRODUCTIVA EN LA ESPECIE OVINA

La melatonina juega un papel primordial en el control de la reproducción en animales estacionales, sincronizando la actividad reproductiva con los cambios ambientales a través del eje hipotálamo-hipofisario-gonadal (Gerlach and Aurich

2000). En el caso de la especie ovina, esta hormona estimula la reproducción durante la época de días cortos, es decir, durante el otoño e invierno (Malpaux et al. 1997), para que los partos se concentren en primavera-verano. De este modo, la melatonina actúa como hormona "progonadal" ya que estimula la liberación de GnRH (*Gonadotropin-Releasing Hormone*) en el hipotálamo, lo que provoca a su vez la secreción de la hormona lutenizante (LH), responsable de la actividad ovárica en la hembra y de la producción de espermatozoides en el macho (Malpaux et al. 1997; Misztal et al. 2002).

En la especie ovina, la estacionalidad es más marcada en la hembra que en el macho, que mantiene una producción continua de espermatozoides a lo largo de todo el año. Sin embargo, durante la época no reproductiva, en el morueco se observa una disminución de la calidad espermática, la fertilidad, la libido, así como del peso y volumen testicular (Karagiannidis et al. 2000; D'Alessandro and Martemucci 2003; Avdi et al. 2004). Estos cambios están relacionados con la reducción de la actividad del eje hipotálamo-hipofisario-gonadal (Lincoln et al. 1990) causado por la baja liberación de melatonina por la glándula pineal durante este periodo (Sheikheldin et al. 1992).

Sin embargo, el tratamiento con melatonina exógena, generalmente mediante implantes subcutáneos, durante la época no reproductiva en sementales ovinos, puede revertir los efectos de los días largos sobre los niveles hormonales(Lincoln and Ebling 1985; Webster et al. 1991; Kokolis et al. 2000), el comportamiento sexual (Rosa et al. 2000), la calidad seminal y parámetros testiculares (Tekpetey and Amann 1988; Coyan 1998; Kaya 2000) y dar lugar a un aumento de la espermatogénesis y la fertilidad (Palacín 2008; Casao et al. 2010c; Rosa 2012). El tratamiento con melatonina exógena también provoca en un aumento en los niveles de testosterona y estradiol en el plasma seminal, además de un incremento de la actividad enzimática de la glutatión peroxidasa y glutatión reductasa, lo que se relacionaría con su papel antioxidante (Casao et al. 2013) y la mejora de la calidad espermática *in vivo*.

2.4.2.4. .EFECTO DE LA MELATONINA EN EL ESPERMATOZOIDE

Numerosos estudios han profundizado en el efecto directo de la melatonina en los espermatozoides de diversas especies, ya que trabajos previos habían descrito su presencia tanto en el plasma seminal (Luboshitzky et al. 2002; Casao et al. 2010a) como en el TRF (Brzezinski et al. 1987), por lo que esta hormona podría regular la funcionalidad de estas células durante su tránsito por el TRF.

Además, se ha descrito que el tratamiento i*n vitro* con melatonina produce un aumento de la motilidad en espermatozoide humano (Ortiz et al. 2011) y una mejora de los parámetros de calidad seminal en ovino (Casao *et al.* 2010c) y porcino (Casao et al. 2010c; Jang et al. 2010). La melatonina, además, produce una disminución del daño oxidativo y de los niveles intracelulares de ROS y NO (Rao and Gangadharan 2008; du Plessis et al. 2010; Jang et al. 2010), así como una reducción de la peroxidación lipídica de la membrana plasmática (Gadella et al. 2008; du Plessis et al. 2010), una disminución de los marcadores apoptóticos (Casao et al. 2010b; Espino et al. 2011) y de la fragmentación de ADN (Sarabia et al. 2009; Espino et al. 2011).. También se ha usado como un aditivo para la refrigeración y congelación de semen (Martin-Hidalgo et al. 2011; Ashrafi et al. 2013).

Todos estos efectos beneficiosos de la melatonina en el espermatozoide han sido identificados tanto en especies no estacionales (humano y porcino) como en especies estacionales de días largos (caballo) y días cortos (ovino y ciervo), lo que sugiere que su acción sobre estas células no estaría relacionada únicamente con la regulación del eje hipotálamo-hipofisario-testicular. El efecto directo de la melatonina sobre el espermatozoide se ha relacionado con su actividad antioxidante y su habilidad para atravesar la membrana plasmática, disminuyendo los niveles de ROS y NOS en el medio intra y extracelular (Reiter et al. 2000), aunque también los receptores para melatonina MT_1 y MT_2 podrían estar implicados.

Los receptores de melatonina MT_1 y MT_2 fueron descritos por primera vez en la membrana de los espermatozoides humanos (van Vuuren et al. 1992). Posteriormente,

estudios de nuestro grupo demostraron también su presencia en espermatozoides de morueco, burro, caballo, perro, ciervo, cerdo y toro (Casao et al. 2012; Gonzalez-Arto et al. 2016b), lo que parece indicar que la melatonina podría tener un papel importante en la reproducción y fertilidad de muchas especies de mamíferos.

Estudios previos de nuestro grupo han demostrado que el tratamiento *in vitro* de melatonina modula la capacitación espermática en la especie ovina, actuando como agente capacitante o descapacitante a concentraciones bajas y altas respectivamente (Casao et al. 2010b). También se ha observado que promueve la capacitación en otras especies como el búfalo (Di Francesco et al. 2009), aumenta el porcentaje de espermatozoides vivos con acrosoma intacto en la especie porcina (Martin-Hidalgo et al. 2011) e induce la hiperactivación en espermatozoides de hámster (Fujinoki 2008).

El efecto dual de la melatonina sobre la capacitación espermática observada en la especie ovina podría deberse a sus propiedades antioxidantes. Las altas concentraciones de melatonina presentes en el plasma seminal podrían proteger al espermatozoide del daño oxidativo (Reiter et al. 2009) eliminando ROS, al igual que actúan enzimas como la superóxido dismutasa o la catalasa (Drevet 2006). Para que se produzca la capacitación son necesarios unos niveles moderados de ROS, ya que actúan como segundos mensajeros en este proceso (de Lamirande and O'Flaherty 2008). Por tanto, al eliminar las ROS, la melatonina a alta concentración es capaz de inhibir la capacitación espermática. Una vez en el TRF, el espermatozoide en el oviducto estaría expuesto a melatonina a bajas concentraciones, presente en el fluido folicular o procedente del folículo ovulatorio (Tamura et al. 2013) . Esta concentración baja de melatonina no eliminaría totalmente las ROS a nivel extracelular, y por tanto no inhibiría la capacitación. Pero la identificación de los receptores de melatonina en el espermatozoide ovino sugiere que esta hormona también podría actuar sobre la capacitación espermática a través de ellos. De hecho, un estudio reciente de nuestro grupo sugiere que la melatonina modularía la capacitación espermática a través del receptor MT_2 (Gonzalez-Arto et al. 2016a), aunque el mecanismo molecular implicado todavía no se conoce completamente.

En células somáticas, la activación de los receptores MT_1 y/o MT_2, como se ha comentado anteriormente, disminuye la producción de cAMP) (Morgan 1989) y la actividad de enzimas como JNK (Chan et al. 2002), PKA (Witt-Enderby et al. 1998; Jockers et al. 2008) y PKC (Hunt et al. 2001) y la fosforilación de MEK1, MEK2, ERK1 y ERK2 (Witt-Enderby et al. 2000). Todas estas moléculas intervienen en la capacitación y la reacción acrosómica (Almog et al. 2008; Iwanaga et al. 2008; Breitbart et al. 2010), por lo que la melatonina podría estar ejerciendo su acción sobre el espermatozoide actuando sobre ellas. Pero, por otro lado, también podría modular la capacitación a través de sus receptores vía señalización del Ca^{2+}, ya que se ha observado que la melatonina modula la movilización de Ca^{2+} intracelular en enterocitos duodenales a través del receptor MT_2 (Sjoblom and Flemstrom 2003). Otra posible vía de acción sería mediante su unión a la proteína calmodulina (Benitez-King et al. 1993; Pozo et al. 1997) implicada en la capacitación espermática ovina (Colas et al. 2009), así como en la hiperactivación (Si and Olds-Clarke 2000), capacitación y RA (Bendahmane et al. 2001; Tulsiani et al. 2007) en otras especies.

A la vista de estos antecedentes, se hace necesario profundizar en los mecanismos moleculares por los que la melatonina ejerce su acción sobre la capacitación espermática.

3. OBJETIVOS

3. OBJETIVOS

El objetivo central de esta tesis doctoral fue profundizar en el papel que ejercen algunas hormonas presentes en el tracto reproductor femenino, en concreto la progesterona, el 17-β estradiol y la melatonina, sobre la funcionalidad del espermatozoide ovino, investigando los mecanismos moleculares mediante los cuales ejercen su acción.

En los últimos años, numerosos estudios han demostrado la presencia de los receptores para estrógenos y progesterona en espermatozoides de varias especies. Se han identificado tanto los receptores nucleares clásicos (los implicados en las acciones genómicas de estas hormonas) (Mosselman et al. 1996; De Amicis et al. 2011), como otros receptores (denominados no clásicos) para ambas hormonas (Sabeur et al. 1996; Luconi et al. 1998; Luconi et al. 1999). Respecto a la progesterona, se ha descrito su implicación en la capacitación en espermatozoide humano (Baldi et al. 2009) y en el proceso de hiperactivación en humano, hámster y macaco (Uhler et al. 1992; Noguchi et al. 2008; Sumigama et al. 2015). Además, se ha demostrado su efecto inductor de la reacción acrosómica en espermatozoides de múltiples especies (Baldi et al. 2009). En relación al 17-β estradiol, se ha descrito su participación en la capacitación en espermatozoides de ratón (Adeoya-Osiguwa et al. 2003; Sebkova et al. 2012) y cerdo (Ded et al. 2010), y su implicación en la hiperactivación y RA del espermatozoide de ratón(Bosakova et al. 2018) . Sin embargo, hasta la fecha no se han descrito los receptores para estrógenos y progesterona en el espermatozoide de morueco ni se conoce la implicación de ambas hormonas en los procesos fisiológicos que sufre el espermatozoide ovino durante su tránsito por el TRF.

En cuanto a la melatonina, en estudios previos de nuestro grupo de investigación se describió la presencia de receptores MT_1 y MT_2 en la membrana del espermatozoide ovino (Casao et al. 2012). Asimismo, nuestro grupo demostró que la melatonina era capaz de modular la capacitación espermática en el espermatozoide ovino, actuando como agente capacitante o descapacitante a concentraciones bajas y altas respectivamente, así como de disminuir los marcadores apoptóticos (Casao et al.

2010b). Además, otros grupos han descrito que la melatonina es capaz de provocar una disminución del daño oxidativo en espermatozoides humanos y porcinos (du Plessis et al. 2010; Jang et al. 2010). Sin embargo, hasta la fecha, se desconocen los mecanismos moleculares implicados en la acción de la melatonina sobre los espermatozoides, y especialmente sobre la capacitación espermática ovina.

A la vista de estos antecedentes, en esta tesis se propusieron los siguientes objetivos concretos:

1. Investigar la presencia y localización de los posibles receptores de progesterona y 17-β estradiol en el espermatozoide ovino *(Artículo 1).*

2. Determinar la implicación de ambas hormonas esteroideas, progesterona y 17-β estradiol, en la funcionalidad del espermatozoide ovino *(Artículo 1).*

3. Descubrir si las acciones que ejercen la progesterona y el 17-β estradiol sobre el espermatozoide ovino están mediadas por su unión a receptores *(Artículo 2).*

4. Profundizar en los mecanismos moleculares por los que las hormonas esteroideas ejercen sus efectos sobre el espermatozoide ovino *(Artículo 3).*

5. Investigar los mecanismos moleculares por los que la melatonina ejerce su acción dual capacitante/descapacitante sobre el espermatozoide ovino *(Artículo 4).*

4. MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. REAGENTS

Unless otherwise stated, all the reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA (a subsidiary of Merck KGaA),Darmstadt, Germany) .

4.2. SEMEN COLLECTION AND HANDLING

4.2.1. SEMEN COLLECTION

All experiments were performed with fresh semen obtained from nine mature *Rasa Aragonesa* rams using an artificial vagina. The rams, aged 2-6 years old, belonged to the National Association of *Rasa Aragonesa* Sheep Breeders (ANGRA) and were housed under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza (Spain). All experimental procedures was performed under Project License PI19/17, approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. Second ejaculates of each ram were collected every two days and pooled to avoid individual differences (Ollero et al. 1996). Samples were kept at 37 °C until sperm processing.

4.2.2. REMOVAL OF SEMINAL PLASMA IN SEMEN SAMPLES: THE DEXTRAN/SWIM-UP METHOD

The removal of seminal plasma is necessary due to its harmful effects on sperm viability and fertility (Mortimer 1994) and the presence of steroids hormones and melatonin (Casao et al. 2010a; Casao et al. 2013) that could interfere with the experimental results. A seminal plasma-free sperm population was obtained by a dextran/swim-up procedure, a method developed in our laboratory by García-López et al. (1996) based on a technique described by Alvarez et al. (1993) for human spermatozoa. The swim-up medium (SM) was composed by 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM

$MgSO_4$, 0.33 mM sodium pyruvate, 0.3 mM K_2HPO_4, pH 6.5 (adjusted by NaOH addition). To prevent premature capacitation of sperm cells, $CaCl_2$ and $NaHCO_3$ were omitted in the SM medium, as described by Grasa et al. (2004).

In the dextran/swim-up method, 500 µL of raw semen were carefully placed in the bottom of a rounded 15 mm diameter tube. Semen was covered by 500 µL of SM-Dextran (30 mg dextran/ml SM), which was carefully overlaid by 1.5 ml SM-A (5 mg BSA/ml MS) (**Fig. 4.1**). After 15 minutes of incubation at 37 ° C, 750 µL of the top layer containing spermatozoa were removed and replaced by 750 µL of fresh SM-A medium.The incubation sequence was repeated three times, and four supernatants were obtained. The first supernatant was discarded due to its high content in low-quality spermatozoa. The following three top layers were combined to obtain 2.25 mL of seminal plasma-free sperm sample, enriched in highly viable and motile spermatozoa (García-López et al. 1996).

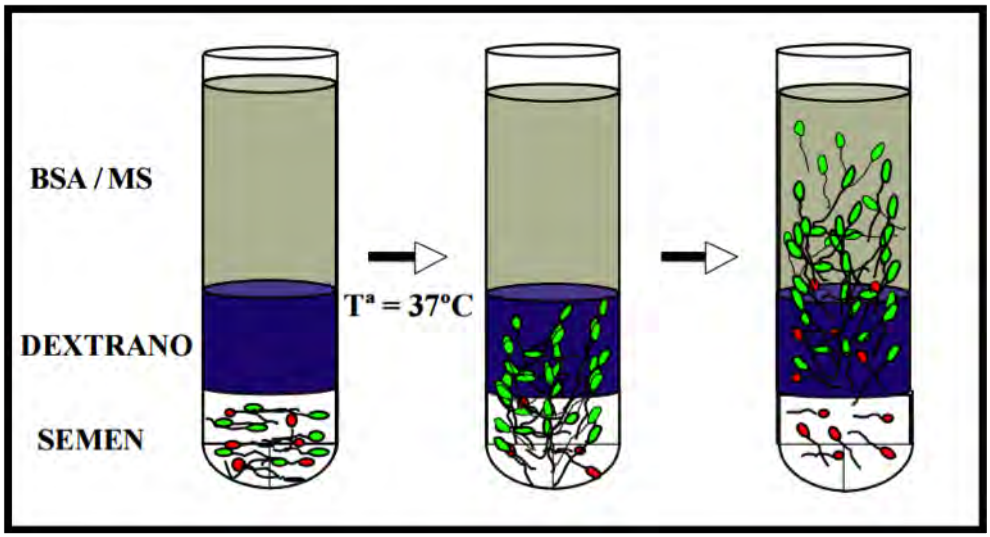


Fig. 4.1. *Schematic representation of the dextran/swim-up procedure.*

The swim-up sample was named “control 0h” in *Article 1* and *Article 4*.

4.2.3. INDUCTION OF THE *IN VITRO* CAPACITATION

In order to induce *in vitro* capacitation, aliquots of swim-up selected spermatozoa (1.6 x10^8 cells/mL) were incubated for 3 hours at 39 °C in an incubator with 5% CO_2 in air and 100% humidity. Incubations were performed in TALP (Tyrode´s Albumin Lactate Pyruvate) medium (Parrish et al. 1988). TALP medium was composed of 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 3 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose and 5 mg/ml bovine serum albumin, pH 7.3 (adjusted with NaOH). A cocktail of cAMP elevating compounds, already proven for capacitating ram spermatozoa (Grasa et al. 2006; Colas et al. 2008) composed of dibutyryl-cAMP (db-cAMP, 1 mM), caffeine and theophylline (both inhibitors of phosphodiesterases, 1 mM each), okadaic acid (OA, a broad spectrum phosphatase inhibitor, 0.2 µM), and methyl-β-cyclodextrin (M-β-CD, 2.5 mM) was added to the TALP medium. The sperm sample capacitated by the addition of these compounds was named "cocktail" in *Article 1, Article 3 and Article 4* or "capacitated control" (Cap-C) in *Article 2*. A non-capacitated sample, maintained under the same conditions as the capacitated samples but without the c-AMP elevating agents, was named "control" (*Article 2 and Article 3*) or "control 3h" (*Article 1 and Article 4*).

4.2.4. INDUCTION OF THE *IN VITRO* ACROSOME REACTION

The acrosome reaction was induced by the addition of lysophosphatidylcholine (LPC) after 3 h of incubation in capacitating conditions. To stimulate *in vitro* acrosome reaction, 5 µL of LPC (300 µg/ml) were added to 95 µl of 1.6 x 10^8 cells/mL capacitated aliquots (Gomez et al. 1997) and incubated at 39 °C, 5 % CO_2 in air and 100% humidity for 20 min. The acrosome reacted sperm sample was named "AR" in *Article 1* or "LPC" in *Article 3.*

4.2.5. EXPERIMENTAL DESIGN

Melatonin, progesterone (P4) and 17-β estradiol (E2) were diluted in DMSO and PBS and added to the sperm samples to yield a final concentration of 1 µM, 10 nM and 100 pM. The final concentration of DMSO in all the samples was 0.1%. Thus, the same DMSO concentration was included in control and capacitated control samples.

In order to determine the involvement of P4 and E2 receptors (PR and ER) on sperm functionality, swim-up-selected spermatozoa were *in vitro* capacitated in the presence of either steroid hormones (1 µM), receptor agonists or antagonists. Assayed receptor agonist were tanaproget (5-(4,4-dimethyl-2-sulfanylidene-2,4-dihydro-1H-3,1-benzoxazin-6-yl)-1-methyl-1H pyrrole-2-carbonitrile, NSP-989) for PR, at 1 µM or 5 µM (Zhang et al. 2005) and resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol, at 5 µM or 10 µM (Li et al. 2018). Used antagonists were mifepristone ((8S,11R,13S,14S,17S)-11-(4-(dimethylamino)phenyl)-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta(a)phenanthren-3-one, RU-486) for PR, at 4 µM or 40 µM (Ko et al. 2014) and tamoxifen ((2-{4-[(1Z)-1,2-diphenylbut-1-en-1-yl]phenoxy}ethyl)dimethylamine) for ER at 5 µM or 10 µM (Morley and Whitfield 1994; Jan et al. 2000) . After the first hour of incubation with the antagonists, steroid hormones (P4 or E2) were added to the samples.

Receptors agonists and antagonists were also diluted in DMSO and PBS, so the final concentration of DMSO in all the samples was 0.1%.

4.3. EVALUATION OF SPERM SAMPLES

4.3.1. CONCENTRATION

Sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany). Dilutions were 1/2000 (v/v) for raw semen and 1/100 (v/v) for swim-up samples. A drop of 6 µL was loaded in a Neubauer's chamber covered by a coverslide. Samples were examined at 100X magnification with a contrast phase microscopy. Cells in 0.1 mm^3 were counted and the following formula was applied:

$$\frac{counted\ spermatozoa}{10000\ ml} \times \frac{1}{dilution} = spermatozoa/ml$$

4.3.2. MOTILITY ANALYSIS

Motility and kinematic parameters were analysed by computer-assisted semen analysis (CASA), using two different software: a commercial one, ISAS (version 1.2.; Proiser SL, Valencia, Spain, *Article 1, Article 2 and Article 4*) and an open source software named OpenCASA (Alquézar-Baeta et al.) (*Article 3*).

In the motility analysis, unless otherwise specified, an aliquot of 6 µL of each sample was placed onto a pre-warmed microscope slide and then covered with 22 x 22 mm pre-warmed coverslip. Samples were kept at 37 °C during all the analysis on a heated slide holder. Two drops of each sample were studied and five fields of each drop were recorded. The recording was performed at 25 frames/s for 1 second with a videocamera (Basler A312f, Basler Vision Components, Exton, PA, USA) mounted on a microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan). The microscope was equipped with a 10x negative-phase contrast lens.

Using the ISAS CASA system, percentage of total motile and progressive motile spermatozoa in all samples were analyzed. In addition, the following kinematic parameters were evaluated (Mortimer 2000) :

- Average path velocity, VAP (µm/s): the average velocity of the sperm head along its average trajectory.
- 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. Sperm cells with less than 20% of deviation were considered with a linear trajectory.
- Beat cross-frequency, BCF (Hz): the frequency with which the actual sperm trajectory crosses the average path trajectory.
- Percentage of linearity, LIN (%): the ratio between VSL and VCL (VSL/VCL).
- Percentage of straightness, STR (%): the ratio between VSL and VAP (VSL/VAP).

- Wobble coefficient, WOB (%): the ratio between VAP and VCL (VAP/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.
- Combination of the lateral and forward movement of the head, DANCE ($\mu m^2/s$): product of VCL and ALH (VCL x ALH).
- Mean angular displacement, MAD (degrees): measure of the curvature trajectory of spermatozoa and is defined as "the time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory".

Hyperactivated spermatozoa were identified according to the thresholds proposed by Colas et al. (2010). Thus, spermatozoa were considered hyperactivated when their LIN was ≤ 45% and their ALH ≥ 3.5 µm.

In *Article 3*, motility was evaluated using the Motility module of the OpenCASA (Alquézar-Baeta et al.), an open-source software developed by our research group. For this analysis, the recording was performed at 60 frames/s for 2 seconds, with a resolution of 800x600 pixels by a video-camera (Basler acA1920, Basler Vision Components, Exton, PA, USA) mounted on a microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan). The microscope was equipped with a 10x negative-phase contrast lens. Spermatozoa were diluted to a final concentration of 3×10^7 cells/mL and two drops of each sample (2 µL) were placed onto Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and kept at 37 °C in during all the analysis on a heated slide holder.

As OpenCASA allows the user to set the analysis parameters freely, the settings used in the assays were: frames per second, 60; number of frames, 120; resolution images, 800x600 pixels; minimum cell size, 10 μm^2; maximum cell size, 100 μm^2; progressive motility, STR>80% and VAP>90%; minimum VCL, 10 µm/s; VCL lower threshold, 100 µm/s; VCL upper threshold, 200 µm/s; minimum track length, 30 frames; maximum displacement between frames, 20 µm.

In addition to the above mentioned kinematic parameters, OpenCASA also provides ALH_{max}, that is the maximum ALH found along the trajectory, and the fractal dimension (FD), which serves the best for characterizing hyperactivated motility and the structure of sperm trajectories (Mortimer et al. 1996).

4.3.3. CAPACITATION STATUS EVALUATED BY CHLORTETRACYCLINE (CTC) STAINING

Chlortetracycline (CTC) is a fluorescent antibiotic that binds to membrane-associated cations, especially Ca^{2+} (Caswell and Hutchison 1971). Upon entering the sperm cell, CTC binds free calcium, becoming more fluorescent (Ericsson 1967). These CTC-Ca^{2+} complexes preferentially bind to the cell membrane, resulting in three staining patterns, which allow us to differentiate non-capacitated, capacitated, and acrosome reacted cells (Fraser 1982).

A modified version of the chlortetracycline (CTC) assay described by Ward and Storey (1984) and validated for ram sperm by Grasa et al. (2006) was used. 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 sterilized through a 0.22 µm filter (Merck Millipore, Darmstadt, Germany). After that, 20 µL of CTC solution were added to 18 µL of sperm sample, fixed with 5 µl of 12.2% (w/v) paraformaldehyde in 0.5 M Tris-HCl, pH 7.8 and incubated at 4 °C in the dark for 30 min. A 6 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 v/v). The samples were covered with 24 x 60 mm coverslips, sealed with colourless enamel, and stored in the dark at -20 °C until evaluation. For the assessment of CTC patterns, samples were examined using a Nikon Eclipse E-400 microscope under epifluorescence illumination using a V-2A filter.

All samples were processed in duplicate and at least 150 spermatozoa/slide were scored. No fluorescence was observed when CTC was omitted from the preparation. Three sperm patterns were identified (Gillan et al. 1997): non-capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-

reacted cells (AR, showing no fluorescence on the head, with or without a bright equatorial band) (**Fig.4.2**).

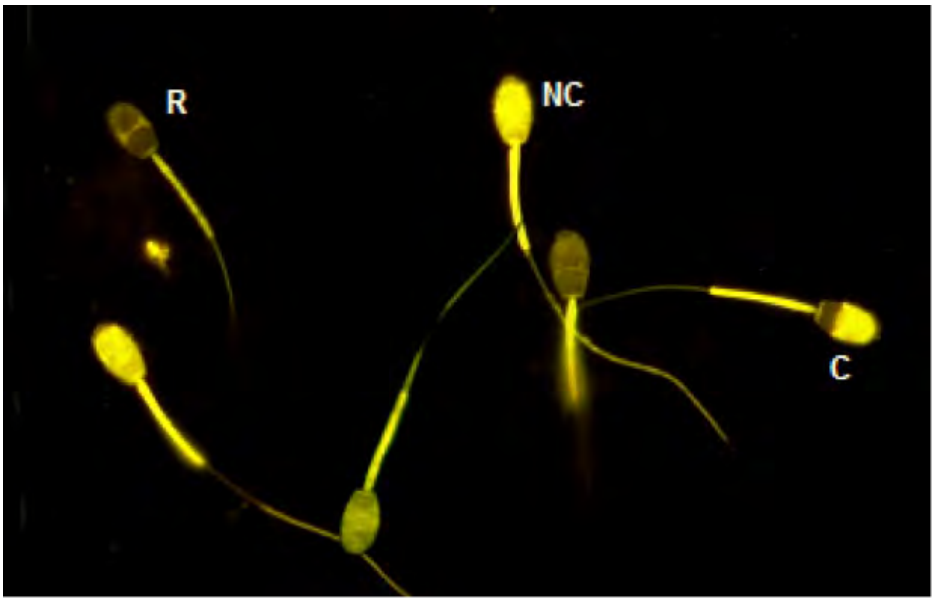


Fig.4.2. *Representative image of ram spermatozoa stained with chlortetracycline (CTC). NC: non capacitated (even head stained), C: capacitated (fluorescence only in acrosome), AR: acrosome reacted (no fluorescence on the head, with or without a bright equatorial band). (V-2A filter, original magnification X1000).*

4.3.4. SPERM VIABILITY (MEMBRANE INTEGRITY)

Sperm viability usually refers to the percentage of sperm with intact plasma membrane. It was assessed by fluorescent staining with carboxyfluorescein diacetate and propidium iodide (CFDA/PI) (Harrison and Vickers 1990) and evaluated by flow cytometry. CFDA/PI staining was based on the differential colouration of spermatozoa depending on the integrity of their plasma membrane. Spermatozoa with an intact plasma membrane, considered as viable cells, are green because their plasma membrane contains esterases which hydrolize carboxyfluorescein diacetate to carboxifluorescein, resulting in green fluorescence. At the same time, the plasma membrane does not let propidium iodide goes through membrane. However, not viable spermatozoa present damaged plasma membrane, with no functional esterases, and therefore, propidium iodide enters inside sperm, staining DNA. Consequently, not viable spermatozoa are red. Among not viable sperm two populations can be

distinguished: not viable, damaged-acrosome cells (red fluorescence), and not viable, intact acrosome cells (green fluorescence in acrosome, red in post-acrosome).

For viability analysis, samples were diluted in SM to a final concentration of 5 x 10^6 cells/mL. Then, 3 µL of 1 mM CFDA (diluted in water) and 3 µL of 1.5 mM PI (diluted in DMSO) were added. Samples were fixed with 5 µL of formaldehyde (0.5% v/v in water) and incubated for 15 minutes at 37 °C in the dark.

All the flow cytometry analysis were performed in a Beckman Coulter FC 500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA) equipped with two lasers of excitation (air-cooled Argon ion laser 488 nm and Red Solid state laser 633 nm), 5 filters of absorbance (FL1-525, FL2-575, FL3-610, FL4-675 and FL5-755, ± 5 nm each bandpass filter) and CXP software. A minimum of 20000 events were recorded in all the experiments. The sperm population was identified for further analysis by its specific forward (FS) and side scatter (SS) properties; thus, a FS area versus SS area density plot was adquired with adjusted gate settings and was used to exclude non-sperm particles from the analysis. Data were represented in logarithmic scale. A flow rate stabilized at 200 - 300 cells/sec was used.

For the sperm viability analysis, the argon laser and filters FL1-525 and FL4-675 nm were used to avoid overlapping. Monitored parameters were FS log, SS log, FL1 log (CFDA) and FL4 log (PI). The percentage of viable spermatozoa (CFDA+/PI-) was studied.

4.3.5. EVALUATION OF SPERM ACROSOMAL MEMBRANE INTEGRITY

To determine the sperm acrosome integrity, *Ricinus communis* agglutinin was used. The method described by Martí *et al*. (2000) was adapted here for flow cytometry evaluation. Briefly, 5 µL of 20 µg/mL *Ricinus communis* agglutinin (FITC-RCA, Vector Laboratories, Burlingame, CA, USA), 5 µL of propidium iodide (PI, 1.5 mM) and 3 µL of formaldehyde (0.5% v/v in water) were added to 300 µL of sperm samples (final concentration 6 x 10^6 cells/mL). Samples were then incubated at 37 °C in darkness for 15 min. For flow cytometry analysis, the argon laser and filters FL1-525 and FL4-675 nm were used to avoid overlapping. Monitored parameters were FS log, SS log, FL1 log (FITC-RCA) and FL4 log (PI). The percentage of viable spermatozoa with damaged

acrosome was evaluated (RCA+/PI-).

4.3.6. MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

The production of ROS was measured by incubating the spermatozoa in the presence of the dye 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA), which is permeable across cell membranes and is incorporated into hydrophobic regions of the cell (Bass et al. 1983). Then is cleaved by cellular esterases, resulting in the formation of the non-permeant, non-fluorescent, 2',7'-dichlorodihydrofluorescein (H_2DCF). The H_2DCF is oxidized by H_2O_2 to dichlorofluorescein (DCF), which emits fluorescence at 530 nm in response to 488 nm excitation (Carter et al. 1994). This fluorescent probe was combined with PI for excluding the nonviable population from the analysis (Guthrie and Welch 2006). Samples aliquots, prepared at a final concentration of 5 x 10^6 cells/mL, were stained with 5 µL of H_2DCFDA (20 µM) and PI (1.5 mM). After 15 min at 37 °C in the dark, the samples were fixed with 5 µL formaldehyde (0.5% v/v in water) and analysed by flow cytometry. The argon laser and filters FL1-525 and FL4-675 nm were used to avoid overlapping. The monitored parameters were FS log, SS log, FL1 log (H_2DCFDA) and FL4 log (PI). Both the mean of the green fluorescence intensity after excluding non-viable (PI+) spermatozoa *(Article 4*) or the percentage of live spermatozoa with high ROS levels (PI-/DCF+, *Article 3*) were considered.

4.3.7. MEASUREMENT OF THE INTRACELLULAR CALCIUM LEVELS

To detect intracellular Ca^{2+} (Gee et al. 2000) simultaneously with sperm viability, 1.4 x 10^7 cells/mL from each sperm sample were stained with 3 µL of the fluorescent probe Fluo-4 AM (ThermoFisher Scientific, Waltham, Massachusetts, USA), previously diluted with DMSO and pluronic acid (0.26% and 0.01% v/v, respectively) and with 3 µL of 1.5 mM propidium iodide (PI). After 15 minutes of incubation at 37 °C in the dark, the samples were fixed with 5 µL of formaldehyde (0.5% v/v in water) and analysed by flow cytometry. The argon laser and filters of FL1-525 and FL4-675 nm were used to avoid overlapping. The monitored parameters were FS log, SS log, FL1 log (Fluo-4AM) and FL4 log (PI). The percentage of viable spermatozoa with high levels of intracellular

calcium (PI-/Fluo-4AM+) were analysed.

4.3.8. EVALUATION OF CALCLIUM DISTRIBUTION INTO CELL COMPARTMENTS

To detect Ca^{2+} into the intracellular compartments, the fluorescent probe Rhod-5N-AM was used. Briefly, 1.4 x 10^7 cells/mL from each sperm sample were stained with 2 µL of 5 µM Rhod-5N-AM (Cayman Chemical Company, Ann Arbor, Michigan, USA), previously diluted with pluronic acid and DMSO (final concentrations in samples 0.008 % and 0.1 ‰ (v/v), respectively). After 15 minutes at 37 °C in the dark, the samples were fixed with 5 µL of formaldehyde (0.5% v/v in water).

For the evaluation of calcium patterns, samples were examined using a Nikon Eclipse E-400 microscope under epifluorescence illumination using a G-2A filter. At least 200 spermatozoa per slide were scored.

4.3.9. MEASUREMENT OF cAMP LEVELS

The intracellular cAMP content was measured using the Direct cAMP enzyme immunoassay kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. This kit uses a polyclonal antibody against cAMP to bind competitively the cAMP in the sample or an alkaline phosphatase molecule that has cAMP covalently attached to it. Due to the very low levels of intracellular cAMP in sperm samples, the acetylated version of the kit was used. According to a previous work (Colas et al. 2010), samples were processed after 5 min of incubation in capacitating conditions. 1.2 x 10^8 spermatozoa were treated with 0.05 M HCl and incubated for 20 min at RT to inhibit endogenous phosphodiesterase activity. The extracted sample was centrifuged at 20000 x g for 5 min at RT, and the supernatant was immediately analyzed or snap-frozen until measurement.

Samples and standards were simultaneously incubated with the alkaline phosphatase conjugate and the anti-cAMP antibody at RT in a secondary antibody-coated multi-well plate. The reagents were then washed away and the p-nitrophenyl phosphate

substrate Solution was added. After 1 h of incubation, the enzyme reaction was stopped with trisodium phosphate and the yellow colour generated was read on a multi-well plate reader at 405 nm. The colour intensity was inversely proportional to the concentration of cAMP in both the standards and the samples. The measured optical density was used to calculate the concentration of cAMP.

4.3.10. PKA ACTIVITY ANALYSIS .....

PKA enzymatic activity was determined using a commercial ELISA kit (PKA Activity Kit K027-H1, Arbor Assay, MI, USA) following the manufacturer's instructions. Sperm samples were processed after 5 min of incubation in capacitating conditions and aliquots of 8×10^7 spermatozoa were centrifuged at 900 x g and then were lysed with the cell lysis buffer provided in the kit. Then, samples were incubated for 30 min on ice with occasional vortexing. Samples were centrifuged at 10000 x g at 4 °C and the supernatants were frozen at -80 °C until evaluation. For PKA activity analysis, samples were diluted 1:2000 (v/v) into the kinase assay buffer provided by the manufacturer.Samples and standards were incubated with ATP at 30°C. After 90 minutes of incubation, the reagents were then washed away and samples and standards were incubated with rabbit phospho-PKA substrate antibody and donkey anti-rabbit IgG-HRP conjugate antibody at RT. After 1 hour, the reagents were then washed away and the TMB substrate was added. After 30 minutes of incubation, the enzyme reaction was stopped with hydrochloric acid 1 M and the optical density was read on a multi-well plate reader at 450 nm . The intensity of the color was directly proportional to the amount of PKA in samples and standars. The measured optical density was used to calculate the activity of PKA.

4.4. IDENTIFICATION AND LOCALIZATION OF HORMONE RECEPTORS

4.4.1. IDENTIFICATION AND LOCALIZATION OF HORMONE RECEPTORS BY AVIDIN-BIOTIN PEROXIDASE ASSAYS

To identify estrogen receptors ERα and ERβ and progesterone receptor (PR) in ram

spermatozoa we used the avidin-biotin-peroxidase immunocytochemical staining method (Vector Laboratories, Inc., Burlingame, CA, USA). Aliquots of 4 x 10^6 cells/ml 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 mM KH_2PO_4, pH 7.2) for 20 min at RT. The cells were then centrifuged at 900 x 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 RT for 3 h to ensure good adhesion onto the slide.

Slides were then rehydrated in PBS, and endogenous peroxidase was inactivated with 1.7% hydrogen peroxide in 100% ethanol (v/v) for 30 min. The slides were then washed in PBS and incubated in horse serum as a blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) for 45 min, followed by incubation with the specific antibody was diluted 1:50 (v/v) in PBS 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 estrogen 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 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 hematoxylin 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 (Nikon, Tokyo, Japan) under bright field illumination at 1000 x magnification. Microscopic images were captured and processed with a Nikon image software.

4.4.2. LOCALIZATION OF HORMONE RECEPTORS BY INDIRECT IMMUNOFLUORESCENCE

In addition, the localization of estrogen receptors ERα and ERβ and progesterone receptor (PR) was investigated by indirect immunofluorescence analyses (IIF) to observe changes easier and faster. Aliquots of 4 x 10^6 spermatozoa were fixed with 50 µL of 0.5% (v/v) formaldehyde in phosphate-buffered saline (PBS) for 20 min at RT. The cells were then centrifuged (6 min, 900 X g) at RT and the pellet resuspended in 500 µL of PBS. After that, 40 µL of cell suspension was smeared onto poly-L-lysine-coated slides. Then, samples were blocked with 5% BSA (w/v) in PBS for 4 h at RT in a wet chamber. After that, the samples were washed three times with PBS and incubated overnight at 4 °C in a wet chamber with the correspondent primary antibody.

To localize estrogen receptors ERα and ERβ and PR, the same antibodies as in the avidin-biotin-peroxidase assays were used. These antibodies were diluted 1:50 (v/v) in PBS containing 1% BSA (w/v). After overnight incubation, the slides were washed with PBS and incubated with the secondary antibody for 75 min at RT in a humidity chamber. For ERα, ERβ and PR, the cells were incubated with chicken Alexa Fluor 488-conjugated anti-rabbit antibody (Cat# A-21441 RRID:AB_2535859; Molecular Probes, Thermo Fisher Scientific Inc., Waltham, MA, USA), diluted 1:800 (v/v) in PBS containing 1% BSA (w/v).

After incubation, slides were washed other three times with PBS before the addition of 5 µl of 0.22 M triethylenediamine (DABCO) in glycerol:PBS (9:1) in order to enhance and preserve cell fluorescence. Samples were covered with 22 x 22 mm coverslips, sealed with colourless enamel and stored in the dark at 4 °C. Cells were visualized under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under epifluorescence illumination using a B-2A filter. At least 200 cells per sample were evaluated and the percentage of cells was calculated. Negative control was performed by processing slides without the primary antibody.

4.4.3. DETECTION AND QUANTIFICATION OF HORMONE RECEPTORS BY WESTERN BLOTTING

4.4.3.1. EXTRACTION OF RAM SPERM PROTEINS

For western blotting, sperm proteins were extracted from raw semen by diluting samples in PBS (10^8 cells/mL) and centrifuging them in a microfuge at 900 g for 6 min at RT. The supernatant was discarded, and the pellet was resuspended with 100 µL of extraction buffer (Colas et al. 2008) composed of 2% sodium dodecyl sulfate (SDS, w/v), 0.0626 mM Tris-HCl (pH 6.8), 0.002% bromophenol blue (W/v) in 10% glycerol (1% final glycerol concentration) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich Corp., St. Louis, MO, USA).. After that, all samples were immediately incubated for 5 min at 100 °C. After centrifugation at 16800 x g for 5 min at RT, the supernatant was recovered and 2-mercaptoethanol and glycerol were added to a final concentration of 5% and 1%, respectively. Lysates were stored at -20 °C.

4.4.3.2. SDS-PAGE AND INMUNOBLOTTING

5×10^6 cells (Article 1) were loaded on 10% (w/v) SDS-PAGE gels and separated in one dimension following the Laemmli method (Laemmli 1970) using a Mini-PROTEAN® II Cell vertical slab gel electrophoresis system (Bio-Rad, Hercules, CA, USA). The conditions of the electrophoresis were 130 V for 90 min at 4 °C. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Trans-Blot Turbo transfer pack, Bio-Rad, Hercules, CA, USA) using a transfer unit (Trans-Blot Turbo Transfer System, Bio-Rad, Hercules, CA, USA). Non-specific sites on the membranes were blocked with 5 % BSA (w/v) in PBS (pH 7.4) for 1 h at RT. After that, incubations with the primary antibodies were performed overnight at 4 °C diluted in in PBS-T (0.1% Tween-20 in PBS) containing 1% BSA (w/v).

For the detection of steroid hormone receptors, we used same antibodies as in the avidin-biotin-peroxidase and IIF assays, all of them diluted 1/1000 (v/v). After extensive washing, the membranes were then incubated for 1 h and 15 min at RT with

a secondary antibody diluted in PBS-T containing 1% BSA (w/v): donkey anti-rabbit antibody for ER, PR (Donkey Anti-Rabbit IgG, IRDye 800CW Conjugated antibody; LI-COR Biosciences, Lincoln, NE, USA; Cat# 926-32213, RRID:AB_621848 Li-COR Biosciences,), dilution 1: 35000 for PR and 1:15000 (v/v) for ER respectively).

For hormone receptors identification, we used the commercial cell lysates specifically recommended and supplied by the antibody manufacturer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as positive controls. MCF7 whole-cell lysate, specifically recommended as the positive control for PR and ERα, is derived from the MCF7 cell line (human breast adenocarcinoma). F9 whole-cell lysate, specifically recommended as the positive control for ERβ, is derived from the F9 cell line (mouse testicular teratoma). Liver protein extract was also used as a positive control 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 and Jahn 2005; Dressing et al. 2011). Liver protein extracts were obtained from ovine liver following the same protocol as sperm protein extracts (4.4.3.1).

After extensive washing with PBS-T, the membranes were scanned using the Odyssey Clx Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA)56.

4.5. LOCALIZATION AND QUANTIFICATION OF TYROSINE-PHOSPHORILATED PROTEINS

Furthermore, changes in the content and localization of proteins phosphorylated at tyrosine residues (TyrP) during *in vitro* capacitation it is considered as capacitation markers. Accordingly, the localization and quantification of tyrosine phosphorilated proteins were detected by IIF and western-blot.

4.5.1. LOCALIZATION OF TYROSINE-PHOSPHORILATED PROTEINS BY INDIRECT IMMUNOFLUORESCENCE

To localize tyrosine phosphorylated proteins, the procedure was the same as described above about but using a monoclonal anti-phosphotyrosine antibody (4G10, Millipore, Temecula, CA, USA; Cat# 05-321, RRID:AB_309678) at 1:200 and a chicken

Alexa Fluor 594-conjugated anti-mouse antibody (Cat# A-21201. RRID; AB_2535787; Molecular Probes, Thermo Fisher Scientific Inc., Waltham, MA, USA) as a secondary antibody diluted at 1:600. The following staining patterns were identified acording to the labeled region: E: equatorial region; A: acrosome; AE: acrosome + equatorial region; AF: acrosome + flagellum; EF: equatorial region + flagellum; AEF: acrosome + equatorial region + flagellum; F: flagellum.

4.5.2. DETECTION AND QUANTIFICATION OF TYROSINE-PHOSPHORILATED OF PROTEINS BY WESTERN BLOTTING

4.5.2.1. EXTRACTION OF RAM SPERM PROTEINS

For the detection and quantification of phosphorylated proteins on tyrosine residues, aliquots of 3.2 x 10^7 spermatozoa /mL swim-up selected or previously incubated under different *in vitro* capacitation conditions, were resuspended in 100 µl of the extraction medium. After that, all samples were immediately incubated for 5 min at 100 °C. After centrifugation at 16800 x g for 5 min at RT, the supernatant was recovered and 2-mercaptoethanol and glycerol were added to a final concentration of 5% and 1%, respectively. Lysates were stored at -20 °C.

4.5.2.2. SDS-PAGE AND INMUNOBLOTTING

Sperm-extracted proteins (15 µl (Article 1, Article 2 and Article 4) were loaded on 10% (w/v) SDS-PAGE gels and separated in one dimension following the Laemmli method (Laemmli 1970) using a Mini-PROTEAN® II Cell vertical slab gel electrophoresis system (Bio-Rad, Hercules, CA, USA). The conditions of the electrophoresis were the same as described above. For the detection of tyrosine-phosphorylated proteins, the procedure was the same as described above but using the same mouse monoclonal anti-phosphotyrosine as IIF assay diluted 1:1000 and Donkey Anti-Mouse IgG IRDye Dylight 800CW conjugated as secondary antibody (Li-Cor Biosciences, Lincoln, NE, USA; Cat# 926-32312, RRID:AB_621847) diluted 1:15000 .

Moreover, Anti-actin (Article 1 and Article 2), (Sigma-Aldrich; Cat# A2066,

RRID:AB_476693) or Anti-tubulin antibody (Article 4), (Santa Cruz Biotechnology Cat# sc-5546, RRID:AB_635001) both produced in rabbit and diluted 1:500 were used as a loading control, followed by incubation with a secondary Donkey Anti-Rabbit antibody for actin and tubulin (Donkey Anti-Rabbit IgG, IRDye 800CW Conjugated antibody; LI-COR Biosciences, Lincoln, NE, USA; Cat# 926-32213, RRID:AB_621848 Li-COR Biosciences,) dilution 1:15000

After extensive washing with PBS-T, the membranes were scanned using the Odyssey Clx Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA). Western-blot images were quantified using Odyssey Clx Infrared Imaging System software (Li-COR Biosciences) to determine the peak 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 or tubulin loading control.

4.6. STATISTICAL ANALYSIS

Results are shown as mean ± standard error of the mean (S.E.M.) of the number of assays indicated in each case. All statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad InStat software version 5.01 (GraphPad software Inc., San Diego, CA, USA).

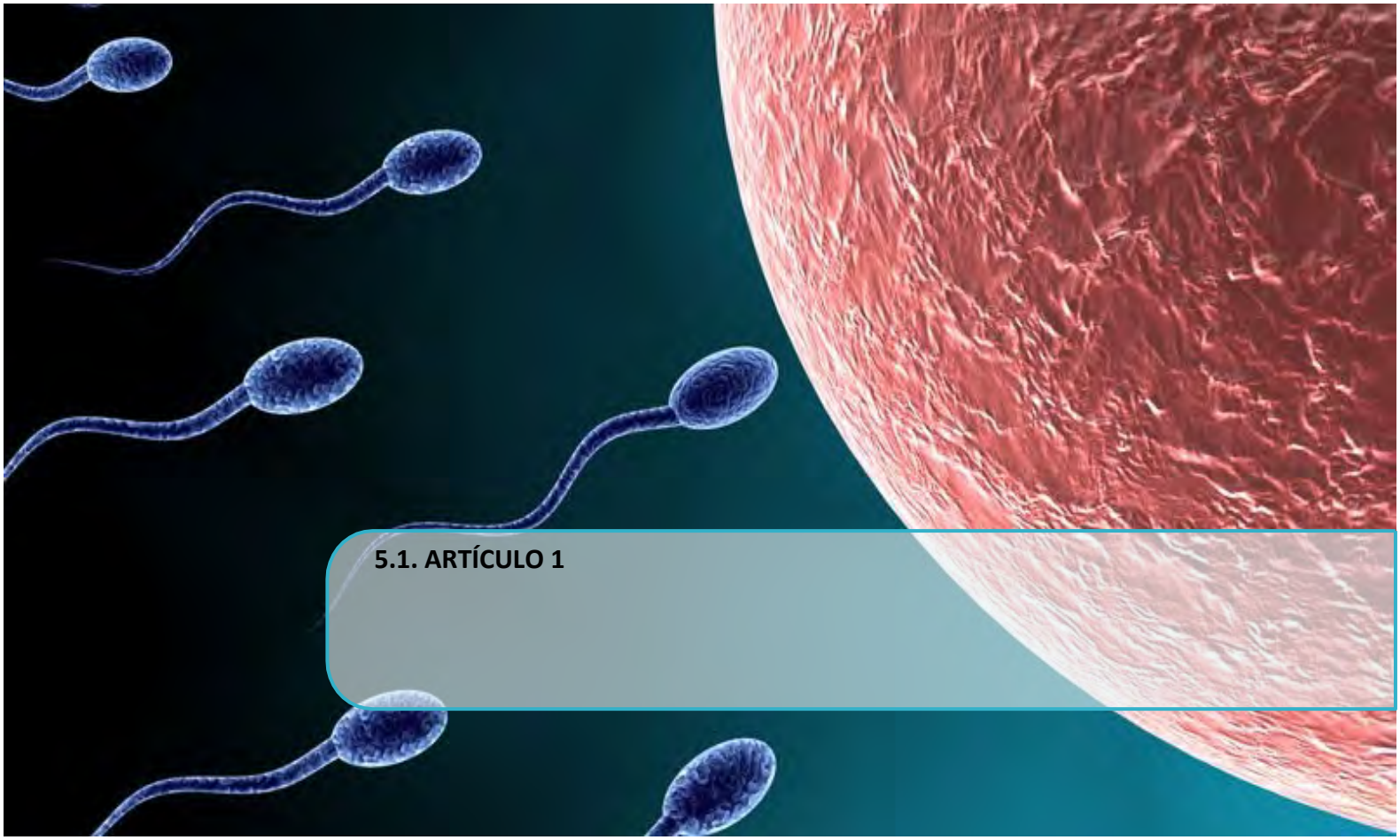
First, data distribution was analyzed by the Kolmogorov-Smirnov normality test. Differences in protein tyrosine phosphorylation, cAMP levels, PKA activity, mean fluorescence of ROS and motility kinematic parameters were analyzed by means of ANOVA followed by the Bonferroni or LSD Fisher *post hoc* tests. In *Article 3*, normality and homoscedasticity of the continuous variables (levels of cAMP, PKA and kinematic parameters) were analysed by the Kolmogorov-Smirnov test and Levene test, respectively. After that, differences between experimental groups were evaluated by ANOVA, followed by Tukey post-hoc test or by the Kruskal-Wallis test followed by Dunn's posttest.

Flow cytometry data (viability, ROS levels, intracellular calcium levels by Fluo-4-AM, capacitation status by CTC, calcium distribution by Rhod5-N, total and progressive

motility parameters, estrogens receptors and TyrP immunolocalization were analyzed by the Pearson´s Chi-Square test by Fisher's exact test (Shan and Gerstenberger 2017). Pearson`s correlation test was used to analyse the correlation between the capacitation state and ERβ or TyrP immunolocalization.

The Principal Component Analysis (PCA) in *Article 4* was performed using the IBM SPSS statistical software (V 21.0; IBM Corp., Chicago, IL, USA). Briefly, all sperm motility and kinetic parameters obtained from CASA assessments (i.e. VSL, VCL, VAP, %LIN, %STR, %WOB, ALH, BCF, DANCE and MAD) were first checked through Shapiro-Wilks and Levene tests to verify normal distribution and homogeneity of variances. All sperm parameters were sorted into two PCA components and the obtained data matrix was rotated using the Varimax procedure with the Kaiser normalisation. Following this analysis, only those variables with a positive loading factor (aij) higher than 0.6 with its respective component and lower than 0.3 with respect to the others in the rotated matrix were selected from the linear combination of j variables (x) in each component yi (yi = ai1x1 + ai2x2 + ... + aijxj). For each spermatozoon, which was considered as an independent statistical case, a regression score was calculated per PCA component. Following PCA, each spermatozoon in each treatment and time point was classified using cluster analysis. This classification was made upon the regression scores per PCA component obtained in the previous step, using the between-groups linkage method based on the squared Euclidean distance. Four subpopulations were obtained. After this, the effects of *in vitro* capacitation treatments upon the sperm subpopulation distribution were determined through a linear mixed model followed by a Bonferroni's post-hoc test. In this model, the incubation time point was the intrasubject factor and the treatment was the fixed-effects factor. All sperm motility parameters were considered as dependent variables. The level of significance was set at P value of 0.05 or lower.

5. RESULTADOS



5.1. ARTÍCULO 1

REPRODUCTION
RESEARCH

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

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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 17β-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, ERα and 55 kDa, ERβ). ERα was located in the postacrosomal region of all the spermatozoa and ERβ on the apical region of 63.7% of the cells. The presence of ERβ 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,

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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 (ERα and ERβ) (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 $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 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% CO_2 in air. Incubations were performed in a TALP medium (Parrish *et al*. 1988) composed of 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 3 mM $CaCl_2$, 0.4 mM $MgCl_2$, 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 µm) 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 µL LPC (300 µg/mL) was added to 95 µL capacitated samples and incubated at 39°C, 5% CO_2 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 µM, 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

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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.

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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-ERα (RRID:AB_640249), anti-ERβ (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 µL 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×***g*** for 6 min at room temperature. The supernatant was discarded, and the pellet was resuspended with 100 µL of extraction buffer (125 mM Tris–HCl (p 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 µL 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×***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-ERα, -ERβ 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 ERα, is derived from the MCF7 cell line (human breast adenocarcinoma). F9 whole-cell lysate, specifically recommended as the positive control for ERβ, 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 ERβ 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

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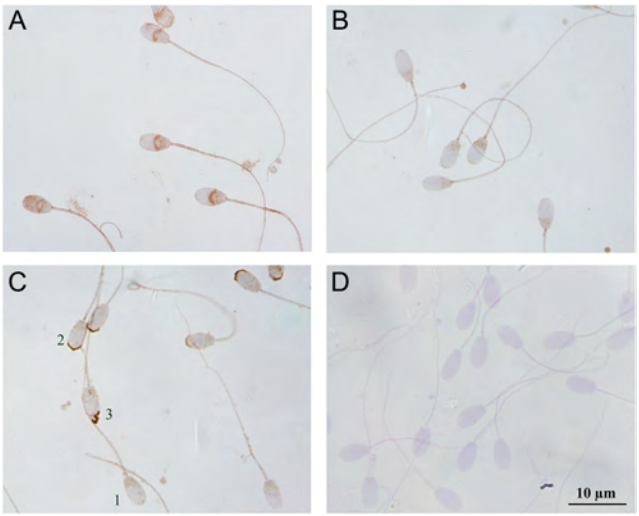

Figure 1 Immunocytochemical localization of progesterone receptor (A), estrogen receptor α (B) and β (C) in ram spermatozoa. Magnification 1000×. Negative controls are shown (D).

appeared faintly on the midpiece of the tail (Fig. 1A). Both estrogen receptors, ERα and ERβ, are also present in ram spermatozoa. The immunocytochemistry assays revealed that ERα 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 ERβ signalling were found: one showing no antibody labelling (ERβ-, sperm 1 in Fig. 1C) and another showing intense reactivity on the apical region of the acrosome (ERβ+, 63.7±4.6%, sperm 2 in Fig. 1C). Curiously, some of the ERβ+ 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.

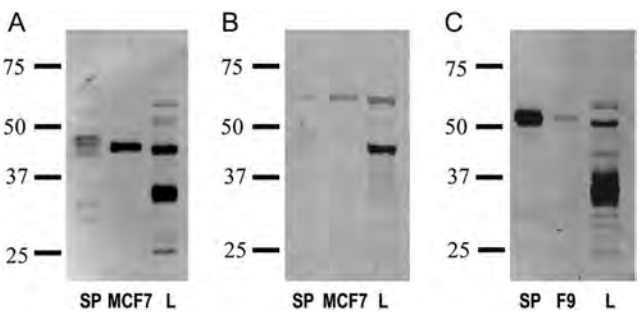

Figure 2 Western blot analysis of the presence of progesterone receptor (A), estrogen receptor α (B) and β (C) in ram sperm protein extracts (SP, lane 1 in A, B and C). Positive controls: MCF7 cell extracts (MCF7, lane 2, A and B), F9 cell extracts (F9, lane 2, C) and ovine liver protein extracts (L, 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 ERα (SP, lane 1 of Fig. 2B) and another of 55 kDa for ERβ (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 ERα and ERβ 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 ERα and ERβ, 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, ERα and ERβ 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 ERα localization after both processes (data not shown). In contrast, significant differences in the percentage of spermatozoa showing ERβ labelling (ERβ+ cells) were evidenced (Table 1). The high ERβ+ sperm rate in control samples (65.2±3.7%) significantly decreased after incubation with either the cocktail (24.6±1.2%; $P<0.05$) or LPC (6.3±1.6%; $P<0.05$). On the basis of these results, we investigated whether there was any correlation between the ERβ 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 ERβ 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 17β-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

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

Sperm sample	ERβ+ cells (%)	Chlortetracycline (CTC) staining patterns		
		NC (%)	C (%)	R (%)
Control	65.2±3.7[a]	72.1±1.3[a]	18.4±1.5[a]	9.2±1.0[a]
In vitro capacitated	24.6±1.2[b]	51.5±3.2[b]	30.9±3.1[b]	18.1±0.7[b]
AR	6.3±1.6[c]	26.7±1.8[c]	27.9±0.9[b]	45.4±1.3[c]

Percentage of spermatozoa with estrogen receptor β labelling (ERβ+) 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±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±4.5%, 68.2±2.3% vs 75.0±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±8.3% and 28.3±9.8% for 100 pM of both hormones vs 13.0±6.4% in control samples, $P<0.001$) (Fig. 5A). Furthermore, 1 μM 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±6.2% vs 30.5±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±1.3% up to 23.5±4.7% with 1 μM P4, $P<0.001$). After 3 h of incubation, the proportion of capacitated cells was increased to 69.7±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±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±9.0% with the highest

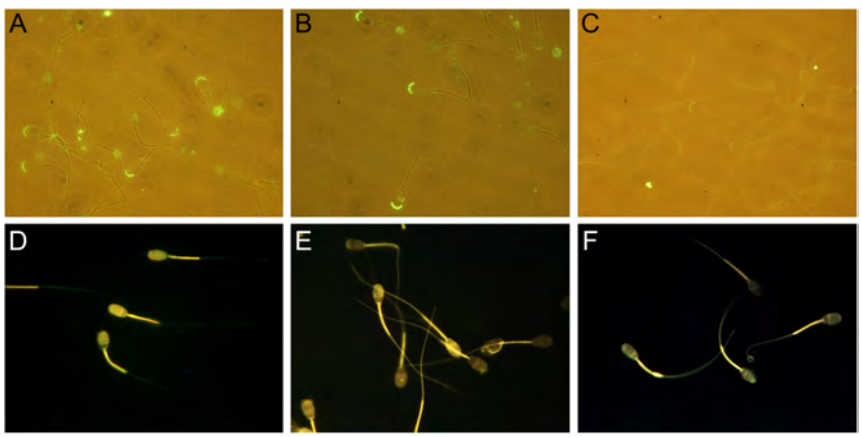

Figure 3 Representative images of immunocytochemical localization of ERβ (bright field and epifluorescence, A, B and C) and capacitation status assessed by chlortetracycline (CTC) staining (epifluorescence, D, E and F) in control (A and D), *in vitro* capacitated (B and E) and acrosome-reacted (C and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

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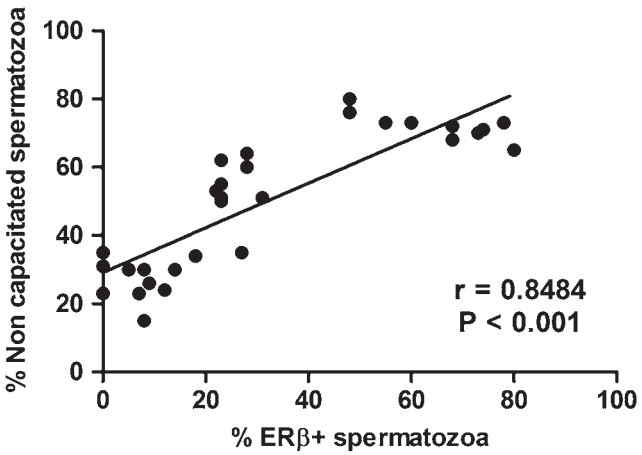

Figure 4 Graphic representation of the correlation found in ram spermatozoa between the presence of estrogen receptor β (ERβ+), localized by indirect immunofluorescence (IFF), and the non-capacitated sperm rate assessed by chlortetracycline (CTC) staining. Experiments were replicated 12 times with control, *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±4.3% in samples with no E2 to 45.7±3.18 and 54.7±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±8.6% with 10 nM E2 vs 13.0±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 17β-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, ERα at the postacrosomal part and ERβ 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 ERα was evidenced at the postacrosomal region of all the spermatozoa, ERβ 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 ERβ 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 ERβ and the percentage of NC spermatozoa. Moreover, the percentage of labelled cells (ERβ+) significantly decreased after *in vitro* capacitation, and nearly disappeared when the acrosome reaction was induced by LPC. All these data indicate that ERβ is involved in capacitation and the acrosome reaction of ram spermatozoa. In contrast, the expression and localization of ERα 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 rapids 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

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

	Control (0 h)	**Control** (3 h)	**Progesterone** (3 h)			**17β-Estradiol** (3 h)		
			100 pM	10 nM	1 μM	100 pM	10 nM	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% CO_2, 100% humidity) in TALP and Cocktail media either with or without different progesterone and 17 β-estradiol concentrations. Mean values ± S.E.M. ($n = 6$). Significant differences related to control 0 h (A) and to control 3 h (B) $P < 0.001$.

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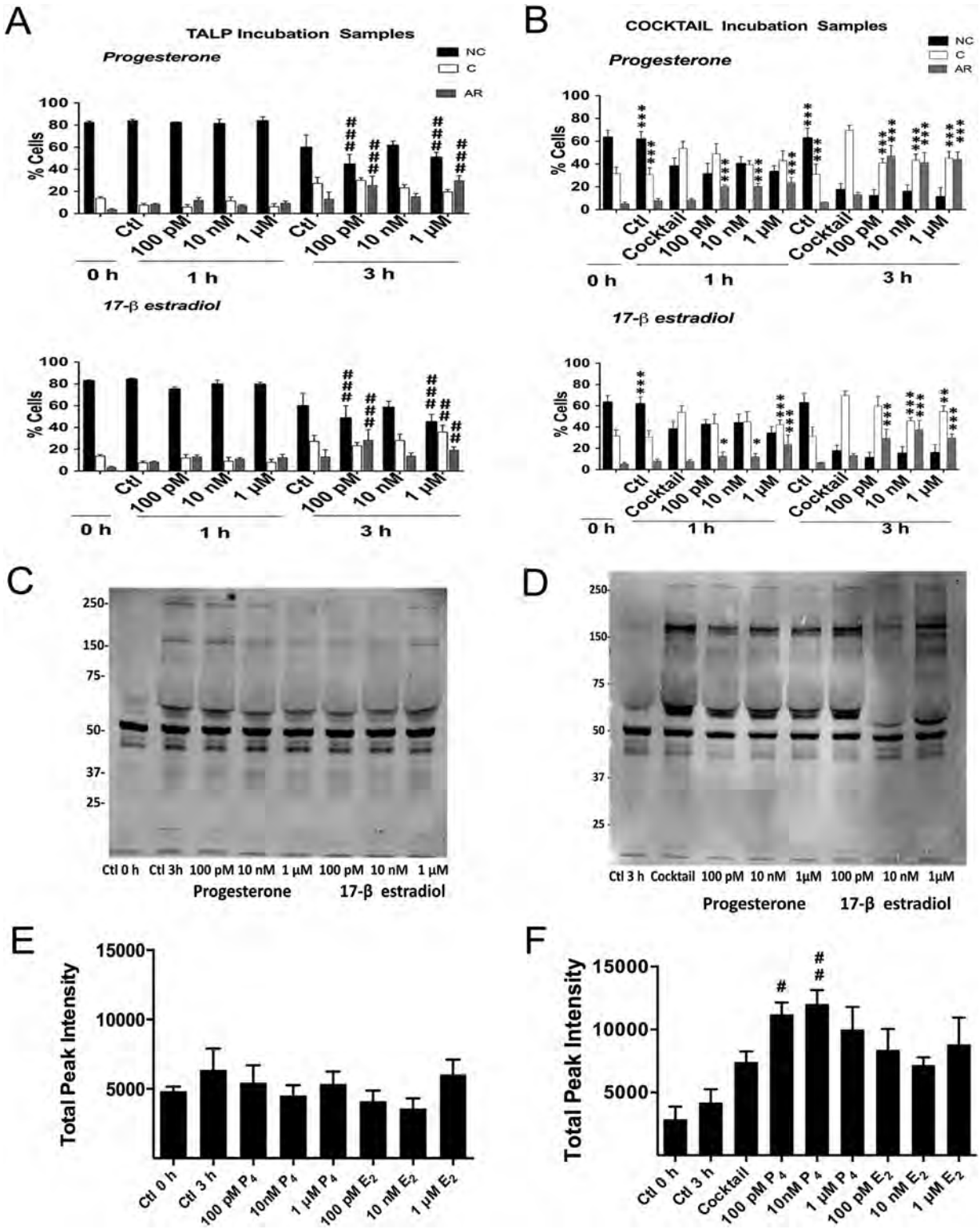

Figure 5 Effect of incubation with different progesterone and 17β-estradiol doses on the capacitation state of ram spermatozoa. Assessment of the capacitation status (CTC staining) of ram spermatozoa of control samples at 0 h, and after 1 h and 3 h of incubation at 39°C and 5% CO_2 with different progesterone and 17β-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 ± 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 ± s.e.m. ($n=4$).

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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 (ERα and ERβ) in ram spermatozoa. A band of about 66 kDa and another of 55 kDa were evidenced when anti-ERα and anti-ERβ antibodies were used, respectively, which were coincident with those described for conventional ERα and ERβ 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 ERβ was much more intense than that matching with ERα, in comparison with their respective positive controls. This is in concordance with results of other authors who have reported that ERβ 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 ERα and 1–150 of ERβ 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 17β-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 17β-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 17β-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 17β-estradiol to induce changes associated to the acrosome reaction by itself, especially when ram spermatozoa are incubated in a medium that increases

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the intracellular cAMP level. Furthermore, 100 pM 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 μM 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. 17β-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 17β-estradiol and CatSper.

In conclusion, our study demonstrates that ovine spermatozoa have progesterone and estrogen receptors and that the presence of ERβ 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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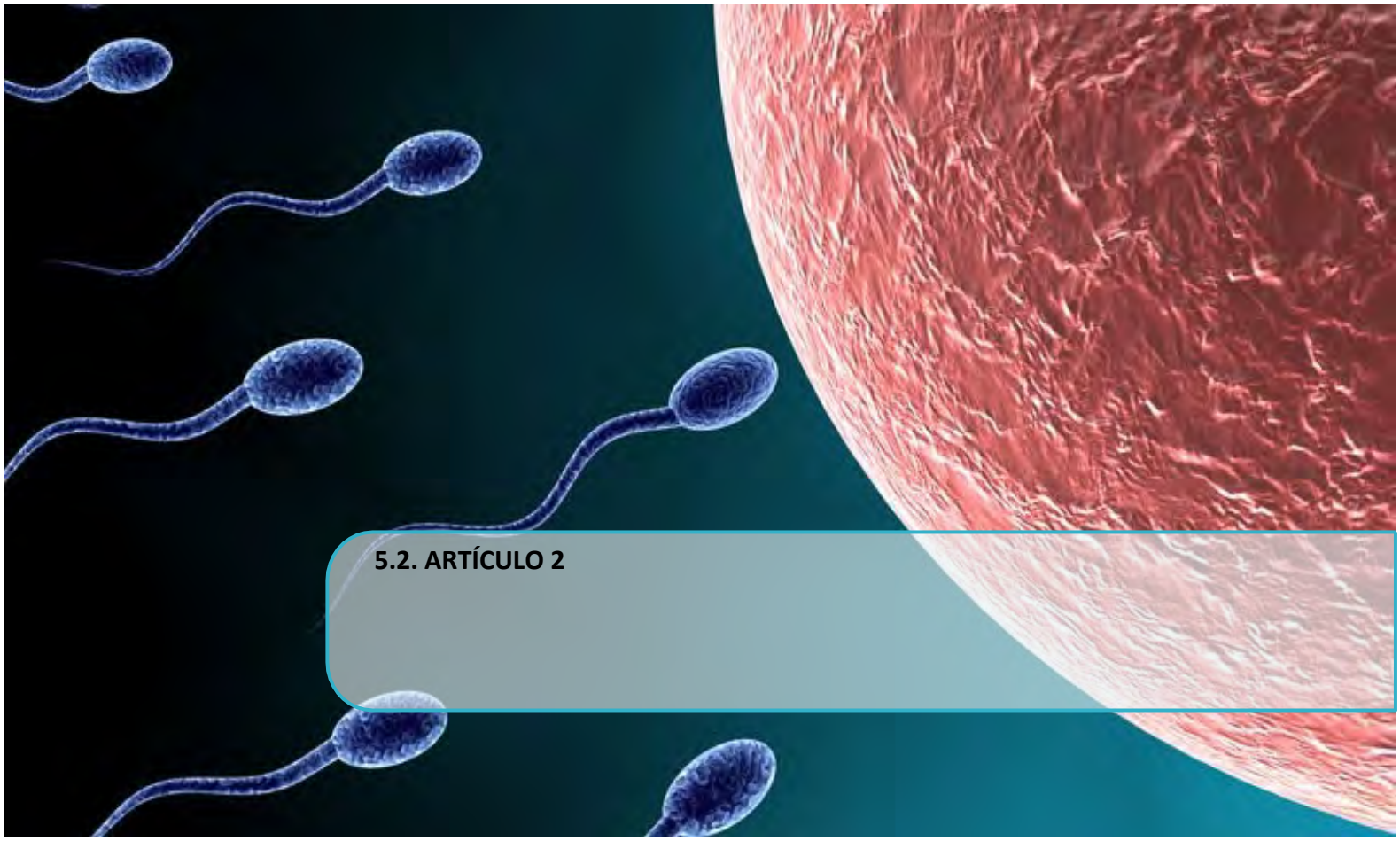

5.2. ARTÍCULO 2

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Involvement of progesterone and estrogen receptors in the ram sperm acrosome reaction

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ABSTRACT

The steroid hormones 17-β estradiol (E2) and progesterone (P4) can regulate capacitation, hyperactive motility, and the acrosome reaction (AR) during the sperm transit through the female tract. Moreover, exogenous P4 and E2 can induce the AR in ovine spermatozoa, and progesterone receptor (PR) and estrogen receptors (ERα and ERβ) are present in these cells. Thus, to investigate whether the effects both steroid hormones in ram sperm capacitation and AR are receptor-mediated, we incubated them with receptor agonists (tanaproget 1 μM and 5 μM for PR or resveratrol 5 μM and 10 μM for ER) or antagonists (mifepristone 4 μM and 40 μM for PR or tamoxifen 5 μM and 10 μM for ER) in capacitating conditions. The addition of receptor modulators did not affect sperm viability or total motility, although changes in progressive motility were detected. The incubation with both receptor agonists increased the percentage of acrosome-reacted spermatozoa, evaluated by chlortetracycline staining, when compared with the capacitated nontreated sample (Cap-C, $P < 0.001$). Moreover, the ER agonist resveratrol 10 μM provoked a greater AR than E2 ($P < 0.01$). Furthermore, the incubation with the receptor antagonists prevented the induction of the AR by P4 or E2, as the antagonists-treated spermatozoa presented a similar CTC pattern to that of Cap-C. In conclusion, these results confirm that P4 and E2 can induce the AR in ram spermatozoa and that this effect is receptor-mediated.

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1. Introduction

Steroid hormones regulate reproductive processes in mammals. During the follicular phase of the estrus cycle, rising levels of 17-β estradiol (E2) are secreted by the growing follicles, whereas progesterone (P4) is synthesized by the corpus luteum during the luteal phase [1]. Both hormones can reach the oviduct by countercurrent transfer from the ovarian vein to the ovarian and uterotubal arteries [2,3]. Moreover, after ovulation, P4 and E2 are also secreted by the cumulus cells surrounding the oocyte [4]. Many studies have described the rapid nongenomic effects of the steroid hormones on the regulation of sperm capacitation, hyperactive motility, and acrosome reaction (AR) during their transit through the female tract (reviewed in [5,6]). Steroid hormones can regulate all these processes by interaction with classical nuclear receptors or other putative novel membrane receptors [7]. A previous study identified a novel membrane progesterone receptor (PR) and the 2 classical nuclear estrogen receptors (ERα and ERβ) in ovine spermatozoa [8]. It also evidenced the decrease of ERβ on the sperm membrane after capacitation and that exogenous P4 and E2 induced the AR in ram spermatozoa. Based on these results, the hypothesis of this study was that the direct effects of P4 and E2 in ram spermatozoa could be receptor-mediated.

Receptor agonists and antagonists are useful tools for investigating the effects of steroid hormones in reproduction [9]. Among these, tanaproget (NSP-989) is a high-

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affinity, nonsteroidal PR agonist with in vitro and in vivo activity and 250-fold selectivity for the PR [10]. Mifepristone (RU-486) was the first effective antiprogestogen developed [11]. In farm animals, it was used to induce premature parturition in cattle [12] and sheep [13]. Moreover, in vitro incubation of human sperm with mifepristone caused hyperactivation, intracellular calcium movement, and the suppression of the P4-induced AR [14,15].

Resveratrol is a natural, nonflavonoid polyphenol and ER agonist present in grapes and red wine, with antioxidant, anticancer, anti-inflammatory, and cardioprotective properties [16]. It has also been demonstrated to have a protective effect against lipid peroxidation and DNA damage after cryopreservation in buffalo [17], bovine [18], boar [19], and ram spermatozoa [20]. Tamoxifen is a synthetic, nonsteroidal type I ER antagonist that also has weak agonist properties. Its agonist or antagonist response depends on the species, organ, tissue, and cell type [9]. In hamster spermatozoa, tamoxifen acts as an antagonist and can inhibit the suppression of P4-enhanced hyperactivation caused by E2 [21].

Thus, to further investigate whether the effects of steroid hormones in ram spermatozoa are receptor-mediated, the aim of this work was to evaluate the impact of PR and ER agonists and antagonists during ram sperm capacitation.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Aldrich (St. Louis, MO).

2.1. Sperm preparation

All experimental procedures were performed under project license PI19/17 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza, in accordance with the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Semen was collected from 8 mature *Rasa Aragonesa* rams (2 to 4 yr old), during the reproductive season using an artificial vagina. All the rams belonged to the National Association of *Rasa Aragonesa* Sheep Breeders (ANGRA) and were housed under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza (Spain). Two successive ejaculates were collected every 2 days, and the second ejaculates were pooled and processed together to avoid individual differences [22]. Concentration by Neubauer chamber, total motility (section 2.4), and viability (section 2.5) of the fresh samples were evaluated to corroborate sperm quality. A seminal plasma-free sperm population was obtained by a dextran/swim-up procedure [23] performed in a medium consisting of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 5 mg/mL BSA, 1.5 IU/mL penicillin, and 1.5 mg/mL streptomycin, with a pH of 6.5 (adjusted by NaOH addition).

2.2. In vitro capacitation

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 an incubator with 5% CO_2 and 100% humidity. Incubations were performed in Tyrode's albumin lactate pyruvate medium [24], composed of 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 3 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose, and 5 mg/mL BSA (pH 7.3 adjusted with NaOH). A cocktail of cAMP-elevating agents (1 mM dibutyryl (db)-cAMP, 1 mM caffeine, 1 mM theophylline, 0.2 μM okadaic acid, and 2.5 mM methyl-β-cyclodextrin), already tested for capacitating ram spermatozoa [25,26], was added to the Tyrode's albumin lactate pyruvate medium to induce in vitro capacitation (capacitated control, Cap-C). A noncapacitated sample (control sample, C), without the addition of the cAMP-elevating agents, was maintained under the same conditions as the capacitated samples.

2.3. Experimental design

2.3.1. Experiment 1: involvement of progesterone receptors in ram sperm functionality

Swim-up–selected spermatozoa were in vitro capacitated with either P4, the PR agonist tanaproget 5-(4,4-dimethyl-2-sulfanylidene-1*H*-3,1-benzoxazin-6-yl)-1-methylpyrrole-2-carbonitrile, NSP-989), or the PR antagonist mifepristone ((8*S*,11*R*,13*S*,14*S*,17*S*)-11-[4-(dimethylamino)phenyl]-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta[a]phenanthren-3-one, RU-486).

In the PR agonist assays, the spermatozoa were incubated in the above mentioned high-cAMP medium with either 1 μM or 5 μM tanaproget [10] or 1 μM P4.

In the PR antagonist experiments, spermatozoa were incubated in the high-cAMP medium with 1 μM P4, or with either 4 μM or 40 μM mifepristone [14], followed by the addition of P4 1 μM after the first hour of incubation.

2.3.2. Experiment 2: involvement of estrogen receptors in ram sperm functionality

This experimental design was similar to that of experiment 1, but the spermatozoa were incubated with the ER agonist resveratrol (5-[(*E*)-2-(4-hydroxyphenyl)ethenyl] benzene-1,3-diol) 5 μM or 10 μM [17,18]) or the antagonist tamoxifen (2-[4-[(*Z*)-1,2-diphenylbut-1-enyl]phenoxy]-*N*,*N*-dimethylethanamine) 5 μM or 10 μM [27,28]). 17-β estradiol was added at a 1 μM concentration.

Hormones and receptor agonists and antagonists were diluted in dimethyl sulfoxide (DMSO) and PBS, and the final concentration of DMSO in all the samples was 0.1%. Thus, the same DMSO concentration was included in the control and capacitated control samples.

2.4. Sperm motility evaluation

Motility parameters were evaluated using a CASA system (ISAS 1.2.; Proiser SL, Valencia, Spain) that consisted of a video camera (Basler A312f, Basler Vision Components, Exton, PA) mounted on a microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan) and equipped with a 10× negative-phase contrast lens. Samples (6 μL) were placed onto prewarmed slides, then covered with standard coverslips (20 × 20 mm) and maintained at 37°C during all the analysis by

a heated slide holder. Two drops of each sample were studied, and 5 fields of each drop were recorded. The recording was performed at 25 frames/s, and 25 consecutive digitalized images were taken from a single field. Percentages of total motile and progressive motile spermatozoa, kinematic parameters, and percentages of hyperactivated spermatozoa in all the samples 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). Hyperactivated spermatozoa were identified in accordance with the thresholds proposed by Colas et al [29].

2.5. Evaluation of sperm membrane integrity

To determine cell membrane integrity (viability), 3 μL of carboxyfluorescein diacetate (CFDA, 1 mM) and propidium iodide (PI, 1.5 mM) and 5 μL of formaldehyde (0.5% v/v in water) were added to 300 μL of sperm samples (final concentration of 5×10^6 cells/mL) based on a modification of the procedure described by Harrison and Vickers [30]. Samples were incubated at 37°C in darkness for 15 min. The analysis was performed on a Beckman Coulter FC 500 flow cytometer (Beckman Coulter Inc., Brea, CA) equipped with 2 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 bandpass filter) and CXP software. The sperm population was identified for further analysis by its specific forward (FS) and side scatter (SS) properties; thus, other nonsperm 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. Monitored parameters were FS log, SS log, FL1 (CFDA), and FL4 (PI). A minimum of 20,000 events were recorded in all the experiments.

2.6. Assessment of capacitation status by chlortetracycline (CTC) staining

Chlortetracycline is a fluorescent antibiotic that binds to membrane proteins in sperm cells. The fluorescence of bound CTC is enhanced by intracellular calcium, and CTC-staining patterns reflect the sperm capacitation state [31]. This fluorescence assay was previously validated for the evaluation of capacitation and AR-like changes in ram spermatozoa [25].

In brief, 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, Darmstadt, Germany). After that, 20 μL of CTC solution and 5 μL of 12.2% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.8) were added to 18 μL of the sperm sample and incubated at 4°C in the dark for 30 min. After incubation, 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) at room temperature and in darkness. The samples were covered with 24 × 60 mm coverslips, sealed with colorless enamel, and stored in the dark at 4°C. They were then examined using a Nikon Eclipse E−400 microscope (Kanagawa, Japan) under epifluorescence illumination with a V-2A filter. Spermatozoa were classified into one of the following 3 patterns [32]: noncapacitated (even distribution of fluorescence on the head, with or without a bright equatorial band; NC pattern), capacitated (with fluorescence in the acrosome; C pattern), and acrosome-reacted cells (showing no fluorescence on the head; AR pattern). All samples were processed in duplicate, and at least 150 spermatozoa were scored per slide.

2.7. Evaluation of tyrosine-phosphorylated proteins (TyrPP)

Aliquots of 3.2×10^7 cells were mixed with 100 μL of an extraction medium composed of 2% SDS (w/v), 0.0626 mM TRIS-HCl (pH 6.8), 0.002% bromophenol blue in 10% glycerol (final glycerol concentration 1%), and protease and phosphatase inhibitors. These were immediately incubated for 5 min at 100°C. After incubation, the samples were centrifuged at 15,000 g for 5 min at room temperature (RT), the supernatant was recovered, and 2-mercaptoethanol and glycerol were added to a final concentration of 5% and 1%, respectively. Lysates were stored at −20°C.

Sperm-extracted proteins (15 μL) were separated by standard SDS-PAGE in 10% (w/v) acrylamide gels and transferred onto a PVDF membrane (Trans-Blot Turbo transfer pack, Bio-Rad, Hercules, CA) using a transfer unit (Trans-Blot Turbo Transfer System, Bio-Rad, Hercules, CA). Nonspecific sites on the membranes were blocked for 1 h with 5% BSA (wt/vol) in PBS (136 mM NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, pH 7.4) at RT. Then, the blots were incubated overnight at 4°C with a mouse monoclonal anti-phosphotyrosine antibody (Clone 4G10; Millipore, Temecula, CA; Cat# 05–321, RRID:AB_309678) diluted 1:1000 (vol/vol) and an anti-actin antibody (Sigma-Aldrich; Cat# A2066, RRID:AB_476693) produced in a rabbit as a loading control diluted 1:500 (vol/vol), both in PBS-T (0.1% Tween-20 in PBS) containing 1% BSA (wt/vol). After extensive washing, the blots were incubated for 75 min at RT with a secondary donkey anti-mouse IgG IRDye DyLight 800CW–conjugated antibody (LI-COR Biosciences, Lincoln, NE; Cat# 926–32,312, RRID:AB_621847) and donkey anti-rabbit IgG, IRDye 680RD–conjugated antibody (LI-COR Biosciences; Cat# 926–32,223, RRID:AB_621845), both diluted 1:15,000 (vol/vol) in the previously mentioned buffer. Finally, after washing, the membranes were scanned using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) to determine the relative intensity

of the TyrPP 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 loading control.

2.8. Statistical analysis

Results are shown as mean ± standard error of the mean. Protein tyrosine phosphorylation and kinematic parameters were analyzed using an ANOVA test after the normality of the data was evaluated by the Kolmogorov-Smirnov test. Total and progressive motility, viability, and CTC staining were compared by means of Pearson's chi-square. GraphPad InStat software (3.01; San Diego, CA) was used for statistical analyses. To corroborate that agonist or antagonist receptors did not affect the spermatozoa quality, the viability and motility of treated samples were compared with control, capacitated control, and hormone-treated groups. For evaluation of the capacitation state by CTC staining and western blot, agonist and antagonist groups were compared only with the Cap-C and hormone-treated groups, the control group remaining solely as a noncapacitated control to confirm the success of the in vitro capacitation.

3. Results

Fresh sperm quality parameters were 90.7% ± 2.9% total motility, 77.5% ± 6.7% viability, and 4.35 ± 0.9 × 10^9 cells/mL concentration.

3.1. Experiment 1: involvement of progesterone receptor in ram sperm capacitation

The incubation in capacitating conditions with the PR agonist tanaproget or antagonist mifepristone at both concentrations did not affect the total motility or the percentage of sperm with intact plasma membrane relative to the control or Cap-C samples (Table 1), except for mifepristone 4 μM which showed a significantly higher viability ($P < 0.01$) when compared with the C and P4 groups, and motility when compared with the C group ($P < 0.05$). The incubation in high-cAMP medium decreased progressive motility ($P < 0.05$) in nearly all the samples. This decrease was even more pronounced in the tanaproget 5 μM sample, which showed significant differences ($P < 0.01$) when compared with the P4 group, whereas incubation with the PR antagonist mifepristone at 40 μM increased progressive motility compared with the Cap-C and P4 groups ($P < 0.05$, Table 1). However, these differences in progressive motility were not accompanied by differences in kinematic parameters, and only a significant decrease in LIN, STR, and BFC was detected in the tanaproget-treated spermatozoa. Finally, the incubation with P4 or modulators of the PR did not affect the percentage of hyperactivated spermatozoa, although this parameter increased when compared with the control group ($P < 0.05$, Table 1).

Incubation in capacitating conditions with 1 μM P4 led to a significant increase in the proportion of acrosome-reacted spermatozoa relative to the Cap-C sample ($P < 0.001$), concomitant with a decrease in noncapacitated and capacitated spermatozoa in both the PR agonist (Fig. 1A) and antagonist (Fig. 1B) sets of experiments. The incubation with the PR agonist tanaproget at both concentrations also increased the proportion of AR spermatozoa compared with the Cap-C sample (30.6 ± 3.27 and 29.1 ± 2.72% vs 20.0 ± 2.71% for tanaproget 1 μM and 5 μM and Cap-C, respectively; $P < 0.001$; Fig. 1A). However, this rise was not as marked as that produced by the addition of P4, and thus, significant differences between tanaproget- and P4-incubated samples were also detected ($P < 0.05$). In addition, the PR agonist provoked a significant decrease of NC and C spermatozoa when compared with the Cap-C sample, but again to a lesser extent than that caused by P4 (Fig. 1A).

On the other hand, the incubation with the PR antagonist mifepristone before P4 addition prevented the AR (Fig. 1B), and sperm samples with mifepristone at 4 and 40 μM presented a lower percentage of acrosome-reacted spermatozoa (17.1 ± 3.11, 15.0 ± 1.56, and 39.9 ± 3.12% with mifepristone 4 μM, 40 μM, and P4, respectively; $P < 0.001$; Fig. 1B), along with a higher proportion of NC and C spermatozoa than the P4 sample. Moreover, the CTC pattern of the sample incubated with mifepristone 40 μM showed no significant differences when compared with the Cap-C sample.

However, despite the great differences detected in the CTC assay evaluation, no significant differences were found in the total intensity of protein tyrosine phosphorylation evaluated by western blot when agonist-, antagonist-, and P4-treated groups were compared with the Cap-C group (Fig. 1C–F).

3.2. Experiment 2: involvement of estrogen receptors in ram sperm capacitation

The incubation in capacitating conditions of sperm samples with the ER agonist resveratrol or the antagonist tamoxifen did not affect sperm viability, and no statistical differences with the control or Cap-C groups were found (Table 2). Regarding total motility, only the tamoxifen-treated sample at the lowest concentration and the E2 sample in the antagonist experiments showed significant differences ($P < 0.05$) with the Cap-C sample. Nonetheless, the main differences between treatment groups were found when progressive motility was analyzed: in the ER agonist experiments, all samples incubated in the high-cAMP medium reduced the progressive motility when compared with the control group (Table 2), and this decrease was more pronounced in the resveratrol 5 μM and E2 samples, where differences with the Cap-C group were also detected. In the antagonist experiments, the tamoxifen-treated spermatozoa increased their progressive motility, showing significant differences when compared with the C group when both concentrations of tamoxifen were used and with the Cap-C and E2 groups when spermatozoa were treated with tamoxifen 10 μM ($P < 0.05$, Table 2). However, no differences were found in the kinematic parameters either in the agonist or the antagonist experiments, except in the percentage of hyperactivated spermatozoa compared with the control group ($P < 0.05$, Table 2).

The incubation in capacitating conditions with E2 resulted in an increased percentage of acrosome-reacted

Table 1
Viability (6-CFDA+/PI-), total and progressive motility, sperm kinematics parameters, and percentage of hyperactivated spermatozoa after 3 h of incubation in capacitating conditions (39°C and 5% CO_2 and 100% humidity) without (control) or with cAMP-elevating compounds (Cap-C), cAMP-elevating compounds with progesterone (P4) 1 μM, with the progesterone receptor agonist tanaproget (1 μM or 5 μM), or with the progesterone receptor antagonist mifepristone (4 μM or 40 μM) plus P4 1 μM added after 1 h of incubation.

Sperm parameter	P4 receptor agonist					P4 receptor antagonist				
	Control	Cap-C	Tanaproget 1 μM	Tanaproget 5 μM	P4 1 μM	Control	Cap-C	Mifepristone 4 μM	Mifepristone 40 μM	P4 1 μM
Viability (%)	68.7 ± 1.87	73.3 ± 1.78	71.8 ± 2.17	71.8 ± 1.76	70.1 ± 3	64.5 ± 1.74	68.3 ± 2.01	71.7 ± 1.42$^{\#\#}$••	64.4 ± 3.66	63.9 ± 3
Total motility (%)	82 ± 2.88	81.8 ± 1.8	83.3 ± 3.05	81.9 ± 3.54	82.4 ± 2.75	69 ± 4.98	72.5 ± 6.03	74.2 ± 6.57$^{\#}$	70.2 ± 6.39	70.2 ± 8.55
Progr. motility (%)	29.6 ± 3.73	24.4 ± 3.62$^{\#}$	24.8 ± 3.04$^{\#}$	21.5 ± 1.91$^{\#\#\#}$••	27.9 ± 2.26	28.7 ± 4.33	21.5 ± 3.25$^{\#\#}$	22.4 ± 4.8$^{\#}$	26.8 ± 5.12*•	21.8 ± 4.45$^{\#\#}$
VCL (μm/s)	96.2 ± 6.15	85.4 ± 6.89	87.7 ± 4.86	87.3 ± 8.11	91.2 ± 7.88	89.6 ± 7.5	80.3 ± 7.71	85.2 ± 5.48	81.7 ± 6.73	81.6 ± 6.58
VSL (μm/s)	55.3 ± 3.88	43.1 ± 5.05	43.2 ± 4.17	40.5 ± 4.37	46.7 ± 5.32	50.2 ± 6.75	40.4 ± 4.68	41.0 ± 3.37	43.0 ± 6.01	37.6 ± 4.94
VAP (μm/s)	74.0 ± 5.2	63.2 ± 6.46	64.5 ± 5.08	62.0 ± 6.86	68.2 ± 7.18	68.0 ± 8.13	59.5 ± 7.01	62.0 ± 5.07	61.6 ± 6.7	57.5 ± 6.32
LIN (%)	57.5 ± 1.6	50.0 ± 3.36	48.7 ± 2.35	46.3 ± 2.2$^{\#}$	50.9 ± 2.87	54.7 ± 3.03	50.4 ± 3.76	48.4 ± 3.12	53.2 ± 4.33	46.0 ± 4.33
STR (%)	74.8 ± 1.08	67.4 ± 2.14$^{\#}$	66.4 ± 1.47$^{\#\#}$	65.5 ± 1.56$^{\#\#}$	68.2 ± 2	73.1 ± 1.24	68.2 ± 2.88	66.4 ± 2.63	70.5 ± 3.06	65.1 ± 3.34
WOB (%)	76.8 ± 1.46	73.4 ± 2.86	73.1 ± 2.04	70.5 ± 2.1	74.3 ± 2.52	74.6 ± 2.95	73.5 ± 3.02	72.6 ± 2.46	74.7 ± 2.99	70.0 ± 3.56
ALH (μm)	3.1 ± 0.2	2.8 ± 0.17	2.9 ± 0.14	3.0 ± 0.16	2.9 ± 0.15	3.1 ± 0.06	2.7 ± 0.12	2.9 ± 0.16	2.8 ± 0.11	2.9 ± 0.17
BCF (Hz)	8.6 ± 0.11	8.3 ± 0.15	8.1 ± 0.11$^{\#\#}$	8.0 ± 0.07$^{\#\#}$	8.1 ± 0.11$^{\#}$	8.3 ± 0.16	8.2 ± 0.16	8.1 ± 0.12	8.4 ± 0.14	8.0 ± 0.33
Hyperactivated spermatozoa (%)	17.3 ± 1.86	25.7 ± 4.37$^{\#\#}$	26.0 ± 2.28$^{\#\#}$	29.7 ± 3.64$^{\#\#\#}$	24.7 ± 5.6$^{\#\#}$	14.9 ± 2.02	24.1 ± 4.21$^{\#\#}$	24.0 ± 4.77$^{\#\#}$	22.0 ± 3.71$^{\#}$	27.8 ± 9.01$^{\#\#}$

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, percentage of linearity; Progr., progressive; STR, percentage of straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL; straight-line velocity; WOB, wobble coefficient.
Values are shown as mean ± SEM (n = 5). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ indicate significant differences related to control sample, $^{*}P < 0.05$ to Cap-C sample and • $P < 0.05$, •• $P < 0.01$ to P4 sample.

spermatozoa, concomitant with a decrease in the proportion of NC compared with the Cap-C sample in both the agonist and antagonist experiments (Fig. 2A, B). The treatment with the ER agonist resveratrol at both concentrations mimicked the effect of this hormone and produced a significant increase in the AR sperm rate while reducing the percentage of NC cells when compared with the Cap-C group. The induction of the AR was enhanced when the highest concentration of resveratrol was used, and the percentage of AR spermatozoa in the resveratrol 10 μM group was significantly higher than that in the Cap-C and the E2 groups (36.8 ± 5.65% vs 30.2 ± 4.73% and 28.6 ± 3.53% for resveratrol 10 μM, Cap-C, and E2, respectively; $P < 0.05$, Fig. 2A). On the other hand, the ER antagonist tamoxifen prevented the induction of the AR, and the spermatozoa incubated with both concentrations of this compound showed a CTC pattern similar to that found in the Cap-C sample, with a significantly higher percentage of NC and C spermatozoa, and a lower rate of AR cells when compared with the E2 sample (17.5 ± 3.69% and 17.0 ± 1.64% vs 30.5 ± 3.25% with resveratrol 5 μM, 10 μM and E2, respectively; $P < 0.01$; Fig. 2B). However, as was the case with the modulators of the P4 receptor, no differences were found in the analysis of protein tyrosine phosphorylation, despite the differences observed in CTC staining (Fig. 2C–F).

4. Discussion

A previous study demonstrated that the incubation with E2 and P4 induced the AR in ram spermatozoa. That work also identified, for the first time, the presence of PRs and ERs in these cells [8]. It also proved that the presence of ERβ decreased after in vitro capacitation and nearly disappeared when the AR was induced [8], which suggests that steroid receptors could play an essential role in ram sperm capacitation and the AR. Therefore, in this study, the role of PRs and ERs on ram sperm capacitation was investigated by using their agonists and antagonists.

First, the PR agonist tanaproget and the antagonist mifepristone were used during ram sperm in vitro capacitation. To the best of our knowledge, this study is the first report on the use of tanaproget to investigate the role of the PR in sperm functionality in any species. The results obtained with either tanaproget or mifepristone showed that neither of them had a detrimental effect on sperm viability, which even increased with the lower concentration of the PR antagonist. This beneficial effect of mifepristone on sperm membrane integrity was previously described in buffalo spermatozoa that had undergone cryopreservation processes [33]. The authors linked this effect of mifepristone with its antioxidant activity during cryopreservation. Because of the sensitivity of all mammalian spermatozoa to oxidative damage [34], it is likely that mifepristone would maintain higher membrane integrity in our ram sperm samples by decreasing the oxidative damage.

The incubation with P4 receptor modulators affected progressive motility, which decreased with the highest concentration of the agonist tanaproget and increased when spermatozoa were previously incubated with the

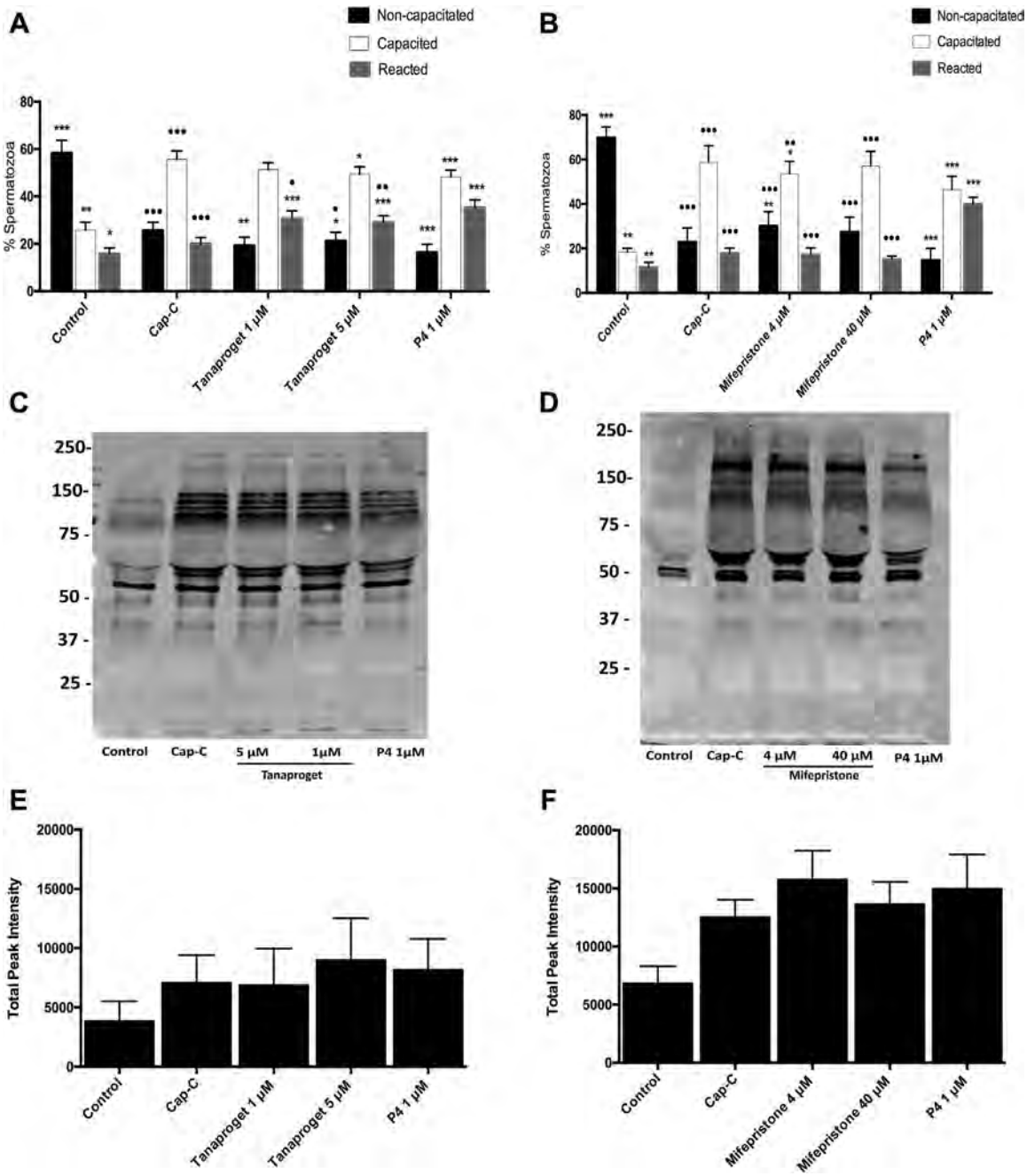

Fig. 1. Assessment of the capacitation status (panels A and B) evaluated by chlortetracycline (CTC) and protein tyrosine phosphorylation (panels C–F) assessed by western blot, in seminal plasma-free spermatozoa after 3 h of incubation in capacitating conditions (39°C and 5% CO_2) without (control) or with cAMP-elevating compounds (Cap-C), cAMP-elevating compounds with progesterone (P4) 1 µM, with the progesterone receptor agonist tanaproget (1 µM or 5 µM, panels A, C, and E), or with the progesterone receptor antagonist mifepristone (4 µM or 40 µM, panels B, D, and F) plus P4 1 µM added after 1 h of incubation. Values are shown as mean ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences relative to the Cap-C sample and $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, and $^{\bullet\bullet\bullet}P < 0.001$ to the P4 sample.

antagonist mifepristone. These changes in sperm progressive motility could be related to sperm hyperactivation. Thus, these results would suggest that, in ram spermatozoa, the P4-induced hyperactivation could be receptor-mediated as in hamster [6] and human [14] spermatozoa. However, the analysis of the percentage of hyperactivated spermatozoa and of the kinematic parameters did not reveal any differences between treatments. This lack of results could be due to the low frame rate (25 fps) and recording time (1 s) used in this study,

Table 2
Viability (6-CFDA+/PI-), total and progressive motility, sperm kinematics parameters, and percentage of hyperactivated spermatozoa after 3 h of incubation in capacitating conditions (39°C and 5% CO_2 and 100% humidity) without (control) or with cAMP-elevating compounds (Cap-C), cAMP-elevating compounds with 17-β estradiol (E2) 1 μM, with the estrogen receptor agonist resveratrol (5 μM or 10 μM), or with the estrogen receptor antagonist tamoxifen (5 μM or 10 μM) plus E2 1 μM added after 1 h of incubation.

Sperm parameter	E2 receptor agonist					E2 receptor antagonist				
	Control	Cap-C	Resveratrol 5 μM	Resveratrol 10 μM	E2 1 μM	Control	Cap-C	Tamoxifen 5 μM	Tamoxifen 10 μM	E2 1 μM
Viability (%)	56.5 ± 2.19	58.4 ± 3.02	57.4 ± 3.13	58.1 ± 2.85	59 ± 1.58	58.4 ± 3.27	58.6 ± 1.42	59.6 ± 1.61	57.8 ± 3	62.6 ± 2.62
Total motility (%)	77.7 ± 7.4	75.5 ± 5.5	78.2 ± 3.81	75.2 ± 4	74 ± 4.23	65.4 ± 9.81	60.4 ± 3.41	66.6 ± 8.12*	64.8 ± 4.77	66.4 ± 5.04*
Progr. motility (%)	28.8 ± 4.53	22.5 ± 4.25#	17.5 ± 2.99###*	18.7 ± 3.86###	17.3 ± 2.53###*	14.2 ± 1.77	15.2 ± 2.24	19.4 ± 4.96#	21 ± 3.49##•*	15.6 ± 4.71
VCL (μm/s)	105.2 ± 8.57	89.1 ± 4.75	89.8 ± 5.29	90 ± 4.76	88 ± 3.5	95.8 ± 6.07	79.5 ± 5	73.5 ± 8.4	65.2 ± 7.3	75.3 ± 4.87
VSL (μm/s)	53.2 ± 5.04	43.4 ± 4.94	39.3 ± 5.72	42.1 ± 4.84	37.7 ± 2.76	42.9 ± 4.63	36.9 ± 3.97	29.4 ± 2.73	26.2 ± 1.55	29.4 ± 2.26
VAP (μm/s)	76.4 ± 7.13	65.3 ± 5.56	62.8 ± 7.26	65.2 ± 5.47	60.5 ± 3.24	64.6 ± 5.82	56.1 ± 5.03	46.6 ± 5.23	41.4 ± 3.42	47.3 ± 2.94
LIN (%)	51.1 ± 3.54	48.1 ± 3.74	42.9 ± 3.6	46.5 ± 4.13	42.9 ± 2.26	44.2 ± 3.13	45.8 ± 2.95	40.3 ± 1.76	42.1 ± 4.35	39 ± 2.04
STR (%)	70 ± 2.95	65.6 ± 2.4	61.7 ± 1.74	63.7 ± 2.87	62.2 ± 1.92	65.6 ± 2.33	65.1 ± 1.9	63.4 ± 1.52	64.2 ± 3.29	62 ± 1.81
WOB (%)	72.6 ± 2.73	72.8 ± 3.2	69.2 ± 3.81	72.2 ± 3.78	68.6 ± 1.67	66.6 ± 2.58	69.7 ± 2.6	63.4 ± 1.5	64.8 ± 3.28	62.8 ± 1.53
ALH (μm)	3.3 ± 0.23	2.9 ± 0.16	3 ± 0.13	2.8 ± 0.18	3 ± 0.06	3.6 ± 0.17	3.1 ± 0.12	3.5 ± 0.18	3.3 ± 0.16	3.4 ± 0.12
BCF (Hz)	8.5 ± 0.15	7.9 ± 0.12	7.6 ± 0.12	7.7 ± 0.39	7.7 ± 0.19	8.3 ± 0.2	7.8 ± 0.14	7.3 ± 0.32	7.1 ± 0.15	7.3 ± 0.27
Hyperactivated spermatozoa (%)	24.0 ± 5.01	33.8 ± 5.88##	34.8 ± 1.92##	32.4 ± 8.12##	32.3 ± 3.84##	22.3 ± 3.03	29.6 ± 3.27##	27.8 ± 8.13#	26.1 ± 3.19#	27.9 ± 6.67#

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, percentage of linearity; Progr., progressive; STR, percentage of straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL; straight-line velocity; WOB, wobble coefficient.
Values are shown as mean ± SEM (n = 5). #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ indicate significant differences related to control sample, *$P < 0.05$ to Cap-C sample and • $P < 0.05$ to E2 sample.

unlike the recording conditions used by other researchers [14,35].

In addition, P4 is known to induce the AR in the spermatozoa of multiple species [36], including ovine [8]. This P4 effect is assumed to be mediated by rapid actions through nongenomic membrane PRs [7,37]. In this work, the incubation with the PR agonist tanaproget increased the rate of acrosome-reacted spermatozoa, although to a lesser extent than the hormone, whereas a previous incubation with the PR antagonist mifepristone prevented the P4 induction of the AR. These results reinforce the idea that the P4 effect on ram spermatozoa is also receptor-mediated. As far as we know, this is the first report on the AR induction by a PR agonist in any species. Nevertheless, mifepristone was also able to suppress the P4-induced AR in other species such as buffalo [33], boars [38], and humans [14].

Unexpectedly, despite the differences found in the CTC staining patterns, no significant differences were found in the protein tyrosine phosphorylation between samples treated with an agonist or antagonist and those capacitated or incubated with P4, which suggests that the effect of P4 on TyrPP in ram spermatozoa that has been previously reported [8] might not be receptor-mediated. Thus, although P4 can increase sperm TyrPP in ovine and other species [6,39,40], and the incubation with mifepristone can decrease phosphorylation of tyrosine proteins in buffalo [33], the involvement of PR in this phosphorylation process in ram spermatozoa remains unclear.

To further the knowledge of the involvement of steroid hormones in ram sperm capacitation and the AR, the role of ERs in these processes was investigated by incubating cells with the ER agonist resveratrol and the ER antagonist tamoxifen. Neither resveratrol nor tamoxifen affected the ram sperm membrane integrity, as found in other studies [20,41]. However, the incubation with either E2 or ER agonist decreased the progressive motility compared with the sample without a hormone or agonist, whereas this parameter increased in spermatozoa previously incubated with the ER antagonist. Most studies on the effect of resveratrol on sperm functionality are focused on its antioxidant effects during sperm cryopreservation. In these conditions, resveratrol had a positive impact on bull [42], boar [43], and goat [44] sperm motility, probably due to its protective effects. Conversely, the resveratrol effect was detrimental in equine sperm [45] or without any effect on buffalo [17] and human [46] motility. It is worth pointing out that this is the first time that resveratrol has been used as an ER agonist to study the involvement of ERs in sperm functionality. The ER antagonist tamoxifen, on the other hand, has been regularly used as a treatment for male infertility [47], although a recent meta-analysis revealed that oral tamoxifen has no effect on human sperm motility [48]. Even so, when tamoxifen was used in vitro, it was able to suppress melatonin- [49] or progesterone-enhanced [21] hyperactivation by E2 in hamster spermatozoa without affecting total motility.

Regarding the capacitation state, previous research has demonstrated that E2 alone can induce the AR in ram spermatozoa incubated in capacitating conditions [8]. Although it is generally accepted that P4 can provoke the

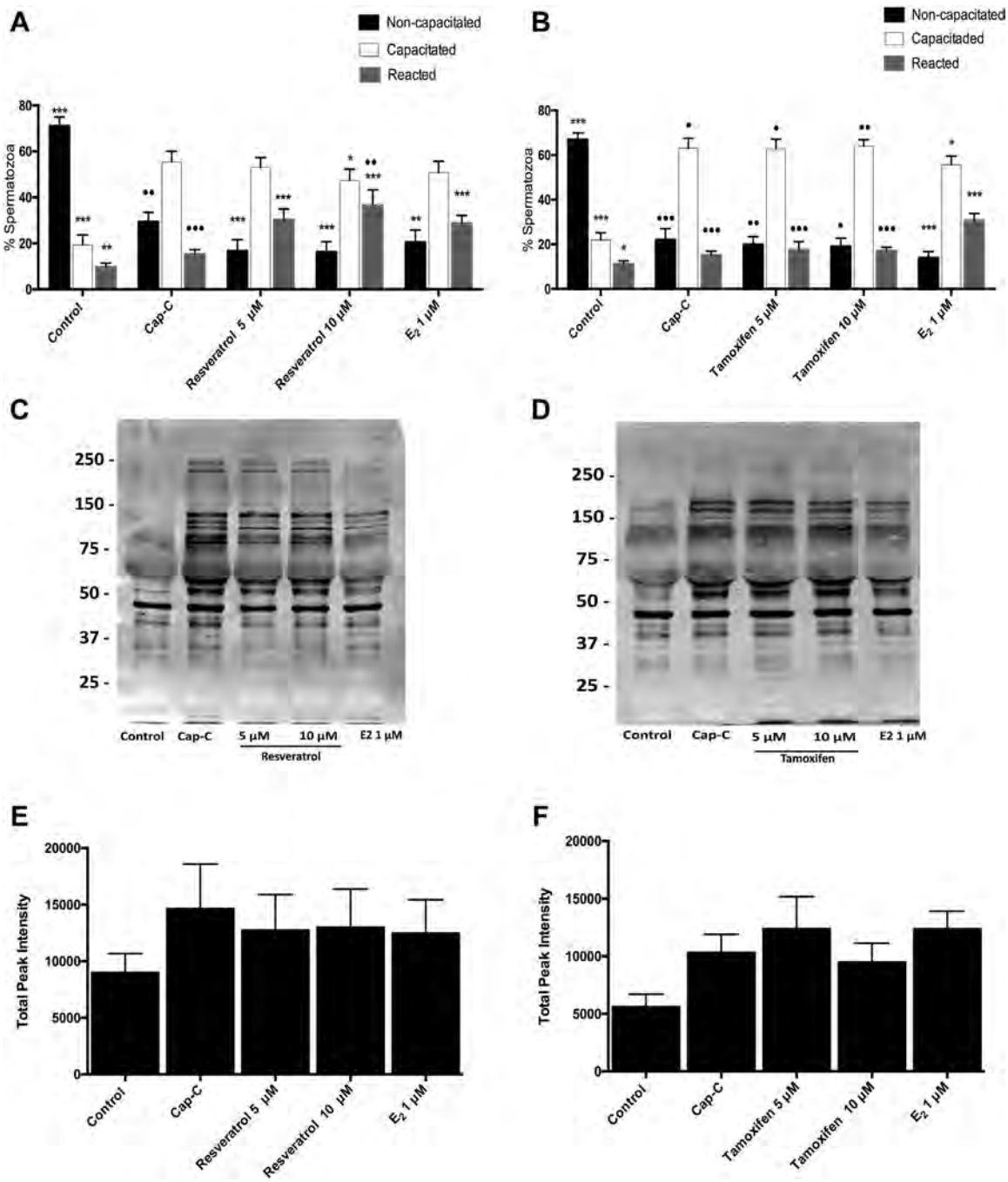

Fig. 2. Assessment of the capacitation status (panels A and B) evaluated by chlortetracycline (CTC) and protein tyrosine phosphorylation (panels C–F) assessed by western blot, in seminal plasma-free spermatozoa after 3 h of incubation in capacitating conditions (39°C and 5% CO_2) without (control) or with cAMP-elevating compounds (Cap-C), cAMP-elevating compounds with 17-β estradiol (E2) 1 μM, with the estrogen receptor agonist resveratrol (5 μM or 10 μM, panels A, C, and E), or with the estrogen receptor antagonist tamoxifen (5 μM or 10 μM, panels B, D, and F) plus E2 1 μM added after 1 h of incubation. Values are shown as mean ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences relative to the Cap-C sample and $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, and $^{\bullet\bullet\bullet}P < 0.001$ to the E2 sample.

AR in most species via receptors [5,37], there is some controversy about the involvement of estradiol and its receptors in this process. Although in some species such as ram [8] and mouse [50] E2 can induce the AR on its own, in other species it has no effect or even decreases the AR induced in response to incubation with P4 [51] or calcium ionophore [52]. Moreover, the involvement of ERs in this process is not clear. In the present study, the incubation with the ER agonist resveratrol provoked the same response as that of the hormone, and we even found a

higher acrosome-reacted sperm rate than with E2 when the highest concentration of the agonist was used. In contrast, tamoxifen maintained the capacitation state at similar levels to those found in the capacitated sample. These results confirm that E2 can induce the AR on its own in ram spermatozoa and that this effect is receptor-mediated.

However, these changes in the CTC staining pattern when the AR was induced by either E2 or ER agonists, or prevented with ER antagonists, were not concomitant with a significant modification in the TyrPP levels. In mouse, E2 increases the overall sperm TyrPP during in vitro capacitation. In this species, the increase in TyrPP on the sperm head [52] has been related to capacitation and zona pellucida binding [53], whereas the presence of phosphotyrosine residues in the sperm flagellum was associated with the onset of hyperactivated movement [54]. In ram spermatozoa, capacitation and hyperactivation are not simultaneous processes [29], and the possibility of differences in TyrPP distribution without changes in the overall protein tyrosine phosphorylation cannot be ruled out.

5. Conclusions

In conclusion, these results indicate that both P4 and E2 can induce the ram sperm AR via membrane receptors. Further research is needed to elucidate the involvement of PRs and ERs in ram sperm hyperactivation and protein tyrosine phosphorylation.

CRediT authorship contribution statement

S. Gimeno-Martos: Investigation, Formal analysis, Data curation, Writing - original draft, Visualization. **M. Santorromán-Nuez:** Investigation, Formal analysis, Data curation. **J.A. Cebrián-Pérez:** Resources, Supervision, Writing - review & editing. **T. Muiño-Blanco:** Supervision, Writing - review & editing. **R. Pérez-Pé:** Funding acquisition, Project administration, Supervision, Conceptualization, Methodology, Resources, Validation, Writing - review & editing. **A. Casao:** Supervision, Conceptualization, Validation, Writing - review & editing, Visualization.

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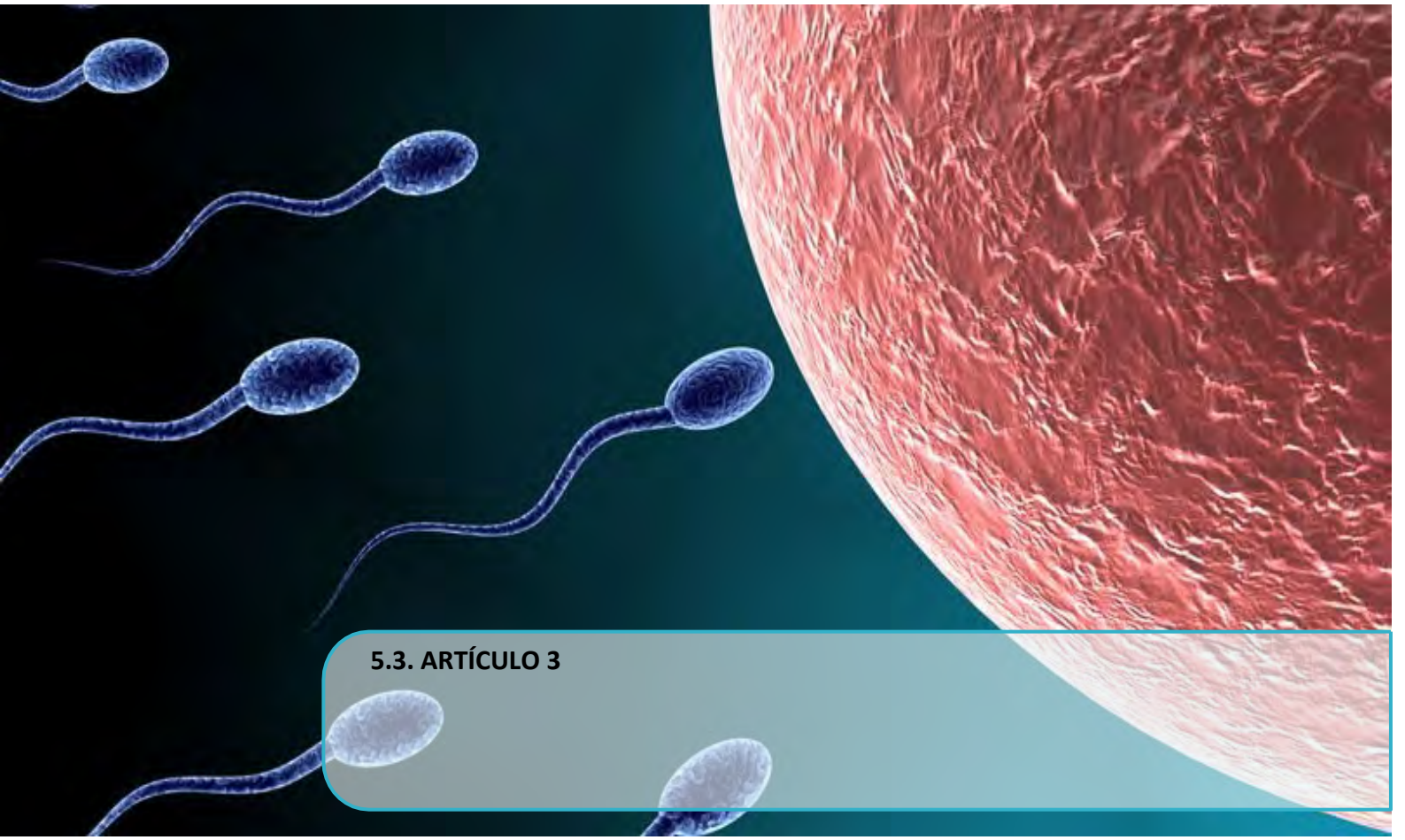

5.3. ARTÍCULO 3

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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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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_i^{2+} 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.

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

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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 × 107 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 200–300 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 × 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-5 N 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

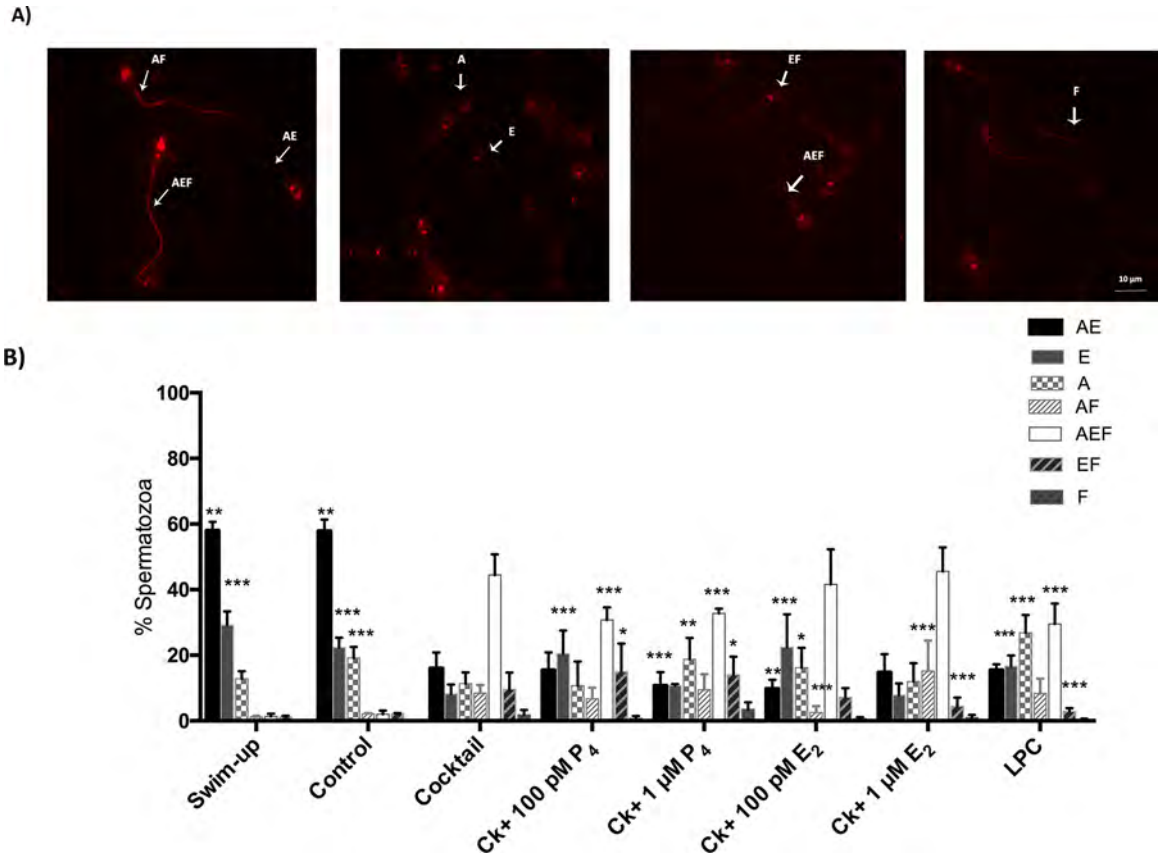

Fig. 1. (a) Representative microscopy images (magnification x1000) of the location of tyrosine-phosphorylated proteins, evaluated by indirect immunofluorescence (IIF) in swim-up selected spermatozoa (control 0 h) and after 3 h of incubation in capacitating conditions (39 °C, 5 % CO_2 and 100 % humidity) 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.; ($n = 5$).

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 Fig. 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).

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

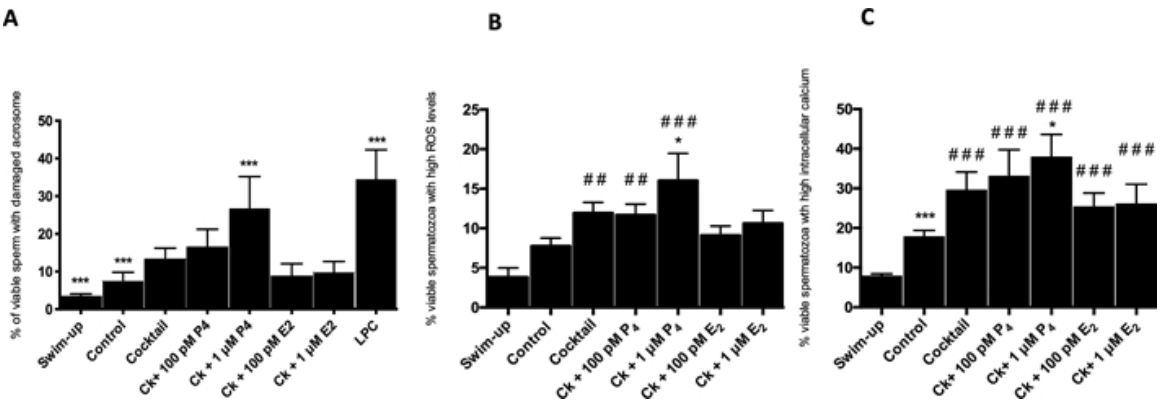

Fig. 2. (a) Percentage of viable spermatozoa with damaged acrosome membrane (RCA+/PI-) evaluated by *Ricinus communis* agglutinin swim-up selected spermatozoa and after 3 h of incubation in capacitating conditions (39 °C, 5 % CO_2 and 100 % humidity) in TALP (control) or TALP with agent that increased 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 h 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 h 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.

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-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

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.

Variable	Swim-up	Control	"Cocktail"-incubated samples (3 h)				
			"Cocktail"	+ 100 pM P4	+ 1 μM P4	+ 100 pM E2	+ 1 μM 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(μm^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}

(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 Fig. 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

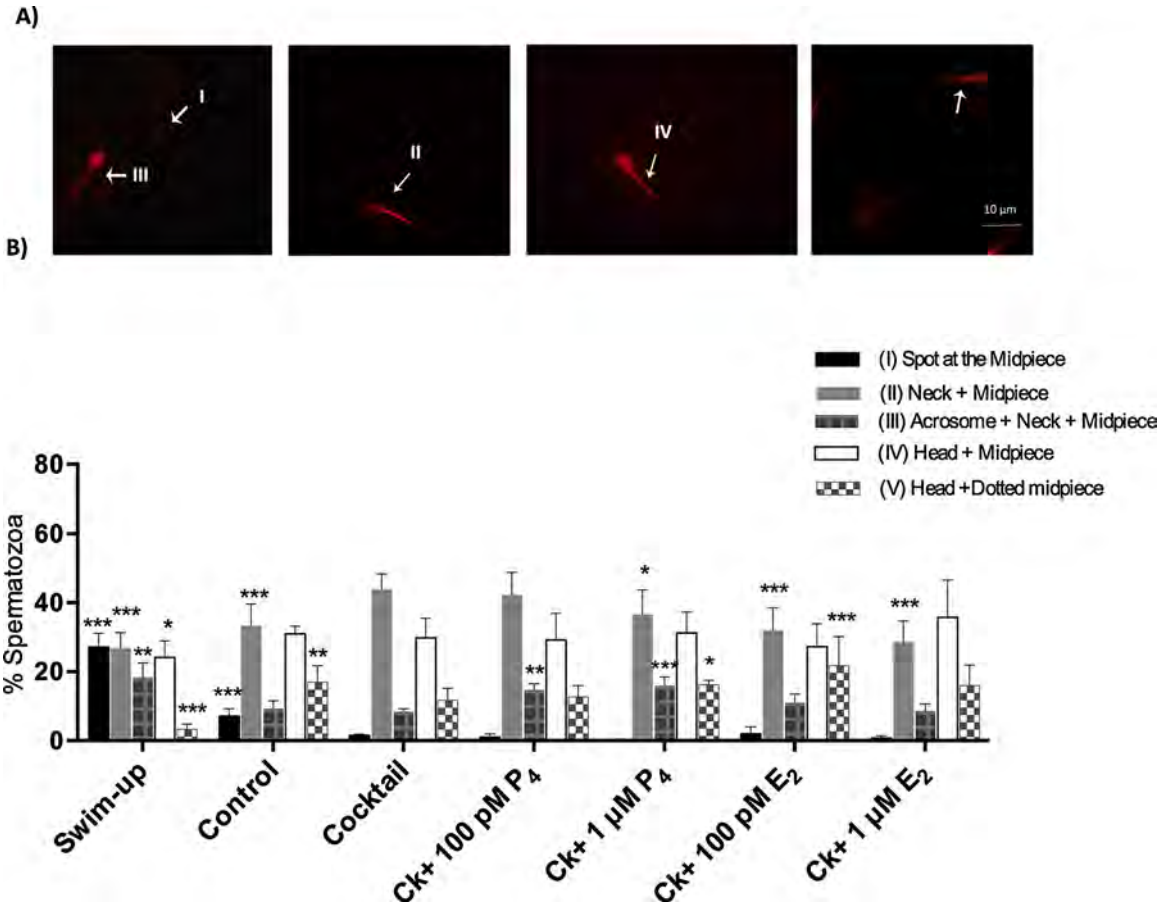

Fig. 3. (a) Representative microscopy images (magnification x1000) of the calcium location into the cell compartments, evaluated using Rhod-5N-AM and **(b)** percentage of the different localization patterns in swim-up selected ram spermatozoa (control 0 h) and after 3 h of incubation in capacitating 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.; ($n = 5$).

(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).

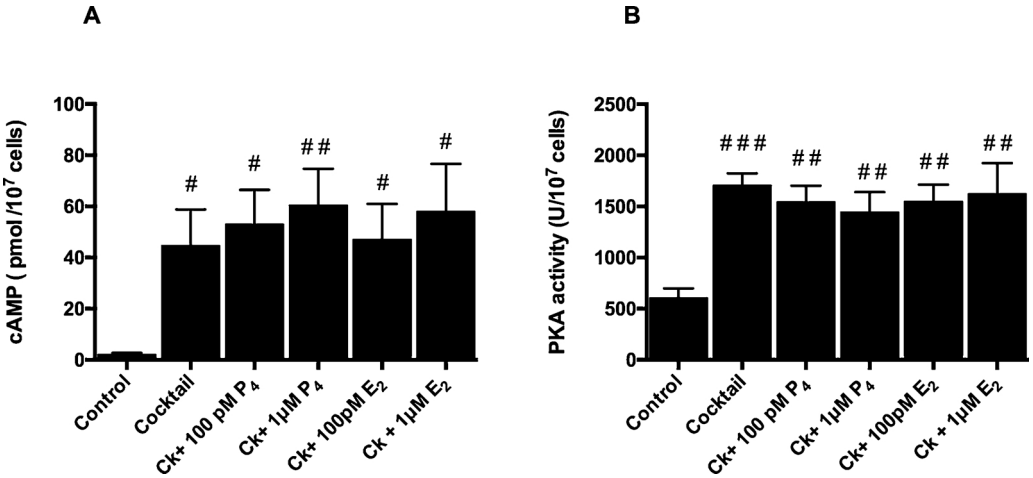

Fig. 4. Assessment of intracellular cAMP content **(a)** and protein kinase A (PKA) activity **(b)** in swim-up selected ram spermatozoa after 5 min of incubation in capacitating conditions (39 °C, 5 % CO_2 and 100 % humidity) in TALP (control) or TALP with agent increasing cAMP concentrations ("cocktail") 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.

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 authorship contribution statement

S. Gimeno-Martos: Methodology, Validation, Investigation, Formal analysis, Writing - original draft. **S. Miguel-Jiménez:** Validation, Investigation. **A. Casao:** Conceptualization, Formal analysis, Supervision, Writing - review & editing. **J.A. Cebrián-Pérez:** Conceptualization, Supervision. **T. Muiño-Blanco:** Conceptualization, Supervision, Writing - review & editing. **R. Pérez-Pe:** Conceptualization, Resources, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2020.106567.

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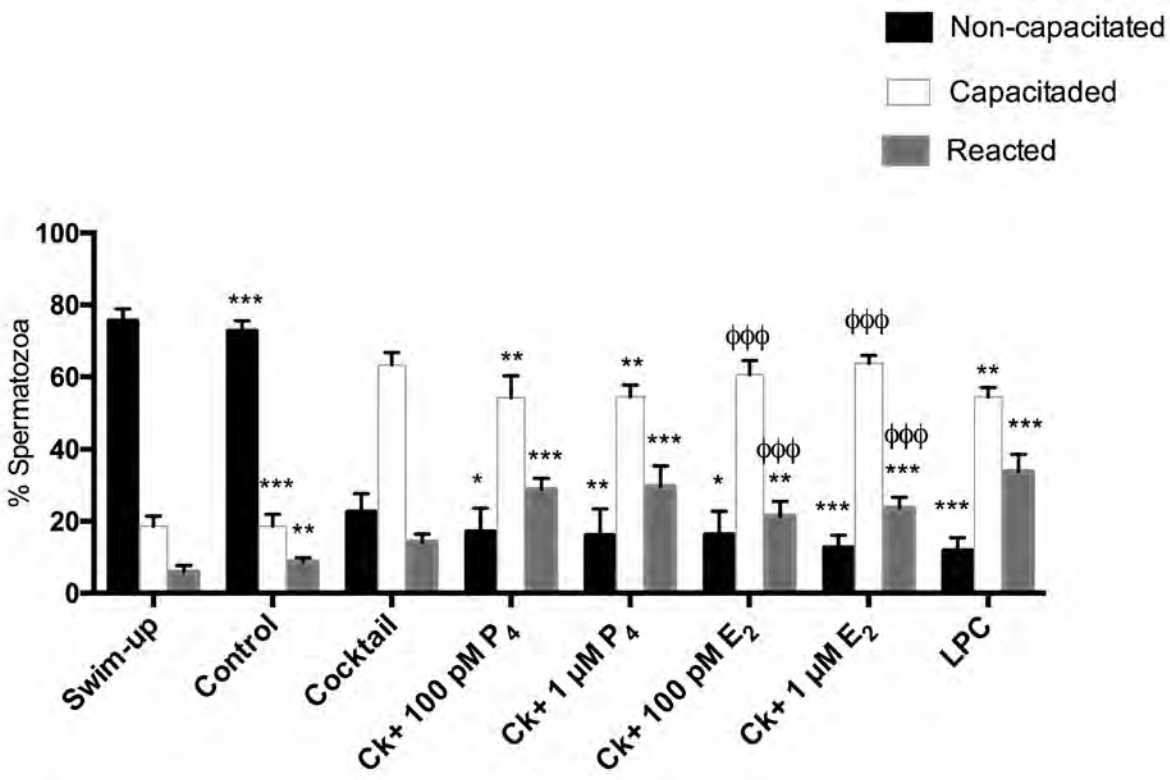
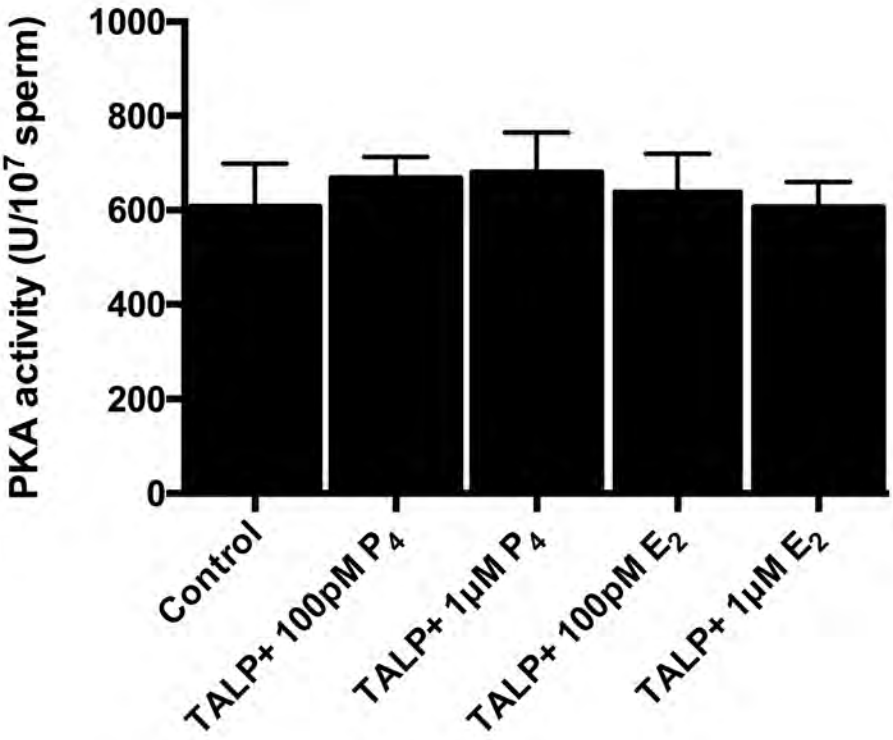
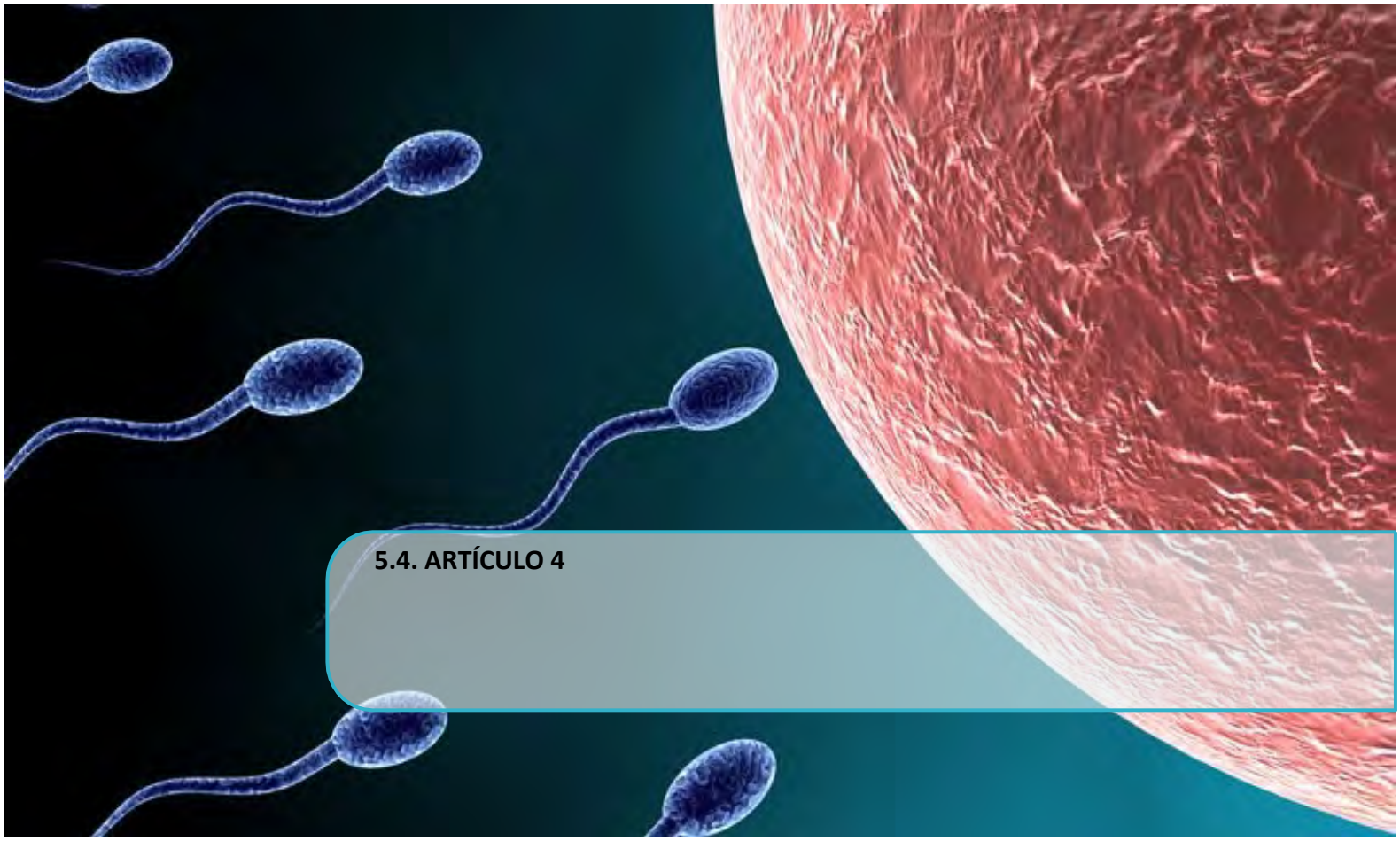
Supplementary figure 1



Supplementary figure 2





5.4. ARTÍCULO 4

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Melatonin reduces cAMP-stimulated capacitation of ram spermatozoa

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Abstract. The presence of melatonin receptors on the surface of ram spermatozoa has led to speculation about melatonin having a role in sperm functionality. The aim of this study was to elucidate the mechanism through which melatonin regulates ram sperm capacitation induced by a cocktail containing cAMP-elevating agents. Cocktail samples capacitated in the presence of 1 μM melatonin showed lower percentages of capacitated spermatozoa (chlortetracycline staining; $P < 0.001$) together with a decrease in protein tyrosine phosphorylation ($P < 0.01$) and lower levels of reactive oxygen species (ROS) and cAMP ($P < 0.05$) compared with cocktail samples without the hormone. Determination of kinematic parameters, together with principal component and cluster analyses, allowed us to define four sperm subpopulations (SP). After 3 h of incubation with cAMP-elevating agents, the percentages of spermatozoa belonging to SP1 (high straightness) and SP4 (less-vigorous spermatozoa with non-linear motility) increased while SP2 and SP3 (rapid spermatozoa starting hyperactivation or already hyperactivated) decreased compared with the control sample. The presence of melatonin at 100 pM and 10 nM restored these subpopulations to values closer to those found in the control sample. These results indicate that melatonin at micromolar concentrations modulates ram sperm capacitation induced by cAMP-elevating agents, reducing ROS and cAMP levels, whereas at lower concentrations melatonin modifies motile sperm subpopulations. These findings warrant further studies on the potential use of melatonin for controlling capacitation in artificial insemination procedures.

Additional keywords: kinematic parameters, protein tyrosine phosphorylation, reactive oxygen species.

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Introduction

Melatonin is a ubiquitous molecule that is present in a wide range of organisms and is involved in multiple functions. In mammals, melatonin produced by the pineal gland is only a fraction of the total melatonin generated in the organism, as it can also be synthesised in various extrapineal tissues, including the gastrointestinal tract, eye, bone marrow, skin and ovarian follicles (Brzezinski *et al.* 1987). We have recently demonstrated that melatonin is synthesised in tissues of the male reproductive tract, especially in the testes, which would be one of the likely sources of the high levels of melatonin found in ram seminal plasma, at least during the day (Gonzalez-Arto *et al.* 2016). One of the main characteristics of the extrapineal melatonin in most of these tissues or fluids, including seminal plasma and follicular fluid, is that concentrations can be higher than those found in blood serum. Therefore, spermatozoa would be exposed to different melatonin concentrations from the male to the female reproductive tract. This fact, together with the presence of melatonin receptors on the sperm surface (van Vuuren *et al.* 1992; Reppert *et al.* 1994; Casao *et al.* 2012) led us to speculate about a direct effect of melatonin on sperm functionality. Among the direct effects of melatonin on spermatozoa, a decrease in oxidative damage (Jang *et al.* 2010) and also in apoptotic markers (Casao *et al.* 2010*a*; Espino *et al.* 2011; Sarabia *et al.* 2009) has been described.

Nevertheless, studies about the relationship between melatonin and sperm capacitation are scarce. Capacitation is a complex process that involves changes in the sperm plasma membrane (Gadella *et al.* 2008), calcium movement (Lishko *et al.* 2011) and a signal transduction cascade (Grasa *et al.* 2006) that leads to the acrosome reaction and the acquisition of the ability to fertilise the oocyte. We have demonstrated that melatonin

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affects the ram sperm capacitation pattern and *in vitro* fertilisation results and that these effects are dose-dependent (Casao *et al.* 2010*b*), promoting capacitation at a picomolar range and preventing it at micromolar concentrations (Casao *et al.* 2010*a*). In contrast, melatonin promotes capacitation in buffalo in a wide range of concentrations (10 μM, 100 μM and 1 mM; Di Francesco *et al.* 2010). Other studies have demonstrated that melatonin regulates other events usually associated with capacitation, such as the acrosome reaction and hyperactivation (Fujinoki 2008). However, the mechanism through which melatonin regulates capacitation is yet to be clarified. After binding to its specific receptors, known as MT1 and MT2, melatonin seems to influence second-messenger cascades that vary depending on the cell type (Sjöblom and Flemström 2003; Markowska *et al.* 2004). Some of these pathways described in somatic cells are coincident with those involved in sperm capacitation. For instance, in several cell types, melatonin binding to its receptors inhibits adenylyl cyclase (AC), which decreases cAMP levels and, consequently, reduces protein kinase A (PKA) activity (Luchetti *et al.* 2010). It is well known that the AC–cAMP–PKA pathway is involved in sperm capacitation (Visconti *et al.* 1998, 2002; Harrison 2004) and that the activation of a soluble adenylyl cyclase increases cAMP levels and activates PKA, resulting in the fast phosphorylation of a subset of proteins (Visconti *et al.* 1995; Harrison 2004). As far as we know, there are no reports on the effects of melatonin on spermatozoa when added to a medium that promotes sperm capacitation by increasing the intracellular cAMP.

Therefore, the main objective of this study was to elucidate the mechanism by which melatonin can regulate cAMP-stimulated ram sperm capacitation. With this purpose in mind, we evaluated the effect of different melatonin concentrations on sperm viability, capacitation status by chlortetracycline staining, changes in protein tyrosine phosphorylation and intracellular levels of reactive oxygen species (ROS) and cAMP. Additionally, we determined the potential changes in the motile sperm subpopulation structure by principal component and cluster analyses.

Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Sperm preparation

All experiments were carried out with fresh semen 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 under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza (Spain).

Two successive ejaculates were collected every 2 days and the second ejaculates were pooled and used for each assay, to avoid individual differences (Ollero *et al.* 1996). A seminal plasma-free sperm population was obtained by a dextran swim-up procedure (García-López *et al.* 1996) performed in a medium consisting of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 10 mM KCl, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 5 mg mL^{-1} bovine serum albumin (BSA), 1.5 IU mL^{-1} penicillin and 1.5 mg mL^{-1} streptomycin, pH 6.5 (adjusted by NaOH addition).

All experimental procedures were performed under Project Licence PI19/17 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza.

In vitro *capacitation*

For the induction of *in vitro* capacitation, aliquots of swim-up-selected spermatozoa (1.6×10^8 cells mL^{-1}) were incubated 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), composed of 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 3 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose and 5 mg mL^{-1} BSA (pH 7.3 adjusted with NaOH). A high-cAMP medium (cocktail) already proven for capacitating ram spermatozoa (Grasa *et al.* 2006; Colas *et al.* 2008) and comprising 1 mM dibutyryl (db)-cAMP, 1 mM caffeine, 1 mM theophylline, 0.2 μM okadaic acid and 2.5 mM methyl-β-cyclodextrin was added to the TALP medium to induce capacitation. A control sample, without the addition of the cocktail, was maintained under the same conditions as the capacitated samples.

Melatonin was solubilised in dimethyl sulphoxide (DMSO) and phosphate-buffered saline (PBS) and added to the sperm samples to yield final concentrations of 1 μM, 10 nM or 100 pM. The final concentration of DMSO in all the melatonin samples was 0.1‰. The same DMSO concentration was included in control samples incubated in capacitating conditions.

Sample analyses described below were performed after 1 h and 3 h of incubation in capacitating conditions, except for cAMP assays (at 5 min) and protein tyrosine phosphorylation determinations (at 3 h).

Assessment of capacitation status by chlortetracycline (CTC) staining

CTC is a fluorescent antibiotic that binds to the membrane proteins of sperm cells. The fluorescence of bound CTC is enhanced by intracellular calcium and CTC staining patterns change in capacitated spermatozoa. Although the molecular basis of CTC staining of sperm cells is not well understood, it is now widely considered to reflect sperm capacitation state (Ward and Storey 1984). In brief, 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). After that, 20 μL of CTC solution and 5 μL of 12.2% (w/v) paraformaldehyde in 0.5 M Tris–HCl (pH 7.8) were added to 18 μL of sperm sample and incubated at 4°C in the dark for 30 min. After incubation, 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) at room temperature and in darkness. Samples were covered with 24 × 60 mm coverslips, sealed with colourless enamel and stored in the dark at 4°C.

The fluorescence assay was previously validated for the evaluation of capacitation and acrosome reaction-like changes

in ram semen (Grasa *et al.* 2006). Spermatozoa were classified into one of the following three patterns (Gillan *et al.* 1997): non-capacitated (even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (with fluorescence in the acrosome) and acrosome-reacted cells (showing no fluorescence on the head). Samples were examined using a Nikon Eclipse E-400 microscope under epifluorescence illumination with a V-2A filter. All samples were processed in duplicate and at least 150 spermatozoa were scored per slide. No fluorescence was observed when CTC was omitted from the preparation.

Sperm motility evaluation

Motility kinematic parameters were evaluated using a computer-assisted sperm analysis (CASA) system (ISAS 1.2.; Proiser SL) that consisted of a video camera (Basler A312f; Basler Vision Components) mounted on a microscope (Nikon Eclipse 50i) equipped with a 10× negative-phase contrast lens. Samples (6 μL) were placed onto pre-warmed slides, then covered with standard coverslips (20 × 20 mm) and maintained at 37°C during all the analysis by a heated slide holder. Two drops of each sample were studied and five fields of each drop were recorded. The recording was performed at 25 frames s^{-1} and 25 consecutive digitalised images were taken from a single field. Percentages of total motile and progressive motile spermatozoa in all samples were recorded.

In addition, kinematic parameters were also recorded for each spermatozoon. These parameters were: curvilinear velocity (VCL, μm s^{-1}; the average path velocity of the sperm head along its actual trajectory), straight-line velocity (VSL, μm s^{-1}; 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^{-1}; 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), beat-cross frequency (BCF, Hz; the frequency with which the actual sperm trajectory crosses the average path trajectory), combination of the lateral and forward movement of the head (DANCE, μm^2 s^{-1}; product of VCL and ALH) and mean angular displacement (MAD, degrees; time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory; Verstegen *et al.* 2002).

Flow cytometry analysis

All the analyses were performed on a Beckman Coulter FC 500 with CXP software (IZASA) 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 minimum of 20 000 events was recorded in all the experiments. The sperm population was identified for further analysis by its specific forward (FS) and side scatter (SS) properties; thus, other non-sperm events were excluded. A flow rate stabilised at 200–300 cells s^{-1} was used.

Evaluation of sperm membrane integrity

To determine cell viability (membrane integrity), 3 μL of 1 mM carboxyfluorescein diacetate (CFDA) and 1.5 mM propidium iodide (PI) and 5 μL formaldehyde (0.5% in water) were added to 300 μL of sperm samples (final concentration of 3×10^7 cells mL^{-1}) based on a modification of the procedure described by Harrison and Vickers (1990). Samples were incubated at 37°C in darkness for 15 min. The argon laser and filters of 525 and 675 nm were used to avoid overlapping. The monitored parameters were FS log, SS log, FL1 (CFDA) and FL4 (PI).

Intracellular ROS

The dye H_2DCFDA is freely permeable across cell membranes and is incorporated into hydrophobic regions of the cell (Bass *et al.* 1983). The acetate moiety is cleaved by cellular esterases, resulting in the formation of non-permeant, nonfluorescent, 2′,7′-dichlorodihydrofluorescein (H_2DCF). The H_2DCF is oxidised by H_2O_2 to dichlorofluorescein (DCF), which emits fluorescence at 530 nm in response to 488 nm excitation (Carter *et al.* 1994). This fluorescent probe was combined with PI for excluding the nonviable population from the analysis (Guthrie and Welch 2006). Aliquots of samples, prepared at a final concentration of 5×10^6 cells mL^{-1}, were stained with 5 μL of each stain, H_2DCFDA (20 μM) and PI (1.5 mM). After 15 min at 37°C in the dark, the samples were fixed with 5 μL formaldehyde (0.5% in water) and analysed by flow cytometry. The argon laser and filters of 525 and 675 nm were used to avoid overlapping. The monitored parameters were FS log, SS log, FL1 (H_2DCFDA) and FL4 (PI). The mean of the green fluorescence intensity was used for analysis, after excluding PI+ events (nonviable spermatozoa).

Cyclic AMP assays

The intracellular cAMP content was measured using a Direct cAMP enzyme immunoassay system (Sigma Aldrich) following the manufacturer's instructions. This kit uses a polyclonal antibody to cAMP to bind competitively the cAMP in the sample or an alkaline phosphatase molecule that has cAMP covalently attached to it.

Because of the very low levels of intracellular cAMP in sperm samples, the acetylated version was used. As in an earlier work (Colás *et al.* 2010), samples were processed after 5 min of incubation in capacitating conditions. Cyclic AMP was extracted from 1.2×10^8 spermatozoa with 0.05 M HCl and incubated for 20 min at room temperature to inhibit endogenous phosphodiesterase activity. The extracted sample was centrifuged at 20 000*g* for 5 min at room temperature, and the supernatant was immediately analysed or snap frozen until measurement.

Samples and standards were simultaneously incubated with the alkaline phosphatase conjugate and antibody at room temperature in a secondary-antibody-coated multiwell plate. The reagents were then washed away and the substrate was added. After 1 h of incubation the enzyme reaction was stopped and the yellow colour generated read on a multiwell plate reader at 405 nm. The colour intensity was inversely proportional to the concentration of cAMP in both the standards and the samples.

The measured optical density was used to calculate the concentration of cAMP.

Extraction of ram sperm proteins

Aliquots of 3.2×10^7 cells of swim-up or capacitated samples were mixed with 100 μL of the same extraction medium previously used (Colas *et al.* 2008) and immediately incubated for 5 min at 100°C. The extraction medium was composed of 2% sodium dodecyl sulphate (SDS; w/v), 0.0626 mM Tris-HCl (pH 6.8), 0.002% bromophenol blue in 10% glycerol (final glycerol concentration 1%) and protease and phosphatase inhibitors. After incubation, samples were centrifuged at 15 000*g* for 5 min at room temperature (RT), the supernatant was recovered and 2- mercaptoethanol and glycerol were added to a final concentration of 5% and 1% respectively. The cell lysates were stored at −20°C.

SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Sperm-extracted proteins (15 μL) were loaded on 10% (w/v) SDS–PAGE gels, separated by standard SDS–PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Trans-Blot pack; Bio-Rad) using a transfer unit (Mini Trans-Blot Electrophoretic Transfer Cell Unit; Bio-Rad).

For the detection of phosphorylated proteins, nonspecific sites on the membranes were blocked for 1 h with 5% BSA (w/v) in PBS (136 mM NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4 and 0.24 g L^{-1} KH_2PO_4, pH 7.4) at RT. The blots were then incubated with a mouse monoclonal anti-phosphotyrosine antibody (Millipore) diluted 1 : 1000 in 0.1% Tween-20 in PBS (PBS-T) containing 1% BSA overnight at 4°C. After extensive washing, this was followed by incubation for 1 h and 15 min at RT with a secondary donkey anti-mouse IgG IRDye Dylight 800CW-conjugated antibody (Li-Cor Biosciences) diluted 1 : 15 000 with the previously mentioned buffer. Anti-tubulin antibody (Santa Cruz) produced in rabbit was used as a loading control (1 : 500 in PBS-T), followed by incubation with a secondary donkey anti-rabbit IgG, IRDye 680RD-conjugated antibody (Li-Cor BioSciences) diluted 1 : 15 000. Finally, the membranes were scanned after washing using the Odyssey Clx Infrared Imaging System (Li-Cor Biosciences) to determine the peak intensity of the tyrosine-phosphorylated protein bands. To prove that the signal was specific, western blotting omitting either primary or secondary antibodies was performed (data not shown).

Statistical analysis

Statistical analyses were performed to determine whether there were significant differences between treatment groups within each incubation period. The results are shown as mean ± standard error of the mean of five experiments, except for cAMP assays ($n = 3$).

The levels of protein tyrosine phosphorylation, cAMP assays and intracellular ROS were analysed using an ANOVA test, whereas viability, motility parameters and CTC staining were compared by means of Pearson's chi-square test using GraphPad InStat software (Version 3.01).

To determine the effects of melatonin upon kinematic parameters of ram spermatozoa incubated under capacitating conditions, a series of statistical analyses were conducted using IBM SPSS statistical software (Version 22.0). Data were checked through Shapiro–Wilks and Levene tests to verify normal distribution and homogeneity of variances.

All sperm kinematic parameters obtained from the CASA assessments (i.e. VSL, VCL, VAP, %LIN, %STR, %WOB, ALH, BCF, DANCE and MAD) were used to run a principal component analysis (PCA). In brief, all the sperm parameters were sorted into two PCA components and the data matrix obtained was rotated using the Varimax procedure with the Kaiser normalisation. Following this analysis, only those variables with a positive loading factor (a_{ij}) higher than 0.6 with its respective component and lower than 0.3 with respect to the others in the rotated matrix were selected from the linear combination of *j* variables (*x*) in each component y_i ($y_i = a_{i1}x_1 + a_{i2}x_2 + \ldots + a_{ij}x_j$). For each spermatozoon, which was considered as an independent statistical case, a regression score was calculated per PCA component.

Following PCA, each spermatozoon in each treatment and time point was classified into a subpopulation using cluster analysis. This classification was made on the basis of the regression scores corresponding to each PCA component, as obtained in the previous step, using the between-groups linkage method based on the squared Euclidean distance. A total of four subpopulations were obtained.

After establishing the sperm subpopulations, the effects of *in vitro* capacitation treatment (control, cocktail and cocktail plus different melatonin concentrations) upon the ram sperm subpopulation distribution were determined through a linear mixed model followed by Bonferroni's post hoc test. In this model, the incubation time point was the intrasubject factor and the treatment (control, cocktail and cocktail plus different melatonin concentrations) was the fixed-effects factor. All the sperm kinematic parameters were considered as dependent variables.

Results

Melatonin reduced sperm capacitation induced by cAMP-elevating agents

The incubation in capacitating conditions with a cocktail that maintains high intracellular cAMP levels led to a significant increase in the capacitated-sperm subpopulation relative to the control sample (i.e. non-capacitating conditions) after 1 h of incubation (34.00 ± 4.37 vs 23.60 ± 3.67%; $P < 0.001$), concomitant with a decrease in the percentage of non-capacitated spermatozoa (Fig. 1). The proportions of CTC-stained spermatozoa did not differ between samples containing 100 pM melatonin and cocktail samples without the hormone. However, when melatonin was added at 10 nM or 1 μM, a significant decrease in the proportion of capacitated spermatozoa was observed ($P < 0.001$ and $P < 0.01$ respectively).

After 3 h of incubation the percentages of capacitated spermatozoa were significantly higher in all the cocktail samples than in the control ($P < 0.001$). However, whereas the value reached 62.80 ± 3.14% in the sample without the hormone,

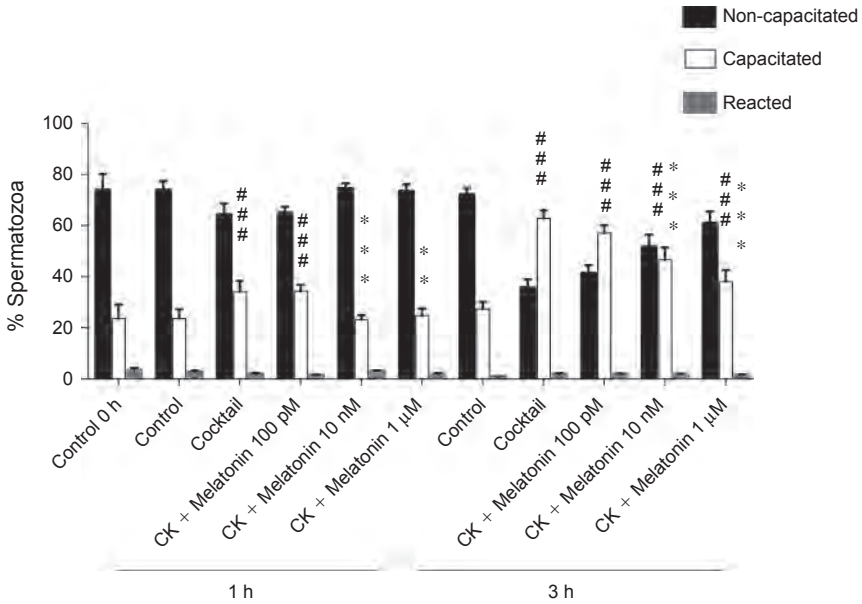

Fig. 1. Assessment of capacitation status of ram spermatozoa, evaluated by chlortetracycline (CTC), in a seminal plasma-free sample before capacitation (control 0 h) and after 1 h and 3 h of incubation at 39°C and 5% CO_2 in capacitating conditions with cocktail (cAMP-elevating agents), cocktail with different melatonin doses or without these compounds (control). Significant differences related to control sample ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) and to cocktail sample (**$P < 0.01$, ***$P < 0.001$) at the same incubation time. Mean values ± s.e.m. ($n = 5$).

Table 1. Effect of melatonin on motility and viability of ram spermatozoa

Percentage of viable (6-CFDA+/PI–), total and progressive motile spermatozoa and kinematic parameters before treatment (control 0 h) and after 3 h of incubation at 39°C and 5% CO_2 in capacitating conditions with cocktail, cocktail plus different melatonin (Mel) concentrations or without these compounds (control 3 h). Values are mean ± s.e.m. ($n = 6$). [a,b,c]Superscript letters indicate significant differences relative to control 0 h (a), control 3 h (b) and cocktail (c)

Parameter	Control (0 h)	Control (3 h)	Cocktail-incubated samples (3 h)			
			Cocktail	+ 100 pM Mel	+10 nM Mel	+1 μM Mel
Viability (%)	81.98 ± 1.43	71.60 ± 7.72[a]	67.90 ± 7.82[a]	74.40 ± 4.33[a,c]	73.22 ± 3.81[a]	67.74 ± 4.98[a]
Total motility (%)	76.44 ± 2.90	61.44 ± 4.71[a]	62.50 ± 5.66[a]	61.56 ± 5.08[a]	63.11 ± 4.20[a]	62.00 ± 5.37[a]
Progr. motility (%)	27.00 ± 2.74	23.67 ± 1.45	22.11 ± 2.01[a,b]	23.33 ± 3.10	25.00 ± 1.64[c]	18.11 ± 3.01[a,b]
VCL (μm s^{-1})	107.43 ± 2.24	99.54 ± 5.40	90.68 ± 5.22[a]	96.74 ± 5.23	97.18 ± 5.50	91.92 ± 4.59[a]
VSL (μm s^{-1})	58.75 ± 2.59	57.37 ± 4.85	52.25 ± 3.82	58.52 ± 5.69	59.65 ± 5.47	50.50 ± 4.97
VAP (μm s^{-1})	82.67 ± 1.80	77.12 ± 5.31	72.02 ± 4.58	72.02 ± 4.58	79.30 ± 5.65	70.60 ± 5.04
LIN (%)	54.86 ± 2.79	57.02 ± 2.65	57.57 ± 2.65	59.57 ± 3.19	60.70 ± 3.02	60.70 ± 3.02
STR (%)	70.87 ± 2.05	73.77 ± 1.65	72.23 ± 1.61	73.92 ± 2.02	74.52 ± 1.93	70.57 ± 2.22
WOB (%)	77.06 ± 1.74	77.04 ± 2.03	79.37 ± 2.19	80.18 ± 2.33	81.15 ± 1.90	76.5 ± 2.85
ALH (μm)	3.05 ± 0.09	3.03 ± 0.12	2.63 ± 0.14[a,b]	2.69 ± 0.12	2.65 ± 0.12[a,b]	2.81 ± 0.18
BCF (Hz)	8.49 ± 0.09	8.33 ± 0.09	8.50 ± 0.16	8.56 ± 0.20	8.58 ± 0.14	8.59 ± 0.21

it was significantly lower in those containing 10 nM or 1 μM melatonin (46.60 ± 4.82% and 38.00 ± 4.60% respectively; $P < 0.001$; Fig. 1). These changes in the capacitation status did not result in alterations in plasma membrane integrity (% PI-negative spermatozoa), although the samples with 100 pM melatonin exhibited higher viability values than the cocktail-incubated samples ($P < 0.05$; Table 1).

Protein tyrosine phosphorylation was analysed by western blotting ($n = 6$) and a representative membrane is shown in Fig. 2. As expected, densitometric evaluation revealed a significant increase in the total band intensity in the cocktail-incubated samples compared with the control (627.01 ± 134.38 vs 240.09 ± 56.85; $P < 0.01$). The presence of melatonin in the cocktail samples resulted in a decrease in the total intensity of

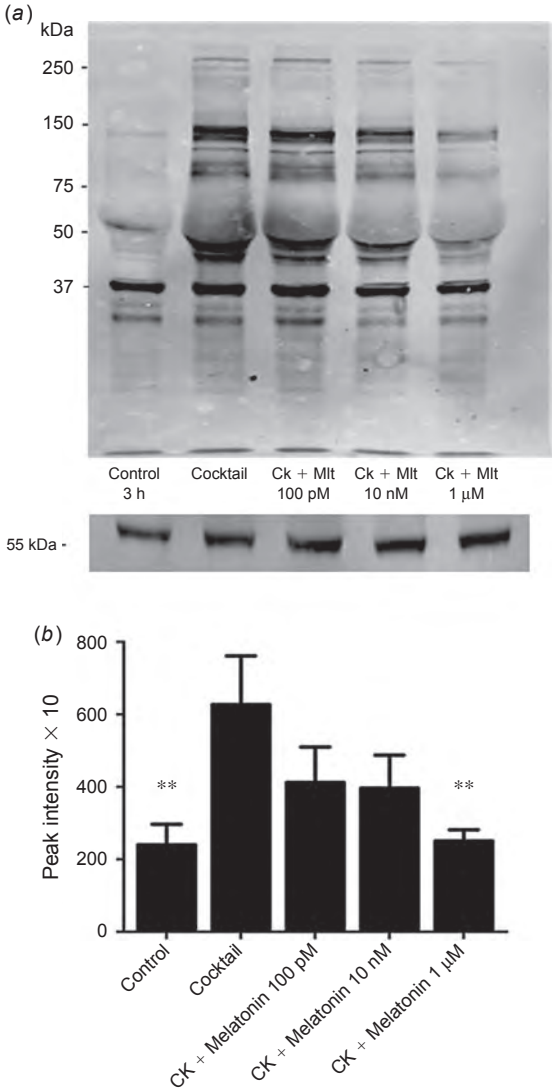

Fig. 2. Protein tyrosine phosphorylation after 3 h of *in vitro* capacitation analysed by (*a*) western blotting (α-tubulin as a loading control is shown in the lower panel) and quantified by (*b*) densitometry. Significant differences related to cocktail samples at 3 h (**$P < 0.01$). Mean values ± s.e.m. ($n = 5$).

the signal, but only the addition of 1 μM melatonin significantly reduced the signal up to the levels observed in the control samples (250.52 ± 31.02; $P < 0.01$ compared with the cocktail sample without hormone; Fig. 2).

Melatonin partially prevented the increase in ROS levels in spermatozoa during capacitation

Intracellular ROS levels expressed as mean of fluorescence intensity (MFI) were significantly increased in cocktail samples with 100 pM and 10 nM melatonin or without hormone after 1 h of incubation in capacitating conditions (values ranging between 10.11 ± 2.14 and 11.21 ± 2.79) compared with the control sample at 0 h (3.88 ± 0.86). However, incubating with 1 μM melatonin showed similar levels (7.27 ± 2.06) to those of the control samples (i.e. non-capacitating conditions; 6.39 ± 0.84) and did not significantly differ from the controls at 0 h (Fig. 3). Melatonin at high concentrations also exerted a protective effect after 3 h of incubation as, despite intracellular ROS levels being higher in all cocktail samples, the values in samples with 10 nM (11.61 ± 2.29) or 1 μM (10.89 ± 2.66) melatonin did not significantly differ from the control at the same time (6.61 ± 0.96).

Effect of melatonin on sperm cAMP levels

As expected, the addition of a cocktail that maintains high intracellular cAMP levels induced a significant increase in cAMP content compared with the control samples (31.32 ± 4.83 vs 0.40 ± 0.19 pmol cAMP per 10^7cells; $P < 0.01$). This effect was already apparent after 5 min of incubation in capacitating conditions (Fig. 4).

The presence of melatonin at 100 pM and 10 nM did not significantly affect the increase in intracellular cAMP content observed in the cocktail-incubated samples (28.25 ± 5.78 and 28.51 ± 8.99 pmol cAMP per 10^7cells with 100 pM and 10 nM respectively). However, the presence of melatonin at 1 μM partially avoided the rise in cAMP intracellular concentration induced by the cocktail, as the cAMP levels were less than half of those found in the cocktail sample without the hormone (14.75 ± 3.93 vs 31.32 ± 4.83 pmol cAMP per 10^7cells; $P < 0.05$; Fig. 4).

Impact of melatonin on sperm motility characteristics and distribution of sperm subpopulations

After 3 h of incubation in capacitating conditions all samples showed a decrease in the percentage of total motility relative to the control at 0 h (Table 1). When percentages of progressive spermatozoa were compared, no significant differences between samples were observed, except a slight decrease in spermatozoa incubated with 1 μM of melatonin. Moreover, the study of the kinematic parameters did not reveal significant differences between cocktail samples with or without melatonin (Table 1).

However, a principal component analysis run with all the kinematic parameters resulted in two extracted components that explained up to 77.49% of the total variance (Table 2). The first principal component was mainly related to straight-line velocity, straightness and beat-cross frequency (VSL, VAP, LIN, STR, WOB, BCF, MAD), whereas the second was strongly linked to curvilinear velocity and amplitude of head displacement (VCL, ALH, DANCE).

On the basis of the regression scores corresponding to each PCA component, a cluster analysis was run resulting in four ram sperm subpopulations. The kinematic parameters (as mean ± standard error of the mean, s.e.m.) for each subpopulation are shown in Table 3. While subpopulations 2 and 3 (SP2 and SP3) corresponded to the fastest spermatozoa with very high values of VCL and VAP (rapid spermatozoa), subpopulation 1 (SP1) showed lower velocity values (medium velocity spermatozoa) and subpopulation 4 (SP4) exhibited the lowest values of these kinetic parameters (slow spermatozoa). In addition, percentages of LIN and STR were higher in SP1 and SP2 and lower in SP3

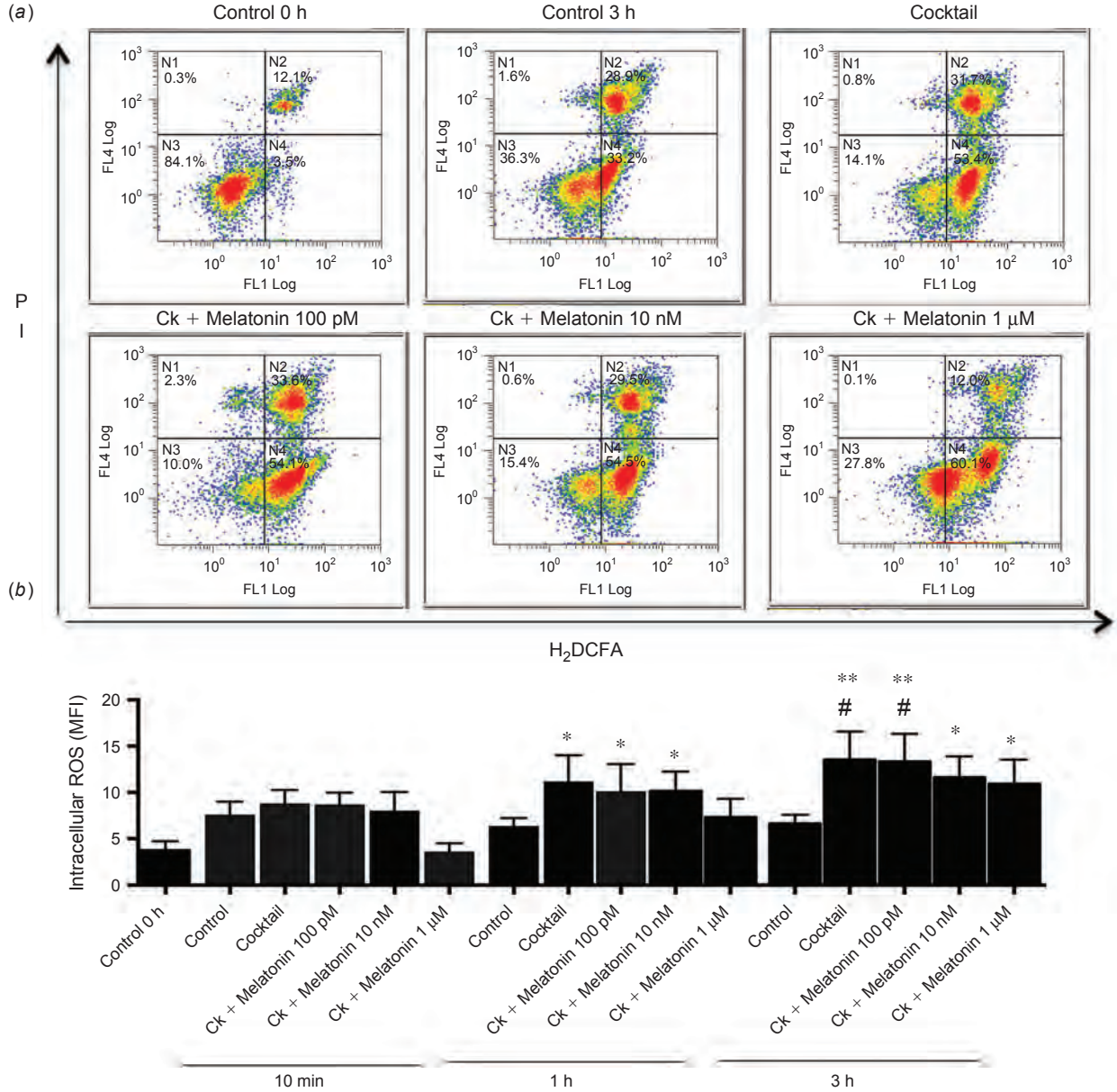

Fig. 3. Assessment of intracellular ROS in a seminal plasma-free sample before capacitation (control 0 h) and in samples incubated for 1 h and 3 h in capacitating conditions with cocktail (cAMP-elevating agents), cocktail with different melatonin concentrations or without these compounds (control). (*a*) Representative flow cytograms in samples at 0 h and after 3 h in capacitating conditions. FL1, H_2DCFDA; FL4, PI. (*b*) Mean values ± s.e.m. ($n = 5$) of the average intensity of fluorescence (MFI) corresponding to H_2DCFDA in live cells (PI–) evaluated by flow cytometry (FL1). Significant differences related to control sample at 0 h (*$P < 0.05$, **$P < 0.01$) and to control sample at 3 h ($^{\#}P < 0.05$).

and SP4, which indicates that the motility of the former was more linear than that of the latter. Thus, SP1 was constituted by spermatozoa with medium velocity and high straightness evidenced by high LIN and STR and low ALH values (71.95%, 83.97% and 2.14 µm respectively). Based on previous studies, which established that spermatozoa are hyperactivated when LIN ≤ 45% and ALH ≥ 3.5 µm (Colás *et al.* 2010), it can be inferred that SP1 represented non-hyperactivated spermatozoa. SP2 included rapid spermatozoa with higher ALH and DANCE (VCL × ALH) values than SP1, so they could start to acquire hypermotility, but they did not reach the above-mentioned limits of hyperactivation. SP3 contained spermatozoa with low LIN and STR coefficients and the highest ALH (34.01%, 50.31% and 5.14 µm respectively), which indicated that they would represent rapid, hyperactivated spermatozoa. Finally, SP4 contained slow spermatozoa with non-linear motility (low values of ALH and DANCE and minimum values of LIN and STR). After establishing the sperm subpopulations, the effects of incubating

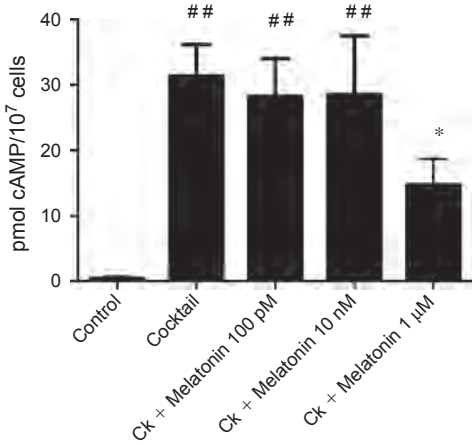

Fig. 4. Assessment of intracellular cAMP content in ram spermatozoa after 5 min of incubation in capacitating conditions with cocktail (cAMP-elevating agents), cocktail with different melatonin concentrations or without these compounds (control). Data are expressed as pmol cAMP per 10^7 cells (mean value ± s.e.m., $n = 3$). Significant differences related to control sample ($^{\#\#}P < 0.01$) and to cocktail sample ($^{*}P < 0.05$).

Table 2. Detail of PCA based on motility descriptors obtained from CASA analysis

The loading factor $\mathbf{a_{ij}^2}$ represents the highest association between a given sperm motility parameter and the corresponding principal component. VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble (VAP/VCL); BCF, beat-cross frequency; MAD (angular degrees); VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; DANCE (VCL × ALH)

Principal component	Variance	Parameter	a_{ij}^2
Component 1	47.49%	VSL	0.76
		VAP	0.46
		LIN	0.90
		STR	0.65
		WOB	0.81
		BCF	0.26
		MAD	0.37
Component 2	30.00%	VCL	0.83
		ALH	0.77
		DANCE	0.94
Total	77.49%		

ram spermatozoa in capacitating conditions, with or without melatonin, were tested throughout a 3 h period. After 3 h of incubation, the control sample was composed of 26.96 ± 3.0% of spermatozoa belonging to SP1 (high straightness), 27.74 ± 3.4% to SP2 (rapid spermatozoa starting hyperactivation), 17.36 ± 2.5% to SP3 (rapid hyperactivated spermatozoa) and 28.42 ± 3.3% to SP4 (less vigorous spermatozoa with non-linear motility). In the presence of cAMP-elevating agents, the sperm percentages belonging to SP1 and SP4 increased while SP2 and SP3 (rapid spermatozoa starting hyperactivation or already hyperactivated) decreased compared with the control. The presence of melatonin at 100 pM and 10 nM reduced SP1 and especially SP4 ($P < 0.05$ for 10 nM) and increased SP2 ($P < 0.05$ for both concentrations) and slightly SP3, modifying the percentages of these subpopulations to values closer to the control (Fig. 5).

Discussion

Our previous results showed that when added to a basal medium with calcium and bicarbonate, melatonin has a dual effect on *in vitro* capacitation of ram spermatozoa, promoting the process at a picomolar dose and preventing it at a micromolar concentration (Casao *et al.* 2010*b*). In the present study, melatonin was added to a medium with cAMP-elevating agents (cocktail), previously proven to capacitate ram spermatozoa *in vitro* (Colas *et al.* 2008).

Sperm incubation in a medium with a cocktail including cAMP analogues (dibutyryl-cAMP), cyclic nucleotide phosphodiesterase (PDE) inhibitors (that prevent cAMP degradation) and phosphatase inhibitors (that prolong PKA-mediated protein phosphorylation), led to an expected increase in sperm cAMP. It has been stated that an increase in cAMP levels activates PKA, resulting in the fast phosphorylation of a subset of proteins that leads to capacitation (Visconti *et al.* 1995; Harrison 2004). In the present study, increases in both the percentage of capacitated spermatozoa assessed by CTC staining and protein tyrosine phosphorylation levels were observed in cocktail-incubated samples. We could also observe an increase in ROS levels in samples incubated with the above-mentioned cocktail compared with the control. Controlled amounts of ROS, such as the superoxide anion ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2), have been shown to be essential for sperm motility, capacitation, the acrosome reaction and fertilising ability (Amaral *et al.* 2013). However, little is known about the regulation of endogenous ROS production during sperm capacitation (de Lamirande and Lamothe 2009; Guthrie and Welch 2012). Therefore, the increase observed here in ROS levels in the cocktail-containing samples could be a consequence of the activation of different mechanisms to ensure adequate ROS levels during the entire capacitation process (e.g. NO^- synthase (NOS) activation, release from mitochondria; de Lamirande and Lamothe 2009). Nonetheless, the study of the mechanism by which ROS increase during cAMP-induced capacitation is not a specific aim of the present work and we can only speculate.

When melatonin was added at a micromolar concentration, the increases both in cAMP and ROS levels that occur during incubation in the presence of cAMP-elevating agents were avoided. On the basis of reports in the literature, and given the nature of melatonin, it is likely that it could exert these effects by different mechanisms (Reiter *et al.* 2000). Being a potent antioxidant molecule, melatonin is able to cross the plasma membrane and reduce the levels of ROS in the intracellular medium (Jang *et al.* 2010). On the other hand, after binding to its specific receptors, known as MT1 and MT2, melatonin seems to influence second-messenger cascades (Sjöblom and Flemström 2003; Markowska *et al.* 2004). For instance, in several cell types melatonin binding to its receptors inhibits adenylyl cyclase (AC), which decreases cAMP levels and, consequently, reduces PKA activity (Luchetti *et al.* 2010) and phosphorylation of several proteins (Koh 2008). Therefore, the decrease in cAMP

Table 3. Kinematic characteristics of the four sperm subpopulations (SP) identified in ram sperm samples

Values are mean ± s.e.m. VSL, straight-line velocity; VCL, curvilinear velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; DANCE (VCL × ALH) and MAD (angular degrees)

Parameter	SP1	SP2	SP3	SP4
No. cells	70 949	77 356	52 962	82 480
Percentage	25.00	27.26	18.67	29.07
VCL ($\mu m\,s^{-1}$)	69.96 ± 0.08	124.37 ± 0.08	135.23 ± 0.11	62.93 ± 0.08
VSL ($\mu m\,s^{-1}$)	51.30 ± 0.08	91.39 ± 0.10	47.38 ± 0.12	18.08 ± 0.04
VAP ($\mu m\,s^{-1}$)	60.58 ± 0.08	110.23 ± 0.09	91.37 ± 0.12	37.40 ± 0.06
LIN (%)	71.95 ± 0.05	72.75 ± 0.05	34.01 ± 0.07	28.33 ± 0.05
STR (%)	83.97 ± 0.04	82.22 ± 0.05	50.31 ± 0.09	48.55 ± 0.08
WOB (%)	85.36 ± 0.04	88.07 ± 0.03	66.93 ± 0.06	57.95 ± 0.05
ALH (μm)	2.14 ± 0.00	3.19 ± 0.00	5.14 ± 0.00	2.97 ± 0.00
BCF (Hz)	7.30 ± 0.01	8.85 ± 0.01	6.94 ± 0.01	5.30 ± 0.01
DANCE ($\mu m^2\,s^{-1}$)	155.83 ± 0.26	398.48 ± 0.45	705.95 ± 1.06	201.94 ± 0.39
MAD (°)	67.03 ± 0.15	58.33 ± 0.14	90.98 ± 0.15	104.98 ± 0.10

levels found in this study could be mediated by melatonin binding to its receptors in the plasma membrane of ram spermatozoa (Casao *et al.* 2012).

By one of these mechanisms, or maybe by both, melatonin at a micromolar concentration was also able to prevent the increase in the percentage of capacitated spermatozoa (CTC staining) together with the protein tyrosine phosphorylation levels that occur in the presence of cAMP-elevating agents. These results are consistent with our previous findings obtained when melatonin was added to a basal medium with calcium and bicarbonate. However, in contrast to these previous results, in the present study melatonin at lower concentrations did not induce any increase in the proportion of capacitated spermatozoa when it was added to the cocktail medium, probably due to the enhanced effect of the cAMP-elevating agents. However, at this low concentration (100 pM), melatonin maintained higher viability values than the control cocktail-incubated samples. Other authors have reported that melatonin ameliorates viability in capacitated human spermatozoa (du Plessis *et al.* 2010), which is consistent with our findings.

Previous studies have demonstrated that incubation with melatonin at 1 mM and 2 mM improves motility of human (Ortiz *et al.* 2011) and bull (Ashrafi *et al.* 2013) spermatozoa respectively. Our results are not consistent with these reported effects. Rather, melatonin at a micromolar concentration led to a slight decrease in progressive motility, which could be due to a depletion of ROS under levels essential for sperm motility, as already reported by other authors (Martín-Hidalgo *et al.* 2011). However, given that mammalian ejaculates are highly heterogeneous (Abaigar *et al.* 1999; Mortimer 2000), motility evaluation based on mean kinematic parameter values results in a loss of a great deal of information (Verstegen *et al.* 2002; Holt *et al.* 2007). Thus, none of the analysed kinematic parameters revealed differences between the samples incubated with cAMP- elevating agents.

Conversely, if the exact movement of each individual spermatozoon was defined, it could be included in a specific subpopulation in accordance with its kinematic characteristics (ESHRE Andrology Special Interest Group 1998). Then the study of these subpopulations would improve the understanding of motility changes that take place during a process. Sperm subpopulations with specific motility characteristics have been identified within ejaculates from different species such as bull (Muiño *et al.* 2008), boar (Abaigar *et al.* 1999), donkey (Miró *et al.* 2005), dog (Núñez Martinez *et al.* 2006), mouse (Goodson *et al.* 2011) and human (Buffone *et al.* 2004). In ram semen, the presence of subpopulations based on differences in motility (García-Álvarez *et al.* 2014) and morphometric characteristics (Maroto-Morales *et al.* 2012; Ramón *et al.* 2013) has also been reported.

In the present study, the PCA and cluster analyses revealed important differences in the sperm subpopulations evidenced in the samples analysed, consistent with a previous study carried out by our group (Luna *et al.* 2017).

Curiously, the presence of cAMP-elevating agents led to significantly higher percentages of spermatozoa belonging to SP1 (high straightness) and lower percentages corresponding to SP2 and SP3 (rapid spermatozoa starting hyperactivation or already hyperactivated) compared with the control sample. Given that the term 'hyperactivation' refers to a characteristic motility pattern that spermatozoa acquire during the process of capacitation (Topper *et al.* 1999; Ho and Suarez 2001), an increase in SP3 (rapid, hyperactivated spermatozoa) in the cocktail-containing samples would, in principle, be expected. These findings are in agreement with previous reports by our group that revealed that the incubation of ram spermatozoa in capacitating conditions with cAMP-elevating agents (Colás *et al.* 2010) or with epidermal growth factor (EGF; Luna *et al.* 2017) resulted in capacitation with no hyperactivation, similar to the cases of bull (Marquez and Suarez 2004) and stallion (McPartlin *et al.* 2009) spermatozoa. All this evidence indicates that although capacitation and hyperactivation can be concomitant processes (Suárez and Osman 1987; García-Álvarez *et al.* 2014), they may not necessarily be interdependent (de Lamirande *et al.* 1997).

The addition of melatonin at low (100 pM) and medium (10 nM) concentrations showed an increase in the proportion of rapid spermatozoa starting to acquire hypermotility (SP2) and a concomitant decrease in the proportion of less-vigorous

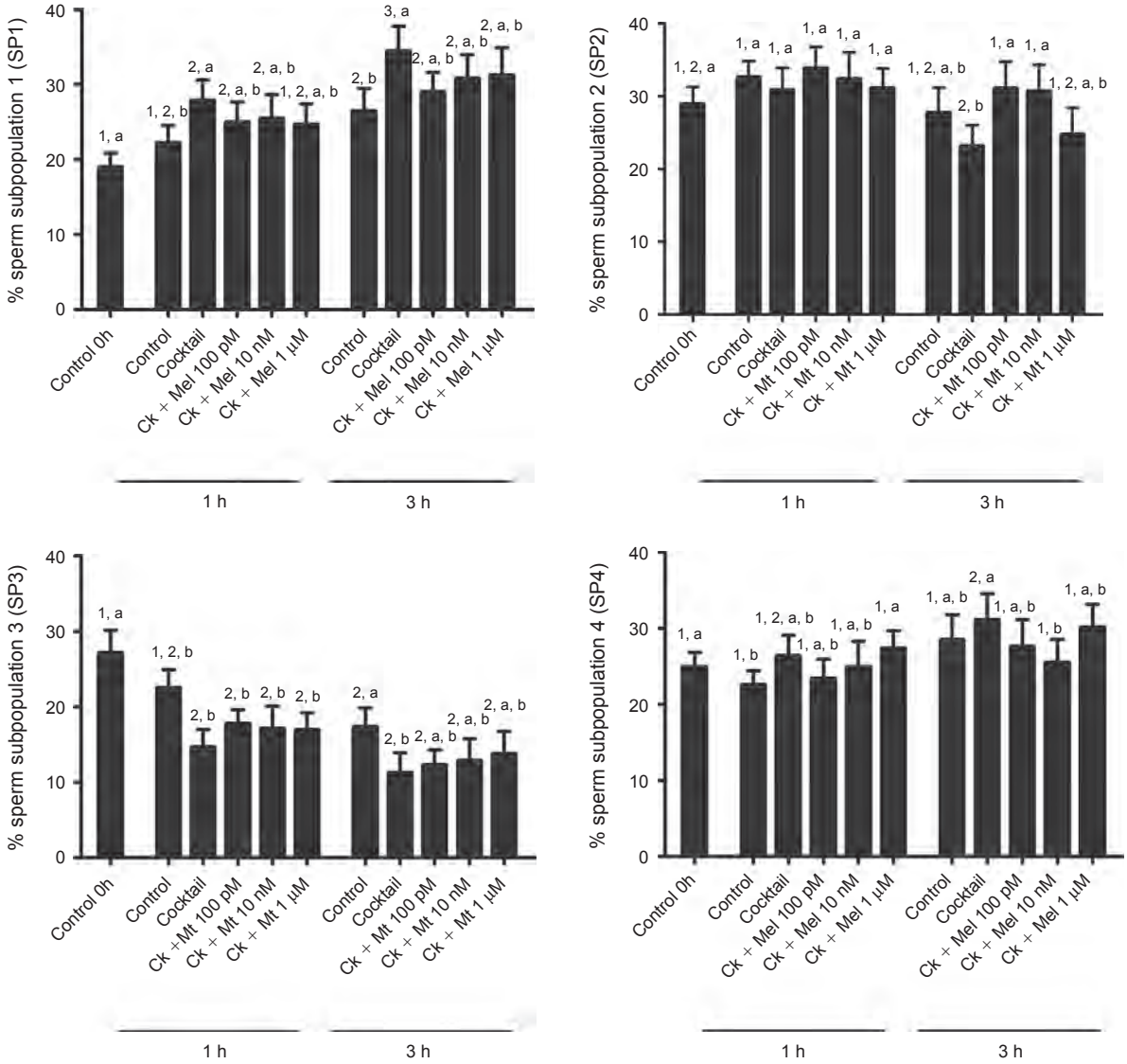

Fig. 5. Effect of incubation in capacitating conditions with cocktail (cAMP-elevating agents), cocktail with different melatonin doses or without these compounds on the evolution of sperm subpopulations identified for PCA in ram sperm samples. Different letters indicate significant differences between treatments at the same time and different numbers indicate significant differences between times for the same treatment.

spermatozoa (SP4), modifying the percentages of these subpopulations to values closer to the control. We can speculate that the melatonin secreted in the female reproductive tract at low concentrations could regulate the process of hyperactivation, as similar results were observed in a study carried out with hamsters (Fujinoki 2008). Furthermore, the addition of 1 µM melatonin did not affect the distribution patterns of motile sperm subpopulations, despite its ability to decrease the CTC-capacitated sperm rate, protein tyrosine phosphorylation and cAMP concentration, which supports the conclusion that capacitation and hyperactivation are not interdependent.

In conclusion, the present study shows that melatonin modulates cAMP-stimulated ram sperm capacitation, modifying motile sperm subpopulations at low concentrations and influencing ROS and cAMP levels at high concentrations. Further experiments would be necessary to determine the biochemical pathways involved in these processes.

Conflicts 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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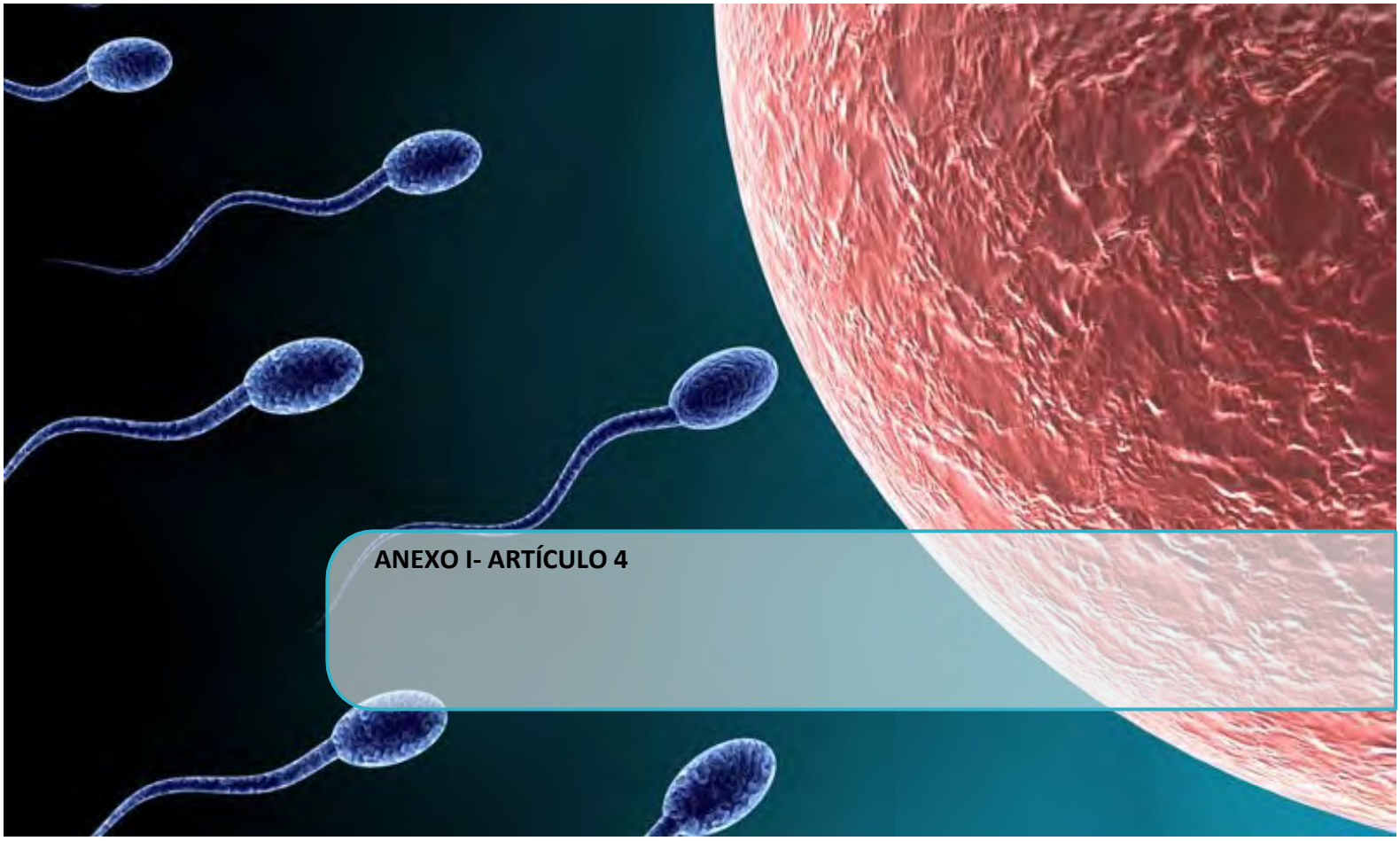

ANEXO I- ARTÍCULO 4

Melatonin modifies the cAMP level with no change on PKA activity on ram spermatozoa

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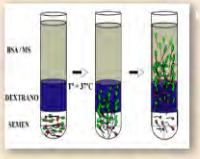
Introduction

Melatonin is an important hormone regulating reproductive functions, and the presence of receptors on the ram sperm surface leads to speculate about a relevant role on sperm functionality. Moreover, in several cell types, the hormone binding to its receptors inhibits adenyl cyclase, which decreases cAMP levels and, consequently, reduces protein kinase A (PKA) activity. Morever, it is well know that the AC-cAMP-PKA pathway is involved in sperm capacitation.

Thus, the objective of this study was to investigate the effect of different melatonin concentration on cAMP levels and PKA activity during the first step of *in vitro* ram sperm capacitation.

Material and Methods

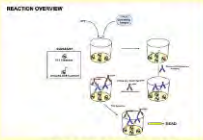
Sperm collection from nine *Rasa Aragonesa* rams



Spermatozoa selection by swim-up /dextran method

TALP | **Cocktail (TALP+ cAMP elevating agents)** | **Cocktail + 100 pM melatonin** | **Cocktail +1 µM melatonin**

***In vitro* capacitation for 5 min at 39 °C, 5% CO_2, 100% humidity**



Direct cAMP enzyme Immunoassay (C-200)(Sigma Aldrich)

Protein Kinase A (PKA) Activity Kit (K027-H1) (Arbor Assays)

Optical density at 405 nm

Optical density at 450 nm

Results

cAMP enzyme Immunoassay

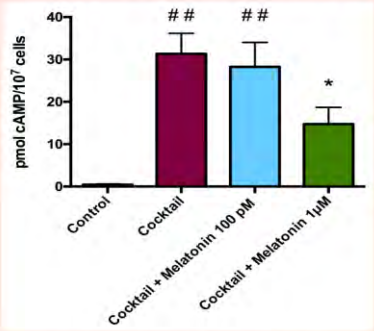


Fig 1. Assessment of intracelullar cAMP content in ram spermatozoa after 5 min of incubation in capacitating condtions with cocktail (cAMP elevating agents), cocktail with different melatonin concentrations or without these compounds (control). Values are expressed as mean ± SEM (n=3). Significant differences related to control sample (##P < 0.01) and to cocktail sample (* P<0.05). ANOVA test was performed.

PKA activity kit

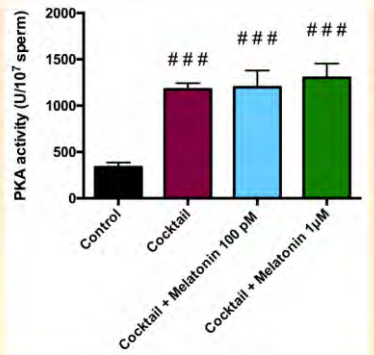


Fig. 2: Assessment of protein kinase A (PKA) activity in ram spermatozoa after 5 minutes of incubation in capacitating conditions with cocktail (cAMP-elevating agents), cocktail with different melatonin concentrations or control (without these compounds). Data are expressed as arbitrary units (U) /10^7 cells (mean values ± S.E.M, n=5). Significant differences related to control sample (### P< 0.001). ANOVA test was performed.

Conclusions

- **The presence of 1 µM of melatonin in capacitating conditions showed lower cAMP leveles (P<0.05) compared with cocktail sample without the hormone.**
- **However, melatonin at both concentrations did not induce any change in PKA activity compared to cocktail sample without hormone.**
- **The preliminary study shows the possibility that melatonin did not regulate ram sperm capacitation by PKA pathway.**

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6. DISCUSIÓN GENERAL

6. DISCUSIÓN GENERAL

6.1. IDENTIFICACIÓN Y LOCALIZACIÓN DE RECEPTORES DE PROGESTERONA Y ESTRÓGENOS

Las hormonas esteroideas, principalmente la progesterona (P4) y el 17-β estradiol (E2), juegan un papel muy importante en la regulación de la reproducción en mamíferos. Su mecanismo de acción clásico es la regulación de la expresión génica a través de su unión a receptores nucleares (PR-A y PR-B para P4 y ERα y ERβ para el E2) en sus células diana. Sin embargo, estas hormonas también pueden ejercer sus efectos de manera no genómica en diferentes tipos celulares, incluyendo los espermatozoides, dando lugar a respuestas rápidas (Bishop and Stormshak 2008). Estos mecanismos de acción no genómicos pueden iniciarse en la membrana o en el citoplasma, implicando la participación de posibles receptores de membrana o citoplásmicos. Estos receptores de membrana podrían tener una estructura muy semejante a los receptores nucleares clásicos o ser diferentes a ellos. La descripción y localización de los receptores de estas hormonas, así como sus acciones no genómicas en espermatozoides, se han estudiado en diferentes especies, principalmente en la humana (Bishop and Stormshak 2008; Baldi et al. 2009), pero también en la equina (Cheng et al. 1998; Arkoun et al. 2014), caprina (Somanath and Gandhi 2002), porcina (Wu et al. 2006; De Amicis et al. 2012) y murina (Sebkova et al. 2012), entre otras. Sin embargo, hasta la fecha, no habían sido descritos en espermatozoides de la especie ovina. Por ello, el primer objetivo de la presente tesis fue **identificar y localizar los posibles receptores de progesterona y 17-β estradiol** en el espermatozoide ovino *(Artículo 1)*.

Nuestros estudios **demostraron, por primera vez, la existencia del receptor de progesterona (PR) en el espermatozoide ovino** mediante ensayos de inmunofluorescencia indirecta, localizándose en la zona ecuatorial y la pieza intermedia. Esta localización coincide con la descrita en la especie humana (Sabeur et al. 1996) pero no con la de otras especies como la caprina o porcina (Somanath and Gandhi 2002; De Amicis et al. 2012). Así mismo, también se constató, **por primera vez**

la presencia de los receptores de estrógenos, ERα y ERβ en el espermatozoide ovino, localizándose en la zona post acrosomal y en la región apical respectivamente. Estos resultados coinciden parcialmente con los descritos en otras especies como la equina (Arkoun et al. 2014), murina (Sebkova et al. 2012) y humana (Solakidi et al. 2005). Sin embargo, otros estudios han circunscrito la presencia de ambos receptores al flagelo (Rago et al. 2007; Imbesi et al. 2008; Guido et al. 2011). Estas diferencias en la localización de PR y ER descritas en los distintos estudios podrían atribuirse a características especie-específicas o al uso de diferentes anticuerpos en los ensayos de inmunolocalización.

Curiosamente, mientras que ERα se evidenció en la región post-acrosomal de todos los espermatozoides, ERβ se localizó en la región apical de solamente una fracción de los mismos, concretamente en el 63% de los espermatozoides, y algunos de ellos parecían estar perdiendo dicho receptor. Las diferencias observadas en la localización de ambos receptores de estrógenos sugieren que ambos jugarían distintos papeles en la funcionalidad espermática, y que ERβ podría sufrir una redistribución relacionada con la capacitación. Esta idea estaría apoyada por la alta correlación encontrada entre la presencia de ERβ y el porcentaje de espermatozoides no capacitados. Además, el porcentaje de células marcadas (ERβ+) disminuyó significativamente tras la capacitación *in vitro*, y prácticamente desapareció tras la inducción de la reacción acrosómica (RA) con lisofosfatidilcolina (LPC). Estos resultados indicarían que el ERβ podría estar implicado en la capacitación y la reacción acrosómica en el espermatozoide ovino. Por el contrario, la localización de ERα, así como de PR no cambiaron ni durante la capacitación ni tras la RA.

Los análisis de *western-blot* para PR de los extractos proteicos espermáticos permitieron identificar tres bandas de un peso molecular comprendido entre 40 y 45 kDa, coincidiendo con las bandas observadas en los controles positivos. Sin embargo, estas bandas no coinciden con las descritas para los receptores nucleares clásicos (94 y 120 kDa para PR-A y PR-B, respectivamente) en la especie humana (De Amicis et al. 2011) ni con las especificaciones de la casa comercial del anticuerpo utilizado (81 y 116 kDa, respectivamente). Sin embargo, dos estudios realizados en el espermatozoide humano, describen un nuevo receptor de P4 en la membrana plasmática con pesos

moleculares similares a los observados en este trabajo de la presente tesis (46-48 y 50-52 kDa, respectivamente) (Sabeur et al. 1996; Luconi et al. 1998). Este nuevo receptor de membrana también ha sido descrito en espermatozoides de perro (Cheng et al. 2005), macho cabrío (Somanath and Gandhi 2002) o cerdo (Wu et al. 2006). Debemos tener en cuenta que el anticuerpo anti-PR utilizado en nuestros experimentos es un anticuerpo policlonal frente al extremo C-terminal del PR humano, y, según algunos autores, ésta podría ser una zona altamente conservada, presente tanto en los receptores nucleares como en el receptor de membrana (Luconi et al. 2004). Por lo tanto, a la vista de nuestros resultados, no podemos concluir si los receptores de P4 identificados pueden corresponder a distintas isoformas del receptor nuclear clásico o bien se trata de dos receptores diferentes y específicos presentes en la membrana del espermatozoide ovino.

En cuanto a los receptores de estrógenos, los análisis de *western-blot* confirmaron la existencia de ambos receptores nucleares clásicos en la membrana del espermatozoide ovino, ERα y ERβ, con pesos moleculares de aproximadamente 66 kDa y 55 kDa, respectivamente, compatibles con los descritos para el espermatozoide humano (Aquila et al. 2004). Sin embargo, tampoco podemos descartar la posibilidad de la presencia de otros receptores de estrógenos en la membrana del espermatozoide ovino diferentes a los receptores nucleares clásicos.

En conclusión, los resultados de este primer trabajo (*Artículo 1)* confirmaron, por primera vez, la existencia de los receptores de progesterona (PR) y estrógenos (ERα y ERβ) en la membrana plasmática del espermatozoide ovino, y describen la implicación del receptor ERβ en los procesos de capacitación y RA.

6.2. EFECTOS DE LAS HORMONAS ESTEROIDEAS EN EL ESPERMATOZOIDE OVINO

Una vez demostrada la presencia de los receptores para progesterona y estrógenos en el espermatozoide de morueco, nos planteamos, como **segundo objetivo**, **determinar la implicación de ambas hormonas esteroideas, progesterona (P4) y 17-β estradiol (E2), en la funcionalidad del espermatozoide ovino** *(Artículo 1)*. Ambas hormonas están presentes en el tracto reproductor femenino, tanto en el fluido folicular durante la ovulación a concentraciones nanomolares, como en el oviductal al que llegan junto

con el ovocito tras la ovulación (Carson et al. 1981), además de ser secretadas por células del *cumulus* (Vanderhyden and Tonary 1995; Chian et al. 1999), a concentraciones micromolares (Frederick et al. 1991). Sin embargo, la concentración exacta de estas hormonas en el tracto reproductor femenino es muy difícil de averiguar debido a varios factores a tener en cuenta, como el momento del ciclo estral, el número de células del *cumulus* presentes, así como diferencias entre especies. Por ello, analizamos el efecto de distintas concentraciones de P4 y E2, 100pM, 10 nM y 1 µM, sobre la funcionalidad de los espermatozoides ovinos. Un aspecto de esa funcionalidad, necesario para alcanzar el ovocito, es una adecuada motilidad espermática durante su tránsito por el tracto reproductor femenino. En el presente trabajo, no se evidenciaron cambios en la motilidad tras la incubación de los espermatozoides ovinos con progesterona y 17-β estradiol en medio TALP. Pero, al analizar el estado de capacitación mediante tinción con clorotetracilina (CTC) observamos una disminución del porcentaje de espermatozoides no capacitados (NC) concomitante con un aumento del porcentaje de espermatozoides reaccionados (AR) tras 3 horas de incubación con ambas hormonas a concentraciones de 100 pM y 1 µM. Estos resultados no fueron acompañados de cambios en el porcentaje de espermatozoides capacitados (C) ni cambios en los niveles de fosforilación de proteínas en residuos de tirosina analizados mediante *western-blot.* Esto podía ser debido a la dificultad de inducir la capacitación *in vitro* en los espermatozoides ovinos, que apenas se capacitan en medio TALP (medio usado en la mayoría de las especies para promover la capacitación). Por ello, llevamos a cabo los mismos experimentos adicionando al medio TALP un conjunto de sustancias elevadoras del cAMP (denominadas comúnmente *cocktail* o Cap-C a lo largo de la tesis*).* Como ya hemos mencionado en la introducción, se trata de un medio específico puesto a punto en nuestro laboratorio y testado como inductor de la capacitación *in vitro* en el espermatozoide ovino (Grasa et al. 2006; Colas et al. 2008). Cuando los espermatozoides se incubaron en condiciones capacitantes en este medio *cocktail,* ya se obvservó una disminución significativa en el porcentaje de espermatozoides capacitados a la hora de incubación con ambas hormonas y a todas las concentraciones ensayadas. Esta disminución fue concomitante con un aumento de los espermatozoides reaccionados. Además, también evidenciamos un aumento en la

fosforilación de proteínas en residuos de tirosina cuando los espermatozoides fueron incubados con P4 a concentraciones 100 pM y 10 nM. En el caso de la incubación con E2, el aumento en el porcentaje de espermatozoides reaccionados fue inferior, y además no produjo modificaciones en la fosforilación de las proteínas en residuos de tirosina. Estas diferencias observadas entre los dos marcadores de capacitación, la tinción con CTC y la cuantificación de la fosforilación de las proteínas en residuos de tirosina, podrían explicarse si tenemos en cuenta que el proceso de capacitación es un proceso secuencial en el que se producen diversos cambios bioquímicos y biofísicos junto con el inicio de la reacción acrosómica (Baldi et al. 2000a). Ambos ensayos son considerados marcadores de capacitación, pero los cambios que ponen en evidencia cada uno de ellos no tienen por qué darse de manera simultánea, de forma que diferentes moléculas podrían desencadenar unos eventos mientras que no otros, como podría ser el caso de la P4 y el E2. Además, ambas hormonas a la concentración más baja ensayada, 100 pM provocaron una disminución de la motilidad total cuando los espermatozoides se incubaron en medio *cocktail*, pero no dieron lugar a cambios en la motilidad progresiva. En el caso de la P4, algunos autores habían observado un efecto positivo de P4 en la motilidad en espermatozoides humanos (Contreras and Llanos 2001), mientras que este efecto no fue confirmado en otros trabajos (Wang et al. 2001). En cuanto al E2, los estudios sobre su efecto en la motilidad son escasos y contradictorios: mientras algunos autores han descrito que produce un incremento de la misma (Guido et al. 2011), otros mostraron un descenso en el porcentaje de espermatozoides con motilidad progresiva (Gautier et al. 2016), pero a concentraciones µM, lo que sugiere que estas diferencias en cuanto a los efectos dependientes de la concentración podrían ser especie-específicas. Por otro lado, con ninguna de las hormonas observamos cambios en los parámetros cinéticos ni en la integridad de la membrana plasmática, en contraposición con otros estudios llevados a cabo en espermatozoides porcinos (De Amicis et al. 2012).

Por lo tanto, nuestros resultados ponen de manifiesto, por primera vez, el papel de la progesterona y el 17-β estradiol en la inducción de la reacción acrosómica en el espermatozoide ovino. En el caso de la P4, esta acción había sido descrita en muchas especies de mamíferos (Osman et al. 1989; Melendrez et al. 1994; Roldan et al. 1994;

Meyers et al. 1995; Somanath et al. 2000; Sumigama et al. 2015), pero en cuanto al E2, los resultados publicados en otras especies eran contradictorios. Así, mientras que algunos autores han descrito un efecto inductor directo (Adeoya-Osiguwa et al. 2003; Ded et al. 2013), otros le atribuyen sólo un papel modulador de los efectos provocados por la P4 (Vigil et al. 2008; Sebkova et al. 2012). En el presente trabajo se ha demostrado que el E2, por sí solo, tiene capacidad de inducir cambios asociados a la reacción acrosómica en el espermatozoide ovino.

Estos resultados abrirían nuevas posibilidades para el uso de las mismas o bien agonistas o antagonistas de sus receptores, para la formulación de diluyentes o medios empleados en inseminación artificial (IA) o en otras técnicas de reproducción asistida, o para el mantenimiento de las dosis seminales.

6.3. IMPLICACIÓN DE LOS RECEPTORES DE PROGESTERONA Y ESTRÓGENOS EN LA REACCIÓN ACROSÓMICA DEL ESPERMATOZOIDE OVINO

Una vez determinados los efectos de la P4 y E2 en la funcionalidad del espermatozoide ovino, nos planteamos estudiar si dichos efectos estaban mediados por su unión a los receptores, PR, ERα y ERβ, que habíamos evidenciado. De esta forma, **el tercer objetivo de esta tesis fue descubrir si las acciones de la progesterona y 17-β estradiol sobre el espermatozoide ovino están mediadas por su unión a receptores *(Artículo 2*).** Para ello, incubamos espermatozoides ovinos, seleccionados por *swim-up*, en condiciones capacitantes en presencia de moduladores (agonistas y antagonistas) de los receptores de progesterona (PR) y estrógenos (ER), tanto agonistas como antagonistas. Para el receptor de progesterona se utilizó como agonista el tanaproget (NSP-989), debido a su alta afinidad por este receptor (Zhang et al. 2005), y como antagonista la mifepristona (RU-486), ya utilizada en espermatozoides humanos (Yang et al. 1994; Ko et al. 2014). Para evaluar la funcionalidad de los ER los espermatozoides se incubaron los espermatozoides con el agonista resveratrol, que ha demostrado efectos antioxidantes en espermatozoides de varias especies (Longobardi et al. 2017; Li et al. 2018), incluida la ovina (Silva et al. 2012) , y con el antagonista tamoxifeno, que puede inhibir el efecto supresor del E2 sobre la hiperactivación inducida por

progesterona en espermatozoides de hámster (Fujinoki 2010; Fujinoki and Takei 2015). Hay que destacar que este es el primer trabajo en el que se han utilizado los agonistas de PR y ER, tanaproget y resveratrol respectivamente, para investigar la implicación de las hormonas esteroideas y su receptores sobre la funcionalidad espermática.

Los resultados de viabilidad celular indicaron que la incubación con moduladores de estos receptores no ejerció un efecto perjudicial sobre los espermatozoides ovinos, como ya se había demostrado en otras especies (Motrich et al. 2007; Silva et al. 2012). La mifepristona a baja concentración (4 µM), incluso fue capaz de mejorar este parámetro, probablemente debido a su actividad antioxidante, descrita previamente en búfalo, (Dalal et al. 2019). En cuanto a la implicación de los PR y ER sobre la motilidad espermática, la incubación con sus respectivos agonistas, tanaproget o resveratrol, provocó un descenso significativo de la motilidad progresiva, mientras que, la incubación con los respectivos antagonistas, mifepristona o tamoxifeno, previa a la adición de las hormonas, provocó un aumento significativo de este parámetro. Esta acción sobre la motilidad podría estar relacionada con la hiperactivación espermática, tal como observaron Fujinoki et al. en espermatozoides de hámster (2010; 2016) , y Ko et al. en espermatozoides humanos (2014). Sin embargo, cuando analizamos el porcentaje de espermatozoides hiperactivados o los parámetros cinéticos no encontramos diferencias significativas entre tratamientos. Es posible que, en el espermatozoide ovino, la unión de esas hormonas a sus receptores no esté relacionada con la hiperactivación flagelar. Pero estas diferencias también podrían ser debidas a los ajustes de la velocidad y el tiempo de grabación (25 fotogramas por segundo y 1 segundo, respectivamente), inferiores a los utilizados en otros trabajos (Noguchi et al. 2008; Ko et al. 2014).

Entre los efectos más relevantes de las hormonas esteroideas sobre la funcionalidad espermática se encuentra la inducción de la RA en múltiples especies, incluida la ovina (*Artículo 1*; Gimeno-Martos *et al.* 2017), que algunos estudios sugieren podría ser ejercida mediante una vía no genómica a través de receptores (Aquila et al. 2004; Luconi et al. 2004). Nuestros resultados apuntan a que este podría ser el caso de la especie ovina, ya que la incubación de los espermatozoides con los

agonistas tanto de PR como de ER provocaron una disminución del porcentaje de espermatozoides capacitados y un aumento del porcentaje de espermatozoides reaccionados, evaluados por CTC. Por el contrario, los antagonistas inhibieron el efecto inductor de P4 y E2 sobre la RA. Aunque la supresión de la RA inducida por progesterona por parte de la mifepristona había sido previamente descrita en espermatozoides de otras especies como búfalo (Dalal et al. 2019), cerdo (Jang and Yi 2002) y humano(Ko et al. 2014), esta es la primera vez que se demuestra que un agonista del PR, en este caso el tanaproget, es capaz de inducir la RA en espermatozoides.

Estos resultados son especialmente interesantes en el caso de la acción del E2 y la implicación de sus receptores sobre la RA, ya que, como se ha comentado anteriormente, los resultados eran contradictorios (Luconi et al. 1999; Sebkova et al. 2012; Gimeno-Martos et al. 2017; Bosakova et al. 2018), y la participación de los receptores en este proceso no estaba del todo clara. En este estudio hemos demostrado que la incubación con un agonista de ER, el resveratrol, es capaz de provocar la misma respuesta que cuando se incubaba E2, e incluso obtuvimos un porcentaje mayor de espermatozoides reaccionados al añadir la concentración más elevada de agonista. Por el contrario, la incubación previa con un antagonista de ER, el tamoxifeno, mantuvo el estado de capacitación de la muestra a unos niveles similares a los del control de capacitación, inhibiendo la RA. Estos resultados confirman que el E2 induce por sí mismo la RA en el espermatozoide ovino, y que este efecto está mediado por su unión a receptores. Sin embargo, a pesar de todas las diferencias observadas en el estado de capacitación, evaluado por CTC, tras la incubación de los espermatozoides ovinos con agonistas y antagonistas de PR y ER, no observamos diferencias en la fosforilación de las proteínas en residuos de tirosina, evaluados por western-blot. Así, aunque se ha descrito que la P4 y/o el E2 pueden aumentar los niveles de proteínas fosforiladas en tirosina (TyrPP) en espermatozoides de algunas especies (De Amicis et al. 2012; Fujinoki et al. 2016; Sajeevadathan et al. 2019), la implicación de sus receptores no queda clara. En el trabajo anteriormente mencionado (*Artículo 1*; Gimeno-Martos *et al.* 2017), habíamos descrito un incremento significativo en los niveles de TyrPP tras la incubación con las concentraciones bajas de P4, pero no

con la aquí ensayada, ni con el E2. En el presente trabajo, no se observa un aumento significativo tras la incubación ni con las hormonas, ni con los agonistas o antagonistas de sus receptores. Sin embargo, no podemos descartar la posibilidad de que se estén produciendo cambios en la distribución de fosfotirosinas sin que se produzcan cambios en sus niveles totales, que a veces son difíciles de detectar mediante el proceso de cuantificación tras el *western-blot.* Estos cambios de localización de TyrPP se han observado en otras especies (Barbonetti et al. 2010; Sebkova et al. 2012; Sagare-Patil and Modi 2017).

En conclusión, estos resultados demuestran que la inducción de la reacción acrosómica provocada tanto P4 como E2 en espermatozoides ovinos está mediada por la unión a sus receptores específicos. Sin embargo, son necesarios más estudios para aclarar la implicación de los mismos sobre la hiperactivación y la fosforilación de las proteínas en residuos de tirosina.

6.4. MECANISMOS MOLECULARES IMPLICADOS EN LA MODULACIÓN DE LA CAPACITACIÓN Y LA REACCIÓN ACROSÓMICA PRODUCIDA POR PROGESTERONA Y ESTRÓGENOS EN EL ESPERMATOZOIDE OVINO

Tras evidenciar la capacidad de las hormonas esteroideas para modular la capacitación y la reacción acrosómica en los espermatozoides ovinos y la implicación de los receptores de estas hormonas en dicha acción, quisimos investigar los mecanismos moleculares subyacentes. Por ello, **el cuarto objetivo de esta tesis fue profundizar en el conocimiento de los mecanismos de acción de la progesterona y el 17-β estradiol sobre el proceso de capacitación y reacción acrosómica en el espermatozoide ovino** *(Artículo 3)*. Como hemos mencionado en apartados anteriores, el espermatozoide recién eyaculado adquiere su capacidad fecundante durante su tránsito por el tracto reproductor femenino mediante el proceso de capacitación. Este fenómeno culmina con la RA (Yanagimachi 1994), e implica cambios en la membrana plasmática (Gadella et al. 2008), movimientos de Ca^{2+} (Lishko et al. 2011), aumento de especies reactivas de oxígeno (ROS) (de Lamirande and O'Flaherty 2008) y la activación de rutas

metabólicas (Visconti et al. 2002) que promueven, en última instancia la fosforilación de las proteínas en residuos de tirosina y cambios asociados al patrón de motilidad.

Aunque ya habíamos evidenciado mediante la tinción con clorotetraciclina (CTC) la capacidad de las hormonas esteroideas para inducir la reacción acrosómica, especialmente en un medio con agentes elevadores del cAMP (*cocktail o Cap-C*) (*Artículo 1 y Artículo 2)*, este método se basa en cambios en la distribución del calcio intracelular asociados a este proceso. Para confirmar el efecto de estas hormonas en la RA, en este estudio evaluamos directamente el estado acrosomal de los espermatozoides usando lectinas. Las lectinas son proteínas que se unen a azúcares con una alta especificidad. En este caso, al unirse a determinadas glicoproteínas de la membrana plasmática o acrosomal, permiten revelar cambios en ella, tal como se describe en espermatozoide humano (Cross and Overstreet 1987) y ovino (Marti et al. 2000) entre otras muchas especies. En este trabajo se utilizó la lectina *Ricinus communis agglutin (RCA)* unida al fluorocromo FITC junto con ioduro de propidio (IP) para diferenciar entre células vivas y muertas con membrana acrosomal intacta o dañada. Esta lectina, al menos en el espermatozoide ovino, se une a glicoproteínas con motivos de galactosa presentes en el acrosoma, y expuestas en la superficie tras la RA (Marti et al. 2000). En nuestro caso, la incubación de los espermatozoides durante tres horas en condiciones capacitantes dio lugar a un aumento del porcentaje de espermatozoides vivos con el acrosoma dañado evaluados por citometría de flujo (IP-/ RCA+). La presencia de P4, únicamente a alta (1 µM) concentración provocó un aumento significativo de esta población (IP-/ RCA+), aunque sin llegar a los valores producidos por la lisofosfatidilcolina (LPC), un inductor de la RA. Sin embargo, en el caso de E2 no observamos diferencias significativas. Este efecto de la P4 no fue debido al incremento de células IP+, como demostraron los ensayos de viabilidad (integridad de membrana), en concordancia con nuestros estudios previos (*Artículo 1)* (Gimeno-Martos et al. 2017).

En ese mismo trabajo (*Artículo 1)* (Gimeno-Martos et al. 2017), junto con el incremento de los espermatozoides reaccionados, también observamos cambios en los niveles de fosforilación de las proteínas en los residuos de tirosina mediante análisis de

western-blot, pero únicamente significativos en presencia de P4 a baja concentración. Durante la capacitación, no solo se ha descrito un incremento en los niveles de proteínas de fosforiladas en tirosina (TyrPP) en varias especies, sino también cambios en su localización en la membrana espermática (Tardif et al. 2001; Asquith et al. 2004; Luno et al. 2013), como bien hemos mencionado anteriormente. Por tanto, para elucidar si la presencia de hormonas esteroideas era capaz de dar lugar a cambios en la localización de TyrPP en el espermatozoide ovino, en este trabajo se llevaron a cabo ensayos inmunofluorescencia indirecta (IFI). En las muestras no sometidas a capacitación *in vitro*, o las incubadas en condiciones capacitantes en medio TALP, el marcaje mayoritario fue en la zona acrosomal y ecuatorial simultáneamente (AE). La adición de sustancias elevadoras del cAMP al medio TALP dio lugar a la aparición de señal de TyrPP en el flagelo del espermatozoide, siendo por tanto la población mayoritaria aquella que presentaba marcaje conjunto en el acrosoma, zona ecuatorial y flagelo (denominada AEF). La presencia de P4 provocó la desaparición de TyrPP del acrosoma, dando lugar a una disminución de las subpoblaciones AEF y AE, y a un aumento de la población con marcaje en la zona ecuatorial y flagelo (EF). Curiosamente, las subpoblaciones que disminuyeron correlacionaron positivamente con espermatozoides no capacitados (AE, $r=0.783$, $P< 0.0001$) y capacitados (AEF, $r=0.571$, $P< 0.0001$) según la tinción con CTC; mientras que la que aumentó lo hizo con los reaccionados (EF, $r=0.295$, $P<0.05$). Estudios llevados a cabo con espermatozoides humanos incubados con P4 demostraron un aumento significativo de TyrPP en la pieza principal del flagelo (Sagare-Patil and Modi 2017), que se relacionó con la activación de motilidad (Lishko et al. 2011; Strunker et al. 2011). Sin embargo, en la especie ovina no observamos dichos efectos.

Cuando comparamos el efecto de la P4 con un inductor de la RA, la LPC, observamos que esta última daba lugar a un aumento de la población con TyrPP en la zona ecuatorial o acrosomal y a una desaparición de la localización en el flagelo. Aunque ambas sustancias, P4 y LPC, inducen la RA en el espermatozoide ovino, los cambios que provocan en la distribución de las TyrPP es distinta, probablemente debido a que la primera la induce de forma fisiológica, y la segunda no.

Por otro lado, la incubación con E2 a baja concentración provocó un descenso de la subpoblación que correlacionó positivamente con espermatozoides no capacitados (AE) y un aumento de las subpoblaciones con localización de TyrPP restringida a la cabeza (subtipos A y E). La incubación con la concentración alta de E2 dio lugar a un aumento del marcaje en el acrosoma y flagelo (AF). Las diferencias de localización en función de la concentración de E2 también se han descrito en el espermatozoide murino (Sebkova et al. 2012). Sin embargo, la incubación con esta hormona no produjo una disminución de la subpoblación correlacionada con los espermatozoides capacitados, ni un aumento de la subpoblación correlacionada con los reaccionados, a diferencia de la progesterona.

Está demostrado que un cierto nivel de especies oxígeno reactivas (ROS) es necesario para que se produzca la capacitación y la RA, entre otros eventos relacionados con la funcionalidad espermática (Amaral et al. 2013), si bien todavía existen aspectos desconocidos sobre la regulación de la producción endógena de ROS en el espermatozoide (de Lamirande and Lamothe 2009; Guthrie and Welch 2012). En el presente trabajo observamos un incremento significativo en los niveles de ROS en las muestras capacitadas en un medio con agentes elevadores del cAMP. Este aumento de ROS podría producirse por la activación de la familia de enzimas NADPH oxidasas, concretamente la NADPH oxidasa 5 (NOX5), descrita en la membrana del espermatozoide humano (Musset et al. 2012), equino (Sabeur and Ball 2007) y canino (Setyawan et al. 2016). Nuestro grupo de investigación ha demostrado recientemente su presencia también en el espermatozoide ovino (Gimeno-Martos et al. 2019c), por lo que esta enzima podría estar implicada en la generación de ROS relacionada con la capacitación. La adición de P4 a alta concentración (1 µM) incrementó todavía más estos niveles de ROS. Estos resultados coinciden con los descritos en la especie humana, donde la P4 también aumenta los niveles de ROS durante la capacitación espermática (de Lamirande et al. 1998; Ghanbari et al. 2018).

Además, la concentración más alta de P4 también aumentó los niveles de calcio intracelular (Ca^{2+}_i), evaluados por citometría de flujo usando Fluo4-AM, en muestras incubadas en condiciones capacitantes. El aumento de Ca^{2+} inducido por la P4 también

se ha observado en espermatozoides de otras especies, como la humana (Blackmore et al. 1991), porcina (Yeste et al. 2015) y murina (Romarowski et al. 2016). Este incremento podía ser debido tanto a una entrada de Ca^{2+} del exterior, como se ha descrito en otras especies debido a la apertura de canales como el CatSper (Lishko et al. 2011; Strunker et al. 2011), o a una salida de los reservorios intracelulares, como el acrosoma (Harper et al. 2004; Lucchesi et al. 2016). Los resultados del presente trabajo, evaluados por microscopía de fluorescencia usando Rhod-5-AM, evidenciaron que la incubación de los espermatozoides ovinos en condiciones capacitantes dio lugar a una movilización de Ca_i^{2+} desde la pieza intermedia del flagelo al cuello, apareciendo una población mayoritaria con un marcaje muy intenso en esta zona. La presencia de P4 incrementó la subpoblación espermática con acúmulo de este ion en acrosoma, cuello y pieza intermedia. Por tanto, se podría inferir que la P4 es capaz de inducir una movilización del Ca^{2+} desde el flagelo hacia el acrosoma, manteniendo la localización de este ion en el cuello y pieza intermedia, que es la predominante en muestras capacitadas.

Sin embargo, la incubación de espermatozoides ovinos en condiciones capacitantes con E2 no provocó, a ninguna de las dos concentraciones ensayadas, cambios en los niveles de ROS y Ca_i^{2+}. Lo que si provocó fueron cambios en la localización del Ca_i^{2+}, disminuyendo también la subpoblación espermática mayoritaria en la muestra capacitada control (con marcaje en la pieza intermedia y cuello). A concentración baja (100 pM), el E2 también dio lugar a una movilización del calcio a toda la cabeza y un marcaje irregular de la pieza intermedia, que podría apuntar a que este ion podría estar perdiéndose gradualmente en esta zona del flagelo. Estos resultados parecen indicar que E2 también produce movilizaciones del calcio, pero no particularmente al acrosoma, como ocurre en el caso de P4.

Para determinar si todos estos efectos provocados por las hormonas esteroideas estaban mediados por la vía del cAMP/PKA, una de las principales descritas en la regulación de la capacitación, evaluamos los niveles de cAMP y la actividad de la enzima PKA mediante ensayos ELISA comerciales. Ni la P4 ni el E2 dieron lugar a un aumento significativo de los niveles de cAMP respecto a la muestra capacitada control (sin hormonas). Este hecho podía ser debido a que el medio de incubación que

utilizamos contiene sustancias elevadoras de cAMP, y por tanto, los niveles de cAMP podrían estar cercanos a la saturación, y las hormonas no serían capaces de incrementar más estos niveles. Además, la presencia de hormonas esteroideas tampoco dio lugar a ningún cambio en la actividad de la PKA. Para asegurarnos de que este hecho no era debido a una saturación en los niveles de cAMP inducido por el medio usado, repetimos las medidas de los niveles de PKA en presencia de P4 y E2, pero incubando a los espermatozoides en medio TALP. El hecho de que tampoco se observaran cambios en estas condiciones parece indicar que la vía cAMP/PKA no estaría directamente implicada en los efectos producidos por ambas hormonas esteroideas. Esta idea se contrapone a otros trabajos realizados en otras especies, donde describen un papel modulador de la P4 en los segundos mensajeros, cAMP y cGMP, y en la enzima PKA (Torres-Flores et al. 2008; Sagare-Patil et al. 2012; Baron et al. 2016)). Sin embargo, estaría en concordancia con otros estudios que demuestran que la P4 no es capaz de aumentar los niveles de cAMP, y que este segundo mensajero no está relacionado directamente con los cambios que produce esta hormona en los niveles de Ca_i^{2+} (Strunker et al. 2011). Por tanto, no podemos obviar la posibilidad de que puedan estar implicadas otras rutas moleculares, como la del PI3K/AKT y la vía MAPKs, las cuales ya han sido relacionadas con la P4 y el E2 en el espermatozoide humano (Aquila et al. 2004; Sagare-Patil et al. 2012; Sagare-Patil et al. 2013).

Las hormonas esteroideas se han relacionado con cambios en la motilidad espermática, generalmente con una inducción de la hiperactivación flagelar (Noguchi et al. 2008; Sagare-Patil et al. 2012; Sumigama et al. 2015). Sin embargo, nuestros resultados anteriores (*Artículo 1)* (Gimeno-Martos et al. 2017) y (*Artículo 2*) sólo revelaron un descenso en la motilidad total asociado a la presencia de las hormonas y cambios en la motilidad progresiva en presencia de moduladores de los receptores de ambas hormonas, sin cambios en los parámetros cinéticos. Ya que el método de análisis y las condiciones de grabación de la motilidad pueden afectar a los resultados obtenidos (Castellini et al. 2011; Valverde et al. 2019), decidimos llevar a cabo los análisis grabando a 60 fps (*frames* o fotogramas por segundo) en lugar de a 25 fps, y usando otro sistema CASA distinto. En este caso, utilizamos un software libre desarrollado recientemente por nuestro grupo de investigación y denominado

OpenCasa (Alquezar-Baeta et al. 2019). Con estas condiciones, en el presente trabajo se evidenciaron cambios en los parámetros cinéticos entre las muestras incubadas en medio TALP sin y con agentes elevadores del cAMP (*cocktail*). Así, la incubación en medio *cocktail* dio lugar a un aumento de VCL, ALH (media y máxima), DANCE y la dimensión fractal (FD), parámetros directamente relacionados con la hiperactivación, y a una disminución de los relacionados con la rectitud del movimiento, como VSL, STR, WOB, LIN y la motilidad progresiva (Mortimer and Swan 1995; Mortimer and Maxwell 1999; Mortimer and Maxwell 2004). Una vez demostrado que el nuevo sistema de análisis de la motilidad permitía evidenciar diferencias relacionadas con la hiperactivación, estudiamos el efecto de las hormonas esteroideas sobre la motilidad espermática con estas mismas condiciones. En este caso, además del descenso en la motilidad total, en concordancia con lo observado en el trabajo anterior (*Artículo 1*) (Gimeno-Martos et al. 2017) también se observó un aumento en la motilidad progresiva con la concentración baja de P4 y la alta de E2. En cuanto a los parámetros cinéticos, sólo la P4 a concentración baja provocó un cambio en LIN, pero, al contrario de lo esperable, ya que aumentó este valor en vez de disminuirlo. Estos resultados no concordarían con los observados en otras especies como en humano, ratón, mono y hámster, donde la incubación con hormonas esteroideas dio lugar a una hiperactivación flagelar (Sagare-Patil et al. 2012; Fujinoki 2014; Sumigama et al. 2015; Bosakova et al. 2018).

En conclusión, el presente estudio muestra que, aunque la P4 y el E2 promueven mecanismos conducentes a la inducción de la reacción acrosómica en los espermatozoides ovinos, estos mecanismos son diferentes. Ambas provocan cambios en la localización de las TyrPP, pero en distintas zonas del espermatozoide. Además, mientras que la P4 produce un aumento en los niveles de calcio y una movilización de este ion hacia el acrosoma, un aumento en ROS y una ruptura significativa del acrosoma, el E2 no da lugar a estos cambios. La vía cAMP-PKA no parece estar involucrada en los efectos provocados por las hormonas esteroideas, y estas hormonas no dan lugar a un aumento de la hiperactivación en espermatozoides ovinos incubados en condiciones de alto AMPc.

6.5. EFECTOS DE LA MELATONINA SOBRE LA CAPACITACIÓN ESPERMÁTICA *IN VITRO* EN LA ESPECIE OVINA

La melatonina, como hemos mencionado en la introducción, es una hormona que, entre otras muchas funciones, juega un importante papel en la reproducción. Entre otros efectos, la melatonina controla la estacionalidad reproductiva a través también del eje hipotálamo-hipofisario-gonadal (Gerlach and Aurich 2000). Esta hormona, además de ser secretada por la hipófisis, se sintetiza en varios tejidos extra-pineales, incluido el tracto reproductor masculino (Tijmes et al. 1996; Luboshitzky et al. 2002) y el femenino (Brzezinski et al. 1987) (revisado en (Cebrian-Perez et al. 2014). Nuestro grupo demostró su presencia en el tracto reproductor del morueco (Gonzalez-Arto et al. 2017), así como en el plasma seminal ovino (Casao et al. 2010a). Así mismo, identificó los receptores de melatonina, MT_1 Y MT_2 , en la membrana plasmática del espermatozoide ovino (Casao et al. 2012).

En base a estos resultados, se especuló sobre el papel de la melatonina en la funcionalidad espermática. Nuestro grupo demostró que esta hormona ejercía una acción protectora, disminuyendo los marcadores apoptóticos, y daba lugar a efectos opuestos, dependientes de la concentración, sobre la capacitación espermática, cuando se añadía a espermatozoides ovinos incubados en un medio no capacitante (Casao et al. 2010b). Más adelante, en otro trabajo se confirmó que los efectos de la melatonina sobre la funcionalidad espermática estaban mediados por interacción con los receptores ya descritos, fundamentalmente con MT_2, (Gonzalez-Arto et al. 2016a). Todos estos trabajos confirman el papel de la melatonina sobre la funcionalidad espermática ovina, especialmente en el proceso de capacitación, pero hasta la fecha se desconoce su mecanismo de acción.

Por ello, **el último objetivo de esta tesis se centró en investigar los mecanismos moleculares por los que la melatonina ejerce su acción dual capacitante/descapacitante sobre el espermatozoide ovino** (*Artículo 4)*, (Gimeno-Martos et al. 2019b). Con este propósito se incubaron los espermatozoides en

condiciones capacitantes, en medio TALP con agentes elevadores del cAMP (*cocktail*), y con distintas concentraciones de melatonina (100 pM, 10 nM y 1 µM).

En el presente trabajo, la concentración baja de melatonina (100 pM) en un medio con agentes elevadores del cAMP no provocó un aumento del porcentaje de espermatozoides capacitados o reaccionados, a diferencia de lo observado en medios carentes de estas sustancias, donde ejercía una acción capacitante (Casao et al. 2010b). Esto puede ser debido a que la capacitación ya está inducida debido a la acción del *cocktail* y la melatonina a baja concentración no sea capaz de incrementar más el porcentaje de espermatozoides capacitados. Sin embargo se observa una mejora de la viabilidad (integridad de la membrana plasmática) respecto a la muestra *cocktail*, tal como se ha descrito en el espermatozoides humano (du Plessis et al. 2010).

La presencia de melatonina a concentraciones 10 nM y 1 µM en el medio *cocktail* disminuyó significativamente el porcentaje de espermatozoides capacitados tras 1 hora de incubación, siendo este efecto mucho más acusado a las 3 horas. En el caso de la concentración alta (1 µM), esta respuesta también se acompañó de una inhibición de la fosforilación de proteínas en residuos de tirosina . Estos resultados demuestran que la melatonina, a alta concentración, también es capaz de ejercer un efecto descapacitante en un medio con agentes elevadores del cAMP, y no sólo en medios carentes de estas sustancias, como ya habíamos demostrado previamente (Casao et al. 2010b). Este efecto inhibitorio de la capacitación podría ser debido a una disminución de los niveles de ROS o de los niveles de cAMP, ya que en este trabajo también se demostró que la melatonina a alta concentración era capaz de producir ambos efectos. El efecto de la melatonina sobre los niveles de ROS ha sido descrito en espermatozoides de otras especies (Ortiz et al. 2011; Ashrafi et al. 2013; Deng et al. 2017). Esta acción sobre los niveles de ROS puede deberse a la acción antioxidante de la melatonina, que es capaz de ejercer atravesando directamente la membrana plasmática del espermatozoide (Reiter et al. 2000; Jang et al. 2010). Respecto al efecto observado sobre el cAMP, este fenómeno se ha descrito en otros tipos celulares, sobre los que la melatonina tiene la capacidad de inhibir la enzima adenilatociclasa (AC), dando lugar a la disminución del cAMP y a una reducción de la actividad de la PKA

(Luchetti et al. 2010). Sin embargo, la melatonina ni a baja (100 pM) ni a alta concentración (1 µM) da lugar a cambios en la actividad de PKA respecto a la muestra control como hemos demostrado en trabajos posteriores *(Anexo-Artículo 4)* (Gimeno-Martos et al. 2019a). Por tanto, la melatonina a concentración 1 µM es capaz de modificar los niveles de cAMP pero no la actividad de la PKA durante la capacitación espermática ovina. Esto implicaría que la melatonina no regula la capacitación por la vía clásica cAMP/PKA. Puede ser que esa disminución en el cAMP no sea suficiente para dar lugar a una disminución de la actividad de PKA, y por tanto, que los efectos observados sean debidos a la disminución de los niveles del ROS. Aunque tampoco podemos obviar que la melatonina pueda ejercer su acción mediante otras vías, como la del EGFR (*epidermal growth factor receptor*) y la vía de las MAPKs (*mitogen-activated protein kinases*). Así, se ha descrito que la unión de melatonina a sus receptores activa las MAPK en varios tipos celulares (Luchetti et al. 2009). Estas vías del EGFR y las MAPKs se han relacionado con la capacitación del espermatozoide ovino por nuestro grupo de investigación (Luna et al. 2012; Luna et al. 2017a), y actualmente se está investigando el efecto de la melatonina sobre estas rutas.

En otras especies, como la humana, se ha descrito un aumento de la hiperactivación espermática dependiente de ROS durante la capacitación (de Lamirande and Gagnon 1993). Por ello, dado que habíamos observado cambios en los niveles de ROS debidos a la melatonina, analizamos su influencia en la motilidad y la hiperactivación del espermatozoide ovino durante la capacitación *in vitro*. Mediante un sistema CASA comercial, pudimos observar que la melatonina alta concentración (1 µM) disminuía la motilidad progresiva. Sin embargo, en espermatozoides humanos (Ortiz et al. 2011) y bovinos (Ashrafi et al. 2013) la incubación con melatonina a concentraciones incluso más altas (≥ 1 mM) aumenta la motilidad espermática.

Al analizar los parámetros cinéticos tras grabar las muestras a 25 fps con el sistema CASA comercial (ya que en ese momento no habíamos desarrollado completamente el *software* libre OpenCASA) no encontramos ninguna diferencia entre las muestras. Esta falta de diferencias, podía ser debida a que los eyaculados de mamíferos son altamente heterogéneos (Abaigar et al. 1999; Mortimer 2000), y una

evaluación de la motilidad basada en los valores medios de los parámetros cinéticos podía dar lugar a una gran pérdida de información (Verstegen et al. 2002; Holt et al. 2007). Por el contrario, si se define el movimiento exacto de cada espermatozoide, éste se puede incluir en una subpoblación específica en base a sus características cinéticas, y el estudio posterior de estas subpoblaciones podría aportar un mayor conocimiento sobre los cambios en la motilidad que tienen lugar durante determinados procesos o condiciones experimentales. La existencia de diferentes subpoblaciones con distintas características de motilidad ha sido descrita en eyaculados de varias especies como toro (Muiño et al. 2008), cerdo (Abaigar et al. 1999), mono (Miro et al. 2005), perro (Nuñez-Martinez et al. 2006), ratón (Goodson et al. 2011), humano (Buffone et al. 2004) y morueco (Garcia-Alvarez et al. 2014). Para el análisis de las diferentes subpoblaciones espermáticas en muestras de espermatozoides ovinos incubados en presencia y ausencia de melatonina, llevamos a cabo un Análisis de Componentes Principales (PCA, *Principal Component Analysis*) en colaboración con el Dr. Marc Yeste del Grupo de *Biotecnología de Reproducción Animal y Humana* en el Departamento de Biología de la Universidad de Girona. Se estudiaron un total de más de 230.000 espermatozoides, cuya motilidad había sido previamente evaluada con el sistema CASA. El estudio de PCA fue aplicado a todos los parámetros cinéticos y reveló dos componentes principales como responsables de más del 77% de la varianza: el primero, asociado a los parámetros de velocidad rectilínea y el batido flagelar, y el segundo, con la velocidad curvilínea y la amplitud lateral de la cabeza. De esta manera se caracterizaron cuatro subpoblaciones (SP; *subpopulation*) de espermatozoides, que coincidieron con las descritas en un estudio previo de nuestro grupo (Luna et al. 2017b): SP1 englobaría a aquellos espermatozoides con velocidad media y alta rectitud en su movimiento (altos valores de LIN y STR y bajos de ALH); SP2, a espermatozoides rápidos iniciando el fenómeno de hiperactivación, mayores valores de ALH y DANCE, pero no llegarían a los límites establecidos para considerarlos hiperactivados según (Colas et al. 2010); SP3, a espermatozoides rápidos e hiperactivados (bajos valores de LIN y STR y muy altos de ALH); y finalmente SP4, los menos vigorosos, con una motilidad no lineal (valores bajos de ALH y DANCE y mínimos de LIN and STR).

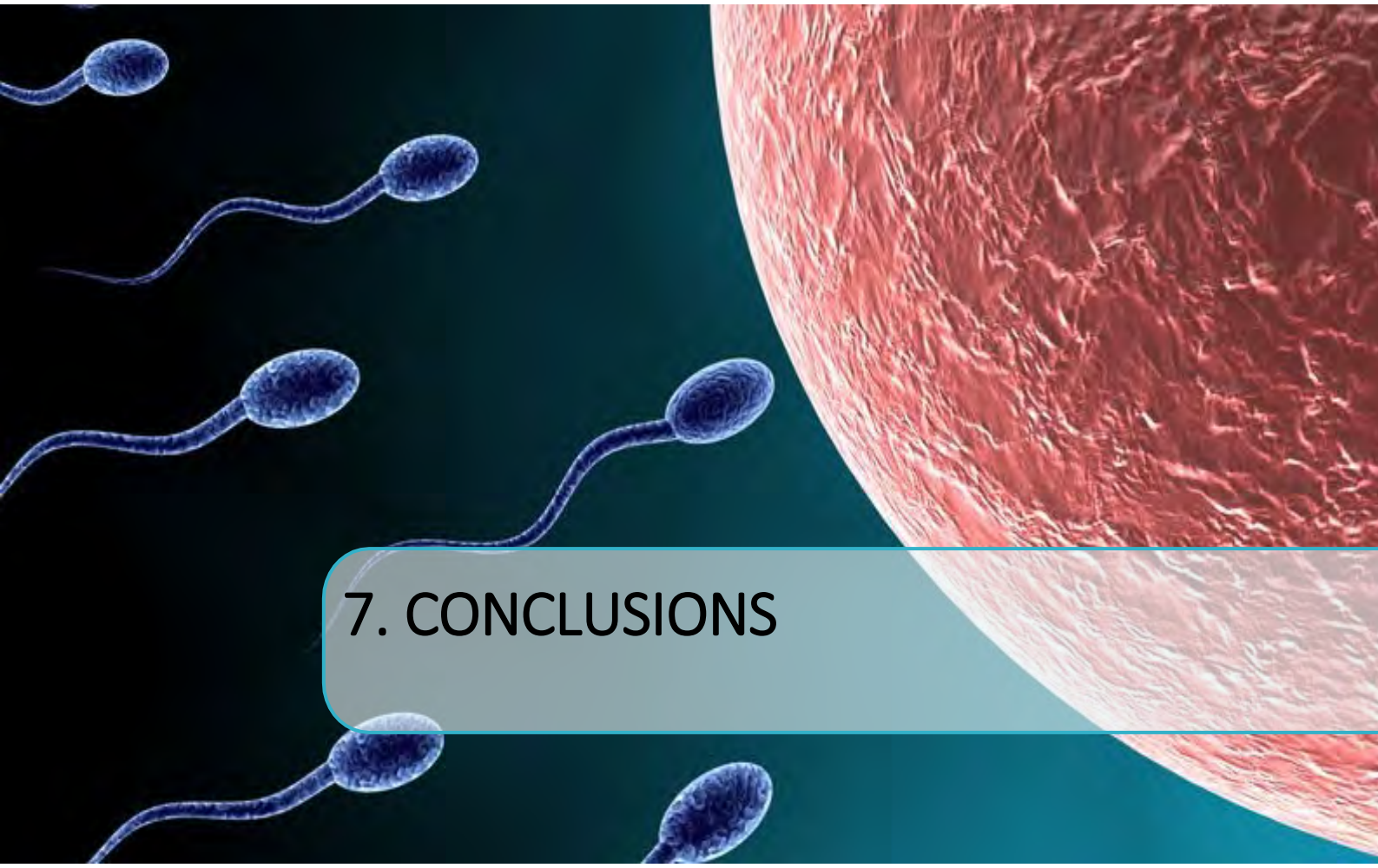
En este trabajo, la adición de melatonina a baja y media concentración (100 pM y 10 nM) a las muestras espermáticas en medio *cocktail* supuso un cambio en los porcentajes de las subpoblaciones observadas, en concreto un aumento de SP2 (rápidos comenzando la hiperactivación) y una disminución de SP4 (menos vigorosos con motilidad no lineal) respecto a las muestras sin hormona. A la vista de estos resultados, podríamos pensar que la melatonina a concentración picomolar, como probablemente se encuentre en el tracto reproductor femenino, podría regular el proceso de hiperactivación como también se ha observado en el espermatozoide de hámster (Fujinoki 2008). Sin embargo, la melatonina a concentración 1 µM no produjo cambios significativos en el porcentaje de las subpoblaciones, a pesar de inducir un descenso del porcentaje de espermatozoides capacitados y de los niveles de fosforilación de proteínas en residuos de tirosina, lo que apoya la idea de que capacitación e hiperactivación pueden no ser interdependientes (de Lamirande et al. 1997; Marquez and Suarez 2004). En conclusión, los resultados de este trabajo mostraron que la melatonina es capaz de modular la capacitación espermática ovina en medios con alto cAMP, modificando las subpoblaciones con distintos parámetros de motilidad a bajas concentraciones y disminuyendo los niveles de ROS y cAMP a alta concentración. Sin embargo, serán necesarios más estudios en un futuro para determinar qué otras rutas metabólicas están implicadas en estos efectos de la melatonina sobre el espermatozoide ovino.

A modo de conclusión final, esta tesis pone de manifiesto los efectos directos de las hormonas presentes en el tracto reproductor femenino, como la melatonina y las hormonas esteroideas, progesterona (P4) y 17-β estradiol (E2), sobre los espermatozoides ovinos. Ambas hormonas esteroideas inducen la RA en el espermatozoide ovino, y cambios en la motilidad, pero no producen hiperactivación flagelar. Estos efectos, como se evidenció por los ensayos con agonistas y antagonistas, están mediados por su unión a receptores, descritos por primera vez en esta tesis en el espermatozoide ovino. En el caso del estradiol, el ERβ parece ser el que está más implicado en la modulación de la capacitación y RA. Sin embargo, aunque ambas hormonas inducen la reacción acrosómica, los mecanismos por la que la regulan son distintos, ya que la P4 produce un aumento en los niveles de calcio y una

movilización de este ion hacia el acrosoma, un aumento en ROS y una ruptura significativa del acrosoma, mientras el E2 no da lugar a estos cambios. Con respecto a la melatonina, los resultados de esta tesis confirman su acción dual dependiente de la concentración, promoviendo la hiperactivación espermática a concentraciones bajas, e inhibiendo el proceso de capacitación y la RA, además de su papel antioxidante, a concentraciones altas. Esto podría indicar un papel protector de esta hormona en el plasma seminal, donde se encuentra a alta concentración, evitando la capacitación prematura y los daños oxidativos en los espermatozoides, mientras que en el tracto reproductor femenino, donde la concentración fisiológica se estima del orden picomolar, prepararía a los espermatozoides para la fecundación. A pesar de que altas concentraciones de melatonina disminuyen los niveles de cAMP, no se observaron cambios en la actividad de la PKA, por lo que la vía clásica cAMP/PKA relacionada con la capacitación no parece estar implicada en sus mecanismos de acción. Por tanto, el estudio de su acción sobre otras vías alternativas continúa abierto en otros experimentos de nuestro grupo.

7. CONCLUSIONES

1. El receptor de progesterona, PR, y los receptores de estrógenos, ERα y ERβ, están presentes en la membrana plasmática del espermatozoide ovino. PR se localiza en la zona ecuatorial y la pieza intermedia, ERα en la zona post-acrosomal y ERβ en la región apical.
2. Existe una correlación positiva y significativa entre el porcentaje de espermatozoides que presentan el receptor ERβ y el de espermatozoides no capacitados.
3. La progesterona y 17-β estradiol tienen la capacidad de inducir la reacción acrosómica en el espermatozoide ovino y lo hacen a través de su unión a sus receptores específicos.
4. La progesterona provoca cambios en la localización de proteínas fosforiladas en tirosinas, un aumento del porcentaje de espermatozoides vivos con acrosoma dañado, de los niveles de especies reactivas de oxígeno y de Ca^{2+} intracelular, así como la movilización de este ion hacia al acrosoma, en el espermatozoide ovino.
5. El 17-β estradiol provoca cambios en la localización de proteínas fosforiladas en tirosinas pero no da lugar a cambios en el porcentaje de espermatozoides vivos con acrosoma dañado, ni a cambios en los niveles de ROS ni de Ca^{2+}.
6. Los efectos producidos por la progesterona y el 17-β estradiol en el espermatozoide ovino no están mediados por la vía cAMP-PKA, ni están relacionados con la estimulación de la hiperactivación espermática, al menos en condiciones de incubación con alto cAMP .
7. La melatonina a alta concentración (1 µM) inhibe la capacitación espermática ovina en medios con alto cAMP, mediante la disminución de los niveles de proteínas fosforiladas en tirosinas, ROS y cAMP, pero no provoca cambios en la actividad de la enzima PKA en condiciones capacitantes.
8. La presencia de melatonina a baja y media concentración (100 pM y 10 nM) promueve el aumento de la subpoblación espermática asociada a motilidad hiperactivada en condiciones capacitantes.



7. CONCLUSIONS

1. Progesterone (PR) and estrogen receptors (ERα y ERβ) are present in ram spermatozoa. PR is located at the equatorial region and midpiece, ERα at the postacrosomal region and ERβ at the apical region.

2. The percentage of spermatozoa showing ERβ receptor labelling has a significant positive correlation with the non-capacitated spermatozoa rate.

3. Progesterone and 17-β estradiol can induce the acrosome reaction in ram spermatozoa, and this effect is receptor-mediated.

4. Progesterone changes in localization of tyrosine-phosphorylated proteins, increases the percentage of live sperm with damaged acrosome, ROS and intracellular Ca^{2+} levels, that is mobilized towards the acrosome.

5. 17-β estradiol changes the localization of tyrosine-phosphorylated proteins, but it does not modified the rate of live sperm with damaged acrosome or ROS and intracellular Ca^{2+} levels.

6. The effects provoked by progesterone and 17-β estradiol in ram spermatozoa are not mediated by the cAMP-PKA pathway, and they are not related to sperm hyperactivation in high-cAMP conditions.

7. Melatonin at micromolar concentration inhibits ram sperm capacitation induced by high-cAMP conditions by a reduction of ROS, cAMP and phosphotyrosine levels. However, it does not changes PKA activity.

8. Melatonin 100 pM and 10 nM increases the hyperactivated spermatozoa subpopulation in high-cAMP conditions.

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9. LISTA DE ABREVIATURAS

Abreviaturas:

1AG 1-araquidonoglicerol

2AG 2-araquidonoglicerol

AANAT Arilaquilamina N-acetiltransferasa

AC *Adenylate cyclase*; Adenilato ciclasa

ABH2 *α-ß hydrolasedomain–containing protein;* enzima serin hidrolada alpha beta hidrolasa 2

ADN Ácido dexirribonucleico

AI *Artificial Insemination*; Inseminación artificial

AKAPs *A-Kinase Anchoring Proteins*; Proteínas de anclaje de la PKA

ANGRA Asociación Nacional de Ganadores de *Rasa Aragonesa*

ANOVA *Analysis of Variance*; Análisis de varianza.

AR *Acrosome-reacted*; Espermatozoide reaccionado

s AR S*pontaneous acrosome-reaction*; Reacción acrosómica espontánea

Arg *Arginine*; Arginina

ASMT N-acetilserotonina O-metiltransferasa

ATP *Adenosine triphosphate*; Adenosín trifosfato

BSA *Bovine Serum Albumin*; Albúmina sérica bovina

BSPs *Binder sperm proteins;* Proteínas unión a espermatozoide

C *Capacitated*; Espermatozoide capacitado

cAMP *Cyclic adenosine 3´-5´-monophosphate*; Adenosín monofosfato-3´, 5´ cíclico-AMP cíclico

CAMKII *Calmodulin Protein Kinase* II; Calmodulina proteín kinasa II

Cap-C Capacitated control; Control de capacitación

CASA *Computer-Assisted Sperm Analysis*; Análisis espermático computerizado

cGMP *cyclic Guanosine monophosphate*

Ca_v *high voltage-gated Ca^{2+} channel*

CAT *Catalase*: Catalasa

Catsper *Cation channel sperm*

CFDA *CarboxyFluorescein DiAcetate;* Diacetato de carboxifluoresceína

CK *Cocktail*

CNG *cyclic nucleotide-gated Ca^{2+} channel*

CREB *cAMP response element-binding*

CT *Chlortetracycline*; Clorotetraciclina

DABCO *1,4-Diazacicyclo[2.2.2]octane*

DAG *Diacylglycerol*; Diacilglicerol

Db-AMPc Diburitil-AMPc

DMSO *Dimethylsulfoxide;* Dimetilsulfóxido

DNA *Deoxyribonucleic acid*; Ácido desoxirribonucleico

E2 17- β estradiol

EDTA *Ethylenediaminetetraacetic acid;* Ácido etilendiaminotetraacético

EGF *Epidermal Growth Factor*; Factor de crecimiento epidérmico

EGF-R *Epidermal Growth Factor Receptor*; Receptor del factor de crecimiento epidérmico

EPAC *Exchange Protein Activated Directly by cAMP*; Proteína de intercambio activada directamente por cAMP.

ER *Estrogen receptors;* Receptor de estrógenos

ER Época reproductiva

ENR Época no reproductiva

ERKs *Extracellular Regulated Kinases*; Quinasas reguladas extracelularmente

F-actina Actina filamentosa

FD *Fractal dimension*; Dimensión fractal

FITC *Fluorescein isothiocyanate*; Isotiocianato de fluoresceína

FL-channels; *Fluorescence channels;* Canales de fluorescencia (citómetro de flujo)

FPS Frames per second; Fotogramas por segundo

FS *Forward Scatter*; Difracción de la luz en sentido frontal (parámetro del citómetro de flujo)

Gly *Glycine*; Glicina

GFP *Green Protein Fluorescent*; Proteína verde fluorescente

GnRH *Gonadotropin-Releasing Hormone*; Hormona liberadora de gonadotropina

GPx *Glutathione Peroxidase*; Glutatión peroxidasa

GPCRs G Protein-Coupled Receptors; Receptores acoplados a proteínas G

GPER *G Protein Estrogen Receptor;* Receptores de estógenos acoplados a proteínas G

GR *Glutathione Reductase*; Glutatión reductasa

HDL *High-Density Lipoproteins*; Lipoproteínas de alta densidad

HRP *Horseradish peroxidase*; Peroxidasa de rábano

IA Inseminación artificial

IFI Inmunofluorescencia indirecta

IIF *Indirect immunofluorescence*

IP_3 *Inositol 1,4,5-triphosphate*; Inositol 1,4,5-trifosfato

IPR_3R *Inositol 1,4,5-triphosphate*; Inositol 1,4,5-trifosfato receptor

IPK_3 *Inositol 1,4,5-triphosphate Kinase*; Inositol 1,4,5-trifosfato kinasa

ISAS *Integrated Semen Analysis System;* Sistema de análisis espermático integrado

IU *Intenational Units;* Unidades internacionales

JNKs *c-Jun N-terminal Kinases*; Quinasas c-Jun N-terminal

JNKi *c-Jun N-terminal Kinase Inhibitor*; Inhibidor de quinasa c-Jun N-terminal

LH *Luteinizing hormone;* Hormona lutenizante

LPA Ácido lisofosfatídico

LPC *Lysophosphatidylcoline*; Lisofosfatidilcolina

mAC *Membrane adenyl cyclase*; Adenilato ciclasa asociada a membrana

MAPK, *Mitogen-Activated Protein Kinase*; Proteín quinasa activada por mitógenos

M- β -CD *Methyl-beta-cyclodextrin*; Metil-beta-ciclodextrina

MCF-7 Cells *Michigan Cancer Foundation-7 cells*

MFI *Mean Fluorescence intensity*; Intesisdad de fluorescencia total

MT_1 *Melatonin Receptor 1*; Receptor de melatonina 1

MT_2 *Melatonin Receptor 2*; Receptor de melatonina 2

MT_3 *Melatonin Receptor 2*; Receptor de melatonina 3

NC *Non-capacitated*; Espermatozoide no capacitado

NO *Nitric Oxide*; Óxido nítrico

NOS *Nitric Oxide Synthase*; Óxido nítrico sintasa

eNOS Endothelial *Nitric Oxide Synthase*; Óxido nítrico sintasa

NADPH *NADPH Nicotinamide adenine dinucleotide phosphate reduced*; Nicotinamida adenina dinucleótido fosfato reducido

NETs *Neutrophil extracelular traps*

NOX *NADPH oxidase*; NADPH oxidasa

OA *Okadic Acid;* Ácido okadaico

OAM *Outer acrosomal membrane*; Membrana acrosomal externa

P4 *Progesterone*; Progesterona

PAGE *PolyAcrilamide Gel Electrophoresis*; Electroforesis en gel de poliacrilamida

PBS *Phosphate Buffer Saline*; Buffer fosfato salino

PBS-T *PBS-Tween*

PCA *Principal Components Analysis*; Análisis de componentes principales

PDEs *Phosphodiesterases*; Fosfodiesterasas

Phi pH intracelular

PI *Propidium Iodide*; Ioduro de propidio

PI3K/Akt *Phosphatidylinositol 3-kinase*; Fosfatidil inositol 3 quinasa

PIP_2 *Phosphatidylinositol*-4,5-biphosphate; Fosfatidilinositol-4,5-bifosfato

PKA *Protein kinase A*; Proteín quinasa A

PKB *Protein kinase B;* Proteín quinasa B

PKC *Protein kinase C;* Proteín quinasa C

PLA_2 *Phospholipase A_2*; Fosfolipasa A_2

PLC *Phospholipase C;* Fosfolipasa

PLD *Phospholipase D*; Fosfolipasa D

PP1 *Protein phosphatase 1*

PP2B *threonine-protein phosphatase 2B*

PR *Progesterone Receptor*; Receptor de progesterona

PRA A *Progesterone Receptor*; Receptor de progesterona A

PRB B *Progesterone Receptor*; Receptor de progesterona B

PVDF *Polyvinylidene fluoride*

RA Reacción Acrosómica

RANTES *Regulated on Activated Normal T Cell expressed and secreted*

RAP1 *Ras related protein-1*; Proteína de unión a RAS 1

RCA *Ricinus communis aglutinin*

RE Retículo endoplasmático

RNA *Ribonucleic acid*; Ácido ribonucleico

RNE *Redudant Nuclear Envelope*; Envoltura nuclear redudante

RNS *Reactive Nitrogen Species*; Especies reactivas de nitrógeno

ROS *Reactive Oxigen Species*; Especies reactivas de oxígeno

RT *Room Temperature*; Temperatura ambiente

RT-PCR *Reverse Transcriptase-Polymerase Chain Reaction*; Reacción en cadena de la polimerasa con transcriptasa inversa

sAC *Soluble Adenylate Cyclase*; Adenilato ciclasa soluble

SDS *Sodium Dodecyl Sulfate;* Dodecil sulfato sódico

SEM *Standard Error of the Mean*; Error estándar de la media

Ser Serine; Serina

SM *Swim-up medium*; Medio de swim-up

SNARE *soluble SNF attachment protein*; Proteína soluble de fijación de NSF

SP *Sperm subpopulation;* Subpoblación espermática

SS *Side Scatter*; Reflexión de la luz en sentido lateral (parámetro del citómetro de flujo)

TALP *Tyrode´s Albumin Lactate Pyruvate*

Thr *Threonine*; Treonina

TRF Tracto reproductor femenino

Tyr *Tyrosine*; Tirosina

TyrP *Tyrosine Proteins*; Proteínas con residuos de tirosina

TyrPP *Tyrosine* phosphorilated proteins; Proteínas fosforiladas en residuos de tirosina

TM *Total Motility*; Motilidad total

UTJ *Utero-tubal junction;* Unión útero-tubárica

UV *UltraViolet*; Ultravioleta

Vmax *Maximum velocity*; Velocidad máxima (enzimática)

VAP *Average path velocity*; Velocidad media

ZP *Zona pellucida*; Zona pelúcida

ZP3 *Zona pellucida sperm-binding protein 3*;Proteína 3 de unión de espermatozoide a zona pelúcida

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