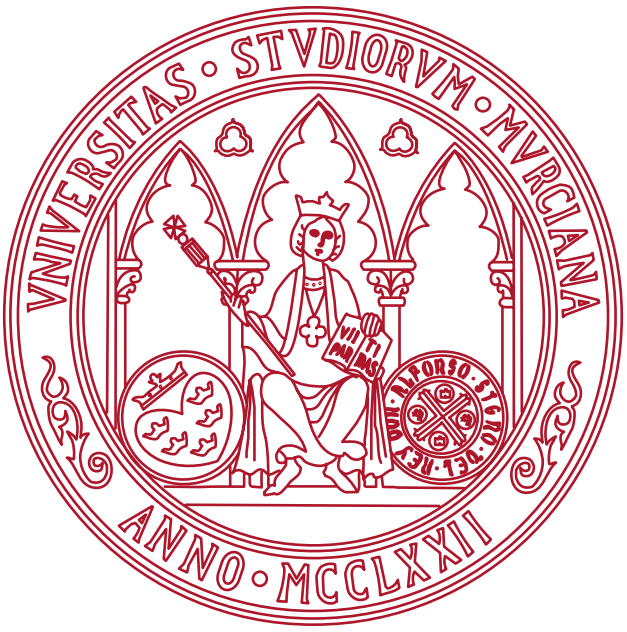


UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Simulation of the Oviductal Environment Modulates *in vitro* Sperm Capacitation and Fertility in Porcine Species

La Simulación del Ambiente Oviductal Modula la Capacitación Espermática y la Fertilidad *in vitro* en la Especie Porcina

Dña. Cristina de las Mercedes Soriano Úbeda

2017

UNIVERSIDAD DE
MURCIA

D. FRANCISCO ALBERTO GARCÍA VÁZQUEZ, Profesor Contratado Doctor del Departamento de Fisiología

AUTORIZA:

La presentación de la Tesis Doctoral titulada "**La simulación del ambiente oviductal modula la capacitación espermática y la fertilidad *in vitro* en la especie porcina**", realizada por **Dª. Cristina de las Mercedes Soriano Úbeda**, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Murcia, a 26 de octubre de 2017.

Fdo. Francisco Alberto García Vázquez

Francisco Alberto García Vázquez
Facultad de Veterinaria
Departamento de Fisiología
Email: fagarcia@um.es
Campus Universitario de Espinardo. 30100 Murcia
T. 868 888009 – F. 868 884147 – **www.um.es/grupo-fisiovet**

UNIVERSIDAD DE MURCIA

Dª. CARMEN MATÁS PARRA, Catedrática de Universidad del Departamento de Fisiología

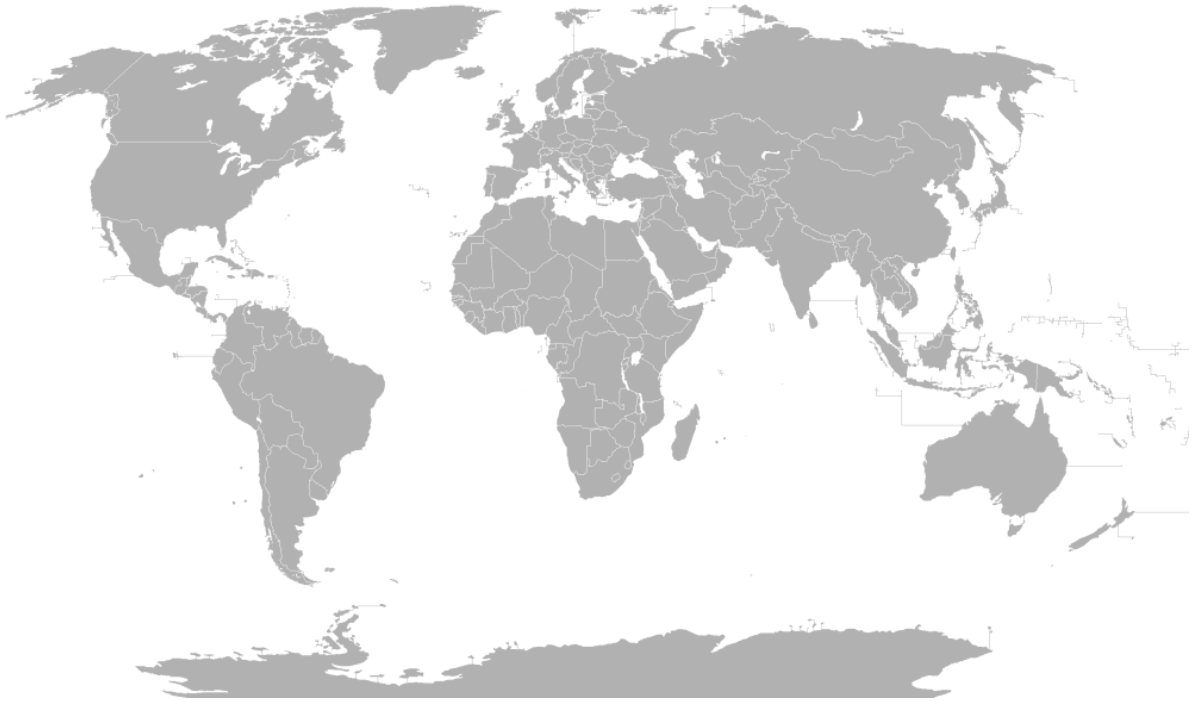
AUTORIZA:

La presentación de la Tesis Doctoral titulada "**La simulación del ambiente oviductal modula la capacitación espermática y la fertilidad *in vitro* en la especie porcina**", realizada por **Dª. Cristina de las Mercedes Soriano Úbeda**, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Amherst, a 26 de octubre de 2017.

Fdo. Carmen Matás Parra

Francisco Alberto García Vázquez
Facultad de Veterinaria
Departamento de Fisiología
Email: fagarcia@um.es
Campus Universitario de Espinardo, 30100 Murcia



Esta tesis doctoral ha sido propuesta para Mención de Doctor Internacional en virtud de las estancias de investigación realizadas y los informes de dos expertos extranjeros.

ESTANCIAS DE INVESTIGACIÓN

School of Biosciences. University of Birmingham. Birmingham (Reino Unido).
Dr. Stephen J. Publicover.
Del 4/4/2016 al 4/6/16

School of Biosciences. University of Birmingham. Birmingham (Reino Unido).
Dr. Stephen J. Publicover.
Del 7/6/2016 al 7/7/16

INFORMES DE EXPERTOS EXTRANJEROS

Dr. José Luis Ros-Santaella
Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources
Czech University of Life Sciences, Prague (Czech Republic)

Dr. Pablo Andrés Ortiz Pineda
Facultad de Ciencias Naturales Exactas
Universidad del Cauca, Popayán (Colombia)

UNIVERSITY OF BIRMINGHAM

School of Biosciences

Dr SJ Publicover
Reader in Reproductive Physiology
Direct Tel: 0121 414 5455
Email s.j.publicover@bham.ac.uk

04.06.2016

Cristina Soriano Úbeda

I am writing to confirm that Cristina Soriano Úbeda has been doing her research work in my laboratory in the School of Biosciences at the University of Birmingham during the period from 4th April 2016 to 4th June 2016.

Yours faithfully,

Dr S J Publicover

SCHOOL OF BIOSCIENCES
THE UNIVERSITY OF BIRMINGHAM
EDGBASTON, BIRMINGHAM B15 2TT
TEL. 0121-414-3386/5896
FAX. 0121-414-5925

[Escudo de la Universidad]

UNIVERSIDAD DE BIRMINGHAM

Facultad de Biociencias

Dr SJ Publicover
Profesor Titular de Fisiologia Reproductiva
Teléfono: 0 121 414 5455
Email s.j.publicover@bham.ac.uk

04.06.2016

Cristina Soriano Úbeda

Este documento sirve para confirmar que Cristina Soriano Úbeda ha desarrollado trabajos de investigación en mi laboratorio en la Facultad de Biociencias en la Universidad de Birmingham durante el periodo comprendido entre el 4 de abril de 2016 y el 4 de junio de 2016.

Saludos cordiales

[Firma ilegible]

Dr SJ Publicover

[Sello] FACULTAD DE BIOCIENCIAS
UNIVERSIDAD DE BIRMINGHAM
EDGBASTON, BIRMINGHAM B15 2T1
TEL. 0121-414-3386/5896
FAX. 0121-414-5925

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UNIVERSITY OF BIRMINGHAM

School of Biosciences

Dr SJ Publicover
Reader in Reproductive Physiology
Direct Tel: 0121 414 5455
Email s.j.publicover@bham.ac.uk

07.07.2016

Cristina Soriano Úbeda

I am writing formally to confirm that Cristina Soriano Úbeda has been doing her research work in my laboratory in the School of Biosciences at the University of Birmingham during the period from June 7th 2016 to July 7th 2016.

Yours faithfully,

Dr S J Publicover

SCHOOL OF BIOSCIENCES
THE UNIVERSITY OF BIRMINGHAM
EDGBASTON, BIRMINGHAM B15 2TT
TEL. 0121-414-3386/5896
FAX. 0121-414-5925

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UNIVERSIDAD DE BIRMINGHAM

Facultad de Biociencias

Dr SJ Publicover
Profesor Titular de Fisiología Reproductiva
Teléfono: 0 121 414 5455
Email s.j.publicover@bham.ac.uk

07.07.2016

Cristina Soriano Úbeda

Este documento sirve para confirmar que Cristina Soriano Úbeda ha desarrollado trabajos de investigación en mi laboratorio en la Facultad de Biociencias en la Universidad de Birmingham durante el periodo comprendido entre el 7 de junio de 2016 y el 7 de julio de 2016.

Saludos cordiales

[Firma ilegible]

Dr SJ Publicover

UNIVERSIDAD DE MURCIA
SERVICIO DE IDIOMAS

[Sello] FACULTAD DE BIOCIENCIAS
UNIVERSIDAD DE BIRMINGHAM
EDGBASTON, BIRMINGHAM B15 2T1
TEL. 0121-414-3386/5896
FAX. 0121-414-5925

Para uso exclusivo de la Universidad de Murcia en la tramitación de la tesis doctoral y la mención de Doctor Europeo/Europeus/Internacional.

Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources

Department of Veterinary Sciencies
Czech University of Life Sciences Prague
Kamýcká 129, 165 00 Praha-Suchdol, Czech Republic
Tel.: +420 224 382 937
e-mail: lhotskar@af.czu.cz, www.culs.cz

Dr. José Luis Ros-Santaella
October 30th 2017

To whom it may concern

Departamento de Fisiología
Facultad de Veterinaria. Universidad de Murcia
Murcia 30100, Spain
Tel. +34868888009

I hereby inform:

I have reviewed the Doctoral Thesis presented by **Cristina de las Mercedes Soriano Úbeda,** entitled *"Simulation of the oviductal environment modulates in vitro sperm capacitation and fertility in porcine species"*, carried out by the supervision of Dr. Francisco Alberto García Vázquez and Dr. Carmen Matás Parra of the Department of Physiology at University of Murcia. I consider that the present thesis should be positively evaluated for the next public defense and evaluation, in order to obtain the academic degree of **Doctor by the University of Murcia** with the mention **"International Doctorate"**.

Por la presente informo:

He revisado la Tesis Doctoral presentada por **Cristina de las Mercedes Soriano Úbeda,** titulada *"La simulación del ambiente oviductal modula la capacitación espermática y la fertilidad in vitro en la especie porcina"* y llevada a cabo bajo la dirección de los Dres. Francisco Alberto García Vázquez y Carmen Matás Parra. Considero que la presente tesis debe ser evaluada positivamente para ser defendida en acto público para obtener el grado académico de **Doctor por la Universidad de Murcia** con mención de **"Doctorado Internacional"**.

Yours sincerely,

CZECH UNIVERSITY OF LIFE SCIENCES
FACULTY OF AGROBIOLOGY,
FOOD AND NATURAL RESOURCES
DEPARTMENT OF VETERINARY SCIENCES
Kamýcká 129, 165 21 Prague 6 - Suchdol
Czech Republic, EU

Dr. José Luis Ros-Santaella
Telephone: +420 224 882 932
E-mail: ros-santaella@ftz.czu.cz

To Whom it may concern:
A quien pueda Corresponder:

Departamento de Fisiología
Facultad de Veterinaria. Universidad de Murcia
Murcia 30100. Spain
Tel 34 868888009

Dr. Pablo Andres Ortiz Pineda
Universida del Cauca
Facultad de Ciencias Naturales Exactas
Carrera 2 No. 2N-35
Popayan
Colombia
+57(315)5803902
Pablo.ortiz@yale.edu

29th October 2017

Dear madam:

Re: Cristina de las Mercedes Soriano Úbeda

Inform:

The project of Doctoral Thesis entitled **"Simulation of the oviductal environment modulates in vitro sperm capacitation and fertility in porcine species"** presented by Cristina de las Mercedes Soriano Úbeda under the research supervision of Dr. Francisco Alberto García Vázquez and Dr. Carmen Matás Parra has been carefully reviewed by me and I approve my support for the next public defense and evaluation by a jury to obtain the academic degree of Doctor by the University of Murcia with the mention "International Doctorate".

Informe:

El proyecto de Tesis Doctoral titulado **"La simulación del ambiente oviductal modula la capacitación espermática y la fertilidad *in vitro* en la especie porcina"** presentada por Cristina de las Mercedes Soriano Úbeda bajo la dirección de los Dres. Francisco Alberto García Vázquez y Carmen Matás Parra ha sido revisada cuidadosamente por mi persona y apruebo darle mi apoyo para ser defendido en acto público ante un tribunal para obtener el grado académico de Doctor por la Universidad de Murcia con mención de "Doctorado Internacional".

Yours faithfully.

Pablo A. Ortiz Pineda (PhD)
Biólogo Molecular
Universidad del Valle
T.P. 76327573

Dr. Pablo Andrés Ortiz Pineda. (Ph.D.)
Profesor Cátedra Universidad del Cauca.
Popayán. Colombia.

Este trabajo experimental ha sido financiado por el Ministerio de Economía y Competitividad y cofinanciado por el Fondo Europeo de Desarrollo Regional (Proyectos AGL2012-40180-C03-01 y AGL2015-66341-R) y la Fundación Séneca, la Agencia de Ciencia y Tecnología de la Región de Murcia (Proyecto ref. 20040/GERM/16).

Cristina M. Soriano Úbeda disfrutó de una Ayuda para Contratos Predoctorales para la Formación de Doctores de 2013 (ref. BES-2013-062739) asociada al proyecto AGL2012-40180-C03-01, concedida por la Secretaría de Estado de Investigación, Desarrollo e Innovación del Ministerio de Economía y Competitividad desde el 01/01/2014 al 31/12/17.

Cristina M. Soriano Úbeda recibió una Ayuda a la Movilidad Predoctoral para la Realización de Estancias Breves en Centros de I+D de 2015 (EEBB-I-16-11724) concedida por la Secretaría de Estado de Investigación, Desarrollo e Innovación del Ministerio de Economía y Competitividad del 04/04/2016 al 04/06/2016.

A mi madre

A mi hermana

A María

En memoria de mi abuela Filo

Acknowledgements / Agradecimientos

ACKNOWLEDGEMENTS / AGRADECIMIENTOS

Me he imaginado muchas veces este momento a lo largo del doctorado. Cada vez ha sido de una manera. Con mayor o menor distancia, no dependiendo necesariamente de la cercanía temporal a este momento, sino de cómo me iba sintiendo. Lo que no me podía imaginar era cómo me sentiría el día que tocara. Ese día ha sido hoy, siete años después y llevándome a la espalda una mochila en la que han tenido cabida muchas cosas, tanto personales como profesionales. Imagino que de eso se trataba. Un día tomé una de las decisiones más importantes que he tomado en mi vida, la de estudiar el Máster en Biología y Tecnología de la Reproducción en Mamíferos. A partir de ahí, muchas personas han pasado por el camino hacia esta tesis, o mejor dicho, con esta tesis he recorrido un camino en el que había muchas personas. Lo que tengo muy claro es que todos han sido importantes, y además sé quiénes han sido imprescindibles. Para vosotros, mi "colección de medallas y arañazos".

En primer lugar, agradecer a mis directores la ardua tarea de dirigir una tesis. No ha sido nada fácil. Sé que confiáis en mí y he intentado estar a la altura. Ojalá lo haya estado al menos en una alta proporción de las ocasiones. Espero haberlo conseguido y que podáis abrir ese libro muchas veces y sentiros orgullosos. Este trabajo es vuestro. Me habéis enseñado mucho y os estaré eternamente agradecida. Fran, eres una persona con una capacidad de trabajo excepcional y sin duda mereces ser un Profesor Titular (por el momento) tan joven y con tanta experiencia, que demuestras que no están reñidas. El tiempo pone a cada uno donde merece, eres un claro ejemplo de ello. Muchas gracias por enseñarme la importancia de la exactitud, el método y la precisión. Te elegí como director de mi TFM porque quería aprender ICSI. Bueno, al final me llevo una tesis, que el cambio no está tan mal, ¿no? Me diste la oportunidad de empezar una tesis, y he ahí otra de las grandes decisiones que he tomado. No olvidaré nunca el primer día que me dijiste "valora el esfuerzo/beneficio", tuve claro que esto no iba a ser fácil. Creo que al final lo hemos conseguido y espero que podamos mirar atrás y nos acordemos de todo lo que hemos hecho juntos y enorgullecernos, yo sin duda lo haré. Gracias por todo lo que has hecho por mí. Carmen, eres una persona muy especial, y me consta que no soy la única que piensa así. Me has enseñado otra manera de ver el trabajo, desde tu punto de vista, y eso sin duda me ha enriquecido muchísimo. Eres muy concreta, sin florituras y al grano. Clara y directa. Con mano izquierda cuando quieres. Y muy sabia quieras o no (porque a veces te gusta hacer como que no, pero he logrado identificar cuándo, así que bien). Gracias por tus consejos profesionales y por darme la oportunidad de estar codo con codo contigo. Tienes muchísima experiencia y he tenido la suerte de poder enriquecerme con ella. Gracias también por el papel que haces con nosotros los becarios, nos es muy muy importante (aunque sé que también obtienes *feedback*). Tú y yo hemos sido madre e hija por accidente (científicamente hablando), aunque no te guste que te pregunten si soy tu hija porque soy muy mayor para ello, y te crees que te están diciendo que eres vieja, y yo lo que creo que es que me están diciendo a mí que parezco tener 25. Cada una tira pa lo suyo. Bueno, solo sé que cada vez te sienta

mejor, y eso a mí me halaga. Has sido muy importante para mí y te lo he dicho en muchas ocasiones. Escribiéndolo aquí quedará para siempre y para que lo pueda leer todo el mundo (o al menos aquellos a quienes les interese el bicarbonato y el pH en la capacitación y FIV porcina). A los dos, muchas gracias por haber sido tan importantes para mí, no os olvidaré nunca.

Al departamento de Fisiología (Veterinaria) en su conjunto. A todos los profesores, me faltan palabras. Vuestros alumnos, pero sobre todo los que empiezan el Máster, no tienen ni idea de la puerta que se les abre. Ojalá se dieran cuenta del esfuerzo, el trabajo y las horas que lleva lo que hacéis, y lo mantenéis y mejoráis con el paso de los años. La calidad de lo que ofrecéis, la sensatez, la humanidad, la cabeza y el corazón que ponéis, que no puede ser de otra manera porque esto desde luego no está pagado. Pilar, Rakel, Joaquín, Salva, Sebas, para mí ha sido una suerte y un placer haberme podido formar con vosotros. Sin duda es algo que podré decir con la boca bien grande. No hay nada que no podáis conseguir, juntos. Pilar, gracias por hacerme beneficiaria de la beca de la que he disfrutado, sin duda ha marcado mi rumbo profesional. Rakel, me has enseñado a ser práctica y poner la cabeza donde hay que ponerla. Ha sido un auténtico placer compartir el trabajo contigo en el laboratorio y aprender de ti. Joaquín, siempre dándome un punto de vista diferente, que a veces no veo. Tu experiencia. He aprendido mucho de ti, y no me refiero solo a científicamente. Salva, siempre tu puerta ha estado abierta, siempre dispuesto a ayudarme. Sebas, muchas gracias por tu apoyo, ha sido un placer trabajar contigo este corto tiempo desde que regresaste. En este apartado tienen su lugar también mis directores. Esta tesis también va para vosotros como grupo.

A los investigadores en formación (porque lo de decir “becarios” a día de hoy es saltarse el esfuerzo de mucha gente en conseguir ciertos derechos que hoy tenemos reconocidos) que han pasado por aquí y con los que he tenido la suerte de vivir miles de momentos. A los primeros, algunos ya doctores, Irene, Karen, Luisito, Aitor, Laura, Rebeca. A los que vinieron un poco después, Sole pequeña, Silvia, Analuce, Eve. A los de la última explosión (literal) de la ITN, Florentín, Jordana. Eve y Jordana, para mí sois todo un hallazgo, por lo mucho que nos reímos y lo dispuestas que estáis siempre a ayudarme. Por vuestro sentido del humor. Y no voy a seguir escribiendo sobre más gente sin deciros que sois más malas que arrancás. Bueno, y dentro de este grupo humano, debo agradecer especialmente a los que habéis sido mis hermanos, Jon y Luis Vieira. Qué suerte he tenido por poder apoyarme en vosotros, cuánto me habéis ayudado, cuánto me habéis enseñado, cuánto hemos hablado y cuánto os he observado. Habéis sido la guía de mis pasos. Si volviera atrás, desearía que fuera siempre con vosotros. Millones de gracias.

Al equipo técnico del departamento, Juan y Sole grande. También Eve. Es importantísima la labor que hacéis y sé que a veces puede verse poco recompensada. Solo deciros que este trabajo (al igual que otras muchas tesis) es también vuestro y que sin vosotros habría sido el doble de difícil. Carlitos, siempre estás salvándonos la vida informática. Aunque tú sí que pides recompensa. No he visto a nadie que tenga la capacidad que tú tienes de relacionarlo todo a esa velocidad, principalmente con lo mismo. A los de la cantina, Raúl y Jose con los que tanto me río cada día en los cafés. La importancia de darle humor a la vida. Así es más fácil.

A Manolo Avilés, María José Izquierdo y María Jiménez Movilla, del departamento de Biología Celular de la Facultad de Medicina, por el trabajo compartido y la ayuda que me habéis prestado.

I would like to thank Prof. Hiroaki Funahashi (Okayama University, Japan) and Prof. Stephen Publicover (University of Birmingham, U.K.) for providing me with the opportunity to enter new laboratories abroad. I thank you for opening the doors of your labs to me and for allowing me to become acquainted with the work that is being carried out there. I deeply thank you for letting me see science from a different point of view. It was, not only professionally but also personally, a very enriching experience. In addition, I would like to mention and specially thank my colleagues at the University of Birmingham, Elis Torrezan, Cosmas Achikanu, Sarah Costello and Sanjana Pillay. I learnt a lot from you during the three-month stay, and I cannot but thank you for your valuable help and support. Finally, I should say many thanks to Elis and Sanjana for the beers and laughs. We will hopefully meet someday for another round, whether in Brazil or India.

A mis amigos, que tengo desperdigados por el mundo, pero os llevo siempre dentro. A Maila, María Gil, Elena Gómez, Belén, Inma Úbeda, Cristina Meseguer, Susana, Tere, Merce, Puri Gómez Abellán, Aida, Pili Valdés, Álex Gómez Soler, Olga, Laura, Nati, las Cristinas, Lucía. En este saco tengo que meter también a Jon, Luis Viera y Carmen. Manolo Sansegundo, (tú fuiste el primero que me hablaste del Máster, que lo sepas). Todos habéis sido importantes en mi vida, en uno u otro momento, en muchos momentos y desde siempre. Sois parte de lo que soy, la familia que eliges. La mayoría no os conocéis entre sí, y sois todos completamente diferentes, pero hay un denominador común. Sobre todo, os elijo por vuestro sentido del humor, vuestra inteligencia y empatía. Con vosotros es mucho más fácil vivir. Ojalá algún día pudiera juntaros a todos, porque sería muy feliz.

Otro punto clave ha sido tener la oportunidad de trabajar en el Centros de Nutrición Garaulet de Molina de Segura durante cinco años. Para los primeros tres fue esencial. No hubiera podido llegar aquí sin haber trabajado allí. Tuve la suerte de tener dos jefas excepcionales, primero Elena Morales y después Puri Gómez Abellán. He vivido y aprendido muchas cosas allí, el trato con el paciente, la administración, etc. Además de lo profesional, mucho ha sido lo que nos aportamos personalmente. Siempre estaréis en mi corazón. Puri, además tú sabes lo que es una tesis, con mayúsculas. Tus consejos han sido clave para mí. Has sido otra guía para mis pasos. Eres una persona muy especial con la que he tenido la suerte de compartir miles de horas, y lo mejor, qué bien lo hemos pasado trabajando, que a veces parece difícil que eso suceda, pero contigo es muy fácil.

A la meva altra família, Mari, Pepe, Sandra, Ruth, Javi, Paco, Javier, Marcos, Ruthy, Andrea i les oncles Begoña i Jose. Moltes gràcies per acollir-me en la vostra família com a una més. Tinc la sort de compartir molts moments amb vosaltres, i tot i haver arribat l'última poder sentir-me com a casa. Per molts anys més. Per recolzar-me tant en aquesta tesi i també contribuir amb el vostre granet de sorra. Sempre us porto en el meu cor.

A mi Pancheras, que ha escrito la tesis conmigo, debajo de mi mesa, al lado de mis pies (o literalmente encima) todo el tiempo. No he estado sola ni un minuto.

A mi familia. Mamá, esto no te lo esperabas. Seguro que todavía piensas que debería haber estudiado Medicina. Esta tesis va para ti por todo lo que has pasado para que yo pueda estar aquí hoy. A mi hermana, que has sido también mi madre, y el espejo en el que mirarme, cuánto he aprendido de ti. Esto es también vuestro, no lo podría haber hecho sin vosotras. A mi abuela Filo, que me ha criado, y a la que siempre han dicho que me parezco. También has pasado mucho para que yo haya llegado aquí. Esto va en tu memoria. Sabéis lo importante que siempre ha sido que estemos las cuatro juntas. Hemos pasado muchas cosas y todas las hemos superado. Me habéis apoyado tanto que hoy no soy yo la que se doctora. A mi abuelo Pepe, que tantas cosas me enseñaba. A mis tíos Alfonso y Angustias. A mi cuñado Miguel, que es como si fuera un hermano. Y a Isaac, porque nos has revolucionado a todos, probablemente cuando más lo necesitábamos. Sois todo lo que soy. Sin vosotros sí que hubiera sido imposible.

Por último a María, no voy a hablar contigo de esta tesis, sino del doctorado, que ha sido mucho más que una tesis. Lo has vivido conmigo en primera persona, de principio a fin. Sus pros y sus contras. Sus altos y sus bajos. Lo has sufrido tanto como yo. Gracias por tu esfuerzo, por tu fuerza, por tu inteligencia, por entenderlo. Sobre todo, y algo que admiro, por tu valentía. Por supuesto, valentía al embarcarte conmigo en cosas solo aptas para valientes, pero claro, eso es una manera de ser, que veo todos los días, incluso en los pequeños detalles. Eres mi pareja, mi compañera y mi mejor amiga. Me lo has demostrado siempre y no puedo más que estarte agradecida. Has sido otra de las elecciones a las que me refería arriba. Si tuviera que volver atrás, elegiría una y mil veces que fuera contigo. Este doctorado va para ti.

- Vi una peli tuya.

- ¿A ti también te gusta Míster Q?

- No, vi una en la que interpretabas a un padre que no conocía a su hijo y se encuentran por primera vez cuando el hijo tiene catorce años.

-¡Pero si nadie vio esa película!

- Hay un diálogo que me encantó. Cuando tu hijo dice "¿Por qué no te quedaste?", y tú le dices "No creí que fuera capaz". En ese momento entendí algo muy importante.

- ¿El qué?

- Que nadie en el mundo se siente preparado, así que no hay que preocuparse.

La juventud
Paolo Sorrentino
Italia, 2015

"Ábrelo, ábrelo despacio.
Di qué ves. Dime qué ves, si hay algo.
Un manantial, breve y fugaz, entre las manos.

Toca afinar, definir de un trazo.
Sintonizar, reagrupar pedazos
en mi colección de medallas y de arañazos.

Ya está aquí, ¿quién lo vio
bailar como un lazo en un ventilador?
¿Quién iba a decir
que sin carbón no hay Reyes Magos?

Aún quedan vicios por perfeccionar
en los días raros.
Los destaparemos en la intimidad
con la punta del zapato.

Ya está aquí, ¿quién lo vio
bailar como un lazo en un ventilador?
¿Quién iba a decir
que sin borrón no hay trato?

El futuro se vistió
con el traje nuevo del Emperador.
¿Quién iba a decir
que sin carbón no hay Reyes Magos?

Nos quedan muchos más
regalos por abrir.
Monedas que, al girar,
descubran un perfil
que empieza en celofán
y acaba en eco."

Los días raros
Vetusta Morla
2011

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Summary / Resumen

SUMMARY

Assisted reproduction techniques (ARTs) have always been of great interest for both research and animal production. In this sense, the porcine species is especially important due to its great anatomical, physiological and genetic similarity with the human species. This gives the pig special relevance as a biomedical model for genetic technology and the development of specific proteins and organs. Moreover, the increasing need to produce human food makes porcine industry especially interesting and the development of techniques to reduce production costs. Such economic strategies focus on producing the largest number of healthy animals with the best reproductive characteristics and within the shortest amount of time. For this purpose, all the critical points during the animal reproduction processes must be controlled and the *in vitro* production of embryos is no exception.

Fertilization is a complex biological process in which innumerable factors are directly and/or indirectly involved. Although the efficiency of *in vitro* fertilization (IVF) is, for healthy animals, lower than *in vivo*, in some mammalian species such as bovine or murine a high yield has been achieved. However, in porcine species the IVF is still suboptimal. In the last decades, different studies have been conducted to improve IVF efficiency by using a variety of methods to prepare gametes, bearing in mind the special requirements of this species. While significant progress has been made in both basic science and assisted reproduction, the results obtained to date have not been sufficient. Currently, the polyspermic penetration of oocytes remains the main obstacle in porcine IVF, and the differences between the *in vitro* and *in vivo* environment in which fertilization occurs seem to play a key role. The study of spermatozoa physiology and functionality, from their origin in the testicle to the fertilization site, and also the oocyte and its interaction with the spermatozoa gains special importance for increasing the efficiency of the processes of *in vitro* sperm capacitation (the acquired ability of spermatozoa to fertilize oocytes) and fertilization. The *in vitro* simulation of *in vivo* conditions of sperm capacitation and fertilization is postulated as the best strategy to improve the IVF output.

The concentration of bicarbonate (HCO_3^-), one of the spermatozoa capacitating effectors which acts through activating the soluble adenylyl cyclase (sAC)/adenosine 3':5'-cyclic monophosphate (cAMP)/protein kinase A (PKA) pathway, varies greatly in the different environments that mature spermatozoa go through from the cauda epididymis (~3-4 mM) to the oviduct (from 10 mM in the isthmus to between 25 and 90 mM in the fertilization site, depending on the stage of the estrous cycle). The molecular changes during *in vivo* sperm capacitation begin as soon as are ejaculated as a result of, among other factors, the HCO_3^- intake and subsequent intracellular pH alkalinization (pH_i) and motility activation (fast capacitation events). Once in the female genital tract, other events occur in a sequential and parallel way for an extended period of time, such as hyperactivation, phosphorylation of tyrosine residues (Tyr-P) and preparation to undergo acrosome reaction (AR) once in contact with the oocyte (slow capacitation events). Both fast and slow events depend, among other factors, on the HCO_3^- and Ca^{2+} concentrations in the medium, but the slow ones also depend on the presence of

albumin as membrane cholesterol acceptor. In the oviduct, the low HCO_3^- concentration in the caudal portion of the isthmus allows the spermatozoa to remain in a quiescent state, attached to the oviductal epithelial cells (OECs) and forming the sperm reservoir (SR). This reservoir maintains the viability of the spermatozoa and ensures that a suitably low number of viable and potentially fertile spermatozoa are available to reach the fertilization site. At ovulation, there is an increase in HCO_3^- concentration, which triggers the hyperactivation of spermatozoa that allows their release from the SR. The plasma membrane is destabilized as part of capacitation process. The pH variations associated to these changes of HCO_3^- can substantially influence the development of the pathways and processes related to sperm capacitation and gamete interaction and, thus, may compromise the success of fertilization. In the porcine oviductal lumen, HCO_3^- secretion provokes a great increase in pH in the periovulatory phase from around 6.5 in the isthmus and to around 8.0 in the oviductal ampulla. An increasingly alkaline milieu in the place where both gametes interact is an indispensable condition for fertilization success, which is taken to mean an oocyte being penetrated by only one spermatozoon (monospermy). Once ovulation occurs, the increasing flow of HCO_3^- and pH enable spermatozoa to complete the capacitation process: spermatozoa hyperactivation and release from SR, transport to the fertilization site at the ampullary-isthmic junction (AIJ), the development of the AR to penetrate the egg vestments and oocyte fertilization. The periovulatory oviductal environment in which these phenomena take place is quite different from the *in vitro* characteristics of the medium usually used for sperm capacitation and IVF which contains a standard concentration of HCO_3^- of 25 mM and pH 7.4. It remains to be studied whether adjusting these parameters to reflect more closely *in vivo* conditions may increase the yield in obtaining viable zygotes in porcine species.

In addition to the HCO_3^- concentration and acid-base equilibrium in which sperm capacitation and fertilization occur, there are also other factors in which differences between *in vivo* and *in vitro* milieus may be important. These biological processes *in vivo* take place in specific fluids of the female genital tract, the uterine fluid (UF) and oviductal fluid (OF), but their exact composition is only partially known, which makes it difficult to mimic them *in vitro*. These biofluids allow spermatozoa transport, modulate sperm capacitation and regulate fertilization and early embryo development. Specifically, several substances of the periovulatory OF act as spermatozoa attractants *in vivo* and these substances are derived from the oviductal epithelium secretions, follicular fluid (FF) and cumulus cells-oocytes complexes (COCs) secretions. Among these molecules are progesterone (P4), nitric oxide, adenosine, hyaluronic acid and glycodelin. It has been described that the inclusion of OF in IVF protocols reduces polyspermy by acting on both gametes, through the action of proteins oviductins in the zona pellucida of oocytes, regulating the capacitation rate and preventing premature spermatozoa AR. However, although it would seem logical to include these biofluids in the current protocols of ARTs to improve performance and provide a more *in vivo*-like environment, their use is currently limited to research purposes and not very widespread in animal species. In porcine, the study of these reproductive secretions is of special interest due to possible benefits regarding the problem of high incidence of polyspermy.

In vitro sperm capacitation and IVF are presently produced in static systems. These systems are made up of: chemically defined media, with diverse grades of complexity but of limited composition and physical

characteristics (probably distant from those encountered in the reproductive secretions), and plastic tubes or dishes in which gametes make contact randomly, rather than by selection and/or sperm competition. Several devices for IVF have been developed in recent decades, and all of them allow a certain degree of selection of the spermatozoa population and expression of their heterogeneity in terms of motility, capacitating status and fertility. They locate the physically separated gametes between them and many of them can include biofluids, other molecules and even OEC cultures. However, none has been widely used in reproduction laboratories or eliminated the great problem of polyspermy in pigs.

The persistence of an unacceptably high incidence of polyspermy in porcine species raises the need to implement new strategies in sperm capacitation and IVF. For this purpose, a study of the *in vivo* factors that influence these processes as well as the interaction of gametes with the environment is especially important. This work focuses on the effect on IVF of factors such as HCO_3^- concentration and pH of the medium (extracellular pH, pH_e), the effect of periovulatory oviductal secretions and a device in which gametes are located physically separated and spermatozoa must swim to contact the oocytes.

In chapter 1, the effects of different HCO_3^- concentrations (0, 5, 15 and 25 mM) were studied on the capacitation of mature spermatozoa that have never been in contact with high concentrations of HCO_3^- (cauda epididymal spermatozoa) and different functionality parameters were determined, including *in vitro* fertility. The pH_i, one of the key factors regulating sperm motility and functionality, increased in spermatozoa after HCO_3^- uptake but only extracellular concentrations of 15 mM and above activated the cAMP/PKA capacitation cascade in the spermatozoa. Moreover, the time required for the high phosphorylation of PKA substrates (PKAs-P) was HCO_3^- concentration-dependent, since at 15 mM it took PKAs-P significantly longer than with the usual concentration of 25 mM. 15 mM of HCO_3^- also stimulated sperm linear motility and increased other late events in capacitation such as Tyr-P. To evaluate spermatozoa fertility according to the HCO_3^- concentration of the medium, two different IVF systems were contemplated: i) a one-phase IVF system (monophasic IVF), in which spermatozoa were directly inseminated without any previous treatment and co-cultured with the oocytes in an IVF medium with the different HCO_3^- concentrations, and ii) a two-phases IVF system (biphasic IVF) in which spermatozoa were first pre-incubated for 60 min in a capacitating medium with different HCO_3^- concentrations and then inseminated in an IVF medium with the oocytes that contained the same or higher HCO_3^- concentration than in pre-incubation. The objective pursued with the biphasic IVF was to simulate and study the increase in HCO_3^- concentration that spermatozoa experience during *in vivo* capacitation in the oviduct. The results of monophasic IVF showed that 15 mM led to higher efficiency (26.2%) than the rest of concentrations (0 mM: 0.0%; 5 mM: 13.2%; 25 mM: 8.6%). In the biphasic IVF, both pre-incubation and IVF at 15 mM achieved 33.9% of viable zygotes (oocytes penetrated by only one spermatozoa), which represented in a significant increase of 25.3% with respect to the usual one-phase system at 25 mM of HCO_3^-. Based on these results, we were able to establish an IVF method in two phases that significantly reduced polyspermy.

Chapter 2 discusses our trial to develop an IVF system based on the *in vivo* oviductal conditions, combining a medium with the pH found in the oviduct during the periovulatory stage (pH_e 8.0), a mixture of oviductal periovulatory components formed by COC secretions, follicular fluid and oviductal periovulatory fluid (OFCM) and a device that interposes a physical barrier between gametes (an inverted screw cap of a Falcon® tube, S) in which spermatozoa must to swim towards the encounter with the oocytes. This system was compared with the classical system at pH_e 7.4, lacking oviductal biological secretions and in a 4-well multidish (W) in which spermatozoa are directly and randomly in contact with the oocytes. The results showed that the combined use of the three factors studied (pH_e 8.0, OFCM addition and S device) reduced polyspermy and increased the final efficiency of viable zygotes obtained to 48.7% compared with the classical system at pH_e 7.4, lacking OFCM and a W device (18.9%). The spermatozoa functionality under these tested conditions during IVF was then studied and, in general terms, we determined that both pH_e and OFCM modulate sperm capacitation. The motility parameters and capacitation status through Tyr-P was lower at pH_e 8.0 and with the addition of OFCM. Moreover, PKAs-P and AR were lower in the presence of OFCM, independently of the pH_e. When the pH_i related to the pH_e was analyzed in the presence or absence of OFCM it was determined that pH_i was always lower than pH_e but always correlated. OFCM did not have any effect on pH_i. Relating the results of IVF with those of spermatozoa functionality, we concluded that the higher efficiency of IVF with pH_e 8.0, in the presence of OFCM and using an S device could be a direct consequence of the action of OFCM components on gametes.

The results obtained in this work suggest that the current suboptimal IVF conditions in porcine species can be improved by simulating the *in vivo* periovulatory milieu in the oviduct. Adjusting the HCO_3^- concentration and acid-base equilibrium of the porcine IVF systems, adding periovulatory oviductal fluids and using a device in which gametes are located physically separated but with a means of contact, increased the efficiency of IVF by reducing polyspermy and obtaining higher proportion of potentially viable zygotes. Despite the innumerable known and unknown factors that influence the capacitation and fertilization processes we identify new paths towards improving IVF in porcine and probably a new model for ARTs in all species.

RESUMEN

Las Técnicas de Reproducción Asistida (ARTs) han sido siempre de gran interés para la investigación y la producción animal. En este sentido, la especie porcina es especialmente importante debido a las grandes similitudes anatómicas, fisiológicas y genéticas con la especie humana. Este hecho dota de especial relevancia al cerdo como modelo en biomedicina para la tecnología genética y el desarrollo de proteínas y órganos específicos. Además, la necesidad de producción de alimentos para consumo humano hace especialmente interesante para la industria porcina el desarrollo de técnicas que reduzcan los costes de producción. Estas estrategias económicas se centran en producir el mayor número de animales sanos con las mejores características reproductivas y en el menor tiempo posible. Con este propósito, todos los puntos críticos durante los procesos de reproducción animal deben ser controlados, y la producción *in vitro* de embriones no es una excepción.

La fecundación es un proceso biológico complejo en el que innumerables factores están directa o indirectamente implicados. Aunque la eficiencia de la fecundación *in vitro* (IVF) es, en animales sanos, menor que *in vivo*, en algunas especies de mamíferos como la bovina o la murina se ha alcanzado un alto rendimiento, sin embargo en la especie porcina el rendimiento de la IVF es aún subóptimo. En las últimas décadas, diversos estudios se han dirigido a la mejora de la eficiencia de la IVF aplicando diferentes métodos de preparación de los gametos con el objetivo de satisfacer los requerimientos de esta especie. Mientras se ha alcanzado un significativo progreso tanto en ciencia básico como en ARTs, los resultados obtenidos hasta ahora no han sido suficientes. Actualmente, la penetración polispérmica de los ovocitos permanece como uno de los principales obstáculos en la IVF porcina. Las diferencias entre el ambiente *in vivo* e *in vitro* en el que ocurre la fecundación parecen desempeñar un papel clave. El estudio de la fisiología y funcionalidad del espermatozoide, desde su origen en el testículo hasta el lugar de fecundación, y también del ovocito y su interacción con el espermatozoide cobran especial importancia en el propósito de incrementar la eficiencia en el proceso de capacitación espermática *in vitro* (la adquisición de la capacidad del espermatozoide de fecundar ovocitos) y fecundación. La simulación de las condiciones *in vivo* de capacitación espermática y fecundación se postula como la mejor estrategia para mejorar los resultados de IVF.

La concentración de bicarbonato (HCO_3^-), uno de los efectores de la capacitación espermática a través de la activación de la vía de la adenilato ciclasa soluble (sAC)/AMP cíclico (cAMP)/proteína kinasa A (PKA), varía enormemente en los diferentes ambientes que los espermatozoides maduros atraviesan desde la cola del epidídimo (~3-4 mM) al oviducto (de 10 mM en el istmo hasta entre 25 y 90 mM en el lugar de fecundación, dependiendo de la fase del ciclo estral). Los cambios moleculares de la capacitación espermática comienzan tan pronto los espermatozoides son eyaculados, como resultado de, entre otros factores, la absorción de HCO_3^- y alcalinización del pH intracelular (pH_i) y la activación de la motilidad (eventos rápidos de la capacitación). Una vez en el tracto genital femenino, ocurren otros eventos de un modo secuencial y paralelo durante un periodo de

tiempo extenso, como la hiperactivación, la fosforilación de tirosina (Tyr-P) y la preparación para experimentar la reacción acrosómica (AR) una vez que contacta con el ovocito (eventos lentos de la capacitación). Tanto los eventos rápidos como los lentos dependen, entre otros factores, de las concentraciones de HCO_3^- y Ca^{2+} en el medio, pero los eventos lentos además dependen de la presencia de albúmina como aceptor del colesterol de membrana. En el oviducto, la baja concentración de HCO_3^- en la porción caudal del istmo permite a los espermatozoides permanecer unidos a las células del epitelio oviductal (OECs) en un estado quiescente y formando el reservorio espermático (SR). Este SR mantiene la viabilidad de los espermatozoides y asegura que un número bajo y adecuado de espermatozoides viables y potencialmente fértiles estén disponibles para alcanzar el lugar de fecundación. Durante la ovulación, acontece un incremento de la concentración de HCO_3^- que desencadena la hiperactivación del espermatozoide y permite su liberación desde el SR. La membrana plasmática es desestabilizada como parte del proceso de capacitación. Las variaciones de pH asociadas a estos cambios de HCO_3^- pueden influir sustancialmente en el desarrollo de vías y procesos relacionados con la capacitación espermática y la interacción entre gametos, por tanto, puede comprometer el éxito de la fecundación. En la luz oviductal porcina, la secreción de HCO_3^- provoca un gran incremento del pH en la fase periovulatoria desde aproximadamente 6,5 en el istmo a incluso 8,0 en la ampolla oviductal. Un ambiente cada vez más alcalino en el lugar donde los gametos interactúan es condición indispensable para el éxito de la fecundación, interpretando éxito como la penetración del ovocito solamente por un espermatozoide (monospermia). Una vez ocurre la ovulación, el flujo creciente de HCO_3^- y pH permite al espermatozoide completar el proceso de capacitación, esto es: la hiperactivación espermática y la liberación del SR, el transporte al lugar de fecundación en la unión ampular-ístmica (AIJ), el desarrollo de la AR para atravesar las envolturas del ovocito y la penetración. El ambiente oviductal periovulatorio en el que estos fenómenos tienen lugar es bastante diferente de las características *in vitro* de los medios habitualmente utilizados para la capacitación espermática y IVF, que contienen una concentración de HCO_3^- estándar de 25 mM y un pH de 7,4. Queda por estudiar si el ajuste de estos parámetros para ser más similares a las condiciones *in vivo* puede incrementar el rendimiento en la obtención de zigotos potencialmente viables en la especie porcina.

Además de la concentración de HCO_3^- y el equilibrio ácido-base en el que la capacitación espermática y la fecundación acontecen, hay otros factores en los que las diferencias entre los ambientes *in vivo* e *in vitro* son significativas. Estos procesos biológicos *in vivo* tienen lugar en fluidos específicos del tracto genital de la hembra, los fluidos uterino (UF) y oviductal (OF), pero su composición concreta es aún parcialmente desconocida, lo que dificulta su mimetización *in vitro*. Estos biofluidos permiten el transporte espermático, modulan la capacitación espermática y regulan la fecundación y el desarrollo embrionario temprano. Específicamente, algunas sustancias del OF periovulatorio actúan como atrayentes *in vivo* de los espermatozoides y están derivadas de las secreciones del epitelio oviductal, del fluido folicular (FF) y del complejo ovocito-células del cúmulus (COCs). Entre estas moléculas se encuentran la progesterona (P4), el óxido nítrico, la adenosina, el ácido hialurónico y la glicodelina. Se ha descrito que la inclusión del OF en los protocolos de IVF reduce la polispermia actuando sobre ambos gametos, a través de la acción de proteínas oviductinas en la zona pelúcida de los ovocitos, regulando la

velocidad de capacitación y previniendo la AR prematura de los espermatozoides. Sin embargo, aunque podría parecer lógico incluir estos biofluidos en los actuales protocolos de ARTs para mejorar su rendimiento y proveer un ambiente más similar al existente *in vivo*, su uso es actualmente muy limitado a fines de investigación y no de un modo muy extendido ni en muchas especies animales. En la especie porcina, es de especial interés estudiar estas secreciones reproductivas debido a su acción reductora de la polispermia.

La capacitación *in vitro* y la IVF se producen actualmente en sistemas estáticos. Estos sistemas integran: medios químicamente definidos, con diversos grados de complejidad pero con composición y características físicas limitadas (probablemente distantes de las que se encuentran en las secreciones reproductivas), y en tubos o placas de plástico en las que los gametos contactan aleatoriamente, más que por selección y/o competición espermática. Se han desarrollado varios dispositivos de IVF en las últimas décadas, y todos ellos permiten cierta selección de la población espermática y la expresión de su heterogeneidad en términos de motilidad, estatus de capacitación y fertilidad. En ellas los gametos se localizan físicamente separados entre sí y muchos pueden incluir biofluidos, moléculas o incluso cultivos de OECs. Sin embargo, ninguno de ellos ha sido extendido ampliamente en los laboratorios de reproducción ni han conseguido eliminar el problema de la polispermia.

La persistencia de una incidencia de polispermia inaceptablemente alta en la especie porcina aumenta la necesidad de implementar nuevas estrategias en la capacitación espermática y IVF. Para ello, es de especial importancia el estudio de los factores *in vivo* que influencian estos procesos, así como de la interacción de los gametos con el ambiente en el que se encuentran. Este trabajo se centra en el efecto que sobre el rendimiento de la IVF tienen factores como la concentración de HCO_3^- y el pH del medio (pH extracelular, pH_e), las secreciones oviductales periovulatorias y un dispositivo en el que los gametos se disponen físicamente separados y los espermatozoides han de nadar para contactar con los ovocitos.

En el Capítulo 1, se estudió el efecto de diferentes concentraciones de HCO_3^- (0, 5, 15 and 25 mM) en la capacitación de espermatozoides maduros que nunca han estado en contacto con altas concentraciones de HCO_3^- (espermatozoides de la cola del epidídimo) y se evaluaron diferentes parámetros de funcionalidad espermática, incluida la fertilidad *in vitro*. El pH_i, uno de los factores clave en la regulación de la motilidad y funcionalidad espermática, se incrementó tras la absorción de HCO_3^-, pero solo concentraciones extracelulares de 15 mM o superiores activaron la cascada de capacitación de sAC/cAMP/PKA en el espermatozoide. Además, el tiempo requerido para producir una alta fosforilación de los sustratos de PKA (PKAs-P) fue dependiente de la concentración de HCO_3^-, dado que con 15 mM fue más tardía que con la concentración habitual de 25 mM. 15 mM de HCO_3^- también estimuló una motilidad lineal en los espermatozoides e incrementó otros eventos de la capacitación tardíos como la Tyr-P. Para evaluar la capacidad fecundante de los espermatozoides dependiendo de la concentración de HCO_3^- del medio, se analizaron dos sistemas: i) un sistema de IVF en una fase (IVF monofásica) en la que los espermatozoides se inseminaron directamente sin ningún tratamiento previo y co-cultivados con los ovocitos en un medio de IVF con las diferentes concentraciones de HCO_3^-, y ii) un sistema de

IVF en dos fases (IVF bifásica) en la que los espermatozoides primero se incubaron durante 60 min en un medio capacitante con diferentes concentraciones de HCO_3^- y después inseminados en un medio de IVF con los ovocitos y la misma concentración de HCO_3^- que en la preincubación o superior. El objetivo perseguido con la IVF bifásica fue simular y estudiar el incremento de la concentración de HCO_3^- que experimenta el espermatozoide durante la capacitación *in vivo* en el oviducto. Los resultados de la IVF monofásica mostraron que 15 mM produjo una eficiencia más alta (26,2%) comparada con el resto de grupos (0 mM: 0,0%; 5 mM: 13,2%; 25 mM: 8,6%). En la IVF bifásica, la preincubación y IVF a 15 mM alcanzó un 33,9% de zigotos viables (ovocitos penetrados por solo un espermatozoide), lo que resultó en un incremento significativo del 25,3% con respecto al habitual sistema de IVF en una fase y con 25 mM HCO_3^-. Basándonos en estos resultados, fuimos capaces de establecer un método de IVF en dos fases que reduce significativamente la polispermia.

El Capítulo 2 trata de nuestro intento de desarrollar un sistema de IVF basado en las condiciones oviductales *in vivo*, combinando un medio con el pH del oviducto durante la fase periovulatoria (pH_e 8,0), una mezcla de componentes oviductales periovulatorios formada por secreciones de los COCs, FF y FO periovulatorio (OFCM) y un dispositivo que interpone una barrera física entre los gametos (un tapón de rosca de tubo Falcon® invertido, S) en el que los espermatozoides han de nadar al encuentro con los ovocitos. Se comparó este sistema con el clásico a pH_e 7,4, carente de secreciones biológicas oviductales y en una placa de 4 pocillos (W) en la que los espermatozoides están directa y aleatoriamente en contacto con los ovocitos. Los resultados mostraron que el uso combinado de los tres factores estudiados (pH_e 8,0, adición de OFCM y el dispositivo S) redujo la polispermia e incrementó la eficiencia final de obtención de zigotos viables a un 48,7% comparado con el sistema clásico a pH_e 7,4, carente de OFCM y con el dispositivo W (18,9%). Posteriormente fue analizada la funcionalidad espermática de los espermatozoides expuestos a las condiciones de IVF descritas y, en términos generales, determinamos que tanto pH_e como OFCM modulan la capacitación espermática. Los parámetros de motilidad y el estatus de capacitación a través de Tyr-P fue menor a pH_e 8,0 y con la adición de OFCM. Además, PKAs-P y RA fueron menores en presencia de OFCM, independientemente del pH_e. El pH_i relativo al pH_e fue analizado en presencia o ausencia de OFCM y se determinó que el pH_i fue siempre inferior al pH_e aunque correlacionado. OFCM no mostró tener ningún efecto sobre el pH_i. Relacionando los resultados de IVF con los de funcionalidad espermática, pudimos concluir que la mayor eficiencia en la IVF con pH_e 8,0, presencia de OFCM y el dispositivo S podría ser una consecuencia directa de la acción de los componentes de OFCM sobre los gametos.

Los resultados obtenidos en este trabajo sugieren que las actuales condiciones subóptimas de la IVF en la especie porcina pueden ser mejoradas simulando el ambiente oviductal periovulatorio *in vivo*. Ajustando la concentración de HCO_3^- y el equilibrio ácido-base de los sistemas de IVF porcina, añadiendo fluidos oviductales periovulatorios y utilizando un dispositivo en el que los gametos son ubicados físicamente separados pero con posibilidad de contactar, incrementa la eficiencia de la IVF, reduce la polispermia y se obtiene una alta proporción de zigotos potencialmente viables. A pesar de los innumerables factores, conocidos y no conocidos,

que influyen en los procesos de capacitación y fecundación, con este trabajo se muestran nuevas vías hacia la mejora de la IVF en la especie porcina y, probablemente, un nuevo modelo de ART para todas las especies.

Literature Review

1. INTRODUCTION

Assisted reproduction techniques (ARTs) have been used since the 14th century in animals and perhaps even in humans (Ombelet and Van Robays, 2015; Sherman, 1978). However, it has not been until the last 40 (in humans) to 80 (in animals) years that these techniques have caused a considerable interest (reviewed by Soriano-Úbeda et al., 2013 and Foote, 2002). In 1978, the birth of Louise Brown, the first born baby conceived through *in vitro* fertilization (IVF) (Steptoe and Edwards, 1978), was a turning point in the development of reproductive technologies in humans. The major contribution in this field has been to help determine many of physico-chemical events around reproductive physiology in animals, with a remarkable increase in mammals and profound implications for human beings. Researchers have been looking for ways to expand the knowledge about the genetic influence in reproduction, the hormonal reproductive cycle regulation, superovulation and embryo collection, culture, freezing and transfer. The achievements have allowed the development of various techniques that have been gaining much interest in recent years in mammals, such as IVF.

In the particular case of porcine species, reproductive technologies have been directed toward both swine production and research. The economic interest of this species has produced the development of numerous systems for production of pigs for human food that are continuously undergoing changes to reduce costs and increase production efficiency (Day, 2000). Moreover, although not classically thought of as an obvious model organism, the pig has recently become relevant in research due to the anatomy, genetics and physiology closer to human biology, more than other classic animal models. Several notable advancements have been achieved in the use of this species as a biomedical model in the genetic technology and have motivated the interest in the possible use of swine as donors of specific proteins and even organs for the improvement of human health (Mourad and Gianello, 2017; Watson et al., 2016). At the same time, the scientific interest on swine reproduction has undergone significant progress in basic science and assisted reproduction techniques. Particular emphasis has been made in the development of different protocols for IVF and *in vitro* production of viable embryos in pigs. For these purpose, numerous studies have been carried out related to the sperm capacitation process signalling, the molecules involved in the gametes interaction and fertilization and in early embryo development, both *in vivo* and *in vitro* (Romar et al., 2016). However, while these techniques have achieved a good efficiency in some mammalian species such as bovine or murine, the results obtained in porcine species are so far not comparable to *in vivo* output. The study of spermatozoa physiology and functionality, from their origin in the testicle to the fertilization site, and also of oocyte and its interaction with the spermatozoa gains special importance in the purpose to increase the efficiency of the processes of *in vitro* sperm capacitation (the ability of spermatozoa to fertilize oocytes) and fertilization.

2. THE JOURNEY OF THE SPERMATOZOA FROM ITS ORIGIN TO THE FERTILIZATION SITE

The spermatozoon is a haploid and motile cell that constitutes the male gamete. Its function is the formation of a totipotent zygote, as the result of the fusion of its nucleus with that of the female gamete, the oocyte. Before encountering the oocyte, the spermatozoon must travel from its site production in the testis, to its site of storage in the epididymis, to its site of deposition (vagina, cervix, or uterus depending on the species), and, finally to the site of fertilization, which is in the ampullary-isthmic junction (AIJ) of the oviduct. Up to now, the journey of the sperm to the egg and fertilization *in vivo* is still poorly understood, but what seems clear is that all the processes and physico-chemical events that the spermatozoon undergoes from its origin in the testis to the fertilization site are a coordinated system in which each step is important for the success of the spread of genetics. Below, what is currently understood about some of the most important events in these processes is detailed.

2.1. The spermatozoa in the male genital tract

2.1.1. Spermatozoa in the testicle and epididymis: origin, maturation and storage

The spermatozoa originate in the germinal epithelium of the seminiferous tubules of the testis during spermatogenesis. Spermatogenesis is the process that takes place in the seminiferous tubules, in which the male produces spermatozoa from spermatogonial stem cells by consecutive mitotic and meiotic divisions. In boars, total spermatogenesis, from spermatogonia to sperm, lasts 40 days approximately (Swierstra, 1968). The spermatozoa developed in the process of spermatogenesis are released into the lumen of the seminiferous tubules, which converge in the rete testis. When the immature spermatozoa leave the rete testis by passing through the efferent ducts, they enter a unique tubule, the epididymis, in which the final stages of spermatozoa differentiation occur (Joseph et al., 2009).

During the transit through the epididymis, there are progressive and sequential modifications of maturing male gametes that have been demonstrated to be essential for the acquisition of motility and fertility (Yanagimachi, 1981). It has been described that the spermatozoon lasts between 9.0 and 11.8 days to go through it (França and Cardoso, 1998; Swierstra, 1968). The epididymal tubule in boar has a total length of 60-70 meters and three anatomical regions are grossly discernible: i) caput, the most anterior or cranial, which receives spermatozoa from the testicular efferent ducts, ii) corpus, the intermediate portion, iii) cauda, the most posterior or caudal, which stores spermatozoa before ejaculation (Figure 1).

Epididymal spermatozoal maturation is the result of complex interactions between the spermatozoon and the specific microenvironment in different epididymal regions (Dacheux et al., 2003). The morphological, biochemical and physiological changes as they move along the different regions of the epididymis basically are: i) changes in motility pattern, ii) modifications in the metabolism and the distribution of tail organelles, iii) chromatin

restructuring, iv) acrosome shape remodeling, v) water reabsorption and an increased concentration of cells, vi) cytoplasmic droplet migration and detachment, and vii) modifications in the spermatozoal plasma membrane. At the same time that spermatozoa move forward in the epididymis, the epididymal epithelia and the composition of the epididymal fluid that surrounds them also change sequentially. A specific composition in proteins (Syntin et al., 1996) and other organic molecules and ions (Setchell et al., 1993; Rodriguez-Martinez et al., 1990; Cooper, 1986) in each epididymal region allows the maturation of spermatozoa. A high proportion of mature spermatozoa are stored in the cauda in a state of quiescence until ejaculation (Briz et al., 1995).

The epididymal influences on spermatozoal maturation and storage have two clear objectives: i) promoting the ability of spermatozoa to respond appropriately to conditions within the female genital tract so they can fertilize the oocyte, and ii) preventing this response within the male tract itself (Cooper, 1986). The successive loss of protein and adhesion to spermatozoa and the interaction with the other epididymal fluid constituents produce a plasma membrane remodeling that modulates the nature of the spermatozoal surface and, once at the fertilization site, allow the recognition of zona pellucida (ZP)-specific components and oocyte fertilization. Once fertilizing ability is acquired in the epididymis, the inhibitory effect of this organ on spermatozoa acts to permit spermatozoal latency and prevents premature capacitation, both within the epididymis and once ejaculated. The epididymal epithelium secretes some factors (mainly sterol sulphates and proteins) that decrease the possibilities of premature sperm activation and acrosome reaction (AR) and maintain spermatozoal fertility but in a quiescent state. This state of inactivity also is associated with low extracellular pH resulting from a low HCO_3^- concentration. The concentration of HCO_3^- in efferent ducts is approximately 30 mM and decreases substantially along the epididymis to the cauda, where the spermatozoa encounter a concentration of ~3-4 mM (Rodriguez-Martinez et al., 1990; Okamura et al., 1985). More than 95% of the testicular HCO_3^- is reabsorbed in the efferent duct and consequently a pH gradient of acidification is produced from efferent ducts (pH 7.2) to cauda epididymis (pH 6.2) (Rodriguez-Martinez et al., 1990). The extracellular fall of HCO_3^-/pH levels inevitably produces a low intracellular concentration of HCO_3^- and an acidification of intracellular pH (Newcombe et al., 2000). Other factors such as high fluid viscosity, osmolarity and cell concentration, the decrease of Na^+, the increase of K^+ and the low O_2 tension in cauda epididymis contribute to the state of mechanical immobilization (Rodriguez-Martinez et al., 1990). Cauda epididymal spermatozoa undergo activation during ejaculation in which they are exposed to some factors that promote sperm activation.

2.1.2. Spermatozoa in the ejaculate

During ejaculation in the boar, spermatozoa emitted together with cauda epididymal fluid are poured into the urethra and sequentially exposed and resuspended in different mixtures of accessory sex gland secretions. The fluid portion of this suspension, the seminal plasma (SP), nourishes, transports and protects spermatozoa during their journey through the female genital tract. The SP regulates spermatozoal motility and acquisition of the ability of spermatozoa to fertilize oocytes (sperm capacitation), modulates the recognition and interaction

between gametes, maintains spermatozoa fertility, buffers the extracellular media, maintains the osmolarity and is a source of energy for spermatozoa metabolism (Mann and Lutwak-Mann, 1981). The molecular changes that SP provokes in the spermatozoa enhance their lifespan and fertilizing capacity but cannot be totally responsible for it. To carry out all of the aforementioned functions, the specific composition of SP in each animal species is important. In porcine, SP is composed principally of water, proteins (fibronectin type II, cysteine-rich secretory proteins, spermadhesins, immunoglobulins), enzymes (proteases, nucleases, phosphatases), hormones (androgens, estrogens, follicle stimulating and luteinizing hormones, chorionic gonadotrophin-like molecules, growth hormone, insulin, glucagon, prolactin, relaxin), ions (Zn^{2+}, Ca^{2+}, Na^{+}, K^{+}, Mg^{2+} and Cl^{-}), sugars (fructose), organic compounds (salts, citric acid, inositol, sorbitol ascorbic acid, ergothioneine) and prostaglandins (Strzezek, 2002; Garner and Hafez, 2000; Zini et al., 1993; Harrison, 1974).

As mentioned, mammalian spermatozoa encounter an acidic media (pH ~6.2) with a low HCO_3^- concentration in the epididymis that favors a spermatozoal state of inactivity. Once spermatozoa are ejaculated and are in contact with the SP, they display one type of physiological motility called 'activated' motility. That is, they become motile and undergo an increase in metabolic activity due to the release from the suppressive conditions in the epididymis. As metabolic activity acidifies the cytosol, spermatozoa develop an intracellular pH (pH_i) regulation system that balance the production, elimination, transport and buffering of H^+. During the ejaculation, spermatozoa are suddenly immersed in a HCO_3^--rich fluid, particularly in the sperm-richest fraction (Rodriguez-Martinez et al., 1990), which achieves an average concentration in the ejaculate of 20 mM (Okamura et al., 1985). As a consequence of the HCO_3^- increase in the SP to which the spermatozoa are suddenly exposed, the pH of the media surrounding the spermatozoa also increases to between 7.3 and 7.8 (Garner and Hafez, 2000).

The main ion transporters for pH_i regulation can be arranged into two groups (Figure 2): membrane H^+ transporters and HCO_3^- transporters. The first group involves the membrane Na^+/H^+ exchangers (sNHE) (Orlowski and Grinstein, 2011) and H^+ channels (H_v) (Lishko et al., 2010). The second group consists of membrane proteins that carry HCO_3^- across the plasma membrane, such as solute carriers 4 and 26 families (SLC4 and SLC26) and the cystic fibrosis transmembrane conductance regulator (CFTR) (Liu et al., 2012) that acts as an ion channel. Moreover, the CO_2/HCO_3^- is equilibrated by membrane associated carbonic anhydrases (CAs) that catalyzes the reversible conversion of cytosolic CO_2 to HCO_3^- and H^+ release ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$) (reviewed by Nishigaki et al., 2014). Upon ejaculation, HCO_3^- is the unique activator that makes the quiescent spermatozoa motile (Tajima et al., 1987; Okamura et al., 1985). The SP activates certain channels in the spermatozoal plasma membrane during ejaculation that causes them to pass from immobile and inactive in the epididymis to mobile and active in SP. However, although the SP activates sperm motility, it does not activate other pathways related to sperm capacitation. Sperm capacitation involves a series of physico-chemical changes occurring in the spermatozoon that give it the ability to fertilize the oocyte (Chang, 1951). These capacitation pathways are activated *in vivo* by specific molecules and factors along the female genital tract, and SP not only

lacks certain of these molecules, but has specific 'decapacitating' factors that prevent the premature capacitation of spermatozoa in the ejaculate.

Chang (1957) described in rabbit that capacitated spermatozoa could return to the uncapacitated state, in a process known as 'decapacitation', by bathing them in SP for a short period of time. The equilibrium between capacitating and decapacitating factors in SP components allows the activation of spermatozoa (motility and metabolism) and at the same time impedes premature capacitation or AR. The components of SP that are considered decapacitating factors are mainly proteins of both epididymal and sex accessory gland origin including glycoproteins (acrosome-stabilizing factor), spermadhesins, ions like Zn^{2+} and other molecules like cholesterol, and it is generally accepted that coating of spermatozoa by these factors stabilizes the plasma membrane, masks antigens exposed to the cell surface and prevents premature AR. Although SP contains important elements for sperm capacitation such as Ca^{2+} and HCO_3^-, the existence of these decapacitating factors can inhibit or even reverse capacitation and impede the binding of the spermatozoa to the ZP of the oocyte (De Lamirande et al., 1997; Fraser, 1990; Sidhu and Guraya, 1989; Okamura et al., 1985; Dukelow et al., 1967).

Molecules contained in the SP in several mammalian species including in boar (Piehl et al., 2013) can stimulate or inhibit the onset of the capacitation process. This is the case of SP vesicles termed 'exosomes' that are rich in cholesterol, proteins like actin, chloride channels and spermadhesins and with a complex composition of lipids such as sphingomyelin and thought to be of cellular origin and are from testis, epididymis, vas deferens, vesicular glands and prostate. Exosomes mediate decapacitation by inhibiting the cholesterol efflux, increasing spermatozoa apical membrane fluidity, delivering Ca^{2+} signalling molecules and controlling the AR (Aalberts et al., 2014). They have been suggested to play an important role once spermatozoa are in the female genital tract by maintaining the spermatozoal function and integrity and reducing their ability to bind to the ZP during gamete interaction (Piehl et al., 2013).

For the sperm capacitation process to occur normally, it is necessary to have SP removal and plasma membrane decoating of the decapacitating factors that are inhibiting or blocking the process. These SP factors will be lost *in vivo* along the spermatozoal journey through the female genital tract.

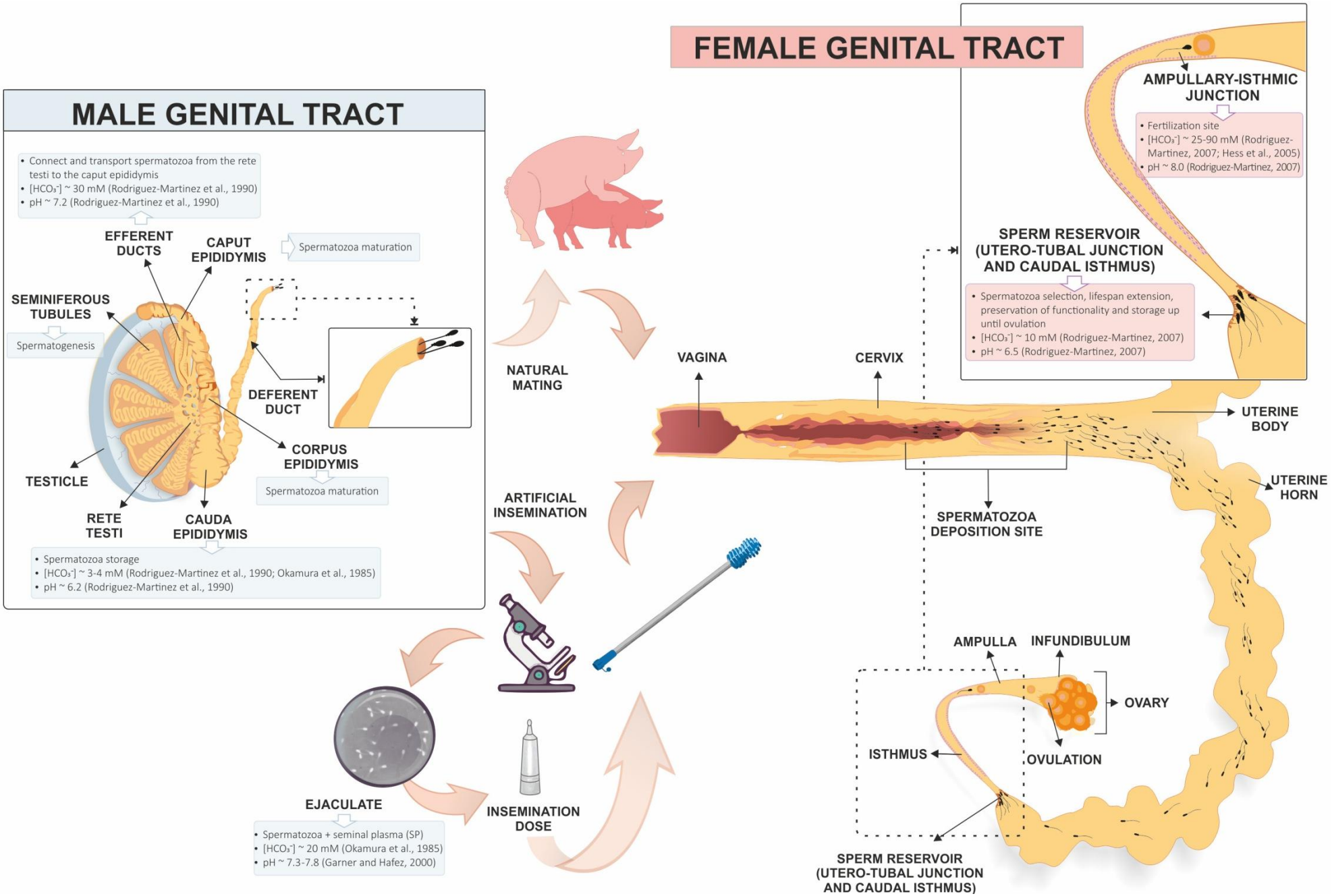

MALE GENITAL TRACT
• Connect and transport spermatozoa from the rete testi to the caput epididymis
• [HCO3-] ~ 30 mM (Rodriguez-Martinez et al., 1990)
• pH ~ 7.2 (Rodriguez-Martinez et al., 1990)
EFFERENT DUCTS
CAPUT EPIDIDYMIS
Spermatozoa maturation
SEMINIFEROUS TUBULES
Spermatogenesis
DEFERENT DUCT
CORPUS EPIDIDYMIS
Spermatozoa maturation
TESTICLE
RETE TESTI
CAUDA EPIDIDYMIS
• Spermatozoa storage
• [HCO3-] ~ 3-4 mM (Rodriguez-Martinez et al., 1990; Okamura et al., 1985)
• pH ~ 6.2 (Rodriguez-Martinez et al., 1990)
NATURAL MATING
ARTIFICIAL INSEMINATION
EJACULATE
• Spermatozoa + seminal plasma (SP)
• [HCO3-] ~ 20 mM (Okamura et al., 1985)
• pH ~ 7.3-7.8 (Garner and Hafez, 2000)
INSEMINATION DOSE
FEMALE GENITAL TRACT
AMPULLARY-ISTHMIC JUNCTION
• Fertilization site
• [HCO3-] ~ 25-90 mM (Rodriguez-Martinez, 2007; Hess et al., 2005)
• pH ~ 8.0 (Rodriguez-Martinez, 2007)
SPERM RESERVOIR (UTERO-TUBAL JUNCTION AND CAUDAL ISTHMUS)
• Spermatozoa selection, lifespan extension, preservation of functionality and storage up until ovulation
• [HCO3-] ~ 10 mM (Rodriguez-Martinez, 2007)
• pH ~ 6.5 (Rodriguez-Martinez, 2007)
VAGINA
CERVIX
UTERINE BODY
UTERINE HORN
SPERMATOZOA DEPOSITION SITE
AMPULLA
INFUNDIBULUM
OVARY
ISTHMUS
OVULATION
SPERM RESERVOIR (UTERO-TUBAL JUNCTION AND CAUDAL ISTHMUS)

Figure 1. The journey of the spermatozoa from its origin to the fertilization site. Functions, HCO_3^- concentration and pH in each section of the male and female genital tract. The spermatozoon must travel first from its site production in the seminiferous tubules of the testis, to the maturation site along the epididymis (caput and corpus) and to its site of storage in the epididymis, where they encounter an acidic environment with ~3-4 mM of HCO_3^- and pH ~6.2. During ejaculation, spermatozoa are emitted together with the seminal plasma (SP) formed by the cauda epididymal fluid and accessory sex glands secretions. SP contains ~20 mM of HCO_3^- and pH 7.3-7.8. During natural mating, the ejaculate is deposited into the anterior cervix. From ejaculate, insemination doses can also be obtained for artificial insemination that can be deposited in anterior cervix or uterine body using the appropriate insemination catheter. Spermatozoa move through the uterus by passive transport favored by myometrial contractions and also active transport by sperm swimming. Billions of spermatozoa are deposited into the female reproductive tract but only a limited number reach de utero-tubal junction (UTJ) and the large majority of those do not even pass through it. In the UTJ and caudal isthmus, spermatozoa attach the oviductal epithelial cells (OECs) forming the sperm reservoir (SR). The SR selects the spermatozoa, extends their lifespan, preserves their functionality and storages them up until ovulation. The HCO_3^- concentration in SR is low (~10 mM) and pH slightly acid (pH 6.5) to allow spermatozoa quiescence. Once ovulation occurs, an increase of HCO_3^- concentration (~25-90 mM) and pH (~8.0) takes place that triggers the hyperactivation of spermatozoa that allows its release from the SR. The spermatozoa enter into the oviductal lumen in which they complete the capacitation process: the transport to the fertilization site in ampullary-isthmic junction (AIJ), the preparation to undergo the acrosome reaction (AR) to go through the egg vestments and the contact with the oocytes and penetration.

2.2. The spermatozoa in the female genital tract: cervix and uterine horns

Spermatozoal transport from the place where semen is deposited to the fertilization site is a complex process in which spermatozoa are progressively exposed to a dynamic and hostile environment and a strict selection process. It involves numerous interactions between semen and the female genital tract that ensures that a sufficient number of fertilization-competent spermatozoa colonize the oviduct. Although the exact mechanism by which spermatozoa are transported through the uterus is still not completely known it seem to be influenced by the mating behavior, semen deposition site, spermatozoal interactions with the uterine environment, presence of SP and characteristics of the uterus (musculature, secretions, epithelial cells, immunological reaction).

2.2.1. Semen deposition and spermatozoal transport

During natural mating or AI in porcine, billions of spermatozoa are deposited in the uterus but only a few thousand reach the oviducts (Sumransap et al., 2007; First et al., 1968) (Figure 1). Therefore, some phenomena take place in the uterus to radically reduce the number of spermatozoa moving forward to the oviduct (García-Vázquez et al., 2015; Hernández-Caravaca et al., 2015; Taylor et al., 2008; Matthijs et al., 2003; Steverink et al., 1998): i) the backflow of a high proportion of spermatozoa after semen deposition (up to 24% within 1 h and 50% in 4 h), and ii) phagocytosis of the spermatozoa by polymorphonuclear neutrophils (PMN) that migrate from blood to the uterine lumen. The fact that these processes happen in the uterus suggests that spermatozoa are subjected to strict selection during their transport to the oviduct. Nevertheless and under normal circumstances, spermatozoa are able to reach the oviduct within 1 h after semen deposition (Hunter, 1981).

The displacement of spermatozoa through the female genital tract to the site of fertilization seems to be a combination of passive and active transport. In the 'passive transport', the action of the uterus is essential to spermatozoa moving forward. The vigorous contractile activity of the myometrium directed forward and backward favors, on one hand, the advance of spermatozoa through the uterine horns towards the oviduct, and on the other hand, contractile activity eliminating part of the spermatozoa in the backflow (García-Vázquez et al., 2015; Langendijk et al., 2005). Progesterone and estrogens control the activity of the myometrium, which reaches its maximum during estrus by the action of estrogens, oxytocin and prostaglandins (Langendijk et al., 2005). Spermatozoal transport through the uterus is also influenced by the specific composition of SP (Robertson, 2005), since some of the component exert a protective effect on the spermatozoa and decrease the inflammatory reaction of the uterus (Katila, 2012), and other SP components such as estrogens, proteins and prostaglandins cause strong myometrial contractions that favor the passive transport of spermatozoa (Kaczmarek et al., 2010; Robertson, 2007; Claus et al., 1990; Claus et al., 1989). The importance of SP to sperm survival and transport through the uterus has been described in other mammals in addition to the pig, such as rodents. Crane and Martin (1991) studied the stimulatory activity of seminal components in females rats in which the incidence of

strong uterine contractions declined when they were mated with vasectomized males, and Kawano et al. (2014) described in mice the importance of the seminal vesicle protein SVS2 to sperm survival in the uterus.

The rate of spermatozoal transport along the uterus is much faster if spermatozoa swim efficiently in the uterine fluid (UF), which is commonly called 'active transport'. This kind of transport is especially important to maintain the spermatozoa in suspension in the UF. Rapid transport of spermatozoa through the uterus can enhance spermatozoal survival by helping them to evade the local immune reaction. Spermatozoa swimming in the UF reduces adhesion to the endometrium and favors that spermatozoa reach the oviduct (Langendijk et al., 2005). Gaddum-Rosse (1981) observed that immotile spermatozoa were unable to reach the last portion of the uterine horns, the utero-tubal junction (UTJ). The UTJ is, after the cervix, the second great anatomical barrier of the uterus for the spermatozoa. The UTJ is extremely narrow and tortuous and only a few thousand manage to pass through it and enter the oviduct.

2.2.2. Spermatozoa interaction with uterine environment

Changes in the immune reaction in the uterus throughout the estral cycle have been extensively studied for years. The epithelial uterine cells act to create an immunological barrier for spermatozoa and biological agents like viruses and bacteria. In the estrus stage of the cycle there is a massive migration of leukocytes (mainly PMN) to the sub-epithelial stroma (reviewed by Taylor et al., 2009). The presence of semen constituents, whether during coitus or AI, enhances the immunological reaction, trying to eliminate the foreign material including any bacteria (Schuberth et al., 2008), and produces an ideal environment for embryo implantation (Rozeboom et al., 1998). Epithelial cells, macrophages, natural killer cells and neutrophils produce chemokines, cytokines and bactericidal and virucidal agents that recruit and activate immune cells and protect the uterus from any foreign agents, including spermatozoa. It remains unknown if spermatozoal phagocytosis by PMN is mediated by a random attachment or involves spermatozoa-specific recognition molecules. It has been postulated that PMN collaborate in spermatozoal selection in the uterus due to their phagocytic action on non-motile or damaged spermatozoa (Matthijs et al., 2003; Tomlinson et al., 1992). One of the mechanisms to avoid the selective action of backflow and neutrophil attack is spermatozoal adhesion to the uterine epithelial cell (UEC) apical membrane and communication with the UECs (Taylor et al., 2008), mainly by spermadhesin-, lectin- or lectin-like protein-mediated interactions (Bergmann et al., 2012; Töpfer-Petersen et al., 1998). Only viable, membrane-intact and motile spermatozoa attach to the UECs (Rath et al., 2008) and it is probable that spermatozoal selection also depends on a specific morphology (reviewed by García-Vázquez et al., 2016).

Along the uterus, spermatozoa are progressively separated from several components of SP and in contact with the UF as they approach the oviduct (Garner and Hafez, 2000). Transport of spermatozoa into the uterus influences capacitation because spermatozoa are separated from the 'decapacitating factors' and other inhibitors and protectors present in the SP such as spermadhesins (Garner and Hafez, 2000; Dostàlovà et al.,

1995). Spermatozoa that prematurely capacitate in the uterus are eliminated by phagocytosis or backflow. However, SP presence in the uterus is beneficial because it may regulate the intensity of the immune response for elimination of both damaged spermatozoa or pathogens that may damage the early embryo (reviewed by Rath et al., 2016). The importance of the presence of SP in the uterus and its influence on the offspring have even been suggested, since altered offspring phenotype was partly attributable to SP deficiency on the female genital tract (Bromfield et al., 2014).

During transport through the uterus, spermatozoa are significantly diluted in luminal secretions and are susceptible to the changes of characteristics or composition of UF. The volume and biochemical composition of the UF varies during the estrus cycle and mainly consists of blood serum proteins and a small amount of uterine-specific proteins. Moreover, the female reproductive secretions have a high concentration of HCO_3^- which conditions the pH of the UF. The acidity or excessive alkalinity immobilizes spermatozoa but moderately alkaline fluid enhances their motility (Garner and Hafez, 2000). The pH is highly variable in the different sections of the uterus and stages of the estral cycle. In general terms, at the time of ovulation cervical mucus is most alkaline (pH ~8.4, Hafez and Hafez, 2000), in which only some of the spermatozoa survive, which contributes to the selection of spermatozoa that are resistant to pH changes. Among other things, around the time of ovulation cervical mucus is most abundant, of lowest viscosity and most conducive to sperm survival. In contrast, the more acidic cervical mucus is encountered until a day or so before ovulation, which is completely unfavorable for spermatozoa. The pH of UF in the uterine body and horns is moderately alkaline (pH ~7.8, Hafez and Hafez, 2000), which contributes to spermatozoal survival post-ovulation during their transport to the UTJ.

After the passage throughout all selective barriers aforementioned, the number of spermatozoa that reach the lower oviduct has been considerably reduced. The oviduct, with the formation of the sperm reservoir (SR) ensures that a suitably low number of viable and potentially fertile spermatozoa are available to reach the fertilization site. Finally, in relation to ovulation, spermatozoa will be slowly released from the SR towards the site of fertilization.

2.3. The spermatozoa in the female genital tract: the oviduct

Once spermatozoa have gone through the hostile environment of the uterus, where they are subjected to a strict selection, those spermatozoa that have succeeded in surviving all the barriers interposed reach the oviduct, the last part in their journey through the female genital tract. The mammalian oviduct provides a suitable environment for spermatozoal transport, storage and capacitation, oocyte pick-up, transport, maturation and fertilization, and early embryo development (Hunter and Rodriguez-Martinez, 2004). The oviducts are tubular conduits connecting the ovaries with the uterus horn, is approximately 20 cm length and is anatomically divided into three parts (from cranial to caudal): i) infundibulum, which collects the oocytes after ovulation, ii) ampulla, where gamete interaction and fertilization take place, iii) isthmus, the caudal region of the oviduct that connects

with the uterine horn through the UTJ, forms the SR, provides a safe environment for spermatozoa, collaborates in the regulation of sperm capacitation and allows the development of the early embryo.

In some species of mammals including pigs and cows, oocytes are rapidly fertilized after ovulation; however, spermatozoa do not always colonize the oviduct at the same time as ovulation occurs. Spermatozoa need to survive and keep their fertility potential while waiting for ovulation within a certain time window. For this purpose, in the caudal isthmus is formed a storage site for spermatozoa, the SR, which has been described to be functional in pigs up to 30 h from onset of estrus (Rodríguez-Martínez et al., 2005). Spermatozoa remain attached to the oviductal (isthmus) epithelial cells (OECs) in the SR and remain in a state of quiescence. After ovulation, spermatozoa are progressively released from OECs, their motility pattern changes (termed hyperactivation) and their plasma membrane is destabilized as part of the sperm capacitation process. In porcine, unlike other mammalian species where fertilization takes place in the ampulla, spermatozoa reach the ampullary-isthmic junction (AIJ) where they interact with the ZP of the oocytes and undergo the AR to fertilize them. Finally, spermatozoa penetrate the zona pellucida and fuse with the oolemma (Yanagimachi, 1994a). During all these events in the oviduct, the specific characteristics and composition of the oviductal fluid (OF) in the periovulatory stage are essential to ensure proper sperm capacitation and gamete interaction.

2.3.1. Spermatozoa-oviduct adhesion and release from the 'Sperm Reservoir'

Only a small part of the inseminated spermatozoa achieves the caudal part of the oviduct, and transiently adhere to the ciliated OECs forming the SR (Figure 1). This allows the selection of spermatozoa with certain qualities, extension of their lifespan and maintaining of spermatozoa functionality. The SR formation has been described as a crucial phase for fertilizing potential before gametes interact, which ensures that a suitable number of viable and potentially fertile spermatozoa are available (Hunter, 1984). The more probable significance of the SR establishment is to sequentially release the attached spermatozoa after ovulation to allow only a small quantity of them to reach the oocyte at any given time and therefore reduce the possibility of polyspermy (Hunter, 1973).

The microenvironment of the SR favors the adhesion of competent spermatozoa that seems to be mediated by molecules exposed on the spermatozoal surface, such as lectin-like proteins of the rostral region of the spermatozoal head. Spermadhesin proteins AQN1 and AWN of the spermatozoal apical head recognize several carbohydrates, mainly mannose and galactose structures, expressed on the surface of OECs in a species-specific manner (Ekhlasi-Hundrieser et al., 2005; Wagner et al., 2002; Green et al., 2001; Gualtieri and Talevi, 2000; Dostàlovà et al., 1995). The OECs have the ability to select viable and uncapacitated spermatozoa and protect them during a certain time window from being damaged and capacitated until the ovulated egg arrives (Töpfer-Petersen et al., 2002). However, not all spermatozoa are able to bind and take part in the SR.

It has been reported in *in vitro* studies that the selective action of OECs only binds mature (Petrunkina et al., 2001a), viable (Yeste et al., 2009; Fazeli et al., 1999), morphologically normal (Yeste et al., 2012; Waberski et al., 2006; Fazeli et al., 1999), uncapacitated (López-Úbeda et al., 2017; Yeste et al., 2009; Petrunkina et al., 2001b; Fazeli et al., 1999) and fertile spermatozoa (López-Úbeda et al., 2017; Holt and Fazeli, 2010), with non-fragmented chromatin (Ardón et al., 2008), good osmoregulatory abilities (Petrunkina et al., 2007) and that express certain surface proteins (Talevi and Gualtieri, 2010). All these strict requisites enable the selection of a higher quality spermatozoa population with a greater fertilizing potential, and is the basis of the spermatozoal competition that has been speculated by several authors in porcine and other mammalian species (López-Úbeda et al., 2017; Gualtieri and Talevi, 2003).

In the SR, spermatozoa are quiescent and capacitation is delayed until specific and partially unknown signals around the time of ovulation that induce the spermatozoal release. Little is known about the mechanism underlying the communication between the spermatozoa-oviduct-ovulated oocyte but the SR establishment seems to be a prerequisite for functional mammalian spermatozoa (Suarez, 1999). Spermatozoal release could be a combination of periovulatory signals secreted by the oviduct itself or stimulated by cumulus-oocyte complexes (COCs), follicular fluid (FF) and the presence of spermatozoa in the oviduct (reviewed by Brüssow et al., 2008; Hunter, 2008; Georgiou et al., 2007), in combination with the progesterone levels in the periovulatory stage (Bureau et al., 2002). Many of these signals or molecules are currently under study and may enhance the ability of spermatozoa to be released from the SR.

Some of the components of the extracellular matrix of the cumulus related to sperm release from the SR are the glycosaminoglycans (GAGs), especially non-sulfated hyaluronan (HA). HA is produced by COCs during cumulus expansion and seems to participate in communication between spermatozoa, OECs and oocyte. These GAGs in general and HA in particular seem to be involved in spermatozoal survival, binding to and release from the SR and capacitation (Brüssow et al., 2008). Moreover, other oviductal factors such as Ca^{2+} concentration in OF are also important to produce the state of hyperactivation in spermatozoa that allows the bound spermatozoa to be released from OECs (Petrunkina et al., 2001b; Flesch and Gadella, 2000; Gadella and Harrison, 2000). The power of the increased bend amplitude of the sperm tail during hyperactivation can overcome spermatozoal-epithelial binding, and it has been demonstrated that only hyperactivated spermatozoa are able to detach from the OEC (Suarez et al., 1992). Interestingly, it has been proposed that proteins involved in spermatozoal binding to OECs are lost during capacitation (Ekhlasi-Hundrieser et al., 2005).

In pigs, spermatozoa begin to be released progressively toward the AIJ where the oocyte is located after ovulation. By initiation of the capacitation process and spermatozoal hyperactivation, associated molecules are shed from the spermatozoal surface and spermatozoa are detached from the OECs and swim freely towards the ampulla (Suarez, 1998).

2.3.2. Function of the oviduct in the sperm capacitation process

Sperm capacitation is a complex and lengthy physiological process that involves a combination of sequential and parallel molecular changes that affect both the spermatozoal head and tail (Suarez, 2007). It takes place during the transport of spermatozoa in the female genital and is critical to the ability of the spermatozoa to fertilize the oocyte. It has been described that uncapacitated spermatozoa are unable to recognize and fuse with the oocyte, but in the immediate vicinity of the oocyte the plasma membrane remodeling allows the capacitated spermatozoa to recognize and bind to the oocyte. When the spermatozoon interacts with the oocyte ZP, the rostral spermatozoal plasma and the outer acrosomal membranes become more unstable, gradually fusing and undergoing the AR. The spermatozoa then become fusogenic and are able to fertilize the oocyte.

The phenomenon of sperm capacitation was first described in the early 1950s when Austin and Chang, independently, described the acquisition of fertilization ability by spermatozoa during a period of time in the female uterine tract (Austin, 1951; Chang, 1951). Since sperm capacitation is absolutely necessary for the success of fertilization, it seems especially logical to deeply study the cellular and molecular events involved in the process. This critical process includes many changes in sperm, such as alterations in plasma membrane fluidity, protein tyrosine phosphorylation (Tyr-P), and acquisition of a hyperactive motility and the ability to undergo the AR. Some of these changes take place as the spermatozoa are ejaculated but others require a longer period of time and develop along the female genital tract, specifically in the oviduct. What seems clear is that all these processes (both fast and slow) are triggered by HCO_3^- and regulated by protein kinase A (PKA) in the soluble adenylyl cyclase (sAC)/adenosine 3':5'-cyclic monophosphate (cAMP) pathway (Visconti, 2009; Visconti et al., 1995a) (Figure 2).

One of the first events of capacitation is the activation of spermatozoal motility by the action of HCO_3^- that stimulates the flagellum (García Herreros et al., 2005), which involves HCO_3^- uptake from the extracellular medium, producing a slight alkalinization of the spermatozoal cytosol. The later events of capacitation take place in the oviduct. This involves first a remodeling of plasma membrane architecture and cholesterol-efflux due to the action of albumin and high-density lipoproteins present in the OF, which increases membrane fluidity. A reorganization of proteins and lipid scrambling take place (Petrunkina et al., 2001b; Gadella and Harrison, 2002; Harrison et al., 1996) and there is a loss of AQN1 from the spermatozoa surface (Ekhlasi-Hundrieser et al., 2005). The consequence of this increase in membrane fluidity is membrane depolarization, which causes an increase in spermatozoal Ca^{2+} permeability (Wennemuth et al., 2003). The spermatozoal motility pattern also changes drastically (hyperactivation) and the spermatozoon acquires the ability to fertilize the oocyte (Visconti, 2009). Glucose from the extracellular medium provides energy via the glycolytic pathway of spermatozoa and ATP production, which is essential for spermatozoa activation and capacitation (Vadnais et al., 2007).

Other molecules also are thought to be related with sperm capacitation in the oviduct. Endogenous nitric oxide (NO) regulates the ciliary beating of the ciliated OECs and induces sperm chemotaxis and also has a significant role in sperm functionality. NO affects sperm motility by mobilizing the stored Ca^{2+} in the sperm

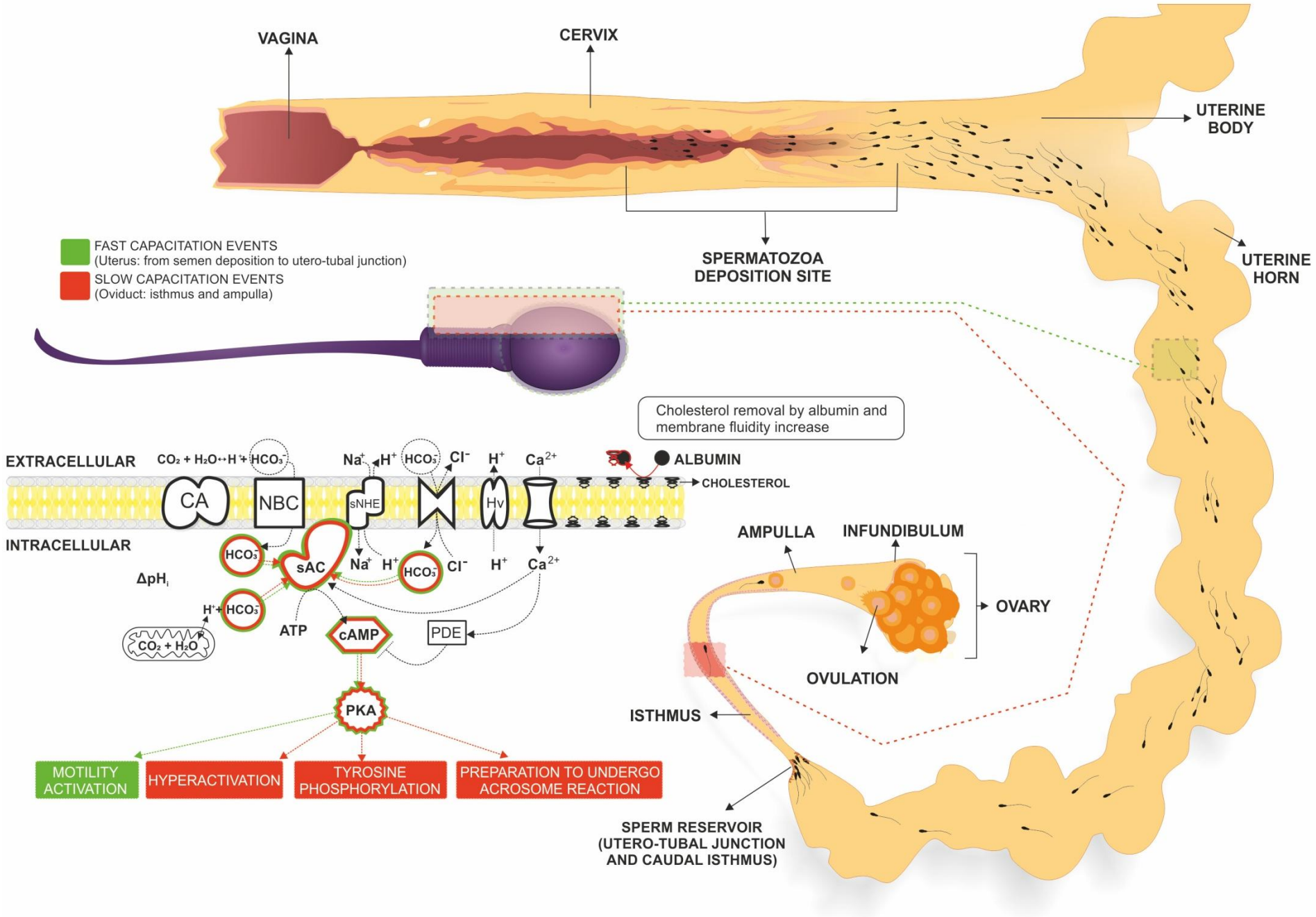

VAGINA
CERVIX
UTERINE BODY
UTERINE HORN
SPERMATOZOA DEPOSITION SITE
FAST CAPACITATION EVENTS
(Uterus: from semen deposition to utero-tubal junction)
SLOW CAPACITATION EVENTS
(Oviduct: isthmus and ampulla)
Cholesterol removal by albumin and membrane fluidity increase
EXTRACELLULAR
INTRACELLULAR
ALBUMIN
CHOLESTEROL
CA
NBC
sNHE
Hv
sAC
ΔpH$_i$
ATP
cAMP
PDE
PKA
AMPULLA
INFUNDIBULUM
OVARY
OVULATION
ISTHMUS
SPERM RESERVOIR (UTERO-TUBAL JUNCTION AND CAUDAL ISTHMUS)
MOTILITY ACTIVATION
HYPERACTIVATION
TYROSINE PHOSPHORYLATION
PREPARATION TO UNDERGO ACROSOME REACTION

Figure 2. Sperm molecular basis of the spermatozoa capacitation pathway dependent of bicarbonate and crosstalk of sperm bicarbonate during capacitation. Fast and slow capacitation events depending on HCO_3^- uptake. The fast capacitation events (represented in green) take place as soon as spermatozoa are capacitated and deposited in the uterus: HCO_3^- uptake and subsequent intracellular pH alkalinization (pH_i) and motility activation (fast capacitation events). The slow capacitation events (represented in red) occur once spermatozoa achieve the oviduct (from isthmus to ampulla): hyperactivation, tyrosine phosphorylation (Tyr-P) and preparation to undergo acrosome reaction (AR) develop in a sequential and parallel way. Both fast and slow events depend on the HCO_3^- and Ca^{2+} concentrations in the medium, but the slow ones also depend on the presence of albumin as membrane cholesterol acceptor. Membrane HCO_3^- transporters: Na^+/HCO_3^- co-transporters (NBC) and Cl^-/HCO_3^- anion exchangers. Membrane H^+ transporters: Na+/H+ exchangers (sNHE), H+ channels (H_v). The CO_2/HCO_3^- is equilibrated by membrane associated carbonic anhydrases (CA) that catalyzes the reversible conversion of cytosolic CO_2 to HCO_3^- and H+ release ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$). After changes in sperm membrane fluidity and lipid scrambling HCO_3^--dependent pathway is activated (Visconti et al., 1995a). In the presence of albumin, HCO_3^- leads to the activation of the soluble adenylyl cyclase (sAC) of the sperm membrane, which catalyzes cAMP metabolism and regulate the protein kinase A substrates phosphorylation (PKAs-P) (Visconti, 2009) on which fast and slow events depend.

Other molecules also are thought to be related with sperm capacitation in the oviduct. Endogenous nitric oxide (NO) regulates the ciliary beating of the ciliated OECs and induces sperm chemotaxis and also has a significant role in sperm functionality. NO affects sperm motility by mobilizing the stored Ca^{2+} in the sperm neck/midpiece, regulates the Tyr-P of different sperm proteins, enhances the sperm-ZP binding and modulates the AR (reviewed by Staicu and Matás, 2017). To carry out these functions, NO acts via three main pathways: i) activating the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway that results in an increase of cGMP which activates the cyclic nucleotide-gated channels (CNG) and allows Ca^{2+} entry into the spermatozoal cytosol, ii) directly phosphorylating tyrosine residues by activating the sAC/cAMP/PKA pathway, iii) regulating the post-translational protein modification in spermatozoa via S-nitrosylation (reviewed by Staicu and Matás, 2017).

The OF and its specific composition provides the optimum medium for sperm capacitation, gamete interaction, fertilization and early embryonic development. OF is composed of the specific secretions of the oviductal epithelium and also the transudates from blood. It is quantitatively and qualitatively specific in the different stages of the estrous cycle and also in oviductal regions in terms of macromolecular content, osmolarity, ionic composition and pH and is regulated by hormones (Leese, 1988). The OF is protein-rich, most of which are referred to as oviduct-specific proteins or 'oviductins' (Killian, 2004) and it is also rich in molecules such as GAGs (Tienthai et al., 2000). The OF has also a high concentration of HCO_3^-, providing an alkaline environment necessary to sperm capacitation and fertilization in the periovulatory phase of the estrous cycle (Zhou et al., 2005).

The environmental features of the SR allow the maintenance of spermatozoal quiescence and prevention of capacitation before ovulation and then modulating sperm capacitation and fertilization. These two aspects are influenced by HCO_3^- concentration and environmental pH. HCO_3^- in the oviduct seems to have an important role in two main functions: i) buffering the environment and maintaining the proper acid-base equilibrium in each estrous phase, and ii) affecting membrane dynamics and triggering the lipid scrambling at the beginning of the sperm capacitation process. Thus, HCO_3^- and the pH in the oviductal lumen are considered key effectors in sperm capacitation and fertilization (Gadella and Van Gestel, 2004; Tienthai et al., 2004). As we mentioned above, HCO_3^- concentration markedly varies along the journey of the mature spermatozoa from the epididymis to the fertilization site. Specifically in the oviduct, spermatozoa are exposed to a wide range of HCO_3^- concentrations from approximately 10 mM in the isthmus to between 25 and 90 mM at the fertilization site (Petrunkina et al., 2001b; reviewed by Hess et al., 2005) (Figure 1). Determination of the exact HCO_3^- concentration *in vivo* in the oviduct is troublesome due to the existence of methodological difficulties. The pH of the oviductal lumen associated with HCO_3^- production by the OEC greatly increases from near 6.5 in the isthmus to around 8.0 in the oviductal ampulla in the periovulatory phase in porcine (Rodriguez-Martinez, 2007). In the middle of the estrous cycle the difference between the pH of the ampulla and isthmus ranges between 0.3 and 0.7 units, increasing a further 0.4 units at the time of ovulation (Nichol et al., 1997). This HCO_3^- and pH gradients in the oviduct from isthmus to the fertilization site seem to enable the HCO_3^-- and pH-dependent state of

activation of spermatozoa. Spermatozoa are firstly inactive in the SR and then activated and free in the oviductal lumen moving towards the ovulated oocyte, ready for ZP binding, undergoing the AR, and fertilizing the oocyte.

2.3.3. Fertilization

Fertilization is the process in which two haploid gametes (spermatozoon and oocyte) unite to form a genetically distinct individual. It comprises a series of sequential and coordinated phases that result in a diploid zygote. In porcine, fertilization takes places in the AIJ (Hunter, 1974) and begins when the capacitated spermatozoa in the immediate vicinity of oocyte pass through the cumulus-oophorus complex and interact with the glycoproteins of the ZP of the oocyte (Kim et al., 2008; Yanagimachi, 1994b). Spermatozoal-ZP recognition and binding is mediated by carbohydrate interactions that can be divided into two phases:

i) Primary binding: the capacitated spermatozoon recognizes the ZP mainly by apical surface spermadhesins and proacrosin and binds to the ZP glycoproteins in a specific manner (Töpfer-Petersen et al., 2008; Töpfer-Petersen et al., 1998; Harris et al., 1994; Töpfer-Petersen and Henschen, 1987; Töpfer-Petersen et al., 1985). This interaction triggers the initiation of the AR, in which acrosomal enzymes are discharged into the ZP (Yanagimachi, 1994b). The hyperactivated spermatozoon then uses the impulse of flagellar beating to pass through the ZP.

ii) Secondary binding: the inner plasma membrane of the acrosome-reacted spermatozoa is exposed, and its equatorial region adheres to and fuses with the oolemma.

The cortical reaction then takes place and the ZP changes the structure of its proteins to a 'hardened' state, which helps to block polyspermy (Funahashi et al., 2000). The spermatozoon next penetrates the oocyte and introduces its nucleus into the ooplasm (Yanagimachi, 1994b). Despite the continuous advance in the knowledge of the physiology of gametes as well as their interaction, there are still many aspects to be clarified to efficiently extrapolate the *in vivo* environment in the oviduct to the *in vitro* conditions in the laboratory. Many strategies and protocols have been carried out so far; however, the efficiency obtaining quality embryos *in vitro* remains lower than that of their *in vivo* counterparts.

3. *IN VIVO VS. IN VITRO* SPERM CAPACITATION AND FERTILIZATION

The rapid advance of assisted reproduction techniques in mammals has led to reliable and standardized protocols of obtaining zygotes and embryos in the laboratory with a relatively reasonable success rate. Establishment of *in vitro* systems has been assisted and encouraged by the ready availability of protocols for preparation of gametes, chemically-defined culture media, disposable plastic dishes and tubes, and computer-controlled incubators set to a predetermine gas concentration, humidity and temperature (Hunter and Rodriguez-

Martinez, 2002). However, conditions applied fruitfully *in vitro* may not be closely representative of those found *in vivo*, and levels of success *in vitro* seldom match those found *in vivo* (Hunter and Rodriguez-Martinez, 2002).

The population of spermatozoa selected or capacitated *in vitro* probably is different from the small proportion of competent spermatozoa that would reach the site of fertilization *in vivo* close to the time of ovulation. As an example, a droplet of culture medium containing spermatozoa and COCs represents, strictly speaking, a post-ovulatory milieu, but is probably much different from the preparatory milieu to which gametes are exposed *in vivo* (Rodríguez-Martínez et al., 2005). The current conditions in porcine IVF are still far from optimal since none has managed to reduce the unacceptably high incidence of polyspermy in this species. *In vitro* techniques have resulted in pregnancies and live offspring through embryo transfer in multiple mammalian species. For decades, research in this field has increased the knowledge in basic science about physiology of gametes and their interaction, even in the complex uterine environment. However, while good performance of these techniques has been achieved in some mammalian species such as bovine or murine, the results obtained in porcine so far are not comparable to the *in vivo* output. In pigs, differences between *in vivo* and *in vitro* environment in which sperm capacitation and fertilization take place could be a determinant of the low success in obtaining potentially viable zygotes (Table 1). More studies are needed trying to find a more efficient method of preparing gametes and performing IVF improve the performance of assisted reproduction in this species.

Spermatozoal fertility usually refers to the ability of spermatozoa to fertilize oocytes, either *in vivo* or *in vitro*. In most mammalian species including porcine, spermatozoa acquire fertilization ability once the reach the corpus epididymis or proximal segment of the cauda epididymis (Yanagimachi, 1994a). In *in vivo* conditions, both in natural mating and in AI, spermatozoa entering the uterus are ejaculated spermatozoa; that is, spermatozoa that have had contact with the SP. There are also studies in porcine in which sows were inseminated with epididymal spermatozoa (fresh or frozen-thawed) but fertilization and conception rates and litter size after AI were much lower than those obtained with ejaculated spermatozoa (Okazaki et al., 2012; Holtz and Smidt, 1976). *In vitro*, two sources of spermatozoa can be used – ejaculated or epididymal – depending on the objective and the technical constraints. In fact, there are numerous studies in which both ejaculated and epididymal spermatozoa have been used and compared (Matás et al., 2010). The fertility and response to treatments usually is variable in ejaculated spermatozoa both within and between individual boars and ejaculates, probably due to the variable effects of SP components on the different subpopulations of spermatozoa. In this sense, epididymal spermatozoa tend to be more 'stable' in their response to selection or capacitation treatments (Matás et al., 2010). It has been reported that epididymal spermatozoa *in vitro* can reach capacitation and fertilize oocytes much easier than ejaculated spermatozoa (Yanagimachi, 1994b), but this depends on the specific treatments used for sperm selection and capacitation.

As discussed, one of the first *in vivo* barriers after semen deposition to which spermatozoa are subjected is spermatozoal selection. In general terms, the uterine cervix, body and horns exert a selective effect on spermatozoa mainly through myometrial contractions, backflow and phagocytosis by PMN, and provide a hostile

environment for spermatozoa (see section 2.2). Only a small proportion of spermatozoa, compared with that initially ejaculated, complete all the events involved in spermatozoal selection in the uterus. Billions of spermatozoa are deposited into the cervix during mating or common AI in porcine, but only a limited number reach the UTJ and the large majority of those do not pass through it (Hunter and Nichol, 1988). The SR (see section 2.3.1.) also provides a selective barrier in the oviduct, although more specific than that of the uterus. The spermatozoa that bind to the SR are the most competent for fertilization based on their functionality (reviewed by Holt and Fazeli, 2010). The spermatozoa remain bound to OEC for up to 36-40 h before ovulation with no reduction in their fertilizing ability (Tienthai, 2015). It has even been suggested that the action of the isthmus on spermatozoa is more to delay the capacitation progress than promote it (Rodriguez-Martinez et al., 2001; Smith, 1998). Over years, different types of *in vitro* spermatozoal selection techniques have been developed with the main objective to eliminate the SP and decapacitating factors, residues of diluents used for spermatozoal preservation, or certain contaminating particles. Many of these selection methods involve the centrifugation (washing with albumin or through colloids and sperm pelleting) or the use of a non-physiological composition of selection media (for example, spermatozoal filtration with Sephadex gel or glass wool) that select spermatozoa by their physical characteristics, but some of these procedures can produce cellular damage (Matás et al., 2011; Holt et al., 2010).

Once spermatozoa are released from SR and enter the oviductal lumen, they are exposed to the specific and dynamic milieu of the oviduct, which due to its particular temperature, oxygen and pH provides a suitable environment to promote sperm capacitation and fertilization (De Lamirande et al., 1997). *In vivo*, sperm capacitation occurs during the transport through the female reproductive tract, is progressive and sequential due to the dynamic environments encountered by the spermatozoa from the site of semen deposition to the site of fertilization in terms of biofluid composition, pH, temperature, percentage of oxygen, etc. The diversity and heterogeneity in responsiveness among spermatozoal subpopulations allow different grades of sperm capacitation in each uterine section. The specific composition of UF and OF in the periovulatory stage is still partially unknown, which makes it difficult to mimic *in vitro*.

In vitro sperm capacitation is conducted in plastic tubes or dishes, in static systems, in chemically defined media of differing complexity but limited and fixed composition and physical characteristics, probably much different from that encountered in OF. One of the main consequences of these static systems is that under *in vitro* conditions most of spermatozoa are capacitated simultaneously (Rodriguez-Martinez, 2007). *In vitro* capacitation can occur by incubating spermatozoa at physiological temperature of 38.5ºC, in the presence of HCO_3^- and Ca^{2+}, with albumin as a protein source, and with energy substrate molecules such as glucose, pyruvate and lactate, in a balanced salt solution in which slightly alkaline pH is maintained, normally at 7.4 (Watson and Green, 2000; Visconti and Kopf, 1998; Harrison, 1996).

Table 1. Differences between *in vivo* and *in vitro* sperm capacitation and fertilization in mammals, with special attention to porcine species.

Parameter / Factor	*In vivo* (*)	*In vitro* (†)	Reference
Source of spermatozoa	Ejaculate	Epididymis or ejaculate	* Yanagimachi, 1994b † Matás et al., 2010
Spermatozoa selection and capacitation	Backflow, phagocytosis by neutrophils and formation of sperm reservoir (SR)	Washes with albumin or through colloids and sperm pelleting, filtration with Sephadex gel or glass wool	* García-Vázquez et al., 2015; Hernández-Caravaca et al., 2015; Holt and Fazeli, 2010; Taylor et al., 2008; Matthijs et al., 2003; Steverink et al., 1998; † Matás et al., 2011; Holt et al., 2010; Matás et al., 2003
Sperm capacitation site	During the transport through the female reproductive tract	Plastic tubes and dishes in computer-controlled incubators set to predetermine gas phase, humidity and temperature	* Yanagimachi, 1994a † Hunter and Rodriguez-Martinez, 2002
Sperm capacitation media	Uterine and oviductal fluids (UF and OF)	Chemically defined media	* Rodriguez-Martinez, 2007. † Rath et al., 1999; Abeydeera and Day, 1997; Mattioli et al., 1988
Spermatozoa response to capacitation	Progressive and sequential. Heterogeneity among spermatozoa subpopulations	Most of spermatozoa at the same time	* Suarez, 2007 † reviewed by Funahashi, 2003
Timing of sperm capacitation	2-3 h depending on the place of deposition	Initiated in max. 60 s. The latter signs occur after 60 min incubation	* Harrison, 1996; Hunter and Dziuk, 1968 † Rath et al., 1999; Harrison, 2004
Source of matured oocytes	Follicle (ovulated oocytes)	Follicle (ovulated oocytes) or *in vitro* matured oocytes in plastic dishes	* Yanagimachi, 1994a † Hunter and Rodriguez-Martinez, 2002
Cumulus oophorus	Present	Present or absent	* Yanagimachi, 1994a † Coy et al., 1993c
Oocytes and zygotes ZP thickness	Thick	Thin	* Funahashi et al., 2001; Wang et al., 1998 † Funahashi et al., 2001
Spermatozoa and oocytes coincubation time	-	From 15 s to 18 h	† Almiñana et al., 2008; Coy et al., 2008b; Matás et al., 2003; Coy et al., 1993a
Spermatozoa concentration for insemination	From 30-60 millions (AI) to hundreds of millions (natural mating) spermatozoa/ml	From tens of thousands to millions spermatozoa/ml	* Waberski et al., 2008 † Ballester et al., 2014; Almiñana et al., 2008; Coy et al., 1993b

Parameter / Factor	*In vivo* (*)	*In vitro* (†)	Reference
Capacitating and fertilizing environment	Dynamic	Static	* Rodriguez-Martinez, 2007 † Nagai, 1994
Fertilization site	Ampullary-isthmic junction (AIJ)	4-wells, microdrops, caps of tubes, straws, microchannels, microfluidic sorters, 3-D OECs cultures (see figure 3)	* Yanagimachi, 1994a † Ferraz et al., 2017; Sano et al., 2010; Clark et al., 2005; Li et al., 2003; Funahashi and Nagai, 2000
Fertilization media	Periovulatory oviductal fluid (OF)	TCM-199, TALP, BO, TBM	* Yanagimachi, 1994b † Coy and Romar, 2002; Coy et al., 2002
Regulation and synchronization of environment	Oviductal and follicular factors	Technical and human factors	* Hunter, 1990 † Hunter and Rodriguez-Martinez, 2002
Environmental HCO_3^- concentration	~10 mM in isthmus and 25-90 mM in AIJ	25 mM	* Rodriguez-Martinez, 2007; Hess et al., 2005; Rodriguez-Martinez et al., 1990 † Parrish, 2014; Yoshioka et al., 2008; Bedu-Addo et al., 2007; Coy et al., 2002; Rath et al., 1999; Quinn, 1995
Environmental pH	~8.0	7.4	* Rodriguez-Martinez, 2007 † Matás et al., 2010; Abeydeera and Day, 1997; Coy et al., 1993b;
Biofluids presence	Uterine or oviductal fluids (UF or OF)	Oviductal of follicular fluids (OF or FF) incorporated to the media	* Leese et al., 2001; Nichol et al., 1992; Leese, 1988 † Coy and Avilés, 2010; Coy et al., 2008b; Coy et al., 2002; Kim et al., 1997; Kim et al., 1996; Funahashi and Day, 1993; Nagai and Moor, 1990

It is difficult to specify the exact timing of the events that take place during the sperm capacitation process. Under *in vivo* conditions, sperm capacitation could last 2 or 3 hours, depending on the place of semen deposition (Harrison, 1996; Hunter and Dziuk, 1968). What seems clear is that OF modulates the rate of sperm capacitation events *in vivo*, and probably also when it is added to an *in vitro* system. Once spermatozoa are sequentially exposed to UF and OF, these fluids should be able to regulate the rate of the process (Rodriguez-Martinez, 2007). However, it has been described that *in vitro* capacitation is initiated within a few seconds or minutes after spermatozoa come into contact with a HCO_3^--enriched capacitating medium because HCO_3^- rapidly stimulates sAC (maximum within 60 s) that increases the levels of intra-spermatozoal cAMP and activates the PKA-dependent protein phosphorylation cascade (Visconti, 2009; Harrison, 2004;). One of the first signals of the stimulation of the PKA-cascade is the activation of spermatozoal motility, but also a scrambling of plasma membrane phospholipids that produces an increase in plasma membrane fluidity (Harrison and Miller, 2000). Significantly later, the membrane fluidity increases again but by the action of the presence of albumin in the incubation medium, which removes or redistributes the cholesterol in the spermatozoal plasma membrane (Flesch et al., 2001). Another late effect (> 60 min) of HCO_3^- in *in vitro* sperm capacitation is the activation of tyrosine kinases and the subsequent Tyr-P (Visconti, 2009).

The process of fertilization *in vivo* is precisely and strictly regulated and synchronized. In the oviduct, oviductal and follicular factors coordinate gamete interaction and function (Hunter, 1990). Only few capacitated spermatozoa reach the COCs and undergo the AR during passage through the cumulus (Mattioli et al., 1998) or ZP (Cummins and Yanagimachi, 1982). However, *in vitro* sperm capacitation and fertilization provides a static system controlled by technical (incubators, thermostats, gas infusion pumps, etc.) and human (time of manipulation of gametes, maintenance of asepsis, contamination, abrupt fluctuations of temperature, etc.) factors (Hunter and Rodriguez-Martinez, 2002) that are far from resembling the dynamic system to which gametes are exposed *in vivo* (Visconti, 2009; Nagai, 1994). In IVF, there is no single medium commonly used by researches, but what seems clear is the importance of Ca^{2+} availability in the medium and its uptake by oocytes and spermatozoa. Currently, conditions used for IVF differ enormously from those *in vivo* but all the *in vivo* parameters should first be determined to be efficiently mimicked and applied *in vitro*.

The strict control by the female reproductive tract of spermatozoal functions and capacitation state enables, in physiological conditions in the oviduct, monospermic penetration of oocytes in porcine of around 95% of total fertilizations (reviewed by Funahashi, 2003). The strategies employed by the uterus to reduce the number of spermatozoa that reach the site of fertilization, select the spermatozoa most suitable for fertilization and modulate their state of activation-quiescence-capacitation are still largely unknown. As previously described, the mechanisms that control the number of porcine spermatozoa that are fertilization competent are well developed *in vivo* and effectively reduce polyspermy but the complexity of these mechanisms make it difficult to reproduce *in vitro* (reviewed by Funahashi, 2003). Thus it seems nearly impossible to obtain the high rate of monospermy in pigs *in vitro* that has been achieved in other mammals. In fact, it has been proposed that polyspermy is the bottleneck to improvements in porcine IVF and this has been related to the induction of partial AR of boar

spermatozoa in IVF media and thus to the use of a highly non-physiological number of spermatozoa which are simultaneously capacitated and ready to fertilize. The percentage of polyspermy *in vitro* is directly related to the number of spermatozoa used, but, unfortunately, simply reducing the number of spermatozoa per oocyte not only does not address the problem of polyspermy but decreases the penetration rate. The current conditions of preparation of the gametes prior to porcine IVF are still far from optimal since none of them has managed to reduce the unacceptably high incidence of polyspermy in this species. One strategy is to modulate the *in vitro* sperm capacitation to impede the simultaneous presence of massive numbers of capacitated spermatozoa around the oocytes at the time of fertilization.

On the other hand, polyspermy has been also related to the significant differences in organization and ultrastructure of ZP proteins in pig between *in vitro* and *in vivo* matured and fertilized oocytes (Coy et al., 2008a). ZP of ovulated oocytes has a rather thick mesh-like fibrillar network structure whereas in *in vitro* matured oocytes the ZP is more compacted and smoother. After the contact with spermatozoa, *in vitro* matured oocytes undergo a delayed or incomplete zona reaction far from the complete polyspermy-blocking reaction that takes place *in vivo* in the oviduct (Funahashi, 2003). Polyspermic penetration in porcine oocytes also occurs *in vivo*; however there are physiological mechanisms, many of them unknown, that modulate this incidence. Additional macromolecules have been reported in the ZP of ovulated pig oocytes (Hedrick et al., 1987), such as oestrogen-dependent oviductal glycoproteins, which are secreted into the oviductal lumen (Brown and Cheng, 1986). With the current media used in the IVF laboratories, it has not yet been possible to reproduce this phenomenon. During final maturation, the ZP of *in vivo* matured oocytes adds oviductal glycoproteins, which allows the change of conformation in the inner ZP necessary to undergo an appropriate ZP reaction when fertilized. Changes in ovulated oocytes that are conferred by OF seem to produce ZP resistance to digestion by pronase (Wang et al., 1998); this ZP resistance can be increased in *in vitro* matured oocytes by exposing them to OF (Coy et al., 2008a).

Despite the development of different spermatozoa-oocyte co-incubation times (Almiñana et al., 2008; Coy et al., 2008b; Matás et al., 2003; Coy et al., 1993a), spermatozoal concentration for insemination (Ballester et al., 2014; Almiñana et al., 2008; Coy et al., 1993b), presence of the cumulus oophorus (Coy et al., 1993c), sperm capacitation methods (Matás et al., 2003) and innovative IVF protocols in which gametes are physically separated (Figure 3) (Sano et al., 2010; Clark et al., 2005; Li et al., 2003; Funahashi and Nagai, 2000), polyspermy in porcine IVF is not yet solved. Moreover, there are numerous studies attempting to improve IVF by developing defined capacitation and fertilization media for pigs (Rath et al., 1999; Abeydeera and Day, 1997; Mattioli et al., 1988) and comparing their efficiency, such as TCM-199, Tyrode's albumin lactate pyruvate (TALP), Brackett-Oliphant solution (BO) or Tris-buffered medium (TBM) (reviewed by Coy and Romar, 2002; Coy et al., 2002), but none of them has achieved an efficiency comparable to fertilization *in vivo*. Other variables studied have been the spermatozoal concentration used for insemination, coincubation time, source of spermatozoa or volume of coculture medium, but there is a lack of conclusive results and standardized protocols in porcine (reviewed by Coy and Romar, 2002).

Therefore, more studies are needed to deepen the knowledge of the oviductal microenvironment in which porcine capacitation and fertilization take place *in vivo* to try to mimic them *in vitro*. At present, the main objectives of the research carried out in our laboratories are aimed at improving media and conditions of culture, the incorporation of biofluids and the development of IVF devices in which gamete functionality is more similar to physiological conditions. Some of these factors, still to be optimized, such as HCO_3^- concentration, pH, use of biofluids and IVF devices that allow much more physiological contact between gametes, are reviewed below.

3.1. Bicarbonate concentration

HCO_3^- has been identified *in vitro* as a key capacitating agent that promotes lipid rearrangement-disorder in the spermatozoal plasma membrane that initiates the capacitation. It acts by activating the sAC/cAMP-dependent pathway (Visconti et al., 1995b). Sperm capacitation in the isthmus is initiated under a concentration of HCO_3^- around 10 mM (Rodriguez-Martinez et al., 1990) and spermatozoa are progressively exposed to higher HCO_3^- as they ascend to the fertilization site. *In vivo*, the number of capacitated spermatozoa at one particular time and place of the oviduct would be low. The capacitated state is transient and irreversible *in vivo*. So, there should be a continuous replacement of capacitated, short-lived spermatozoa leading to low spermatozoal number per area at any one time, ensuring the availability of capacitated spermatozoa for an extended period between spermatozoal deposition and ovulation (Rodriguez-Martinez, 2007). However, far from these dynamic environments that spermatozoa go through and the progressive and sequential events in capacitation of spermatozoal subpopulations *in vivo*, currently most of the *in vitro* capacitation and fertilization protocols result in the majority of spermatozoa being capacitated and available to fertilize at the same time. The most common media for *in vitro* sperm capacitation and IVF contain a fix concentration of 25 mM of HCO_3^-, whatever the origin of the spermatozoa (epididymal or ejaculated) and the treatments received (dilution in a extender, refrigeration, selection process) (Parrish, 2014; Yoshioka et al., 2008; Bedu-Addo et al., 2007; Coy et al., 2002; Rath et al., 1999; Quinn, 1995). HCO_3^-, as a key effector in sperm capacitation, needs to be adjusted in the medium in an attempt to produce more physiological sperm capacitation and combat the problem of polyspermy.

3.2. pH

In addition to be a capacitating effector, HCO_3^- acts to buffer the reproductive environments in which gametes are found. One of the consequences of the exposure of spermatozoa to HCO_3^- is the alkalinization of the intracellular fluid, which in the oviduct activates spermatozoal motility and triggers the cascade of molecular events of capacitation (Visconti, 2009).

The pH in the oviductal lumen, due to the production of HCO_3^- in the OEC, greatly increases in the periovulatory stage, reaching around pH 8.0 at the site of fertilization at the time of the encounter between

gametes (Rodriguez-Martinez, 2007). However, media commonly used for *in vitro* sperm capacitation and IVF have a fixed pH of 7.4 (Matás et al., 2010; Abeydeera and Day, 1997; Coy et al., 1993b). It is known that most of biological processes in organisms are regulated by pH (Lodish et al., 2000), and mammalian capacitation, fertilization and polyspermy blocking could be some of them. For this reason, it is especially interesting to study the effects of the application of a more physiological pH in these procedures.

3.3. Biofluids

As discussed, under *in vitro* conditions, the pattern of sperm capacitation and fertilization is not similar to the situation *in vivo*. The media used in laboratories is not similar to OF and there is not a single medium commonly used in all laboratories that allows standardization of IVF in porcine (Coy et al., 2002). Chemically defined media are currently used for these techniques, with a more or less complex composition based in salts, sugars, proteins such as albumin and other components. However, the media composition has not been able to equate with OF, as it is still unknown. In order to increase the performance of IVF in pigs, several protocols have been developed that include OF in the medium (Coy et al., 2010) although the lack of knowledge of OF composition remains a handicap in achieving consistency.

The intraluminal periovulatory OF is the optimal medium to transport gametes and allow the interaction between them and also to promote early embryonic development (Yanagimachi, 1994b). Periovulatory OF is formed by specific OEC secretions and by transudation of blood serum, but OF differs from serum in terms of ionic composition, osmolarity, pH and macromolecular content (Nichol et al., 1992; Leese, 1988). Regional differences also exist in the composition of OF and this seems to be related with the process of gamete preparation for fertilization (Leese et al., 2001).

Incubation of spermatozoa with undefined oviductal components before or during IVF as has been done for OECs, has demonstrated that OF or FF improve IVF (Kim et al., 1997; Funahashi and Day, 1993; Nagai and Moor, 1990). In addition, specific oviductal components such as glycoproteins (Coy et al., 2008a; Kouba et al., 2000), GAGs such as HA (Suzuki et al., 2000) or proteins such as osteopontin (Hao et al., 2006) have shown beneficial effects. *In vitro*, the preincubation of porcine oocytes in OF from early-luteal phase increased ZP hardening and pronase digestion time, which has been linked with higher monospermy rate (Coy and Avilés, 2010; Coy et al., 2008a; Coy et al., 2002; Kim et al., 1996). It has been described that the inclusion of OF in IVF systems reduces polyspermy, probably by preventing the spermatozoal AR (Kim et al., 1997), regulating the capacitation rate and making it slower before ovulation and faster after it (Avilés, 2011; Coy et al., 2010; Rodriguez-Martinez et al., 1998).

Other difference between *in vivo* and *in vitro* interactions between porcine gametes is that many IVF protocols remove the cumulus cells that surround the oocyte. In these cases, the secretory products of cumulus cells derived from their metabolism are absent in many of the current IVF systems. In pigs, the presence of

cumulus cells has been shown to improve fertility and monospermy ratios (Coy et al., 1993c). The FF, which is a product of the follicular wall, is also important in the oviductal milieu as during ovulation it is poured into the oviduct and forms part of the OF. In bovine, granulosa cells obtained from the follicular wall increase the fertilization rate and decrease polyspermy (Fukui and Ono, 1989). In porcine, the effect of including FF in the pre-fertilization media for spermatozoal incubation has been studied and during IVF results in a reduction of polyspermy and number of spermatozoa bound to the ZP (Funahashi and Day, 1993).

There is a large gap in knowledge of the exact composition of OF, but what seems clear is that despite the use of chemically defined culture media has not been able to match the results of OF *in vivo* or the addition of biofluids to IVF systems. More studies are needed to analyze in depth the composition of OF with the objective to establish a chemically defined culture medium or system for sperm capacitation and IVF that more closely resembles the physiological environment.

3.4. *In vitro* fertilization devices

With the objective to mimic the strict spermatozoal selection process that takes place in the female genital tract and to allow the establishment of competition between spermatozoa, several methods and devices have been developed: IVF by climbing-over-a-wall (Funahashi and Nagai, 2000), using biomimetic microfluidic technology (Clark et al., 2005), IVF in straws (Li et al., 2003), applying modified swim-up for spermatozoal selection (Park et al., 2009), using a microfluidic spermatozoal sorter (Sano et al., 2010) or 3D OEC culture systems for IVF and embryo production (reviewed by Ferraz et al., 2017) (Figure 3). The majority of these methods reduce the number of spermatozoa that contact the oocytes, allowing a certain selection of spermatozoa population and expression of their heterogeneity in terms of motility, capacitation state and fertility. Many of them can include biofluids that can be replaced over time to mimic the dynamic oviductal environment in terms of composition of proteins, hormones and other molecules, and they even have different compartments for male and female gametes and/or OECs. Normally, they are methods or devices in which gametes, female and male, are physically distant from each other and spermatozoa have to cross an artificial obstacle to reach the oocytes, more similar to the situation *in vivo*. However, none of them has managed to eliminate the problem of polyspermy under *in vitro* conditions in the pig. Many studies are currently underway to develop new devices in which spermatozoa are guided to the oocytes through the so-called 'taxis': chemo-, rheo- and thermotaxis. In each one of them, spermatozoa are attracted by a different stimulus gradient: chemical, fluid flow or temperature, respectively (Pérez-Cerezales et al., 2015).

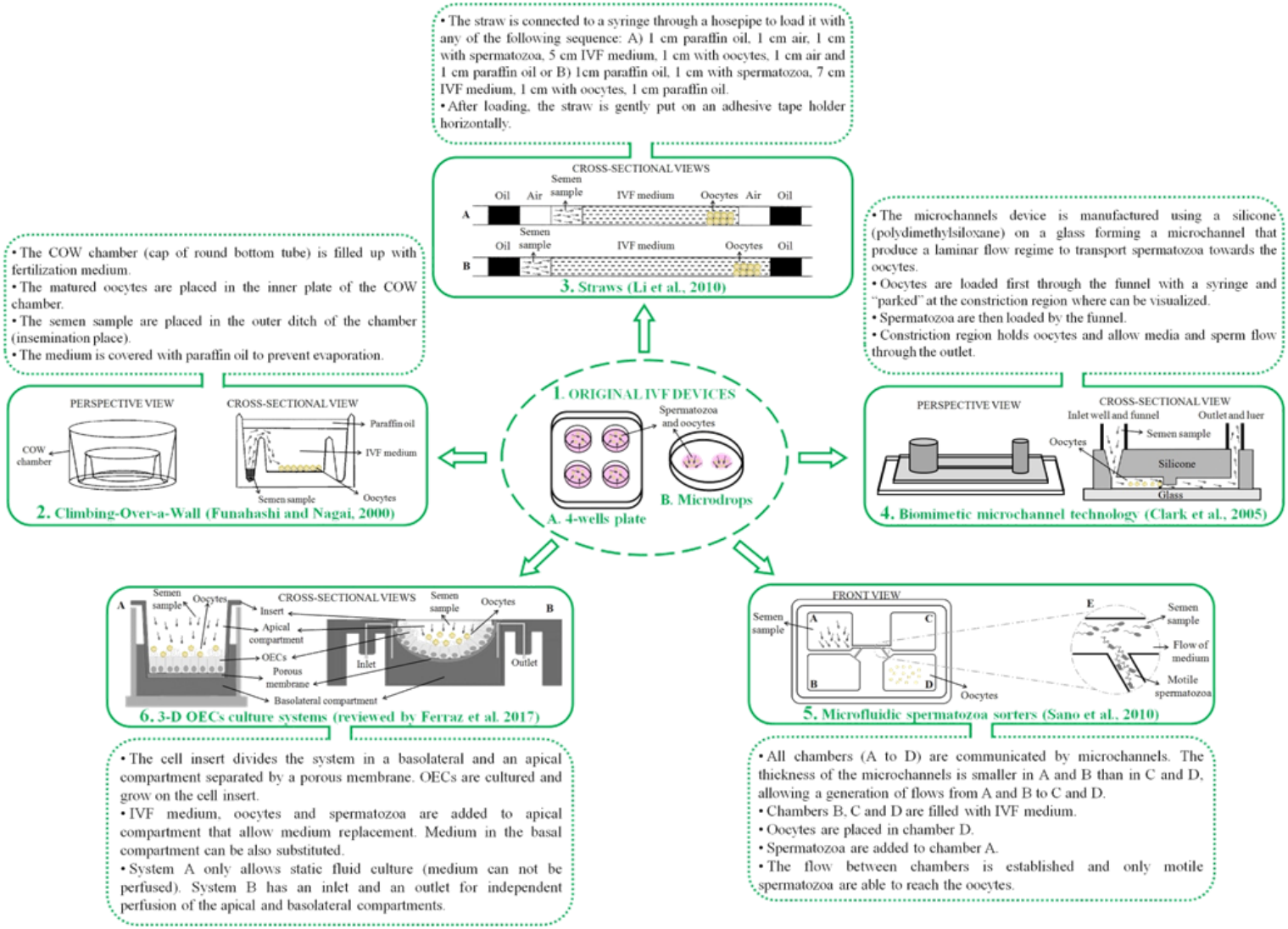


Figure 3. Different devices and systems for porcine IVF improvement. The middle circle shows the most commonly used devices in porcine IVF: 4-well plate and microdroplets (1A and B). The continuous-line boxes show some of the devices used for porcine IVF efficiency improvement: Climbing-Over-a-Wall (COW) (2), straws (3A and B), biomimetic microchannel technology (4), microfluidic spermatozoa sorter (5) and 3-D OECs culture systems (6A and B). Each of the drawings is a modified one from the originals in the indicated references. The dotted boxes summarize the operation of each device.

4. CONCLUDING REMARKS

From the studies reviewed, major research progress has been made in reproductive physiology and technology in swine. However, attempts to improve *in vitro* reproduction in pigs have not been sufficient. Lack of knowledge of many factors involved in *in vitro* sperm capacitation and fertilization and its regulation could contribute to the abnormally high incidence of polyspermy in this species. The use of simple *in vitro* systems of pre-treatment of gametes and IVF have been well demonstrated to be valid to analyze the complex processes of fertilization and evaluate several sperm, oocyte and embryo parameters. However, they suffer from serious limitation for the *in vitro* production of embryos due to fact that the available capacitation media produce a large percentage of capacitated spermatozoa that ready for fertilize at the same time. The direction in which we must advance is to allow oocytes to express their natural ability to block polyspermy, with more studies on the physiology of gametes, as well as with the development of new strategies for modulation of sperm capacitation. To do this, it would seem logical to first adjust the known parameters involved in *in vitro* sperm capacitation and fertilization processes that are far from their counterparts *in vivo* and that could firmly establish the basis of these techniques in the porcine species.

5. REFERENCES

Aalberts, M., Stout, T. A. E. and Stoorvogel, W. (2014). Prostasomes: extracellular vesicles from the prostate. *Reproduction* **147(1)**, R1–R14.

Abeydeera, L. R. and Day, B. N. (1997). Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol. Reprod.* **57(4)**, 729–734.

Almiñana, C., Gil, M. A., Cuello, C., Parrilla, I., Roca, J., Vazquez, J. M. and Martinez, E. A. (2008). Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization. *Anim. Reprod. Sci.* **106(3)**, 393–401.

Ardón, F., Helms, D., Sahin, E., Bollwein, H., Töpfer-Petersen, E. and Waberski, D. (2008). Chromatin-unstable boar spermatozoa have little chance of reaching oocytes *in vivo*. *Reproduction* **135(4)**, 461–470.

Austin, C. R. (1951). Observations on the penetration of the sperm in the mammalian egg. *Aust. J. Biol. Sci.* **4(4)**, 581–596.

Avilés, K. (2011). Estudio de la funcionalidad espermática, interacción de gametos y análisis proteico en espermatozoides epididimarios y eyaculados en la especie porcina. PhD Thesis. Universidad de Murcia.

Ballester, L., Romero-Aguirregomezcorta, J., Soriano-Úbeda, C., Matás, C., Romar, R. and Coy, P. (2014). Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine *in vitro* fertilization efficiency. *Fertil. Steril.* **102(6)**, 1762–1768.

Bedu-Addo, K., Barratt, C. L. R., Kirkman-Brown, J. C. and Publicover, S. J. (2007). Patterns of $[Ca^{2+}]_i$ mobilization and cell response in human spermatozoa exposed to progesterone. *Dev. Biol.* **302(1)**, 324–332.

Bergmann, A., Taylor, U. and Rath, D. (2012). Sperm binding to porcine uterine epithelial cells might be lectin mediated. *Reprod. Fertil. Dev.* **25(1)**, 152–152.

Briz, M. D., Bonet, S., Pinart, B., Egozcue, J. and Camps, R. (1995). Comparative study of boar sperm coming from the caput, corpus, and cauda regions of the epididymis. *J. Androl.* **16(2)**, 175–188.

Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J. and Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc. Natl. Acad. Sci.* **111(6)**, 2200–2205.

Brown, C. R. and Cheng, W. K. (1986). Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo. *J. Embryol. Exp. Morphol.* **92(1)**, 183–191.

Brüssow, K.-P., Rátky, J. and Rodriguez-Martinez, H. (2008). Fertilization and early embryonic development in the porcine fallopian tube. *Reprod. Domest. Anim.* **43(s2)**, 245–51.

Bureau, M., Bailey, J. L. and Sirard, M.-A. (2002). Binding regulation of porcine spermatozoa to oviductal vesicles *in vitro*. *J. Androl.* **23(2)**, 188–193.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into the Fallopian tubes. *Nature* **168(4277)**, 697–698.

Chang, M. C. (1957). A detrimental effect of seminal plasma on the fertilizing capacity of sperm. *Nature* **179(4553)**, 258–259.

Clark, S. G., Haubert, K., Beebe, D. J., Ferguson, C. E. and Wheeler, M. B. (2005). Reduction of polyspermic penetration using biomimetic microfluidic technology during *in vitro* fertilization. *Lab Chip* **5(11)**, 1229–1232.

Claus, R., Moshammer, T., Aumüller, R. and Weiler, U. (1989). Replenishment of AI-doses with oestrogens in physiological amounts: effect on sow prolificacy in a field trial. *J. Vet. Med. Ser. A* **36(1-10)**, 797–800.

Claus, R., Meyer, H. D., Gimenez, T., Hoangvu, C. and Munster, E. (1990). Effect of seminal estrogens of the boar on prostaglandin-F2-alpha release from the uterus of the sow. *Anim. Reprod. Sci.* **23(2)**, 145–156.

Cooper, T. G. (1986). *The epididymis, sperm maturation and fertilization*. Berlin: Springer-Verlag.

Coy, P. and Avilés, M. (2010). What controls polyspermy in mammals, the oviduct or the oocyte? *Biol. Rev.* **85(3)**, 593–605.

Coy, P. and Romar, R. (2002). *In vitro* production of pig embryos: a point of view. *Reprod. Fertil. Dev.* **14(5)**, 275–286.

Coy, P., Martínez, E., Ruiz, S., Vázquez, J. M., Roca, J., Matás, C. and Pellicer, M. T. (1993a). *In vitro* fertilization of pig oocytes after different coincubation intervals. *Theriogenology* **39(5)**, 1201–1208.

Coy, P., Martínez, E., Ruiz, S., Vázquez, J. M., Roca, J. and Matas, C. (1993b). Sperm concentration influences fertilization and male pronuclear formation *in vitro* in pigs. *Theriogenology* **40(3)**, 539–546.

Coy, P., Martínez, E., Ruiz, S., Vázquez, J. M., Roca, J. and Gadea, J. (1993c). Environment and medium volume influence *in vitro* fertilisation of pig oocytes. *Zygote* **1(3)**, 209–13.

Coy, P., Gadea, J., Romar, R., Matás, C. and García, E. (2002). Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. *Reproduction* **124(2)**, 279–288.

Coy, P., Cánovas, S., Mondéjar, I., Saavedra, M. D., Romar, R., Grullón, L., Matás, C. and Avilés, M. (2008a). Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc. Natl. Acad. Sci.* **105(41)**, 15809–15814.

Coy, P., Grullon, L., Canovas, S., Romar, R., Matas, C. and Aviles, M. (2008b). Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction* **135(1)**, 19–27.

Coy, P., Lloyd, R., Romar, R., Satake, N., Matas, C., Gadea, J. and Holt, W. V. (2010). Effects of porcine pre-ovulatory oviductal fluid on boar sperm function. *Theriogenology* **74(4)**, 632–642.

Crane, L. H. and Martin, L. (1991). Postcopulatory myometrial activity in the rat as seen by video-laparoscopy. *Reprod. Fertil. Dev.* **3(6)**, 685–698.

Cummins, J. M. and Yanagimachi, R. (1982). Sper-egg ratios and the site of the acrosome reaction during *in vivo* fertilization in the hamster. *Gamete Res.* **5(3)**, 239–256.

Dacheux, J. L., Luc Gatti, J. and Dacheux, F. (2003). Contribution of epididymal secretory proteins for spermatozoa maturation. *Microsc. Res. Tech.* **61(1)**, 7–17.

Day, B. N. (2000). Reproductive biotechnologies: current status in porcine reproduction. *Anim. Reprod. Sci.* **60–61**, 161–172.

De Lamirande, E., Leclerc, P. and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* **3(3)**, 175–194.

Dostàlovà, Z., Töpfer-Petersen, E. and Calvete, J. J. (1995). Interaction of non-aggregated boar AWN-1 and AQN-3 with phospholipid matrices. A model for coating of spermadhesins to the sperm surface. *Biol. Chem. Hoppe. Seyler.* **376(4)**, 237–242.

Dukelow, W. R., Chernoff, H. N. and Williams, W. L. (1967). Properties of decapacitation factor and presence in various species. *J. Reprod. Fertil.* **14(3)**, 393–399.

Ekhlasi-Hundrieser, M., Gohr, K., Wagner, A., Tsolova, M., Petrunkina, A. and Töpfer-Petersen, E. (2005). Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. *Biol. Reprod.* **73(3)**, 536–545.

Fazeli, A., Duncan, A. E., Watson, P. F. and Holt, W. V. (1999). Sperm-oviduct interaction: Induction of

capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol. Reprod.* **60(4)**, 879–886.

Ferraz, M. A. M. M., Henning, H. H. W., Stout, T. A. E., Vos, P. L. A. M. and Gadella, B. M. (2017). Designing 3-Dimensional *in vitro* oviduct culture systems to study mammalian fertilization and embryo production. *Ann. Biomed. Eng.* **45(7)**, 1731–1744.

First, N. L., Short, R. E., Peters, J. B. and Stratman, F. W. (1968). Transport and loss of boar spermatozoa in the reproductive tract of the sow. *J. Anim. Sci.* **27(4)**, 1037–1040.

Flesch, F. M. and Gadella, B. M. (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim. Biophys. Acta - Rev. Biomembr.* **1469(3)**, 197–235.

Flesch, F. M., Brouwers, J. F., Nievelstein, P. F., Verkleij, a J., van Golde, L. M., Colenbrander, B. and Gadella, B. M. (2001). Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *J. Cell Sci.* **114(19)**, 3543–3555.

Foote, R. H. (2002). The history of artificial insemination: selected notes and notables. *J. Anim. Sci.* **80(1968)**, 1–10.

França, L. R. and Cardoso, F. M. (1998). Duration of spermatogenesis and sperm transit time through the epididymis in the Piau boar. *Tissue Cell* **30(5)**, 573–582.

Fraser, L. R. (1990). Sperm capacitation and its modulation. In *Fertilization in Mammals* (ed. Bavister, B. D., Cummings, J. and Roldán, E. R.), pp. 141–153. Norwell (Milan).

Fukui, Y. and Ono, H. (1989). Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. *J. Reprod. Fertil.* **86(2)**, 501–506.

Funahashi, H. (2003). Polyspermic penetration in porcine IVM-IVF systems. *Reprod. Fertil. Dev.* **15(3)**, 167–177.

Funahashi, H. and Day, B. (1993). Effects of follicular fluid at fertilization *in vitro* on sperm penetration in pig oocytes. *J. Reprod. Fertil.* **99(1)**, 97–103.

Funahashi, H. and Nagai, T. (2000). Sperm selection by a climbing-over-a-wall IVF method reduces the incidence of polyspermic penetration of porcine. *J. Reprod. Dev.* **46(5)**, 319–324.

Funahashi, H., Ekwall, H. and Rodriguez-Martinez, H. (2000). Zona reaction in porcine oocytes fertilized *in vivo* and *in vitro* as seen with scanning electron microscopy. *Biol. Reprod.* **63(5)**, 1437–1442.

Funahashi, H., Ekwall, H., Kikuchi, K. and Rodriguez-Martinez, H. (2001). Transmission electron microscopy studies of the zona reaction in pig oocytes fertilized *in vivo* and *in vitro*. *Reproduction* **122(3)**, 443–452.

Gaddum-Rosse, P. (1981). Some observations on sperm transport through the uterotubal junction of the rat. *Am. J. Anat.* **160(3)**, 333–341.

Gadella, B. M. and Harrison, R. A. P. (2000). The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development* **127(11)**, 2407–2420.

Gadella, B. M. and Harrison, R. A. P. (2002). Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biol. Reprod.* **67(1)**, 340–350.

Gadella, B. M. and Van Gestel, R. A. (2004). Bicarbonate and its role in mammalian sperm function. *Anim. Reprod. Sci.* **82–83**, 307–319.

Gadella, B. M., Tsai, P. S., Boerke, A. and Brewis, I. A. (2008). Sperm head membrane reorganisation during capacitation. *Int. J. Dev. Biol.* **52(5-6)**, 473–480.

García-Vázquez, F. A., Hernández-Caravaca, I., Matás, C., Soriano-Úbeda, C., Abril-Sánchez, S. and Izquierdo-Rico, M. J. (2015). Morphological study of boar sperm during their passage through the female genital tract. *J. Reprod. Dev.* **61(5)**, 407–413.

García-Vázquez, F. A., Gadea, J., Matás, C. and Holt, W. V (2016). Importance of sperm morphology during sperm transport and fertilization in mammals. *Asian J. Androl.* **18(6)**, 844–850.

García Herreros, M., Aparicio, I. M., Núñez, I., García-Marín, L. J., Gil, M. C. and Peña Vega, F. J. (2005). Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. *Theriogenology* **63(3)**, 795–805.

Garner, D. L. and Hafez, E. S. E. (2000). Spermatozoa and seminal plasma. In *Reproduction in Farm Animals* (ed. Balado, D.), pp. 96–109. Baltimore: Lippincott Williams and Wilkins.

Georgiou, A. S., Snijders, A. P. L., Sostaric, E., Aflatoonian, R., Vazquez, J. L., Vazquez, J. M., Roca, J., Martinez, E. A., Wright, P. C. and Fazeli, A. (2007). Modulation of the oviductal environment by gametes. *J. Proteome Res.* **6(12)**, 4656–4666.

Green, C. E., Bredl, J., Holt, W. V., Watson, P. F. and Fazeli, A. (2001). Carbohydrate mediation of boar sperm

binding to oviductal epithelial cells *in vitro*. *Reproduction* **122(2)**, 305–315.
Gualtieri, R. and Talevi, R. (2000). *In vitro*-cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. *Biol. Reprod.* **62(2)**, 1754–1762.
Gualtieri, R. and Talevi, R. (2003). Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium *in vitro*. *Reproduction* **125(2)**, 251–258.
Hafez, E. S. E. and Hafez, B. (2000). Transport and survival of gametes. In *Reproduction in Farm Animals* (ed. Balado, D.), pp. 82–95. Baltimore: Lippincott Williams and Wilkins.
Hao, Y., Mathialagan, N., Walters, E., Mao, J., Lai, L., Becker, D., Li, W., Critser, J. and Prather, R. S. (2006). Osteopontin reduces polyspermy during *in vitro* fertilization of porcine oocytes. *Biol. Reprod.* **75(5)**, 726–733.
Harris, J., Hilber, D., Fontenot, G., Hsu, K., Yurewicz, E. and Sacco, A. (1994). Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA Seq. - J. Seq. Mapp.* **4(6)**, 361–393.
Harrison, R. A. P. (1974). The detection of hexokinase, glucosephosphate isomerase and phosphoglucomutase activities in polyacrylamide gels after electrophoresis: A novel method using immobilized glucose 6-phosphate dehydrogenase. *Anal. Biochem.* **61(2)**, 500–507.
Harrison, R. A. P. (1996). Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod. Fertil. Dev.* **8(4)**, 581–594.
Harrison, R. A. P. (2004). Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol. Reprod. Dev.* **67(3)**, 337–352.
Harrison, R. A. P. and Miller, N. G. A. (2000). cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Mol. Reprod. Dev.* **55(2)**, 220–228.
Harrison, R. A. P., Ashworth, P. J. C. and Miller, N. G. A. (1996). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **45(3)**, 378–391.
Hedrick, J. L., Wardrip, N. J. and Berger, T. (1987). Differences in the macromolecular composition of the zona pellucida isolated from pig oocytes, eggs, and zygotes. *J. Exp. Zool.* **241(2)**, 257–262.
Hernández-Caravaca, I., Soriano-Úbeda, C., Matás, C., Izquierdo-Rico, M. J. and García-Vázquez, F. A. (2015). Boar sperm with defective motility are discriminated in the backflow moments after insemination. *Theriogenology* **83(4)**, 655–661.
Hess, K. C., Jones, B. H., Marquez, B., Chen, Y., Ord, T. S., Kamenetsky, M., Miyamoto, C., Zippin, J. H., Kopf, G. S., Suarez, S. S., et al. (2005). The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev. Cell* **9(2)**, 249–259.
Holt, W. V. and Fazeli, A. (2010). The oviduct as a complex mediator of mammalian sperm function and selection. *Mol. Reprod. Dev.* **77(11)**, 934–943.
Holt, W. V., Hernandez, M., Warrell, L. and Satake, N. (2010). The long and the short of sperm selection *in vitro* and *in vivo*: swim-up techniques select for the longer and faster swimming mammalian sperm. *J. Evol. Biol.* **23(3)**, 598–608.
Holtz, W. and Smidt, D. (1976). The fertilizing capacity of epididymal spermatozoa in the pig. *J Reprod Fertil* **46(1)**, 227–229.
Hunter, R. H. F. (1973). Polyspermic fertilization in pigs after tubal deposition of excessive numbers of spermatozoa. *J. Exp. Zool.* **183(1)**, 57–62.
Hunter, R. H. (1974). Chronological and cytological details of fertilization and early embryonic development in the domestic pig, Sus scrofa. *Anat. Rec.* **178(2)**, 169–85.
Hunter, R. H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil.* **63(1)**, 109–117.
Hunter, R. H. F. (1984). Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct. *J. Reprod. Fertil.* **72(1)**, 203–211.
Hunter, R. H. F. (1990). Fertilization of pig eggs *in vivo* and *in vitro*. *J. Reprod. Fertil.* **Suppl. 40**, 211–226.
Hunter, R. H. F. (2008). Sperm release from oviduct epithelial binding is controlled hormonally by peri-ovulatory graafian follicles. *Mol. Reprod. Dev.* **75(1)**, 167–174.
Hunter, R. H. F. and Dziuk, P. J. (1968). Sperm penetration of pig eggs in relation to the timing of ovulation and insemination. *J.Reprod.Fertil.* **15(2)**, 199–208.
Hunter, R. H. F. and Nichol, R. (1988). Capacitation potential of the fallopian tube: a study involving surgical insemination and the subsequent incidence of polyspermy. *Gamete Res.* **21(3)**, 255–266.

Hunter, R. H. and Rodriguez-Martinez, H. (2002). Analysing mammalian fertilisation: reservations and potential pitfalls with an *in vitro* approach. *Zygote* **10(1)**, 11–15.

Hunter, R. H. F. and Rodriguez-Martinez, H. (2004). Capacitation of mammalian spermatozoa *in vivo*, with a specific focus on events in the Fallopian tubes. *Mol. Reprod. Dev.* **67(2)**, 243–250.

Joseph, A., Yao, H. and Hinton, B. T. (2009). Development and morphogenesis of the Wolffian/epididymal duct, more twists and turns. *Dev. Biol.* **325(1)**, 6–14.

Kaczmarek, M. M., Krawczynski, K., Blitek, A., Kiewisz, J., Schams, D. and Ziecik, A. J. (2010). Seminal plasma affects prostaglandin synthesis in the porcine oviduct. *Theriogenology* **74(7)**, 1207–1220.

Katila, T. (2012). Post-mating inflammatory responses of the uterus. *Reprod. Domest. Anim.* **47(s5)**, 31–41.

Kawano, N., Araki, N., Yoshida, K., Hibino, T., Ohnami, N., Makino, M., Kanai, S., Hasuwa, H., Yoshida, M., Miyado, K., et al. (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proc. Natl. Acad. Sci.* **111(11)**, 4145–4150.

Killian, G. J. (2004). Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Anim. Reprod. Sci.* **82–83**, 141–153.

Kim, N. H., Funahashi, H., Abeydeera, L. R., Moon, S. J., Prather, R. S. and Day, B. N. (1996). Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes *in vitro*. *J. Reprod. Fertil.* **107(1)**, 79–86.

Kim, N. H., Day, B. N., Lim, J. G., Lee, H. T. and Chung, K. S. (1997). Effects of oviductal fluid and heparin on fertility and characteristics of porcine spermatozoa. *Zygote* **5(1)**, 61–65.

Kim, E., Yamashita, M., Kimura, M., Honda, A., Kashiwabara, S. I. and Baba, T. (2008). Sperm penetration through cumulus mass and zona pellucida. *Int. J. Dev. Biol.* **52(5-6)**, 677–682.

Kouba, A. J., Abeydeera, L. R., Alvarez, I. M., Day, B. N. and Buhi, W. C. (2000). Effects of the porcine oviduct-specific glycoprotein on fertilization, polyspermy, and embryonic development *in vitro*. *Biol. Reprod.* **63(1)**, 242–250.

Langendijk, P., Soede, N. M. and Kemp, B. (2005). Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. *Theriogenology* **63(2)**, 500–513.

Leese, H. J. (1988). The formation and function of oviduct fluid. *J. Reprod. Fertil.* **82(2)**, 843–856.

Leese, H. J., Tay, J. I., Reischl, J. and Downing, S. J. (2001). Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction* **121(3)**, 339–346.

Li, Y.-H., Li, M., Hou, Y., Jiao, L.-H. and Wang, W.-H. (2003). Reduced polyspermic penetration in porcine oocytes inseminated in a new *in vitro* fertilization (IVF) system: straw IVF. *Biol. Reprod.* **69(5)**, 1580–1585.

Lishko, P. V., Botchkina, I. L., Fedorenko, A. and Kirichok, Y. (2010). Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell* **140(3)**, 327–337.

Liu, Y., Wang, D.-K. and Chen, L.-M. (2012). The physiology of bicarbonate transporters in mammalian reproduction. *Biol. Reprod.* **86(4)**, 99–99.

Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D. and Darnell, J. (2000). *Molecular Cell Biology*. 4th ed. (ed. Company, W. H. F. and) New York.

López-Úbeda, R., García-Vázquez, F. A., Gadea, J. and Matás, C. (2017). Oviductal epithelial cells selected boar sperm according to their functional characteristics. *Asian J. Androl.* **19(4)**, 396–403.

Mann, T. and Lutwak-Mann, C. (1981). *Male reproductive function and semen. Themes and trends in physiology, biochemistry and investigative andrology*. Berlin: Springer-Verlag.

Matás, C., Coy, P., Romar, R., Marco, M., Gadea, J. and Ruiz, S. (2003). Effect of sperm preparation method on *in vitro* fertilization in pigs. *Reproduction* **125(1)**, 133–141.

Matás, C., Sansegundo, M., Ruiz, S., García-Vázquez, F. A., Gadea, J., Romar, R. and Coy, P. (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. *Theriogenology* **74(8)**, 1327–1340.

Matás, C., Vieira, L., García-Vázquez, F. A., Avilés-López, K., López-Úbeda, R., Carvajal, J. A. and Gadea, J. (2011). Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. *Anim. Reprod. Sci.* **127(1-2)**, 62–72.

Matthijs, A., Engel, B. and Woelders, H. (2003). Neutrophil recruitment and phagocytosis of boar spermatozoa after artificial insemination of sows, and the effects of inseminate volume, sperm dose and specific additives in the extender. *Reproduction* **125(3)**, 357–367.

Mattioli, M., Galeati, G., Bacci, M. L. and Seren, E. (1988). Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. *Gamete Res.* **21(3)**, 223–232.

Mattioli, M., Lucidi, P. and Barboni, B. (1998). Expanded cumuli induce acrosome reaction in boar sperm. *Mol.*

Reprod. Dev. **51(4)**, 445–453.
Mourad, N. I. and Gianello, P. (2017). Gene editing, gene therapy, and cell xenotransplantation: cell transplantation across species. *Curr. Transplant. Reports* **4(3)**, 193–200.
Nagai, T. (1994). Current status and perspectives in IVM-IVF of porcine oocytes. *Theriogenology* **41(1)**, 73–78.
Nagai, T. and Moor, R. M. (1990). Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized *in vitro*. *Mol. Reprod. Dev.* **26(4)**, 377–382.
Newcombe, N., Clulow, J., Man, S. Y. and Jones, R. C. (2000). pH and bicarbonate in the ductuli efferentes testis of the rat. *Int. J. Androl.* **23(1)**, 46–50.
Nichol, R., Hunter, R. H. F., Gardner, D. K., Leese, H. J. and Cooke, G. M. (1992). Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. *J. Reprod. Fertil.* **96(2)**, 699–707.
Nichol, R., Hunter, R. H. F. and Cooke, G. M. (1997). Oviduct fluid pH in intact and unilaterally ovariectomized pigs. *Can. J. Physiol. Pharmacol.* **75(9)**, 1069–1074.
Nishigaki, T., José, O., González-Cota, A. L., Romero, F., Treviño, C. L. and Darszon, A. (2014). Intracellular pH in sperm physiology. *Biochem. Biophys. Res. Commun.* **450(3)**, 1149–1158.
Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J. Biol. Chem.* **260(17)**, 9699–9705.
Okazaki, T., Akiyoshi, T., Kan, M., Mori, M., Teshima, H. and Shimada, M. (2012). Artificial insemination with seminal plasma improves the reproductive performance of frozen-thawed boar epididymal spermatozoa. *J. Androl.* **33(5)**, 990–998.
Oliphant, G. (1974). Role of seminal plasma in sperm capacitation. In *Federation Proceedings*, p. 289. Rockville Pike, Bethesda, Maryland.
Ombelet, W. and Van Robays, J. (2015). Artificial insemination history: hurdles and milestones. *Facts, views Vis. ObGyn* **7(2)**, 137–43.
Orlowski, J. and Grinstein, S. (2011). Na^+/H^+ exchangers. *Compr. Physiol.* **1(4)**, 2083–2100.
Park, C. H., Lee, S. G., Choi, D. H. and Lee, C. K. (2009). A modified swim-up method reduces polyspermy during *in vitro* fertilization of porcine oocytes. *Anim. Reprod. Sci.* **115(1-4)**, 169–181.
Parrish, J. J. (2014). Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81(1)**, 67–73.
Pérez-Cerezales, S., Boryshpolets, S. and Eisenbach, M. (2015). Behavioral mechanisms of mammalian sperm guidance. *Asian J. Androl.* **17(4)**, 628.
Petrunkina, A. M., Gehlhaar, R., Drommer, W., Waberski, D. and Töpfer-Petersen, E. (2001a). Selective sperm binding to pig oviductal epithelium *in vitro*. *Reproduction* **121(6)**, 889–896.
Petrunkina, A. M., Friedrich, J., Drommer, W., Bicker, G., Waberski, D. and Töpfer-Petersen, E. (2001b). Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic Ca^{2+} concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction* **122(3)**, 469–480.
Petrunkina, A. M., Waberski, D., Günzel-Apel, A. R. and Töpfer-Petersen, E. (2007). Determinants of sperm quality and fertility in domestic species. *Reproduction* **134(1)**, 3–17.
Piehl, L. L., Fischman, M. L., Hellman, U., Cisale, H. and Miranda, P. V. (2013). Boar seminal plasma exosomes: Effect on sperm function and protein identification by sequencing. *Theriogenology* **79(7)**, 1071–1082.
Quinn, P. (1995). Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J. Assist. Reprod. Genet.* **12(2)**, 97–105.
Rath, D., Long, C. R., Dobrinsky, J. R., Welch, G. R., Schreier, L. L. and Johnson, L. A. (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77(12)**, 3346–3352.
Rath, D., Schuberth, H. J., Coy, P. and Taylor, U. (2008). Sperm interactions from insemination to fertilization. *Reprod. Domest. Anim.* **43(s5)**, 2–11.
Rath, D., Knorr, C. and Taylor, U. (2016). Communication requested: Boar semen transport through the uterus and possible consequences for insemination. *Theriogenology* **85(1)**, 94–104.
Robertson, S. A. (2005). Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res.* **322(1)**, 43–52.
Robertson, S. A. (2007). Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs.

J. Anim. Sci. **85(s13)**, E36–E44.
Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation. *Theriogenology* **68(s1)**, 138–146.
Rodriguez-Martinez, H., Ekstedt, E. and Einarsson, S. (1990). Acidification of epididymal fluid in the boar. *Int. J. Androl.* **13(3)**, 238–243.
Rodriguez-Martinez, H., Larsson, B., Pertoft, H. and Kjellén, L. (1998). GAGs and spermatozoon competence *in vivo* and *in vitro*. In *Gametes: Development and Function* (ed. Lauria, A., Gandolfi, F., Enne, G. and Gianaroli, L.), pp. 239–274. Italy: Serono Symposia.
Rodriguez-Martinez, H., Tienthai, P., Suzuki, K., Funahashi, H., Ekwall, H. and Johannisson, A. (2001). Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reprod. Suppl.* **58**, 129–145.
Rodríguez-Martínez, H., Saravia, F., Wallgren, M., Tienthai, P., Johannisson, A., Vázquez, J. M., Martínez, E., Roca, J., Sanz, L. and Calvete, J. J. (2005). Boar spermatozoa in the oviduct. *Theriogenology* **63(2)**, 514–535.
Romar, R., Funahashi, H. and Coy, P. (2016). *In vitro* fertilization in pigs: new molecules and protocols to consider in the forthcoming years. *Theriogenology* **85(1)**, 125–134.
Rozeboom, K. J., Troedsson, M. H. T. and Crabo, B. G. (1998). Characterization of uterine leukocyte infiltration in gilts after artificial insemination. *J. Reprod. Fertil.* **114(2)**, 195–199.
Sano, H., Matsuura, K., Naruse, K. and Funahashi, H. (2010). Application of a microfluidic sperm sorter to the in-vitro fertilization of porcine oocytes reduced the incidence of polyspermic penetration. *Theriogenology* **74(5)**, 863–870.
Satake, N., Elliott, R. M. A., Watson, P. F. and Holt, W. V (2006). Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. *J. Exp. Biol.* **209(8)**, 1560–72.
Schuberth, H. J., Taylor, U., Zerbe, H., Waberski, D., Hunter, R. and Rath, D. (2008). Immunological responses to semen in the female genital tract. *Theriogenology* **70(8)**, 1174–1181.
Setchell, B. P., Sånchez-Partida, L. G. and Chairussyuhur, A. (1993). Epididymal constituents and related substances in the storage of spermatozoa: a review. *Reprod. Fertil. Dev.* **5(6)**, 601–612.
Sherman, J. K. (1978). History of artificial insemination and the development of human semen banking. In *The Integrity of Frozen Spermatozoa*, pp. 201–207. Washington, D.C.: National Academies Press, National Academy of Sciences.
Sidhu, K. S. and Guraya, S. S. (1989). Cellular and molecular biology of capacitation and acrosome reaction in mammalian spermatozoa. *Int. Rev. Cytol.* **118**, 231–80.
Smith, T. T. (1998). The modulation of sperm function by the oviductal epithelium. *Biol. Reprod.* **58(5)**, 1102–4.
Soriano-Úbeda, C., Matás, C. and Fa, G. (2013). An overview of swine artificial insemination: retrospective, current and prospective aspects. *J. Exp. Appl. Anim. Sience* **1(1)**, 67–97.
Staicu, F. D. and Matás, C. (2017). Nitric oxide: key features in spermatozoa. In *Nitric Oxide Synthse-Simple Enzyme-Complex Roles* (ed. Dr. Seyed Soheil Saeedi Saravi), pp. 75–100. InTech.
Steptoe, P. C. and Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet* **7**, 366.
Steverink, D. W. B., Soede, N. M., Bouwman, E. G. and Kemp, B. (1998). Semen backflow after insemination and its effect on fertilisation results in sows. *Anim. Reprod. Sci.* **54(2)**, 109–119.
Strzezek, J. (2002). Secretory activity of boar seminal vesicle glands. *Reprod. Biol.* **2(3)**, 243–266.
Suarez, S. S. (1998). The oviductal sperm reservoire in mammals: mechanisms of formation. *Biol. Reprod.* **58(5)**, 1105–1107.
Suarez, S. S. (1999). Regulation of sperm transport in the mammalian oviduct. In *The male gamete* (ed. Gagnon, C.), pp. 71–80. Vienna: Cache River Press.
Suarez, S. S. (2007). Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. *Reprod. Fertil. Dev.* **19(1)**, 103–110.
Suarez, S. S., Dai, X.-B., DeMott, R. P., Redfern, K. and Mirando, M. A. (1992). Movement characteristics of boar sperm obtained from the oviduct or hyperactivated *in vitro*. *J. Androl.* **13(1)**, 75–80.
Sumransap, P., Tummaruk, P. and Kunavongkrit, A. (2007). Sperm distribution in the reproductive tract of sows after intrauterine insemination. *Reprod. Domest. Anim.* **42(2)**, 113–117.
Suzuki, K., Eriksson, B., Shimizu, H., Nagai, T. and Rodriguez-Martinez, H. (2000). Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized *in vitro*. *Int. J. Androl.* **23(1)**, 13–21.
Swierstra, E. E. (1968). Cytology and duration of the cycle of the seminiferous epithelium of the boar; duration of spermatozoan transit through the epididymis. *Anat. Rec.* **161(2)**, 171–185.
Syntin, P., Dacheux, F., Druart, X., Gatti, J. L., Okamura, N. and Dacheux, J. L. (1996). Characterization and

identification of proteins secreted in the various regions of the adult boar epididymis. *Biol. Reprod.* **55(5)**, 956–974.

Tajima, Y., Okamura, N. and Sugita, Y. (1987). The activating effects of bicarbonate on sperm motility and respiration at ejaculation. *Biochim. Biophys. Acta* **924(3)**, 519–529.

Talevi, R. and Gualtieri, R. (2010). Molecules involved in sperm-oviduct adhesion and release. *Theriogenology* **73(6)**, 796–801.

Taylor, U., Rath, D., Zerbe, H. and Schuberth, H. J. (2008). Interaction of intact porcine spermatozoa with epithelial cells and neutrophilic granulocytes during uterine passage. *Reprod. Domest. Anim.* **43(2)**, 166–175.

Taylor, U., Schuberth, H. J., Rath, D., Michelmann, H. W., Sauter-Louis, C. and Zerbe, H. (2009). Influence of inseminate components on porcine leucocyte migration *in vitro* and *in vivo* after pre- and post-ovulatory insemination. *Reprod. Domest. Anim.* **44(2)**, 180–188.

Tienthai, P. (2015). The porcine sperm reservoir in relation to the function of hyaluronan. *J. Reprod. Dev.* **61(4)**, 245–50.

Tienthai, P., Kjellén, L., Pertoft, H., K, S. and Rodriguez-Martinez, H. (2000). Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. *Reprod. Fertil. Dev.* **12(4)**, 173–182.

Tienthai, P., Johannisson, A. and Rodriguez-Martinez, H. (2004). Sperm capacitation in the porcine oviduct. *Anim. Reprod. Sci.* **80(1-2)**, 131–146.

Tomlinson, M., White, A., Barratt, C., Bolton, A. and Cooke, I. (1992). The removal of morphologically abnormal sperm forms by phagocytes: a positive role for seminal leykocytes? *Hum Reprod.* **7(4)**, 517–22.

Töpfer-Petersen, E. and Henschen, A. (1987). Acrosin shows zona and fucose binding, novel properties for a serine proteinase. *FEBS Lett.* **226(1)**, 38–42.

Töpfer-Petersen, E., Friess, A. E., Nguyen, H. and Schill, W. B. (1985). Evidence for a fucose-binding protein in boar spermatozoa. *Histichemistry* **83(2)**, 139–145.

Töpfer-Petersen, E., Romero, A., Varela, P. F., Ekhlasi-Hundrieser, M., Dostàlovà, Z., Sanz, L. and Calvete, J. J. (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* **30(4-5)**, 217–224.

Töpfer-Petersen, E., Wagner, A., Friedrich, J., Petrunkina, A., Ekhlasi-Hundrieser, M., Waberski, D. and Drommer, W. (2002). Function of the mammalian oviductal sperm reservoir. *J. Exp. Zool.* **292(2)**, 210–215.

Töpfer-Petersen, E., Ekhlasi-Hundrieser, M. and Tsolova, M. (2008). Glycobiology of fertilization in the pig. *Int. J. Dev. Biol.* **52(5-6)**, 717–736.

Vadnais, M. L., Galantino-Homer, H. L. and Althouse, G. C. (2007). Current concepts of molecular events during bovine and porcine spermatozoa capacitation. *Arch. Androl.* **53(3)**, 109–123.

Visconti, P. E. (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proc. Natl. Acad. Sci.* **106(3)**, 667–668.

Visconti, P. E. and Kopf, G. S. (1998). Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod.* **59(1)**, 1–6.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995a). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121(4)**, 1139–1150.

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995b). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121(4)**, 1129–1137.

Waberski, D., Magnus, F., Ardón, F., Petrunkina, A. M., Weitze, K. F. and Töpfer-Petersen, E. (2006). Binding of boar spermatozoa to oviductal epithelium *in vitro* in relation to sperm morphology and storage time. *Reproduction* **131(2)**, 311–318.

Waberski, D., Petrunkina, A. M. and Töpfer-Petersen, E. (2008). Can external quality control improve pig AI efficiency? *Theriogenology* **70(8)**, 1346–1351.

Wagner, A., Ekhlasi-Hundrieser, M., Hettel, C., Petrunkina, A., Waberski, D., Nimtz, M. and Töpfer-Petersen, E. (2002). Carbohydrate-based interactions of oviductal sperm reservoir formation - Studies in the pig. *Mol. Reprod. Dev.* **61(2)**, 249–257.

Wang, W., Abeydeera, L. R., Prather, R. S. and Day, B. N. (1998). Morphologic comparison of ovulated and *in vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. *Mol. Reprod. Dev.* **49(3)**, 308–316.

Watson, P. F. and Green, C. E. (2000). Cooling and capacitation of boar spermatozoa: what do they have in common? In *Boar Semen Preservation IV* (ed. Johnson, L. A. and Guthrie, H. D.), pp. 53–60. Lawrence: Allen Press.

Watson, A. L., Carlson, D. F., Largaespada, D. A., Hackett, P. B. and Fahrenkrug, S. C. (2016). Engineered swine models of cancer. *Front. Genet.* **7(78)**, 1–16.

Wennemuth, G., Carlson, A. E., Harper, A. J. and Babcock, D. F. (2003). Bicarbonate actions on flafellar and Ca^{2+}-channel responses: initial events in sperm activation. *Development* **130(7)**, 1317–1326.

Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. In *Fertilization and embrionic development* in vitro (ed. Mastroianni, L. and Biggers, J.), pp. 81–182. New York: Plenum Press.

Yanagimachi, R. (1994a). Fertility of mammalian spermatozoa: its development and relativity. *Zygote* **2(4)**, 371–372.

Yanagimachi, R. (1994b). Mammalian fertilization. In *The Physiology of Reproduction* (ed. Kenobil, E. and Neill, J. D.), pp. 189–317. New York: Raven Press.

Yeste, M., Lloyd, R. E., Badia, E., Briz, M., Bonet, S. and Holt, W. V. (2009). Direct contact between boar spermatozoa and porcine oviductal epithelial cell (OEC) cultures is needed for optimal sperm survival *in vitro*. *Anim. Reprod. Sci.* **113(1-4)**, 263–278.

Yeste, M., Castillo-Martín, M., Bonet, S. and Briz, M. D. (2012). Direct binding of boar ejaculate and epididymal spermatozoa to porcine epididymal epithelial cells is also needed to maintain sperm survival in *in vitro* co-culture. *Anim. Reprod. Sci.* **131(3-4)**, 181–193.

Yoshioka, K., Suzuki, C. and Onishi, A. (2008). Defined system for *in vitro* production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54(3)**, 208–213.

Zhou, C. X., Wang, X. F. and Chan, H. C. (2005). Bicarbonate secretion by the female reproductive tract and its impact on sperm fertilizing capacity. *Acta Physiol. Sin.* **57(2)**, 115–124.

Zini, A., De Lamirande, E. and Gagnon, C. (1993). Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int. J. Androl.* **16(3)**, 183–188.

Hypothesis and Objectives

The working hypothesis is that by simulating the *in vivo* porcine oviductal environment through certain factors of the periovulatory stage, the efficiency of *in vitro* fertilization in this species could be increased.

To confirm this hypothesis, the main objective of this thesis was to focus on how the oviductal environment modulates *in vitro* sperm capacitation and fertilization. For this purpose, the behavior of spermatozoa subjected to different *in vivo* periovulatory oviductal conditions, such as bicarbonate concentration, pH and biofluids components, were studied. To achieve this main objective, the following specific objectives were established:

Objective 1 (Chapter 1). Effect of the different bicarbonate concentrations (0, 5, 15 and 25 millimolar) in:

1.1. Spermatozoa functionality related to protein kinase A substrates phosphorylation, intracellular pH, motility, tyrosine phosphorylation, viability and acrosome reaction.

1.2. *In vitro* fertilization performed in a one-phase (monophasic) process, in which spermatozoa and oocytes were co-incubated in the same bicarbonate concentration, or in two phases (biphasic) in which spermatozoa were first pre-incubated in a given bicarbonate concentration and then co-incubated with oocytes in a medium with the same or higher bicarbonate concentration.

Objective 2 (Chapter 2). Effect of the pH of the medium, periovulatory oviductal secretions and a selective device (spermatozoa must swim to overcome a physical barrier to contact the oocytes) in:

2.1. *In vitro* fertilization efficiency in a medium of different pH values (8.0 *vs.* 7.4), periovulatory oviductal secretions (present or absent) in a selective or non-selective device.

2.2. Spermatozoa functionality related to motility, protein kinase A substrates and tyrosine phosphorylation, acrosome reaction and intracellular pH.

Chapter 1

A 15 millimolar concentration of bicarbonate is sufficient to allow epididymal sperm capacitation and improve IVF output in porcine

The concentration of bicarbonate (HCO_3^-), one of the sperm capacitating effectors through activating the soluble adenylyl cyclase (sAC)/protein kinase A (PKA) pathway, varies greatly in the different environments spermatozoa go through from the cauda epididymis (~3-4 mM) to the oviduct (from 10 mM in the isthmus to ~25 mM in the fertilization site). However, the *in vitro* sperm capacitation and fertilization media usually contain a standard concentration of 25 mM. This work studied the effects of different HCO_3^- concentrations (0, 5, 15 and 25 mM) on epididymal sperm functionality, including *in vitro* fertility. The intracellular pH (pH_i) of spermatozoa increased after HCO_3^- uptake, but only extracellular concentrations of and above 15 mM activate the PKA capacitation cascade, although it took significantly longer than the usual concentration of 25 mM. 15 mM of HCO_3^- also stimulated sperm linear motility and increased other late events in capacitation such as tyrosine phosphorylation (Tyr-P). Based in this knowledge, we were able to establish an IVF method in two phases (biphasic) in which spermatozoa are first pre-incubated in a given HCO_3^- concentration and then co-incubated with oocytes in the same HCO_3^- concentration or higher. Both pre-incubation and IVF at 15 mM of HCO_3^- significantly reduced polyspermy. This system achieved a 33.9% of viable zygotes, which supposed a significant increase of 25.3% with respect to the usual one-phase system in which spermatozoa are directly inseminated and co-incubated with oocytes at 25 mM.

1. INTRODUCTION

Fertilization is considered a multifactorial process in which many factors are directly and/or indirectly involved. The success rate of this physiological process depends to a high degree on the ability of spermatozoa to undergo capacitation and penetrate the oocyte vestments (Yanagimachi, 1994). *In vivo* sperm capacitation is a gradual event that is actively and progressively coordinated within succeeding segments of the female genital tract (Hunter and Rodriguez-Martinez, 2004). The changes occurring in spermatozoa during *in vivo* capacitation begin as soon as they are ejaculated as a result of, among other reasons, the bicarbonate (HCO_3^-) intake, subsequent intracellular pH alkalinization (pH_i) and motility activation (fast capacitation events) (Visconti, 2009). Once inside the female genital tract, other events occur in a sequential and parallel way for an extended period of time, such as hyperactivation, tyrosine phosphorylation (Tyr-P) and preparation to undergo acrosome reaction (AR) (slow capacitation events). Both fast and slow events depend on the HCO_3^- and Ca^{2+} concentrations, but the slow ones also depend on the presence of cholesterol acceptors like albumin (reviewed by Visconti, 2009). The inward transport of HCO_3^- from the surrounding medium during capacitation depends on the specific electrogenic Na^+/HCO_3^- co-transporter (NBC) or Cl^-/HCO_3^- anion exchanger (Liu et al., 2012). After changes in sperm membrane fluidity and lipid scrambling, protein kinase A (PKA) activation is regulated by the soluble adenylyl cyclase (sAC)/cyclic AMP (cAMP) HCO_3^--dependent pathway (Visconti et al., 1995a). In the presence of albumin, HCO_3^- leads to the activation of the sAC of the sperm membrane, which catalyzes cAMP metabolism. Numerous studies have indicated that cAMP plays a major secondary messenger role in PKA substrates phosphorylation (PKAs-P) and the initiation of many changes that depend on it, such as motility, Tyr-P and AR (Visconti, 2009). Previous studies in porcine species suggest that PKAs-P rapidly increases after about 160 s through the action of

HCO_3^-, after which the activity increases slowly to reach a maximum at 21 min (Harrison, 2004). The HCO_3^- seems to act as a key capacitating agent, and its importance in mediating changes in spermatozoa has been demonstrated in multiple studies in mice and humans. Knock-out mice that fail to express HCO_3^- responsive proteins were infertile (Wang et al., 2004) mainly because of the failure to activate sAC (reviewed by Gadella and Van Gestel, 2004), and females with defective HCO_3^- secretion in the uterus produced low sperm capacitation and fertility (Wang et al., 2004).

After maturing as they pass through the epididymis, the spermatozoa are stored in the epididymal cauda in low HCO_3^- conditions (~3-4 mM, Rodriguez-Martinez et al., 1990). During ejaculation, the spermatozoa come into contact with the seminal plasma (SP), which contains higher concentrations of HCO_3^- (about 20 mM, Okamura et al., 1985) but which also contains decapacitating factors, mainly spermadhesins from accessory sex glands, that coat and stabilize the sperm surface to prevent premature capacitation (Töpfer-Petersen et al., 1998). Once in the female genital tract, sperm is subjected to different selection mechanisms that remove immature or damaged spermatozoa. As they move forward into the uterus the spermatozoa have to swim through several environments increasingly rich in HCO_3^- (~25 mM) (Hess et al., 2005) and the decapacitating factors are gradually removed, enabling membrane destabilization and capacitation. When in the oviduct, they remain attached to the epithelium after utero-tubal junction, in the caudal isthmus, establishing the sperm reservoir (SR). Once ovulation occurs, the spermatozoa are gradually released from the SR and swim to the fertilization site at the ampullary-isthmic junction (Hunter, 1981). However, many of the signs of ovulation and mechanisms of gradual sperm release from the reservoir are still unknown, and, *in vitro*, conditions are far from ideal for exposing spermatozoa to capacitating conditions gradually. Currently, most of the *in vitro* capacitation and fertilization (IVF) methods, in porcine and also other species, expose all the spermatozoa to a fixed HCO_3^- concentration of 25 mM at the same time (Parrish, 2014; Yoshioka et al., 2008; Bedu-Addo et al., 2007; Coy et al., 2002; Rath et al., 1999; Quinn, 1995), providing a static IVF system that little resembles the dynamic system of fluids to which gametes are exposed *in vivo*. Despite the development of chemically defined fertilization media (Rath et al., 1999; Abeydeera and Day, 1997; Mattioli et al., 1988), different spermatozoa and oocytes co-incubation times (Coy et al., 1993), sperm capacitation methods (Matás et al., 2003) and the use of innovative IVF methods in straws or in devices in which both gametes are physically separated (Li et al., 2003), one of the major problems in porcine IVF – polyspermy – persists. The microenvironment of *in vitro* capacitation and fertilization currently used in laboratories is probably far from optimal for porcine species, and further studies of the special features of pig gametes are necessary to increase the yield of these techniques.

Although HCO_3^- concentrations in the epididymis are ~3-4 mM and can reach >20 mM in the oviduct, current systems of sperm capacitation and IVF use media with a fixed concentration of 25 mM. The objective of this work was to study the effect of increasing concentrations of HCO_3^- on sperm capacitation, mimicking as far as possible the increase that takes place *in vivo*. For this purpose, different HCO_3^- concentrations were tested on PKAs activation, pH_i and sperm motility, and on several subsequent events to PKAs activation in the cascade of capacitation, such as Tyr-P and AR. Finally, in an evaluation of the effect of HCO_3^- on sperm functionality, *in vitro*

fertility parameters were studied under different HCO_3^- concentrations. The results of this study could increase our knowledge of sperm physiology and lead to an improvement in *in vitro* sperm capacitation and oocyte fertilization output in porcine species.

2. MATERIAL AND METHODS

2.1. Ethics

The study was carried out following the Spanish Policy for Animal Protection RD 53/2013, which meets European Union Directive 2010/63/UE on animal protection. All the procedures carried out in this work were approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (ref. no. A13160609).

2.2. Sperm collection

Epididymes from mature boars were obtained from a slaughterhouse and transported to the laboratory within 30 min of slaughter. The cauda epididymis was dissected and the epididymal duct was isolated. A 24G BD Insyte™ catheter (381212, Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah, USA) adapted to a syringe was inserted into the epididymal duct, and sperm samples were obtained by pressurized air through the deferens duct.

2.3. Western blotting (WB)

Isolated proteins from 1 x 10^6 spermatozoa were obtained as described (Navarrete et al., 2015). The primary polyclonal antibodies used were anti-phospho-PKA substrates (9624, Cell Signaling Technology, Beverly, USA, 1:2,000) and anti-β-actin HRP (β-act) (AB20272, Abcam PLC, Cambridge, UK, 1:5,000) or anti-β-tubulin (β-tub) (T0198, Sigma-Aldrich®, Madrid, Spain, 1:5,000). For membranes stripping, a solution of 2% SDS, 0.74% β-mercaptoethanol, 62.5 mM Tris and pH 6.5 was used at 60ºC for 20 min. Blots were visualized by chemiluminescence (Amersham Imager 600, GE Healthcare) using a Pierce® ECL 2 Western Blotting Substrate (80196, Lumigen Inc, Southfield, MI, USA). The relative amount of signal in each membrane was semi-quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK).

2.4. Measurement of spermatozoa intracellular pH (pH_i)

The analysis of pH_i was carried out in spermatozoa (30 x 10^6 cells/ml) loaded with 5 µM of the pH-sensitive dye acetoxymethyl ester of bis-carboxyethyl-carboxyfluorescein (BCECF-AM, B1150, Sigma-Aldrich®, Madrid, Spain) incubated for 30 min at 38.5ºC. The samples were centrifuged at 700g for 3 min to remove the excess of dye and resuspended in PBS without Ca^{2+} and Mg^{2+} and incubated again for 15 min at 38.5ºC for the de-esterification of the dye. Finally, the samples were centrifuged and resuspended in the corresponding medium according to the experimental group. The fluorescence was monitored using a spectrofluorometer (FP-6300, Jasco®, Cremella, Italy). A calibration of the system was first performed using BCECF-AM stained and equilibrated spermatozoa at pH 6.0, 6.5, 7.0, 7.5 and 8.0 in the presence of 0.1% Triton X-100 and adjusting the pH with HCl and NaOH (Marquez and Suarez, 2007). The emitted fluorescence ratio from the excitation at 490/440 nm was calculated and the regression line for pH_e *vs.* the 490/440 nm ratio was obtained. The pH_i of sperm cells was estimated from the regression line.

2.5. Spermatozoa motion assay

Motility parameters were evaluated using a computer-assisted spermatozoa motility analysis (CASA), the ISAS® system (PROISER R+D S.L., Valencia, Spain), connected to a negative phase-contrast microscope (magnification x100; Leica DMR, Wetzlar, Germany) and a digital camera (Basler Vision, Ahrensburg, Germany). For this purpose, 4 µl of samples were placed on a warmed (38.5ºC) 20 micron SpermTrack® chamber (STP-21006, PROISER R+D, S.L., Valencia, Spain).

2.6. Immunocytochemistry: tyrosine phosphorylation detection by indirect immunofluorescence (IIF)

IIF was performed as described Luño et al., (2013) to study sperm Tyr-P. The primary antibody used was anti-phosphotyrosine (4G10, Millipore, CA, USA, 1:300) and the secondary antibody was fluorescein-conjugated goat anti-mouse (Bio-Rad Laboratories, Madrid, Spain, 1:400).

2.7. *In vitro* fertilization (IVF)

Porcine oocytes were isolated from ovaries obtained from 6- to 7-month old animals at a local slaughterhouse and transported to the laboratory in saline solution at 38.5ºC. Once in the laboratory, the ovaries were washed in 0.04% cetrimide solution (w/v) and saline. Cumulus-oocytes complexes (COCs) were collected from antral follicles (3-6 mm diameter) and washed twice with Dulbecco's PBS. Groups of 50 COCs with complete and dense cumulus oophorus were *in vitro* matured in 500 µl NCSU-37 medium (Petters and Wells, 1993) for 42 h, as previously described Funahashi et al. (1997). After *in vitro* maturation (IVM), the COCs were

gently pipetted to remove the cumulus. The medium used in IVF was Tyrode's albumin lactate pyruvate medium (TALP) (Rath et al., 1999) with different HCO_3^- concentrations: 0, 5, 15 or 25 mM. Prior to IVF, all media were adjusted to pH 7.4. The pH of the medium containing 0 mM was adjusted using NaOH and HCl, and the pH of the media containing 5, 15 and 25 mM was adjusted in an incubator for almost 3 h at 38.5ºC, saturation humidity and 1.0, 3.0 or 5.0% of CO_2 atmosphere in air, respectively, according to the Henderson-Hasselbalch equation. Groups of 50 oocytes were transferred to a 4-well multidish containing 500 µl per well of the corresponding TALP medium. The sperm were added to give a final concentration of 5 x 10^4 cells/ml. After 18 h of co-culture, putative zygotes were fixed and evaluated as previously described by Matás et al. (2010) under an epifluorescence microscope at x400 (Leica® DMR, USA).

3. EXPERIMENTAL DESIGN

HCO_3^- is believed to be the key effector in sperm capacitation due to its role in plasma membrane modifications during the sperm capacitation process (Flesch and Gadella, 2000) and it directly stimulates, among other things, the sAC of sperm (Litvin et al., 2003). In an attempt to optimize the HCO_3^- concentration in a chemically defined medium for porcine *in vitro* sperm capacitation and fertilization, this study used matured spermatozoa that had not been in contact with high concentrations of HCO_3^-. Spermatozoa from the cauda epididymis were exposed to different HCO_3^- concentrations similar to the existing when they are ejaculated and mixed with the accessory sex gland secretions and placed in the vicinity of the oocyte in the oviduct. Indicators of sperm functionality and capacitation status (PKAs-P, pH_i, motility, Tyr-P, viability and AR) were evaluated in capacitating conditions. Finally, since the capacitation process is a regulatory event that precedes the encounter with oocytes, this study determined the *in vitro* fertilizing ability of spermatozoa in the different HCO_3^- conditions studied. The experimental groups of spermatozoa incubated in a capacitating medium (TALP) were established according to the HCO_3^- concentration of that medium: 0 mM, 5 mM, 15 mM and 25 mM. Moreover, a non-capacitating control group was incubated in PBS (NCAP). For this purpose, two experiments were performed (Figure 1):

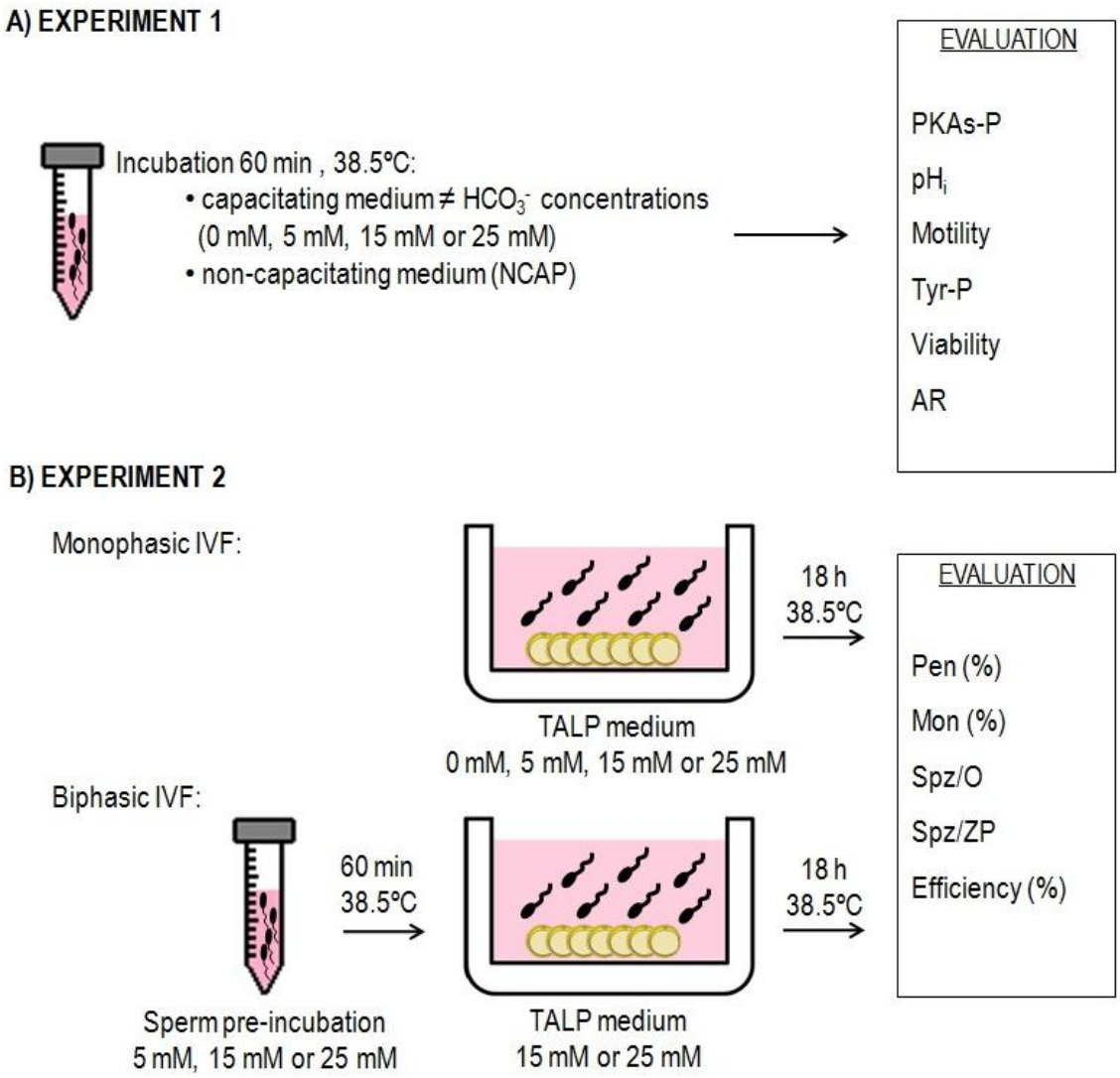


Figure 1. Experimental design: effect of HCO_3^- concentration on sperm functionality and fertility in capacitating conditions. A) Experiment 1: Analysis of sperm functionality. Spermatozoa were incubated in capacitating medium (TALP) with different HCO_3^- concentrations (0 mM, 5 mM, 15 mM and 25 mM) or in non-capacitating medium (NCAP) at 38.5ºC for 60 min. Protein kinase A substrates phosphorylation (PKAs-P) was evaluated and compared in NCAP, 0 mM, 5 mM, 15 mM and 25 mM after 60 min of incubation in 4 replicates. Moreover, spermatozoa were incubated in 0 mM, 5 mM, 15 mM and 25 mM for different times (1, 5, 15, 30 and 60 min) and PKAs-P was compared for times of incubation in the same medium in 4 replicates. In each blot, a lane of spermatozoa incubated in PBS was included as non-capacitating control (NCAP), and in 0 mM and 5 mM blots a lane for spermatozoa incubated in 25 mM for 60 min was included as positive control. β-act or β-tub were used as loading control. After 60 min of incubation intracellular pH (pH_i), motility, tyrosine phosphorylation (Tyr-P), viability and acrosome reaction (AR) were evaluated in the NCAP, 0 mM, 5 mM, 15 mM and 25 mM groups after 60 min of incubation. **B) Experiment 2: Analysis of sperm fertility *in vitro.* Monophasic IVF:** performed in 0 mM, 5 mM, 15 mM and 25 mM at 38.5ºC for 18 h of co-culture. **Biphasic IVF (Sperm pre-incubation + monophasic IVF):** sperm were pre-incubated in TALP medium at 38.5ºC for 60 min with different HCO_3^- concentrations (5 mM, 15 mM or 25 mM) and then the IVF was performed in 5 mM, 15 mM or 25 mM and maintained at 38.5ºC for 18 h of co-culture. The experimental groups established were: sperm pre-incubation in 5 mM and IVF in 15 mM; sperm pre-incubation in 5 mM and IVF in 25 mM; sperm pre-incubation in 15 mM and IVF in 15 mM; sperm pre-incubation in 15 mM and IVF in 25 mM; sperm pre-incubation in 25 mM and IVF in 25 mM. The IVF parameters studied were: the percentage of penetrated oocytes (Pen, %), the percentage of monospermy of penetrated oocytes (Mon, %), the number of spermatozoa penetrating each oocyte (Spz/O), the number of sperm bound to ZP (Spz/ZP) and the efficiency (%), which represents the final number of putative zygotes in each group per 100 penetrated oocytes.

3.1. Experiment 1: Effect of HCO_3^- concentration on spermatozoa functionality

The samples were incubated for up to 60 min at 38.5ºC in NCAP, 0 mM, 5 mM, 15 mM or 25 mM and several parameters of sperm functionality were determined (Figure 1A):

3.1.1. Spermatozoa protein kinase A substrates phosphorylation (PKAs-P)

To evaluate PKAs activation in relation to capacitating conditions and HCO_3^- concentrations, blotting for PKAs-P was compared in the different experimental groups (NCAP, 0 mM, 5 mM, 15 mM and 25 mM) after 60 min of incubation in four replicates. Moreover, to ascertain the exact timing of PKAs-P in the experimental groups, spermatozoa were incubated in 0 mM, 5 mM, 15 mM and 25 mM for different times (1, 5, 15, 30 and 60 min) in four replicates. In each blot, a lane of spermatozoa incubated in PBS was included as non-capacitating control (NCAP) and in the 0 mM and 5 mM blots a lane for spermatozoa incubated in 25 mM for 60 min was included as positive control. β-act or β-tub were used as loading control.

3.1.2. Spermatozoa intracellular pH (pH_i)

Epididymal spermatozoa were stained with BCECF-AM and incubated in NCAP, 0 mM, 5 mM, 15 mM and 25 mM for 1 and 60 min. The fluorescence was monitored by spectrofluorimetry every 2 s for a total time of 300 s. The 490/440 nm ratio and the regression line for the different media used were recorded. The pH_i was determined from the regression line in four replicates.

3.1.3. Spermatozoa motility

Spermatozoa motility was determined in the 0 mM, 5 mM, 15 mM and 25 mM groups at 1, 15, 30 and 60 min of incubation in three replicates. Motility was measured in three different fields per sample. The following parameters were determined per field: the percentage of total motile spermatozoa (Mot, %), motile progressive spermatozoa (MotPro, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, µm), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), and beat cross-frequency (BCF, Hz). Moreover, the spermatozoa of each replicate were classified in three different clusters per experimental group and incubation time to identify groups of spermatozoa with a high degree of similarity in their motility pattern: i) *Cluster 1:* spermatozoa with the poorest motility (lowest values in all CASA parameters), ii) *Cluster 2:* spermatozoa with the most linear trajectory (high values of VSL,

LIN, STR and WOB), and iii) *Cluster 3:* spermatozoa with the most curvilinear trajectory (high values of VCL and ALH and low values of VSL, LIN and STR).

3.1.4. Spermatozoa tyrosine phosphorylation (Tyr-P)

The Tyr-P location in the spermatozoa was evaluated by IIF after 60 min of incubation in NCAP, 0 mM, 5 mM, 15 mM and 25 mM. Two-hundred spermatozoa per sample were analyzed in four replicates. Then, the Tyr-P location of spermatozoa proteins were classified and grouped into four different categories according to the four patterns described by Luño et al. (2013): Pattern I (low capacitation), which included spermatozoa without fluorescence, with phosphorylated acrosome, tail or acrosome and tail, Pattern II, (medium capacitation), which included spermatozoa with fluorescence in the equatorial subsegment with or without the presence of signal in the flagellum, and Pattern III (high capacitation), which included spermatozoa with signal in the equatorial subsegment and acrosome area and with or without the presence of signal in the flagellum. A fourth pattern was established related to the motility activation (Pattern IV), which included those spermatozoa with a signal in the flagellum regardless of other locations (hyperactivation pattern).

3.2. Experiment 2: Effect of HCO_3^- concentration on IVF

In experiment 1 the effect of different HCO_3^- concentrations on sperm functionality was studied in *in vitro* conditions. In experiment 2, the fertilizing ability of porcine epididymal spermatozoa was tested *in vitro* using the same HCO_3^- concentrations. Each insemination dose comprised a pool of epididymal spermatozoa from three different boars. The IVF parameters studied were: the percentage of penetrated oocytes (Pen, %), the percentage of monospermy of penetrated oocytes (Mon, %), the number of spermatozoa penetrating each oocyte (Spz/O), the number of spermatozoa bound to ZP (Spz/ZP) and the Efficiency (%), which represents the final number of putative zygotes in each group per 100 penetrated oocytes (Figure 1B).

3.2.1. Monophasic IVF

IVF was performed in the 0 mM, 5 mM, 15 mM and 25 mM groups for 18 h of co-culture. A total of 616 oocytes were used in four replicates.

3.2.2. Biphasic IVF (spermatozoa pre-incubation + monophasic IVF)

Before IVF, the sperm were pre-incubated for 60 min in 5 mM, 15 mM or 25 mM. Then, IVF was performed in TALP medium containing the same or higher HCO_3^- concentration for 18 h of co-culture. In this way, five experimental groups were established: sperm pre-incubation in 5 mM and IVF in 15 mM, sperm pre-incubation in 5 mM and IVF in 25 mM, sperm pre-incubation in 15 mM and IVF in 15 mM, sperm pre-incubation in 15 mM and IVF in 25 mM, sperm pre-incubation in 25 mM and IVF in 25 mM. A total of 843 oocytes were used in four replicates.

4. STATISTICAL ANALYSIS

All statistical analyses were performed using IBM SPSS v20.0 (SPSS Inc. Armonk, NY, USA) and data were analyzed by one-way ANOVA. In the motility experiment, two types of analysis were carried out per field: i) data were analyzed using the general linear repeated-measures procedure, and ii) data were grouped by a non-hierarchical k-means clustering for each time of incubation and a repeated-measures procedure was followed for each cluster. In both sets of analyses, the results obtained were compared by one-way ANOVA. Data are expressed as the mean ± standard error of the mean (SEM). Differences were considered statistically significant at $p<0.05$, and when these analyses revealed a significant effect, values were compared using the least significant difference pairwise multiple comparison post-hoc test (Tukey).

5. RESULTS

5.1. Experiment 1: Effect of HCO_3^- concentration on spermatozoa functionality

Among the ions existing along the route spermatozoa must follow from the epididymis, HCO_3^- has an important role in two main functions that affect sperm: i) collaborating in the functional maturation of spermatozoa and affecting membrane dynamics, and ii) buffering the environment (Gangwar and Atreja, 2015; Gadella and Van Gestel, 2004; Bhattacharyya and Yanagimachi, 1988). Moreover, HCO_3^- is a key factor in inducing sperm capacitation by direct stimulation of sAC before triggering the cascade of fast and slow events of capacitation. In the present study, the effect of different HCO_3^- concentrations on these capacitating events was investigated.

5.1.1. Spermatozoa protein kinase A substrates phosphorylation (PKAs-P)

The effect of HCO_3^- concentration on PKAs-P after 60 min in NCAP, 0 mM, 5 mM, 15 mM and 25 mM are shown by WB in Figure 2. The results showed that 15 mM and 25 mM produced a significantly higher degree

of PKAs-P compared with the rest of the groups ($p<0.05$). Moreover, the effect of HCO_3^- at different times of incubation (1, 5, 15, 30 and 60 min) is shown in Figure 3. When spermatozoa were incubated in HCO_3^--free capacitating medium (0 mM, Figures 3A and B) or in 5 mM (Figures 3C and D) PKAs-P remained in a low state of activation throughout the 60 min of incubation, and was statistically different ($p<0.05$) from the positive control in 25 mM for 60 min. PKAs-P in 15 mM (Figures 3E and F) was statistically higher than in NCAP from 1 to 30 min of incubation, and again significantly higher ($p<0.05$) at 60 min of incubation. When sperm incubation was carried out in 25 mM (Figures 3G and H), the highest degree of PKAs-P was achieved from 15 min of incubation ($p<0.05$) with no statistically significant differences with 1 and 5 min incubations.

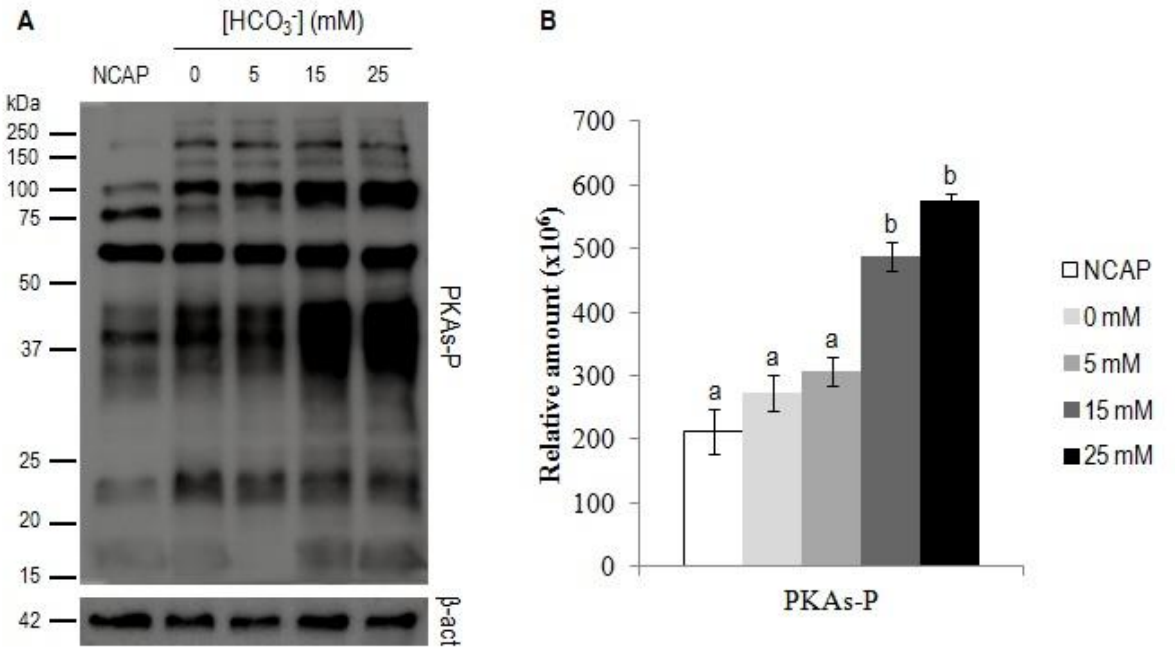


Figure 2. Effect of HCO_3^- concentration on protein kinase A substrates phosphorylation (PKAs-P). A) PKAs-P of sperm samples incubated for 60 min in capacitating medium (TALP) with different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and in PBS as non-capacitating control (NCAP) in 4 replicates. β-actin (β-act) was used as loading control. **B)** Relative amount of semi-quantified signal for blots. Results are shown as mean ± SEM. Different letters (a, b) indicate statistically significant differences ($p<0.05$).

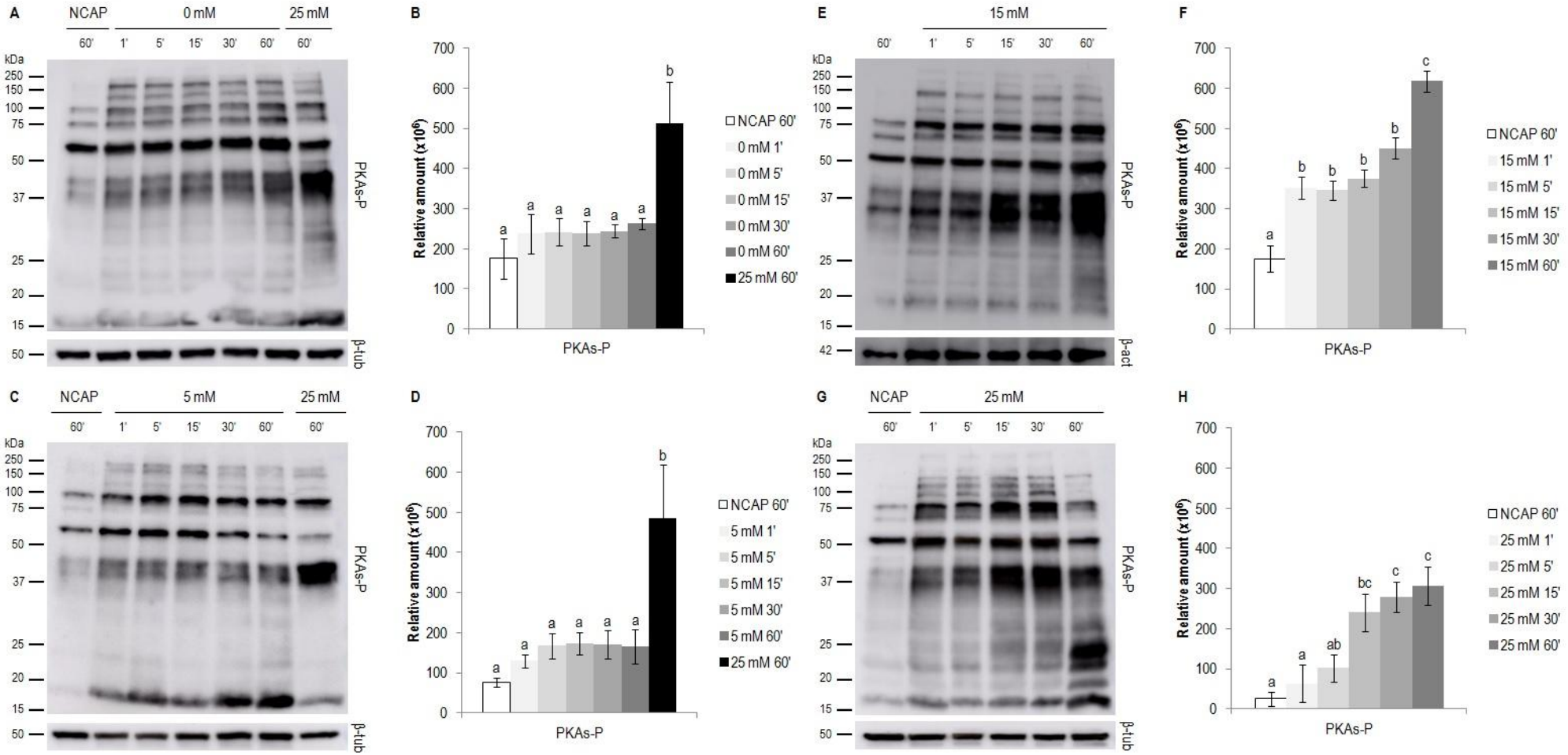


Figure 3. Kinetics of different HCO_3^- concentrations on protein kinase A substrates phosphorylation (PKAs-P). Sperm were incubated for 1, 5, 15, 30 and 60 min in capacitating medium (TALP) containing 0 mM (**A**), 5 mM (**C**), 15 mM (**E**) and 25 mM (**G**) of HCO_3^- in 4 replicates. In each blot a lane of spermatozoa incubated in PBS was included as non-capacitating control (NCAP). In 0 mM (**A**) and 5 mM blots (**C**) a lane for spermatozoa incubated in 25 mM for 60 min was included as positive control. β-actin (β-act) or β-tubulin (β-tub) was used as loading control. Relative amount of semi-quantified signal for 0 mM, 5 mM, 15 mM and 25 mM blots are shown in graphics **B**, **D**, **F** and **H**, respectively. Results are shown as mean ± SEM. Different letters (a-c) indicate statistically significant differences ($p<0.05$) between groups.

5.1.2. Spermatozoa intracellular pH (pH_i)

PKAs activation and all sAC/cAMP cascade events are boosted by the intracellular alkalinization of spermatozoa associated with the intake of external HCO_3^-, but the relation with the specific HCO_3^- concentration in capacitating conditions is still unclear. The pH_i of the spermatozoa exposed to NCAP, 0 mM, 5 mM, 15 mM and 25 mM are shown in Figure 4 as mean ± SEM. At 1 min of incubation, there were no differences in the pH_i between groups (NCAP: 6.65 ± 0.03, 0 mM: 6.56 ± 0.02, 5 mM: 6.56 ± 0.02, 15 mM: 6.55 ± 0.02, 25 mM: 6.55 ± 0.03, $p>0.05$). After 60 min of incubation, the pH_i of spermatozoa increased in all groups, but only those that contained HCO_3^- were statistically higher than NCAP (NCAP: 6.65 ± 0.03, 0 mM: 6.77 ± 0.01, 5 mM: 6.93 ± 0.02, 15 mM: 7.00 ± 0.03, 25 mM: 7.06 ± 0.05), but with no statistical differences between them.

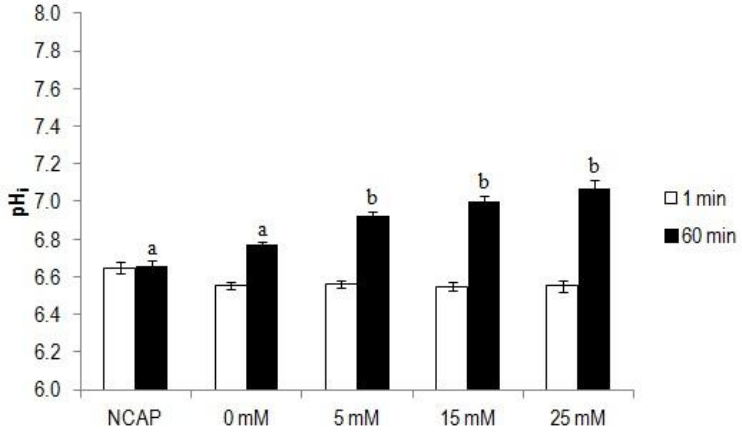


Figure 4. Effect of HCO_3^- on boar epididymal sperm intracellular pH (pH_i). Sperm were incubated for 1 and 60 min in capacitating medium (TALP) containing different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and non-capacitating medium (NCAP) in 4 replicates. Results are shown as mean ± SEM. Different letters (a, b) in the same time of incubation indicate statistically significant differences ($p<0.05$).

5.1.3. Spermatozoa motility

One of the first events related with sAC/PKA pathway activation by HCO_3^- is sperm motility stimulation. Thus, the effect of HCO_3^- on sperm motility and trajectory was studied in capacitating conditions in 0 mM, 5 mM, 15 mM and 25 mM (Figures 5, 6 and 7). The results showed that the HCO_3^- concentration did not affect Mot (ranged from 84.9 and 92.9% in all groups), MotPro (between 83.2 and 92.4% in all groups) or VCL (between 138.2 and 165.0 µm/s in all groups) but stimulated straight and average path velocities (VSL and VAP, $p<0.05$) (Figure 5). Moreover, the 15 mM concentration produced significantly higher LIN, STR and WOB than 0 mM and 5 mM but 25 mM did not induce any extra improvement (Figure 5). There were no statistical differences in ALH and BCF between groups. A cluster analysis revealed that the proportion of spermatozoa with poorer motility (cluster 1) was similar between groups, regardless of the HCO_3^- concentration and length of incubation (Figure 7). The proportion of spermatozoa with the most rectilinear trajectory (cluster 2) was significantly higher ($p<0.05$) in the 5 mM, 15 mM and 25 mM groups than in 0 mM. In addition, and despite a certain tendency ($p = 0.051$) to decrease in the presence of HCO_3^-, there were no statistical differences in the proportion of spermatozoa with the most curvilinear trajectory (cluster 3) between groups.

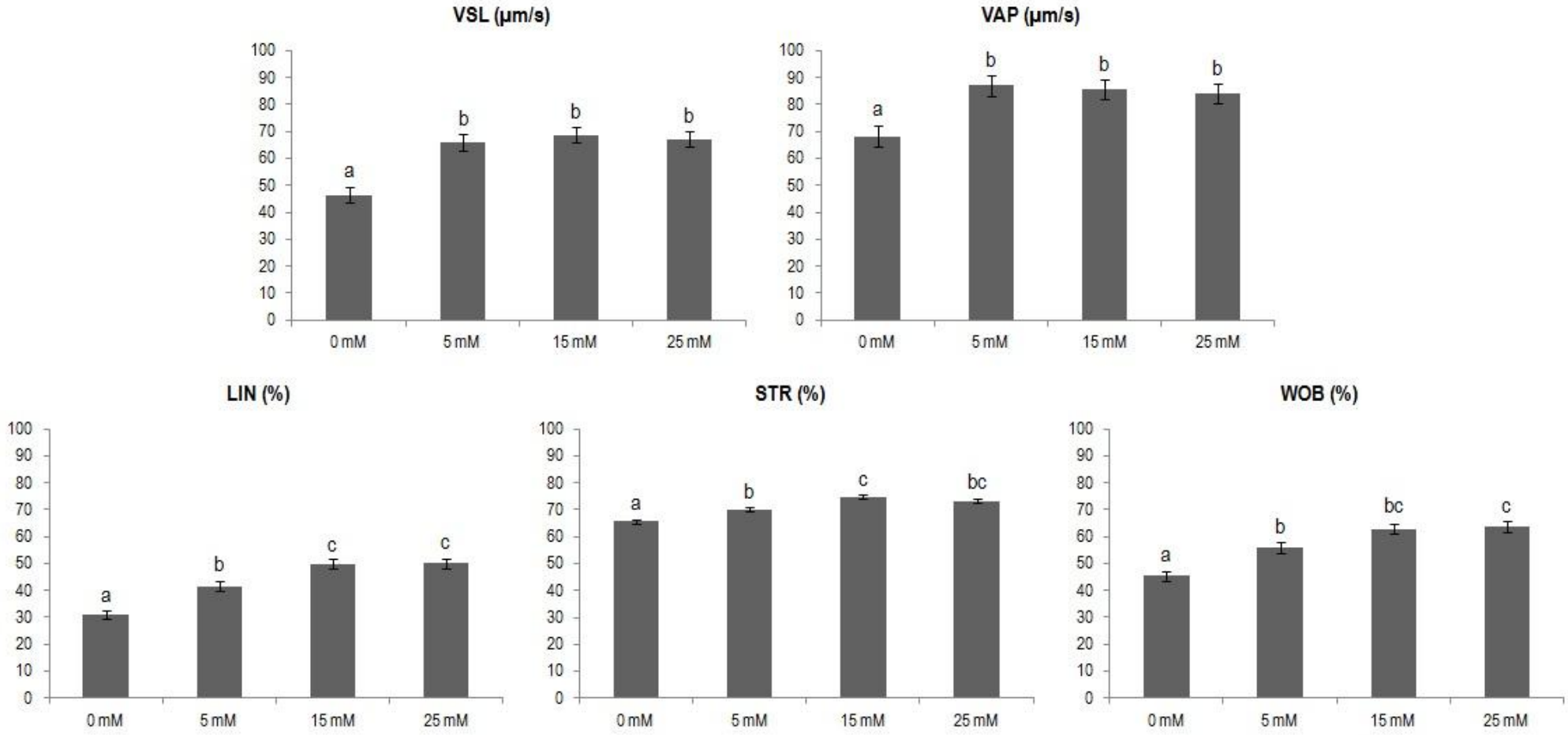


Figure 5. **Effect of HCO_3^- on epididymal sperm motility in capacitating conditions.** Boar epididymal spermatozoa were incubated in capacitating medium (TALP) containing different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and CASA parameters were determined at 1, 15, 30 and 60 min of incubation in 3 replicates. VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory (VSL/VCL, %); STR: straightness (VSL/VAP, %); WOB: wobble (VAP/VCL, %). The data per field were analyzed by the general linear repeated-measures model and a Tukey test. Results expressed as mean ± SEM. Different letters (a-c) for graph bars indicate statistical differences ($p<0.05$).

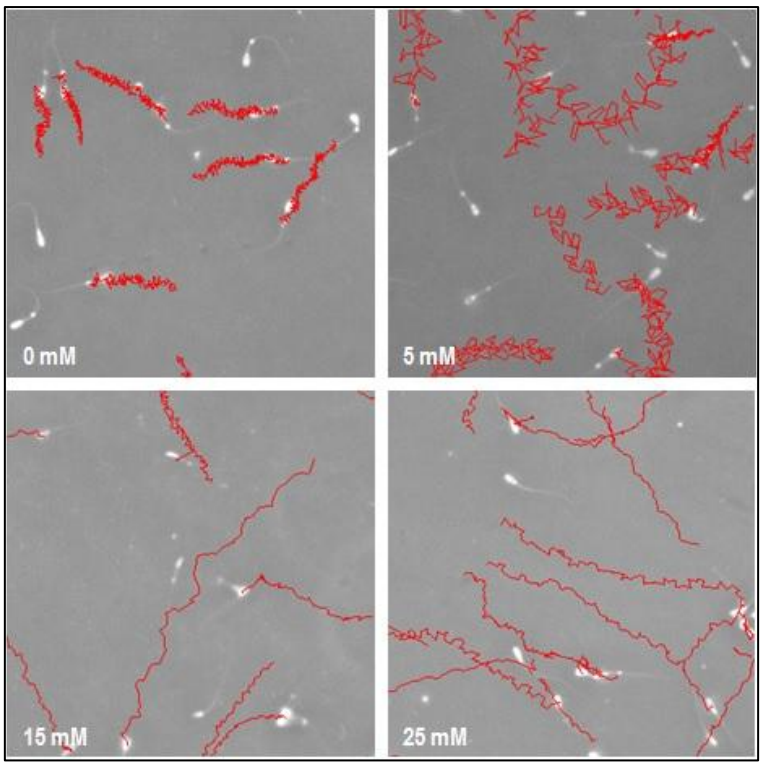


Figure 6. Representative images of swimming trajectories of epididymal sperm in capacitating conditions with different HCO_3^- concentrations. Boar epididymal spermatozoa were incubated in capacitating medium (TALP) containing different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) for 60 min. Sequences of 2 s were obtained with CASA (50 frames/s).

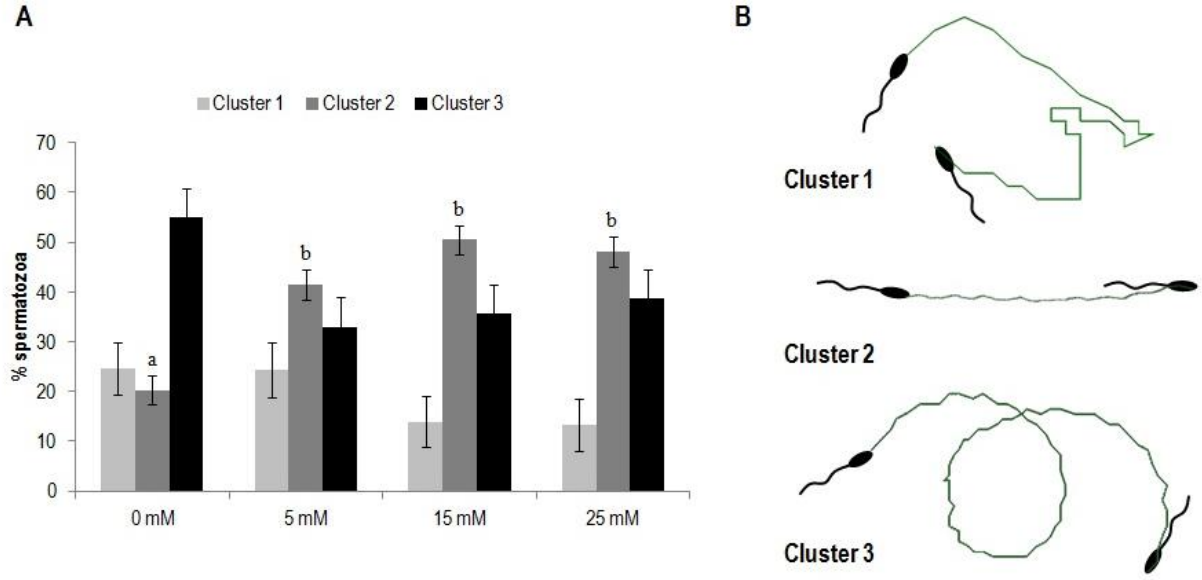


Figure 7. Effect of HCO_3^- on the epididymal sperm trajectory in capacitating conditions. A) Boar epididymal spermatozoa were incubated in capacitating medium (TALP) containing different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and CASA parameters were determined at 1, 15, 30 and 60 min of incubation in 3 replicates. Clustering of spermatozoa was carried out per field. Cluster 1: lowest values in all CASA parameters (poorest motility). Cluster 2: high values of VSL, LIN STR and WOB (the most linear trajectory). Cluster 3: high values of VCL and ALH and low of VSL, LIN and STR (the most curvilinear trajectory). Data were analyzed by one-way ANOVA and a Tukey test. Results expressed as mean ± SEM. Different letters (a-c) in the same parameter or cluster indicate statistically significant differences ($p<0.05$). **B)** Illustrations are representative of the three clusters defined.

5.1.4. Spermatozoa tyrosine phosphorylation (Tyr-P)

The pH_i seems to favor the cascade of slow and fast events in spermatozoa, including the activation of tyrosine kinases and the subsequent Tyr-P of proteins. In fact, the capacitation status was described to be strongly correlated with Tyr-P (Visconti et al., 1995a) as a direct consequence of the action of HCO_3^-, whose omission not only inhibits Tyr-P (Visconti et al., 1995a), but also diminishes the ability of spermatozoa to bind to ZP (Harkema et al., 1998). This slow capacitation response must be preceded by lipid scrambling and cholesterol removal. Different compartments have been described as undergoing a specific sequence of phosphorylation events during both capacitation and binding to ZP (Sakkas et al., 2003). The present experiment tried to evaluate the effect of HCO_3^- concentration on Tyr-P immunolocation in epididymal porcine spermatozoa (Figure 8).

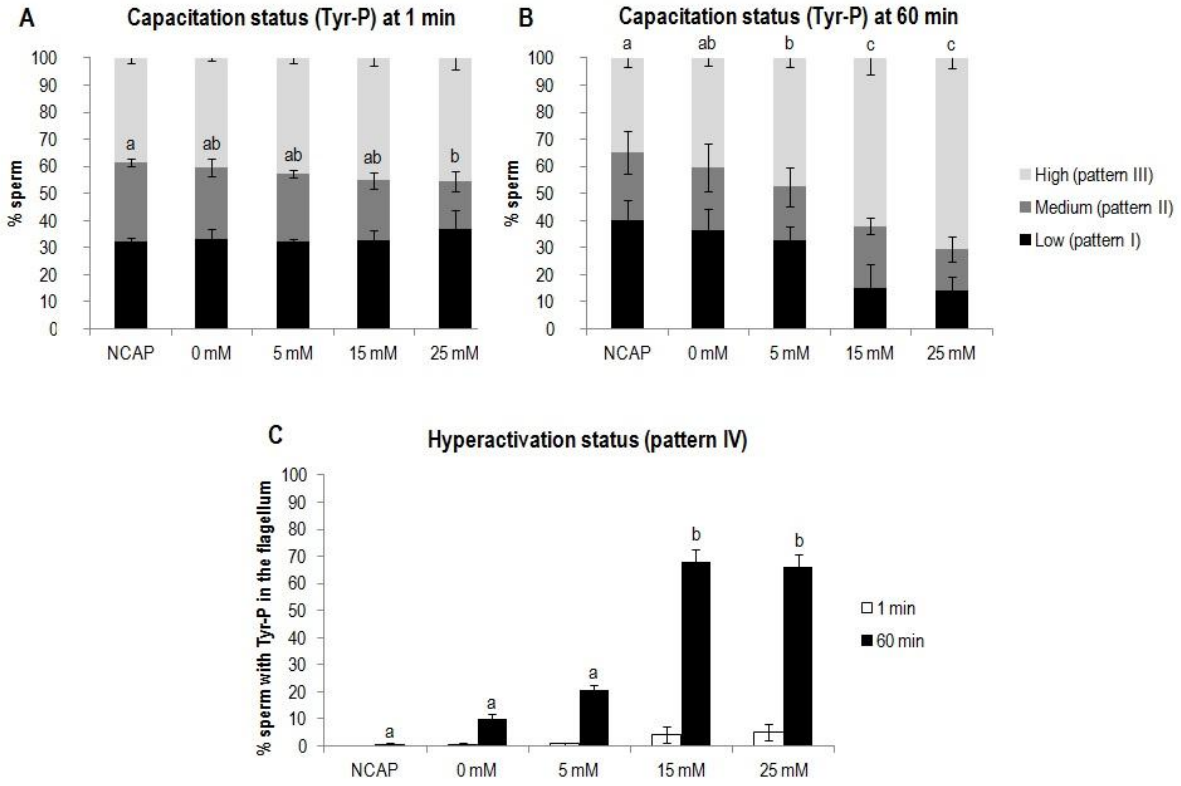


Figure 8. Effect of HCO_3^- concentration on boar epididymal sperm capacitation status and hyperactivation according to the immunolocation of protein tyrosine phosphorylation (Tyr-P). Sperm were incubated for 1 and 60 min in capacitating medium (TALP) containing different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and non-capacitating medium (NCAP) in 4 replicates. **A** and **B)** Capacitation status at 1 and 60 min of incubation, respectively: percentage of sperm with low capacitation status (pattern I: non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa), medium capacitation status (pattern II: equatorial segment or equatorial segment and flagellum phosphorylated) or high capacitation status (pattern III: equatorial segment and head and/or flagellum phosphorylated). **C)** Hyperactivation status at 1 and 60 min of incubation: percentage of hyperactivated sperm (pattern IV: flagellum phosphorylation regardless of phosphorylation in other locations). Results are shown as mean ± SEM. Different letters (a-c) for the same time of incubation and pattern indicate statistically significant differences ($p<0.05$).

At 1 min of incubation, all the experimental groups had the same proportion of spermatozoa with patterns I and III (low and high capacitation status, respectively). Compared with NCAP, the 25 mM concentration had a significantly reduced proportion of spermatozoa with pattern II (medium capacitation status) (Figure 8A). At

60 min of incubation, the proportion of pattern I and II (low and medium capacitation status, respectively) did not show any statistical difference between the media used ($p>0.05$). However, the proportion of pattern III (high capacitation status) was significantly higher in 15 mM and 25 mM ($p<0.05$) (Figure 8B). As regards pattern IV (hyperactivation status), 15 mM and 25 mM produced the highest level of hyperactivation in spermatozoa, which differed significantly from the rest of the groups ($p<0.05$; Figure 8C). Taken together, these results showed that a 15 mM concentration of HCO_3^- in the capacitation medium is sufficient to attain a high level of Tyr-P and hyperactivation.

5.2. Experiment 2: Effect of HCO_3^- concentration on IVF

As described above, the concentration of HCO_3^- affects sperm functionality and modulates sperm capacitation, a regulatory event that prepares spermatozoa for fertilization prior to encountering the oocyte. Based on this, experiment 2 tried to determine the fertility of spermatozoa and the interaction between gametes in the same conditions as studied in experiment 1.

5.2.1. Monophasic IVF

The IVF output for penetration, monospermy, Spz/O, Spz/ZP and Efficiency are shown in Table 1A as mean ± SEM. In the absence of HCO_3^- (0 mM) no oocyte was penetrated, which confirms that HCO_3^- is an indispensable component of the IVF medium for the spermatozoa to bind to ZP and to penetrate the oocyte. When 5 mM was used, all the studied parameters were significantly lower than when 15 mM or 25 mM was used. The 25 mM concentration had lower efficiency (8.6 ± 1.7%) than 15 mM because, although penetration was high, monospermy was low. The use of 15 mM significantly increased the efficiency of the technique to 26.2 ± 3.1%, with a lower penetration but higher monospermy ($p<0.05$) than at 25 mM. In light of these results, it is clear that sperm fertility was affected by the mere presence of HCO_3^- and also its concentration. This study then turned to the task of identifying an IVF method that increased the efficiency of obtaining potentially viable zygotes by optimizing the HCO_3^- concentration to which gametes are gradually exposed.

5.2.2. Biphasic IVF (spermatozoa pre-incubation + monophasic IVF)

The pre-incubation of sperm in a capacitating medium affected all the parameters studied (Table 1B). Penetration increased proportionally to the concentration of HCO_3^- in sperm pre-incubation, although monospermy was more closely related to the HCO_3^- concentration in the IVF medium, reaching between 21.5 and 25.4% when IVF was carried out at 25 mM and between 36.2 and 48.7% when it was carried out at 15 mM. Thus, the higher efficiency achieved when both pre-incubation and IVF was performed in 15 mM (33.9 ± 3.7%, $p<0.05$).

Table 1. Effect of HCO_3^- concentration on *in vitro* sperm fertility. A) Monophasic IVF: performed in TALP medium containing different HCO_3^- concentrations (0 mM, 5 mM, 15 mM and 25 mM) for 18 h of co-culture at 38.5ºC. **B) Biphasic IVF (spermatozoa pre-incubation + monophasic IVF):** sperm were pre-incubated in TALP for 60 min in 5 mM, 15 mM or 25 mM and then IVF was performed in TALP medium with the same or higher HCO_3^- concentration for 18 h of co-culture at 38.5ºC.

A

[HCO_3^-] IVF (mM)	n	Pen (%)	Mon (%)	Spz/O	Spz/ZP	Efficiency (%)
0	216	0^{a}	-	-	0.3 ± 0.1^{a}	0^{a}
5	204	21.6 ± 2.9^{b}	61.4 ± 7.4^{a}	1.7 ± 0.2^{a}	2.8 ± 0.2^{b}	13.2 ± 2.4^{b}
15	206	79.6 ± 2.8^{c}	32.9 ± 3.7^{b}	4.0 ± 0.3^{b}	4.9 ± 0.5^{c}	26.2 ± 3.1^{c}
25	280	97.5 ± 0.1^{d}	8.8 ± 1.7^{c}	6.6 ± 0.3^{c}	8.8 ± 0.5^{d}	8.6 ± 1.7^{b}

B

[HCO_3^-] (mM)		n	Pen (%)	Mon (%)	Spz/O	Spz/ZP	Efficiency (%)
Sperm pre-incubation	IVF						
5	**15**	171	46.8 ± 3.8^{a}	36.2 ± 5.4^{ab}	3.0 ± 0.2^{ab}	3.4 ± 0.2^{a}	17.0 ± 2.9^{a}
5	**25**	172	54.1 ± 3.8^{a}	21.5 ± 4.3^{a}	4.4 ± 0.3^{c}	4.1 ± 0.3^{abc}	11.6 ± 2.4^{a}
15	**15**	165	69.7 ± 3.6^{b}	48.7 ± 4.7^{b}	2.4 ± 0.2^{b}	3.6 ± 0.2^{ab}	33.9 ± 3.7^{b}
15	**25**	164	76.8 ± 3.3^{bc}	25.4 ± 3.9^{a}	3.6 ± 0.2^{ac}	4.5 ± 0.2^{bc}	19.5 ± 3.1^{a}
25	**25**	171	86.6 ± 2.6^{c}	24.3 ± 3.5^{a}	3.4 ± 0.2^{a}	4.8 ± 0.3^{c}	21.0 ± 3.1^{a}

Results are expressed as mean ± SEM. Different superscripts ($^{a-c}$) in the same column indicate significant differences ($p<0.05$). Pen (%): penetration percentage of total oocytes used; Mon (%): monospermy percentage calculated from penetrated oocytes; Spz/O: number of spermatozoa penetrating each oocyte; Spz/ZP: number of sperm bound to ZP; Efficiency (%): final number of putative zygotes (monospermic) in each group per 100 penetrated oocytes.

6. DISCUSSION

Maturation in the epididymis comprises progressive and sequential modifications of maturing spermatozoa that have been demonstrated to be essential for the acquisition of motility and fertility (Yanagimachi, 1981). During this maturation process in the epididymis, the HCO_3^- concentration decreases from the caput to the cauda (Rodriguez-Martinez et al., 1990) while the plasma membrane is substantially remodeled. Once in the cauda, where the HCO_3^- concentration is minimal (~3-4 mM), matured spermatozoa remain in a quiescent state. Epididymal spermatozoa incubated *in vitro* in HCO_3^--enriched media have the ability to undergo a series of molecular events that initiates sperm capacitation and includes the stimulation of sAC (Visconti et al., 1995b),

which promotes the activation of the PKA-dependent phosphorylation cascade (Harrison, 2004). Gadella and Van Gestel (2004) mention that sAC during capacitation is activated by a relatively high concentration of HCO_3^- (>20 mM), and Harrison (2004) described how sperm capacitation in ejaculated spermatozoa is quickly activated after 80 s exposure to HCO_3^--enriched medium. In *in vivo* conditions, the sperm capacitation process is completed in the female isthmus and with a HCO_3^- concentration of 10 mM (Rodriguez-Martinez, 2007) and spermatozoa are progressively exposed to increasing amounts of the effector HCO_3^- (Tienthai et al., 2004). The present study attempts to elucidate the sperm response to different HCO_3^- concentrations and to optimize the concentration for use *in vitro*.

This study was carried out using mature spermatozoa that had never been in contact with high concentrations of HCO_3^- or with decapacitant agents from accessory sex glands. Epididymal spermatozoa were incubated in a capacitating medium containing 0, 5, 15 or 25 mM of HCO_3^- and in non-capacitating medium. The results showed that the highest degree of PKAs activation was achieved by incubation in a capacitating medium containing 15 mM of HCO_3^- or above, although the 25 mM concentration did not perform any better than 15mM. In spite of the presence of other capacitating agents such as calcium and albumin, concentrations lower than 15 mM (for example the 5 mM, which is similar to that existing in epididymis) produced a low PKAs-P, similar to that produced in the absence of HCO_3^- (0 mM). It is known that defective HCO_3^- secretion into the uterus may be related to impaired sperm capacitation and low female fertility (Wang et al., 2003). Therefore, a 15 mM concentration of HCO_3^- is postulated as being sufficient to achieve the high degree of PKAs activation that is essential in the sperm capacitation process. The differences in the results obtained with the different HCO_3^- concentrations were not dependent on the viability of the cells analyzed. Although the capacitation process affected viability (which, in the presence of HCO_3^-, was reduced by approximately 20%, supplementary file 1), all the experimental groups maintained similar cell viability levels during incubation. Therefore, although this study showed the dose-dependent effect of HCO_3^- on sperm capacitation, above 15 mM there was no extra effect. However, the kinetic of PKAs activation allowed us to understand that the required time to activate PKAs does depend on HCO_3^- concentration, which was rapidly activated at 1 min of incubation at concentrations of 15 and 25 mM, which agrees with Harrison (2004). PKAs was highly phosphorylated after 15 min of incubation with 25 mM and after 60 min with 15 mM, demonstrating that a concentration 15 mM can produce a high degree of PKAs-P, although it takes longer. The absence (0 mM) or low concentrations of HCO_3^-, similar to that existing in the cauda epididymis (5 mM) did enable PKAs activation even after 60 min of incubation, which shows that not only is the presence of HCO_3^- essential in this pathway but that a minimum concentration is required. HCO_3^- is a key activator of a cascade of events in capacitation and a minimum concentration and incubation time is necessary to trigger the capacitation process and produce a high PKAs-P, despite the presence of other capacitating agents like calcium and albumin. 15 mM of HCO_3^- seems to be a sufficient concentration to allow epididymal sperm capacitation. Nevertheless, the importance of HCO_3^- not only lies in the activation of PKAs but also the fact that it produces another series of events essential for sperm physiology. The increase in intracellular pH conditions the

phenomena during capacitation (reviewed by Nishigaki et al., 2014) and the sAC/cAMP/PKA pathway is not an exception.

Epididymal spermatozoa in the cauda epididymis remain in a quiescent state favored by low HCO_3^- content and acidic pH (Rodriguez-Martinez et al., 1990). The present study showed that, in the absence of HCO_3^- and the presence of other capacitating agents like calcium an albumin, the pH_i remained at 6.7-6.8. In the presence of HCO_3^-, the pH_i increased 0.3-0.4 units, confirming that the increase depends exclusively on HCO_3^-. This increase of around 0.3-0.4 units in pH_i may seem small, but, it has been demonstrated that an increase of 0.2 units of pH may modify enzymatic activity more than 20-fold (Trivedi and Danforth, 1966). The activity of intra- and extracellular enzymes taking part in the cellular metabolism are pH-sensitive. One of those enzymes is phosphofructokinase, the limiting enzyme in glycolysis that is the main route of ATP production in porcine spermatozoa. Studies that have acidified the sperm intracellular medium reported that capacitation was inhibited (Galantino-Homer et al., 2004). The activity of this enzyme strongly and directly increases with the rise in intracellular pH in a small physiological range (Madshus, 1988). This favors the protein phosphorylation cascade in spermatozoa that becomes operative after HCO_3^- challenge (Harrison, 2004) and facilitates sperm motility activation (Rodriguez-Martinez et al., 1990).

The effect of HCO_3^- has been widely studied and there is evidence that extracellular HCO_3^- influences the motility of epididymal spermatozoa of several species (Visconti et al., 1995a; Rojas et al., 1993; Rodriguez-Martinez et al., 1990; Babcock et al., 1983). This study attempts to elucidate whether motility activation is also HCO_3^--dependent, at least in porcine. HCO_3^- concentration did not affect the proportion of motile spermatozoa produced during incubation in a capacitating medium, or their progressivity, but did affect parameters related to sperm trajectory. The straight and average velocities, straightness and linearity significantly increased with the HCO_3^- concentration in the medium. As stated by Holt and Harrison (2002), the present study shows that HCO_3^- produces a change in sperm trajectory, which becomes much more linear and rapid. HCO_3^- seems not only to be important for motility activation in a balanced salt solution but also that the type of movement produced is concentration-dependent. A concentration of 15 mM HCO_3^- during sperm capacitation produced a more linear movement, which has previously been related with the state of capacitation in boar (García Herreros et al., 2005; Holt and Harrison, 2002). This observation of an increase in LIN in capacitating conditions are in clear contrast with the results of other studies in porcine (Schmidt and Kamp, 2004) and in other species (Kinukawa et al., 2003; Cancel et al., 2000), in which LIN decreased during incubation in capacitating conditions. In our study, HCO_3^- concentrations lower than 15 mM produced a more curvilinear trajectory, while higher concentrations (25 mM) did not produce any extra improvement over the value obtained at 15 mM. However, each individual spermatozoon responds differently to HCO_3^- (Satake et al., 2006), and a clustering analysis identified three different sperm subpopulations according to their trajectory in response to HCO_3^-. In this case, the proportion of spermatozoa with a more linear trajectory was higher in the presence of HCO_3^-. While a concentration of HCO_3^- from 15 mM produced a high level of hyperactivation and a change in the type of trajectory, no additional effect was obtained

with 25 mM. Thus, 15 mM is postulated as the optimal HCO_3^- concentration in the capacitation medium to stimulate the characteristic motility of capacitation status in porcine epididymal spermatozoa.

The role of HCO_3^- in sperm capacitation is not only restricted to the immediate effects on spermatozoa (increased pH_i, activation of motility and PKAs-P) but is also important in slow capacitation events such as proteins phosphorylation and AR. *In vitro* sperm capacitation is accompanied by a time-dependent increase in the Tyr-P of a subset of proteins, as one of the final steps of the PKA-dependent cascade of capacitation (Visconti et al., 1995a). Sperm incubated in a medium lacking HCO_3^- fail to undergo capacitation, but the process can be recovered by adding HCO_3^- in a concentration-dependent way (Visconti et al., 1995a). This study showed that a concentration of 15 mM is sufficient to produce an intracellular alkalinization and a high degree of Tyr-P in spermatozoa but no extra effect was observed when 25 mM was used. Something similar occurred with Tyr-P in the flagellum of spermatozoa as regard sperm hyperactivation. A high degree of hyperactivation was observed at 15 mM and above, although no significantly improvement was observed in 25 mM. The overall of this study agree with those of other authors (Galantino-Homer et al., 2004), who showed that HCO_3^- is the link to the intracellular alkalinization required to activate sAC, which increases intracellular cAMP, promotes PKA activity and subsequent Tyr-P. An HCO_3^- concentration of 15 mM is a therefore sufficient to trigger this cascade of events and produce a high degree of capacitation in epididymal spermatozoa in *in vitro* conditions.

The above mentioned observations concerning the role of HCO_3^- concentration on sperm functionality have shown the modulating power of HCO_3^- in sperm physiology in capacitation conditions. However, whether or not this modulating effect influences the fertilizing ability of sperm in IVF has not been elucidated. In this study, the effect of HCO_3^- concentration on sperm fertility was investigated (Table 1A). Taking in to account the results obtained concerning the modulating effect of HCO_3^- on sperm physiology, it was hypothesized that varying the HCO_3^- concentration in the porcine sperm capacitation process could regulate their functionality and probably affect IVF output. Indeed, IVF was directly and strongly affected by the HCO_3^- concentration in the medium. The importance of HCO_3^- in the fertilization process was confirmed by the fact that no oocyte was penetrated by a spermatozoon in the absence of HCO_3^- (0 mM). This agrees with Suzuki et al. (1994), who described how the absence of HCO_3^- in fertilization media makes oocyte fertilization impossible. In our study, we found that HCO_3^- exerts a dose-dependent impact on fertilization as penetration increased as the HCO_3^- concentration was increased. The Spz/O also increased with the HCO_3^- concentration, which resulted in a reduced monospermy. The high incidence of polyspermy in porcine has been widely related with an unacceptably low efficiency of IVF (Coy and Romar, 2002). Hunter and Nichol (1988) hypothesized that the incidence of polyspermy is a direct consequence of the number of capacitated spermatozoa in the immediate vicinity of ovulated porcine oocytes *in vivo*. Later, Abeydeera and Day (1997) also described this correlation under *in vitro* conditions. HCO_3^- seems to be necessary to prepare spermatozoa for ZP-binding and the effect on IVF of a specific HCO_3^- concentration could be related with the timing of the capacitation events shown in experiment 1 that could influence the fertilization success (monospermic fertilization). Figures 3C and D showed that 5 mM did not activate PKAs while 25 mM seemed to produce massive sperm capacitation at the same time as and faster than 15 mM. Figures 3F to

3H show that PKAs activation in sperm at 15 mM and 25 mM is rapid (after 1 min of incubation) but achieves maximum activation at 60 and 15 min, respectively, in capacitating conditions. The slower PKAs activation of 15 mM could be responsible for the lower penetration and the higher monospermy than observed with 25 mM in the fertilization experiments. So, HCO_3^- concentration affects the timing of capacitation events and subsequently could play a key role in fertilization. Moreover, Funahashi (2003) described that one of the strategies to reduce polyspermy in porcine is to induce full AR in partially reacted spermatozoa. However, the results of the AR assay (supplementary file 2) showed that polyspermy is not related with the proportion of acrosome-reacted spermatozoa surrounding the egg. Concentrations of 15 and 25 mM HCO_3^- in the capacitating media produce the same proportion of acrosome-reacted sperm after 60 min of incubation.

In the same way as penetration and monospermy parameters, HCO_3^- had a determining action in the primary recognition between gametes, since practically no spermatozoon was able to bind to ZP in the absence of HCO_3^-. The recognition of and binding of spermatozoa to ZP is essential for fertilization (Yanagimachi, 1994). As Flesch et al. (2001) suggested, it is probable that the absence of HCO_3^- hindered certain capacitation events like the Tyr-P of plasma membrane proteins, which resulted in a failed binding affinity of spermatozoa for ZP. The loss of this sperm function in HCO_3^--free capacitating medium would result in a failure to generate reactive oxygen species, impair the capacity of proteins to be tyrosine phosphorylated and incapacitate spermatozoa from producing the normal calcium peaks. The affinity of spermatozoa for ZP increased with increasing HCO_3^- concentrations. The Spz/ZP was higher in 5 mM, 15 mM and 25 mM than in 0 mM probably because changes in the plasma membrane are HCO_3^--dependent.

It would seem important to know whether the influence of HCO_3^- in IVF is mainly exerted on spermatozoa or on gamete co-incubation (Table 1B). After sperm pre-incubation with 15 mM, which produced a moderate penetration, when IVF was also carried out at 15 mM, monospermy was significantly higher and the Spz/O was significantly lower than in the rest of groups, which resulted in a higher final efficiency (33.9%). What is clear is that oocyte penetration directly increased with the HCO_3^- concentration in the sperm pre-incubation medium, which leads us to think that the action of HCO_3^- is exerted more on spermatozoa than on oocytes. Very few studies have described the effect of HCO_3^- on *in vitro* matured oocytes, but Suzuki et al. (1994) suggested that, although HCO_3^- was apparently affecting gamete interaction, it is highly probable that HCO_3^- affects fertilization through its effect on spermatozoa rather than through any effect on the oocytes. We have showed that spermatozoa pre-incubation in a capacitating medium with 15 mM HCO_3^- maintains their fertilizing ability and maximizes IVF in porcine, probably because 15 mM allows slower capacitation and gives a chance to spermatozoa to express the individual progression of capacitation events. Lower concentrations than 15 mM produce lower penetration, which is probably related with the lower state of capacitation demonstrated in the experiments described previously in this work. On the other hand, higher concentrations than 15 mM decrease monospermy, most probably because more spermatozoa achieve a high capacitation status at the same time and are ready for fertilization. Taking these results into account, 15 mM would be a sufficient HCO_3^- concentration in IVF media because it produces a high degree of sperm capacitation and maximizes IVF efficiency in porcine.

In conclusion, adjusting the HCO_3^- concentration of the medium during *in vitro* epididymal sperm capacitation and fertilization allowed us to increase the efficiency of these *in vitro* processes in porcine, which are currently suboptimal. A concentration of 15 mM HCO_3^- in the capacitating medium is sufficient to activate the sperm capacitation cascade and induce the slower progression of the sAC/cAMP/PKA pathway. A concentration of 15 mM produces a sufficient level of activation of sperm motility, pH_i increase and Tyr-P, maintaining high levels of fertilizing ability and increased rates of monospermy. There are innumerable factors that influence the capacitation and fertilization processes and few are completely understood. More studies are necessary to improve our knowledge of reproductive physiology and to optimize current conditions of *in vitro* sperm capacitation and fertilization in porcine.

7. REFERENCES

Abeydeera, L. R. and Day, B. N. (1997). Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol. Reprod.* **57(4)**, 729–734.

Babcock, D. F., Rufo, G. A. and Lardy, H. A. (1983). Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm (intracellular pH/carboxyfluorescein/cyclic AMP/proton permeability). *Cell Biol.* **80(5)**, 1327–1331.

Bedu-Addo, K., Barratt, C. L. R., Kirkman-Brown, J. C. and Publicover, S. J. (2007). Patterns of $[Ca^{2+}]_i$ mobilization and cell response in human spermatozoa exposed to progesterone. *Dev. Biol.* **302(1)**, 324–332.

Bhattacharyya, A. and Yanagimachi, R. (1988). Synthetic organic pH buffers can support fertilization of guinea pig eggs, but not as efficiently as bicarbonate buffer. **19(2)**, 123–129.

Cancel, A. M., Lobdell, D., Mendola, P. and Perreault, S. D. (2000). Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Hum. Reprod.* **15(6)**, 1322–1328.

Coy, P. and Romar, R. (2002). *In vitro* production of pig embryos: a point of view. *Reprod. Fertil. Dev.* **14(5)**, 275–286.

Coy, P., Martínez, E., Ruiz, S., Vázquez, J. M., Roca, J. and Gadea, J. (1993). Environment and medium volume influence *in vitro* fertilisation of pig oocytes. *Zygote* **1(3)**, 209–13.

Coy, P., Gadea, J., Romar, R., Matás, C. and García, E. (2002). Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. *Reproduction* **124(2)**, 279–288.

Flesch, F. M. and Gadella, B. M. (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim. Biophys. Acta - Rev. Biomembr.* **1469(3)**, 197–235.

Flesch, F. M., Wijnand, E., Van De Lest, C. H. A., Colenbrander, B., Van Golde, L. M. G. and Gadella, B. M. (2001). Capacitation dependent activation of tyrosine phosphorylation generates two sperm head plasma membrane proteins with high primary binding affinity for the zona pellucida. *Mol. Reprod. Dev.* **60(1)**, 107–115.

Funahashi, H. (2003). Polyspermic penetration in porcine IVM-IVF systems. *Reprod. Fertil. Dev.* **15(3)**, 167–177.

Funahashi, H., Cantley, T. C. and Day, B. N. (1997). Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization. *Biol. Reprod.* **57(1)**, 49–53.

Gadella, B. M. and Van Gestel, R. A. (2004). Bicarbonate and its role in mammalian sperm function. *Anim. Reprod. Sci.* **82–83**, 307–319.

Galantino-Homer, H. L., Florman, H. M., Storey, B. T., Dobrinski, I. and Kopf, G. S. (2004). Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-

associated protein tyrosine phosphorylation. *Mol. Reprod. Dev.* **67(4)**, 487–500.

Gangwar, D. K. and Atreja, S. K. (2015). Signalling events and associated pathways related to the mammalian sperm capacitation. *Reprod. Domest. Anim.* **50(5)**, 705–711.

García Herreros, M., Aparicio, I. M., Núñez, I., García-Marín, L. J., Gil, M. C. and Peña Vega, F. J. (2005). Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. *Theriogenology* **63(3)**, 795–805.

Harkema, W., Harrison, R. A. P., Miller, N. G., Topper, E. K. and Woelders, H. (1998). Enhanced binding of zona pellucida proteins to the acrosomal region of the intact boar spermatozoa in response to fertilizing conditions: a flow cytometric study. *Biol. Reprod.* **58(2)**, 421–30.

Harrison, R. A. P. (2004). Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol. Reprod. Dev.* **67(3)**, 337–352.

Hess, K. C., Jones, B. H., Marquez, B., Chen, Y., Ord, T. S., Kamenetsky, M., Miyamoto, C., Zippin, J. H., Kopf, G. S., Suarez, S. S., et al. (2005). The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev. Cell* **9(2)**, 249–259.

Holt, W. and Harrison, R. (2002). Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: between cell and between-ejaculated differences are not due deficies in protein kinase A activation. *J. Androl.* **23(4)**, 557–565.

Hunter, R. H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil.* **63(1)**, 109–117.

Hunter, R. H. F. and Nichol, R. (1988). Capacitation potential of the fallopian tube: a study involving surgical insemination and the subsequent incidence of polyspermy. *Gamete Res.* **21(3)**, 255–266.

Hunter, R. H. F. and Rodriguez-Martinez, H. (2004). Capacitation of mammalian spermatozoa *in vivo*, with a specific focus on events in the Fallopian tubes. *Mol. Reprod. Dev.* **67(2)**, 243–250.

Kinukawa, M., Nagata, M. and Aoki, F. (2003). Changes in flagellar bending during the course of hyperactivation in hamster spermatozoa. *Reproduction* **125(1)**, 43–51.

Li, Y.-H., Li, M., Hou, Y., Jiao, L.-H. and Wang, W.-H. (2003). Reduced polyspermic penetration in porcine oocytes inseminated in a new *in vitro* fertilization (IVF) system: straw IVF. *Biol. Reprod.* **69(5)**, 1580–1585.

Litvin, T. N., Kamenetsky, M., Zarifyan, A., Buck, J. and Levin, L. R. (2003). Kinetic properties of "soluble" adenylyl cyclase: Synergism between calcium and bicarbonate. *J. Biol. Chem.* **278(18)**, 15922–15926.

Liu, Y., Wang, D. K. and Chen, L. M. (2012). The physiology of bicarbonate transporters in mammalian reproduction. *Biol. Reprod.* **86(4)**, 99–99.

Luño, V., López-Úbeda, R., García-Vázquez, F. A., Gil, L. and Matás, C. (2013). Boar sperm tyrosine phosphorylation patterns in the presence of oviductal epithelial cells: *in vitro*, *ex vivo*, and *in vivo* models. *Reproduction* **146(4)**, 315–24.

Madshus, I. H. (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250(1)**, 1–8.

Marquez, B. and Suarez, S. S. (2007). Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. *Biol. Reprod.* **76(4)**, 660–665.

Matás, C., Coy, P., Romar, R., Marco, M., Gadea, J. and Ruiz, S. (2003). Effect of sperm preparation method on *in vitro* fertilization in pigs. *Reproduction* **125(1)**, 133–141.

Matás, C., Sansegundo, M., Ruiz, S., García-Vázquez, F. A., Gadea, J., Romar, R. and Coy, P. (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. *Theriogenology* **74(8)**, 1327–1340.

Mattioli, M., Galeati, G., Bacci, M. L. and Seren, E. (1988). Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. *Gamete Res.* **21(3)**, 223–232.

Navarrete, F. A., García-Vázquez, F. A., Alvau, A., Escoffier, J., Krapf, D., Sánchez-Cárdenas, C., Salicioni, A. M., Darszon, A. and Visconti, P. E. (2015). Biphasic role of calcium in mouse sperm capacitation signaling pathways. *J. Cell. Physiol.* **230(8)**, 1758–1769.

Nishigaki, T., José, O., González-Cota, A. L., Romero, F., Treviño, C. L. and Darszon, A. (2014). Intracellular pH in sperm physiology. *Biochem. Biophys. Res. Commun.* **450(3)**, 1149–1158.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J. Biol. Chem.* **260(17)**, 9699–9705.

Parrish, J. J. (2014). Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81(1)**, 67–73.

Petters, R. M. and Wells, K. D. (1993). Culture of pig embryos. *J. Reprod. Fertil.* **Suppl. 48**, 61–73.

Quinn, P. (1995). Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J. Assist. Reprod. Genet.* **12(2)**, 97–105.

Rath, D., Long, C. R., Dobrinsky, J. R., Welch, G. R., Schreier, L. L. and Johnson, L. A. (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77(12)**, 3346–3352.

Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation. *Theriogenology* **68(s1)**, 138–146.

Rodriguez-Martinez, H., Ekstedt, E. and Einarsson, S. (1990). Acidification of epididymal fluid in the boar. *Int. J. Androl.* **13(3)**, 238–243.

Rojas, F. J., Patrizio, P., Do, J., Silber, S., Asch, R. H. and Moretti-Rojas, I. (1993). Evidence for a novel adenylyl cyclase in human epididymal sperm. *Endocrinology* **133(6)**, 3030–3033.

Sakkas, D., Leppens-Luisier, G., Lucas, H., Chardonnens, D., Campana, A., Franken, D. R. and Urner, F. (2003). Localization of tyrosine phosphorylated proteins in human sperm and relation to capacitation and zona pellucida binding. *Biol. Reprod.* **68(4)**, 1463–1469.

Satake, N., Elliott, R. M. A., Watson, P. F. and Holt, W. V (2006). Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. *J. Exp. Biol.* **209(8)**, 1560–72.

Schmidt, H. and Kamp, G. (2004). Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. *Reproduction* **128(2)**, 171–179.

Suzuki, K., Ebihara, M., Nagai, T., Clarke, N. G. E. and Harrison, R. A. P. (1994). Importance of bicarbonate/CO_2 for fertilization of pig oocytes *in vitro* and synergism with caffeine. *Reprod. Fertil. Dev.* **6(2)**, 221–227.

Tienthai, P., Johannisson, A. and Rodriguez-Martinez, H. (2004). Sperm capacitation in the porcine oviduct. *Anim. Reprod. Sci.* **80(1)**, 131–146.

Töpfer-Petersen, E., Romero, A., Varela, P. F., Ekhlasi-Hundrieser, M., Dostàlovà, Z., Sanz, L. and Calvete, J. J. (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* **30(4-5)**, 217–224.

Trivedi, D. and Danforth, W. (1966). Effect of pH on the kinetics of frog muscle phosphofructokinase. *J. Biol. Chem.* **241(17)**, 4110–4114.

Visconti, P. E. (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proc. Natl. Acad. Sci.* **106(3)**, 667–668.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995a). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121(4)**, 1139–1150.

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995b). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121(4)**, 1129–1137.

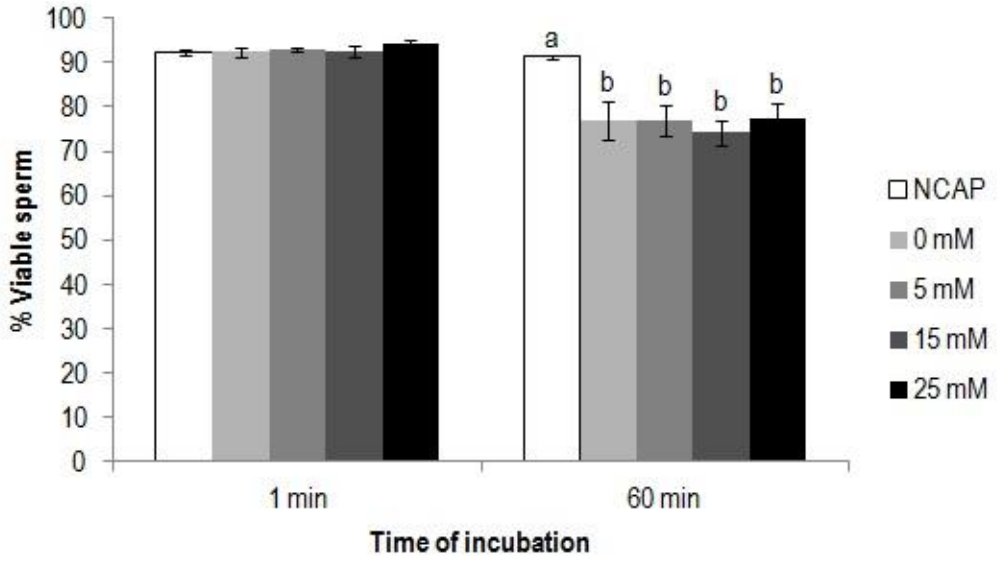
Wang, X. F., Zhou, C. X., Shi, Q. X., Yuan, Y. Y., Yu, M. K., Ajonuma, L. C., Ho, L. S., Lo, P. S., Tsang, L. L., Liu, Y., et al. (2003). Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. *Nat Cell Biol* **5(10)**, 902–906.

Wang, L., Beserra, C. and Garbers, D. L. (2004). A novel aminophospholipid transporter exclusively expressed in spermatozoa is required for membrane lipid asymmetry and normal fertilization. *Dev. Biol.* **267(1)**, 203–215.

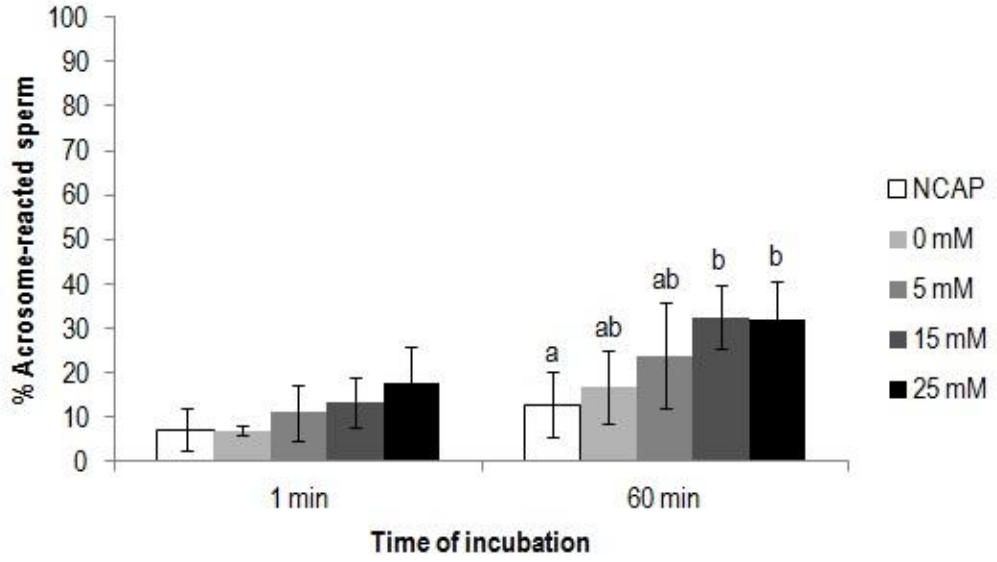
Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. In *Fertilization and embrionic development in vitro* (ed. Mastroianni, L. and Biggers, J.), pp. 81–182. New York: Plenum Press.

Yanagimachi, R. (1994). Mammalian fertilization. In *The Physiology of Reproduction* (ed. Kenobil, E. and Neill, J. D.), pp. 189–317. New York: Raven Press.

Yoshioka, K., Suzuki, C. and Onishi, A. (2008). Defined system for *in vitro* production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54(3)**, 208–213.



Supplementary file 1. Effect of HCO_3^- concentration on sperm viability. Epididymal sperm samples were incubated at 38.5ºC for 1 and 60 min in a capacitating medium (TALP) with different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and in non-capacitating medium (NCAP). Sperm plasma membrane integrity was evaluated as a reflection of sperm viability using the eosin-nigrosin staining technique. 7 replicates were performed, in which 200 spermatozoa per experimental group were classified as viable (membrane-intact) or non-viable (membrane-altered). Results are shown as mean ± SEM. Data were analyzed by one-way ANOVA and a Tukey test of multiple comparisons. Different letters (a-b) indicate statistically significant differences between groups in the same time of incubation ($p<0.05$).



Supplementary file 2. Effect of HCO_3^- concentration on acrosome reaction (AR). Epididymal sperm samples were incubated at 38.5ºC for 1 and 60 min in a capacitating medium (TALP) with different concentrations of HCO_3^- (0 mM, 5 mM, 15 mM and 25 mM) and in non-capacitating medium (NCAP). AR was assessed by staining with 1% FITC-conjugated peanut agglutinin from *Arachis hypogaea* (PNA-FITC L7381, Sigma-Aldrich®, Madrid, Spain) 20 µg/ml. Samples were fixed at 1% formol-saline, smeared onto glass slides and coverslips and analyzed by epifluorescence microscopy (blue filter, BP 480/40; emission BP 527/30; Leica® DM4000 B LED, USA) at x400. 4 replicates were performed, in which 200 spermatozoa per sample were analyzed, classifying the sperm in to reacted or non reacted-sperm sperm (with or without fluorescence in the acrosomal region, respectively). Results are shown as mean ± SEM. Data were analyzed by one-way ANOVA and a Tukey test of multiple comparisons. Different letters (a-b) in the same time of incubation indicate statistically significant differences ($p<0.05$).

Chapter 2

Improving porcine *in vitro* fertilization output by simulating the oviductal environment

Differences between the *in vitro* and *in vivo* environment in which fertilization occurs seem to play a key role in the low efficiency of porcine *in vitro* fertilization (IVF). This work proposes an IVF system based on the *in vivo* oviductal periovulatory environment. The combined use of an IVF medium at the pH found in the oviduct in the periovulatory stage (pH_e 8.0), a mixture of oviductal components (cumulus-oocyte complex secretions, follicular fluid and oviductal periovulatory fluid, OFCM) and a device that interposes a physical barrier between gametes (an inverted screw cap of a Falcon® tube, S) was compared with the classical system at pH_e 7.4, in a 4-well multidish (W) lacking oviduct biological components. The results showed that the new IVF system reduced polyspermy and increased the final efficiency by more than 48%. This higher efficiency seems to be a direct consequence of a reduced sperm motility and lower capacitating status and it could be related to the action of OFCM components over gametes and to the increase in the sperm intracellular pH (pH_i) caused by the higher pH_e used. In conclusion, a medium at pH 8.0 supplemented with OFCM reduces polyspermy and improves porcine IVF output.

1. INTRODUCTION

In pigs, unlike other species, the efficiency of *in vitro* embryo production is very low because of the high incidence of polyspermy that occurs during *in vitro* fertilization (IVF) (Grupen, 2014). Such polyspermic fertilization might be related with the high number of spermatozoa required to attain an acceptable penetration rate compared with the number that reaches the oviduct *in vivo*. Another probable cause is that spermatozoa capacitation *in vitro* is not a sequential process that provides capacitated and partially reacted spermatozoa around fertilization time (Rodriguez-Martinez, 2007) as occurs *in vivo*. Numerous IVF systems have been developed using different spermatozoa capacitation methods (Matás et al., 2010; Matás et al., 2003), fertilization media (Coy et al., 2002) or IVF devices (Grupen, 2014). However, despite the improvements achieved, there is no efficient and replicable method that can be used easily in IVF laboratories.

Under *in vivo* conditions, millions of spermatozoa are deposited in the female genital tract, but only a small subpopulation will reach the oviduct and site of fertilization. In the oviduct, spermatozoa bind to the epithelium and form the reservoir, where they will remain in a state of low-activity or uncapacitated status (Rodriguez-Martinez, 2007). When ovulation occurs, spermatozoa separate from the epithelium and swim toward the ampullary region, where fertilization takes place (Suarez, 2008a). For this purpose, spermatozoa acquire a type of special movement named hyperactivation, which is characterized by a high amplitude and an asymmetrical tail bending behaviour which results in a significantly higher swimming force than normal motility (Suarez, 2008a).

Mammalian sperm encounter an acidic medium with a low HCO_3^- concentration in the epididymis, which keeps them quiescent, and in the female the pH changes from acid in the vagina to basic in the oviduct, where the luminal fluid has a high concentration of HCO_3^-, the increasingly alkaline condition being necessary for

fertilization (Liu et al., 2012). The pH of the oviductal lumen, due to the production of HCO_3^- in the epithelial cells lining it, greatly increases upon ovulation, and reaching a pH of around 8.0 in the oviductal ampulla in the periovulatory phase in of porcine (Rodriguez-Martinez, 2007).

Spermatozoa intracellular pH (pH_i) is a key regulator of spermatozoa motility and fertilizing ability (Lishko and Kirichok, 2010). It should be noted that several unique sperm ion transporters and enzymes, whose absence causes infertility, are either pH_i dependent or in some way related to pH_i regulation (Nishigaki et al., 2014). It has been shown that a reduction in $[H^+]_i$ and an increase in $[Ca^{2+}]_i$ are required for the hyperactive movement, which is controlled by the opening of H^+ and Ca^{2+} channels in the spermatozoa membrane (Lishko and Kirichok, 2010). These channels are in part regulated by the progesterone (P4) released by the ovaries and the cumulus cells surrounding the egg, by the glycoproteins of the ZP and by albumin, the main protein of the oviductal fluid (OF) (Darszon et al., 1999). The hyperactive movement allows spermatozoa to exit the epithelium folds, swim through the viscous OF and penetrate the egg vestments. However, less than 20% of the spermatozoa population develops this kind of movement (Suarez, 2008b).

Once the spermatozoa are released from the reservoir they must find the oocyte, but the chances of such a low number of spermatozoa successfully reaching the egg without a guidance mechanism are very slim. For this reason, it is believed that spermatozoa must be guided along the oviduct in order to reach the oocyte. Indeed, it seems that spermatozoa are equipped with a mechanism for moving towards the oocyte in response to thermotaxis, rheotaxis and chemotaxis, each depending on a specific stimulus: a temperature gradient, fluid flow and a chemoattractant gradient, respectively (Pérez-Cerezales et al., 2015).

Several substances that act as spermatozoa attractants *in vivo* have been identified within the follicular fluid (FF) and OF, while others are secreted by cumulus cells and the oocyte (COCs). Among these molecules are P4, which is considered the main chemoattractant, nitric oxide (Miraglia et al., 2007), adenosine (Fraser, 2008), hyaluronic acid (HA) (Brüssow et al., 2006) and glycodelin (Seppälä et al., 2007). Spermatozoa chemoattractants, which are not only secreted prior to ovulation within the follicle, but also after oocyte maturation outside the same, have two origins: the mature oocyte and the surrounding cumulus cells (Sun et al., 2011).

In *in vitro* conditions the preincubation of oocytes in OF has been demonstrated as a good method for increasing IVF efficiency in pigs, as the ZP is modified through the binding of specific components from the OF (Coy et al., 2008). It has also been shown that OF contains P4 (Ballester et al., 2014) and improves embryo quality (Lloyd et al., 2009).

In order to improve IVF efficiency in pig, we have designed a system based on some of these *in vivo* conditions. Spermatozoa were physically separated from the oocyte into an alkaline medium supplemented with OF and FF, where the capacitation process could start. After capacitation, the sperm population, it was thought, would be able to respond to the attractants released by COCs and, consequently, swim toward the oocyte and fertilize it.

2. MATERIAL AND METHODS

2.1. Ethics

The study was carried out following the Spanish Policy for Animal Protection RD 53/2013, which meets European Union Directive 2010/63/UE on animal protection. All the procedures carried out in this work have been approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (ref. no. A13160609).

2.2. *In vitro* maturation of oocytes (IVM)

COCs were collected from antral follicles (3-6 mm diameter) and washed with Dulbecco's PBS (DPBS). Only COCs with complete and dense cumulus oophorus, in groups of 50, were cultured for 42 h in 500 µl NCSU-37, as previously described (Funahashi et al., 1997).

2.3. Conditioned medium (CM), follicular fluid (FF) and periovulatory oviductal fluid (OF) collection

After maturation, COCs were pipetted to mix their secretions with the surrounding NCSU-37 medium. The whole content of the wells was collected and centrifuged at 7,000g for 10 min at 4ºC, discarding the oocytes and cumulus cells to obtain the supernatant (CM). The FF was collected from antral follicles (3-6 mm diameter) of prepuberal gilts, as previously described (Funahashi et al., 1994). The OF was obtained from a pool of porcine oviducts with ovaries close to ovulation, as described (Carrasco et al., 2008). The FF and OF were centrifuged as described for CM. The CM, FF and OF were aliquoted and stored at -20ºC until use.

2.4. Sperm collection

The sperm-rich fraction was collected from boars with proven fertility by the manual method. Sperm concentration, motility, acrosome integrity, and normal morphology were microscopically evaluated by standard laboratory techniques.

2.5. *In vitro* fertilization (IVF)

The medium used for IVF was Tyrode's albumin lactate pyruvate (TALP) (Rath et al., 1999) (supplemented or not with OFCM) equilibrated for almost 3 h at 38.5ºC and under 5 or 1.5% of CO_2 to adjust the pH of the medium to 7.4 or 8.0, respectively (according to the Henderson-Hasselbalch equation). IVF was

performed with 2 types of device: i) a 4-well multi-dish containing 500 µl TALP per well (W) (Matás et al., 2011), and ii) inverted screw cap of a conical centrifuge tube (Falcon® 15 ml high-clarity polypropylene conical centrifuge tubes with polyethylene dome seal screw caps) containing 1000 µl TALP (S). For a full description of the device see (Funahashi and Nagai, 2000). After IVM, the oocytes (with or without cumulus cells) were gently deposited in W or in the inner plate of the S device, depending on the experimental group (Figure 1). The sperm cells were added to W or to the outer plate of the S device to give a final concentration of 1 x 10^5 cells/ml. After 18 h of co-culture the putative zygotes were fixed and evaluated as previously described (Matás et al., 2010).

2.6. Assessment of zona pellucida (ZP) solubility

The IVM oocytes, before or after IVF, were washed by pipetting to remove surrounding cells and added to 100 µl of 0.1% (w/v) pronase solution in PBS. ZPs were continuously observed for dissolution under an inverted microscope equipped with a warm plate at 37 °C (Coy et al., 2002). The ZP dissolution time of each oocyte was registered as the time elapsing between placing the samples in the pronase solution and the time when the ZP was no longer visible at a magnification of x200.

2.7. Chemiluminiscent microparticle immunoassay (CMIA)

The P4 concentration in FF, OF and CM was assessed by chemiluminiscent microparticle immunoassay (CMIA; Architect, Abbott), as described Ballester et al. (2014).

2.8. Spermatozoa motion assay

Computer-assisted spermatozoa motility analysis (CASA) was performed using ISAS® system (PROISER R+D S.L., Valencia, Spain). For this propose, a 6 µl drop of the sample was placed on a warmed (38.5ºC) 20 micron Leja® slide (SC-20-01-02-B, Leja Products B.V., Nieuw Vennep, The Netherlands) and was analyzed using a phase-contrast microscope (magnification x200; Leica DMR, Wetzlar, Germany).

2.9. Western blotting (WB)

Isolated proteins from 1 x 10^6 spermatozoa samples were obtained and immunoblotted as described (Navarrete et al., 2015). The antibodies used were anti-protein kinase A (9624, Cell Signaling Technology, Beverly, USA, 1:2,000), anti-phosphotyrosine (4G10, Millipore, CA, USA, 1:10,000) and anti-β-tubulin (T0198, Sigma-Aldrich®, Madrid, Spain, 1:5,000). Blots were visualized by chemiluminescence (Amersham Imager 600,

GE Healthcare) using a Pierce® ECL 2 Western Blotting Substrate (80196, Lumigen Inc, Southfield, MI, USA). The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK).

2.10. Immunocytochemistry: Tyrosine Phosphorylation (Tyr-P) detection by Indirect Immunofluorescence (IIF)

IIF was carried out to study Tyr-P location as described (Matás et al., 2011) using the same anti-phosphotyrosine as used for Tyr-P detection by WB (1:300 in BSA 1%). The secondary antibody used was fluorescein-conjugated goat anti-mouse (Bio-Rad Laboratories, Madrid, Spain, 1:400 in BSA 1%).

2.11. Acrosome Reaction (AR) assay

The AR was assessed by staining with FITC-conjugated peanut agglutinin from *Arachis hypogaea* (PNA-FITC L7381, Sigma-Aldrich®, Madrid, Spain) as previously used Kawano et al. (2014). Samples were analyzed under an epifluorescence microscope (blue filter, BP 480/40; emission BP 527/30; Leica® DM4000 B LED, USA) at x400 magnification.

2.12. Measurement of sperm pH_i

Spermatozoa (30 x 10^6 cells/ml) were loaded with 5 µM of the pH-sensitive dye acetoxymethyl ester of bis-carboxyethyl-carboxyfluorescein (BCECF-AM, B1150, Sigma-Aldrich®, Madrid, Spain) for 30 min incubation at 38.5ºC. Extracellular dye was removed by centrifugation at 700g for 3 min. The spermatozoa were resuspended in PBS without Ca^{2+} and Mg^{2+} and incubated again for an additional 15 min at 38.5ºC to allow de-esterification of the dye. After that, the samples were centrifuged and resuspended in the corresponding medium, depending on the experimental group. The fluorescence was immediately monitored using a spectrofluorometer (FP-6300, Jasco®, Cremella, Italy). The system was first calibrated using BCECF-AM equilibrated spermatozoa at pH 7.0, 7.4, 8.0 and 8.5 in the presence of 0.1% Triton X-100 and adjusting the pH with HCl and NaOH (Marquez and Suarez, 2007). The cells were excited at both 490 and 440 nm wavelength and the emission spectra were recorded at 535 nm. The emitted fluorescence ratio from the excitation at 490/440 nm was calculated and the regression line for pH_e *vs.* the 490/440 nm ratio was plotted. Finally, the pH_i values of the sperm cells were estimated from that regression line.

3. EXPERIMENTAL DESIGN

This study aimed to mimic several *in vivo* conditions during fertilization to improve IVF efficiency. In pursuit of these conditions, we used TALP medium supplemented with OFCM, pH 8.0 and a device (S) which separated sperm and COCs. The OFCM was composed of OF (1%) and CM (2%). The OF concentration was based on previous studies (Lopera-Vasquez et al., 2015; Kim et al., 1996). However, the reasons to supplement with 2% CM were basically mechanical (because 50 COCs were taken in a volume of 20 µl) and to ensure that the P4 concentration was within the range of values that occur in the oviduct during fertilization (Ballester et al., 2014). The spermatozoa capacitation status after incubation at pH_e 8.0 and the addition of OFCM was also evaluated. For this purpose, two experiments were performed (Figure 1).

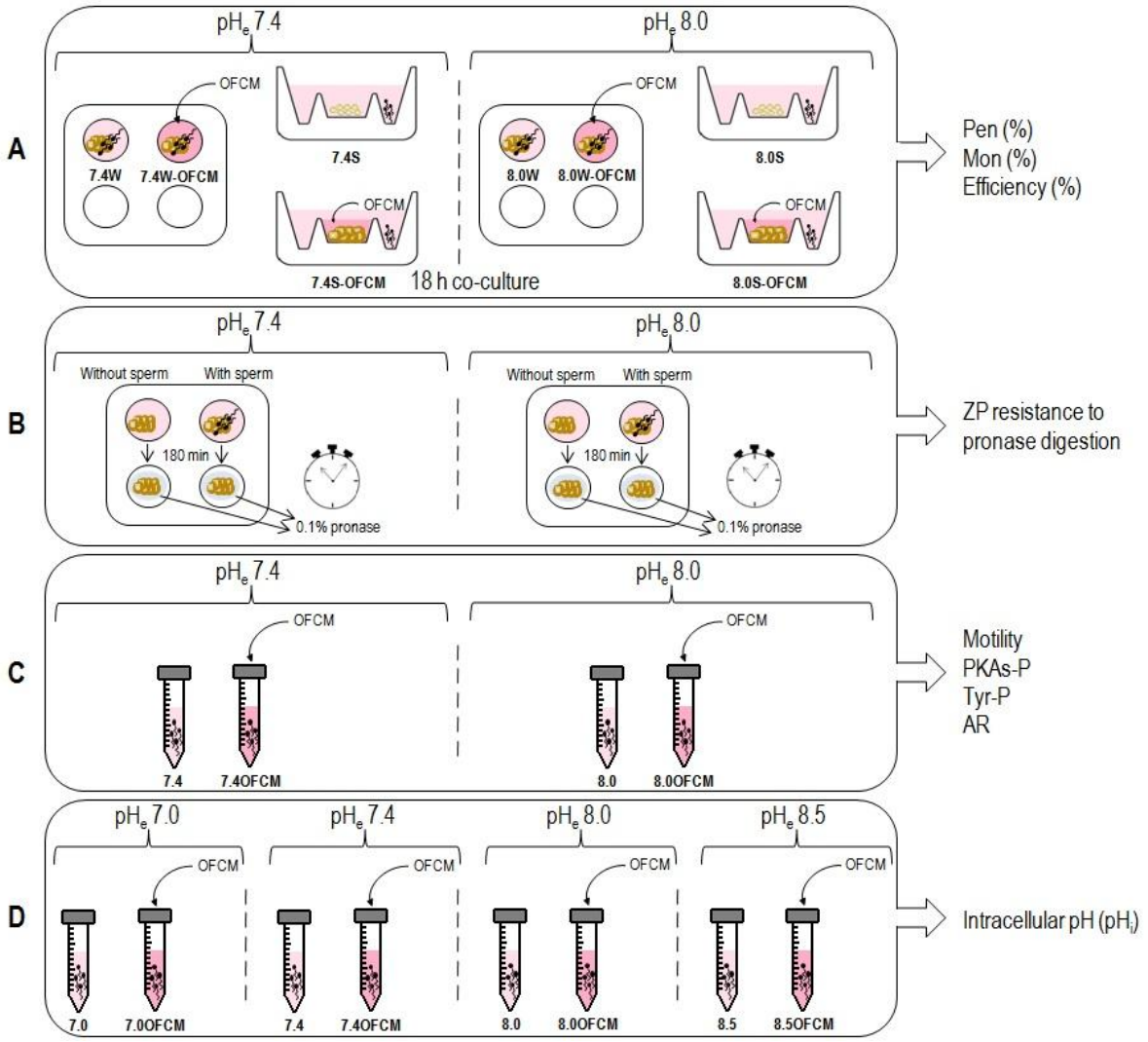


Figure 1. Experimental design. (A) Analysis of the effect of pH_e (7.4; 8.0), device (W: 4-well multi-dish; S: inverted screw cap of a conical tube) and OFCM (1% OF and 2% CM) on IVF parameters (percentages of penetration, monospermy and efficiency). **(B)** Analysis of the effect of pH_e on zona pellucida solubility after 180 min of incubation with or without spermatozoa. **(C)** Assessment of the effect of pH_e and OFCM addition on spermatozoa functionality: motility, tyrosine phosphorylation (Tyr-P), protein kinase A substrates phosphorylation (PKAs-P) and acrosome reaction (AR). **(D)** Assessment of the effect of pH_e and OFCM addition on spermatozoa pH_i.

3.1. Experiment 1: Effect of pH_e, device and medium supplementation on IVF

3.1.1. IVF

The IVF experimental groups were established depending on the pH of the medium (7.4 or 8.0), the device used (W or S) and the presence or absence of OFCM as additive to the medium (Table 1 and Figure 1A).

Table 1. Experimental groups for IVF (experiment 1).

Experimental groups	pH_e	Device	OFCM addition
7.4W	7.4	W	no
7.4S	7.4	S	no
8.0W	8.0	W	no
8.0S	8.0	S	no
7.4W-OFCM	7.4	W	yes
7.4S-OFCM	7.4	S	yes
8.0W-OFCM	8.0	W	yes
8.0S-OFCM	8.0	S	yes

A total of 1079 oocytes were used in 4 replicates to determine the percentage of penetrated oocytes (Pen, %), monospermy percentage of penetrated oocytes (Mon, %) and efficiency (Efficiency, %), which represents the final number of putative zygotes in each group per 100 penetrated oocytes.

3.1.2. ZP solubility

In vitro matured oocytes incubated for 3 h in TALP (at pH 7.4 and 8.0) and oocytes collected 3 h post-IVF (at pH 7.4 and 8.0) were subjected to ZP solubility analysis. 20 oocytes/zygotes per experimental group were used (4 replicates) (Figure 1B).

3.2. Experiment 2: Effect of pH_e and OFCM on spermatozoa functionality and pH_i

Spermatozoa were incubated in TALP medium at pH 7.4 or 8.0 and supplemented or not with OFCM (Table 2 and Figure 1C).

Table 2. Experimental groups for the spermatozoa functionality analysis (experiment 2).

Experimental groups	pH_e	OFCM addition
7.4	7.4	no
7.4OFCM	7.4	yes
8.0	8.0	no
8.0OFCM	8.0	yes

In the experimental groups defined several parameters of spermatozoa functionality were determined at different times of incubation:

3.2.1. Spermatozoa motility

Spermatozoa motility was determined at 30 min of incubation (4 replicates) in 3 different fields per sample. The parameters observed were the percentage of total motile spermatozoa (Mot, %), motile progressive spermatozoa (MotPro, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, µm), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), and beat cross-frequency (BCF, Hz).

3.2.2. Western blotting (WB): Protein Kinase A substrates and Tyrosine Phosphorylation (PKAs-P and Tyr-P)

After 180 min of incubation, PKAs-P and Tyr-P were determined by WB and semi-quantified in each experimental group in 4 replicates. β-tubulin (β-tub) was used as loading control.

3.2.3. Immunocytochemistry: Tyrosine Phosphorylation (Tyr-P) by Indirect Immunofluorescence (IIF)

The Tyr-P location in the spermatozoa was evaluated by IIF after 180 min incubation. Two-hundred spermatozoa per sample were analyzed in 4 replicates. Then, the Tyr-P location of the spermatozoa proteins was classified and grouped into three different categories according to the patterns given by Luño et al. (2013): Pattern I (low capacitation) includes spermatozoa without fluorescence, those with phosphorylated acrosome, those with phosphorylated tail and those with phosphorylated acrosome and tail. Pattern II (medium capacitation) includes spermatozoa with fluorescence in the equatorial subsegment with or without the presence of signal in the flagellum. Pattern III (high capacitation) includes spermatozoa with signal in the equatorial subsegment and acrosome area and with or without the presence of signal in the flagellum. In addition, a fourth pattern was established related with the hypermotility capacity, which included those spermatozoa with a signal in the flagellum regardless of other locations (hyperactivation pattern).

3.2.4. Acrosome Reaction (AR)

The AR was analyzed at 0 and 180 min of incubation in 4 replicates. Two-hundred spermatozoa per sample were analyzed and classified as reacted-sperm or non reacted-sperm (without or with fluorescence in the acrosomal region, respectively).

3.2.5. Spermatozoa pH_i

BCECF-AM stained and de-esterified spermatozoa were resuspended in TALP medium at pH 7.0, 7.4, 8.0 or 8.5 in presence or absence of OFCM (Figure 1D). The fluorescence was monitored for 90 s by spectrofluorimetry. The 490/440 nm ratio was recorded and the regression line for presence or absence of OFCM for all pH_e studied was calculated. The pH_i was determined from the regression lines in 4 replicates.

4. STATISTICAL ANALYSIS

All statistical analyses were performed using IBM SPSS v20.0 (IBM SPSS Inc. Armonk, NY, USA). Data are expressed as the mean ± standard error of the mean (SEM) and were fitted to the binomial variable model. In Experiment 1, the rates of oocyte penetration, monospermy and efficiency were analyzed by three-way ANOVA, considering pH_e, device and OFCM addition as the main variables. Efficiency was defined as the rate of monospermic oocytes with two pronuclei arising from the total number of inseminated oocytes. In Experiment 2.1, the data were analyzed by two-way ANOVA, considering pH_e and OFCM addition as the main variables. When ANOVA revealed a significant effect, values were compared using the least significant difference pairwise multiple comparison post hoc test (Tukey). In Experiment 2.2, regression lines were plotted and a non-parametric Kolmogórov-Smirnov test for normality of variables was applied. Mean values were analyzed and compared by Student's t-test for two independent samples and correlation coefficients of Pearson were calculated. For all experiments, the level of significance was set at $p<0.05$.

5. RESULTS

5.1. Experiment 1: Effect of pH_e, device and OFCM on *in vitro* fertilization (IVF)

5.1.1. IVF

During fertilization the environment in which gamete interaction takes place has a pH close to 8.0 (Leemans et al., 2014; Rodriguez-Martinez, 2007) but this encounter of gametes does not occur by a random

process, rather the spermatozoa must be guided towards the oocyte by different mechanisms (Pérez-Cerezales et al., 2015) through the tortuous path that is the isthmus.

So, this experiment was interested to simulate the *in vivo* fertilization conditions, studying the effect of increasing pH_e (from 7.4 to 8.0), adding OFCM (formed by FF, OF and COCs secretions) and separating the gametes using an S device in which the spermatozoa face a barrier and are physically far from the oocytes (simulating isthmus-ampulla conditions). The IVF results for penetration, monospermy and efficiency are shown in Table 3, where they are expressed as percentages.

The pH increase in the IVF medium and the addition of OFCM affected all three studied parameters ($p<0.05$). Although penetration rates were lower when pH_e 8.0 was used, monospermy was higher, meaning higher efficiency. However, using the S device did not affect any of the three parameters studied ($p>0.05$). The addition of OFCM to IVF only affected the penetration results when pH_e 7.4 and the W device were used, penetration in this case decreasing from 76.7% to 58.8%. Monospermy and efficiency rates increased in the presence of OFCM, reaching the highest percentages at pH_e 8.0 with the S device. In short, the use of pH_e 8.0 and OFCM in an S device resulted in the highest efficiency (putative zygotes) (48.7 ± 4.7%).

Table 3. Effect of pH_e, device and OFCM addition to TALP medium on pig IVF results. The IVF was carried out at pH 7.4 or 8.0, in a 4-well multidish (W) or an inverted screw cap of a tube (S) and in absence (-) or presence (+) of OFCM (1% OF and 2% CM).

pH_e		Device	OFCM	Pen (%)	Mon (%)	Efficiency (%)
7.4		W	-	76.7 ± 2.6^{a}	24.6 ± 3.0^{a}	18.9 ± 2.4^{a}
			+	58.8 ± 3.8^{b}	53.0 ± 5.0^{b}	31.2 ± 3.6^{abc}
		S	-	54.0 ± 3.6^{b}	25.5 ± 11.2^{a}	13.8 ± 6.1^{a}
			+	54.6 ± 4.4^{b}	59.1 ± 5.9^{b}	32.3 ± 4.1^{bcd}
8.0		W	-	54.3 ± 3.4^{b}	65.8 ± 4.5^{bc}	35.7 ± 3.3^{bcd}
			+	45.9 ± 3.7^{b}	77.1 ± 4.6^{bc}	35.4 ± 3.6^{bcd}
		S	-	55.9 ± 3.7^{b}	69.7 ± 4.6^{bc}	39.0 ± 3.7^{cd}
			+	54.9 ± 4.7^{b}	88.7 ± 4.0^{c}	48.7 ± 4.7^{d}
p-value	pH_e			0.002	0.000	0.000
	Device			0.122	0.183	0.273
	OFCM			0.011	0.000	0.000

Results are expressed as mean ± SEM. Different superscripts ($^{a-c}$) in the same column indicate significant differences ($p<0.05$). Pen (%): penetration percentage of total oocytes used; Mon (%): monospermy percentage calculated from penetrated oocytes; Efficiency (%): final number of putative zygotes (monospermic) in each group per 100 penetrated oocytes.

5.1.2. ZP solubility

It has been established that the ZP of oocytes matured *in vivo* is highly resistant to pronase digestion (Wang et al., 1998). This hardening may be the result of the deposition of oviductal secretions and is reversible (Coy et al., 2008). However, it is not known if pH_e affects ZP solubility. Therefore, we measured ZP solubility under different conditions.

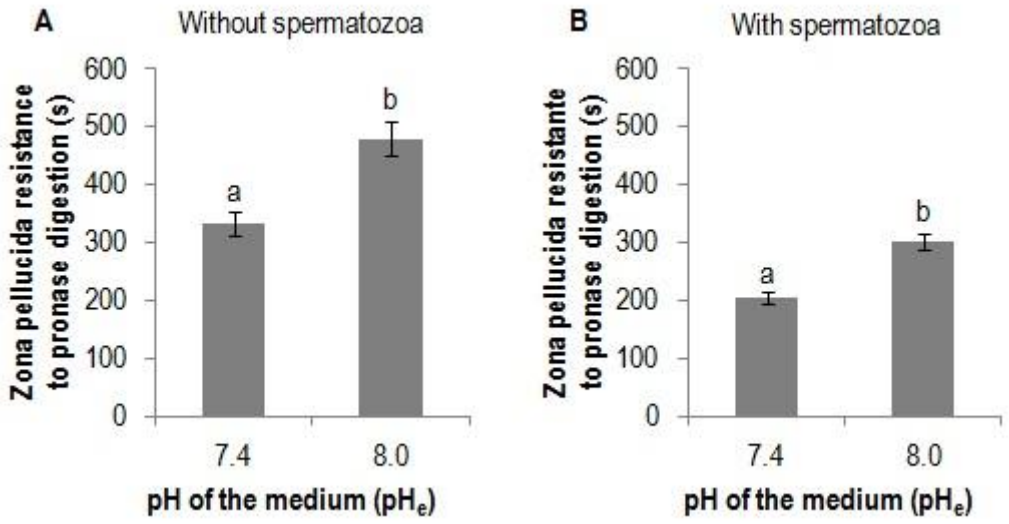


Figure 2. Zona pellucida resistance to pronase digestion in pig oocytes incubated for 180 min in TALP medium at two pH values (7.4 and 8.0). (A) Without spermatozoa co-culture. **(B)** With spermatozoa co-culture. Results expressed as mean ± SEM. Different letters (a, b) show statistical differences ($p<0.05$).

ZP resistance to pronase digestion was significantly higher ($p<0.05$) in oocytes incubated at pH_e 8.0 than at pH_e 7.4, taking 477.8 ± 30.1 and 332.4 ± 20.3 s, respectively, to dissolve (Figure 2a). In the presence of spermatozoa, pH_e-dependent differences were noted: ZP resistance after the co-culture of oocytes and spermatozoa at pH_e 7.4 was significantly lower than at pH_e 8.0 (203.6 ± 8.9 *vs.* 299.0 ± 14.0 s, $p<0.05$) (Figure 2b). The results from the present study indicate that pH_e affects ZP solubility both in the presence and absence of spermatozoa. Shorter dissolution times were recorded when the pH_e used was 7.4, with or without the presence of spermatozoa.

5.1.3. P4 concentration

The effects of P4 on spermatozoa include inducing AR, attracting the spermatozoa and/or modulating their hyperactivation in a dose-dependent manner. When P4 concentration was checked in FF (n = 6), OF (n = 5) and CM (n = 7), the results were 36.9 ± 0.5, 54.0 ± 2.4 and 105.1 ± 4.5 ng/ml (mean ± SEM), respectively. So, P4 concentration in OFCM (1% OF and 2% CM), where spermatozoa were incubated to assess functionality, was 2.6 ± 0.2 ng/ml.

5.2. Experiment 2: Effect of pH_e and OFCM on spermatozoa functionality and pH_i

In *in vivo* conditions and before gamete encounter, sperm are exposed to different environments in preparation for fertilization. In experiment 1 it was noticed that the presence of pH_e 8.0 and oviductal substances modified IVF output, so the effect of these factors on sperm functionality was investigated. The samples were incubated in a capacitating medium at pH 7.4 or 8.0 and in the presence or absence of OFCM. Several parameters of spermatozoa functionality were determined at different times of incubation:

5.2.1. Spermatozoa motility

When ovulation occurs, spermatozoa are released from the sperm reservoir before they plunge into the oviduct and swim to the fertilization site at the ampullary-isthmic junction (Hunter, 1981). During this process, the oviductal environment prepares the spermatozoa for fertilization, and, among other events, the oviductal pH increases and the motility of the spermatozoa increases (hyperactivation) (Eisenbach, 1999). This experiment studied the effect that such a periovulatory environment (pH_e 8.0 and the presence of OFCM) has on spermatozoa motility, as evaluated by CASA (Table 4). All the motility parameters studied were affected by pH_e ($p<0.05$) except MotPro and WOB. In general terms, pH_e 8.0 produced lower motility parameters than pH_e 7.4, with lower velocities (VCL, VSL and VAP), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF). When OFCM was added, the motility pattern was similar to that just described or even lower since all the motility parameters except WOB were affected by its addition. The combined use of pH_e 8.0 and OFCM (8.0OFCM) affected all motility parameters which were all significantly reduced ($p<0.05$) compared with the 7.4 group, except WOB.

Table 4. Effect of pH_e and OFCM addition on sperm motility parameters. Spermatozoa were incubated in TALP medium for 30 min at pH 7.4 or 8.0 in presence or absence of OFCM (1% OF and 2% CM) and motility were measured by CASA. Results are expressed as mean ± SEM.

pH_e		OFCM	Mot (%)	MotPro (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
7.4		-	75.3 ± 1.3^{a}	69.8 ± 1.4^{a}	107.2 ± 1.4^{a}	83.2 ± 1.3^{a}	90.1 ± 1.2^{a}	74.6 ± 0.8^{a}	87.6 ± 0.6^{a}	83.0 ± 0.5	1.8 ± 0.0^{a}	16.9 ± 0.2^{a}
		+	54.6 ± 1.5^{b}	44.8 ± 1.5^{b}	94.0 ± 2.0^{b}	70.2 ± 1.8^{b}	77.4 ± 1.7^{b}	69.8 ± 1.0^{b}	84.1 ± 0.8^{b}	80.8 ± 0.7	1.7 ± 0.03^{b}	14.7 ± 0.3^{b}
8.0		-	76.9 ± 1.3^{a}	67.7 ± 1.5^{a}	101.1 ± 1.7^{a}	75.1 ± 1.6^{b}	83.5 ± 1.5^{c}	69.7 ± 0.9^{b}	83.0 ± 0.8^{bc}	81.4 ± 0.6	1.7 ± 0.0^{ab}	15.1 ± 0.3^{b}
		+	60.6 ± 1.6^{c}	41.4 ± 1.6^{b}	75.0 ± 2.3^{c}	55.3 ± 2.0^{c}	62.8 ± 2.0^{d}	68.0 ± 1.1^{b}	80.8 ± 0.9^{c}	81.6 ± 0.7	1.4 ± 0.0^{c}	11.1 ± 0.4^{c}
p-value	pH_e		0.008	0.066	0.000	0.000	0.000	0.000	0.000	0.565	0.000	0.000
	OFCM		0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.114	0.000	0.000

Two-way ANOVA in which dependent variables were: Mot: percentage of total motile spermatozoa; MotPro: percentage of motile progressive spermatozoa; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity of the curvilinear trajectory; STR: straightness; WOB: Wobble (VAP/VCL); ALH: amplitude of lateral head displacement; BCF: beat cross-frequency. Different superscripts ($^{a-c}$) in the same column indicate statistically significant differences ($p<0.05$).

5.2.2. Western blotting (WB): Protein Kinase A substrates and Tyrosine Phosphorylation (PKAs-P and Tyr-P)

Spermatozoa need to undergo a series of changes before they can fertilize, in a process known as capacitation. This phenomenon involves the early activation of protein kinases and the inactivation of protein phosphatases. To investigate the effect of the oviductal periovulatory environment (pH_e 8.0 and presence of OFCM) on the *in vitro* spermatozoa capacitation process, PKAs-P and Tyr-P were analyzed and quantified by WB (Figure 3) since they are indicative of spermatozoa capacitation status (Visconti, 2009).

The WB for PKAs-P showed the same degree of phosphorylation in spermatozoa incubated at pH_e 7.4 and 8.0. However, when spermatozoa were incubated with OFCM, the signal intensity was lower than without it (Figures 3a and b). In the same way, the results showed that increasing pH_e is not necessarily associated with an increase or decrease in Tyr-P, while adding substances such as OFCM leads to a significantly lower degree of activation of this protein or a lower number of spermatozoa with Tyr-P (Figures 3d and e).

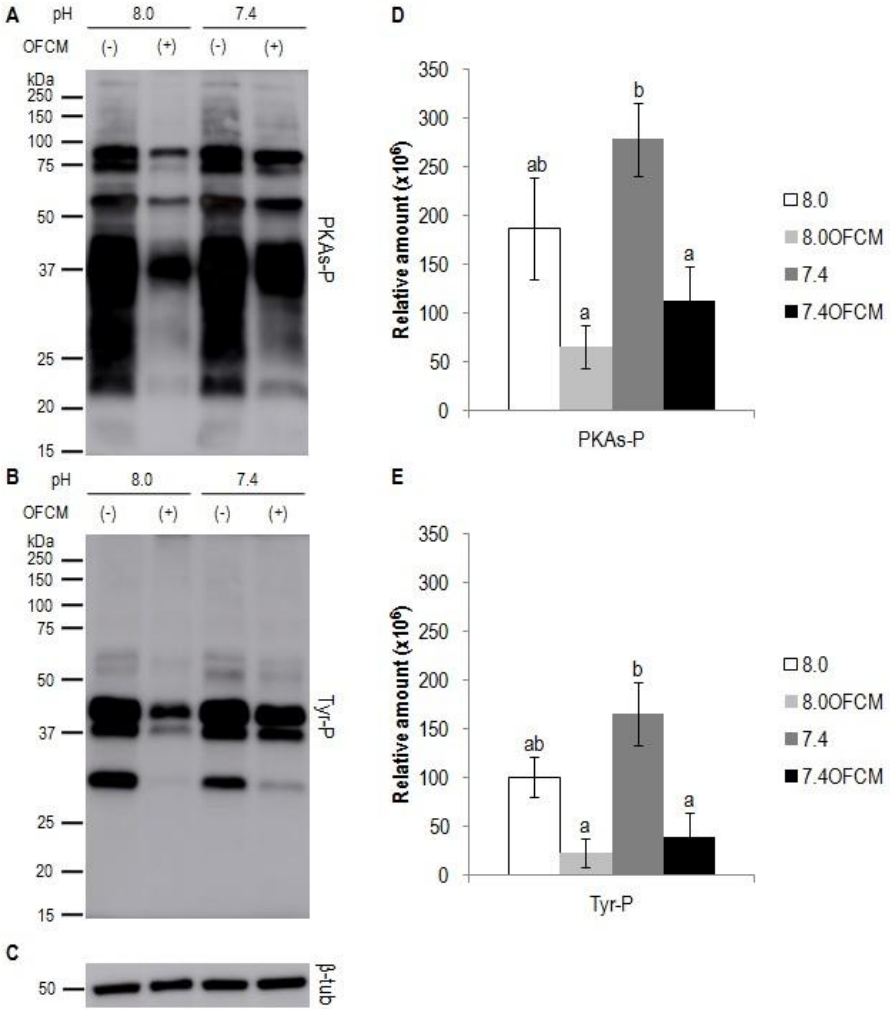


Figure 3. Effect of pH_e and OFCM on both PKA substrates and Tyrosine phosphorylation (PKAs-P and Tyr-P). Sperm were incubated for 180 min under capacitating conditions using both different pH_e values (7.4 and 8.0) and in the presence (or not) of OFCM. **(A and B)** Sperm protein extracts were analyzed for phosphorylation by western blotting using α-pPKAs or α-pTyr as first antibodies, respectively. (**C**) Loading control with β-tubulin (β-tub). **(D and E)** Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. Different letters (a, b) indicate statistically significant differences ($p<0.05$) between groups.

5.2.3. Immunocytochemistry: Tyrosine Phosphorylation (Tyr-P) observed by Indirect Immunofluorescence (IIF)

Tyr-P localization in the spermatozoa is related to their degree of capacitation (capacitation status) (Luño et al., 2013). In this experiment, the immunolocation of Tyr-P was analyzed by IIF, which allowed different spermatozoa subpopulations to be identified within a sample according to their degree of capacitation and hyperactivation (Figures 4a and b).

There were no statistical differences between groups as regard the percentage of sperm with a low capacitation status (pattern I, $p>0.05$). In the case of pattern II (considered as medium capacitation status), it was observed that at pH_e 8.0 the number of sperm showing this pattern increased. It was also recorded that when pattern II increased, pattern III (considered as high capacitation status) decreased, especially with the addition of OFCM. This means that pH_e 8.0 maintains spermatozoa in a state of low capacitation, especially in the presence of OFCM.

Figure 4b shows the Tyr-P in the flagellum, independently of other locations related with sperm hypermotility (hyperactivation pattern). According to the motion parameters described above, the 7.4 group had the highest rate of Tyr-P in the flagellum ($p<0.05$).

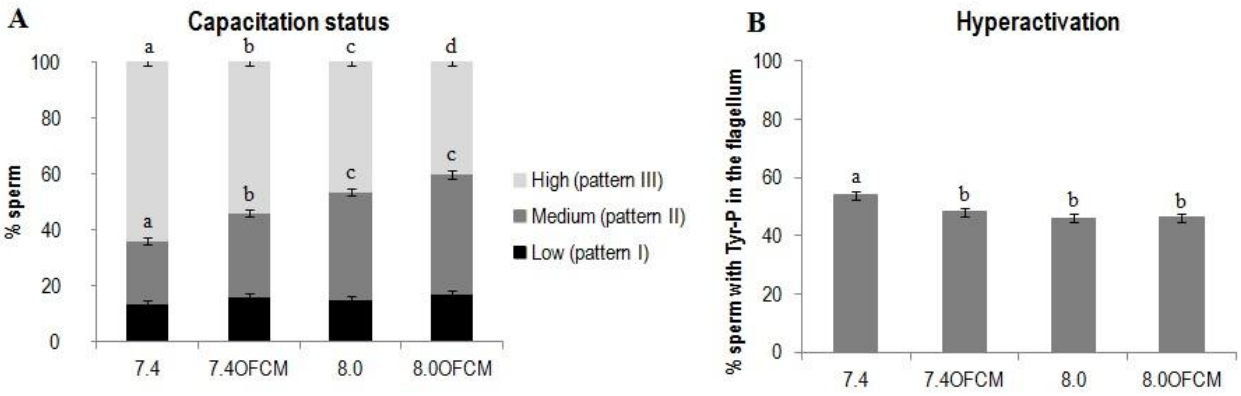


Figure 4. Effect of pH_e and OFCM on sperm capacitation status according to immunolocation of protein tyrosine phosphorylation (Tyr-P). Spermatozoa were incubated for 180 min under capacitating conditions using both different pH_e values (7.4 and 8.0) and in the presence (or not) of OFCM. Results are shown as mean ± SEM. Different letters (a-d) in the same pattern of each column indicate statistically significant differences ($p<0.05$). **(A)** Percentage of sperm with low capacitation status (pattern I: non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa), medium capacitation status (pattern II: equatorial segment or equatorial segment and flagellum phosphorylated) or high capacitation status (pattern III: equatorial segment and head and/or flagellum phosphorylated). **(B)** Percentage of hyperactivated sperm (pattern IV: flagellum phosphorylated regardless in other locations).

5.2.4. Acrosome Reaction (AR)

Following spermatozoa capacitation, the AR takes place in response to an agonist such as P4 or ZP (De Lamirande et al., 1997). *In vivo*, the oviductal environment to which spermatozoa are exposed protects the spermatozoa from a premature AR (Ghersevich et al., 2015; Zumoffen et al., 2010), which the spermatozoa only

undergo when in contact with the oocyte. Therefore, this experiment studied the *in vitro* effect of pH_e 8.0 and the presence of OFCM on spermatozoa AR at 0 and 180 min of incubation (Figure 5).

At the beginning of incubation (0 min), as expected, all groups showed a low proportion of acrosome-reacted spermatozoa with no significant differences between them ($p>0.05$). After 180 min of incubation in the medium, the proportion of acrosome-reacted spermatozoa had increased in all groups but in different ways. The pH_e had no statistically relevant effect but the addition of OFCM made a great difference considerably lowering the percentage of acrosome-reacted sperm.

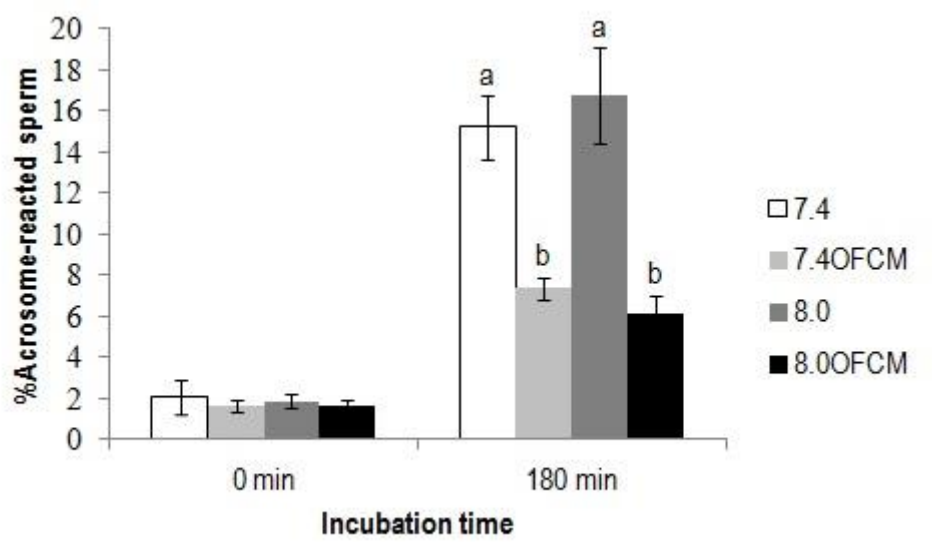


Figure 5. Effect of pH_e and OFCM on sperm acrosome reaction (AR). Spermatozoa were incubated for 0 and 180 min in capacitating medium using both different pH_e values (7.4 and 8.0) and in the presence (or not) of OFCM. Different letters (a, b) in the same time of incubation indicate statistically significant differences ($p < 0.05$).

5.2.5. Spermatozoa pH_i

During fertilization process spermatozoa are exposed to a varying pH_e and capacitation is associated to an intracellular alkalinisation in sperm of many mammalian species. On the other hand, the metabolic activity acidifies the cytosol and changes in pH_i affect the ionization state of the weak acids and bases present in most proteins and many biomolecules. For the reason explained above, it was decided to analyse whether OFCM modified the pH_i when the pH_e increased.

The results showed the fluorescence ratio at 490/440 nm and the pH_i when spermatozoa were exposed to different pH_e (Figures 6a and b). The spermatozoa had a lower pH_i than pH_e but they were correlated ($r = 0.954$) since when pH_e increased, the pH_i increased too. However, for all values of pH_e studied the presence of OFCM had no effect on pH_i ($p = 0.752$).

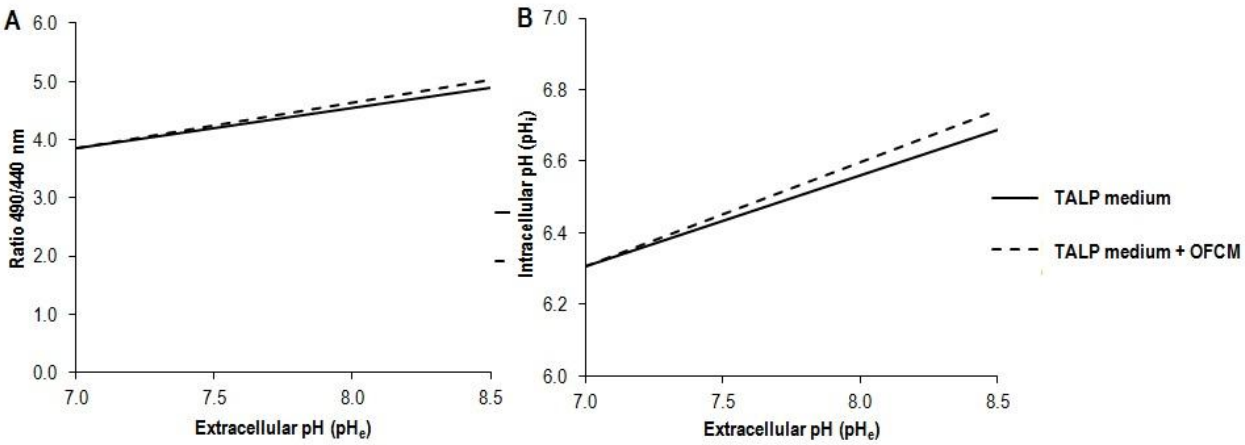


Figure 6. Effect of extracellular pH (pH_e) and OFCM on intracellular pH (pH_i) in boar spermatozoa incubated in TALP medium. (A) Fluorescence emission ratio with BCECF-AM when samples were exposed to different pH_e values in a range from 7.0 to 8.5 in the absence or presence of OFCM and excited at 490 and 440 nm. **(B)** pH_i calculated from the emission ratio (490/440 nm) when spermatozoa are exposed to different pH_e in a range from 7.0 to 8.5 in absence or presence of OFCM.

6. DISCUSSION

The current *in vitro* production of potentially viable porcine embryos is not efficient mainly due to the high incidence of polyspermy (Grupen, 2014) and it is clear that the microenvironment in which fertilization occurs plays a key role (Coy et al., 2009; Coy et al., 2008). The present study attempts to improve previous results by ensuring the presence of conditioned medium [COCs secretions, oviductal and follicular fluids (OFCM)], using an IVF device in which spermatozoa are physically separated from the oocytes, but in which they can swim towards them, and, finally, the exposing the gametes to specific oviductal factors at different pH values.

The pH in the porcine oviductal ampulla is close to 8.0 during the periovulatory phase (Rodriguez-Martinez, 2007). For this reason, the first factor analyzed in the present study was the effect of increasing the pH_e from 7.4 (current IVF system) to 8.0. The IVF results showed that efficiency was higher at pH_e 8.0, which seems reasonable because most biological processes that occur in living organisms are regulated by the pH level (Lodish et al., 2000) and fertilization is no exception. The slight increase in pH_e may be acting in two different ways to decrease polyspermy. On the one hand, it could be slowing down the enzymatic activity of the acrosomal content over ZP proteins, which would reduce the number of spermatozoa that completely dissolve the ZP proteins in the short time available for fertilization, resulting in a protective effect against multiple penetrations. On the other hand, pH_e 8.0 could be improving the release of cortical granules to the perivitelline space, promoting the correct conformational changes of ZP proteins that prevent polyspermic penetration (Coy et al., 2002; Iwamoto et al., 1999). In order to test this possibility, a ZP hardening assay was performed. The results showed that ZP resistance to dissolution was higher in oocytes incubated at pH_e 8.0 after culture both in the presence and

in absence of spermatozoa. Nonetheless, the effect of pH_e on ZP hardening was not comparable to the effect of using periovulatory OF (Fraser, 2008).

Porcine IVF is usually carried out without barriers between the spermatozoa and oocytes and with a high number of spermatozoa per oocyte. Therefore, the *in vitro* penetration of oocytes is more a matter of coincidence than a competition and selection process that occurs *in vivo* (Holt and Fazeli, 2016). In IVF systems, each oocyte has contact with many spermatozoa at the same time. Besides, it has been shown that the presence of a large number of spermatozoa at an early stage of the spontaneous AR in co-culture (Funahashi and Nagai, 2001) results in high polyspermy rates. Therefore, using a device in which gametes are separated and which spermatozoa have to swim up to find oocytes could be considered a good way to improve monospermy. When we used an S device, as used in (Funahashi and Nagai, 2001), neither monospermy nor efficiency improved. However, these authors used frozen semen, which could mean limited sperm survival. In the present work, fresh semen was used, and it is known that the corresponding spermatozoa can penetrate oocytes for up to 23 h of co-culture (Martinez et al., 1996), enabling spermatozoa to undergo capacitation and swim toward the oocyte for a longer time.

The third condition that could improve efficiency was to mimic the *in vivo* fertilization environment by using secretions produced in the oviduct during the periovulatory stage. So, OFCM medium was added to the IVF system. Our results showed that components of the OFCM seemed to be acting on the gametes and/or their interaction, although this effect was only relevant when IVF was performed at pH_e 7.4. This result agrees with those of other authors, who reported the hardening effect of OF on ZP and the consequent prevention of multiple penetration (Coy et al., 2008). When a pH_e of 8.0 was used, the addition of OFCM did not increase the efficiency. Perhaps pH_e 8.0 in itself is enough to improve the IVF output, although it has been shown that OF has any beneficial effect on subsequent embryo quality (Lloyd et al., 2009).

Spermatozoa motility, PKA activity, Tyr-P and AR were analysed in order to clarify whether the IVF results described above could be attributed to the spermatozoa capacitation status. As has been reported, the pattern of spermatozoa movement varies among species and with the physical environment in which the spermatozoa swim. However in mammals the motility of recently ejaculated spermatozoa is characterized by a relatively low-amplitude and symmetrical tail bending, but, when hyperactivated, this motility changes to a high-amplitude accompanied by asymmetrical tail bending (observed close to the site of fertilization) (Suarez, 2008a). The results of our study showed that pH_e 8.0 with OFCM led to a decrease in all motility parameters except for WOB although the main factor responsible was the addition of OFCM. Some authors (Zhou et al., 2015) observed that a pH_e below 7.2 affected sperm motility and capacitation but there was no effect between pH_e 7.2 and 8.2. This apparent inconsistency with our results could be explained by the different experimental conditions used in the studies, the sperm source (human sperm *vs.* porcine sperm) and the incubation times.

When spermatozoa were incubated with OFCM, the values of all motility parameters decreased, perhaps because this medium reverses the influence of the capacitating factors present in TALP medium.

Besides, it has been shown that the pattern of hyperactivation is greatly influenced by both the surrounding microstructure and the viscosity of the medium (Pérez-Cerezales et al., 2016; Yanagimachi, 1994). It is known that certain components of OFCM medium, such as P4, are chemoattractants and produce lower hyperactivation (Armon and Eisenbach, 2011). OF and HA prolong spermatozoa survival and delay destabilization processes at the plasmalemma i.e. spermatozoa capacitation (Rodriguez-Martinez et al., 2016) and resulting hyperactivation. In fact, the sperm reservoir regulates the physiological status of the spermatozoa, particularly the processes of capacitation and hyperactivation within the SR to ensure that spermatozoa are in the proper state when ovulation occurs (Suarez, 2002). The other factor to consider is the viscosity of the medium since viscoelastic fluids can reduce the swimming velocity of spermatozoa (Pérez-Cerezales et al., 2016). The OFCM medium is more viscous than TALP (Coy et al., 2009), and an increase in the viscoelasticity has been associated with a lower percentage of motile spermatozoa as well as with lower VCL, VAP and ALH (Elzanaty et al., 2002). Some authors (Nosrati et al., 2015; Coy et al., 2009) also showed that viscosity affected the spermatozoa motility parameters but the results varied depending on the degree of the viscosity of the medium.

Capacitation status has been associated with an increase in PKA activity and phosphorylation in tyrosine residues of general sperm proteins (Visconti et al., 1999). Although increasing the pH_e to 8.0 provoked an increase of 0.2 points in pH_i, it seemed to have no significant effect on sperm capacitation and pH_e 7.4 is enough to reach a high grade of PKAs-P and Tyr-P. However, pH_e 8.0 had a significant effect, increasing ZP resistance to digestion and probably playing a key role in gametes interaction. In the present study, we showed that the presence of OFCM in the capacitation medium decreased PKA activity and the Tyr-P of sperm proteins, suggesting that OFCM interferes with the cyclic AMP (cAMP)/PKA/Tyr-P pathway but in a way that is still unknown. In this same sense, the localization of Tyr-P showed that the presence of OFCM produced a lower percentage of highly capacitated spermatozoa, especially at pH_e 8.0, supporting the idea that their components could act by preserving their function and fertilizing ability.

In *in vivo* conditions, capacitation is inhibited while the spermatozoa remain in the oviductal reservoir (Zumoffen et al., 2010; Tienthai et al., 2004) despite the abundant presence of bicarbonate (35-90 mM) (Rodriguez-Martinez, 2007) which would normally be sufficient to induce capacitation and rapid flagellar activity *in vitro*. Apart from bicarbonate-induced effects during sperm capacitation, it is also important to notice how oviduct proteins and fluids may affect the composition and organization of proteins on the sperm surface (Brewis and Gadella, 2009), acting to sequentially activate different signalling pathways. It is known that the adenosine present in the FF regulates the soluble adenylyl cyclase (sAC)/cAMP signal transduction pathway stimulating cAMP production in uncapacitated cells but inhibiting it in capacitated ones (Fraser, 2008). The hyaluronic acid (HA) from the OF and FF present in the oviduct can modulate sperm capacitation and promote sperm survival (Rodriguez-Martinez, 2007), and HA from cumulus cells promote the AR close to the oocyte (Suzuki et al., 2002). Besides, it has been suggested, some oviductal proteins could be involved in modulating sperm function and fertilization (Zumoffen et al., 2010); for example, Glycodelin-A, -S, -F and -C bind to the sperm head during their passage through the female genital tract and first act by inhibiting the sperm-zona pellucida binding and

suppressing the P4-induced AR and finally stimulate sperm-zona pellucida binding (Seppälä et al., 2007). Additionally, the OF contains soluble proteins such as oviduct glutathione peroxidase, superoxide dismutase and catalase, that may regulate the balance between reactive oxygen species and antioxidants in sperm – which is important for sperm viability and motility (Lapointe, 2003) – and so regulate the capacitation process (Baker and Aitken, 2004).

The oviduct environment acts as the sperm reservoir, maintaining spermatozoa viability and modulating the subpopulation of spermatozoa that initiates the capacitation process, preventing a premature AR and resulting in a lower rate of polyspermy, as previously described (Ghersevich et al., 2015; Zumoffen et al., 2010). The results of the present study showed that OFCM components decrease the induced AR after incubation of the spermatozoa in capacitation conditions and maintain their fertilizing potential probably via sAC/cAMP, as has been previously reported (Fraser, 2008). This could be attributed to a decrease in PKA activation and Tyr-P. It also has been determined that PKA, together with inositol-trisphosphate, activates Ca^{2+} channels in the outer acrosomal membrane, which leads to an increase in cytosolic Ca^{2+}. The depletion of Ca^{2+} in the acrosome will activate a store-operated Ca^{2+} entry mechanism in the plasma membrane, leading to increase in cytosolic Ca^{2+} and resulting in membrane fusion and AR (Breitbart, 2002). Therefore, the effect of OFCM on PKA activity and AR might help to explain the decrease in polyspermy percentage observed. P4, another component of OFCM, induces AR but this effect seems to be prevented by adenosine and glycodelins in OFCM (Seppälä et al., 2007). The low concentration of P4 detected in OFCM (much lower concentration that that used for *in vitro* AR induction (Wu et al., 2006) was probably responsible for guiding the spermatozoa to the oocytes but did not produce a higher level of capacitation or induce the AR (Publicover et al., 2008). However, the proteins of the OFCM could have been responsible for decreasing the number of P4 receptors in the sperm membrane, as has been suggested (Munuce et al., 2009).

Therefore, the main effect on spermatozoa of the substances present in OFCM seems to be keeping them in a state of low capacitation, thus regulating the number of spermatozoa ready for fertilization, leading to an increase in efficiency.

In conclusion, the results obtained in this work suggest that the IVF conditions proposed are more similar to the *in vivo* periovulatory environment in the oviduct than can be obtained with the currently used IVF protocol. Since the success of fertilization is a multifactorial process that is difficult to control *in vitro*, setting several periovulatory oviductal factors (presence of cumulus cells, follicular and oviductal fluids and adjusted pH) reduces polyspermy and increases IVF efficiency in pig. Manipulation of these components has enabled us to propose a new way of performing IVF, resulting in a greater number of potentially viable zygotes. However, more studies are necessary to clarify the improvements and to identify new conditions that might contribute to a better understanding of swine reproductive physiology.

7. REFERENCES

Armon, L. and Eisenbach, M. (2011). Behavioral mechanism during human sperm chemotaxis: Involvement of hyperactivation. *PLoS One* **6(12)**, e28359.

Baker, M. A. and Aitken, R. J. (2004). The importance of redox regulated pathways in sperm cell biology. *Mol. Cell. Endocrinol.* **216(1)**, 47–54.

Ballester, L., Romero-Aguirregomezcorta, J., Soriano-Úbeda, C., Matás, C., Romar, R. and Coy, P. (2014). Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine *in vitro* fertilization efficiency. *Fertil. Steril.* **102(6)**, 1762–1768.

Breitbart, H. (2002). Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol. Cell. Endocrinol.* **187(1-2)**, 139–144.

Brewis, I. A. and Gadella, B. M. (2009). Sperm surface proteomics: From protein lists to biological function. *Mol. Hum. Reprod.* **16(2)**, 68–79.

Brüssow, K. P., Torner, H., Rátky, J., Manabe, N. and Tuchscherer, A. (2006). Experimental evidence for the influence of cumulus-oocyte-complexes on sperm release from the porcine oviductal sperm reservoir. *J. Reprod. Dev.* **52(2)**, 249–57.

Carrasco, L. C., Romar, R., Avilés, M., Gadea, J. and Coy, P. (2008). Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. *Reproduction* **136(6)**, 833–842.

Coy, P., Gadea, J., Romar, R., Matás, C. and García, E. (2002). Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. *Reproduction* **124(2)**, 279–288.

Coy, P., Cánovas, S., Mondéjar, I., Saavedra, M. D., Romar, R., Grullón, L., Matás, C. and Avilés, M. (2008). Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc. Natl. Acad. Sci.* **105(41)**, 15809–15814.

Coy, P., Gadea, J., Rath, D. and Hunter, R. H. F. (2009). Differing sperm ability to penetrate the oocyte *in vivo* and *in vitro* as revealed using colloidal preparations. *Theriogenology* **72(9)**, 1171–1179.

Darszon, A., Labarca, P., Nishigaki, T. and Espinosa, F. (1999). Ion channels in sperm physiology. *Physiol. Rev.* **79(2)**, 481–510.

De Lamirande, E., Leclerc, P. and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* **3(3)**, 175–194.

Eisenbach, M. (1999). Mammalian sperm chemotaxis and its association with capacitation. *Dev. Genet.* **25(2)**, 87–94.

Elzanaty, S., Richthoff, J., Malm, J. and Giwercman, A. (2002). The impact of epididymal and accessory sex gland function on sperm motility. *Hum. Reprod.* **17(11)**, 2904–2911.

Fraser, L. R. (2008). The role of small molecules in sperm capacitation. *Theriogenology* **70(8)**, 1356–1359.

Funahashi, H. and Nagai, T. (2000). Sperm selection by a climbing-over-a-wall IVF method reduces the incidence of polyspermic penetration of porcine. *J. Reprod. Dev.* **46(5)**, 319–324.

Funahashi, H., Cantley, T. and Day, B. N. (1994). Different hormonal requirements of pig oocyte-cumulus complexes during maturation *in vitro*. *J. Reprod. Fertil.* **101(1)**, 159–165.

Funahashi, H., Cantley, T. C. and Day, B. N. (1997). Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization. *Biol. Reprod.* **57(1)**, 49–53.

Funahashi, H & Nagai, T. (2001). Regulation of *in vitro* penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. *Mol. Reprod. Dev.* **58(4)**, 424–431.

Ghersevich, S., Massa, E. and Zumoffen, C. (2015). Oviductal secretion and gamete interaction. *Reproduction* **149(1)**, R1–R14.

Grupen, C. G. (2014). The evolution of porcine embryo invitro production. *Theriogenology* **81(1)**, 24–37.

Holt, W. V. and Fazeli, A. (2016). Sperm selection in the female mammalian reproductive tract. Focus on the oviduct: hypotheses, mechanisms, and new opportunities. *Theriogenology* **85(1)**, 105–112.

Hunter, R. H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil.* **63(1)**, 109–117.

Iwamoto, K., Ikeda, K., Yonezawa, N., Noguchi, S., Kudo, K., Hamano, S., Kuwayama, M. and Nakano, M. (1999). Disulfide formation in bovine zona pellucida glycoproteins during fertilization: evidence for the involvement of cystine cross-linkages in hardening of the zona pellucida. *J. Reprod. Fertil.* **117(2)**, 395–402.

Kawano, N., Araki, N., Yoshida, K., Hibino, T., Ohnami, N., Makino, M., Kanai, S., Hasuwa, H., Yoshida, M.,

Miyado, K., et al. (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proc. Natl. Acad. Sci. U. S. A.* **111(11)**, 4145–50.

Kim, N. H., Funahashi, H., Abeydeera, L. R., Moon, S. J., Prather, R. S. and Day, B. N. (1996). Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes *in vitro*. *J. Reprod. Fertil.* **107(1)**, 79–86.

Lapointe, J. (2003). Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biol. Reprod.* **68(4)**, 1157–1164.

Leemans, B., Gadella, B. M., Sostaric, E., Nelis, H., Stout, T. A. E., Hoogewijs, M. and Soom, A. Van (2014). Oviduct binding and elevated environmental pH induce protein tyrosine phosphorylation in stallion spermatozoa. *Biol. Reprod.* **9113(1)**, 1–12.

Lishko, P. V and Kirichok, Y. (2010). The role of Hv1 and CatSper channels in sperm activation. *J. Physiol.* **588(23)**, 4667–4672.

Liu, Y., Wang, D. K. and Chen, L. M. (2012). The physiology of bicarbonate transporters in mammalian reproduction. *Biol. Reprod.* **86(4)**, 99–99.

Lloyd, R. E., Romar, R., Matás, C., Gutiérrez-Adán, A., Holt, W. V. and Coy, P. (2009). Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced *in vitro*. *Reproduction* **137(4)**, 679–687.

Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D. and Darnell, J. (2000). *Molecular Cell Biology*. 4th ed. (ed. Company, W. H. F. and) New York.

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Lloreda, V., Coy, P., Gutierrez-Adan, A., Bermejo-Alvarez, P. and Rizos, D. (2015). Effect of bovine oviductal fluid on development and quality of bovine embryos produced *in vitro*. *Reprod. Fertil. Dev.* **29(3)**, 621–629.

Luño, V., López-Úbeda, R., García-Vázquez, F. A., Gil, L. and Matás, C. (2013). Boar sperm tyrosine phosphorylation patterns in the presence of oviductal epithelial cells: *in vitro*, *ex vivo*, and *in vivo* models. *Reproduction* **146(4)**, 315–24.

Marquez, B. and Suarez, S. S. (2007). Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. *Biol. Reprod.* **76(4)**, 660–665.

Martinez, E. a, Vazquez, J. M., Matas, C., Gadea, J., Alonso, M. I. and Roca, J. (1996). Oocyte penetration by fresh or stored diluted boar spermatozoa before and after *in vitro* capacitation treatments. *Biol. Reprod.* **55(1)**, 134–40.

Matás, C., Coy, P., Romar, R., Marco, M., Gadea, J. and Ruiz, S. (2003). Effect of sperm preparation method on *in vitro* fertilization in pigs. *Reproduction* **125(1)**, 133–141.

Matás, C., Sansegundo, M., Ruiz, S., García-Vázquez, F. A., Gadea, J., Romar, R. and Coy, P. (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. *Theriogenology* **74(8)**, 1327–1340.

Matás, C., Vieira, L., García-Vázquez, F. A., Avilés-López, K., López-Úbeda, R., Carvajal, J. A. and Gadea, J. (2011). Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. *Anim. Reprod. Sci.* **127(1-2)**, 62–72.

Miraglia, E., Rullo, M. L., Bosia, A., Massobrio, M., Revelli, A. and Ghigo, D. (2007). Stimulation of the nitric oxide/cyclic guanosine monophosphate signaling pathway elicits human sperm chemotaxis *in vitro*. *Fertil. Steril.* **87(5)**, 1059–1063.

Munuce, M. J., Serravalle, A., Caille, A. M., Zumoffen, C., Botti, G., Cabada, M. and Ghersevich, S. (2009). Human tubal secretion can modify the affinity of human spermatozoa for the zona pellucida. *Fertil. Steril.* **91(2)**, 407–413.

Navarrete, F. A., García-Vázquez, F. A., Alvau, A., Escoffier, J., Krapf, D., Sánchez-Cárdenas, C., Salicioni, A. M., Darszon, A. and Visconti, P. E. (2015). Biphasic role of calcium in mouse sperm capacitation signaling pathways. *J. Cell. Physiol.* **230(8)**, 1758–1769.

Nishigaki, T., José, O., González-Cota, A. L., Romero, F., Treviño, C. L. and Darszon, A. (2014). Intracellular pH in sperm physiology. *Biochem. Biophys. Res. Commun.* **450(3)**, 1149–1158.

Nosrati, R., Driouchi, A., Yip, C. M. and Sinton, D. (2015). Two-dimensional slither swimming of sperm within a micrometre of a surface. *Nat. Commun.* **6**, 8703.

Pérez-Cerezales, S., Boryshpolets, S. and Eisenbach, M. (2015). Behavioral mechanisms of mammalian sperm guidance. *Asian J. Androl.* **17(4)**, 628.

Pérez-Cerezales, S., López-Cardona, A. P. and Gutiérrez-Adán, A. (2016). Progesterone effects on mouse sperm kinetics in conditions of viscosity. *Reproduction* **151(5)**, 501–507.

Publicover, S. J., Giojalas, L. C., Teves, M. E., de Oliveira, G. S. M. M., Garcia, A. A. M., Barratt, C. L. R. and Harper, C. V. (2008). Ca^{2+} signalling in the control of motility and guidance in mammalian sperm. *Front. Biosci.* **13**, 5623–37.

Rath, D., Long, C. R., Dobrinsky, J. R., Welch, G. R., Schreier, L. L. and Johnson, L. A. (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77(12)**, 3346–3352.

Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation. *Theriogenology* **68(s1)**, 138–146.

Rodriguez-Martinez, H., Tienthai, P., Atikuzzaman, M., Vicente-Carrillo, A., Rubér, M. and Alvarez-Rodriguez, M. (2016). The ubiquitous hyaluronan: Functionally implicated in the oviduct? *Theriogenology* **86(1)**, 182–186.

Seppälä, M., Koistinen, H., Koistinen, R., Chiu, P. C. N. and Yeung, W. S. B. (2007). Glycosylation related actions of glycodelin: gamete, cumulus cell, immune cell and clinical associations. *Hum. Reprod. Update* **13(3)**, 275–287.

Suarez, S. S. (2002). Formation of a reservoir of sperm in the oviduct. *Reprod. Domest. Anim.* **37(3)**, 140–143.

Suarez, S. S. (2008a). Regulation of sperm storage and movement in the mammalian oviduct. *Int. J. Dev. Biol.* **52(5-6)**, 455–462.

Suarez, S. S. (2008b). Control of hyperactivation in sperm. *Hum. Reprod. Update* **14(6)**, 647–657.

Sun, T. T., Chung, C. M. and Chan, H. C. (2011). Acrosome reaction in the cumulus oophorus revisited: Involvement of a novel sperm-released factor NYD-SP8. *Protein Cell* **2(2)**, 92–98.

Suzuki, K., Asano, A., Eriksson, B., Niwa, K., Nagai, T. and Rodriguez-Martinez, H. (2002). Capacitation status and *in vitro* fertility of boar spermatozoa: Effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int. J. Androl.* **25(2)**, 84–93.

Tienthai, P., Johannisson, A. and Rodriguez-Martinez, H. (2004). Sperm capacitation in the porcine oviduct. *Anim. Reprod. Sci.* **80(1-2)**, 131–146.

Visconti, P. E. (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proc. Natl. Acad. Sci.* **106(3)**, 667–668.

Visconti, P. E., Ning, X., Fornés, M. W., Alvarez, J. G., Stein, P., Connors, S. A. and Kopf, G. S. (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev. Biol.* **214(2)**, 429–43.

Wang, W., Abeydeera, L. R., Prather, R. S. and Day, B. N. (1998). Morphologic comparison of ovulated and *in vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. *Mol. Reprod. Dev.* **49(3)**, 308–316.

Wu, J. Te, Chiang, K. C. and Cheng, F. P. (2006). Expression of progesterone receptor(s) during capacitation and incidence of acrosome reaction induced by progesterone and zona proteins in boar spermatozoa. *Anim. Reprod. Sci.* **93(1-2)**, 34–45.

Yanagimachi, R. (1994). Mammalian fertilization. In *The Physiology of Reproduction* (ed. Kenobil, E. and Neill, J. D.), pp. 189–317. New York: Raven Press.

Zhou, J., Chen, L., Li, J., Li, H., Hong, Z., Xie, M., Chen, S., Yao, B. and Drevet, J. R. (2015). The semen pH affects sperm motility and capacitation. *PLoS One* **10(7)**, 1–15.

Zumoffen, C. M., Caille, A. M., Munuce, M. J., Cabada, M. O. and Ghersevich, S. A. (2010). Proteins from human oviductal tissue-conditioned medium modulate sperm capacitation. *Hum. Reprod.* **25(6)**, 1504–1512.

Conclusions

1. An *in vitro* concentration of 15 millimolar of bicarbonate is sufficient to achieve the maximum degree of sperm capacitation in terms of protein kinase A substrates and tyrosine phosphorylation, intracellular pH, hyperactivation and linear motility.

2. *In vitro* fertilization is modulated by the concentration of bicarbonate in the medium during the co-incubation gametes, reaching the maximum efficiency with 15 millimolar of bicarbonate.

3. The pre-incubation of spermatozoa in a medium with lower concentration of bicarbonate than that used as *in vitro* fertilization medium does not improve the efficiency of the system, whereas maintaining 15 millimolar of bicarbonate in the biphasic system maximizes it.

4. Mimicking the *in vivo* periovulatory oviductal environment (pH 8.0, presence of oviductal and follicular fluids and secretions of cumulus cells) and using a selective device in which spermatozoa must swim to overcome a physical barrier to contact the oocytes, increases the *in vitro* fertilization output.

5. The pH 8.0 of the capacitating medium and the presence of periovulatory oviductal secretions maintain the spermatozoa in a low capacitation status as regard motility and tyrosine phosphorylation. Periovulatory oviductal secretions also decrease the acrosome reaction and protein kinase A phosphorylation.

Abbreviations

AI: artificial insemination

AIJ: ampullary-isthmic junction

ALH: amplitude of lateral head displacement

AR: acrosome reaction

ARTs: artificial reproductive technologies

BCECF-AM: acetoxymethyl ester of bis-carboxyethyl-carboxyfluorescein

BCF: beat cross-frequency

BO: Brackett- Oliphant solution

CA: carbonic anhydrase

cAMP: adenosine 3':5'-cyclic monophosphate

CASA: computer-assisted sperm analysis

CFTR: cystic fibrosis transmembrane conductance regulator

cGMP: cyclic guanosine monophosphate

CNG: cyclic nucleotide-gated channels

COC: cumulus-oocyte complex

FF: follicular fluid

GAGs: glycosaminoglycans

HA: hyaluronic acid (non-sulphated hyaluronan)

HCO_3^-: bicarbonate ion

Hv: H^+ channel

IIF: indirect immunofluorescence

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

LIN: linearity of the curvilinear trajectory

mM: millimolar

Mot: total motile spermatozoa

MotPro: motile progressive spermatozoa

NBC: specific electrogenic Na^+/HCO_3^- co-transporter

NCAP: non-capacitating

NO: nitric oxide

OEC: oviductal epithelial cell

OF: oviductal fluid

OFCM: oviductal periovulatory secretions

P4: progesterone

PBS: phosphate buffered solution

pH_e: extracellular pH

pH_i: intracellular pH

PKA: protein kinase A

PKAs-P: protein kinase A substrates phosphorylation

PMN: polymorphonuclear neutrophils

S: inverted screw cap of a Falcon® tube

sAC: soluble adenylyl cyclase

SEM: standard error of the mean

sGC: soluble guanylate cyclase

SLC26: solute carrier 26

SLC4: solute carrier 4

sNHE: membrane NA^+/H^+ exchangers

SP: seminal plasma

Spz/O: spermatozoa penetrating each oocyte

Spz/ZP: spermatozoa bound to ZP

SR: spermatozoa reservoir

STR: straightness

TALP: tyrodes's albumin lactate pyruvate medium

TBM: tris-buffered medium

Tyr-P: tyrosine phosphorylation

UEC: uterine epithelial cell

UTJ: utero-tubal junction

VAP: average path velocity

VCL: curvilinear velocity

VSL: straight line velocity

W: 4-well multidish

WOB: wobble of the curvilinear trajectory

ZP: zona pellucida

β-act: beta-actin

β-tub: beta-tubulin