UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Selección de los espermatozoides en la especie porcina: relación con su funcionalidad y capacidad de fecundación *in vitro*

Spermatozoa selection in porcine species: relation with their functionality and *in vitro* fertilization competence

Dña. Rebeca López Úbeda

2015

UNIVERSIDAD DE
MURCIA

Dña. CARMEN MATÁS PARRA, Profesora Titular del Departamento de Fisiología

AUTORIZA:

La presentación de la Tesis Doctoral titulada ‘‘**Selección de los espermatozoides en la especie porcina: relación con su funcionalidad y capacidad de fecundación *in vitro*”**, realizada por **Dña. Rebeca López Úbeda**, bajo su inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Murcia, a 22 de Julio de 2015.

Fdo. Carmen Matás Parra

Dra. Carmen Matás Parra
Facultad de Veterinaria
Departamento de Fisiología
Email: cmatas@um.es
Campus Universitario de Espinardo. 30.100 Murcia
T. 868 88 7256 – F. 868 88 4147 – **www.um.es/grupo-fisiovet**

UNIVERSIDAD DE
MURCIA

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

AUTORIZA:

La presentación de la Tesis Doctoral titulada ‘‘**Selección de los espermatozoides en la especie porcina: relación con su funcionalidad y capacidad de fecundación *in vitro*”**, realizada por **Dña. Rebeca López Úbeda**, bajo su inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Murcia, a 22 de Julio de 2015.

Fdo. Francisco Alberto García Vázquez

Dr. Francisco Alberto García Vázquez
Facultad de Veterinaria
Departamento de Fisiología
Email: fagarcia@um.es
Campus Universitario de Espinardo. 30100 Murcia
T. 868 88 8009 – F. 868 88 4147 – **www.um.es/grupo-fisiovet**

The experimental part of this thesis has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER), Grant AGL2009-12512-C02-01 and AGL2012-40180-C03-01 and by Fundación Séneca 08752/PI/08.

Acknowledgements/ Agradecimientos

Agradecimientos

La vida consiste en ir hacia delante quemando etapas, con esta memoria cierro una etapa muy importante en mi vida durante la cual he tenido la suerte de conocer a personas maravillosas y de la que me llevo grandes amigos.

Quiero dar las gracias a todos los componentes del departamento de Fisiología por abrirme sus puertas y colaborar, cada uno a su manera, en esta tesis. Sin duda sois un gran equipo científico, pero si en algo destacáis todos es en la gran valía personal. Realmente espero que nuestro contacto no termine aquí y que podamos seguir colaborando en el futuro.

A Carmen por aceptar iniciar conmigo esta dura e increíble etapa, por su constancia, su apoyo, su inestimable ayuda y por creer en todo momento que esta tesis llegaría a su fin. Gracias también por haberme dado la oportunidad de trabajar en "esto de la reproducción". Por último, gracias por tu paciencia en los momentos de desánimo y por enseñarme otra forma de ver la vida.

A Fran al que estoy enormemente agradecida por haber aceptado formar parte de este proyecto y ser un ejemplo de que con ganas las cosas se pueden hacer rápido y bien.

A Pilar por confiar en mí y darme la oportunidad de trabajar en cosas nuevas. A Joaquín por sus consejos y soluciones, y por todos los momentos que le he robado con mis consultas. A Raquel por su forma de ver las cosas, por tener siempre abiertas las puertas de su despacho, por tratar de solucionarme cualquier problema en el momento y hacerme ver que al final todo saldría bien (gracias Jefa!!). A Salvador por hacer que muchas cosas funcionen desde "el fondo del pasillo".

A nuestros técnicos (Juan, Sole y Darío) sin los que este trabajo no habría podido realizarse, a Juan por prepararme numerosos litros de Percoll y por estar dispuesto a solucionar cualquier problema, y a Sole por preocuparse por mí y porque siempre, sin fallar un sólo día, me ha preguntado cómo iba.

A los doctorandos que estaban cuando llegué que me acogieron y me enseñaron muchas cosas de buena gana y a los que poco a poco he visto convertirse en doctores, Irene, Karen, Jon y, en especial, a Luis Vieira por estar dispuesto a colaborar en todo y tener siempre palabras de ánimo (se te echa mucho de menos amigo). A los que llegaron conmigo como Cristina, o después como Laura, Silvia y Analuce que han sufrido conmigo y han alegrado el despacho, los congresos y las horas de laboratorio.

Cómo no recordar a todos los alumnos y visitantes que han pasado por el departamento y que han traído ilusión, alegría y muchas ganas de hacer cosas: Noly, Fernando, Ernesto, Luis, Ángela, Irene, Roberto, Mercedes. En especial, quiero dar las gracias a Virginia que llegó en un momento de caos y trabajó muchísimo para que todo saliese adelante. A Sonia por su forma de ser, por ver la vida con optimismo y hacer que los momentos de trabajo pasasen a ser de ocio, sin ti habría sido un año muchísimo más duro. A Rodrigo por estar siempre de buen humor y sacarme una sonrisa en cualquier momento, muchas gracias por ayudarme a pesar de tu tiempo limitado.

A Manuel Avilés por su enorme entusiasmo, a Mª José Izquierdo, Carla Moros, Blanca Algarra y Ascensión Guillen del departamento de Biología Celular e Histología, por confiar en mí y permitirme trabajar con ellos. Gracias por vuestros consejos y por enseñarme otra manera de hacer las cosas.

A todo el departamento de Anatomía por abrirme sus puertas y dejarme usar sus instalaciones, especialmente a Octavio por su entusiasmo y su total disponibilidad para prestarme su ayuda en cualquier momento.

A todos mis amigos por acompañarme en los distintos momentos de mi vida y hacerlos mejor. A los de siempre: Carol, Alfred, Lidia, Nieves, Beatriz, Lorena, Tania y Sarai con los que aprendí que hay que luchar por lo que se quiere sin importar el tiempo que cueste ni lo lejos que debamos ir para lograrlo. A los que se unieron más tarde para quedarse: Pepa, Mónica, Rosa y, en especial, a Eva por hacer del infierno un lugar mejor y verme donde nadie más. Sin olvidar a los recién llegados, todos los componentes de “Il pulcino pio” por enseñarme que siempre se puede celebrar algo y que cualquier momento es bueno para ir de tapas. Y a todos los que me han acompañado en algún momento del camino haciéndolo mejor y me han hecho ser quien soy.

A Vicky por ser de esas personas que el destino pone en tu vida para que forme parte importante de ella. Muchísimas gracias por creer en mí, darme fuerzas y estar a mi lado desde la distancia. No quiero olvidarme tampoco de su familia por acogerme en su casa y tratarme como un miembro más.

Gracias especialmente a toda mi familia por el tiempo y la paciencia que les he robado. A mi tíos Mª Sol y Tomás por su cariño y apoyo. A mis primos Noelia y Tomás que llegaron para alegrar y revolucionar nuestro mundo.

A mi abuela Antonia, que siempre estuvo orgullosa de mí, por su constancia y cariño, por sus buenas palabras y estar siempre de buen humor. Muchísimas gracias por cuidar de mí, por tus juegos, tus besos y tus manos que nunca olvidaré. Gracias yaya por darlo todo y más.

A Isabel, mi hermana, mi amiga y mi cómplice, por ser ejemplo de constancia, paciencia, dedicación y esfuerzo. Por ser fuente inagotable de sabiduría y enseñarme algo cada día, muchísimas gracias por estar en los buenos y no tan buenos momentos, reír conmigo y luchar junto a mí en la vida, sin ti todo habría sido imposible!!!

A Fran por encontrarme y quererme como soy sin intentar cambiarme. Gracias por creer que podía lograrlo y empujarme para hacer aquello que me hiciese feliz, por hacer buenos los días malos y los buenos mejor. Sin olvidarme de toda su familia por aceptarme desde el primer momento.

A mis padres sin los cuales nada de esto habría sido posible, por ayudarme en todos los sentidos a llegar aquí. Mil gracias por apoyarme incondicionalmente en todas las decisiones, por tirar de mí hacia delante y por todo lo que me habéis dado en la vida.

Gracias a todos y cada uno por formar parte de mi vida y por colaborar en este trabajo, haciéndolo una realidad.

"Toda mente debe aprender por sí misma la lección entera, debe recorrer todo el terreno. Lo que no ve, lo que no vive, no lo sabrá"

Ralph Waldo Emerson

A mis padres

A Isabel

A Fran

Index

Summary/Resumen

SUMMARY

Once spermatozoa are deposited in the female after natural mating or artificial insemination they ascend through the female genital tract. During their ascent, sperm cells acquire the ability to fertilize through a process called capacitation. The modifications produced by this process have several consequences for the male gamete such as changes in the pattern of movement, the generation of free radicals, increased calcium levels, modification of lipids of the membrane or protein phosphorylation.

Several different mechanisms along the female genital tract allow the gradual selection of the most suitable sperm for fertilization purposes by establishing different sperm subpopulations. These subpopulations are partially or totally deficient in one or more phenotypic or functional aspects necessary to participate in the fertilization process. This means that if the same sperm concentration is used, the number of spermatozoa in the oviduct varies from one individual to another, because each male produces its particular ratio of subpopulations. Among these phenotypic variations that determine different sperm populations, some affect the morphology of the head, motility or flagellar amplitude, and others involve the efficiency of transduction and the integrity of the genetic and epigenetic information that is sent to the oocyte.

The utero-tubal junction is one of the main mechanisms of sperm selection, since about 96% of the sperm in the oviduct are located here. Only those spermatozoa with a progressive motility and a specific biochemical composition membrane can cross this area. The molecular integrity of the surface of the sperm is crucial for entry into the oviduct, so, processes such as capacitation, which produces structural changes, reduce the ability of sperm to reach and bind to the oviductal epithelial cells.

In the oviduct, only a small proportion of sperm is able to bind to epithelial cells of the oviduct in the so-called sperm reservoir (located in the caudal part of the oviductal isthmus). The spermatozoa remain in this area until ovulation, when they are released sequentially mainly due to endocrine changes, which involve a number of changes in the plasma membrane of the oviduct epithelium or in the intraluminal fluid, known as oviductal fluid. The oviduct plays an important modulatory role in the reproductive process as it monitors and synchronizes gametes until their union.

For many years, attempts have been made to control capacitation and sperm selection in order to simulate under *in vitro* conditions. The used methods eliminate both the seminal plasma and the spermatozoa of low quality, but the results are highly variable and are far from *in vivo* ones. So, a selected sperm *in vitro,* which is *a priori*

morphologically normal and able to fertilize *in vitro*, could not be selected by the oviductal epithelial cells to bind them. All this leads us to the aim of this thesis: the study of sperm subpopulations that have been previously selected by different methods: *in vitro* (Percoll gradients in Chapter 1, oviductal cells culture in Chapter 2 and by combining both in Chapter 3), *ex vivo* (in oviductal explants, Chapter 2) and *in vivo* (in the oviduct of the sow after insemination, Chapter 2), and their relationship with functionality, sperm capacitation status and fertilizing capacity. To achieve this main objective, different specific objectives for each of the chapters of this thesis were established.

One of the *in vitro* methods used in many laboratories for sperm selection and to start the capacitation process is centrifugation through density gradients. In theory, the seminal plasma and sperm of lower quality are eliminated by this method. This led us to consider whether different subpopulations obtained from an ejaculate after separation with different density gradients might present different sperm parameters and fertilization competence.

In the first study (**Chapter 1**) centrifugation by Percoll gradients was analysed as a sperm selection and capacitation method, and its efficiency for selecting sperm was evaluated. Three different Percoll gradient combinations (45/60, 60/75 and 45/90%) were used, in order to separate different sperm populations that form an ejaculate.

Firstly, in *experiment 1*, several parameters related to sperm function (morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation and intracellular calcium concentration) were analysed in the different sperm populations obtained through the three experimental techniques (Percoll 45/60, 60/75 and 45/90%). The results of this experiment showed that as the Percoll gradient increased, the number of morphologically abnormal sperm or sperm with DNA fragmentation is reduced, recovering especially those sperm that have begun the process of sperm capacitation, since 95% of spermatozoa present tyrosine phosphorylation in any of the studied areas (acrosome, equatorial segment and/or tail). So, we can say that the separation of sperm by Percoll gradients is primarily based on their functional differences.

Second, in *experiment 2*, the different sperm populations were used in an *in vitro* fertilization system to evaluate the penetration rate, checking which Percoll gradient selects the best sperm for fertilization purposes. The penetration rate and the mean number of spermatozoa found in each oocyte were evaluated. The results showed that spermatozoa obtained from the most restrictive Percoll gradient (45/90%) had higher levels of penetration, since a greater amount of sperm were selected with high levels of capacitation, which initially must be more prepared to interact favourably with the oocytes.

Although sperm selection by Percoll gradients provides good results in *in vitro* fertilization, there are still differences with the results obtained *in vivo.* In pig, the main problem is a high degree of polyspermy, because under current working conditions a lot of spermatozoa reach, very close to the eggs and in the same time, a high state of capacitation, without any barrier or selection mechanism, which prevents polyspermy. This led us to focus our interest and go one step further in sperm selection through a study of the effect of the oviductal epithelial cells, which are responsible for selecting and binding the spermatozoa in the sperm reservoir.

That is why in the second study (**Chapter 2**) the capacitation status of sperm selected by the oviductal epithelial cells was evaluated (by measuring levels of tyrosine phosphorylation) under three conditions: 1) *In vitro:* by culture of epithelial cells obtained from the oviducts of slaughtered animals. 2) *Ex vivo:* through the use of oviduct explants, also collected from the slaughterhouse. 3) *In vivo:* in physiological conditions in the oviduct of the sow after artificial insemination.

The main contribution of the results of this experiment is the observation that the phosphorylation pattern changes when sperm are incubated with the epithelial cells of the oviduct, with differences between sperm bound or unbound to the cells. Among bound spermatozoa, the unusual presence of phosphorylation in the acrosome, which is indicative of an advanced stage of capacitation was observed, it was concluded that the epithelial cells of the oviduct are able to select spermatozoa with a low level of tyrosine phosphorylation in any of the studied conditions (*in vitro*, *ex vivo* or *in vivo*).

Based on the results from previous experiments (Chapter 1 and 2) it is clear that it is possible to separate different populations *in vitro* of an ejaculate by their morphology, and that oviductal epithelial cells exercise a second sperm selection process and are capable of binding sperm with certain characteristics. This led us to consider how the oviductal epithelial cells exercise this second sperm selection, the features of the selected sperm, and what be the fertilization competence of these doubly selected sperm might be.

In the final study (**Chapter 3**), sperm selected by Percoll gradients (45/90%) were incubated with *in vitro* cultured oviductal epithelial cells. This incubation resulted in two distinct sperm populations: sperm bound to cells and sperm unbound. The study was divided into two experiments.

In *experiment 1* the ability of the two populations to fertilize was evaluated using different parameters (penetration rate, number of sperm bound to the zona pellucida, number of sperm per oocyte penetrated, the number of swollen spermatozoa and the formation of the male pronucleus). The results showed that the doubly selected sperm

(using gradients and later bound by oviductal cells) led to better results in the *in vitro* fertilization systems, significantly improving penetration rates.

In *experiment 2* the functional characteristics of sperm from the two populations (tyrosine phosphorylation, translocation of phosphatidylserine in the plasma membrane, DNA fragmentation and chromatin condensation) were analysed. The results indicated that the oviductal epithelial cells bind higher quality sperm according to different functional parameters and perform their own sperm selection.

In summary, the results of this thesis show that sperm selection by Percoll gradients (45/90%) is a suitable technique to obtain sperm subpopulations with higher levels of functionality from an ejaculate. However, this sperm selection may be more restrictive with the use of oviductal epithelial cells, which perform a second more rigorous selection based on numerous parameters. This improves the results of fertilization. Finally, it is concluded that the oviductal epithelial cells are able to distinguish those sperm with a higher fertilization capacity. In the future this could provide a solution to obtaining the best sperm for fertilization and embryo development *in vitro*. Using a non-invasive technique that does not damage or impair the sperm for later use would avoid the subjective selection that currently exists in conventional sperm selection techniques.

RESUMEN

Los espermatozoides una vez depositados en la hembra tras una monta natural o una inseminación artificial ascienden por el tracto genital femenino. Durante este recorrido los espermatozoides adquieren la capacidad para fecundar a través de un proceso denominado capacitación. Las modificaciones producidas por este proceso tienen varias consecuencias en el gameto masculino como son, entre otras, la modificación en el patrón de movimiento, la generación de radicales libres, el incremento del nivel de calcio intracelular, la modificación de lípidos de membrana o la fosforilación de proteínas.

A lo largo del tracto genital femenino existen diferentes mecanismos que realizan una selección progresiva de la población espermática más apta para fecundar, de tal manera que se establecen diferentes subpoblaciones espermáticas. Estas subpoblaciones son parcial o totalmente deficientes en uno o más aspectos fenotípicos o funcionales necesarios para participar en los diferentes aspectos del proceso de fecundación. Esto quiere decir que, si inseminamos con la misma concentración de espermatozoides, aquellos capaces de acceder al oviducto variará de un individuo a otro ya que, cada macho produce su particular proporción de subpoblaciones. Entre estas variaciones fenotípicas que determinan las diferentes poblaciones espermáticas, podemos encontrar desde aquellas que afectan a la forma de la cabeza, la motilidad o la amplitud flagelar, a otras que involucran la eficiencia de las señales de transducción y la integridad de la información genética y epigenética que se transmite al ovocito.

El paso por la unión útero-tubárica es uno de los principales mecanismos de selección espermática, ya que alrededor del 96% de los espermatozoides localizados en el oviducto se encuentran en dicha unión. Esta zona sólo será atravesada por aquellos que presenten motilidad progresiva y una determinada composición bioquímica a nivel de membrana, la cual se encuentra relacionada con el estado de capacitación. La integridad molecular de la superficie del espermatozoide es determinante para su entrada al oviducto, por lo que procesos como la capacitación que producen modificaciones estructurales hacen que disminuya la capacidad de los espermatozoides para llegar al oviducto y unirse a las células epiteliales.

Una vez en el oviducto solo una pequeña proporción de espermatozoides será capaz de unirse a las células epiteliales en el denominado reservorio espermático (localizado en la parte caudal del istmo oviductal). Los espermatozoides permanecerán en esta zona del oviducto hasta la ovulación, momento en el que serán liberados de manera secuencial gracias principalmente a modificaciones endocrinas, las cuales provocan una serie de cambios a nivel de la membrana plasmática del epitelio oviductal o en el fluido intraluminal, conocido como fluido oviductal.

De todas las partes que recorren los espermatozoides durante su ascenso, el oviducto juega un importante papel modulador en el proceso reproductivo, ya que controla y sincroniza a los gametos hasta el momento de su unión.

Durante muchos años se ha tratado de simular bajo condiciones *in vitro* el proceso de capacitación y selección espermática que lleva a cabo el oviducto en condiciones fisiológicas y para ello se han desarrollado diferentes sistemas. Aunque mediante estos sistemas se ha logrado eliminar tanto el plasma seminal como la mayor parte de los espermatozoides de baja calidad de las muestras espermáticas, los resultados obtenidos son muy variables y distan mucho de la selección que ejerce el oviducto *in vivo.* De modo que, un espermatozoide seleccionado *in vitro*, que *a priori* es morfológicamente normal y capaz de fecundar en condiciones *in vitro*, podría no ser seleccionado por las células epiteliales del oviducto como un espermatozoide apto para unirse a ellas. Todo esto nos lleva a plantearnos el objetivo principal de esta tesis doctoral: el estudio de las subpoblaciones espermáticas previamente seleccionadas a través de métodos *in vitro* (por gradientes de Percoll, Capítulo 1; mediante cultivo de células oviductales, Capítulo 2; y por la combinación de ambos sistemas, Capítulo 3), *ex vivo* (en explantes oviductales, Capítulo 2) e *in vivo* (en el oviducto de la cerda tras una inseminación artificial, Capítulo 2), y su relación con la funcionalidad y el estado de capacitación espermática, así como su capacidad fecundante. Para alcanzar este objetivo principal se establecieron diferentes objetivos específicos para cada uno de los capítulos que conforman esta tesis doctoral.

Uno de los métodos utilizados de rutina *in vitro* en muchos laboratorios para llevar a cabo la selección espermática y para iniciar la capacitación es la centrifugación a través de gradientes de densidad. En teoría, mediante este método se elimina el plasma seminal así como los espermatozoides de menor calidad. Esto nos llevó a plantearnos si las diferentes subpoblaciones que se obtienen de un eyaculado tras la separación mediante diferentes gradientes de densidad presentarían diferencias entre ciertos parámetros espermáticos y cuál sería la fertilidad de estas subpoblaciones en un sistema de fecundación *in vitro*.

Para lograr esto, en el primero de los estudios (**Capítulo 1**) se analizó la centrifugación mediante gradientes de Percoll como método de selección y capacitación espermática y se evaluó la eficacia en la selección de espermatozoides en función del gradiente utilizado. Se emplearon tres combinaciones diferentes de Percoll (45/60, 60/75 y 45/90%), con el fin de separar diferentes poblaciones espermáticas que forman parte del eyaculado, y con ellas se realizaron dos experimentos.

En primer lugar, en el *experimento 1*, se analizaron diferentes parámetros relacionados con la funcionalidad espermática (morfología, estado del acrosoma, motilidad y parámetros motiles, generación de ROS, condensación de la cromatina, fragmentación del ADN, fosforilación de la tirosina y concentración de calcio intracelular) de las

diferentes poblaciones obtenidas a través de los 3 grupos experimentales (Percoll 45/60, 60/75 y 45/90%). Los resultados alcanzados en este experimento demostraron que a medida que aumentamos la diferencia en la densidad del gradiente de Percoll se reduce el número de espermatozoides morfológicamente anormales o con fragmentación del ADN, de manera que se recuperan, principalmente, aquellos espermatozoides que han iniciado el proceso de capacitación espermática ya que un 95% de éstos presentan fosforilación de tirosina en alguna de las zonas estudiadas (acrosoma, segmento ecuatorial y/o flagelo). De modo que podemos decir que la separación de espermatozoides mediante gradientes de Percoll se fundamenta principalmente en sus diferencias funcionales.

En segundo lugar, en el *experimento 2*, se emplearon las diferentes poblaciones espermáticas en un sistema de fecundación *in vitro* para evaluar la capacidad de penetración de cada una de ellas, y determinar qué gradiente de Percoll selecciona los espermatozoides más aptos para fecundar y su posible relación con los parámetros estudiados en el experimento 1. Para ello, se evaluó el porcentaje de penetración y el número medio de espermatozoides que presentaba cada ovocito. Los resultados mostraron que aquellos espermatozoides obtenidos a partir del gradiente de Percoll más restrictivo (45/90%) dan lugar a mayores niveles de penetración, ya que se selecciona una mayor cantidad de espermatozoides con mejores propiedades para completar el proceso de capacitación que, en un principio, deben corresponder a los más preparados para interactuar de forma favorable con los ovocitos y posteriormente penetrarlos.

Aunque mediante la selección de espermatozoides a través de gradientes de Percoll se logran unos buenos resultados de fecundación *in vitro*, aún existen diferencias con los resultados obtenidos *in vivo*. En el caso de la especie porcina el principal problema es un alto grado de polispermia, el cual puede deberse a las actuales condiciones de trabajo. Bajo estas condiciones una gran cantidad de espermatozoides alcanzan, muy próximos a los ovocitos y de manera simultánea, un alto estado de capacitación. Dado que no existe ningún mecanismo barrera o de selección, la gran cantidad de espermatozoides capacitados imposibilitan el correcto funcionamiento de los mecanismos de bloqueo de la polispermia del ovocito entre otros. Esto nos llevó a centrar nuestro interés en el efecto que tienen las células epiteliales del oviducto, encargadas de seleccionar y unir los espermatozoides en el reservorio espermático, sobre la capacitación espermática.

Es por ello que en el segundo de los estudios (**Capítulo 2**) se evaluó el estado de capacitación (mediante la distribución de la fosforilación de la tirosina) de los espermatozoides seleccionados mediante células epiteliales del oviducto bajo tres condiciones: 1) *In vitro*: mediante cultivos de células epiteliales obtenidas de oviductos de matadero. 2) *Ex vivo*: a través del uso de explantes de oviducto recolectados de

animales de matadero. 3) *In vivo*: en condiciones lo más fisiológicas posible como es en el propio oviducto de la cerda tras una inseminación artificial. Los espermatozoides utilizados fueron previamente seleccionados mediante lavados por gradientes de Percoll (en condiciones *in vitro* y *ex vivo*) o directamente por el propio tracto genital femenino (*in vivo*).

La principal aportación de los resultados de este experimento es que el patrón de fosforilación de los espermatozoides se modifica cuando se incuban con las células epiteliales del oviducto. Entre los espermatozoides unidos o no unidos a las células existen diferencias significativas en los patrones de fosforilación. En los espermatozoides unidos destaca la ausencia de fosforilación en el acrosoma, lo que es indicativo de un avanzado estado de capacitación. De estos resultados se puede concluir que las células epiteliales del oviducto son capaces de seleccionar los espermatozoides con un bajo grado de fosforilación en tirosina en cualquiera de las condiciones estudiadas (*in vitro*, *ex vivo* e *in vivo*).

A partir de los estudios anteriores (Capítulo 1 y 2) queda claro que es posible separar *in vitro* las diferentes poblaciones de un eyaculado mediante su morfología, que las células oviductales ejercen una segunda selección y son capaces de unir espermatozoides con determinadas características. Esto nos llevó a plantearnos cómo ejercen esta segunda selección espermática las células epiteliales del oviducto, qué características presentan estos espermatozoides para ser seleccionados por las células oviductales y cuál sería la capacidad de fecundación de estos espermatozoides doblemente seleccionados al emplearlos en un sistema de fecundación *in vitro*.

Para tratar de contestar a las preguntas planteadas en el párrafo anterior, en el último de los estudios (**Capítulo 3**) se emplearon espermatozoides seleccionados a través de gradientes de Percoll (45/90%) que posteriormente fueron incubados sobre cultivos *in vitro* de células epiteliales del oviducto. Esta incubación dio lugar a dos poblaciones espermáticas claramente diferenciadas: espermatozoides unidos a las células y espermatozoides no unidos. El estudio fue dividido en dos experimentos.

En el *experimento 1* se evaluó la capacidad para fecundar de las dos poblaciones de espermatozoides obtenidas, mediante el análisis de diferentes parámetros de fecundación (tasa de penetración, número de espermatozoides unidos a la zona pelúcida, número de espermatozoides por ovocito penetrado, el número de espermatozoides descondensados y la formación del pronúcleo masculino). Los resultados del experimento 1 demostraron que los espermatozoides doblemente seleccionados (mediante gradientes y posteriormente unidos por las células oviductales) dan lugar a mejores resultados en los sistemas de fecundación *in vitro*, con una mejora significativa de las tasas de penetración.

En el *experimento 2* se analizaron las características funcionales (fosforilación de la tirosina, translocación de la fosfatidilserina en la membrana plasmática, fragmentación del ADN y condensación de la cromatina) que presentan las diferentes poblaciones de espermatozoides (espermatozoides unidos o no unidos). Los resultados del experimento 2 mostraron que los espermatozoides unidos presentaban menor fosforilación de tirosina así como menores niveles de translocación de fosfatidilserina en sus membranas. Esto nos lleva a pensar que las células epiteliales del oviducto unen los espermatozoides de mayor calidad en base a diferentes parámetros funcionales y que llevan a cabo su propia selección incluso partiendo de espermatozoides previamente seleccionados.

En resumen, los resultados de esta tesis doctoral muestran que la selección espermática a través de gradientes de Percoll (45/90%) es una técnica adecuada para obtener de un eyaculado la subpoblación espermática con una mayor funcionalidad. Sin embargo, esta selección espermática puede ser más restrictiva con el empleo de células epiteliales del oviducto, que ejercen una segunda selección mucho más rigurosa basada en diversos parámetros funcionales, lo que mejora los resultados de fecundación. Así, a partir de todos los resultados obtenidos, se concluye que las células epiteliales del oviducto son capaces de distinguir aquellos espermatozoides con una capacidad de fecundación superior, lo que en un futuro podría representar una solución a la obtención de los mejores espermatozoides para la fecundación y el desarrollo embrionario *in vitro*. A todo lo anterior, se añade la ventaja de ser una técnica no invasiva, que no daña los espermatozoides lo que permite su uso posterior y que evitaría la selección subjetiva que existe actualmente en las técnicas convencionales de selección de espermatozoides.

Literature review

LITERATURE REVIEW

An approach to the factors related with the sperm capacitation process

---

1. INTRODUCTION

Once sperm are in the genital tract of the female they begin a long journey to meet the oocyte, during which they will have to overcome numerous morphological and functional barriers, so that only those spermatozoa with certain characteristics will reach their goal.

Most spermatozoa that come into contact with the female reproductive tract are unable to fertilize an oocyte, and it is on their way through the genital tract that sperm cells undergo a series of functional and molecular changes that enable them to be able to complete the process of fertilization. The biological changes that sperm undergo in the female genital tract are jointly known as "capacitation" (Austin, 1952), which appears to be controlled by crosstalk between different pathways (de Lamirande *et al.*, 1997; Fraser, 2010). Although the process of capacitation was discovered and described many years ago (Austin, 1951; Chang, 1951), and although sperm capacitation has been reproduced in many species under *in vitro* conditions, many of the factors and molecular pathways directly involved in the regulation of this complex process remain unknown.

While it is true that under physiological conditions the oviduct plays an important role in sperm capacitation, it is also able to perform a much more important role – the selection of high quality spermatozoa from a heterogeneous sperm population (Immler, 2008), favouring very efficient fertilization (Fitzpatrick & Lüpold, 2014). Only selected spermatozoa will bind to form the oviductal sperm reservoir, maintaining their viability and avoiding full premature capacitation until the time of ovulation (Brüssow *et al.*, 2006). Therefore, despite the large number of sperm which are released into the reproductive tract after ejaculation (37.5 billion in the case of boar), only few will be able to reach the site of fertilization (5000) (Avilés *et al.*, 2015).

Despite being a very important physiological process since it leads to highly successful fertilization, how this process is regulated and the functional status of spermatozoa after the sperm selection, have been poorly studied. Any increase in our knowledge of the capacitation process and sperm selection, which will allow us to simulate what happens *in vivo*, should provide information on the specific characteristics of these spermatozoa and therefore could significantly improve the different techniques of assisted reproduction used in both livestock and human fertility processes.

2. SPERM JOURNEY IN THE FEMALE GENITAL TRACT

After mating or artificial insemination, millions of sperm are deposited in the female genital tract, of which only a small proportion is able to reach the oviduct (Rodriguez-Martinez *et al.*, 2005; Holt, 2009). During the ascent of the reproductive tract, most sperm (>70%) are eliminated in the uterine lumen (Figure 1A) (Einarsson & Viring, 1972), either by retrograde reflux (25-45%) (Matthijs *et al.*, 2003; Hernández-Caravaca *et al.*, 2012) or by action of polymorphonuclear (Rozeboom *et al.*, 1999). This removal mainly is mediated by leukocytes that migrate from the lumen of endometrium within 30 minutes after mating (Matthijs *et al.*, 2003). This latency period allows some sperm to pass through the uterus which cannot be phagocytosed (Rodriguez-Martinez *et al.*, 2005). During such advancement there is also a progressive modification of some factors derived from the seminal plasma, which are attached to the surface of the sperm plasma (Hunter & Rodriguez-Martinez, 2004). These factors that prevent premature capacitation are involved in binding the oviductal cells (Rodriguez-Martinez *et al.*, 2009) and have also been shown as a requirement for sperm survival in the uterus, at least in mouse (Kawano *et al.*, 2014). Finally, only a small proportion will pass through the utero-tubal junction (UTJ) (revised by Soriano-Úbeda *et al.*, 2013).

From the UTJ sperm travel to the caudal portion of the isthmus, where the population of motile sperm encounters a sticky secretion of glycoprotein that modifies the sperm surface (Rodriguez-Martinez *et al.*, 2000). Motility decreases in this viscous medium and facilitates the sperm adhesion to the epithelium (Figure 1B). Experimental studies in domestic animals like goat, cow and pig have shown that in the pre-ovulatory period (between 25-40 hours) sperm are unable to move and so, they stay together until near ovulation. Between 1-2 hours before ovulation, a small number of sperm will be reactivated and will go progressively toward fertilization site (Hunter, 2008). As the number of sperm is released and activated consequently, there are increased progressively drift towards the ampullary-isthmic junction. This brief description of sperm activity in the caudal portion of the isthmus suggests that there is an influence of ovaries on oviduct and on sperm bounded (Hunter, 2008).

Sperm remains in the caudal portion of the oviductal isthmus, during the pre and peri-ovulatory time, forming the sperm reservoir (SR) (Suarez *et al.*, 1991; Töpfer-Petersen *et al.*, 2002). Several factors may be involved in the formation of this SR. The binding occurs through direct contact via ligand-receptor interaction between the molecules present in the rostral aspect of the sperm and membrane carbohydrates of oviductal cells. This interaction is species-specific (Lefebvre *et al.*, 1997; Lapointe *et al.*, 1998). The binding is a reversible process involving, in all species studied, different sugars (Dobrinski *et al.*, 1996; Suarez, 1998). Other factors such as mucus, the chemical properties of oviductal fluid and temperature gradients could contribute to the formation of the SR (revised by Soriano-Úbeda *et al.*, 2013).

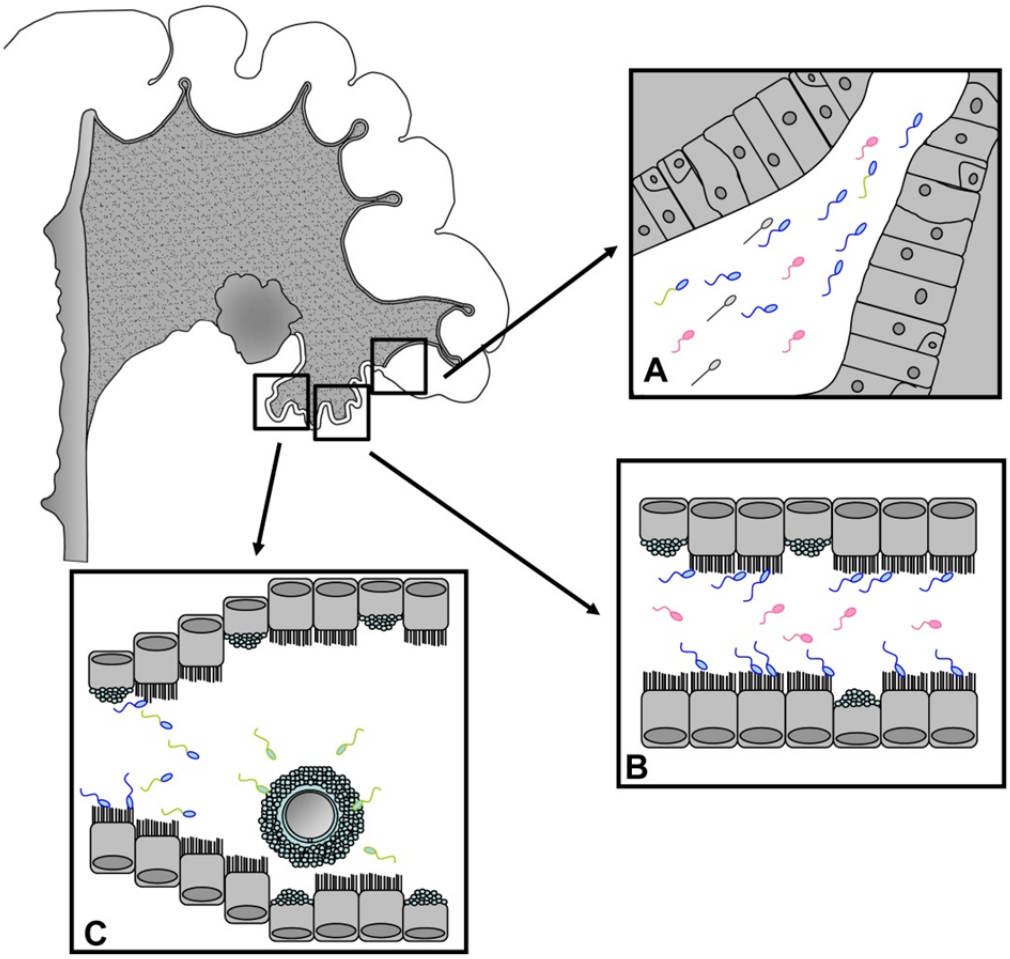

Figure 1. Sperm events within female genital tract. **A)** After sperm deposition, a heterogeneous sperm population starts a journey into the reproductive tract. **B)** Only a few sperm achieves the oviduct and are attached to the oviductal epithelial cells, creating the sperm reservoir (SR) in the caudal portion of the isthmus. **C)** During peri-ovulatory time, sperm release from the SR and those who complete a correct sperm capacitation are able to contact with the oocyte and fertilize it. Different colours indicate distinct types of sperm: dead (grey), damaged (red), normal (blue), hyperactivated (green-blue) and successfully capacitated (green).

The sequential release of a limited number of sperm from the SR to the ampullary-isthmic junction ensures fertilization of oocytes in a time interval (Figure 1C), even if ovulation occurs over a long period of time (Rodriguez-Martinez *et al.*, 2008). The peri-ovulatory progression of sperm is a complex system concerning various processes such as the opening of the oviductal lumen due to declining hormone levels, dissolution of rich mucus in hyaluronate, sperm hyperactivation, and increased flow of tubal fluid and redirection of uterine contractions (Rodriguez-Martinez *et al.*, 2008). However, the mechanisms which sperm are released from SR are unknown.

The presence in the oviductal microenvironment signals for the release of the sperm may be modulated using alternative or synergistic events such as: i) the pre-ovulatory

phase of the endocrine environment, ii) the presence of gametes, which can change the secretory activity of the oviduct; and iii) the effect of *cumulus-oocyte* complex. In this sense, it has been shown that injection of progesterone or preovulatory follicular fluid on the serosal layer or directly into the SR causes a massive release of spermatozoa, leading to high rates of polyspermy (Hunter, 2008). This has led to the assumption that progesterone may be a direct or an indirect signal to the release of the spermatozoa (Talevi & Gualtieri, 2010). Furthermore unsulfated glycosaminoglycans (components of the extracellular matrix, whose accumulation in pig oocytes increases around ovulation), could also be involved in sperm capacitation and the release of SR (Brüssow *et al.*, 2008).

3. FUNCTIONAL CHANGES AND MOLECULAR PATHWAYS DURING SPERM CAPACITATION

During the capacitation process, spermatozoa undergo a series of functional changes, which enables them to bind to the extracellular matrix of the oocyte and consequently require the acrosome reaction. Although the latter is under discussion as recently shown by Jin *et al.* (2011) that the acrosome reaction in mouse sperm occurs before binding to the zona pellucida. Besides, the pattern of movement of sperm flagellum changes allowing penetration of the zona pellucida (Suarez, 2008).

Capacitation process implied several changes sequentially. Some of these changes are rapid and occur at the moment of ejaculation. Others require a longer period of time in the female genital tract (*in vivo*) or in a medium capable of supporting this process (*in vitro*). All these processes (both rapid and slow), appear to be regulated by protein kinase A (PKA) and HCO_3^-, soluble adenylate cyclase (SACY or sAC), and cyclic adenosine 3',5'-monophosphate (cAMP) participate in this process (revised by Visconti, 2009).

Traditionally, reactive oxygen species (ROS) are considered to be injurious by products of cellular metabolism but also fundamentally participants in cell signalling and regulation mechanisms (Finkel, 2001). This apparent paradox also is true for spermatozoa, which are particularly susceptible to ROS-induced damage because their plasma membranes contain relatively large amounts of polyunsaturated fatty acids and their cytoplasm contains relatively low concentrations of scavenging enzymes (Aitken & Fisher, 1994), but require low concentration of ROS to acquire the fertilizing ability (Aitken, 1997; de Lamirande *et al.*, 1997). The essential role of ROS as modulators of capacitation is recognized in human (Herrero *et al.*, 2003), bovine (O'Flaherty *et al.*, 2006), mouse (Herrero *et al.*, 2003), and boar spermatozoa (Funahashi, 2002; Aquila *et al.*, 2011).

Some authors consider that capacitation occurs in two steps, fast and low (Visconti, 2009):

***Facts during fast sperm capacitation**:* An early event during capacitation is the activation of sperm motility. Although sperm stored in the cauda epididymis being practically immobile consume oxygen in large proportions. The flagellum movement starts immediately after sperm are released from the epididymis and contact has been made with seminal plasma. This is due to exposure of sperm to the HCO_3^- (Wennemuth *et al.*, 2003).

***Facts during slow sperm capacitation**:* In contrast to the rapid activation of motility, other processes associated with capacitation require a longer period of time. During slow capacitation, sperm acquire the ability to fertilize, which is preceded by the preparation of the sperm to undergo the acrosome reaction and change the pattern of motility called hyperactivation. Components in oviductal fluid such as high weight molecular proteins and high density lipoproteins promote cholesterol efflux resulting in an increased capacitation and tyrosine phosphorylation (TP) using the cAMP signalling pathway/PKA (Visconti *et al.*, 1999). Additionally, these slow processes also are achievable *in vitro* by incubation of spermatozoa in defined media, which contain a protein source (usually bovine serum albumin-BSA), and different ions, including HCO_3^- and Ca^{2+}.

4. MOLECULES AND MECHANISMS INVOLVED IN THE PROCESS OF CAPACITATION

As we mentioned above, HCO_3^-, Ca^{2+} and cholesterol acceptor are essential during capacitation process entirely (Figure 2). These substances induce modifications lipid membrane, loss of cholesterol, activation of cAMP/PKA pathway, increase Ca^{2+} uptake and intracellular pH (pH_i), hyperpolarisation of membrane potential, and TP (Aitken & Nixon, 2013). However, there are other pathways in relation to capacitation as NO/sGC/cGMP or protein nitrosylation are being studied.

4.1. Bicarbonate and sperm capacitation

Several studies have shown that HCO_3^- plays a key role in sperm capacitation and therefore achieve fertilization under both *in vivo* and *in vitro* (Okamura *et al.*, 1985; Visconti *et al.*, 1995; Harrison *et al.*, 1996; Harrison, 2004; Boerke *et al.*, 2013). Epididymal spermatozoa are exposed to low HCO_3^- concentrations (3-4 mM). However, when they arrive before the capacitation, takes place (oviduct). They are found in much higher level (>20 mM) (Rodriguez-Martinez *et al.*, 1990). Movement of HCO_3^- through the membrane has been associated with increased pH_i during capacitation (Zeng *et al.*, 1996). Moreover, another likely target for the action of HCO_3^- on sperm metabolism is the regulation of cAMP (Garbers *et al.*, 1982) by activation of sAC

(Visconti, 2009). This in turn stimulates PKA to phosphorylate substrates, thereby allowing TP (Visconti *et al.*, 1998; Gadella & Harrison, 2002). Furthermore, activation of the PKA results in activation of phospholipase D (PLD), which stimulates the polymerization of F-actin (Cohen *et al.*, 2004), which is an event associated with the process of acrosome reaction.

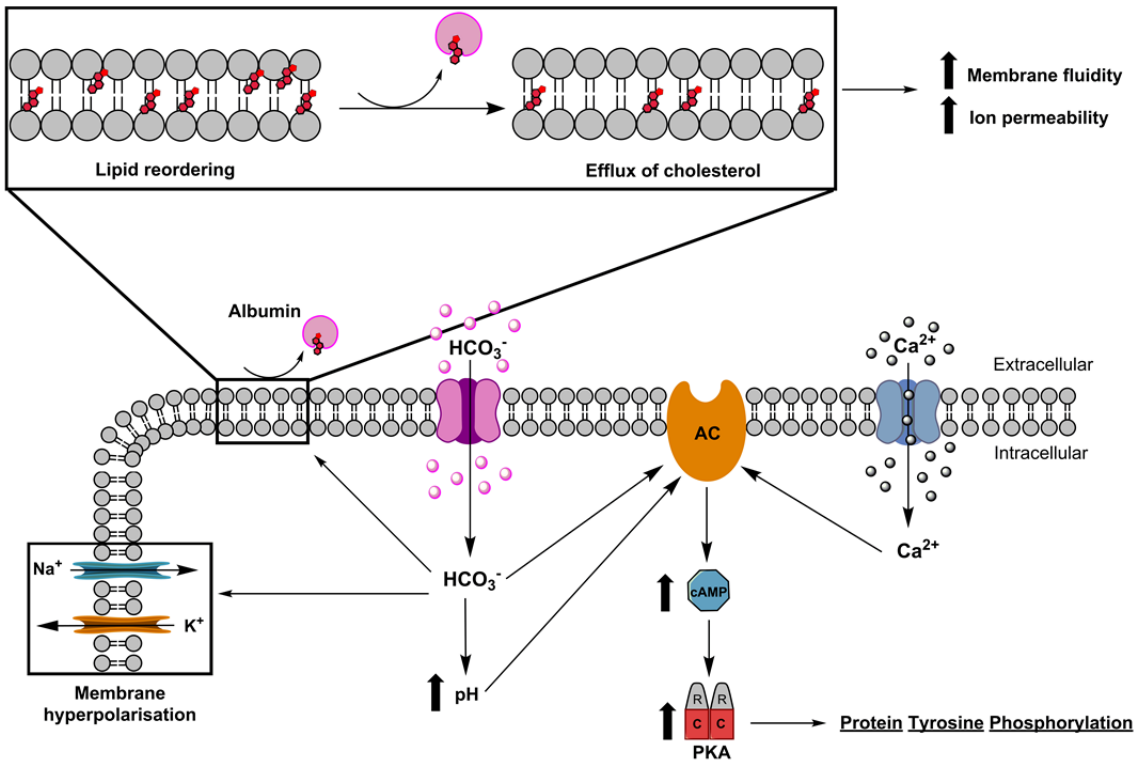

Figure 2. HCO_3^- input leads to hyperpolarisation of the membrane potential, sAC activation (directly, or indirectly by increasing the pH) and reordering of the lipids in the membrane which changes the position of cholesterol in the apical part. This facilitates their removal using albumin, increasing membrane fluidity and promotes Ca^{2+} entry. Calcium activates sAC, increases the protein tyrosine phosphorylation through the cAMP/PKA pathway.

Bicarbonate and lipid membrane structure: HCO_3^- also modifies the lipid structure of the sperm plasma membrane during capacitation and it is a reversible phenomenon (Figure 2) (Harrison *et al.*, 1996). Gadella and Harrison (2000) showed that influx HCO_3^- during capacitation produces change in the lipid membrane structure using path sAC/cAMP/PKA and so is augmented by inhibitors of phosphatases (Harrison & Miller, 2000). These changes lead to a reordering of membrane phospholipids phosphatidylethanolamine, phosphatidylserine, sphingomyelin and phosphatidylcholine. Lipid reordering allows to relocate the cholesterol in the apical part of the sperm head. Apparently, this relocation has the function of removal of cholesterol (Flesch *et al.*, 2001a). Albumin, high-density lipoprotein (HDL), and β-cyclodextrines promote sperm capacitation acting as acceptors of cholesterol by

removing it from the plasma membrane (Vadnais *et al.*, 2007). As a result of this process, decrease ratio of cholesterol/phospholipid consequently contributes to an increased membrane fluidity promoting increase of ion permeability (Davis, 1976; Cross, 1998; Visconti *et al.*, 1999; Travis & Kopf, 2002).

Bicarbonate and sterol depletion: Albumin acts in synergy with HCO_3^- by mediating efflux of sterols from the sperm surface (Boerke *et al.*, 2008; Brouwers *et al.*, 2011). Flesch *et al.* (2001a) observed that the addition of albumin causes cholesterol efflux (Figure 2), but only in HCO_3^--responding cells that exhibited virtually no filipin labelling in the sperm head area. In the absence of HCO_3^-, albumin had no effect on other lipid components and no affinity to cholesterol. HCO_3^- also induces sperm surface oxysterol formation by activation of signalling pathway of the ROS, which can be inhibited or blocked by addition of antioxidants as vitamin E or vitamin A (Boerke *et al.*, 2013). These sterols oxidation products (oxysterols), which are more hydrophilic can be extracted using albumin (Brouwers *et al.*, 2011) or can facilitate an oxysterol dependent scavenger-sensitive transport of free sterols to albumin (Jessup *et al.*, 2006).

Bicarbonate and sperm plasma membrane potential: Under normal conditions, spermatozoa maintain intracellular ion concentration markedly different from extracellular environment and these differences provide the resting membrane potential (Salicioni *et al.*, 2007). When spermatozoa are exposed to different environments during transport through the male and female genital tracts, they find different extracellular ion concentration. For example, the epididymal fluid contains high K^+, low Na^+, and even lower concentrations of HCO_3^-. After ejaculation there is a drastic change in the concentrations of these ions in the seminal fluid and finally into the female tract, where there concentrations of low potassium and high HCO_3^- are present (Brooks, 1983; Setchell *et al.*, 1994). As a result of changes in extracellular ion concentrations, there will be changes in intracellular concentrations of these ions leaving alterations in membrane potential (Muñoz-Garay *et al.*, 2001; Demarco *et al.*, 2003) which consequently occurs in the hyperpolarisation of sperm plasma membrane (Zeng *et al.*, 1995). It has been shown in mouse sperm that changes in membrane potential do not occur in BSA or HCO_3^- absence (Demarco *et al.*, 2003). These results suggest that HCO_3^- present in capacitation media as well as cholesterol efflux may have a direct or indirect function of events allowing hyperpolarisation of the sperm plasma membrane (Salicioni *et al.*, 2007). Arnoult *et al.* (1999) showed that only hyperpolarized sperm populations are capable of undergoing the acrosome reaction in presence of solubilised zona pellucida material.

4.2. Calcium and sperm capacitation

In 1915, Loeb was the first to demonstrate that Ca^{2+} is required in the extracellular medium for fertilization to occur in invertebrates. Of all intracellular signalling

mechanisms, perhaps the most studied and best characterized one is the mobilization of Ca^{2+}. This pathway involves transitory increase of intracellular Ca^{2+} concentrations produced by multitude intercellular messengers.

One of the most important consequences of cholesterol efflux from membranes is a massive influx of extracellular Ca^{2+}, which is considered a prerequisite for the acrosome reaction process (Flesch & Gadella, 2000). This Ca^{2+} influx may be due to changes occurring in the membrane fluidity. The intracellular Ca^{2+} increase in sperm can activate one or more enzymatic pathways (Figure 2). For example, the adenylate cyclase (sAC) increases during capacitation in response to Ca^{2+}, this enzyme will catalyse the conversion of ATP to cAMP (revised by Vadnais *et al.*, 2007).

In 1998, Visconti & Kopf suggested a cooperative effect of Ca^{2+} and HCO_3^- in modulating sperm capacitation requiring the presence of both as well as increase in cAMP levels and the subsequent phosphorylation of different proteins. In swine, both Ca^{2+} and HCO_3^- appear to be required for capacitation and their roles are synergistic, since it has been shown that the HCO_3^- will stimulate the entry of Ca^{2+} in this species (Harrison *et al.*, 1993). However, in mouse spermatozoa, Tateno *et al.* (2013) showed that Ca^{2+} ionophore A23187 can make spermatozoa capable of fertilizing *in vitro* without activation of cAMP-dependent phosphorylation pathways in media HCO_3^- free.

Ca^{2+} is important to sperm hyperactivation during capacitation. CatSperm 1 and 2 are voltage dependent Ca^{2+}channels that are located in the tail of the sperm. Sperm from mice deficient in these Ca^{2+} channels are infertile and do not exhibit hyperactivation during capacitation despite having TP (Carlson *et al.*, 2003).

Another aspect that influences capacitation related to Ca^{2+} is pH_i. Sperm not capacitated maintain an acidified intracellular pH (Parrish *et al.*, 1989). This fact acts as a regulator of Ca^{2+} influx (Florman *et al.*, 1992) preventing capacitation and acrosome reaction. Intracellular pH becomes more alkaline during capacitation (Vredenburgh-Wilberg & Parrish, 1995). Today it is believed that increasing intracellular Ca^{2+}, HCO_3^- and the pH during sperm capacitation produce sAC activation and consequently cAMP (Travis & Kopf, 2002; Breitbart, 2003; Harrison & Gadella, 2005; Hess *et al.*, 2005a).

In addition, calmodulin, which is a protein binding Ca^{2+} considered to be an important transducer of Ca^{2+} signals, appears to be diminished during capacitation. This mechanism could be based on inhibition of Ca^{2+}-ATPase plasma membrane by increasing cAMP levels through PDE1 inhibition (reviewed by Bailey, 2010).

4.3. Tyrosine phosphorylation of sperm proteins

Protein phosphorylation or de-phosphorylation is controlled by activity of protein kinases and protein phosphatases, which provide cells a “switch” through which they can activate function of various proteins (Signorelli *et al.*, 2013). Phosphorylation

occurs in serine, threonine, and tyrosine. TP is related to capacitation process and sperm hyperactivation in many mammal species (human (Baldi *et al.*, 2002), bovine (Galantino-Homer *et al.*, 1997), murine (Visconti & Kopf, 1998) or porcine (Tardif *et al.*, 2001)). In opposite, it has been shown that protein phosphatases decrease their activity during capacitation (Signorelli *et al.*, 2013).

Increasing TP during capacitation is regulated by a cAMP-dependent pathway which involves PKA (Kalab *et al.*, 1998). cGMP-PKG pathway is also involved in this process (Cisneros-Mejorado *et al.*, 2014). In 2002, Visconti *et al.* described the possible mechanisms, which could regulate the TP dependent signalling pathway cAMP/PKA: i) the direct or indirect stimulation of a tyrosine kinase by PKA, ii) the direct or indirect inhibition of a tyrosine phosphatase, and iii) direct or indirect phosphorylation of proteins by PKA on serine or threonine residues to prepare these proteins for subsequent phosphorylation on tyrosine residues.

TP is specific for each species. For example, in man TP during sperm capacitation requires the presence of BSA, and HCO_3^- but no Ca^{2+} (Muratori *et al.*, 2010). In the case of stallion TP during capacitation requires HCO_3^- but neither BSA nor Ca^{2+} (González-Fernández *et al.*, 2012). Another factor to consider in TP is time. TP in boar sperm occurs close to 1 hour after the addition of HCO_3^- (Gadella & Van Gestel, 2004), whereas in bull sperm it occurs 4 hours after addition of heparin (Galantino-Homer *et al.*, 1997).

Although TP is an important key in capacitation, it is not yet entirely clear how the phosphorylation of these proteins is involved in sperm-zona recognition, gamete interaction, or exocytosis of acrosomal content (Flesch *et al.*, 2001b). The level of TP in human sperm correlates strongly with the sperm-zona-binding capacity (Liu *et al.*, 2006) and alterations in TP have been found in subfertile subjects (Buffone *et al.*, 2009) indicating its physiological role in fertilization. In pigs ejaculated spermatozoa selected in the oviduct adhere to the epithelial cells and suppress TP of sperm proteins. This modulation by the oviductal epithelium on TP and, therefore capacitation could help synchronize sperm functions to the time of ovulation.

4.4. Nitric oxide (NO) and sperm capacitation

Previous papers showed that NOS (nitric oxide synthase) is present in the oviduct (Rosselli *et al.*, 1996; Ekerhovd *et al.*, 1999; Lapointe *et al.*, 2006), oocyte, *cumulus* and corona cells (Reyes *et al.*, 2004; Tao *et al.*, 2004) of different species (Bryant *et al.*, 1995; Rosselli *et al.*, 1996; Ekerhovd *et al.*, 1997). NOS isoforms are hormonally regulated in the oviduct and expresses differently throughout the oestrous cycle. In the oviduct, nitric oxide (NO) has been shown to regulate contractility (Rosselli *et al.*, 1994), ciliary beating of the ciliated epithelial cells, the sperm motility or even inducing

chemotaxis (Miraglia *et al.*, 2007). For this reason NO also module sperm capacitation although the pathway is not known totally.

NO is synthesized *in vivo* from L-arginine by the action of NOS (Figure 3), an enzyme existing in three isoforms: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), also referred to as constitutive NOS, responsible for the continuous basal release of NO, and both require Ca^{2+}/calmodulin for activation (Griffith & Stuehr, 1995; Snyder, 1995). A third isoform is an inducible Ca^{2+}-independent form (iNOS or NOS2). NOS activity is dependent on substrate availability and the co-factors NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4). The availability of these factors determines the cellular rates of NO synthesis (Rosselli *et al.*, 1998).

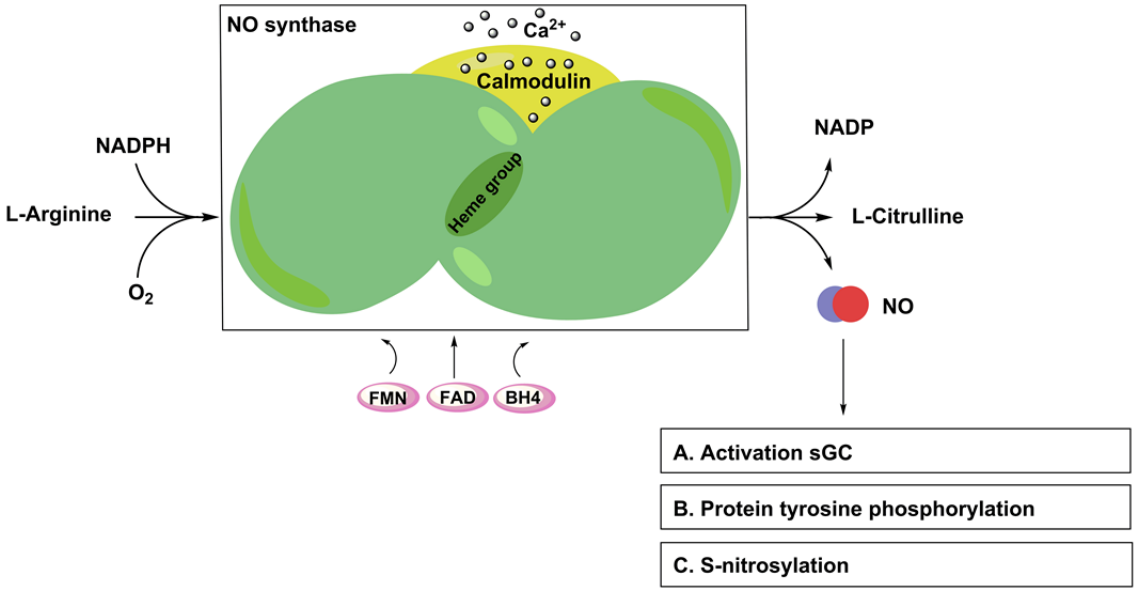

Figure 3. Nitric oxide synthase produces NO from L-arginine and different co-factors (NADPH, FMN, FAD and BH_4). NO activates three different pathways. **A)** Activation of soluble guanylate cyclase (sGC); **B)** Protein tyrosine phosphorylation and **C)** Modulation of protein function by S-nitrosylation. This figure represents Ca^{2+} dependent isoforms (nNOS and eNOS); in the case of Ca^{2+} independent isoform (iNOS) the calmodulin is not present.

Different NOS isoforms were detected in mammalian spermatozoa such as mouse (Herrero *et al.*, 1997), bull (Meiser & Schulz, 2003), human (Herrero *et al.*, 1996; O'Bryan *et al.*, 1998) and boar spermatozoa (Hou *et al.*, 2008) activating the biosynthesis of NO. NO was able to affect sperm motility (Lewis *et al.*, 1996; Donnelly *et al.*, 1997; O'Bryan *et al.*, 1998), acrosomal reaction (Revelli *et al.*, 1999, 2001), acts on TP of sperm proteins (Herrero *et al.*, 1999; Thundathil *et al.*, 2003) and enhancement of sperm-zona pellucida binding ability (Sengoku *et al.*, 1998).

NO has different functions in the spermatozoa, acting on different pathways that result in sequential and parallel processes (Figure 3). The main actions of NO are:

A. <u>Activation of soluble guanylate cyclase (sGC)</u>

The most important intracellular signalling role for NO in the spermatozoa is its capacity to activate the soluble isoform of guanylate cyclase (sGC) (Murad, 1993). Activation of sGC (Figure 4) leads to increase in intracellular levels of cGMP, which has been implicated in several sperm signalling pathway functions, such as capacitation, acrosome reaction, chemotaxis, and sperm-egg interaction (Revelli *et al.*, 2001, 2002; Herrero *et al.*, 2003; Miraglia *et al.*, 2007).

There are at least three targets of cGMP: A1) Cyclic nucleotide-gated (CNG) channels, A2) cGMP-dependent protein kinase (PKG) and A3) phosphodiesterase (PDE), involved in several physiological events in mammalian spermatozoa. All of these targets result in increased levels of intracellular Ca^{2+} and the phosphorylation of different proteins causing sperm hyperactivation and acrosome reaction. Calcium influx together with increased protein phosphorylation brings about the capacitation response (Figure 4).

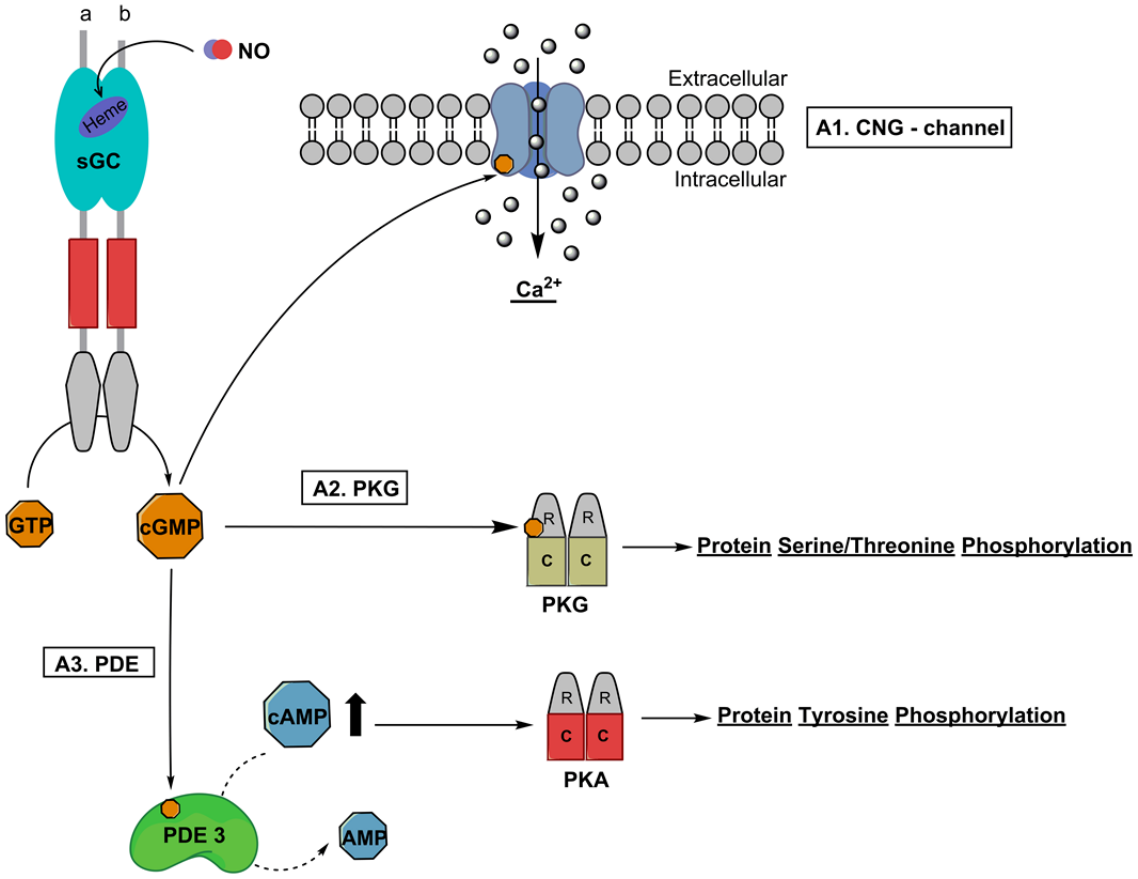

Figure 4. Implications of activation of soluble guanylate cyclase (sGC). NO activates the sCG, which increases the production of cGMP. The cGMP can act on three targets: CNG-channel **(A1)**, cGMP-dependent protein kinase **(A2)** and phosphodiesterase **(A3)**.

B. <u>Protein tyrosine phosphorylation</u>

NO appears to be involved in TP through different mechanisms, acting on two essential pathways for sperm capacitation: on cAMP/PKA by activation of sAC with a subsequent increase in cAMP levels (Herrero *et al.*, 2000) to increase TP by activation of PKA or extracellular signal regulated kinase (ERK) pathway (Figure 5). These mechanisms for the control of TP are not mutually exclusive neither excluding, both pathways act in parallel (O'Flaherty *et al.*, 2006).

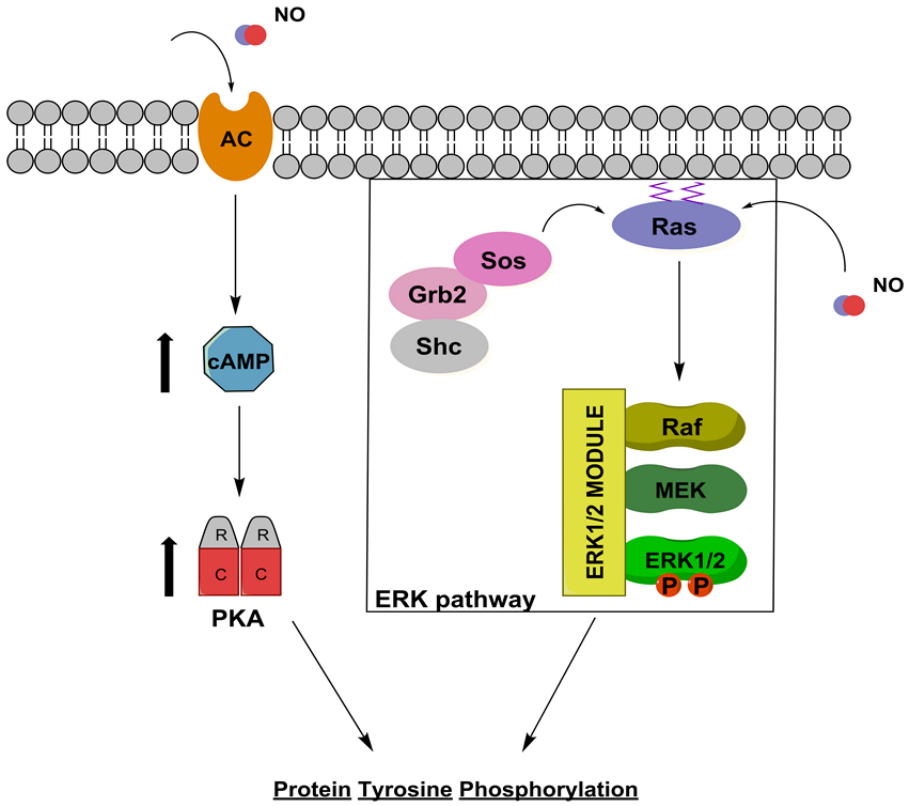

Figure 5. NO is directly involved in tyrosine phosphorylation: modulating adenylate cyclase (AC) activating the cAMP/PKA pathway or modifying RAS protein. Ras activates ERK1/2 module, Thr and Tyr residues in the active sites of ERK1/2 are phosphorylated allowing subsequent protein tyrosine phosphorylation.

C. <u>Direct modulation of protein function by S-nitrosylation of exposed cysteine residues</u>

Mature sperm lack the necessary machinery for the transcription or protein modification and thereby require post-translational modifications to control the activity of proteins. NO participates in protein regulation, which acts directly on protein targets (exposed cysteine residues) via S-nitrosylation (Davis *et al.*, 2001; Ahern *et al.*, 2002). S-nitrosylation is a regulated post-translational protein modification (Figure 6), analogous to phosphorylation and acetylation (Foster & Stamler, 2004; Hess *et al.*, 2005b), which involves the covalent incorporation of a NO

into thiol groups (-SH), to form S-nitrosothiol (S-NO). This modification is selective, reversible and stabilizes NO in a uniquely bioactive form. Lefièvre *et al.* (2007) described numerous sperm proteins that can be nitrosylated in human sperm. Some of the S-nitrosylated proteins are involved in processes related to sperm capacitation as energy generation, sperm motility (Lefièvre *et al.*, 2007) or hyperactivation (Bedu-Addo *et al.*, 2008; Machado-Oliveira *et al.*, 2008). However, how some of nitrosylated proteins perform their function remains unknown.

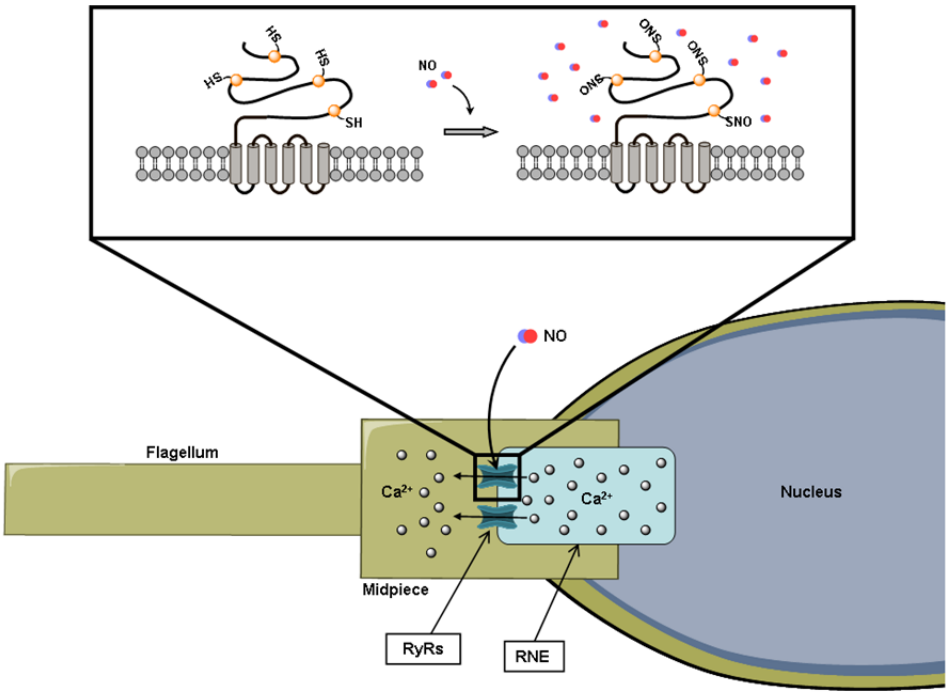

Figure 6. S-nitrosylation process. NO acts on the thiol groups (-SH) of the cysteines in proteins to form S-nitosothiol (S-NO). In the sperm neck/midpiece occurs S-nitrosylation in ryanodine receptors (RyRs) allowing the release of Ca^{2+} from the redundant nuclear envelope (RNE), which is required for sperm hyperactivation.

5. SUMMARY AND PERSPECTIVE

From the moment they are deposited in the female genital until they reach the place of fecundation, sperm undergo a series of changes known classically as capacitation, which enables them to fertilization purposes. However, of all the sperm deposited in the female genital, just a few are able to reach the oviduct, and of these only a small part forms the sperm reservoir and finally reaches the oocyte. These selected spermatozoa must show certain characteristics that distinguish them from the rest of sperm, enabling them to overcome all the physiological barriers within the female reproductive tract and make them suitable for fertilization. Knowledge of the functional characteristics that present the selected spermatozoa and understanding of the complex process of sperm capacitation could lead to improvements in *in vitro* fertilization techniques.

REFERENCES

Ahern GP, Klyachko VA & Jackson MB (2002). cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends in neurosciences.* 25: 510-517. (doi: 10.1016/S0166-2236(02)02254-3).

Aitken RJ & Fisher H (1994). Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays.* 16: 259-267. (doi: 10.1002/bies.950160409).

Aitken RJ (1997). Molecular mechanisms regulating human sperm function. *Molecular human reproduction.* 3: 169-173. (doi: 10.1093/molehr/3.3.169).

Aitken RJ & Nixon B (2013). Sperm capacitation: a distant landscape glimpsed but unexplored. *Molecular human reproduction.* 19: 785-793. (doi: 10.1093/molehr/gat067).

Aquila S, Giordano F, Guido C, Rago V & Carpino A (2011). Nitric oxide involvement in the acrosome reaction triggered by leptin in pig sperm. *Reproductive Biology and Endocrinology.* 9: 133. (doi: 10.1186/1477-7827-9-133).

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M & Florman HM (1999). Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proceedings of the National Academy of Sciences.* 96: 6757-6762. (doi: 10.1073/pnas.96.12.6757).

Austin CR (1951). Activation and the correlation between male and female elements in fertilization. *Nature.* 168: 558-559. (doi: 10.1038/168558c0).

Austin CR (1952). The "Capacitation" of the Mammalian Sperm. *Nature.* 170: 326-326.

Avilés M, Coy P & Rizos D (2015). The oviduct: a key organ for the success of early reproductive events. *Animal Frontiers.* 5: 25-31.

Bailey JL (2010). Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine.* 56: 334-348. (doi: 10.3109/19396368.2010.512377).

Baldi E, Luconi M, Bonaccorsi L & Forti G (2002). Signal transduction pathways in human spermatozoa. *Journal of reproductive immunology.* 53: 121-131. (doi: 10.1016/S0165-0378(01)00089-4).

Bedu-Addo K, Costello S, Harper C, Machado-Oliveira G, Lefievre L, Ford C, Barratt C & Publicover S (2008). Mobilisation of stored calcium in the neck region of human sperm-a mechanism for regulation of flagellar activity. *International Journal of Developmental Biology.* 52: 615-626. (doi: 10.1387/ijdb.072535kb).

Boerke A, Tsai P, Garcia-Gil N, Brewis I & Gadella BM (2008). Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology.* 70: 1188-1196. (doi: 10.1016/j.theriogenology.2008.06.021).

Boerke A, Brouwers JF, Olkkonen VM, van de Lest CH, Sostaric E, Schoevers EJ, Helms JB & Gadella BM (2013). Involvement of bicarbonate-induced radical signaling in oxysterol formation and sterol depletion of capacitating mammalian sperm during *in vitro* fertilization. *Biology of reproduction.* 88: 1-18. (doi: 10.1095/biolreprod.112.101253).

Breitbart H (2003). Signaling pathways in sperm capacitation and acrosome reaction. *Cellular and molecular biology (Noisy-le-Grand, France).* 49: 321-327.

Brooks D (1983). Epididymal functions and their hormonal regulation. *Australian Journal of Biological Sciences.* 36: 205-222. (doi: 10.1071/BI9830205).

Brouwers JF, Boerke A, Silva PF, Garcia-Gil N, van Gestel RA, Helms JB, van de Lest CH & Gadella BM (2011). Mass spectrometric detection of cholesterol oxidation in bovine sperm. *Biology of reproduction.* 85: 128-136. (doi: 10.1095/biolreprod.111.091207).

Brüssow KP, Torner H, Rátky J, Manabe N & Tuchscherer A (2006). Experimental evidence for the influence of *cumulus-oocyte*-complexes on sperm release from the porcine oviductal sperm reservoir. *Journal of Reproduction and Development.* 52: 249-257.

Brüssow KP, Ratky J & Rodriguez-Martinez H (2008). Fertilization and early embryonic development in the porcine fallopian tube. *Reproduction in Domestic Animals.* 43: 245-251. (doi: 10.1111/j.1439-0531.2008.01169.x).

Bryant C, Tomlinson A, Mitchell J, Thiemermann C & Willoughby D (1995). Nitric oxide synthase in the rat fallopian tube is regulated during the oestrous cycle. *Journal of endocrinology.* 146: 149-157. (doi: 10.1677/joe.0.1460149).

Buffone MG, Verstraeten SV, Calamera JC & Doncel GF (2009). High cholesterol content and decreased membrane fluidity in human spermatozoa are associated with protein tyrosine phosphorylation and functional deficiencies. *Journal of andrology.* 30: 552-558. (doi: 10.2164/jandrol.108.006551).

Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL & Babcock DF (2003). CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. *Proceedings of the National Academy of Sciences.* 100: 14864-14868. (doi: 10.1073/pnas.2536658100).

Cisneros-Mejorado A, Hernández-Soberanis L, Islas-Carbajal M & Sánchez D (2014). Capacitation and Ca^{2+} influx in spermatozoa: role of CNG channels and protein kinase G. *Andrology.* 2: 145-154. (doi: 10.1111/j.2047-2927.2013.00169.x).

Cohen G, Rubinstein S, Gur Y & Breitbart H (2004). Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. *Developmental biology.* 267: 230-241. (doi: 10.1016/j.ydbio.2003.10.034).

Cross NL (1998). Role of cholesterol in sperm capacitation. *Biology of reproduction.* 59: 7-11. (doi: 10.1095/biolreprod59.1.7).

Chang M (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*. 168: 697-698 (doi: 10.1038/168697b0).

Davis B (1976). Inhibitory effect of synthetic phospholipid vesicles containing cholesterol on the fertilizing ability of rabbit spermatozoa. *Experimental Biology and Medicine.* 152: 257-261. (doi: 10.3181/00379727-152-39374).

Davis KL, Martin E, Turko IV & Murad F (2001). Novel effects of nitric oxide. *Annual review of pharmacology and toxicology.* 41:203-236. (doi: 10.1146/annurev.pharmtox.41.1.203).

de Lamirande E, Leclerc P & Gagnon C (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular human reproduction.* 3: 175-194. (doi: 10.1093/molehr/3.3.175).

Demarco IA, Espinosa F, Edwards J, Sosnik J, de la Vega-Beltran JL, Hockensmith JW, Kopf GS, Darszon A & Visconti PE (2003). Involvement of a Na^+/HCO_3^- cotransporter in mouse sperm capacitation. *Journal of Biological Chemistry.* 278: 7001-7009. (doi: 10.1074/jbc.M206284200).

Dobrinski I, Ignotz G, Thomas P & Ball B (1996). Role of carbohydrates in the attachment of equine spermatozoa to uterine tubal (oviductal) epithelial cells *in vitro*. *American journal of veterinary research.* 57: 1635-1639.

Donnelly ET, Lewis S, Thompson W & Chakravarthy U (1997). Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. *Molecular human reproduction.* 3: 755-762. (doi: 10.1093/molehr/3.9.755).

Einarsson S & Viring S (1972). Effect of boar seminal plasma on the porcine uterus and the isthmus part of oviducts *in vitro*. *Acta veterinaria Scandinavica.* 14: 639-641.

Ekerhovd E, Brännström M, Alexandersson M & Norström A (1997). Evidence for nitric oxide mediation of contractile activity in isolated strips of the human Fallopian tube. *Human reproduction.* 12: 301-305. (doi: 10.1093/humrep/12.2.301).

Ekerhovd E, Brännström M, Weijdegård B & Norström A (1999). Localization of nitric oxide synthase and effects of nitric oxide donors on the human Fallopian tube. *Molecular human reproduction.* 5: 1040-1047. (doi: 10.1093/molehr/5.11.1040).

Finkel T (2001). Reactive oxygen species and signal transduction. *IUBMB life.* 52: 3-6. (doi: 10.1080/15216540252774694).

Fitzpatrick JL & Lüpold S (2014). Sexual selection and the evolution of sperm quality. *Molecular human reproduction.* 20: 1180-1189. (doi: 10.1093/molehr/gau067).

Flesch FM & Gadella BM (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes.* 1469: 197-235. (doi: 10.1016/S0304-4157(00)00018-6).

Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B & Gadella BM (2001a). Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of cell science.* 114: 3543-3555.

Flesch FM, Wijnand E, Van de Lest C, Colenbrander B, van Golde LM & Gadella BM (2001b). Capacitation dependent activation of tyrosine phosphorylation generates two sperm head plasma membrane proteins with high primary binding affinity for the zona pellucida. *Molecular reproduction and development.* 60: 107-115. (doi: 10.1002/mrd.1067).

Florman HM, Corron ME, Kim TD-H & Babcock DF (1992). Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Developmental biology.* 152: 304-314. (doi: 10.1016/0012-1606(92)90137-6).

Foster MW & Stamler JS (2004). New insights into protein S-nitrosylation. Mitochondria as a model system. *Journal of Biological Chemistry.* 279: 25891-25897. (doi: 10.1074/jbc.M313853200).

Fraser LR (2010). The “switching on” of mammalian spermatozoa: molecular events involved in promotion and regulation of capacitation. *Molecular reproduction and development.* 77: 197-208. (doi: 10.1002/mrd.21124).

Funahashi H (2002). Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. *Reproduction.* 124: 857-864. (doi: 10.1530/rep.0.1240857).

Gadella BM & Harrison RA (2000). The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development.* 127: 2407-2420.

Gadella BM & Harrison RA (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. *Biology of reproduction.* 67: 340-350. (doi: 10.1095/biolreprod67.1.340).

Gadella BM & Van Gestel R (2004). Bicarbonate and its role in mammalian sperm function. *Animal Reproduction Science.* 82: 307-319. (doi: 10.1016/j.anireprosci.2004.04.030).

Galantino-Homer HL, Visconti PE & Kopf GS (1997). Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3',5'-monophosphate-dependent pathway. *Biology of reproduction.* 56: 707-719. (doi: 10.1095/biolreprod56.3.707).

Garbers D, TUbb D & Hyne R (1982). A requirement of bicarbonate for Ca^{2+}-induced elevations of cyclic AMP in guinea pig spermatozoa. *Journal of Biological Chemistry.* 257: 8980-8984.

González-Fernández L, Macías-García B, Velez IC, Varner DD & Hinrichs K (2012). Calcium–calmodulin and pH regulate protein tyrosine phosphorylation in stallion sperm. *Reproduction.* 144: 411-422. (doi: 10.1530/REP-12-0067).

Griffith OW & Stuehr DJ (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annual Review of Physiology.* 57: 707-734. (doi: 10.1146/annurev.ph.57.030195.003423).

Harrison RA, Mairet B & Miller N (1993). Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations. *Molecular reproduction and development.* 35: 197-208.

Harrison RA, Ashworth P & Miller N (1996). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular reproduction and development.* 45: 378-391. (doi: 10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V).

Harrison RA & Miller N (2000). cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Molecular reproduction and development.* 55: 220-228. (doi: 10.1002/(SICI)1098-2795(200002)55:2<220::AID-MRD12>3.0.CO;2-I).

Harrison RA (2004). Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Molecular reproduction and development.* 67: 337-352.

Harrison RA & Gadella BM (2005). Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology.* 63: 342-351.

Hernández-Caravaca I, Izquierdo-Rico MJ, Matás C, Carvajal JA, Vieira L, Abril D, Soriano-Úbeda C & García-Vázquez FA (2012). Reproductive performance and backflow study in cervical and post-cervical artificial insemination in sows. *Animal Reproduction Science.* 136: 14-22. (doi: 10.1016/j.anireprosci.2012.10.007).

Herrero MB, Pérez Martínez S, Viggiano J & Polak J (1996). Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reproduction, fertility and development.* 8: 931-934. (doi: 10.1071/RD9960931).

Herrero MB, Goin J, Boquet M, Canteros M, Franchi A, Pérez Martínez S, Polak J, Viggiano J & Gimeno M (1997). The nitric oxide synthase of mouse spermatozoa. *FEBS letters.* 411: 39-42. (doi: 10.1016/S0014-5793(97)00570-X).

Herrero MB, de Lamirande E & Gagnon C (1999). Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation *in vitro*. *Biology of reproduction.* 61: 575-581. (doi: 10.1095/biolreprod61.3.575).

Herrero MB, Chatterjee S, Lefièvre L, de Lamirande E & Gagnon C (2000). Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. *Free Radical Biology and Medicine.* 29: 522-536.

Herrero MB, de Lamirande Ed & Gagnon C (2003). Nitric oxide is a signaling molecule in spermatozoa. *Current pharmaceutical design.* 9: 419-425. (doi: 10.2174/1381612033391720).

Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS & Suarez SS (2005a). The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Developmental cell.* 9: 249-259. (doi: 10.1016/j.devcel.2005.06.007).

Hess DT, Matsumoto A, Kim S-O, Marshall HE & Stamler JS (2005b). Protein S-nitrosylation: purview and parameters. *Nature Reviews Molecular Cell Biology.* 6: 150-166. (doi: 10.1038/nrm1569).

Holt WV (2009). Is semen analysis useful to predict the odds that the sperm will meet the egg? *Reproduction in Domestic Animals.* 44:31-38. (doi: 10.1111/j.1439-0531.2009.01363.x).

Hou M-L, Huang S-Y, Lai Y-K & Lee W-C (2008). Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. *Animal Reproduction Science.* 104: 56-68. (doi: 10.1016/j.anireprosci.2007.01.006).

Hunter RHF & Rodriguez-Martinez H (2004). Capacitation of mammalian spermatozoa *in vivo*, with a specific focus on events in the Fallopian tubes. *Molecular reproduction and development.* 67: 243-250. (doi: 10.1002/mrd.10390).

Hunter RHF (2008). Sperm release from oviduct epithelial binding is controlled hormonally by peri-ovulatory graafian follicles. *Molecular reproduction and development.* 75:167-174. (doi: 10.1002/mrd.20776).

Immler S (2008). Sperm competition and sperm cooperation: the potential role of diploid and haploid expression. *Reproduction.* 135: 275-283. (doi: 10.1530/REP-07-0482).

Jessup W, Gelissen IC, Gaus K & Kritharides L (2006). Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages. *Current opinion in lipidology.* 17: 247-257. (doi: 10.1097/01.mol.0000226116.35555.eb).

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K & Hirohashi N (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proceedings of the National Academy of Sciences.* 108: 4892-4896. (doi: 10.1073/pnas.1018202108).

Kalab P, Pěknicová J, Geussova G & Moos J (1998). Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Molecular reproduction and development.* 51: 304-314. (doi: 10.1002/(SICI)1098-2795(199811)51:3<304::AID-MRD10>3.0.CO;2-2).

Kawano N, Araki N, Yoshida K, Hibino T, Ohnami N, Makino M, Kanai S, Hasuwa H, Yoshida M & Miyado K (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proceedings of the National Academy of Sciences.* 111: 4145-4150. (doi: 10.1073/pnas.1320715111).

Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA & Bilodeau J-Fo (2006). Hormonal and spatial regulation of nitric oxide synthases (NOS)(neuronal NOS,

inducible NOS, and endothelial NOS) in the oviducts. *Endocrinology.* 147: 5600-5610. (doi: 10.1210/en.2005-1548).

Lapointe S, Sullivan R & Sirard M-A (1998). Binding of a bovine oviductal fluid catalase to mammalian spermatozoa. *Biology of reproduction.* 58: 747-753. (doi: 10.1095/biolreprod58.3.747).

Lefebvre R, Lo MC & Suarez SS (1997). Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biology of reproduction.* 56: 1198-1204. (doi: 10.1095/biolreprod56.5.1198).

Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL & Barratt CL (2007). Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics.* 7: 3066-3084. (doi: 10.1002/pmic.200700254).

Lewis S, Donnelly E, Sterling E, Kennedy M, Thompson W & Chakravarthy U (1996). Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Molecular human reproduction.* 2: 873-878. (doi: 10.1093/molehr/2.11.873).

Liu D, Clarke G & Baker H (2006). Tyrosine phosphorylation on capacitated human sperm tail detected by immunofluorescence correlates strongly with sperm–zona pellucida (ZP) binding but not with the ZP-induced acrosome reaction. *Human reproduction.* 21: 1002-1008. (doi: 10.1093/humrep/dei435).

Loeb J (1915). On the Nature of the Conditions Which Determine or Prevent the Entrance of the Spermatozoon Into the Egg. *The American Naturalist.* 49: 257-285. (doi: 10.2307/2456166).

Machado-Oliveira G, Lefièvre L, Ford C, Herrero MB, Barratt C, Connolly TJ, Nash K, Morales-Garcia A, Kirkman-Brown J & Publicover S (2008). Mobilisation of Ca^{2+} stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development.* 135: 3677-3686. (doi: 10.1242/dev.024521).

Matthijs A, Engel B & 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: 357-367. (doi: 10.1530/rep.0.1250357).

Meiser H & Schulz R (2003). Detection and localization of two constitutive NOS isoforms in bull spermatozoa. *Anatomia, histologia, embryologia.* 32: 321-325. (doi: 10.1111/j.1439-0264.2003.00459.x).

Miraglia E, Rullo ML, Bosia A, Massobrio M, Revelli A & Ghigo D (2007). Stimulation of the nitric oxide/cyclic guanosine monophosphate signaling pathway elicits human sperm chemotaxis *in vitro*. *Fertility and sterility.* 87: 1059-1063. (doi: 10.1016/j.fertnstert.2006.07.1540).

Muñoz-Garay C, De la Vega-Beltrán JL, Delgado R, Labarca P, Felix R & Darszon A (2001). Inwardly rectifying K^+ channels in spermatogenic cells: functional expression and implication in sperm capacitation. *Developmental biology.* 234: 261-274. (doi: 10.1006/dbio.2001.0196).

Murad F (1993). The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. *Recent progress in hormone research.* 49: 239-248.

Muratori M, Marchiani S, Tamburrino L, Forti G, Luconi M & Baldi E (2010). Markers of human sperm functions in the ICSI era. *Frontiers in bioscience (Landmark edition).* 16: 1344-1363. (doi: 10.2741/3793).

O'Flaherty C, de Lamirande E & Gagnon C (2006). Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. *Free Radical Biology and Medicine.* 40: 1045-1055. (doi: 10.1016/j.freeradbiomed.2005.10.055).

O'Bryan MK, Zini A, Cheng CY & Schlegel PN (1998). Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. *Fertility and sterility.* 70: 1143-1147. (doi: 10.1016/S0015-0282(98)00382-3).

Okamura N, Tajima Y, Soejima A, Masuda H & Sugita Y (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *Journal of Biological Chemistry.* 260: 9699-9705.

Parrish J, Susko-Parrish J & First N (1989). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. *Biology of reproduction.* 41: 683-699. (doi: 10.1095/biolreprod41.4.683).

Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, Bosia A & Ghigo D (1999). Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. *Journal of cellular physiology.* 178: 85-92. (doi: 10.1002/(SICI)1097-4652(199901)178:1<85::AID-JCP11>3.0.CO;2-Y).

Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, Massobrio M, Lindblom B & Ghigo D (2001). Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. *Biology of reproduction.* 64: 1708-1712. (doi: 10.1095/biolreprod64.6.1708).

Revelli A, Ghigo D, Moffa F, Massobrio M & Tur-Kaspa I (2002). Guanylate cyclase activity and sperm function. *Endocrine reviews.* 23: 484-494. (doi: 10.1210/er.2001-0020).

Reyes R, Vázquez M & Delgado N (2004). Detection and bioimaging of nitric oxide in bovine oocytes and sperm cells. *Systems Biology in Reproductive Medicine.* 50: 303-309. (doi: 10.1080/01485010490448471).

Rodriguez-Martinez H, Ekstedt E & Einarsson S (1990). Acidification of epididymal fluid in the boar. *International journal of andrology.* 13: 238-243. (doi: 10.1111/j.1365-2605.1990.tb00982.x).

Rodriguez-Martinez H, Tienthai P, Suzuki K, Funahashi H, Ekwall H & Johannisson A (2000). Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reproduction (Cambridge, England) Supplement.* 58: 129-145.

Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, Martínez E, Roca J, Sanz L & Calvete JJ (2005). Boar spermatozoa in the oviduct. *Theriogenology.* 63: 514-535. (doi: 10.1016/j.theriogenology.2004.09.028).

Rodriguez-Martinez H, Saravia F, Wallgren M, Roca J & Peña F (2008). Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. *Theriogenology.* 70: 1242-1250. (doi: 10.1016/j.theriogenology.2008.06.007).

Rodriguez-Martinez H, Kvist U, Wallgren M, Johannisson A & Sanz L (2009). The physiological roles of the boar ejaculate. *Control of Pig Reproduction VIII.* 66: 1-21.

Rosselli M, Imthurn B, Macas E, Keller P & Dubey RK (1994). Endogenous nitric oxide modulates endothelin-1 induced contraction of bovine oviduct. *Biochemical and biophysical research communications.* 201: 143-148. (doi: 10.1006/bbrc.1994.1680).

Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, Keller P & Imthurn B (1996). Identification of nitric oxide synthase in human and bovine oviduct. *Molecular human reproduction.* 2: 607-612. (doi: 10.1093/molehr/2.8.607).

Rosselli M, Keller R & Dubey RK (1998). Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Human reproduction update.* 4: 3-24. (doi: 10.1093/humupd/4.1.3).

Rozeboom K, Troedsson M, Molitor T & Crabo B (1999). The effect of spermatozoa and seminal plasma on leukocyte migration into the uterus of gilts. *Journal of animal science.* 77: 2201-2206. (doi: 1999.7782201x).

Salicioni A, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J & Visconti PE (2007). Signalling pathways involved in sperm capacitation. *Society of Reproduction and Fertility supplement.* 65: 245-259.

Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T & Ishikawa M (1998). Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. *Fertility and sterility.* 69: 522-527. (doi: 10.1016/S0015-0282(97)00537-2).

Setchell B, Maddocks S & Brooks D (1994). Anatomy, vasculature, innervation, and fluids of the male reproductive tract. *The physiology of reproduction.* 1: 1063-1175.

Signorelli JR, Diaz ES, Fara K, Baron L & Morales P (2013). Protein phosphatases decrease their activity during capacitation: a new requirement for this event. *PloS one.* 8 (doi: 10.1371/journal.pone.0081286).

Snyder SH (1995). No endothelial NO. *Nature.* 377: 196-197. (doi: 10.1038/377196a0).

Soriano-Úbeda C, Matás C & García-Vázquez F (2013). An overview of swine artificial insemination: retrospective, current and prospective aspects. *Journal of Experimental and Applied Animal Sciences.* 1: 67-97.

Suarez S, Redfern K, Raynor P, Martin F & Phillips D (1991). Attachment of boar sperm to mucosal explants of oviduct *in vitro*: possible role in formation of a sperm reservoir. *Biology of reproduction.* 44: 998-1004. (doi: 10.1095/biolreprod44.6.998).

Suarez SS (1998). The oviductal sperm reservoir in mammals: mechanisms of formation. *Biology of reproduction.* 58: 1105-1107. (doi: 10.1095/biolreprod58.5.1105).

Suarez SS (2008). Control of hyperactivation in sperm. *Human reproduction update.* 14: 647-657. (doi: 10.1093/humupd/dmn029).

Talevi R & Gualtieri R (2010). Molecules involved in sperm-oviduct adhesion and release. *Theriogenology.* 73: 796-801. (doi: 10.1016/j.theriogenology.2009.07.005).

Tao Y, Fu Z, Zhang M, Xia G, Yang J & Xie H (2004). Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. *Molecular and cellular endocrinology.* 222: 93-103. (doi: 10.1016/j.mce.2004.04.014).

Tardif S, Dubé C, Chevalier S & Bailey JL (2001). Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of reproduction.* 65: 784-792. (doi: 10.1095/biolreprod65.3.784).

Tateno H, Krapf D, Hino T, Sánchez-Cárdenas C, Darszon A, Yanagimachi R & Visconti PE (2013). Ca^{2+} ionophore A23187 can make mouse spermatozoa capable of fertilizing *in vitro* without activation of cAMP-dependent phosphorylation pathways. *Proceedings of the National Academy of Sciences.* 110: 18543-18548. (doi: 10.1073/pnas.1317113110).

Thundathil J, de Lamirande E & Gagnon C (2003). Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. *Biology of reproduction.* 68: 1291-1298. (doi: 10.1095/biolreprod.102.008276).

Töpfer-Petersen E, Wagner A, Friedrich J, Petrunkina AM, Ekhlasi-Hundrieser M, Waberski D & Drommer W (2002). Function of the mammalian oviductal sperm reservoir. *Journal of Experimental Zoology.* 292: 210-215. (doi: 10.1002/jez.1157).

Travis AJ & Kopf GS (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *Journal of Clinical Investigation.* 110: 731-736. (doi: 10.1172/JCI16392.).

Vadnais ML, Galantino-Homer HL & Althouse GC (2007). Current concepts of molecular events during bovine and porcine spermatozoa capacitation. *Systems Biology in Reproductive Medicine.* 53: 109-123. (doi: 10.1080/01485010701329386).

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS (1995). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development.* 121: 1129-1137.

Visconti PE, Galantino-Homer H, Moore GD, Bailey JL, Ning X, Fornes M & Kopf GS (1998). The molecular basis of sperm capacitation. *Journal of andrology.* 19: 242-248. (doi: 10.1002/j.1939-4640.1998.tb01994.x).

Visconti PE & Kopf GS (1998). Regulation of protein phosphorylation during sperm capacitation. *Biology of reproduction.* 59: 1-6. (doi: 10.1095/biolreprod59.1.1).

Visconti PE, Ning X, Fornés MW, Alvarez JG, Stein P, Connors SA & Kopf GS (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Developmental biology.* 214: 429-443. (doi: 10.1006/dbio.1999.9428).

Visconti PE, Westbrook V, Chertihin O, Demarco I, Sleight S & Diekman A (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *Journal of reproductive immunology.* 53: 133-150. (doi: 10.1016/S0165-0378(01)00103-6).

Visconti PE (2009). Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences.* 106: 667-668. (doi: 10.1073/pnas.0811895106).

Vredenburgh-Wilberg W & Parrish J (1995). Intracellular pH of bovine sperm increases during capacitation. *Molecular reproduction and development.* 40: 490-502. (doi: 10.1002/mrd.1080400413).

Wennemuth G, Carlson AE, Harper AJ & Babcock DF (2003). Bicarbonate actions on flagellar and Ca^{2+}-channel responses: initial events in sperm activation. *Development.* 130: 1317-1326. (doi: 10.1242/dev.00353).

Zeng Y, Clark EN & Florman HM (1995). Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Developmental biology.* 171: 554-563. (doi: 10.1006/dbio.1995.1304).

Zeng Y, Oberdorf JA & Florman HM (1996). pH regulation in mouse sperm: identification of Na^+-, Cl^--, and HCO_3^--dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Developmental biology.* 173: 510-520. (doi: 10.1006/dbio.1996.0044).

Objectives/Objetivos

OBJECTIVES

The main objective of this thesis was the study of sperm subpopulations that have previously been selected by different methods: ***in vitro*** (Percoll gradients in Chapter 1, oviductal cells culture in Chapter 2 and by combining both in Chapter 3), ***ex vivo*** (in oviductal explants, Chapter 2) and ***in vivo*** (in the oviduct of the sow after insemination, Chapter 2), and the relationship with the functionality, sperm capacitation status and their fertilizing capacity.

To achieve the main objective the following specific objectives were established:

CHAPTER 1

Objective 1. Characterization of sperm populations selected through three different discontinuous Percoll gradients (45/60, 60/75 and 45/90%).

1.1 Analysis of different sperm function parameters: morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation and intracellular calcium concentration.

1.2 Evaluation of the sperm penetrating capacity using an *in vitro* fertilization system.

CHAPTER 2

Objective 2. Comparison of sperm capacitation status (by evaluation of protein tyrosine phosphorylation) in sperm selected by oviductal epithelial cells (bound and unbound sperm) under three different experimental conditions.

2.1 Sperm incubated with oviductal epithelial cells culture: "*in vitro* model".

2.2 Sperm incubated into oviductal explants: "*ex vivo* model".

2.3 Sperm found in the oviduct of the sow after insemination: "i*n vivo* model".

CHAPTER 3

Objective 3. Evaluation of sperm characteristics of sperm selected by Percoll gradients after their incubation with oviductal epithelial cells (bound *vs* unbound sperm).

3.1 Evaluation of their ability to fertilization purposes in an *in vitro* fertilization system.

3.2 Assessment of sperm functionality (tyrosine phosphorylation, plasma membrane phosphatidylserine translocation, DNA fragmentation and chromatin condensation).

OBJETIVOS

El objetivo principal de esta tesis es el estudio de las subpoblaciones espermáticas previamente seleccionadas a través de métodos ***in vitro*** (gradientes de Percoll-Capítulo 1, mediante cultivo de células oviductales-Capítulo 2 y por la combinación de ambos-Capítulo 3), ***ex vivo*** (en explantes oviductales-Capítulo 2) e ***in vivo*** (en el oviducto de la cerda tras una inseminación-Capítulo 2), y su relación con la funcionalidad y estado de capacitación espermática, así como su capacidad fecundante.

Para alcanzar el objetivo principal se establecieron los siguientes objetivos específicos:

CAPÍTULO 1

Objetivo 1. Caracterización de las poblaciones espermáticas seleccionadas a través de tres gradientes discontinuos de Percoll (45/60, 60/75 y 45/90%).

1.1 Análisis de los parámetros espermáticos funcionales: morfología, estado del acrosoma, motilidad y parámetros mótiles, generación de ROS, condensación de la cromatina, fragmentación del ADN, fosforilación de la tirosina y concentración de calcio intracelular.

1.2 Evaluación de la capacidad de penetración espermática en un sistema de fecundación *in vitro*.

CAPÍTULO 2

Objetivo 2. Comparación del estado de capacitación (a través de la evaluación de los patrones de fosforilación de tirosina) en espermatozoides seleccionados por las células epiteliales (espermatozoides unidos a las células o no unidos) bajo tres condiciones experimentales.

2.1: Espermatozoides incubados sobre cultivos de células epiteliales del oviducto: "modelo *in vitro*".

2.2: Espermatozoides incubados en explantes de oviducto: "modelo *ex vivo*".

2.3: Espermatozoides localizados en el oviducto tras una inseminación artificial: "modelo *in vivo*"

CAPÍTULO 3

Objetivo 3. Evaluación de las características de espermatozoides seleccionados a través de gradientes de Percoll, después de su incubación sobre cultivos de células epiteliales (espermatozoides unidos a las células o no unidos).

3.1 Evaluación de su capacidad para fecundar en un sistema de fecundación *in vitro*.

3.2 Evaluación de la funcionalidad espermática (fosforilación de la tirosina, translocación de la fosfatidilserina en la membrana plasmática, fragmentación del ADN y condensación de la cromatina).

Chapters

CHAPTER 1

Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function

ABSTRACT

In this study, different combinations of 2-step, discontinuous gradient centrifugation were used, consisting of three different combinations of isotonic Percoll (45/60, 60/75 and 45/90%) that allowed us to select different sperm subpopulations from fertile and normozoospermic boars. Our objective in this study is to evaluate the effects of centrifugation through three different discontinuous Percoll gradients on sperm function parameters (morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation and intracellular calcium concentration) and the sperm penetrating capacity in an IVF system. All the Percoll treatments evaluated increased the percentage of spermatozoa with normal morphology, the proportion of undamaged DNA, normal chromatin condensation, motion parameters measured by CASA and the percentage of capacitated spermatozoa with tyrosine phosphorylated proteins compared to control group. Finally, the *in vitro* oocyte penetrating capacity of boar spermatozoa was significantly affected by Percoll centrifugation. All the Percoll treatments increased the penetration rates and mean number of sperm per penetrated oocyte. Despite the efficiency of all three of the sperm treatments tested in selecting spermatozoa with improved sperm parameters and capacity to penetrate oocytes *in vitro*, the optimum performance of this system was demonstrated after preselecting spermatozoa by centrifugation on a discontinuous 45/90 Percoll gradient. The P45/90 treatment leads to obtain a higher percentage of spermatozoa which develop properly the capacitation process as it was shown measuring tyrosine phosphorylation and intracellular calcium concentration.

1. INTRODUCTION

Porcine semen is composed of a heterogeneous population of sperm with varying degrees of structural and functional differentiation and normality (Thurston *et al.*, 2001; Quintero-Moreno *et al.*, 2004; Holt, 2009). In order to enhance the sperm parameters of ejaculates with low sperm quality or to optimize porcine IVF, sperm selection methods have been used to isolate sperm subpopulations with high fertilizing capacity for use in animal breeding (Rodriguez-Martinez *et al.*, 1997; Morrell, 2006). According to Henkel & Schill (2003), the ideal sperm separation technique should i) be quick, easy and cost-effective, ii) isolate as many motile spermatozoa as possible, iii) not cause sperm damage or non-physiological alterations of the separated sperm cells, iv) eliminate dead spermatozoa and other cells, including leukocytes and bacteria, v) eliminate toxic or bioactive substances such as decapacitation factors or reactive oxygen species (ROS), and vi) allow processing of larger volumes of ejaculates. Among these separation methods in pigs are swim-up (Berger & Parker, 1989), Percoll discontinuous gradient (PDG) (Berger & Horton, 1988; Berger & Parker, 1989; Mattioli *et al.*, 1989; Grant *et al.*, 1994; Jeong & Yang, 2001; Matás *et al.*, 2003; Suzuki & Nagai, 2003) and other colloid gradients (Morrell *et al.*, 2009), Sephadex gel filtration (Bussalleu *et al.*, 2009) and glass wool filtration (Berger & Horton, 1988). Gradient density centrifugation is the method most frequently used because it is reported to meet many of these requirements (Morrell, 2006).

Percoll solution consists of silica beads coated with polyvinylpyrrolidone (PVP) to protect the cells from the toxic action of the silica, used for the purification of cells and isolation of subcellular particles (Pertoft, 2000). PDG has been used for separating spermatozoa from different species (Pertoft, 2000) but the technique was withdrawn from human clinical use in 1996, because it could damage spermatozoa (Biotech, 1996).

Since the pioneering studies in the late 1980s in porcine IVF (Berger & Horton, 1988; Berger & Parker, 1989; Mattioli *et al.*, 1989), PDG has been used for the selection of different kinds of boar spermatozoa (frozen-thawed, ejaculated or epidydimal) (Grant *et al.*, 1994; Jeong & Yang, 2001; Matás *et al.*, 2003, 2010; Suzuki & Nagai, 2003).

Percoll has been used for the selection of several sperm subpopulations from normozoospermic human seminal samples (Gomez *et al.*, 1996; Buffone *et al.*, 2004). However, a lack of information is related to pig spermatozoa. In the literature different conditions for PDG with different Percoll concentrations, number of layers and centrifugation regimen are described. However, no data exist on the effect of such PDG on sperm functional parameters of fertile, normozoospermic boars.

Our objective in this study was to evaluate the effects of centrifugation through three different discontinuous Percoll gradients on sperm function (morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation and intracellular calcium concentration). Additionally, fertilization *in vitro* provides information on gamete interaction and the ability of sperm to bind and fertilize homologous oocytes. IVF therefore gives both qualitative and quantitative information on sperm functionality that cannot be tested by these other laboratory tests. Our study therefore aimed at including IVF as an important supplement to those.

2. MATERIALS AND METHODS

2.1. Ethics

This study was developed following institutional approval from the University of Murcia, and it was performed in accordance with the Animal Welfare regulations of that institution.

2.2. Reagents

All chemicals were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain) unless otherwise indicated.

2.3. Sperm collection and semen handling

Semen was collected from mature fertile boars (2-4 years old) from an Artificial Insemination Centre using the manual method and a dummy (King & Macpherson, 1973). We evaluated 12 ejaculates from 6 different boars. The sperm-rich fraction was collected in a pre-warmed thermos, while the gel fraction was held on a gauze tissue covering the thermos opening. The semen was then diluted 1:2 with isothermal Beltsville Thawing Solution (BTS) extender (Pursel & Johnson, 1975) and transported to the laboratory. Once in the laboratory, the sample was split in four fractions and added to the different groups (control and 3 Percoll treatments).

The Percoll-group involved layering a 0.5 ml aliquot of spermatozoa on a discontinuous 45 and 60%, 60 and 75% or 45 and 90% (v/v) Percoll (Pharmacia, Uppsala, Sweden) gradient (Buffone *et al.*, 2004). For Percoll 45/90 (P45/90), in a 12 ml conic centrifuge tube, 2 ml of 45% Percoll was layered on top of 2 ml of 90% Percoll. Finally, 0.5 ml diluted semen was added, with care taken to avoid mixing the solutions (Matás *et al.*, 2003). The same procedure was used for Percoll 60/75 (P60/75) and Percoll 45/60 (P45/60). The three experimental groups were then centrifuged at 700 *g* for 30 minutes. The sperm pellet was resuspended in TALP medium (10 ml; Rath *et al.*, 1999)

previously pre-equilibrated at 38.5°C in 5% CO_2 in 100% humidified air and washed by centrifugation at 700 *g* for 10 minutes. For the control group, the spermatozoa were directly diluted in TALP medium and finally sperm concentration adjusted to 2×10^8 spermatozoa/ml in all experimental groups.

Seminal parameters were evaluated immediately after sperm treatment: morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation, intracellular calcium concentration and *in vitro* oocyte penetrating capacity.

2.4. Morphologic and acrosome sperm evaluation

Wet mounts of semen fixed in buffered 2% glutaraldehyde solution were examined under a phase-contrast microscope (1000× magnification, Leica, Wetzlar, Germany) to analyse morphology and acrosomes (Pursel *et al.*, 1972). The proportion of spermatozoa with a normal apical ridge (NAR) was determined on two slides per sample and a total of 200 spermatozoa per slide. Four hundred spermatozoa were categorized according to sperm morphology into those with normal morphology, cells with attached cytoplasmic droplets, tail defects (folded tail, coiled tail) and others (abnormal heads, etc.) (Gadea *et al.*, 2004).

2.5. Analysis of motion parameters

Motion parameters were determined using a CASA system (ISAS®, Proiser, Valencia, Spain). The CASA-derived motility characteristics studied were total motility (%), progressive motility (%), 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,%), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL,%), amplitude of lateral head displacement (ALH, µm) and beat cross-frequency (BCF, Hz). A 7 µl drop of the sample was placed on a warmed (37°C) slide and covered with a 24 mm × 24 mm cover slip.

The setting parameters were: 100 frames in which spermatozoa had to be present in at least 15 in order to be counted, 50 images per second. Images were obtained at 200× magnification using a negative contrast phase objective (Nikon, Tokyo, Japan). Spermatozoa with a VAP <10 µm/s were considered immotile. A minimum of five fields per sample was evaluated, counting a minimum of 200 spermatozoa per sub-sample. For statistical analysis the mean measurements of CASA for each field were entered into the ANOVA model.

2.6. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analysed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

Production of reactive oxygen species

Production of ROS was measured by incubating the spermatozoa in TALP medium in the presence of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (0.5 µm) for 60 minutes at 37°C (Gadea *et al.*, 2005a). This dye is a fluorogenic probe commonly used to detect cellular ROS production. H_2DCFDA is a stable cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescin upon oxidation. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter. Measurements were expressed as mean green intensity fluorescence units (mean channel in the FL1) and this was used as an index of ROS generation.

Determination of chromatin condensation

Sperm chromatin was stained with propidium iodide (PI) for the determination of sperm chromatin condensation (Gadea *et al.*, 2005b). Sperm samples were centrifuged (1200 *g*, 3 min) and the pellet was resuspended in a solution of ethanol and phosphate buffered saline (PBS) (70/30, v/v) for 30 min for sperm membrane permeabilization and stored at −20°C until analysis. After thawing, the samples were centrifuged, the supernatant was discarded and the pellet was resuspended in a PI solution (10 mg/ml) in PBS. Samples were maintained in darkness for 1 hour before the flow cytometric analysis. Red PI fluorescence was collected with a FL3 sensor using a 650 nm band-pass filter. Measurements were expressed as mean red intensity fluorescence units (mean channel in the FL3) and this was used as an index of the state of the chromatin condensation, as this is directly related to the PI uptake by DNA.

Evaluation of sperm DNA fragmentation by TUNEL

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining was used to determine sperm DNA fragmentation according to the method described previously (Gadea *et al.*, 2008). In brief, the cells were concentrated by centrifugation, fixed in a solution of ethanol and PBS (70/30, v/v) for 30 minutes for sperm membrane permeabilization, and stored at −20°C. Cells (approximately 1×10^6) were washed twice with PBS and resuspended in 50 µl terminal

deoxynucleotidyltransferase (TdT) reaction buffer containing: 10 µl 5× concentrated buffer solution, 1 µl (15 units) TdT, 0.25 µl (0.25 nmol) fluorescein-dUTP (BODIPY@-FL-X-14-dUTP, Invitrogen SA, Barcelona, Spain) and 39 µl distilled water.

Negative controls were incubated in fluorescein-dUTP in the absence of enzyme terminal transferase. The cells were incubated with the reaction buffer for 60 minutes at 37°C, then rinsed twice and measured by flow cytometry. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter and two populations were determined. The cells with fragmented DNA presented an intense green nuclear fluorescence, in comparison with spermatozoa with non-fragmented DNA which presented low green fluorescence. Measurements were expressed as the percentage of cells with high green intensity fluorescence and this was used as an index of the DNA fragmentation, as it is directly related to the dUTP uptake by DNA.

2.7. Localization of proteins phosphorylated in tyrosine residues

Immunofluorescence was employed to determine the localization of proteins phosphorylated in tyrosine residues (Tardif *et al.*, 2001). Sperm from the different Percoll and control groups were washed with PBS and centrifuged at 270 *g* for 10 minutes. Spermatozoa were fixed in 2% formaldehyde solution for 60 minutes at 4°C. Spermatozoa were washed once in PBS and blocked with 2% (w/v) BSA-PBS overnight at 4°C before addition of primary antibody. The sperm were washed and resuspended in PBS, smeared onto a microscope slide, and allowed to air dry. Slides were then incubated for 1 hour with anti-phosphotyrosine monoclonal antibody at 4°C (clone 4G10, 1:200, Millipore, Madrid, Spain) rinsed with PBS, and incubated for an additional 1 hour with fluorescein-conjugated goat anti-mouse antibodies (1:400, Bio-Rad Laboratories, Madrid, Spain). After rinsing with PBS, samples were mounted on the slides with 90% glycerol/PBS (v/v). Sperm were observed with a Nikon microscope equipped with fluorescent optics (excitation 450-490 nm: B2-A filter, 4003) for anti-phosphotyrosine antibody labelling.

Two hundred spermatozoa per slide and two 2 slides per sample were classified into four groups according to the localization of the anti-phosphotyrosine monoclonal antibody signal (Figure 1): i) no signal of fluorescence; ii) fluorescent signal only presents in the equatorial subsegment, triangular in appearance (Jones *et al.*, 2008), iii) signal in equatorial segment and in acrosome area or/and tail and iv) signal only in acrosome or/and tail.

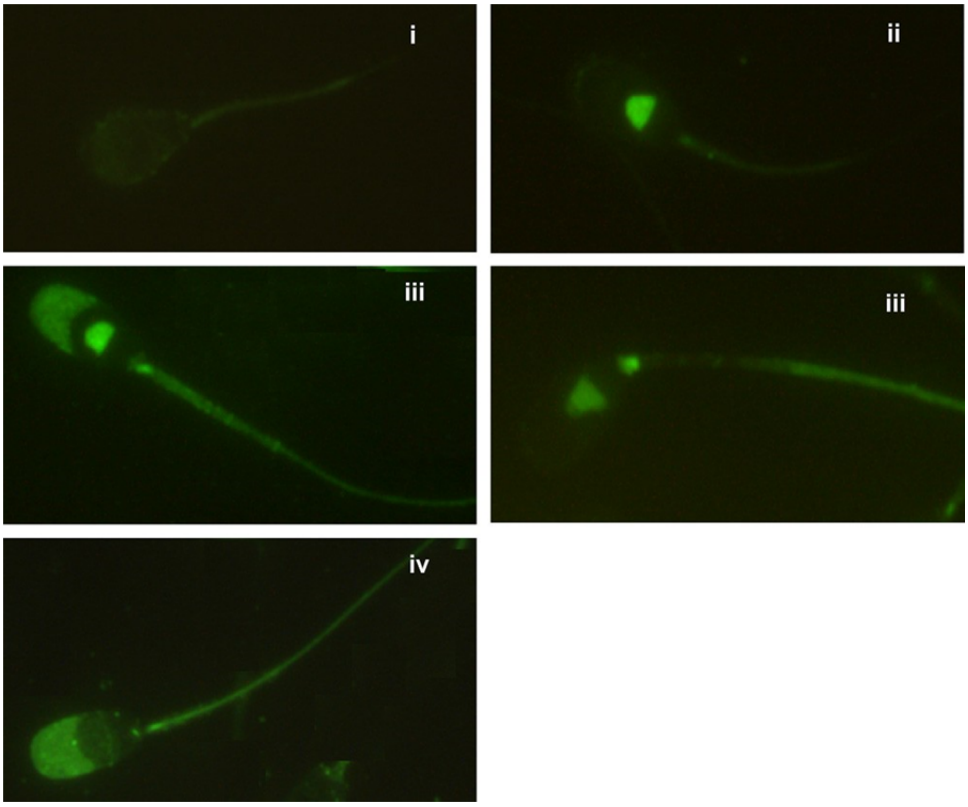

Figure 1. Boar spermatozoa classified into four groups according to the localization of the anti-phosphotyrosine monoclonal antibody signal: **i**) no signal, **ii**) signal only in the equatorial subsegment, triangular in appearance, **iii**) signal in equatorial segment and in acrosome area and/or tail and **iv**) signal only in acrosome and/or tail.

2.8. Determination of intracellular calcium concentration

Calcium concentration was measured according to the method reported previously (Tardif *et al.*, 2003; Matás *et al.*, 2010). To measure intracellular free Ca^{2+}, spermatozoa were incubated with 2.5 µM fura-2/AM in a buffer medium consisting of 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose, and 1 mM pyruvate for 45 minutes at 37°C. The extracellular unloaded fura-2 was removed by centrifugation (700 *g*, 5 min). Washed sperm were resuspended in the same buffer to a concentration of 3×10^8 cells/ml and incubated at 37°C for 15 minutes in dark. Then, spermatozoa were centrifuged (700 *g*, 5 min) and resuspended in TALP medium. As a negative control, spermatozoa were also resuspended in PBS media without calcium (control PBS). Fluorescence was monitored using Jasco FP-6300 spectrofluorometer (Jasco, Madrid, Spain) for a further 30 minutes. Excitation wavelengths alternated between 340 and 380 nm with emission held at 510 nm. At the end of the experiments, sperm were lysed with Triton X-100 (0.5%), and then calcium was depleted by addition of 25 mM EGTA. Intracellular Ca^{2+} concentration $[Ca^{2+}]i$ was calculated as previously described (Grynkiewicz *et al.*, 1985).

The equation used for calculation was as follows: $[Ca^{2+}]i = K_d \times (R - R_{min}/R_{max} - R) \times S_f/S_b$ where R indicates the fluorescence ratio 340/380 nm λ; R_{min} indicates the minimum values of fluorescence; R_{max} indicates the maximal fluorescence values; S_f indicates the fluorescence intensity in the Ca^{2+}-free medium (excitation 380 nm λ); S_b indicates the fluorescence intensity under Ca^{2+} saturation conditions and K_d (224 nmol) is the Ca^{2+} constant dissociation for Fura-2AM. For the statistical analysis the calcium concentration (nM/l) was recorded t from 0 to 1800 s and every 30 s for every experimental group and replicate. Finally the mean value during the incubation period was calculated.

2.9. *In vitro* maturation and fertilization

Oocyte collection and in vitro maturation

Within 30 minutes of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline (0.9% (w/v) NaCl) containing 100 µg/ml kanamycin sulphate at 37°C, washed once in 0.04% (w/v) cetrimide solution and then twice in saline. C*umulus-oocyte* complexes were collected from non-atretic follicles (3-6 mm diameter), washed twice in DPBS supplemented with 4 mg/ml polyvinyl alcohol (PVA), and twice more in maturation medium previously equilibrated for at least 3 hours at 38.5°C under 5% CO_2 in 100% humidified air. Only oocytes harvested within 2 hours of slaughter with a complete, dense *cumulus oophorus* were matured (Matás *et al.*, 1996). The medium used for oocyte maturation was NCSU-37 (Petters & Wells, 1993); supplemented with 0.57 mmol/l cysteine, 1 mmol/l dibutyryl cAMP, 5 µg/ml insulin, 50 µmol/l β-mercaptoethanol, 10 IU/ml equine chorionic gonadotrophin (Foligon, Intervet International BV, Boxmeer), 10 IU/ml human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, Barcelona, Spain), and 10% (v/v) pig follicular fluid. Groups of 50 oocytes were cultured in 500 µl maturation medium for 20-22 hours at 38.5°C and 5% CO_2 in air. Once cultured, the oocytes were washed twice, transferred to fresh maturation medium without hormones or dibutyryl cAMP, and cultured for a further 20-22 hours. Under these conditions the efficiency of oocyte maturation is higher than 90% (Coy *et al.*, 1999; Coy & Romar, 2002).

In vitro fertilization

After maturation oocytes were mechanically stripped of *cumulus* by gentle aspiration with a pipette. Denuded oocytes were washed three times in TALP medium and groups of 25-30 oocytes transferred to each well of four-well Nunc multidishes (Nunc, Roskilde, Denmark) containing 250 µl TALP medium previously equilibrated at 38.5°C under 5% CO_2 (Matás *et al.*, 2003, 2010). Sperm suspensions (250 µl) from each treatment group were added to the fertilization wells to obtain a final concentration of 1×10^5 cells/ml. At 18-20 hours post-insemination, putative zygotes were fixed for 30

minutes (0.5% glutaraldehyde in PBS), stained for 15 minutes (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/ml polyvinylpyrrolidone, mounted on glass slides and examined under an epifluorescence microscope at 400× magnification for evidence of sperm penetration. Penetration rate and mean number of spermatozoa per penetrated oocyte were assessed for each group (Gadea *et al.*, 1998; Matás *et al.*, 2007).

2.10. Statistical analysis

Data are expressed as the mean ± S.E.M. and analysed by ANOVA, considering the specific sperm treatment as the main variable and ejaculate as covariable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison *post-hoc* test (Tukey). Differences were considered statistically significant at $p < 0.05$. *In vitro* penetration values (categorical data) were modeled according to the binomial model of parameters by arcsine transformation of the data and were analysed by ANOVA. Pearson correlation index was calculated for chromatin condensation and TUNEL assay values.

3. RESULTS

3.1. Sperm morphology and acrosome status

Centrifugation in a discontinuous Percoll gradient 45/90 increased the percentage of spermatozoa with normal morphology to values higher than 95% from the 82% in the control group ($p < 0.01$, Table 1). The reduction in morphological abnormalities achieved by density centrifugation was based on a reduction in the percentage of spermatozoa with cytoplasmic droplets and tail defects. However, the use of P45/60 and P60/75 was not efficient enough to increase the percentage of spermatozoa with normal-morphology. On the other hand, the acrosome status evaluated as NAR was not affected by the Percoll treatment (ranged from 84 to 88.80%, $p = 0.73$, Table 1).

Table 1. Percentages of morphologically normal sperm, sperm with abnormal morphology and normal apical ridge (NAR) recovered from different Percoll discontinuous gradient systems (mean values ± S.E.M.).

Treatment	Normal (%)	Cytoplasmic droplets (%)	Altered tail (%)	Other abnormalities (%)	NAR (%)
Control	82.35 ± 2.79^{a}	11.25 ± 1.96^{a}	6.30 ± 2.09^{a}	0.10 ± 0.07	88.80 ± 2.85
P45/60	80.75 ± 2.97^{a}	13.50 ± 2.33^{a}	5.60 ± 1.77^{ab}	0.15 ± 0.11	84.00 ± 2.92
P60/75	87.35 ± 2.34^{a}	8.60 ± 1.81^{ab}	3.70 ± 1.37^{ab}	0.35 ± 0.26	87.40 ± 4.46
P45/90	96.25 ± 0.89^{b}	3.30 ± 0.80^{b}	0.45 ± 0.17^{b}	0.00 ± 0.00	88.80 ± 3.22
p-Value	<0.01	<0.01	0.04	0.40	0.73

Different letters (a, b) in the same column indicate significant differences ($p < 0.05$).

3.2. Motility and motion parameters

The percentage of total sperm motility was not affected by the selection by Percoll density gradient (ranged from 60.62 to 67.53%, p = 0.06, Table 2). However, progressive motility was higher for the spermatozoa selected by P60/75 than control (62.71% *vs* 53.45%, p = 0.03, Table 2), while progressive motility for P45/60 and P45/90 offered intermediate values. All the motion parameters analysed (velocity, straightness, linearity, etc.) were increased after Percoll treatment compared to the control group (p <0.01, Table 2) without differences between the Percoll groups, except for VSL which was higher for P60/75 than P45/60 and P45/90 (p <0.05, Table 2).

Table 2. Motility parameters measure by CASA obtained from sperm recovered from different Percoll discontinuous gradient systems (mean values ± S.E.M.).

Treatment	Motility (%)	Progressive motility (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)
Control	65.65 ± 1.86	53.45 ± 2.36^{a}	59.66 ± 2.73^{a}	33.85 ± 2.71^{a}	43.57 ± 2.64^{a}
P45/60	62.50 ± 2.04	58.56 ± 2.59^{ab}	86.79 ± 2.70^{b}	61.42 ± 2.89^{bc}	71.26 ± 2.74^{b}
P60/75	67.53 ± 1.75	62.71 ± 2.23^{b}	89.39 ± 2.45^{b}	67.51 ± 2.47^{c}	76.05 ± 2.44^{b}
P45/90	60.62 ± 2.10	56.98 ± 2.67^{ab}	82.97 ± 3.15^{b}	57.98 ± 2.82^{b}	67.18 ± 2.80^{b}
p-Value	0.06	0.03	<0.01	<0.01	<0.01

Treatment	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Control	50.79 ± 2.21^{a}	68.08 ± 1.84^{a}	69.36 ± 1.59^{a}	1.32 ± 0.04^{a}	11.41 ± 0.39^{a}
P45/60	67.00 ± 2.11^{b}	79.57 ± 1.62^{b}	80.00 ± 1.54^{b}	1.48 ± 0.05^{b}	15.23 ± 0.41^{b}
P60/75	71.01 ± 1.27^{b}	82.68 ± 0.81^{b}	82.37 ± 0.93^{b}	1.45 ± 0.03^{b}	14.76 ± 0.26^{b}
P45/90	65.99 ± 1.84^{b}	80.01 ± 1.26^{b}	78.51 ± 1.29^{b}	1.51 ± 0.05^{b}	14.59 ± 0.47^{b}
p-Value	<0.01	<0.01	<0.01	0.01	<0.01

VCL: curvilinear velocity; **VSL**: straight-line velocity; **VAP**: average path velocity; **LIN**: linearity of the curvilinear trajectory (VSL/VCL); **STR**: straightness (VSL/VAP); **WOB**: wobble of the curvilinear trajectory (VAP/VCL); **ALH**: amplitude of lateral head displacement; **BCF**: beat cross-frequency.
Different letters (a, b, c) in the same column indicate significant differences (p <0.05).

3.3. ROS generation, chromatin condensation and DNA fragmentation

Measurements of the ROS generated revealed higher values for sperm treated by Percoll 45/60 than control (p <0.01, Table 3) while no differences were found for the other Percoll groups (P60/75 and P45/90) compared to control (p >0.05, Table 3).

Sperm chromatin condensation was affected by the Percoll treatments; the chromatin after Percoll treatments was less condensed than control, with higher values of fluorescence units that correspond with higher IP uptake measured by flow cytometry

(p <0.01, Table 3). At the same time, the Percoll treatment reduced the percentage of spermatozoa with fragmented DNA (p <0.01, Table 3). When we studied the relation between DNA fragmentation and chromatin condensation we observed a significant relation between these parameters (r = −0.63, p <0.01), the samples with highest chromatin condensation showed the highest DNA fragmentation.

Table 3. ROS generation, chromatin decondensation and DNA fragmentation (TUNEL) obtained from sperm recovered from different Percoll discontinuous gradient systems.

Treatment	ROS generation (UF)	Chromatin decondensation (UF)	DNA fragmentation (%)
Control	21.28 ± 0.85^{a}	13.83 ± 0.64^{a}	5.26 ± 0.31^{a}
P45/60	27.63 ± 1.86^{b}	22.82 ± 0.84^{b}	2.97 ± 0.11^{b}
P60/75	25.09 ± 1.71^{ab}	21.66 ± 0.75^{b}	3.08 ± 0.25^{b}
P45/90	21.98 ± 1.27^{a}	21.57 ± 0.79^{b}	2.44 ± 0.31^{b}
p-Value	<0.01	<0.01	<0.01

UF: arbitrary units of fluorescence.
Different letters (a, b) in the same column indicate significant differences (p <0.05).

3.4. Tyrosine protein phosphorylation

Indirect phosphotyrosine immunolocalization showed that the treatment by Percoll increased the percentage of cells with phosphorylation of tyrosine, used as an index of capacitation (p <0.01, Table 4). So, while in the control group most of the spermatozoa (85.50%) have not undergone the tyrosine phosphorylation, the P45/60 and the P60/75 groups displayed phosphorylation in 80-90% of the spermatozoa and finally in P45/90 group more than 95% of the spermatozoa showed phosphotyrosine signal (Table 4). The pattern of staining is also different in the P45/90 group with a higher proportion of cells with signal in equatorial region, acrosome and tail compared to the P45/60 and P60/75 groups which show a similar proportion of only equatorial segment staining (Table 4).

Table 4. Percentage of sperm with phosphotyrosine immunoreactive signal obtained from sperm recovered from different Percoll discontinuous gradient systems (mean values ± S.E.M.).

Treatment	No signal (%)	Only equatorial segment (%)	Equatorial segment and acrosome and/or tail (%)	Acrosome or/and tail (%)
Control	85.50 ± 2.66^{a}	7.25 ± 2.28^{a}	2.75 ± 1.06^{a}	4.50 ± 1.95^{a}
P45/60	19.50 ± 5.01^{b}	46.80 ± 3.00^{b}	32.90 ± 5.63^{b}	0.80 ± 0.21^{b}
P60/75	7.70 ± 3.43^{bc}	51.75 ± 5.95^{b}	40.00 ± 6.65^{b}	0.55 ± 0.19^{b}
P45/90	3.15 ± 1.15^{c}	20.05 ± 4.99^{a}	75.40 ± 6.05^{c}	1.40 ± 0.44^{b}
p-Value	<0.01	<0.01	<0.01	0.01

Different letters (a, b, c) in the same column indicate significant differences (p <0.05).

3.5. Intracellular calcium concentration

Since intracellular Ca^{2+} influx is one of the crucial biochemical events occurring during capacitation, this experiment was performed to study changes in Ca^{2+} uptake between sperm groups. Calcium intake by the sperm increased through the incubation time (30 min) in the presence of TALP medium, while incubation in a calcium-free medium (PBS), used as negative control, resulted in a constant calcium concentration at a basal level during the time of study (mean value = 84.26 ± 7.52 nM/l; Figure 2). Treatment with Percoll affected the calcium concentration ($p < 0.01$) (Figure 2). P45/90 induced the highest values in calcium concentration (mean value = 658.72 ± 65.27 nM/l), values significantly higher ($p < 0.01$) than in the control TALP group (mean value = 276.14 ± 16.68 nM/l), P45/60 group (299.26 ± 21.20 nM/l) and P60/75 group (341.96 ± 23.53) without differences between these last three groups.

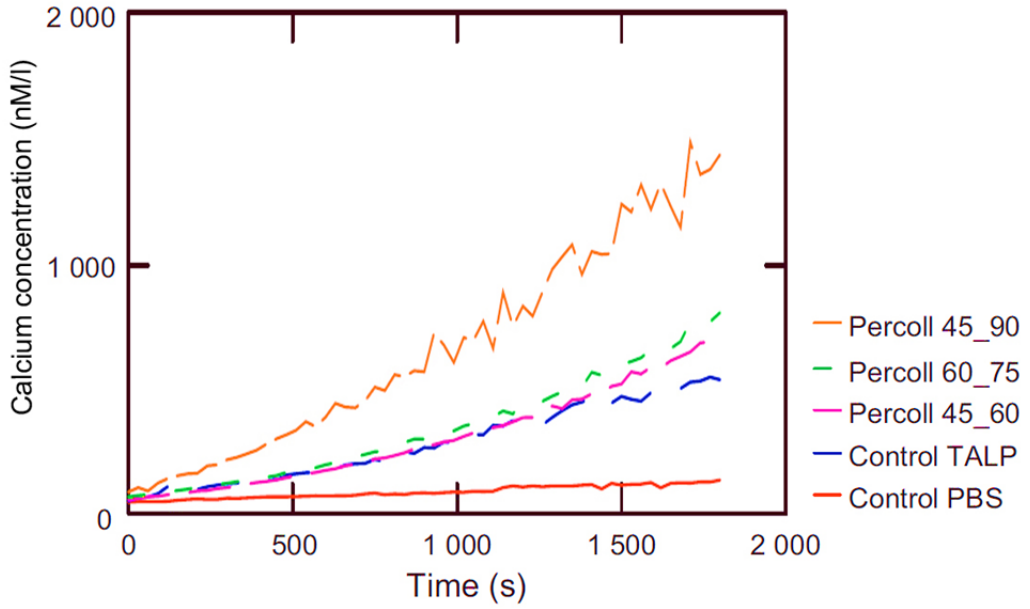

Figure 2. Calcium concentration (nM/l) in boar spermatozoa recovered from different Percoll discontinuous gradient systems and diluted in TALP medium. Fluorescence was measured by Fura-2 and monitored using spectrofluorimeter for 60 minutes.

3.6. *In vitro* oocyte penetrating capacity

The *in vitro* fertilizing capacity of boar sperm was significantly affected by Percoll centrifugation. All the Percoll treatments increased the penetration rates and mean number of sperm per penetrated oocyte ($p < 0.01$, Table 5). The penetration rate increased from 50% in control group to 87% for P45/90 and P60/75 groups, and the highest value in penetration rate was for P45/90 group, at close to 100% (Table 5). The mean number of penetrated sperm per oocyte was affected by Percoll treatment in a similar way to penetration rate (Table 5). However, in all the groups male pronuclear formation was near to 100% without differences between groups.

Table 5. IVF results obtained from sperm recovered from different Percoll discontinuous gradient systems (mean values ± S.E.M.).

Treatment	N	PEN (%)	SPZ/O*
Control	205	49.76 ± 3.50[a]	1.78 ± 0.11[a]
P45/60	246	87.40 ± 2.12[b]	3.20 ± 0.16[b]
P60/75	158	87.34 ± 2.65[b]	4.17 ± 0.20[c]
P45/90	204	98.04 ± 0.97[c]	4.31 ± 0.20[c]
p-Value		<0.01	<0.01

N: number of inseminated oocytes; **PEN**: rate of penetration; **SPZ/O**: mean number of penetrated sperm per oocyte.

*Based on penetrated oocytes.

Different letters (a, b, c) in the same column indicate significant differences (p <0.05).

4. DISCUSSION

Under *in vivo* conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus. During this process, not only are progressively motile spermatozoa selected but male germ cells also undergo physiological changes in a process called capacitation, which is a fundamental prerequisite for the sperm's functional competence with regard to acrosome reaction (Bedford, 1983; Yanagimachi, 1998).

Under *in vitro* conditions, fractionation of sperm by density gradient centrifugation can separate these subpopulations, resulting in a considerable improvement in the quality of sperm recovered in the pellet. Several reports indicate that higher percentages of motile and morphologically normal human sperm can be recovered from the pellet, in comparison with lower density fractions (Menkveld *et al.*, 1990; Mortimer & Mortimer, 1992; Chen & Bongso, 1999; Ollero *et al.*, 2000). Furthermore, sperm collected from the pellet exhibit less DNA damage (Larson *et al.*, 1999; Sakkas *et al.*, 1999) and produce less reactive oxygen species (ROS) than those recovered from the lower density layers, where abnormal sperm are predominantly found (Ollero *et al.*, 2001). However in boar spermatozoa there is a lack of information about these sperm parameters after Percoll centrifugation and how the Percoll gradient conditions could affect the sperm parameters. In this study, we used different combinations of isotonic Percoll (P45/60, 60/75 and 45/90%) from fertile and normozoospermic boars that led us to select different sperm subpopulations that could be characterized by different sperm parameters.

In relation to sperm morphology, in human spermatozoa, Percoll gradient centrifugation reduces the percentage of morphological alterations, particularly when teratospermia is not severe (Hall *et al.*, 1995). In boar sperm, Percoll density gradient has been used to isolate sperm subpopulations with cytoplasmic droplet (low-density) from spermatozoa without this droplet (higher density) (de Vries & Colenbrander, 1990). On the contrary, a reduction in sperm abnormalities other than cytoplasmic droplets, such as tail loops, after Percoll 35/70 has been previously reported (Waberski *et al.*, 2006). Under our experimental conditions only Percoll 45/90 is able to induce a significant reduction in both kinds of morphoanomalies, results that are in agreement with those reported in humans (Buffone *et al.*, 2004). Analysis of the NAR showed that just after Percoll centrifugation no changes were detected in acrosome status, in agreement with data obtained previously (Grant *et al.*, 1994), although when frozen-thawed spermatozoa were used and showed severe acrosome damage the use of Percoll centrifugation improved the NAR status compared to swim-up selection (Zheng *et al.*, 1992).

The effect of Percoll in selecting porcine sperm with higher motility has been well documented (Berger & Parker, 1989; Horan *et al.*, 1991; Grant *et al.*, 1994; Waberski *et al.*, 2006). However, other studies have not detected significant differences. This is probably related to the high variability that could be present in some boars, differences in the Percoll density procedures (Berger & Horton, 1988; Suzuki & Nagai, 2003) or differences in the system of measurement. In this study, we used a high speed camera (50 images/s, Basler AG, Ahrensburg, Germany) and a high number of images (100 images) per analysis, to more extensively and accurately follow the sperm motility pattern unlike previous studies (camera 25 pictures/s, 25 pictures analysed). As a consequence of this setting, the ALH values are lower and BCF higher than in previous studies (Gadea *et al.*, 2005b). On the other hand, the use of a capacitating media as TALP medium (Rath *et al.*, 1999), with a high level of bicarbonate and calcium in the composition, induced an early capacitation process that implies the presence of aggregates or pairs of spermatozoa. These sperm aggregates are not well recognized by the software because the total area of the particles is higher than setup. In consequence, the percentage of total and progressive motility could be underestimated, but it did not affect the motion parameters.

Only the gradient 60/75 selected higher percentage of progressive motility spermatozoa than control and it could be related to a selective effect on motility produced by the top layer of Percoll 60% that is not done by Percoll 45%. However, the differences in the pattern of movement reported in this study showed that the Percoll selection is important to optimize recovery of a higher percentage of spermatozoa with a fast-linear movement which could be related to the capacitation process as a significant increase of tyrosine phosphorylation and calcium intake was observed. This sperm subpopulation with fast motion characteristics could be related to the fertilizing

capacity (Davis *et al.*, 1995; Quintero-Moreno *et al.*, 2004; Jedrzejczak *et al.*, 2005).

The effect of Percoll on ROS generation could be complex. Centrifugation and resuspension of the resulting pellet subject cells to mechanical forces due to close packing (Abidor *et al.*, 1994), and centrifugation could lead to sublethal damage (Alvarez *et al.*, 1993). Centrifugation can also be detrimental to the spermatozoa because of the production of a burst of reactive oxygen species by a discrete subpopulation of cells characterized by significantly diminished motility and fertilizing capacity (Aitken & Clarkson, 1988) and the elimination of the antioxidant compounds that are present in the seminal plasma. On the other hand, Percoll separation could compensate by eliminating dead cells, cells with cytoplasmic droplets and debris that generate a high proportion of ROS. These data overall suggest that the best balance is for P45/90 and the worse results are seen for P45/60, probably because it is not the most effective procedure for eliminating the damaged cells or because the packing is closer and the subsequent mechanical forces higher. This is in concordance with studies in humans that show that these fractions display higher ROS production (Aitken & Clarkson, 1988; Ollero *et al.*, 2001).

Another interesting point is the fact that capacitating spermatozoa produce controlled amounts of ROS that regulate downstream events such as an increase in cAMP, protein kinase A (PKA) activation and phosphorylation of PKA substrates (de Lamirande & O'Flaherty, 2008). It is possible that ROS generation in spermatozoa after Percoll 45/90 could be related to the capacitation process while in the other experimental groups ROS generation could be related to the presence of dead cells and cytoplasmic droplets. However this hypothesis must be confirmed in further studies.

The subpopulations of spermatozoa selected by Percoll gradient differ not only in terms of conventional criteria such as motility and morphology, but also in terms of their DNA integrity as measured in human studies by different techniques such as the SCSA (Ollero *et al.*, 2001), Comet assay (Van Kooij *et al.*, 2004) and TUNEL (Mitchell *et al.*, 2011). In humans the selection of spermatozoa by density gradient centrifugation also increases the percentage of mature spermatozoa with normal chromatin condensation compared to control (Le Lannou & Blanchard, 1988; Hammadeh *et al.*, 2001; Mitchell *et al.*, 2011). We observed that chromatin condensation and DNA fragmentation were similar among Percoll separated samples but better than in the control group, which suggests that when Percoll density is used to select motile and viable spermatozoa it indirectly selects spermatozoa with a nuclear normal structure and DNA (Mitchell *et al.*, 2011). We also reported in this study a significant relationship between chromatin condensation and DNA fragmentation, as previously studied in different species (Fraser & Strzeżek, 2007; Gadea *et al.*, 2008; Mitchell *et al.*, 2011) that suggests that abnormally condensed spermatozoa chromatin is also related with higher DNA fragmentation.

Spermatozoa washed on a Percoll gradient are partly capacitated (Berger & Parker, 1989) or have undergone the early stages of the acrosome reaction (de Maistre *et al.*, 1996). Protein tyrosine phosphorylation has been associated with the ability of normal sperm to undergo capacitation and fertilize an egg (Urner & Sakkas, 2003). The pattern of tyrosine phosphorylation in boar is different from other species where the flagellum appears to be the principal sperm compartment presenting tyrosine-phosphorylated proteins. In boar the flagellum is less important (Petrunkina *et al.*, 2001; Tardif *et al.*, 2001) and the most important area is the equatorial area, which shows a specific triangular pattern of tyrosine phosphorylated proteins (Jones *et al.*, 2008). Fluorescence is also present over the anterior acrosome, partly due to nonspecific binding of the second layer antibody and partly due to phosphoproteins within the acrosomal matrix (Tardif *et al.*, 2001; Jones *et al.*, 2008). In this study we confirmed that Percoll increases the percentage of cells with phosphorylated tyrosine and that the pattern of staining is different in the different Percoll procedures, which may play a key role in sperm-zona pellucida interaction (Flesch *et al.*, 2001). In Percoll 45/90 the percentage of cells with any kind of tyrosine phosphorylation is near to 100% and the signal extends to most of the spermatozoon (acrosome, equatorial segment and tail). In other Percoll groups (45/60 and 60/75), although the percentage of phosphorylated cells is higher than 75%, the extension of the signal is lower than P45/90. In accordance with other authors (Petrunkina *et al.*, 2001; Urner & Sakkas, 2003), we suggest that the phosphorylation-positive spermatozoa could have a competitive advantage for penetrating the oocytes. However, this fact must be confirmed in further studies, including, for example, *in vitro* production of embryos.

Changes in intracellular calcium concentrations are associated with different aspects of sperm function such as capacitation, acrosome reaction, sperm chemotaxis and hyperactivation (Bedu-Addo *et al.*, 2008; Costello *et al.*, 2009). In this study we measured calcium in spermatozoa treated by Percoll density gradient and obtained results that follow the same pattern as those obtained in the study of tyrosine-phosphorylated proteins. The higher values obtained in P45/90 group suggests that the spermatozoa completed the capacitation process properly and are more capable of proceeding with the fertilization process.

The Percoll procedure yields pure fractions of spermatozoa, whereas the washing technique produces cell pellets that contain a heterogeneous population of spermatozoa along with amorphous material and seminal plasma (Grant *et al.*, 1994). Percoll centrifugation not only selected a sperm subpopulation but also allowed some “decapacitation” factors to be removed from the sperm membrane and/or seminal plasma (Furimsky *et al.*, 2005), which could facilitate subsequent sperm capacitation and acrosome reaction (Nolan & Hammerstedt, 1997).

Several studies have previously demonstrated that Percoll centrifugation leads to selection of a subpopulation of sperm with a higher ability to penetrate oocytes *in vitro* (Berger & Horton, 1988; Berger & Parker, 1989; Grant *et al.*, 1994; Jeong & Yang, 2001; Suzuki & Nagai, 2003). Previously, we have shown that fresh spermatozoa treated with Percoll showed faster oocyte penetration and higher penetrability than control; the time of female and male pronucleus formation was affected by the sperm treatment and was faster for the Percoll group, while cleavage rate and rate of blastocyst formation were also higher after Percoll treatment (Matás *et al.*, 2003). These differences are related to changes in some important spermatozoa parameters such as motility parameters, calcium intake, ROS generation, lipid membrane disorder and acrosome reaction (Matás *et al.*, 2010). However, Percoll gradient did not improve embryo development after ICSI injection (García-Roselló *et al.*, 2006).

Among all the sperm tests that have been developed, IVF tests are the most suitable for assessing overall sperm function during fertilization. The binding and penetration of the zona pellucida is one of the most important barriers the spermatozoa must overcome in the fertilization process. Also, the interaction with the oocyte plasma membrane appears to explain much of the variability in sperm fertilizing potential (Gadea *et al.*, 2005b). We used the IVF system to evaluate the penetration capacity and assumption that an elevated number of sperm could penetrate the oocytes. As we expected, the highest values for penetration were obtained in Percoll 45/90 because all the sperm parameters were better than or equal to control. However for Percoll 45/60, where similar values for morphology, worse values for in ROS generation and better motility, chromatin condensation, DNA and capacitation than control were observed, there was a higher penetration rate than control.

In the present study, three combinations of Percoll gradient density were tested and as expected, the highest penetration rates were observed after pretreatment with Percoll 45/90. Firstly, this procedure selected the highest number of morphologically normal spermatozoa with less alteration in the chromatin structure and less DNA damage; the motion characteristics are also improved and the percentage of capacitated spermatozoa steadily increases throughout incubation. All these characteristics lead to the highest proportion of penetration of oocytes *in vitro*.

ACKNOWLEDGEMENTS

This research was supported by MEC-FEDER (AGL2009-12512-C02-01) and Fundación Séneca (08752/PI/08). Thanks to Ruth Tunn (University of Oxford) for help with the language in the text.

REFERENCES

Abidor I, Li L & Hui S (1994). Studies of cell pellets: I. Electrical properties and porosity. *Biophysical journal.* 67: 418-426. (doi: 10.1016/S0006-3495(94)80497-7).

Aitken R & Clarkson JS (1988). Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *Journal of andrology.* 9: 367-376. (doi: 10.1002/j.1939-4640.1988.tb01067.x).

Alvarez JG, Lasso JL, Blasco L, Nuñez RC, Heyner S, Caballero PP & Storey BT (1993). Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Human reproduction.* 8: 1087-1092.

Bedford J (1983). Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biology of reproduction.* 28: 108-120. (doi: 10.1095/ biolreprod28.1.108).

Bedu-Addo K, Costello S, Harper C, Machado-Oliveira G, Lefievre L, Ford C, Barratt C & Publicover S (2008). Mobilisation of stored calcium in the neck region of human sperm-a mechanism for regulation of flagellar activity. *International Journal of Developmental Biology.* 52: 615-626. (doi: 10.1387/ijdb.072535kb).

Berger T & Horton M (1988). Evaluation of assay conditions for the zona-free hamster ova bioassay of boar sperm fertility. *Gamete research.* 19: 101-111. (doi: 10.1002/mrd.1120190110).

Berger T & Parker K (1989). Modification of the zona-free hamster ova bioassay of boar sperm fertility and correlation with *in vivo* fertility. *Gamete research.* 22: 385-397. (doi: 10.1002/mrd.1120220405).

Biotech (1996). Important Notice: Percoll NOT to be Used in Assisted Reproduction Techologies in Humans. *Pharmacia Biotech Inc.*

Buffone M, Doncel G, Briggiler CM, Vazquez-Levin M & Calamera J (2004). Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation. *Human reproduction.* 19: 139-146. (doi: 10.1093/humrep/deh040).

Bussalleu E, Pinart E, Rivera M, Briz M, Sancho S, Yeste M, Casas I, Fàbrega A, Rigau T & Rodriguez-Gil J (2009). Effects of matrix filtration of low-quality boar semen doses on sperm quality. *Reproduction in Domestic Animals.* 44: 499-503. (doi: 10.1111/j.1439-0531.2008.01221.x).

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J & Publicover S (2009). Ca^{2+}-stores in sperm: their identities and functions. *Reproduction.* 138: 425-437. (doi: 10.1530/REP-09-0134).

Coy P, Ruiz S, Romar R, Campos I & Gadea J (1999). Maturation, fertilization and complete development of porcine oocytes matured under different systems. *Theriogenology.* 51: 799-812. (doi: 10.1016/S0093-691X(99)00028-X).

Coy P & Romar R (2002). *In vitro* production of pig embryos: a point of view. *Reproduction, fertility and development.* 14: 275-286. (doi: 10.1071/RD01102).

Chen MJ & Bongso A (1999). Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. *Human reproduction.* 14: 759-764. (doi: 10.1093/humrep/14.3.759).

Davis R, Drobnis E & Overstreet J (1995). Application of multivariate cluster, discriminate function, and stepwise regression analyses to variable selection and predictive modeling of sperm cryosurvival. *Fertility and sterility.* 63: 1051-1057.

de Lamirande E & O'Flaherty C (2008). Sperm activation: role of reactive oxygen species and kinases. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics.* 1784: 106-115. (doi: 10.1016/j.bbapap.2007.08.024).

de Maistre E, Bene M, Foliguet B, Touati F & Faure G (1996). Centrifugation on Percoll gradient enhances fluorescent lectin binding on human sperm: a flow cytometric analysis. *Systems Biology in Reproductive Medicine.* 37: 179-187. (doi: 10.3109/01485019608988520).

de Vries AC & Colenbrander B (1990). Isolation and characterization of boar spermatozoa with and without a cytoplasmic droplet. *International Journal of Biochemistry.* 22: 519-524. (doi: 10.1016/0020-711X(90)90267-7).

Flesch F, Wijnand E, Van de Lest C, Colenbrander B, van Golde LM & Gadella BM (2001). Capacitation dependent activation of tyrosine phosphorylation generates two sperm head plasma membrane proteins with high primary binding affinity for the zona pellucida. *Molecular reproduction and development.* 60: 107-115. (doi: 10.1002/mrd.1067).

Fraser L & Strzeżek J (2007). Is there a relationship between the chromatin status and DNA fragmentation of boar spermatozoa following freezing–thawing? *Theriogenology.* 68: 248-257. (doi: 10.1016/j.theriogenology.2007.05.001).

Furimsky A, Vuong N, Xu H, Kumarathasan P, Xu M, Weerachatyanukul W, Khalil MB, Kates M & Tanphaichitr N (2005). Percoll gradient-centrifuged capacitated mouse sperm have increased fertilizing ability and higher contents of sulfogalactosylglycerolipid and docosahexaenoic acid-containing phosphatidylcholine compared to washed capacitated mouse sperm. *Biology of reproduction.* 72: 574-583. (doi: 10.1095/ biolreprod.104.036095).

Gadea J, Matás C & Lucas X (1998). Prediction of porcine semen fertility by homologous *in vitro* penetration (hIVP) assay. *Animal Reproduction Science.* 54: 95-108. (doi: 10.1016/S0378-4320(98)00144-4).

Gadea J, Sellés E, Marco MA, Coy P, Matás C, Romar R & Ruiz S (2004). Decrease in glutathione content in boar sperm after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology.* 62: 690-701. (doi: 10.1016/j.theriogenology.2003.11.013).

Gadea J, García-Vazquez F, Matás C, Gardón JC, Cánovas S & Gumbao D (2005a). Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. *Journal of andrology.* 26: 396-404. (doi: 10.2164/jandrol.04155).

Gadea J, Gumbao D, Matás C & Romar R (2005b). Supplementation of the thawing media with reduced glutathione improves function and the *in vitro* fertilizing ability of boar spermatozoa after cryopreservation. *Journal of andrology.* 26: 749-756. (doi: 10.2164/jandrol.05057).

Gadea J, Gumbao D, Canovas S, García-Vázquez FA, Grullón LA & Gardón JC (2008). Supplementation of the dilution medium after thawing with reduced glutathione

improves function and the *in vitro* fertilizing ability of frozen-thawed bull spermatozoa. *International journal of andrology.* 31: 40-49. (doi: 10.1111/j.1365-2605.2007.00756.x.).

García-Roselló E, Matás C, Cánovas S, Moreira PN, Gadea J & Coy P (2006). Influence of sperm pretreatment on the efficiency of intracytoplasmic sperm injection in pigs. *Journal of andrology.* 27: 268-275. (doi: 10.2164/jandrol.05110).

Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS & Aitken RJ (1996). Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *Journal of andrology.* 17: 276-287. (doi: 10.1002/j.1939-4640.1996.tb01783.x).

Grant S, Long S & Parkinson T (1994). Fertilizability and structural properties of boar spermatozoa prepared by Percoll gradient centrifugation. *Journal of reproduction and fertility.* 100: 477-483. (doi: 10.1530/jrf.0.1000477).

Grynkiewicz G, Poenie M & Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry.* 260: 3440-3450.

Hall J, Fishel S, Timson J, Dowell K & Klentzeris L (1995). Andrology: human sperm morphology evaluation pre-and post-Percoll gradient centrifugation. *Human reproduction.* 10: 342-346.

Hammadeh M, Dehn C, Hippach M, Zeginiadou T, Stieber M, Georg T, Rosenbaum P & Schmidt W (2001). Comparison between computerized slow-stage and static liquid nitrogen vapour freezing methods with respect to the deleterious effect on chromatin and morphology of spermatozoa from fertile and subfertile men. *International journal of andrology.* 24: 66-72. (doi: 10.1046/j.1365-2605.2001.00270.x).

Henkel RR & Schill W-B (2003). Sperm preparation for ART. *Reproductive Biology and Endocrinology.* 1: 108. (doi: 10.1186/1477-7827-1-108).

Holt WV (2009). Is semen analysis useful to predict the odds that the sperm will meet the egg? *Reproduction in Domestic Animals.* 44:31-38. (doi: 10.1111/j.1439-0531.2009.01363.x).

Horan R, Powell R, McQuaid S, Gannon F & Houghton J (1991). Association of foreign DNA with porcine spermatozoa. *Systems Biology in Reproductive Medicine.* 26: 83-92. (doi: 10.3109/01485019108987631).

Jedrzejczak P, Rzepczynska I, Taszarek-Hauke G, Pawelczyk L, Kotwicka M & Warchot W (2005). Effect of sperm subpopulation's kinetics on human fertilization *in vitro*. *Systems Biology in Reproductive Medicine.* 51: 185-193. (doi: 10.1080/014850190898791).

Jeong BS & Yang X (2001). Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization *in vitro*. *Molecular reproduction and development.* 59: 330-335. (doi: 10.1002/mrd.1038).

Jones R, James PS, Oxley D, Coadwell J, Suzuki-Toyota F & Howes EA (2008). The equatorial subsegment in mammalian spermatozoa is enriched in tyrosine phosphorylated proteins. *Biology of reproduction.* 79: 421-431. (doi: 10.1095/biolreprod.107.067314).

King G & Macpherson J (1973). A comparison of two methods for boar semen collection. *Journal of animal science.* 36: 563-565. (doi: 10.2134/jas1973.363563x).

Larson K, Brannian J, Timm B, Jost L & Evenson D (1999). Density gradient centrifugation and glass wool filtration of semen remove spermatozoa with damaged chromatin structure. *Human reproduction.* 14: 2015-2019. (doi: 10.1093/humrep/14.8.2015).

Le Lannou D & Blanchard Y (1988). Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure. *Journal of reproduction and fertility.* 84: 551-556. (doi: 10.1530/jrf.0.0840551).

Matás C, Martinez E, Vazquez J, Roca J & Gadea J (1996). *In vitro* penetration assay of boar sperm fertility: effect of various factors on the penetrability of immature pig oocytes. *Theriogenology.* 46: 503-513. (doi: 10.1016/0093-691X(96)00172-0).

Matás C, Coy P, Romar R, Marco M, Gadea J & Ruiz S (2003). Effect of sperm preparation method on *in vitro* fertilization in pigs. *Reproduction.* 125: 133-141. (doi: 10.1530/rep.0.1250133).

Matás C, Decuadro G, Martinez-Miro S & Gadea J (2007). Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. *Theriogenology.* 67: 1087-1091. (doi: 10.1016/j.theriogenology.2006.11.010).

Matás C, Sansegundo M, Ruiz S, García-Vázquez F, Gadea J, Romar R & Coy P (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. *Theriogenology.* 74: 1327-1340. (doi: 10.1016/j.theriogenology.2010.06.002).

Mattioli M, Bacci M, Galeati G & Seren E (1989). Developmental competence of pig oocytes matured and fertilized *in vitro*. *Theriogenology.* 31: 1201-1207. (doi: 10.1016/0093-691X(89)90089-7).

Menkveld R, Swanson R, Kotze T & Kruger T (1990). Comparison of a discontinuous Percoll gradient method versus swim-up method: effects on sperm morphology and other semen parameters. *Andrologia.* 22: 152-158. (doi: 10.1111/j.1439-0272.1990.tb01957.x).

Mitchell L, De Iuliis G & Aitken RJ (2011). The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *International journal of andrology.* 34: 2-13. (doi: 10.1111/j.1365-2605.2009.01042.x).

Morrell JM (2006). Update on semen technologies for animal breeding. *Reproduction in Domestic Animals.* 41: 63-67. (doi: 10.1111/j.1439-0531.2006.00621.x).

Morrell JM, Saravia F, Van Wienen M, Wallgren M & Rodriguez-Martinez H (2009). Selection of boar spermatozoa using centrifugation on a glycidoxypropyltrimethoxysilane-coated silica colloid. *Journal of Reproduction and Development.* 55: 547-552. (doi: 10.1262/jrd.20243).

Mortimer D & Mortimer S (1992). Methods of sperm preparation for assisted reproduction. *Annals of the Academy of Medicine, Singapore.* 21: 517-524.

Nolan JP & Hammerstedt R (1997). Regulation of membrane stability and the acrosome reaction in mammalian sperm. *The FASEB journal.* 11: 670-682. (doi: 0892-6638/97/0011-0670/$01.50).

Ollero M, Powers RD & Alvarez JG (2000). Variation of docosahexaenoic acid content in subsets of human spermatozoa at different stages of maturation: implications for

sperm lipoperoxidative damage. *Molecular reproduction and development.* 55: 326-334. (doi: 10.1002/(SICI)1098-2795(200003)55:3<326::AID-MRD11>3.0.CO;2-A).

Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, Evenson D, Thomas AJ & Alvarez JG (2001). Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Human reproduction.* 16: 1912-1921. (doi: 10.1093/humrep/16.9.1912).

Pertoft H (2000). Fractionation of cells and subcellular particles with Percoll. *Journal of biochemical and biophysical methods.* 44:1-30. (doi: 10.1016/S0165-022X(00)00066-X).

Petrunkina AM, Friedrich J, Drommer W, Bicker G, Waberski D & Töpfer-Petersen E (2001). 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: 469-480. (doi: 10.1530/rep.0.1220469).

Petters R & Wells K (1993). Culture of pig embryos. *Journal of reproduction and fertility. Supplement.* 48: 61-73.

Pursel V, Johnson L & Rampacek G (1972). Acrosome morphology of boar spermatozoa incubated before cold shock. *Journal of animal science.* 34: 278-283. (doi: 10.2134/jas1972.342278x).

Pursel V & Johnson L (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *Journal of animal science.* 40: 99-102.

Quintero-Moreno A, Rigau T & Rodríguez-Gil JE (2004). Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. *Theriogenology.* 61: 673-690. (doi: 10.1016/S0093-691X(03)00248-6).

Rath D, Long C, Dobrinsky J, Welch G, Schreier L & Johnson L (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *Journal of animal science.* 77: 3346-3352. (doi: 1999.77123346x).

Rodriguez-Martinez H, Larsson B & Pertoft H (1997). Evaluation of sperm damage and techniques for sperm clean-up. *Reproduction, fertility, and development.* 9: 297-308. (doi: 10.1071/R96081).

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG & Bianchi U (1999). Origin of DNA damage in ejaculated human spermatozoa. *Reviews of reproduction.* 4: 31-37. (doi: 10.1530/ror.0.0040031).

Suzuki K & Nagai T (2003). *In vitro* fertility and motility characteristics of frozen–thawed boar epididymal spermatozoa separated by Percoll. *Theriogenology.* 60: 1481-1494. (doi: 10.1016/S0093-691X(03)00115-8).

Tardif S, Dubé C, Chevalier S & Bailey JL (2001). Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of reproduction.* 65: 784-792. (doi: 10.1095/biolreprod65.3.784).

Tardif S, Dubé C & Bailey JL (2003). Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. *Biology of reproduction.* 68: 207-213. (doi: 10.1095/ biolreprod.102.005082).

Thurston L, Watson P, Mileham A & Holt WV (2001). Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *Journal of andrology.* 22: 382-394. (doi: 10.1002/j.1939-4640.2001.tb02194.x).

Urner F & Sakkas D (2003). Protein phosphorylation in mammalian spermatozoa. *Reproduction.* 125: 17-26. (doi: 10.1530/rep.0.1250017).

Van Kooij R, De Boer P, Vreeden-Elbertse D, Ganga N, Singh N & Te Velde E (2004). The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. *International journal of andrology.* 27: 140-146. (doi: 10.1111/j.1365-2605.2004.00463.x).

Waberski D, Magnus F, Ardon F, Petrunkina AM, Weitze K & Töpfer-Petersen E (2006). Binding of boar spermatozoa to oviductal epithelium *in vitro* in relation to sperm morphology and storage time. *Reproduction.* 131: 311-318. (doi: 10.1530/rep.1.00814).

Yanagimachi R (1998). Mammalian fertilization. In: Knobil E, Ewing L, Markert C, Greenwald G S and Pfaff D, *The physiology of reproduction*. Raven Press, New York, 135-185.

Zheng Y, Fiser P & Sirard M (1992). The use of ejaculated boar semen after freezing in 2 or 6% glycerol for *in vitro* fertilization of porcine oocytes matured *in vitro*. *Theriogenology.* 38: 1065-1075. (doi: 10.1016/0093-691X(92)90120-G).

CHAPTER 2

Boar sperm tyrosine phosphorylation patterns in the presence of oviductal epithelial cells: *in vitro*, *ex vivo*, and *in vivo* models

ABSTRACT

Spermatozoa transport through the oviduct is a controlled process that regulates sperm capacitation. A crucial event involved in capacitation is protein tyrosine phosphorylation (TP). This study was undertaken to determine whether similarities exist in protein TP distribution between spermatozoa bound or unbound to oviductal epithelial cells (OEC) in three different conditions: i) *in vitro*, spermatozoa coincubated with OEC cultures; ii) *ex vivo*, spermatozoa deposited in porcine oviductal explants from slaughtered animals; iii) *in vivo*, in which sows were inseminated and the oviduct was recovered. The localization of phosphotyrosine protein was determined using indirect immunofluorescence. The distribution of protein TP was significantly ($p < 0.05$) different between bound and unbound cell populations in all experiments. In sows inseminated close to ovulation, spermatozoa were found mainly in the utero-tubal junction, where spermatozoa exhibited higher proportion of flagellum phosphorylation. Spermatozoa not bound to OEC exhibited high levels of protein phosphorylation (phosphorylated equatorial subsegment and acrosome and/or phosphorylated flagellum) in the *ex vivo* and *in vivo* experiments ($p < 0.05$). However, unbound spermatozoa coincubated with OEC in *in vitro* conditions tended to show intermediate levels of TP (equatorial subsegment with or without phosphorylated flagellum). In spermatozoa bound to OEC, protein TP was located in the equatorial subsegment or presented no phosphorylation ($p < 0.05$). Although sperm capacitation conditions *in vivo* were not reproducible *in vitro* in our experimental conditions, sperm and OEC binding seemed to be a mechanism for selecting spermatozoa with a low level of TP in *in vivo*, *ex vivo*, and *in vitro* experiments.

1. INTRODUCTION

After mating, millions of sperm cells are deposited in the female reproductive tract, where only a lesser number of these bind to oviductal epithelial cells (OEC) in the sperm reservoir (SR; Hunter *et al.*, 1987). This interaction is a mechanism that allows selection of the optimal sperm population and the maintenance of fertilization competence (Hunter & Rodriguez-Martinez, 2004). Sperm-OEC adhesion is mediated by molecules exposed on the sperm rostral surface, and carbohydrates localized on the oviductal cell plasma membrane in a species-specific manner (Green *et al.*, 2001). Close to ovulation time, bound spermatozoa begin a progressive and continuous release due to endocrine changes that produce modifications in the plasma membrane of epithelial cells, secretory activity, and oviductal fluid composition (Fazeli *et al.*, 1999; Georgiou *et al.*, 2007).

During transit through the female genital tract, spermatozoa undergo a remodelling process known as capacitation, which provides spermatozoa with the ability to fertilize an oocyte (Austin, 1951; Chang, 1951).

Capacitation is characterized by complex biochemical and biophysical changes that produce structural and morphological modifications in the sperm (Gadella, 2008). Different events accompany the capacitation of mammalian spermatozoa, such as changes in intracellular ion concentration, plasma membrane fluidity, intracellular free calcium concentration, and protein tyrosine phosphorylation (TP; de Lamirande & Cagnon, 1993; Visconti *et al.*, 1995; Matás *et al.*, 2011). The mechanism by which capacitation is controlled is not completely known, but the main factors that mediate this process are sterol acceptors, bicarbonate, and calcium (Harrison *et al.*, 1996). Together, these signalling molecules activate the protein kinase A (PKA) and protein tyrosine kinase (PTK) pathways controlling protein phosphorylation (Visconti *et al.*, 1995).

In several species, sperm capacitation is associated with an increase in protein TP (Visconti *et al.*, 1995; Tardif *et al.*, 2001; Pommer *et al.*, 2003; Grasa *et al.*, 2006). The presence of tyrosine-phosphorylated proteins in the flagellum suggests changes in motility parameters, especially in the midpiece, which has been correlated with the acquisition of hypermotility (Nassar *et al.*, 1999; Petrunkina *et al.*, 2003). In addition, during capacitation, there is a redistribution of tyrosine-phosphorylated proteins localized in the acrosomal region of boar sperm (Flesch *et al.*, 2001). These proteins may be involved in zona pellucida recognition and oocyte penetration (Dubé *et al.*, 2005). Inside the equatorial segment in the head of mammalian spermatozoa, an area enriched in tyrosine-phosphorylated proteins, called the equatorial subsegment, has been described. It has been suggested that this region could be an organizing centre involved in gamete fusion (Jones *et al.*, 2008).

The adhesion between sperm and OEC allows the maintenance of sperm viability, motility, and a fertile life span because the oviduct modulates and controls sperm capacitation until the time of ovulation (Suarez *et al.*, 1991; Töpfer-Petersen *et al.*, 2002). The main criterion for selective binding to the oviduct to ensure a suitable number of potentially fertile spermatozoa available for fertilization appears to be the uncapacitation status of the sperm population (Lefebvre & Suarez, 1996; Fazeli *et al.*, 1999). Other features, such as intact acrosomes, superior morphology, normal chromatin structure, low internal free calcium content, and reduced membrane protein TP, are necessary for binding (Ellington *et al.*, 1998; Fazeli *et al.*, 1999; Petrunkina *et al.*, 2001, 2003; Gualtieri & Talevi, 2003).

In vitro studies have shown that interactions between cultured OEC and sperm suppress and modulate TP protein status (Petrunkina *et al.*, 2001, 2003; Zumoffen *et al.*, 2010). We hypothesized that the sperm TP pattern is modulated by incubation with OEC and during the transit through the different sections of the oviduct in *in vitro*, *ex vivo*, and *in vivo* conditions. Our aim was to evaluate the distribution of TP protein in spermatozoa bound and unbound to OEC in three different experimental conditions: i) *in vitro*, with spermatozoa coincubated with OEC cultures during 1 hour; ii) *ex vivo*, with spermatozoa deposited in porcine oviductal explants from slaughtered animals and incubated for 1 hour; and iii) *in vivo*, in which sows were inseminated and the oviduct was recovered by surgery after 24 hours.

2. MATERIALS AND METHODS

2.1. Media

Unless indicated, all chemicals used in this study were purchased from Sigma-Aldrich Química SA. Beltsville thawing solution (BTS) was used as seminal extender (Pursel & Johnson, 1975). The medium used to capacitate spermatozoa was Tyrode Albumin Lactate Pyruvate (TALP; Rath *et al.*, 1999). The culture medium for OEC (199-OEC) consisted of TCM 199 supplemented with 13% FCS, 150 IU/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL).

2.2. Ethics

The study was performed following approval by the Veterinary Ethical Committee of University of Murcia. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

2.3. Sperm collection and preparation

The sperm-rich fraction was collected from mature fertile boars using the gloved-hand method (King & Macpherson, 1973). The semen was extended 1:2 with isothermal BTS. Sperm concentration, motility, acrosome integrity, and normal morphology were microscopically evaluated by standard laboratory techniques (Gadea & Matás, 2000), selecting only high-quality semen for the experiments. After dilution in extender, a pool of semen from four different boars was used for all the experiments to avoid the effect of individual boars on the results.

The spermatozoa were washed through a two-step gradient of 45 and 90% iso-osmotic Percoll saline (Pharmacia) and centrifuged (700 *g*, 30 min) (Matás *et al.*, 2011). The pellet was washed in TALP medium by centrifugation (700 *g*, 10 min) previously pre-equilibrated overnight at 38.5°C in 5% CO_2 in 95% humidified air and with a final pH of 7.4. The remaining sperm pellet was resuspended in TALP medium. The concentration was adjusted according to the experiment.

2.4. Sperm incubation with oviductal cells

"In vitro model": OEC culture

***Oviduct collection*.** Oviducts from commercial cycling sows (Landrace × Large White) were collected in a local abattoir and transported to the laboratory in saline solution (0.9% w/v NaCl) containing 100 µg/ml kanamycin at 37°C within 2 hours post-mortem. In the laboratory, the oviducts were rinsed once with 0.04% cetrimide solution and twice with PBS supplemented with 4 mg/ml polyvinyl alcohol (PVA). The oviducts were trimmed of surrounding connective tissue and ovaries on Petri dishes under sterile conditions.

***OEC culture*.** OEC were cultured as described by Romar *et al.* (2001). The oviducts were closed at one end with a clip, filled with a Trypsin-EDTA solution for endothelial cell culture (500 BAEE units of porcine Trypsin and 180 µg EDTA), closed at the other end, immersed in a Petri dish containing fresh solution, and incubated at 38.5°C for 45 minutes. The cells used for culture were collected in a Petri dish after a second flush with fresh Trypsin-EDTA solution. The cell groups were dissociated by a repeated pipetting and transferred to a sterile conical centrifuge tube containing 199-OEC medium. Cell suspensions were centrifuged at 700 *g* for 5 minutes, the supernatant was discarded, and the pellet was resuspended in fresh OEC culture medium at a final concentration of 1×10^7 cells/ml. One hundred microliters of the cell suspension were seeded into Petri dishes previously equilibrated for 2 hours at 38.5°C under 5% CO_2 containing 1900 µl of 199-OEC medium. The culture medium was replaced every 48 hours, until confluence was reached (typically after 6-7 days).

***Spermatozoa and OEC co-culture*.** Petri dishes with a monolayer of OEC were washed under sterile conditions in TALP medium and spermatozoa were added (final concentration 1×10^5 cells/ml). Both were coincubated for 1 hour (5% CO_2, 38.5°C, and 95% humidified air). After this period, the medium with the unbound spermatozoa was collected with a pipette. Petri dishes with sperm bound to OEC were fixed in 2% p-formaldehyde-PBS for 60 minutes at 4°C. Both bound and unbound sperm were processed and analysed by immunocytochemistry to determine TP.

"Ex vivo model": oviductal explants

***Oviductal explants and sperm coincubation*.** Oviducts were collected and handled as in the *in vitro* model. The oviducts used showed ovaries close to ovulation (presence of hemorrhagicum corpus and preovulatory follicles). Sperm samples (0.5 ml) were deposited in the final section of the uterine horn (close to the utero-tubal junction; UTJ) (1×10^5 cells/ml), followed by clamping of the ends of the tract. Oviducts were incubated for 1 hour at 38.5°C in a humidified atmosphere of 95% air and 5% CO_2. Then, the oviduct was cut into four parts: ampulla, ampullary-isthmic junction (AIJ), isthmus, and UTJ, and unbound sperm were removed by washing with warm BTS and analysed by immunocytochemistry assay.

***Tissue sections and histological protocol*.** After collection of unbound sperm, the different oviductal pieces were fixed in Bouin's solution for 24 hours. Fixed tissues were washed repeatedly in 75% v/v methanol for 48 hours. Samples were dehydrated, embedded in a paraffin block, and later sectioned transversely to a thickness of 10 µm. Sections were mounted on poly-L-lysine coated slides and deparaffinised. The samples were then processed by immunocytochemistry.

"In vivo model": artificial insemination and oviduct collection

***Animals*.** Crossbred multiparous sows (Landrace × Large White) with an average parity number of 3 were used in the experiment. The animals were kept in individual pens in a temperature and humidity-controlled environment. Estrus detection was performed twice daily by experienced workers allowing sows to contact nose-to-nose with a mature boar and applying back pressure. The occurrence of estrus was defined by the standing reflex in front of a teaser boar, reddening, and swelling of the vulva. The sows were cervically inseminated (Import-vet S.A., Barcelona, Spain) at 12 and 24 hours after the onset of estrus with 3×10^9 spermatozoa per dose in 80 ml BTS.

***Oviduct recovery*.** The sows were anesthetized and laparotomized ~24 hours after insemination (Tummaruk *et al.*, 2007). Preoperative anesthetic and analgesic induction of animals was carried out by a combination of ketamine (100 mg/ml) 10 mg/kg (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain), medetomidine (1.0 mg/ml) 0.2 mg/kg (Domtor, Pfizer S.A., Madrid, Spain), midazolam (5 mg/5 ml) 0.2

mg/kg (Dormicum, Roche), and morphine hydrochloride (20 mg/ml) 0.2 mg/kg (Morphine Braun 2%, B. Braun Medical S.A., Barcelona, Spain) administered i.m. The anesthetic level was maintained using isoflurane (Isoflo, Laboratorios Dr Esteve S.A., Barcelona, Spain) vaporized in oxygen at 2-3% (García-Vázquez *et al.*, 2010). Ovaries and oviducts from animals were exposed through a mid-ventral incision. Oviducts were dissected and collected in Petri dishes. The number of follicles and corpora hemorrhagica for each ovary was recorded. After surgery, animals were killed by i.v. administration of sodium thiopental (10 mg/kg) (Pentotal Lab. Abbott S.A., Madrid, Spain).

***Sperm recovery from the oviduct*.** The method of sperm recovery was a modification of the method described by Kunavongkrit *et al.* (2003) and others (Hunter, 1981, 1984; Mburu *et al.*, 1996). The oviducts on each side were tied and cut into four anatomical parts: ampulla, AIJ, isthmus, and UTJ. Every section was intraluminally flushed with 1 ml BTS at 37°C from the cranial to the caudal section with a pipette. The fluid recovered was processed by immunocytochemistry assay. After flushing, every piece of the oviducts was fixed in Bouin's solution for 24 hours for further histological processing as described earlier.

2.5. Immunolocation of TP protein in spermatozoa

The localization of TP protein in spermatozoa was analysed by indirect immunofluorescence, with a method described by Tardif *et al.* (2001) and adapted by Matás *et al.* (2011). Sperm suspensions from different experimental groups were washed with PBS and centrifuged (270 *g*, 10 min). Spermatozoa were fixed in 2% p-formaldehyde-PBS for 60 minutes at 4°C. Samples were washed once in PBS and blocked with 2% (w/v) BSA-PBS and incubated overnight at 4°C. The samples were centrifuged again, resuspended in PBS, and spread on microscope slides. Then, the spermatozoa were incubated for 1 hour at 4°C with monoclonal anti-phosphotyrosine antibodies (4G10, Millipore, Madrid, Spain) at 1:200 dilution in 0.1% BSA-PBS. The slides were rinsed with PBS and incubated for an additional 1 hour at 4°C with FITC goat anti-mouse antibodies (Bio-Rad Laboratories, Madrid, Spain) at 1:300 dilution in 0.1% BSA-PBS. After rinsing with PBS, coverslips were mounted on the slides with fluorescence medium (Dako, Carpinteria, CA, USA). Petri dishes and slides with histological samples were processed following a similar protocol.

Sperm were observed with a Leica DMR microscope equipped with fluorescent optics (excitation 450-490 nm: I3 filter) and bright field. Images were obtained using a microscope digital camera system (Zeis AxioCam HRc) and saved and edited using Axiovision Release 4.8 Software.

2.6. Experimental design

Three different experiments were designed to study the level and distribution of tyrosine phosphorylated protein in spermatozoa in contact with OEC.

"In vitro model": effect of in vitro cultured OEC on sperm TP protein location

Sperm were incubated in the presence of *in vitro* cultured OEC. The sperm used in this experiment were previously selected by Percoll gradient simulating the sperm subpopulation that reaches the oviduct under physiological conditions (spermatozoa with normal morphology and without seminal plasma). Sperm selected by Percoll gradient but not incubated with oviductal cells were used as control (n = 1001). A total of eight replicates were made and 4705 sperm cells were analysed (bound = 2352, unbound = 2353).

"Ex vivo model": effect of oviductal explants on sperm TP protein distribution

In this second study, spermatozoa were deposited in porcine oviductal explants from slaughtered animals to analyse the influence of the environment of the different oviductal regions. The sperm used for this experiment were similar to those used in *in vitro* model. Four oviducts were used in this experiment, analysing a total of 3574 spermatozoa (bound = 1320, unbound = 2254). Sperm selected by Percoll gradient but not incubated with oviductal explants were used as a control group (n = 799).

"In vivo model": sperm TP protein location in sperm collected from oviduct after artificial insemination

Sows around ovulation were inseminated. The average parity number of sows in this experiment was 4.0 ± 1.5. The number of ovulations per sow was 11.0 ± 3.2.

Oviducts were recovered and the effect of epithelial cells and oviductal fluid composition on spermatozoa was studied. Sperm before insemination were used as a control (n = 301). Eight sows were used for the experiment and a total of 2166 sperm were analysed (bound = 19, unbound = 2147).

In all the three experiments, bound and unbound sperm were collected, processed, analysed, and classified according to their TP protein pattern. Phosphorylation of the sperm proteins was detected in the acrosomal region, in the equatorial subsegment (triangular in appearance), and/or in the flagellum (Jones *et al.*, 2008). For a better analysis, the eight possible combinations of TP signal in the spermatozoa were grouped according to localization into three different categories (Figure 1):

Pattern I "low capacitation level": spermatozoa with no fluorescence signal in the equatorial subsegment, with or without a signal in the acrosome region or the flagellum.

Pattern II "medium capacitation level": spermatozoa with a signal in the equatorial subsegment, no signal in the acrosome area and with or without a signal in the flagellum.

Pattern III "high capacitation level": spermatozoa with signal in the equatorial subsegment and acrosome area, and with or without a signal in the flagellum (Petrunkina *et al.*, 2003).

An additional classification was made, according to the presence or absence of signal in the flagellum alone, regardless of other localizations as an index of "hypermotility capacity" (Pattern IV) (Petrunkina *et al.*, 2003; Kumaresan *et al.*, 2012a).

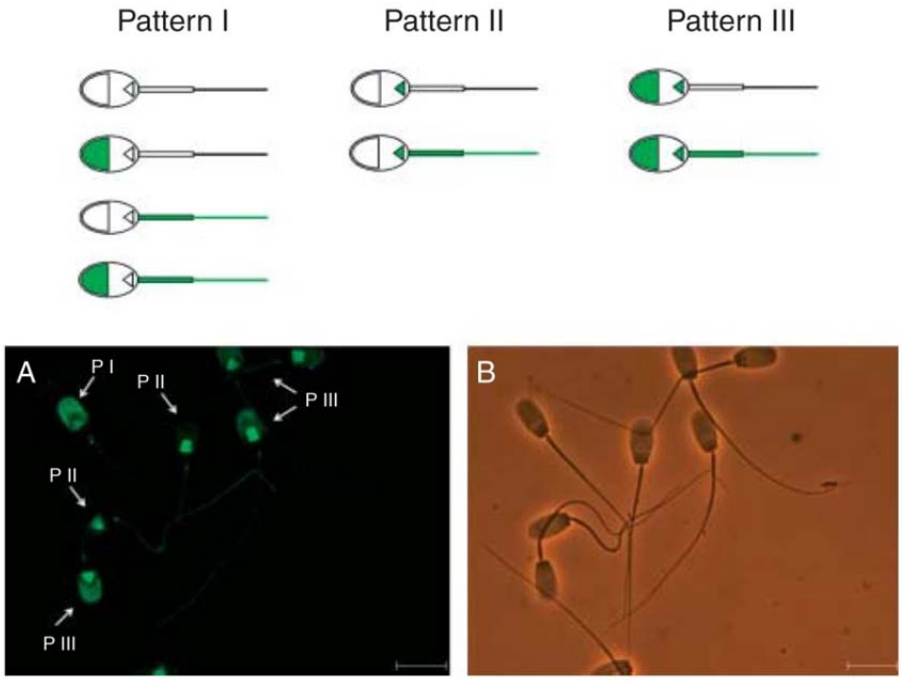

Figure 1. Classification of tyrosine phosphorylation (TP) patterns. **Pattern I** "low capacitation level": this includes spermatozoa without fluorescence in the equatorial subsegment, with or without the presence of signal in the acrosome region or flagellum. **Pattern II** "medium capacitation level": this includes spermatozoa with signal in the equatorial subsegment, no signal in the acrosome area, and with or without the presence of signal in the flagellum. **Pattern III** "high capacitation level": this includes spermatozoa with signal in the equatorial subsegment and acrosome area and with or without the presence of signal in the flagellum. **Image A** shows fluorescence capture and **Image B** the corresponding bright field image (100×) of different patterns of protein TP on boar sperm detected by indirect immunofluorescence. The arrows indicate different TP distribution: pattern I (PI), pattern II (PII), and pattern III (PIII). Scale bars, 10 µm.

2.7. Statistical analysis

The data were first examined using the Shapiro-Wilk test to assess normality distribution. In view of the non-Gaussian distribution of most of the data gathered, a non-parametric test was used. Data were expressed as mean ± S.E.M. and compared by a non-parametric Kruskal-Wallis test and a Conover-Inman test for all pairwise comparisons was used to compare groups of samples. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. "*In vitro* model": effect of *in vitro* cultured OEC on sperm TP protein location

The protein TP pattern changes when the sperm are incubated with OEC, especially as regard patterns II and III. As Table 1 indicates, bound and unbound sperm after OEC incubation showed less pattern II phosphorylation than non-incubated sperm (control group), although an increase in pattern III was observed in unbound sperm and a decrease in the same phosphorylation pattern in bound sperm compared with the control group. The distribution of fluorescence patterns was significantly different ($p < 0.05$) between bound and unbound cell populations (Table 1). The most common pattern of TP in OEC-bound sperm was pattern II (77.4%). OEC binding was most prevalent in sperm with non-phosphorylated heads and tails (Figure 2), while higher proportions of subsegment and head and/or flagellum phosphorylated cells were found in the unbound population (36.4%). The lowest proportion of pattern IV was observed in sperm bound to OEC (17.9%) compared with unbound sperm (81.8%) and sperm not incubated with OEC (84.6%).

Table 1. Immunolocation of protein tyrosine phosphorylation patterns in boar sperm bound and unbound to OEC cultured *in vitro* (data shown mean ± S.E.M.).

Sperm source (N)	Bound (N)	Pattern I (%)	Pattern II (%)	Pattern III (%)	Pattern IV (%)
Not incubated sperm (1001)		3.5 ± 1.8^{a}	85.8 ± 3.5^{a}	10.6 ± 3.1^{a}	84.6 ± 3.6^{a}
Incubated sperm OEC (4705)	No (2352)	2.8 ± 1.6^{a}	60.7 ± 4.8^{b}	36.4 ± 4.8^{b}	81.8 ± 3.9^{a}
	Yes (2353)	18.4 ± 3.8^{b}	77.4 ± 4.1^{c}	4.1 ± 1.9^{c}	17.9 ± 3.8^{b}

N: number of spermatozoa evaluated; **Pattern I**: non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; **Pattern II**: equatorial segment or equatorial segment and flagellum phosphorylated; **Pattern III**: equatorial segment and head and/or flagellum phosphorylated; **Pattern IV**: flagellum phosphorylation independent of phosphorylation at other locations.
Different letters (a, b, c) in the same column indicate significant differences ($p < 0.05$).

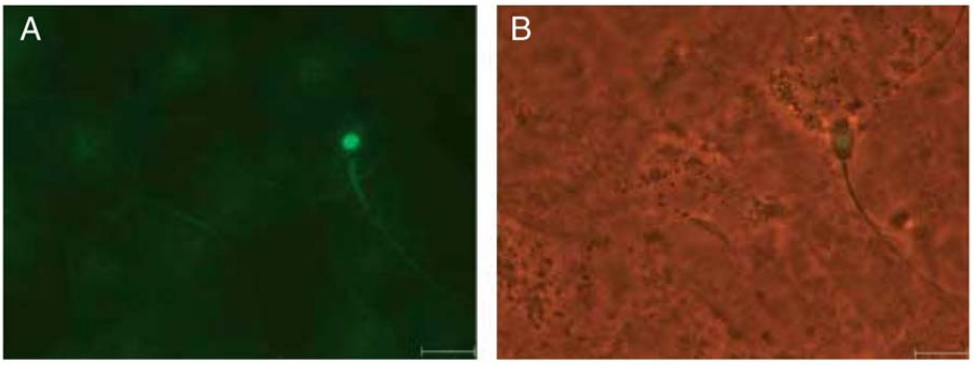

Figure 2. Immunolocation of protein tyrosine phosphorylation in boar spermatozoa attached to OEC cultures. **Image A** shows fluorescence captures and **Image B** the corresponding bright field image (100×). Scale bars, 10 µm.

3.2. "*Ex vivo* model": effect of oviductal explants on sperm TP protein distribution

Spermatozoa bound to the OEC

The bound sperm with TP pattern I were more abundant in the UTJ than in other oviductal section ($p < 0.05$) (see Table 2). In contrast, the fewest cells showing pattern II were observed in the UTJ. The percentage of sperm showing pattern III was similar in the isthmus, AIJ, and ampulla, but no sperm with this pattern were found attached to the UTJ. Finally, as regards pattern IV, there were no significant differences in the percentage of sperm attached to the isthmus, AIJ, and ampulla (37.0, 39.7, and 35.0% respectively); however, in sperm bound to the UTJ this proportion was the lowest (22.5%) ($p < 0.05$).

When the different bound sperm TP patterns (I, II and III) were compared in each oviductal section, the highest proportion corresponded to pattern II, while patterns I and III were similar. In general terms, the proportion of patterns I and III was very low (or even non-existent in the case of pattern III in the UTJ) except for pattern I in the UTJ (close to 30%).

Unbound spermatozoa recovered from oviductal lumen by flushing

The patterns of fluorescence in unbound sperm are summarized in Table 2. The tyrosine-phosphorylated protein pattern I was similar in each section ($p < 0.05$) and showed a low percentage (range 4.8-8.4%). The highest percentage of pattern II was observed in the AIJ (82.6%), while similar percentages of pattern II were found in the UTJ and ampulla sections (64.6 and 60.9% respectively). Flushed sperm samples displayed the highest percentage of pattern III in the isthmus (40.5%) and the lowest level in the AIJ (10.0%); the UTJ and ampulla showed similar percentages (30.4 and 30.5% respectively). Almost all the unbound sperm (75.7-85.6%) showed pattern IV.

When the different unbound sperm TP patterns were compared in each oviductal section, flushed samples showed a high percentage of pattern II in the UTJ, AIJ, and ampulla, which was different from the percentages shown by patterns I and III. Only in the isthmus section were sperm with pattern II and III observed in the same proportion. In general terms, very few pattern I sperm were observed in the different oviductal sections.

Table 2. A) Distribution of protein tyrosine phosphorylation in boar spermatozoa bound to oviductal epithelial explants from different oviductal segments. **B)** Protein tyrosine phosphorylation distribution in boar spermatozoa unbound to oviductal epithelial explants from different oviductal segments (data shown mean ± S.E.M.).

Oviductal segment	N	Pattern I (%)	Pattern II (%)	Pattern III (%)	Pattern IV (%)
A)					
Non-incubated sperm	799	4.6 ± 0.7	88.3 ± 1.1	7.0 ± 0.9	80.3 ± 1.4
UTJ	120	27.5 ± 4.0^{a}	72.5 ± 4.0^{a}	–	22.5 ± 3.8^{a}
Isthmus	400	8.0 ± 1.3^{b}	86.7 ± 1.7^{b}	5.2 ± 1.4	37.0 ± 2.4^{b}
AIJ	400	5.7 ± 1.1^{b}	83.7 ± 1.8^{b}	10.5 ± 1.5	39.7 ± 2.4^{b}
Ampulla	400	10.5 ± 1.5^{b}	81.0 ± 1.9^{ab}	8.5 ± 1.1	35.0 ± 2.3^{b}
B)					
Non-incubated sperm	799	4.6 ± 0.7	88.3 ± 1.1	7.0 ± 0.9	80.3 ± 1.4
UTJ	82	4.8 ± 2.3	64.6 ± 5.0^{a}	30.4 ± 5.1^{a}	84.1 ± 4.0^{ab}
Isthmus	614	7.6 ± 1.0	51.7 ± 2.0^{b}	40.5 ± 1.9^{b}	85.6 ± 1.4^{a}
AIJ	766	7.3 ± 0.9	82.6 ± 1.3^{c}	10.0 ± 1.0^{c}	75.7 ± 1.5^{b}
Ampulla	792	8.4 ± 0.9	60.9 ± 1.7^{ab}	30.5 ± 1.6^{a}	81.1 ± 1.3^{ab}

N: number of spermatozoa evaluated; **Pattern I**: non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; **Pattern II**: equatorial segment or equatorial segment and flagellum phosphorylated; **Pattern III**: equatorial segment and head and/or flagellum phosphorylated; **Pattern IV**: flagellum phosphorylation independent of phosphorylation at other locations.
Different letters (a, b, c) in the same column indicate significant differences ($p < 0.05$).

3.3. "*In vivo* model": sperm TP protein location in sperm collected from oviduct after artificial insemination

Spermatozoa bound to the OEC

Nineteen spermatozoa were found bound to the epithelial cells in 16 oviducts from 8 sows. No statistical analysis was made for this group because of the low number of spermatozoa found. Most of them were localized in the AIJ (n = 8, 42.1%) and the isthmus (n = 6, 31.5%); only two (10.5%) and three (15.7%) spermatozoa were found in the ampulla and the UTJ respectively.

With respect to the TP pattern, 84.2% of the sperm did not show any fluorescence signal (Figure 3) and only three of them (two in UTJ and one in isthmus) showed equatorial subsegment TP (pattern II). Head phosphorylation was not found in sperm bound to OEC, which agrees with the data from the *in vitro* and *ex vivo* experiments. In contrast to the *ex vivo* experiment (oviductal explants), no flagellum TP was observed.

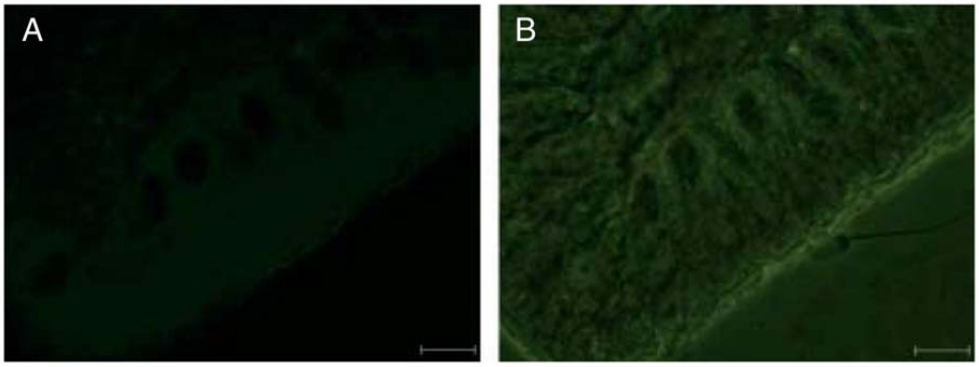

Figure 3. Tyrosine phosphorylation distribution in boar spermatozoa bound to OEC in the isthmus 24 hours after insemination. **Image A** shows fluorescence captures and **Image B** shows the corresponding bright field (100×). Scale bars, 10 µm.

Unbound spermatozoa recovered from the oviductal lumen by flushing

A total of 2147 spermatozoa were collected and evaluated from the different parts of the oviduct. The highest number of spermatozoa was recovered from the UTJ section (1742). The proportion of each TP protein pattern in flushed sperm samples is represented in Table 3. In the UTJ, only 5.8% of spermatozoa showed fluorescence pattern I, which was lower than those in the other sections and in the control group (spermatozoa evaluated before insemination) ($p < 0.05$). In the case of pattern II, no significant differences were observed when the isthmus, AIJ, and ampulla sections were compared (46.0, 36.0, and 43.5% respectively), all being lower than those in the UTJ (84.6%). No differences were found in the proportion of pattern III in the isthmus, AIJ, and ampulla, but, as in the case of pattern I, a low proportion (9.5%) of the spermatozoa observed in the UTJ had phosphorylated equatorial segment and head and/or flagellum (pattern III). When the sperm signal was classified according to the presence or absence of tyrosine protein phosphorylation in the flagellum (pattern IV), a lower percentage of sperm flagellum signal was observed in the isthmus (67.1%) than in the ampulla (85.5%), while intermediate values were found for the UTJ (76.4%) and AIJ (74.5%).

When the different unbound sperm TP patterns were compared in each oviductal section, the UTJ presented a higher percentage of pattern II than of patterns I and III. The isthmus represents the next section during sperm transit toward the fertilization site. Here, sperm pattern II was less common than in the UTJ, while pattern III

increased (although not significantly different from pattern II). When the sperm were in the fertilization site (ampulla) or close to it (AIJ), patterns I, II, and III were present in the same proportions.

Table 3. Proportion of tyrosine phosphorylation patterns in boar spermatozoa recovered from different parts of the oviduct of sows 24 hours after insemination and before insemination (data shown mean ± S.E.M.).

Oviductal segment	N	Pattern I (%)	Pattern II (%)	Pattern III (%)	Pattern IV (%)
Sperm ejaculated	301	63.4 ± 2.7^{a}	34.5 ± 2.7^{a}	1.9 ± 0.8^{a}	8.9 ± 1.1^{a}
UTJ	1742	5.8 ± 0.5^{b}	84.6 ± 0.8^{b}	9.5 ± 0.7^{a}	76.4 ± 1.0^{bc}
Isthmus	152	21.7 ± 3.5^{c}	46.0 ± 4.0^{c}	32.2 ± 4.1^{b}	67.1 ± 3.8^{b}
AIJ	122	26.2 ± 3.9^{c}	36.0 ± 4.3^{ac}	37.7 ± 4.4^{b}	74.5 ± 3.9^{bc}
Ampulla	131	21.3 ± 3.3^{c}	43.5 ± 4.3^{ac}	35.1 ± 3.8^{b}	85.5 ± 3.0^{c}

N: number of spermatozoa evaluated; **Pattern I**: non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; **Pattern II**: equatorial segment or equatorial segment and flagellum phosphorylated; **Pattern III**: equatorial segment and head and/or flagellum phosphorylated; **Pattern IV**: flagellum phosphorylation independent of phosphorylation at other locations.
Different letters (a, b, c) in the same column indicate significant differences ($p < 0.05$).

4. DISCUSSION

The essential steps of capacitation are modulated in the caudal isthmus of the oviduct, where sperm are selected and stored in close contact with the epithelium. A crucial event involved in capacitation and the acquisition of fertilizing potential is protein TP (Töpfer-Petersen, 1999). In this study, we analyzed different distribution patterns of protein TP in boar spermatozoa and their relationship with the ability to bind to OEC under *in vitro*, *ex vivo*, and *in vivo* conditions.

The results showed significantly ($p < 0.05$) different patterns of protein TP in the sperm bound to epithelial cells in all experiments (*in vivo*, *ex vivo*, and *in vitro*), indicating a selective function in the interaction. Sperm after ejaculation showed very-low protein phosphorylation levels (Table 3), which only increased when the samples had been washed through Percoll gradients and the seminal plasma had been eliminated (Table 2). Fresh semen retains most of the seminal plasma proteins that stabilize the cells and prevent capacitation (Töpfer-Petersen *et al.*, 1998). The molecules present on the sperm surface that come from seminal plasma, for example the spermadhesins AQN-1, AQN-2, and AWN, create a protective layer around the plasma membrane, stabilizing it. The cysteine-rich secreted proteins such as CRISP1 appear to be related to maturation processes and inhibit premature sperm capacitation (Udby *et al.*, 2005). Incubation with 10% seminal plasma appears to be sufficient to inhibit TP, acrosome reaction, and several additional key steps in capacitation (Vadnais & Althouse, 2011).

Several authors have demonstrated that unphosphorylated spermatozoa show a preferential binding ability to OEC after a short incubation period (Lefebvre & Suarez, 1996; Fazeli *et al.*, 1999). Furthermore, Petrunkina *et al.* (2001, 2003) demonstrated that long-term coincubations with oviductal cells maintain sperm viability and motility and slow the process of sperm membrane destabilization, when spermatozoa are bound to OEC. Spermatozoa attached to epithelial cells showed constantly low levels of protein TP (mainly pattern I), while unbound spermatozoa showed a high degree of phosphorylation (Yeste *et al.*, 2009). In our study, not only spermatozoa bound to OEC cultures exhibited a low level of TP but also spermatozoa attached to the oviducts of inseminated sows before ovulation and preovulatory porcine oviductal explants presented this pattern ($p < 0.05$).

We found that different TP patterns were associated with different sperm functions (Urner & Sakkas, 2003). In spermatozoa bound to OEC, the protein TP was located in the equatorial subsegment or did not show any fluorescence. Such labeling may represent the first stage of phosphorylation during capacitation (Figure 2). In boar testicular sperm, TP protein is distributed through the entire equatorial segment. During epididymal maturation, dephosphorylation and/or redistribution of phosphorylation occur and the *cauda* spermatozoa exhibit only an intense triangular labeling in the posterior part of the acrosome and the equatorial subsegment (Jones *et al.*, 2008; Fàbrega *et al.*, 2011). SPACA1 and HSPA1A proteins have been determined in this event (Spinaci *et al.*, 2005; Jones *et al.*, 2008). The equatorial subsegment might be an organizing centre that initiates membrane fusion during the fertilization process.

Phosphorylation in the acrosome, equatorial subsegment, and flagellum is related to an advanced stage of capacitation (Petrunkina *et al.*, 2003). The presence of tyrosine-phosphorylated proteins in the flagellum is a preferential pattern of sperm capacitation in several species (Leclerc *et al.*, 1997; Pommer *et al.*, 2003; Grasa *et al.*, 2006; Jagan Mohanarao & Atreja, 2011). This phosphorylation is associated with a fibrous sheath, suggesting that it may be involved in hyperactivation (Pommer *et al.*, 2003; Kumaresan *et al.*, 2012b). Our data showed that spermatozoa washed through Percoll gradients and spermatozoa unbound to oviductal cells had higher proportions of flagellum phosphorylation (pattern IV, $p < 0.05$) in all the experiments. Flagellum TP may be related to the release of bound spermatozoa from the epithelium (Lefebvre & Suarez, 1996). However, these findings contrast with others that point to the recovery of faint labeling in the tail in boar spermatozoa (Petrunkina *et al.*, 2001; Fàbrega *et al.*, 2011). Equatorial subsegment and/or flagellum phosphorylation (pattern II) corresponds to an intermediate capacitation level (Petrunkina *et al.*, 2001).

Acrosome phosphorylation was an unusual pattern and was not found in sperm bound to OEC in any of the three experiments. Suppressed acrosome phosphorylation may be a requirement for binding to oviductal cells (Petrunkina *et al.*, 2003) because it

represents an advanced stage of capacitation. Sp32 is a (pro) acrosin binding protein localized on the acrosome surface. During incubation in capacitating conditions, sp32 is phosphorylated and transformed into p32, a tyrosine phosphorylated protein (Dubé *et al.*, 2005). These changes may affect the sperm's ability to bind to OEC. Moreover, during the acrosome reaction, phosphorylation in the acrosomal region decreased due to the redistribution of phosphotyrosine residues (Tardif *et al.*, 2001; Dubé *et al.*, 2005).

In vivo, boar spermatozoa are deposited in the female genital tract, pass through the UTJ, and accumulate in the isthmus of the oviduct, where a functional SR is established (Hunter & Rodriguez-Martinez, 2004). The sperm distribution results showed that spermatozoa in sows inseminated around ovulation are mainly found in the UTJ where they exhibit a high proportion of flagellum phosphorylation. In contrast, in oviductal explants, spermatozoa were found along the oviduct in the same proportion. The UTJ is a physical barrier that controls the quality and quantity of spermatozoa present in the oviduct. Only sperm cells with an intact acrosome, low internal free calcium content, reduced membrane protein phosphorylation, and normal chromatin structure are able to bind to OEC (Ellington *et al.*, 1998; Fazeli *et al.*, 1999; Petrunkina *et al.*, 2001; Gualtieri & Talevi, 2003). In our study, unbound sperm exhibited a high percentage of protein TP in the equatorial subsegment, acrosome, and flagellum ($p < 0.05$). Kumaresan *et al.* (2012a) speculated that an early increase in the population of spermatozoa with such a phosphorylation pattern would increase the chances of fertilization. The adhesion between sperm and OEC is mediated by molecules exposed on the sperm rostral surface and carbohydrates localized on the oviductal cell plasma membrane in a species-specific manner (Green *et al.*, 2001). Capacitated sperm lose binding sites during capacitation, and our *in vivo* results showed that only sperm with unphosphorylated heads are able to bind to cells.

At the time of ovulation, changes in the oviductal environment occur, promoting the ascent of sperm cells toward the ampulla and the site of fertilization (Hunter, 1981). Recent studies have shown that the arrival of spermatozoa initiates a mutual sperm-epithelial signaling dialog (Fazeli *et al.*, 2004). In an *in vivo* experiment, a small number of spermatozoa were seen bound to OEC around the ovulation stage. Most spermatozoa were in the lumen of the oviduct with a high level of TP, especially when the sperm were close to the fertilization site. Spermatozoa modified protein synthesis and oviductal fluid composition remodeling the binding sites in oviductal cells due to the production of a gradient of calcium during periovulatory moments (Gadella & Harrison, 2000). In sows, several *de novo* synthesized proteins have been identified, and oviduct-specific glycoprotein is related to increased fertilization rates and polyspermy control (Coy *et al.*, 2008).

Porcine oviductal fluid in the follicular phase promotes sperm viability and acrosomal integrity, decreases plasma membrane fluidity, and increases zona binding and polyspermy during IVF (Coy *et al.*, 2010). However, other authors observed no differences in sperm motility during incubation except at 2 hours, when spermatozoa incubated with preovulatory fluid showed higher motility (Kumaresan *et al.*, 2012b). In the same study, preovulatory oviductal fluid induced TP in a higher proportion of boar spermatozoa than post-ovulatory fluid (Kumaresan *et al.*, 2012b). These contradictory results may be related to the boar in question, incubation time, or oviductal fluid treatment. Our results showed that unbound spermatozoa exhibited high levels of tyrosine protein phosphorylation (patterns II and III) in all the experiments.

The final objective of the capacitation process is fertilization. Spermatozoa incubated with OEC cultures showed higher penetration and monospermy rates when short time periods were used (Hunter, 1981). Moreover, the fertilization and cleavage rates of spermatozoa unbound to oviductal cells culture were lower than those for spermatozoa attached to the OEC (Kon *et al.*, 2009). These results indicate that the ability of spermatozoa to bind to oviductal cells is related to their fertilizing ability.

In conclusion, sperm capacitation conditions *in vivo* are not reproducible in *in vitro* models under our experimental conditions. However, sperm-OEC binding was seen to be a mechanism for selecting sperm populations with a low degree of TP, as demonstrated in the *in vivo*, *ex vivo*, and *in vitro* experiments. Further studies are required to obtain more information about the mechanism by which the oviduct modulates sperm capacitation.

ACKNOWLEDGEMENTS

This work was supported by Fundación Séneca 08752/PI/08 and MICINN-FEDER (AGL 2009-12512-C02-01).

REFERENCES

Austin C (1951). Observations on the penetration of the sperm into the mammalian egg. *Australian Journal of Biological Sciences.* 4: 581-596. (doi: 10.1071/BI9510581).

Coy P, Cánovas S, Mondéjar I, Saavedra MD, Romar R, Grullón L, Matás C & Avilés M (2008). Oviduct-specific glycoprotein and heparin modulate sperm–zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences.* 105: 15809-15814. (doi: 10.1073/pnas.0804422105).

Coy P, Lloyd R, Romar R, Satake N, Matás C, Gadea J & Holt WV (2010). Effects of porcine pre-ovulatory oviductal fluid on boar sperm function. *Theriogenology.* 74: 632-642. (doi: 10.1016/j.theriogenology.2010.03.005).

Chang M (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*. 168: 697-698. (doi: 10.1038/168697b0).

de Lamirande E & Cagnon C (1993). Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radical Biology and Medicine.* 14: 157-166. (doi: 10.1016/0891-5849(93)90006-G).

Dubé C, Leclerc P, Baba T, Reyes-Moreno C & Bailey JL (2005). The proacrosin binding protein, sp32, is tyrosine phosphorylated during capacitation of pig sperm. *Journal of andrology.* 26: 519-528. (doi: 10.2154/jandrol.04163).

Ellington JE, Evenson DP, Fleming JE, Brisbois RS, Hiss GA, Broder SJ & Wright RW (1998). Coculture of human sperm with bovine oviduct epithelial cells decreases sperm chromatin structural changes seen during culture in media alone. *Fertility and sterility.* 69: 643-649. (doi: 10.1016/S0015-0282(98)00023-5).

Fàbrega A, Puigmulé M, Yeste M, Casas I, Bonet S & Pinart E (2011). Impact of epididymal maturation, ejaculation and *in vitro* capacitation on tyrosine phosphorylation patterns exhibited of boar (Sus domesticus) spermatozoa. *Theriogenology.* 76: 1356-1366. (doi: 10.1016/j.theriogenology.2011.06.007).

Fazeli A, Duncan A, Watson P & Holt WV (1999). Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of reproduction.* 60: 879-886. (doi: 10.1095/biolreprod60.4.879).

Fazeli A, Affara NA, Hubank M & Holt WV (2004). Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biology of reproduction.* 71: 60-65. (doi: 10.1095/biolreprod.103.026815).

Flesch F, Wijnand E, Van de Lest C, Colenbrander B, van Golde LM & Gadella BM (2001). Capacitation dependent activation of tyrosine phosphorylation generates two sperm head plasma membrane proteins with high primary binding affinity for the zona pellucida. *Molecular reproduction and development.* 60: 107-115. (doi: 10.1002/mrd.1067).

Gadea J & Matás C (2000). Sperm factors related to *in vitro* penetration of porcine oocytes. *Theriogenology.* 54: 1343-1357. (doi: 10.1016/S0093-691X(00)00458-1).

Gadella BM & Harrison RA (2000). The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development.* 127: 2407-2420.

Gadella BM (2008). Sperm membrane physiology and relevance for fertilization. *Animal Reproduction Science.* 107: 229-236. (doi: 10.1016/j.anireprosci.2008.05.006).

García-Vázquez FA, Ruiz S, Matás C, Izquierdo-Rico MJ, Grullón LA, De Ondiz A, Vieira L, Avilés-López K, Gutiérrez-Adán A & Gadea J (2010). Production of transgenic piglets using ICSI–sperm-mediated gene transfer in combination with recombinase RecA. *Reproduction.* 140: 259-272. (doi: 10.150/REP-10-0129).

Georgiou AS, Snijders AP, Sostaric E, Aflatoonian R, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Wright PC & Fazeli A (2007). Modulation of the oviductal environment by gametes. *Journal of proteome research.* 6: 4656-4666. (doi: 10.1021/pr070349m).

Grasa P, Cebrián-Pérez JA & Muiño-Blanco T (2006). Signal transduction mechanisms involved in *in vitro* ram sperm capacitation. *Reproduction.* 132: 721-732. (doi: 10.1530/rep.1.00770).

Green C, Bredl J, Holt WV, Watson P & Fazeli A (2001). Carbohydrate mediation of boar sperm binding to oviductal epithelial cells *in vitro*. *Reproduction.* 122: 305-315. (doi: 10.1530/rep.0.1250251).

Gualtieri R & Talevi R (2003). Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium *in vitro*. *Reproduction.* 125: 251-258. (doi: 10.1530/rep.0.1220305).

Harrison RA, Ashworth P & Miller N (1996). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular reproduction and development.* 45: 378-391. (doi: 10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V).

Hunter RHF (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *Journal of reproduction and fertility.* 63: 109-117. (doi: 10.1530/jrf.0.0630109).

Hunter RHF (1984). Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct. *Journal of reproduction and fertility.* 72: 203-211. (doi: 10.1530/jrf.0.0720203).

Hunter RHF, Fléchon B & Fléchon J-E (1987). Pre-and peri-ovulatory distribution of viable spermatozoa in the pig oviduct: a scanning electron microscope study. *Tissue and Cell.* 19: 423-436. (doi: 10.1016/0040-8166(87)90037-1).

Hunter RHF & Rodriguez-Martinez H (2004). Capacitation of mammalian spermatozoa *in vivo*, with a specific focus on events in the Fallopian tubes. *Molecular reproduction and development.* 67: 243-250. (doi: 10.1002/mrd.10390).

Jagan Mohanarao G & Atreja S (2011). Identification of capacitation associated tyrosine phosphoproteins in buffalo (Bubalus bubalis) and cattle spermatozoa. *Animal Reproduction Science.* 123: 40-47. (doi: 10.1016/j.anireprosci.2010.11.013).

Jones R, James PS, Oxley D, Coadwell J, Suzuki-Toyota F & Howes EA (2008). The equatorial subsegment in mammalian spermatozoa is enriched in tyrosine phosphorylated proteins. *Biology of reproduction.* 79: 421-431. (doi: 10.1095/biolreprod.107.067314).

King G & Macpherson J (1973). A comparison of two methods for boar semen collection. *Journal of animal science.* 36: 563-565. (doi: 10.2134/jas1973.363563x).

Kon Y, Iwata H, Shiono H, Matsubara K, Kurita A, Sakaguchi Y, Kuwayama T & Monji Y (2009). Effect of carbohydrates on the ability of bull sperm to bind to bovine oviduct epithelial cells. *Reproduction in Domestic Animals.* 44: 365-370. (doi: 10.1111/j.1439-0531.2007.01013.x).

Kumaresan A, Johannisson A, Humblot P & Bergqvist AS (2012a). Oviductal fluid modulates the dynamics of tyrosine phosphorylation in cryopreserved boar spermatozoa during capacitation. *Molecular reproduction and development.* 79: 525-540. (doi: 10.1002/mrd.22058).

Kumaresan A, Johannisson A, Saravia F & Bergqvist AS (2012b). The effect of oviductal fluid on protein tyrosine phosphorylation in cryopreserved boar spermatozoa differs with

the freezing method. *Theriogenology.* 77: 588-599. (doi: 10.1016/j.theriogenology.2011.08.035).

Kunavongkrit A, Sang-Gasanee K, Phumratanaprapin C, Tantasuparuk W & Einarsson S (2003). A study on the number of recovered spermatozoa in the uterine horns and oviducts of gilts, after fractionated or non-fractionated insemination. *Journal of veterinary medical science.* 65: 63-67. (doi: 10.1292/jvms.65.63).

Leclerc P, De Lamirande E & Gagnon C (1997). Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. *Free Radical Biology and Medicine.* 22: 643-656. (doi: 10.1016/S0891-5849(96)00379-6).

Lefebvre R & Suarez SS (1996). Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biology of reproduction.* 54: 575-582. (doi: 10.1095/biolreprod54.3.575).

Matás C, Vieira L, García-Vázquez F, Avilés-López K, López-Úbeda R, Carvajal J & Gadea J (2011). Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. *Animal Reproduction Science.* 127: 62-72. (doi: 10.1016/S0015-0282(99)00013-8).

Mburu J, Einarsson S, Lundeheim N & Rodriguez-Martinez H (1996). Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. *Animal Reproduction Science.* 45: 109-121. (doi: 10.1016/S0378-4320(96)01566-7).

Nassar A, Mahony M, Morshedi M, Lin M-H, Srisombut C & Oehninger S (1999). Modulation of sperm tail protein tyrosine phosphorylation by pentoxifylline and its correlation with hyperactivated motility. *Fertility and sterility.* 71: 919-923. (doi: 10.1016/S0015-0282(99)00013-8).

Petrunkina AM, Friedrich J, Drommer W, Bicker G, Waberski D & Töpfer-Petersen E (2001). 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: 469-480. (doi: 10.1530/rep.0.1220469).

Petrunkina AM, Simon K, Günzel-Apel AR & Töpfer-Petersen E (2003). Specific order in the appearance of protein tyrosine phosphorylation patterns is functionally coordinated with dog sperm hyperactivation and capacitation. *Journal of andrology.* 24: 423-437. (doi: 10.1002/j.1939-4640.2003.tb02691.x).

Pommer AC, Rutllant J & Meyers SA (2003). Phosphorylation of protein tyrosine residues in fresh and cryopreserved stallion spermatozoa under capacitating conditions. *Biology of reproduction.* 68: 1208-1214. (doi: 10.1095/biolreprod.102.011106).

Pursel V & Johnson L (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *Journal of animal science.* 40: 99-102.

Rath D, Long C, Dobrinsky J, Welch G, Schreier L & Johnson L (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *Journal of animal science.* 77: 3346-3352. (doi: 1999.77123346x).

Romar R, Coy P, Campos I, Gadea J, Matás C & Ruiz S (2001). Effect of co-culture of porcine sperm and oocytes with porcine oviductal epithelial cells on *in vitro* fertilization. *Animal Reproduction Science.* 68: 85-98. (doi: 10.1016/S0378-4320(01)00133-6).

Spinaci M, Volpe S, Bernardini C, De Ambrogi M, Tamanini C, Seren E & Galeati G (2005). Immunolocalization of heat shock protein 70 (Hsp 70) in boar spermatozoa and its role during fertilization. *Molecular reproduction and development.* 72: 534-541. (doi: 10.1002/mrd.20367).

Suarez S, Katz D, Owen D, Andrew J & Powell R (1991). Evidence for the function of hyperactivated motility in sperm. *Biology of reproduction.* 44: 375-381. (doi: 10.1095/biolreprod44.2.375).

Tardif S, Dubé C, Chevalier S & Bailey JL (2001). Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of reproduction.* 65: 784-792. (doi: 10.1095/biolreprod65.3.784).

Töpfer-Petersen E, Romero A, Varela P, Ekhlasi-Hundrieser M, Dostalova Z, Sanz L & Calvete J (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia.* 30: 217-224. (doi: 10.1111/j.1439-0272.1998.tb02263.x).

Töpfer-Petersen E (1999). Molecules on the sperm's route to fertilization. *Journal of Experimental Zoology.* 285: 259-266. (doi: 10.1002/(SICI)1097-010X(19991015)285:3<259::AID-JEZ9<3.0.CO;2-B).

Töpfer-Petersen E, Wagner A, Friedrich J, Petrunkina AM, Ekhlasi-Hundrieser M, Waberski D & Drommer W (2002). Function of the mammalian oviductal sperm reservoir. *Journal of Experimental Zoology.* 292: 210-215. (doi: 10.1002/jez.1157).

Tummaruk P, Sumransap P, Techakumphu M & Kunavongkrit A (2007). Distribution of spermatozoa and embryos in the female reproductive tract after unilateral deep intra uterine insemination in the pig. *Reproduction in Domestic Animals.* 42: 603-609. (doi: 10.1111/j.1439-0531.2006.00830.x).

Udby L, Bjartell A, Malm J, Egesten A, Lundwall Å, Cowland JB, Borregaard N & Kjeldsen L (2005). Characterization and localization of cysteine-rich secretory protein 3 (CRISP-3) in the human male reproductive tract. *Journal of andrology.* 26: 333-342. (doi: 10.2164/jandrol.04132).

Urner F & Sakkas D (2003). Protein phosphorylation in mammalian spermatozoa. *Reproduction.* 125: 17-26. (doi: 10.1530/rep.0.1250017).

Vadnais ML & Althouse GC (2011). Characterization of capacitation, cryoinjury, and the role of seminal plasma in porcine sperm. *Theriogenology.* 76: 1508-1516. (doi: 10.1016/j.theriogenology.2011.06.021).

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS (1995). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development.* 121: 1129-1137.

Yeste M, Lloyd R, Badia E, Briz M, Bonet S & Holt WV (2009). Direct contact between boar spermatozoa and porcine oviductal epithelial cell (OEC) cultures is needed for optimal sperm survival *in vitro*. *Animal Reproduction Science.* 113: 263-278. (doi: 10.1016/j.anireprosci.2008.08.018).

Zumoffen C, Caille A, Munuce M, Cabada M & Ghersevich S (2010). Proteins from human oviductal tissue-conditioned medium modulate sperm capacitation. *Human reproduction. 25:* 1504-1512. (doi: 10.1093/humrep/deq063).

CHAPTER 3

Sperm selection by oviductal epithelial cells is not merely a morphological-based phenomenon

ABSTRACT

The interaction of oviductal epithelial cells with the spermatozoa has beneficial effects on the sperm functions. The objective of this study was to evaluate the *in vitro* fertilizing capacity of incubating spermatozoa previously selected by density gradient in oviductal epithelial cells (OEC) and determinate some sperm characteristics that could explain the results obtained. In this study, we assessed *in vitro* fertilization (IVF), tyrosine phosphorylation (TP), phosphatidylserine translocation (PS), nuclear DNA fragmentation and chromatin decondensation. Three sets of sperm, previously selected by density gradient, were used in the different experiments: i) washed spermatozoa (W30 group): sperm incubated for 30 minutes without oviductal epithelial cells; ii) unbound (NB group): spermatozoa not bound to OEC after 30 minutes of coincubation; iii) bound (B group): spermatozoa bound to OEC after 30 minutes of coincubation. We found that sperm from the NB group led to a statistically significant lower IVF yield, accompanied by low penetration rates and problems of nuclear decondensation. Moreover, significantly higher levels of TP and PS were observed in the NB group compared with the W30 and B groups. These results demonstrate that the OEC exert a rigorous degree of sperm selection, even within an already highly selected population of spermatozoa, and are able to capture the best functional spermatozoa for fertilization.

1. INTRODUCTION

Once deposited in the female genital tract, sperm undergo a selection process that starts in the lower part of the uterus (cervix), continues in the uterus horns and finishes in the upper part of the female genital tract, in the oviduct, where fertilization takes place. Sperm reaching the oviduct are selected according to their morphological intactness (Waberski *et al.*, 2006), maturity (Petrunkina *et al.*, 2001), uncapacitated status (Fazeli *et al.*, 1999; Luño *et al.*, 2013) and high quality chromatin (Ardón *et al.*, 2008). When sperm come into contact with oviductal epithelial cells that line the female tract and its secretions, some spermatozoa are stored (Hunter, 1981), allowing the selection of sperm with certain qualities (Talevi & Gualtieri, 2010). A highly fertile subpopulation of the original ejaculate will bind to oviductal epithelial cells, mainly in the spermatic reservoir (Gualtieri & Talevi, 2003). Therefore, two functionally distinct sperm subpopulations with the ability to reach and enter into the oviduct can be discerned: i) those bound to the epithelium, which are considered to have been selected because of their high quality (Talevi & Gualtieri, 2010) and found at the bottom of the crypts of the oviductal folds, where they form a reservoir; and ii) those found in the lumen, not bound to the epithelium, and showing membrane modifications or poor vitality (Mburu *et al.*, 1996, 1997; Rodriguez-Martinez *et al.*, 2001; Tienthai *et al.*, 2004). The binding of sperm to oviductal cells is mediated by carbohydrates on the oviductal cell apical membranes and lectin-like molecules on the sperm rostral surface (Gualtieri & Talevi, 2000). During the capacitation of spermatozoa, these lectin-like molecules may be released from the sensitive acrosomal region of the sperm head, allowing the sperm to abandon the epithelium (Töpfer-Petersen *et al.*, 2002) in a sequential process after ovulation (Brüssow *et al.*, 2008) and swim into the ampulla region, where fertilization occurs (Fazeli *et al.*, 1999).

The interaction of oviductal epithelial cells with the spermatozoa has beneficial effects on the sperm functions. Several authors have shown that the coincubation of spermatozoa with oviductal epithelial cells or their conditioned media maintains sperm viability and motility (Zhu *et al.*, 1994; Kervancioglu *et al.*, 2000; Quintero *et al.*, 2005; Munuce *et al.*, 2009; Zumoffen *et al.*, 2010), enhances the fertilization potential by protecting them from oxidative stress (Huang *et al.*, 2013) and even, in some species, stabilizes the sperm chromatin structure (Ellington *et al.*, 1998).

Numerous *in vitro* techniques have been developed for sperm selection and capacitation in an attempt to obtain the best spermatozoa for fertilization (viable and motile) (Matás *et al.*, 2003, 2011; Buffone *et al.*, 2004). All these systems base their selection on sperm motility and morphology, and have certainly improved the yields of IVF. But with the progress of assisted reproduction technologies (ART), these techniques are insufficient to identify the most suitable spermatozoa for fertilization since they do not select sperm populations free of phosphatidylserine translocation.

All the above led as to believe that if an initial selection of sperm could be made in a physical medium such as a Percoll gradient (mimicking the uterus), subsequent selection by means of a biological system such as oviductal cell culture (mimicking the oviduct's action) might provide a more suitable sperm subpopulation for use in ART, particularly when semen quality is poor or when intracytoplasmic sperm injection (ICSI) is to be performed. To develop this hypothesis, IVF was carried out using sperm bound and unbound to oviductal cells. Besides, to determine putative differences between these kinds of spermatozoa, sperm functionality parameters such as tyrosine phosphorylation (TP), phosphatidylserine translocation (PS), DNA fragmentation and chromatin condensation, were be determined.

2. MATERIALS AND METHODS

2.1. Culture media

Unless otherwise indicated, all the chemicals used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). The medium used for oocyte maturation was NCSU-37 supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 µg/ml insulin, 50 µM β-mercaptoethanol, 1 mM glutamine, 10 IU/ml eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml hCG (Chorulon, Intervet International B.V., Boxmeer, Holland), 10 ng/ml EGF and 10% (v/v) porcine follicular fluid (Matás *et al.*, 2003). The fertilization medium was modified TALP supplemented with 3 mg/ml fatty acid-free BSA and 1.10 mM Na-pyruvate (Matás *et al.*, 2003). Oviductal epithelial cells were cultured in TCM 199 with Earle's salts, l-glutamine and $NaHCO_3$ supplemented with 13% (v/v) of foetal calf serum, 150 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Paisley, UK).

2.2. Ethics

The study was carried out in strict accordance with the recommendations in the Guiding Principles for the Care and Use of Animals (DHEW Publication, NIH, 80-23) and was performed following approval by the Veterinary Ethical Committee of University of Murcia. The care and the use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other specifics purposes.

2.3. Culture of porcine oviductal epithelial cells (OEC)

The procedure used to culture OEC was described previously by Ouhibi *et al.* (1991) with the following minor modifications. Oviducts from commercial cycling gilts were recovered from slaughterhouse material. They were rinsed once in saline and twice in

phosphate buffered saline (PBS) at 37°C in the laboratory. In a Petri dish, fat pads and connective tissues were mechanically removed from the oviducts with sterile forceps and fine scissors. The oviducts were closed at one end with a clip, filled with a trypsin-EDTA solution for endothelial cell culture (500 BAAE units of porcine trypsin and 180 µg EDTA), before being closed and incubated for 45 minutes at 38.5°C (Romar *et al.*, 2005).

After incubation, the wall of the oviduct was gently squeezed and its contents flushed into a Petri dish containing pre-equilibrated cell culture medium. The epithelial cell clusters were dissociated by gentle, repeated pipetting followed by centrifugation at 700 *g* for 5 minutes. The supernatant was discarded and the pellet resuspended with fresh cell culture medium and seeded at a final concentration of approximately 1×10^5 cells/ml into Nunc plates (Nunc, Roskilde, Denmark) and cultured at 38.5°C under 5% CO_2. The medium was changed 72 hours after the beginning of the incubation and every 2 days until cell confluence was observed (6-7 days after initial seeding).

2.4. Preparation of spermatozoa

Semen was collected from mature fertile boars (2-4 years old) in an artificial insemination centre using the manual method and a dummy (King & Macpherson, 1973). The sperm-rich fraction was collected in a pre-warmed thermo, while the gel fraction was held on a gauze tissue covering the thermos opening. The semen was then diluted 1:2 with isothermal Beltsville Thawing Solution (BTS) extender (Pursel & Johnson, 1975) and transported to the laboratory. Only high-quality semen samples were used: more than 80% motility, <10% abnormal morphology, <5% damaged acrosomes. Once in the laboratory, sperm cells were selected by centrifugation on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous gradient (45-90%) in a 12 ml conic centrifuge tube, with 2 ml of 45% Percoll layered on top of 2 ml of 90% Percoll. Finally, 0.5 ml diluted semen was added, taking care to avoid mixing the solutions (Matás *et al.*, 2003), and centrifuged at 700 *g* for 30 minutes. The sperm pellet was resuspended in 10 ml TALP medium (Rath *et al.*, 1999) previously pre-equilibrated at 38.5°C in 5% CO_2 in 100% humidified air and later washed by centrifugation at 700 *g* for 10 minutes. Finally, the sperm concentration was adjusted to 1×10^6 spermatozoa/ml.

2.5. Coincubation of spermatozoa with porcine OEC

Nunc plates, with OEC monolayers cultured for 7 days, were washed twice with culture medium. Then, the culture medium was replaced by 2 ml TALP medium, and 1×10^6 washed sperm cells/ml were added. Washed spermatozoa were coincubated with OEC monolayers at 38.5°C and 5% CO_2 in air for 30 minutes. After coincubation, Nunc plates were washed several times with TALP medium to retrieve all the sperm not bound to OEC (NB). The sperm in the supernatant (NB) were collected and replaced with fresh TALP medium.

The number of spermatozoa from the NB group was calculated using a counting chamber. The difference between added and collected sperm was used to estimate the number of sperm remaining bound to the OEC (B group) and was subsequently used to calculate the sperm concentration for IVF.

2.6. In vitro maturation and fertilization

Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were transported at 37°C from a local abattoir to the laboratory in physiological saline containing 100 mg/ml kanamycin. *Cumulus-oocyte* complexes were collected from follicles (3-6 mm in diameter) by aspiration, and washed twice in modified PBS supplemented with 4 mg/ml polyvinyl alcohol (PVA). Oocytes with evenly granulated cytoplasm and several layers of *cumulus oophorus* cells were selected and rinsed twice in maturation medium previously equilibrated for at least 3 hours under 5% CO_2 in maximally humidified air at 38.5°C. Groups of 50 oocytes were matured in 500 µl maturation medium for 22 hours under 5% CO_2 in air at 38.5°C. After the maturation period, the oocytes were washed three times and transferred to hormone-free maturation medium for another 22 hours (Matás *et al.*, 2003).

In vitro fertilization

After maturation, oocytes were mechanically stripped of *cumulus* by gentle aspiration with a pipette. Denuded oocytes were washed three times in TALP medium and groups of 25-30 oocytes were transferred to a Nunc plate containing 2 ml TALP medium previously equilibrated at 38.5°C under 5% CO_2 (Matás *et al.*, 2003, 2010) with or without OEC according to the experiment. The sperm concentration used for IVF was approximately the same in all the experimental groups (W30, B and NB). Among different replicates, the concentration ranged from 3.5 to 5×10^5 cells/ml, depending on the number of sperm bound to OEC in each one.

At 18-20 hours post-insemination, putative zygotes were fixed for 30 minutes (0.5% glutaraldehyde in PBS), stained for 15 minutes (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/ml polyvinylpyrrolidone, mounted on glass slides and examined under an epifluorescence microscope at 400× magnification for evidence of sperm penetration.

2.7. Tyrosine phosphorylation (TP) evaluated by indirect immunofluorescence and Western blot

Immunolocation by indirect immunofluorescence

An immunofluorescence technique was used to determine the localization of proteins phosphorylated in tyrosine residues (Tardif *et al.*, 2001; Matás *et al.*, 2011; Luño *et al.*, 2013). Sperm from the different groups were washed with PBS and centrifuged at 270 *g* for 10 minutes and then fixed in 2% formaldehyde solution for 60 minutes at 4°C. Spermatozoa were washed once in PBS and blocked with 2% (w/v) BSA-PBS and incubated overnight at 4°C before addition of the primary antibody. The sperm were washed and resuspended in PBS, smeared onto a microscope slide, and allowed to dry in air. Slides were then incubated for 1 hour with anti-phosphotyrosine monoclonal antibody at 4°C (clone 4G10, 1:200, Millipore, Madrid, Spain) rinsed with PBS, and incubated for an additional hour with fluorescein-conjugated goat anti-mouse antibody (1:400, Bio-Rad Laboratories, Madrid, Spain). After rinsing with PBS, samples were mounted on the slides with 90% glycerol/PBS (v/v). Sperm were observed with a microscope equipped with fluorescent optics (Leica DMLS, Barcelona, Spain) for antibody detection. After removing the supernatant, the Petri dish samples were processed following a similar protocol (PBS, formaldehyde, primary antibody and secondary antibody added in an amount sufficient to cover the plate and under continuous stirring).

The state of the proteins phosphorylated in the tyrosine residues of sperm bound to the zona pellucida of oocytes was also assessed by indirect immunofluorescence 3 hours after IVF. The protocol employed for this purpose was the same as that used to evaluate TP in spermatozoa, but conjugating the secondary antibody with Alexa Fluor 568 (1:400, Life technologies, Madrid, Spain).

Western blot

Samples were resuspended in lysis buffer and boiled for 5 minutes. Protein extracts equivalent to 1×10^6 sperm were loaded per lane (Jha *et al.*, 2006) and resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, CA, USA). Nonspecific binding sites were blocked by incubation in TBS with 0.1% Tween 20 (TBS-T) and 5% BSA (A-9647, Sigma-Aldrich, Madrid, Spain) for 1 hour, washed in TBS-T and then incubated with anti-phosphotyrosine antibody (4G10, Millipore, Madrid, Spain, 1:10,000) at 4°C overnight. Then, the membrane was washed in TBS-T and incubated with peroxidase conjugate secondary antibody (1721011, Bio-Rad Laboratories, CA, USA, 1:10,000) for 1 hour at room temperature. Protein bands were visualised using an enhanced chemiluminescence detection kit (ECL Plus, Amersham, GE Healthcare) according to the manufacturer's instructions. The Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, CA, USA) was used as a molecular weight standard.

2.8. Evaluation of membrane phosphatidylserine translocation (PS)

Translocation of PS residues to the outer leaflet of the plasma membrane was detected by Annexin V-Cy3^{TM} Apoptosis Detection Kit (Sigma, Madrid, Spain). For this assay, 1µl Annexin V with 5 µl CFDA in 450 µl of binding buffer (commercial kit) was mixed with 50 µl of each sperm sample. After 10 minutes of incubation in the dark, at room temperature, samples were fixed with 10 µl formaldehyde (10% in PBS) (Marti *et al.*, 2008).

2.9. Evaluation of sperm DNA fragmentation by TUNEL

Terminal deoxynucleotidyl transferase-mediated BrdUTP nick-end labelling (TUNEL) was used to determine sperm DNA fragmentation, following the method described previously (Matás *et al.*, 2011). In brief, the cells were concentrated by centrifugation, fixed in a solution of ethanol and PBS (70/30, v/v) for 30 minutes to induce sperm membrane permeabilization, and stored at -20°C until analysis. Cells (approximately 1×10^6) were washed twice with PBS and resuspended in 50 µl of DNA-labelling solution containing 10 µl of reaction buffer, 0.75 µl TdT enzyme, 8.0 µl of BrdUTP (5-Bromo-2'-deoxyuridine 5'-triphosphate) (APO-BrdU™ TUNEL Assay Kit, Invitrogen SA, Barcelona, Spain) and 31.25 µl of dH_2O. The cells were incubated in the DNA-labelling solution for 120 minutes at 37°C. At the end of incubation 1 ml of rinse buffer was added and the mixture was centrifuged twice. Finally, the pellet was incubated with anti-BrdU antibody for 30 minutes at 37°C in a temperature-controlled bath. Negative controls were incubated in the absence of enzyme terminal transferase.

Samples were measured by flow cytometry (Epics XL, Beckman Coulter, L'Hospitalet de Llobregat, Barcelona, Spain). Green fluorescence was collected with an FL1 sensor using a 525 nm band-pass filter, determining two populations.

2.10. Determination of chromatin condensation

Sperm chromatin was stained with propidium iodide (PI) to determine sperm chromatin condensation (Matás *et al.*, 2011). Seminal samples were centrifuged (1200 *g*, 3 min) and the pellet was resuspended in a solution of ethanol and phosphate buffered saline (PBS) (70/30 v/v) for 30 minutes to induce sperm membrane permeabilization. The samples were then centrifuged, the supernatant was discarded and the pellet was resuspended in a PI solution (10 mg/ml) in PBS. Samples were maintained in darkness for 1 hour before flow cytometric analysis. Red PI fluorescence was collected with an FL3 sensor using a 650 nm band-pass filter.

2.11. Experimental design

A semen sample (ejaculated spermatozoa) was washed by centrifugation through discontinuous Percoll gradients and incubated in fertilization medium (TALP) with or without OEC for 30 minutes (Figure 1A). The spermatozoa incubated without OEC was denominated W30 group. From the plates incubated with OEC, we obtained the supernatant with sperm cells unbound to OEC (NB group) and spermatozoa bound to OEC (B group).

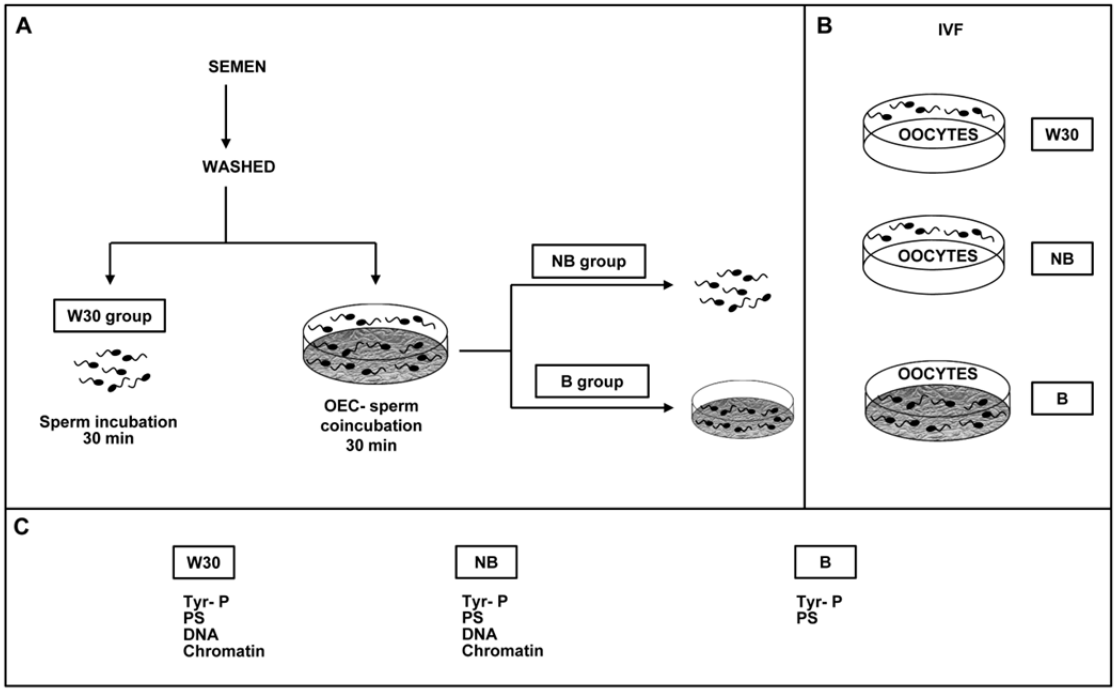

Figure 1. Graphical representation of the experimental design. The shaded plates indicate those with OEC monolayers. **A)** Different experimental groups of spermatozoa. Ejaculated sperm was washed through a Percoll gradient. An aliquot of sperm was incubated in TALP medium without OEC for 30 minutes (**W30 group**). A second sperm aliquot was coincubated with OEC for 30 minutes, and two sperm populations were obtained: sperm bound to OEC (**B group**) and sperm from the supernatant not bound to OEC (**NB group**). **B)** IVF experiment. Sperm from **W30** (**W30 group**) and sperm unbound to OEC (**NB group**) were co-incubated with oocytes in the absence of OEC. In addition, oocytes were co-incubated with sperm bound to OEC (**B group**) (the sperm-oocyte incubation was performed in presence of OEC). After 18 hours of coincubation, IVF parameters were evaluated. **C)** Functionality sperm assays.

Experiment 1. IVF with sperm recovered under different conditions of incubation

Three different experimental groups (W30, NB and B) were established according to the origin of the sperm used for IVF (Figure 1B): i) Spermatozoa from W30 group were incubated with oocytes in the absence of oviductal epithelial cells. ii) Unbound

spermatozoa (NB) were incubated with oocytes, in the absence of OEC. iii) Bound spermatozoa (B) were incubated with oocytes in the OEC plates (IVF carried out on OEC).

Oocytes were collected 18 hours post insemination and were fixed for subsequent evaluation of the IVF parameters. The penetration rate, the number of spermatozoa bound to the zona pellucida, the number of spermatozoa per penetrated oocyte, the number of swollen spermatozoa and male pronuclear formation were assessed for each group (Matás *et al.*, 2003). A total of 5 repetitions were performed, using 689 oocytes.

Experiment 2. Assessment of sperm functionality selected by OEC

Tyrosine protein phosphorylation (TP), plasma membrane phosphatidylserine translocation (PS), sperm DNA fragmentation and chromatin condensation were evaluated in the three experimental sperm groups (W30, NB and B) (Figure 1C). For the sperm groups in suspension (W30 and NB) protein tyrosine phosphorylation (by indirect immunofluorescence and Western blot), PS translocation, DNA fragmentation and chromatin condensation were studied. In the case of sperm from the B group, due to the impossibility of unbinding them, phosphorylation by indirect immunofluorescence and PS translocation were evaluated *in situ*, but it was not possible to perform the rest of the assays.

TP of the sperm proteins evaluated by indirect inmunofluorescence, was classified and grouped into three different categories (Figure 2) according to the patterns given by Luño *et al.* (2013), following a hierarchical sequence of capacitation events, which includes phosphorylation in the different regions of the sperm (acrosome, equatorial subsegment and flagellum). Pattern I includes spermatozoa with no fluorescence signal in the equatorial subsegment (Figure 2-A1), with phosphorylated acrosome (Figure 2-A2), with phosphorylated tail (Figure 2-A3) or with both acrosome and tail phosphorylated (Figure 2-A4). Pattern II includes spermatozoa with a signal in the equatorial subsegment, no signal in the acrosome area and with (Figure 2-B1) or without (Figure 2-B2) a signal in the flagellum. Finally, pattern III includes spermatozoa with signal in the equatorial subsegment and acrosome area, and with (Figure 2-C1) or without (Figure 2-C2) signal in the flagellum. Four repetitions were performed using 1650 spermatozoa.

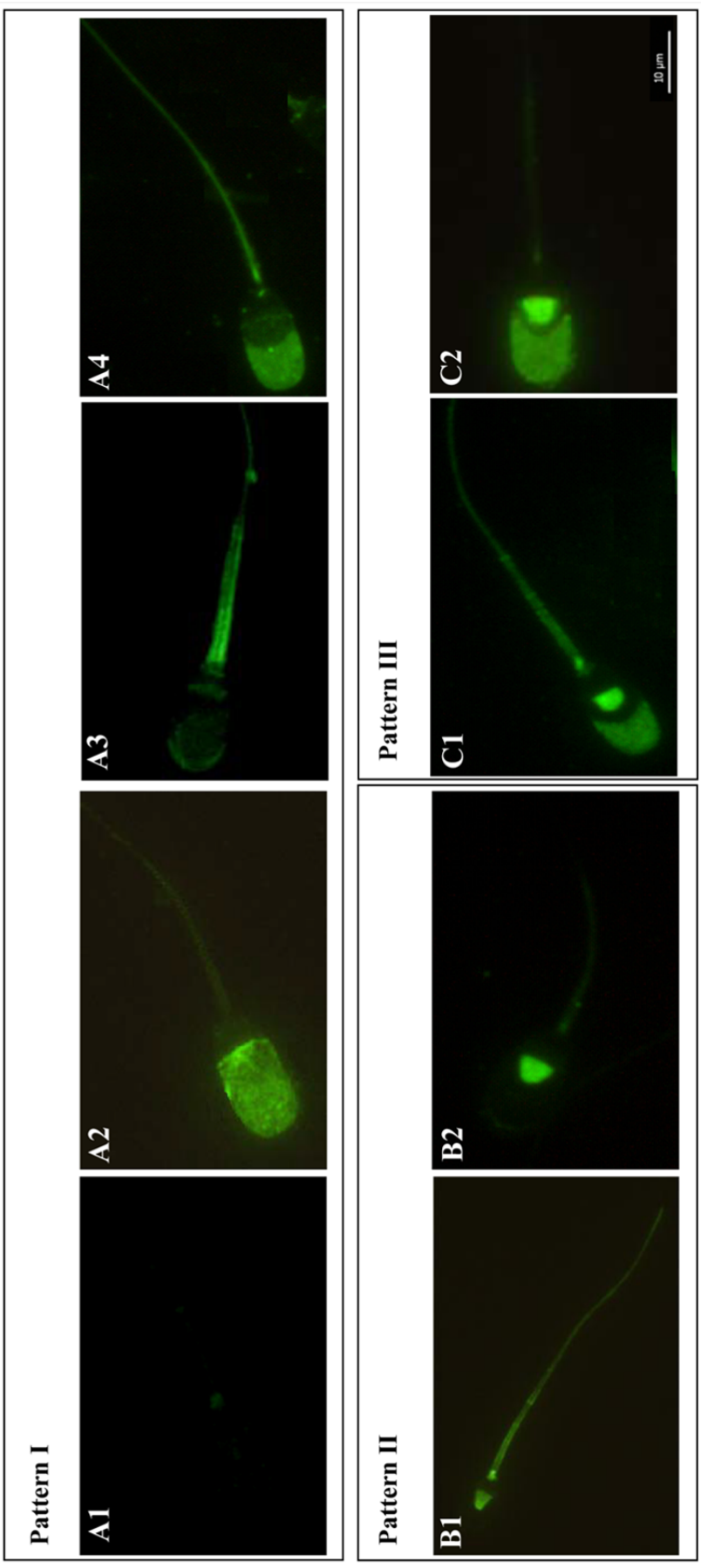

Figure 2. Tyrosine phosphorylation (TP) patterns in boar spermatozoa. Monoclonal anti-phosphotyrosine antibody and FITC-conjugated goat anti-mouse IgG were used to detect TP sperm patterns by indirect immunofluorescence. **Pattern I**: includes spermatozoa without fluorescence (**A1**), with phosphorylated acrosome (**A2**), with phosphorylated tail (**A3**) and with phosphorylated acrosome and tail (**A4**). **Pattern II**: includes spermatozoa with fluorescence in equatorial subsegment with (**B1**) or without (**B2**) the presence of a signal in the flagellum. **Pattern III**: includes spermatozoa with a signal in the equatorial subsegment and acrosome area and with (**C1**) or without (**C2**) the presence of signal in the flagellum.

To check the maximum level of sperm capacitation, which takes place moments before fertilization (Sakkas *et al.*, 2003), we also analysed the localization of proteins phosphorylated in tyrosine residues of sperm bound to the zona pellucida 3 hours after IVF by indirect immunofluorescence (Figure 3B). Three repetitions were performed using 60 oocytes.

In the evaluation of TP proteins by Western blot eight repetitions were performed.

Dual sperm staining was used for PS and the samples were examined by epifluorescence microscopy. Sperm with functional esterases (CFDA+) were visualized in green fluorescence (fluorescein filter) and sperm with PS exposed (Annexin +) in red fluorescence (rhodamine filter) (Marti *et al.*, 2008). Five repetitions were performed using 4800 spermatozoa.

For the DNA fragmentation assay, cells were classified into two categories according to the intensity of fluorescence: low green fluorescence (low DNA damage) and high green fluorescence (high DNA damage). In addition, measurements were expressed as the mean green intensity fluorescence units, which were used as an index of the DNA fragmentation (Matás *et al.*, 2011). A total of 8 repetitions were performed.

For the chromatin condensation assay, measurements were expressed as the mean red intensity fluorescence units, which were used as index of the state of the chromatin condensation, as this is directly related to the PI uptake by DNA. A total of 8 repetitions were performed.

2.12. Statistical analysis

Data are expressed as the mean ± S.E.M. and analysed by ANOVA, considering the specific sperm treatment as the main variable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison *post hoc* test (Tukey). Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Experiment 1. IVF with sperm recovered under different conditions of incubation

IVF provides information on gamete interaction and is the ultimate goal for many reproductive studies. For this reason, IVF was performed using sperm from different incubation conditions (Figure 1B).

The studied IVF parameters changed depending on the origin of the sperm used (W30, NB or B) (Table 1). The penetration rate (PEN) significantly differed among the three study groups, being highest for the sperm bound to oviductal cells (B group: 94.9%), followed by the W30 group (62.9%) and finally the NB group (19.6%) ($p < 0.05$).

The number of spermatozoa bound to the zona pellucida (SPZ/ZP) followed the same trend (B group: 52.9%, W30: 25.7% and NB: 12.4%; $p < 0.05$). However, although binding to the zona pellucida was greatest in the group B, the number of spermatozoa per penetrated oocyte (SPZ/O) was similar to that observed in the washed spermatozoa (W30), and higher than in the NB group. Although male pronuclear formation was observed in all groups studied, the number of swollen spermatozoa (SWO) was lower in the NB group than in the others (Table 1, $p < 0.05$).

Table 1. IVF outcomes using different sperm sources: **W30 group**: sperm after 30 minutes of incubation without oviductal epithelial cells (OEC); **NB group**: sperm not bound to OEC after previous coincubation of 30 minutes. For W30 and NB groups IVF was performed in the absence of OEC; **B group**: sperm bound to OEC after a previous coincubation of 30 minutes; IVF was performed in the presence of OEC (data shown mean ± S.E.M.).

Sperm source	N	PEN (%)	SPZ/ZP	SPZ/O*	SWO*
W30	200	62.9 ± 4.2^{a}	25.7 ± 1.8^{a}	6.1 ± 0.5^{a}	4.1 ± 0.5^{a}
NB	270	19.6 ± 2.4^{b}	12.4 ± 0.7^{b}	1.5 ± 0.1^{b}	0.4 ± 0.1^{b}
B	219	94.9 ± 1.5^{c}	52.9 ± 2.7^{c}	6.1 ± 0.4^{a}	4.4 ± 0.4^{a}

N: number of oocytes used for fertilization ability (five replicates); **PEN**: rate of penetration; **SPZ/ZP**: mean number of sperm bound to zona pellucida; **SPZ/O**: mean number of spermatozoa per penetrated oocyte; **SWO**: swollen spermatozoa.

*Based on penetrated oocytes.

Different subscript letters (a, b, c) in the same column indicate significant differences ($p < 0.05$).

3.2. Experiment 2. Assessment of sperm functionality selected by OEC

A great difference was found in the phosphorylation patterns (patterns II and III) (measured by indirect immunofluorescence) between the sperm subpopulations of the bound (B) and unbound (NB) groups (Figure 3A) ($p < 0.05$).

When sperm were incubated in the absence of OEC (W30) the principal pattern exhibited was pattern II, followed by pattern III. However, pattern II was much more prevalent and pattern III much less prevalent in B than in NB and W30. The unbound sperm group (NB) showed a high level of TP in pattern III. This pattern III was significantly reduced when sperm cells bound to OEC (group B). The localization of

phosphorylated proteins showed that coincubation with OEC modifies the sperm staining pattern. No differences were observed in pattern I among the experimental groups.

Finally, spermatozoa bound to the zona pellucida of oocytes had a high level of protein phosphorylation (pattern III), i.e. with a signal in the acrosome, equatorial subsegment and tail area (Figure 3B, arrows).

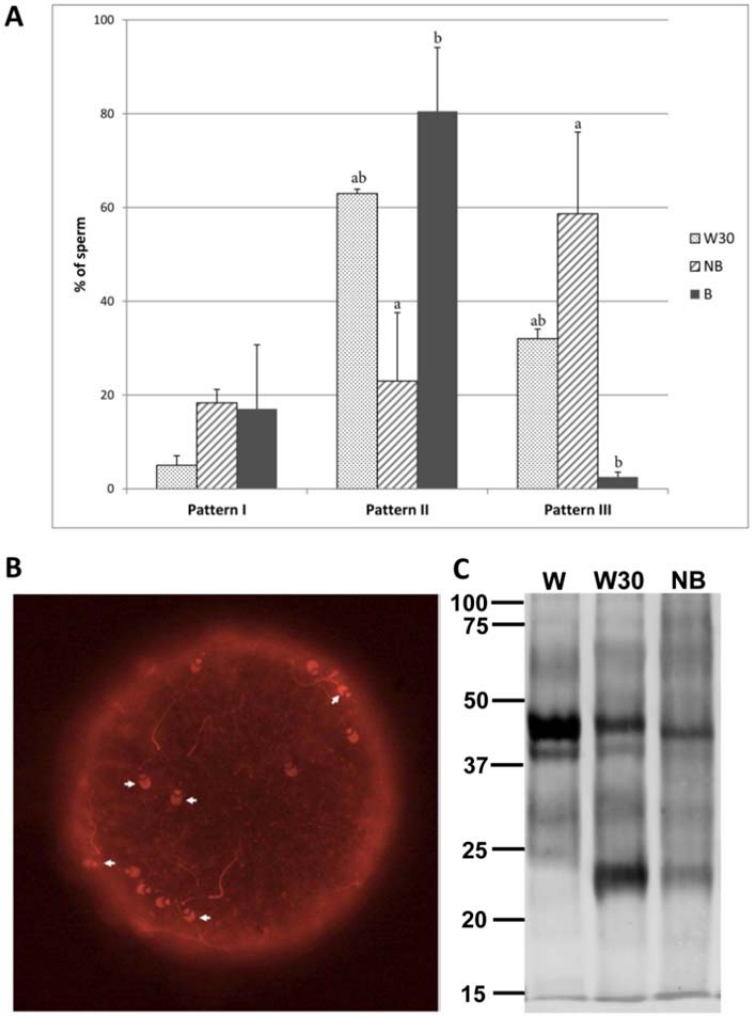

Figure 3. Protein tyrosine phosphorylation (TP) in isolated sperm (previously co-incubated or not with OEC), sperm bound to OEC, sperm bound to zona pellucida or whole sperm population. **A)** Immunolocation by indirect immunofluorescence. Data shown mean ± S.E.M. and the letters a, b in different bars denote significant differences ($p < 0.05$). **B)** Tyrosine phosphorylation in boar spermatozoa bound to zona pellucida of oocytes. Monoclonal anti-phosphotyrosine antibody and Alexa Fluor 568 goat anti-mouse IgG were used in order to detect TP by indirect immunofluorescence. Arrows show spermatozoa with a high level of protein phosphorylation (pattern III). **C)** Western-blotting detection. Molecular weight is expressed in kDa. **W**: washed spermatozoa without incubation. **W30**: washed spermatozoa after 30 minutes of incubation. **NB**: spermatozoa not bound to OEC after 30 minutes of coincubation.

Besides location, we identified the molecular weights of the phosphorylated proteins in the different sperm experimental groups by western blot (Figure 3C), since a single location may contain proteins of different molecular weights. As occurred with phosphorylated proteins evaluated by immunocytochemistry, bands with different signal patterns between the experimental groups were observed by western blot. Note that a band of approximately 45 kDa had a strong signal in the W group but faded after 30 minutes of incubation (W30) and was even weaker in the unbound sperm group (NB). Something similar occurred in another band of 40 kDa. Furthermore, after 30 minutes, washed sperm (line 2, W30) showed a phosphorylated protein band with a molecular weight of about 23 kDa, which was absent before incubation (line 1, W). This phosphorylation band also appeared in the NB sperm (line 3), but with much less intensity. Overall, the unbound sperm group (line 3, NB) showed lower intensity/signal bands than the other groups.

When the translocation of phosphatidylserine (PS), a marker of sperm capacitation and/or apoptosis, was analysed for the different sperm sources, significant differences were observed (Figure 4). The levels of PS reached 44.2% in the W30 group and 93.8% in the unbound sperm group (NB). Conversely, sperm that remained bound to OEC (B) had a PS translocation of 5.8%, which is significantly lower than the other sperm groups ($p < 0.05$).

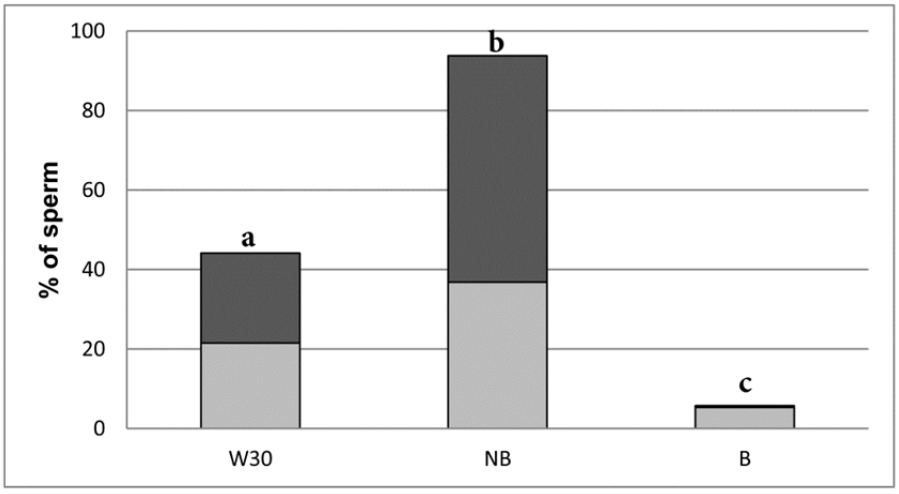

Figure 4. Phosphatidylserine translocation of sperm labelled with Annexin V-Cy3. The bars show percentage of annexin positive sperm. The light grey colour corresponds to the CFDA positive and dark grey correspond to CFDA negative. **W30**: washed spermatozoa after 30 minutes of incubation. **NB**: spermatozoa not bound to OEC after 30 minutes of coincubation. **B**: spermatozoa bound to OEC after 30 minutes of coincubation. Data shown mean ± S.E.M. and the letters a, b, c in different bars denote significant differences ($p < 0.01$).

The number of spermatozoa (CFDA +/annexin +) compared with the total number of spermatozoa (annexin +) decreased slightly with the time of incubation (W30: 48.6%; Figure 4). However this distribution was greatly impaired in groups of sperm coincubated with OEC, while 91.4% of the sperm were CFDA and annexin positive in group B, a value that fell to 39.2% in unbound sperm (NB).

The degree of chromatin condensation measured by PI staining was higher in the unbound group (NB) than in W30 (17.8 ± 0.9 *vs* 23.3 ± 0.8; $p < 0.05$), and inversely related with PI uptake by DNA. When DNA damage was measured by TUNEL in these groups, no differences were found (5.60 ± 0.69 *vs* 4.46 ± 0.75).

4. DISCUSSION

The insemination of large numbers of sperm translates into a selection of a few highly fertile sperm able to reach and interact with the oviduct and subsequently with oocytes. For this, complex natural sperm selection mechanisms, beside the well known barriers in the female tract, are essential. However, although some factors have been identified as fundamental for spermatozoa to reach the sperm reservoir (revised by Soriano-Úbeda *et al.*, 2013), how exactly oviductal cells select the best sperm for successful fertilization remains unknown.

When sperm are prepared for use in IVF for ART, seminal plasma is removed in order to select the most suitable subpopulation of sperm for fertilization purposes (Matás *et al.*, 2011). Although this process attempts to mimic what happens *in vivo*, the final performance, in terms of the quality of embryos produced, is far lower.

Various investigators have characterized some aspects of the superior sperm quality associated with oviductal binding (revised by Holt & Fazeli, 2010), concluding that sperm-oviduct binding plays a key role in the maintenance of sperm fertilizing ability. So, an IVF system based on OEC incubation and further collection of the sperm bound to OEC should be of great use for selecting the best spermatozoa for ART, an ICSI technique widely used in human infertility clinics. In this study, we aim to evaluate the fertilizing capacity of spermatozoa, previously selected by Percoll gradient, incubated in OEC. IVF was carried out with sperm bound and unbound to the OEC and some characteristics of sperm that could explain the fertility results obtained were determined.

The IVF results show that sperm bound to the OEC (B group) were able to fertilize oocytes to a greater extent than those not are in contact with the OEC (W30 group). This was expected because other authors had already mentioned the same (Romar *et al.*, 2005). However, we did not expect the unbound sperm group (NB) to have such low levels of fertilization. We believe that sperm not bound to OEC are less capable of

fertilizing due to the high levels of capacitation, as seen in experiment 2, and subsequent apoptosis (Aitken, 2011). The number of spermatozoa bound to the zona pellucida showed the same trend as the penetration rate (highest values in the B group). The ability of sperm to bind to the zona pellucida is one of the most important characteristics in the sperm fertilizing capacity (Oehninger *et al.*, 1997). Defective binding of spermatozoa to the zona pellucida reflects multiple sperm dysfunctions (Larsson & Rodriguez-Martinez, 2000), which would be consistent with decreased penetration rates. Spermatozoa with high levels of capacitation (Oehninger *et al.*, 1997) or loss of plasma membrane functions (Barroso *et al.*, 2000), as shown here by NB sperm, could contribute to a low incidence of sperm-zona pellucida binding, which would affect the whole process of fertilization. In the same way, it seems to be a prerequisite for such sperm-zona pellucida interaction that spermatozoa must bind to the OEC (Waberski *et al.*, 2005), because this enables the spermatozoa to penetrate the oocytes investments (Hunter, 1984).

Although male pronuclear formation was evident in all the groups studied, the number of swollen spermatozoa (SWO) was lower in the NB group than in the others. Despite problems with binding to the zona pellucida and penetrating oocytes, some NB sperm are able to penetrate oocytes and can form male pronucleus because the oocyte has the capacity to repair pre-existing damage in the sperm (Evenson *et al.*, 2002). However, in some cases, this oocyte repair capacity may be insufficient to repair highly damaged sperm, resulting in non-swollen spermatozoa. The IVF results obtained support the hypothesis that OEC select the best spermatozoa for fertilization, although many of the qualities which spermatozoa must present to be selected by the OEC remain unknown.

It is well documented that sperm protein phosphorylation is associated with capacitation (Visconti *et al.*, 1995) and that sperm treatment prior to IVF increases the percentage of cells with phosphorylated tyrosine (Matás *et al.*, 2011). Besides, the sperm areas that show tyrosine phosphorylation depend on their capacitation status. In boar sperm, the equatorial area is the most important area for tyrosine phosphorylation (Jones *et al.*, 2008), but it is the phosphorylation of the acrosome which reflects high levels of sperm capacitation (Luño *et al.*, 2013). Our results show that an important factor in sperm selection by the OEC is the capacitation status of the sperm population, as mentioned by other authors (Lefebvre & Suarez, 1996; Fazeli *et al.*, 1999; Luño *et al.*, 2013). The sperm population bound to the epithelial cell population showed a degree of fluorescence in the equatorial segment but no fluorescence in the acrosomal area. However, in sperm unbound to the cells (NB group), this pattern was reversed, i.e. they showed fluorescence in the acrosomal area. In human spermatozoa, it has been established that a high degree of TP is considered a prerequisite for fertilization (Sakkas *et al.*, 2003). In this way, sperm must exhibit the

highest degree of phosphorylation at the moment prior to contact with the zona pellucida of the oocyte to activate the acrosome reaction. Conversely, if these levels are reached earlier, far from the oocyte, sperm initiate the apoptosis pathway.

In this paper, the sperm with the highest level of protein TP (pattern III) were those in the NB group, which, nevertheless, was the group with the lowest percentage of penetration, precisely because they reached a high level of phosphorylation very early. However, when we analyzed the pattern of TP in sperm bound to the zona pellucida, like Sakkas *et al.* (2003), the highest level of phosphorylation was exhibited (pattern III: acrosome, equatorial subsegment and flagellum).

On the one hand, during the capacitation process, there is a redistribution of tyrosine-phosphorylated proteins localized in the acrosomal region of boar sperm (Flesch *et al.*, 2001) and, on the other, the oviductal epithelium secretes proteins capable of reversing sperm capacitation (Ghersevich *et al.*, 2015). This redistribution is also evident in the western blot, where the incubation of washed spermatozoa leads to the appearance of new bands due to increased levels of phosphorylation of the principal sperm pieces (Harayama *et al.*, 2004), mainly the equatorial subsegment. The same bands were also found in the NB group, indicating that phosphorylation levels were also increased. This protein redistribution may help explain the change in the intensity of the observed bands. However, we cannot affirm this because only localization patterns were analysed without distinguishing the intensities of fluorescence, so the amount of phosphorylated protein in each piece was not ascertained. We suggest that the changes observed in sperm phosphorylation after coincubation with OEC are not only due to the redistribution of proteins but also to modifications in phosphorylation-dephosphorylation in the proteins. It is still unknown whether OEC select the sperm bound to them based on other criteria and then adjust their levels of phosphorylation, or whether only sperm with a low capacitation pattern are bound.

It is still a matter of controversy too, whether PS translocation is due to capacitation (Barroso *et al.*, 2000; Duru *et al.*, 2001; de Vries *et al.*, 2003; Martin *et al.*, 2005), the apoptosis process (Aitken & Baker, 2013) or both (Aitken, 2011). Our results showed significant differences in levels of PS translocation between the different experimental groups of sperm (the lowest values were observed in the B group), indicating that OEC exercise a selective function and that binding does not occur randomly. It is not surprising that after 30 minutes of incubation, the percentage of spermatozoa with PS translocation increased (Kotwicka *et al.*, 2002) due to an increase in the capacitated sperm population in the sample, which leads to destabilization of the membrane and thus the translocation of PS (Vermes *et al.*, 1995). Accordingly, if OEC bind mainly uncapacitated spermatozoa (Fazeli *et al.*, 1999), this population in our study would correspond to spermatozoa without PS translocation (annexin -), while sperm with PS translocation (annexin +) showed not be able to bind to OEC, remaining in the

supernatant after coincubation, because they are capacitated or apoptotic. OEC would also be able to discriminate between annexin negative spermatozoa, with their morphologically superior quality, and annexin positive sperm (Hoogendijk *et al.*, 2009). Moreover, PS translocation is associated with a decrease in the adenosine triphosphate (ATP) level of spermatozoa (Kotwicka *et al.*, 2013) and with a lower mitochondrial potential (Barroso *et al.*, 2006), and so the OEC do not bind these spermatozoa because they have insufficient energy reserves to remain in the sperm reservoir and perform fertilization. Another explanation is that phospholipids (including PS) are incorporated into the sperm membrane by OEC (Evans *et al.*, 1980) as a selection mechanism. This mechanism would result in a negative selection, similar to that reported previously (Teijeiro *et al.*, 2011), for detecting early phases of disturbed membrane functions and marking for elimination those sperm which probably have a reduced life span (Watson, 1995, 2000), and hence are less suitable for storage in the oviduct because they have ceased to be functional. This could explain the increase in PS translocation observed in NB sperm. Nevertheless, the mechanisms that lead OEC to select and bind with live spermatozoa without phosphatidylserine translocation in the plasma membrane (annexin -) remains unknown.

When reproduction assisted techniques are used, it is necessary to select sperm in an attempt to eliminate sperm with poor quality and seminal plasma, despite the prejudicial effects this may have. In this sense, it has been shown that during centrifugation to remove seminal plasma ROS are generated and these, in turn, induce capacitation, apoptosis and DNA damage (Aitken & Koppers, 2011). In this study, we observed that washing sperm through a Percoll gradient induced chromatin decondensation. After 30 minutes of incubating sperm in a medium with OEC, the sperm that had not been bound to the OEC had the same chromatin condensation as ejaculated spermatozoa (S) even though the sperm had been selected through a Percoll gradient, leaving only sperm of the highest quality (Matás *et al.*, 2011). It has been shown that during sperm capacitation chromatin decondensation occurs, which prepares the sperm for the subsequent formation of pronucleus after fertilization (de Lamirande *et al.*, 2012). The reason for the greater compactness of the unbound sperm is unknown although it has been attributed to defects in the disulphide bonds of proteins to prevent chromatin decondensation. This hypothesis is reinforced by the IVF results since the unbound sperm showed the lowest level of nucleus decondensation in oocyte (SWO). In this regard, it has been shown that alterations in the protamine packing of sperm DNA may contribute to a delay in the initiation of the zygotic S-phase (Eid *et al.*, 1994), increase the length of the zygotic G2-phase (Eid & Parrish, 1995), and block blastocyst formation (Fatehi *et al.*, 2006). On the other hand, Ardón *et al.* (2008) suggested that chromatin instability may be associated with plasma membrane characteristics that hinder the binding of such sperm to the oviduct.

Another important factor related to the nucleus is DNA fragmentation and its effect on fertility. Although there is still controversy regarding this, it has been shown that DNA fragmentation in sperm and poor quality samples increase and are correlated with failed fertilization in intracytoplasmic sperm injection (Lopes *et al.*, 1998). However, in normozoospermic samples fertility failure has been observed caused by spermatozoa with damaged DNA (Aitken & De Iuliis, 2007). The results presented in this paper show that spermatozoa selected by Percoll gradient showed less DNA fragmentation. However, after 30 minutes of incubation with or without OEC, DNA fragmentation was in fact unaffected, so selection with Percoll could be efficient because the selective effect by the OEC was not evident in the case of DNA fragmentation.

Although sperm selection by density gradient initially selects the best spermatozoa from a sperm population, such selection is insufficient to identify the most suitable spermatozoon for the subsequent fertilization process. This paper shows that oviductal epithelial cells (OEC) exert a more rigorous second selection. We conclude that it is OEC that are responsible for real spermatic selection. Such selection by OEC could in the future represent a solution to obtaining the best spermatozoa for fertilization and embryo development. Using a non-invasive technique that does not damage or impair the sperm for later use would avoid the subjective selection that currently exists in conventional sperm selection techniques.

ACKNOWLEDGEMENTS

The authors thank Virginia Reig for her assistance at the start of the experiments. We also thank Rodrigo Diego for his technical support with the use of Annexin V-Cy3^{TM} and Juan Carvajal for technical assistance. This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER), Grant AGL2012-40180-C03-01.

REFERENCES

Aitken RJ & De Iuliis GN (2007). Origins and consequences of DNA damage in male germ cells. *Reproductive BioMedicine Online.* 14: 727-733. (doi: 10.1016/S1472-6483(10)60676-1).

Aitken RJ (2011). The capacitation-apoptosis highway: oxysterols and mammalian sperm function. *Biology of reproduction.* 85: 9-12. (doi: 10.1095/biolreprod.111.092528).

Aitken RJ & Koppers AJ (2011). Apoptosis and DNA damage in human spermatozoa. *Asian Journal of Andrology.* 13: 36-42. (doi: 10.1038/aja.2010.68).

Aitken RJ & Baker MA (2013). Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. *The international journal of developmental Biology.* 57: 265-272. (doi: 10.1387/ijdb.130146ja).

Ardón F, Helms D, Sahin E, Bollwein H, Töpfer-Petersen E & Waberski D (2008). Chromatin-unstable boar spermatozoa have little chance of reaching oocytes *in vivo*. *Reproduction.* 135: 461-470. (doi: 10.1530/REP-07-0333).

Barroso G, Morshedi M & Oehninger S (2000). Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Human reproduction.* 15: 1338-1344. (doi: 10.1093/humrep/15.6.1338).

Barroso G, Taylor S, Morshedi M, Manzur F, Gaviño F & Oehninger S (2006). Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. *Fertility and sterility.* 85: 149-154. (doi: 10.1016/j.fertnstert.2005.06.046).

Brüssow KP, Ratky J & Rodriguez-Martinez H (2008). Fertilization and early embryonic development in the porcine fallopian tube. *Reproduction in Domestic Animals.* 43: 245-251. (doi: 10.1111/j.1439-0531.2008.01169.x).

Buffone M, Doncel G, Briggiler CM, Vazquez-Levin M & Calamera J (2004). Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation. *Human reproduction.* 19: 139-146. (doi: 10.1093/humrep/deh040).

de Lamirande E, Gabriel M & Zini A (2012). Human sperm chromatin undergoes physiological remodeling during *in vitro* capacitation and acrosome reaction. *Journal of andrology.* 33: 1025-1035. (doi: 10.2164/jandrol.111.015982).

de Vries K, Wiedmer T, Sims P & Gadella BM (2003). Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biology of reproduction.* 68: 2122-2134. (doi: 10.1095/biolreprod.102.012500).

Duru NK, Morshedi MS, Schuffner A & Oehninger S (2001). Cryopreservation-thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *Journal of andrology.* 22: 646-651. (doi: 10.1002/j.1939-4640.2001.tb02225.x).

Eid L, Lorton S & Parrish J (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biology of reproduction.* 51: 1232-1237. (doi: 10.1095/biolreprod51.6.1232).

Eid L & Parrish J (1995). Duration of G2-phase and onset of M-phase during the first cell cycle of the bovine embryo is dependent on bull *in vivo* fertility. *Theriogenology.* 43: 205. (doi: 10.1016/0093-691X(95)92359-H).

Ellington JE, Evenson DP, Fleming JE, Brisbois RS, Hiss GA, Broder SJ & Wright RW (1998). Coculture of human sperm with bovine oviduct epithelial cells decreases sperm chromatin structural changes seen during culture in media alone. *Fertility and sterility.* 69: 643-649. (doi: 10.1016/S0015-0282(98)00023-5).

Evans RW, Weaver DE & Clegg ED (1980). Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, in utero-, and *in vitro*-incubated porcine spermatozoa. *Journal of lipid research.* 21: 223-228.

Evenson DP, Larson KL & Jost LK (2002). Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other

techniques. *Journal of andrology.* 23: 25-43. (doi: 10.1002/j.1939-4640.2002.tb02599.x).

Fatehi A, Bevers M, Schoevers E, Roelen B, Colenbrander B & Gadella BM (2006). DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. *Journal of andrology.* 27: 176-188. (doi: 10.2164/jandrol.04152).

Fazeli A, Duncan A, Watson P & Holt WV (1999). Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of reproduction.* 60: 879-886. (doi: 10.1095/biolreprod60.4.879).

Flesch F, Wijnand E, Van de Lest C, Colenbrander B, van Golde LM & Gadella BM (2001). Capacitation dependent activation of tyrosine phosphorylation generates two sperm head plasma membrane proteins with high primary binding affinity for the zona pellucida. *Molecular reproduction and development.* 60: 107-115. (doi: 10.1002/mrd.1067).

Ghersevich S, Massa E & Zumoffen C (2015). Oviductal secretion and gamete interaction. *Reproduction.* 149: R1-R14. (doi: 10.1530/REP-14-0145).

Gualtieri R & Talevi R (2000). *In vitro*-cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. *Biology of reproduction.* 62: 1754-1762. (doi: 10.1095/biolreprod62.6.1754).

Gualtieri R & Talevi R (2003). Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium *in vitro*. *Reproduction.* 125: 251-258. (doi: 10.1530/rep.0.1220305).

Harayama H, Muroga M & Miyake M (2004). A cyclic adenosine 3',5'-monophosphate-induced tyrosine phosphorylation of syk protein tyrosine kinase in the flagella of boar spermatozoa. *Molecular reproduction and development.* 69: 436-447. (doi: 10.1002/mrd.20176).

Holt WV & Fazeli A (2010). The oviduct as a complex mediator of mammalian sperm function and selection. *Molecular reproduction and development.* 77: 934-943. (doi: 10.1002/mrd.21234).

Hoogendijk CF, Kruger TF, Bouic PJ & Henkel RR (2009). A novel approach for the selection of human sperm using annexin V-binding and flow cytometry. *Fertility and sterility.* 91: 1285-1292. (doi: 10.1016/j.fertnstert.2008.01.042).

Huang VW, Zhao W, Lee C-L, Lee CY, Lam KK, Ko JK, Yeung WS, Ho P-C & Chiu PC (2013). Cell membrane proteins from oviductal epithelial cell line protect human spermatozoa from oxidative damage. *Fertility and sterility.* 99: 1444-1452. e1443. (doi: 10.1016/j.fertnstert.2012.11.056).

Hunter RHF (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *Journal of reproduction and fertility.* 63: 109-117. (doi: 10.1530/jrf.0.0630109).

Hunter RHF (1984). Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct. *Journal of reproduction and fertility.* 72: 203-211. (doi: 10.1530/jrf.0.0720203).

Jha K, Salicioni A, Arcelay E, Chertihin O, Kumari S, Herr J & Visconti P (2006). Evidence for the involvement of proline-directed serine/threonine phosphorylation in sperm capacitation. *Molecular human reproduction.* 12: 781-789. (doi: 10.1093/molehr/gal085).

Jones R, James PS, Oxley D, Coadwell J, Suzuki-Toyota F & Howes EA (2008). The equatorial subsegment in mammalian spermatozoa is enriched in tyrosine phosphorylated proteins. *Biology of reproduction.* 79: 421-431. (doi: 10.1095/biolreprod.107.067314).

Kervancioglu ME, Saridogan E, Aitken RJ & Djahanbakhch O (2000). Importance of sperm-to-epithelial cell contact for the capacitation of human spermatozoa in fallopian tube epithelial cell cocultures. *Fertility and sterility.* 74: 780-784. (doi: 10.1016/S0015-0282(00)01514-4).

King G & Macpherson J (1973). A comparison of two methods for boar semen collection. *Journal of animal science.* 36: 563-565. (doi: 10.2134/jas1973.363563x).

Kotwicka M, Jendraszak M & Warchoł J (2002). Plasma membrane translocation of phosphatidylserine in human spermatozoa. *Folia histochemica et cytobiologica/Polish Academy of Sciences, Polish Histochemical and Cytochemical Society.* 40: 111-112.

Kotwicka M, Jendraszak M, Skibinska I, Jedrzejczak P & Pawelczyk L (2013). Decreased motility of human spermatozoa presenting phosphatidylserine membrane translocation-cells selection with the swim-up technique. *Human cell.* 26: 28-34. (doi: 10.1007/s13577-011-0024-1).

Larsson B & Rodriguez-Martinez H (2000). Can we use *in vitro* fertilization tests to predict semen fertility? *Animal Reproduction Science.* 60: 327-336. (doi: 10.1016/S0378-4320(00)00089-0).

Lefebvre R & Suarez SS (1996). Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biology of reproduction.* 54: 575-582. (doi: 10.1095/biolreprod54.3.575).

Lopes S, Sun J-G, Jurisicova A, Meriano J & Casper RF (1998). Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertility and sterility.* 69: 528-532. (doi: 10.1016/S0015-0282(97)00536-0).

Luño V, López-Úbeda R, García-Vázquez FA, Gil L & 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: 315-324. (doi: 10.1530/REP-13-0159).

Marti E, Perez-Pe R, Colas C, Muino-Blanco T & Cebrian-Perez J (2008). Study of apoptosis-related markers in ram spermatozoa. *Animal Reproduction Science.* 106: 113-132. (doi: 10.1016/j.anireprosci.2007.04.009).

Martin G, Sabido O, Durand P & Levy R (2005). Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. *Human reproduction.* 20: 3459-3468. (doi: 10.1093/humrep/dei245).

Matás C, Coy P, Romar R, Marco M, Gadea J & Ruiz S (2003). Effect of sperm preparation method on *in vitro* fertilization in pigs. *Reproduction.* 125: 133-141. (doi: 10.1530/rep.0.1250133).

Matás C, Sansegundo M, Ruiz S, García-Vázquez F, Gadea J, Romar R & Coy P (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. *Theriogenology.* 74: 1327-1340. (doi: 10.1016/j.theriogenology.2010.06.002).

Matás C, Vieira L, García-Vázquez F, Avilés-López K, López-Úbeda R, Carvajal J & Gadea J (2011). Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. *Animal Reproduction Science.* 127: 62-72. (doi: 10.1016/S0015-0282(99)00013-8).

Mburu J, Einarsson S, Lundeheim N & Rodriguez-Martinez H (1996). Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. *Animal Reproduction Science.* 45: 109-121. (doi: 10.1016/S0378-4320(96)01566-7).

Mburu J, Rodriguez-Martinez H & Einarsson S (1997). Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. *Animal Reproduction Science.* 47: 137-148. (doi: 10.1016/S0378-4320(96)01631-4).

Munuce MJ, Serravalle A, Caille AM, Zumoffen C, Botti G, Cabada M & Ghersevich S (2009). Human tubal secretion can modify the affinity of human spermatozoa for the zona pellucida. *Fertility and sterility.* 91: 407-413. (doi: 10.1016/j.fertnstert.2007.11.077).

Oehninger S, Mahony M, Özgür K, Kolm P, Kruger T & Franken D (1997). Clinical significance of human sperm-zona pellucida binding. *Fertility and sterility.* 67: 1121-1127. (doi: 10.1016/S0015-0282(97)81449-5).

Ouhibi N, Benet G & Menezo Y (1991). Fetal bovine oviduct epithelial cell monolayers: method of culture and identification. *Journal of tissue culture methods.* 13: 289-294.

Petrunkina AM, Gehlhaar R, Drommer W, Waberski D & Töpfer-Petersen E (2001). Selective sperm binding to pig oviductal epithelium *in vitro*. *Reproduction.* 121: 889-896. (doi: 10.1530/rep.0.1210889).

Pursel V & Johnson L (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *Journal of animal science.* 40: 99-102.

Quintero I, Ghersevich S, Caille A, Munuce MJ, Daniele S & Morisoli L (2005). Effects of human oviductal *in vitro* secretion on spermatozoa and search of sperm–oviductal proteins interactions. *International journal of andrology.* 28: 137-143. (doi: 10.1111/j.1365-2605.2005.00525.xv).

Rath D, Long C, Dobrinsky J, Welch G, Schreier L & Johnson L (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *Journal of animal science.* 77: 3346-3352. (doi: 1999.77123346x).

Rodriguez-Martinez H, Tienthai P, Suzuki K, Funahashi H, Ekwall H & Johannisson A (2001). Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reproduction (Cambridge, England) Supplement.* 58: 129-145.

Romar R, Coy P, Gadea J & Rath D (2005). Effect of oviductal and cumulus cells on zona pellucida and cortical granules of porcine oocytes fertilized *in vitro* with epididymal spermatozoa. *Animal Reproduction Science.* 85: 287-300. (doi: 10.1016/j.anireprosci.2004.04.040).

Sakkas D, Leppens-Luisier G, Lucas H, Chardonnens D, Campana A, Franken D & Urner F (2003). Localization of tyrosine phosphorylated proteins in human sperm and relation to capacitation and zona pellucida binding. *Biology of reproduction.* 68: 1463-1469. (doi: 10.1095/biolreprod.102.011023).

Soriano-Úbeda C, Matás C & García-Vázquez F (2013). An overview of swine artificial insemination: retrospective, current and prospective aspects. *Journal of Experimental and Applied Animal Sciences.* 1: 67-97.

Talevi R & Gualtieri R (2010). Molecules involved in sperm-oviduct adhesion and release. *Theriogenology.* 73: 796-801. (doi: 10.1016/j.theriogenology.2009.07.005).

Tardif S, Dubé C, Chevalier S & Bailey JL (2001). Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of reproduction.* 65: 784-792. (doi: 10.1095/biolreprod65.3.784).

Teijeiro JM, Dapino DG & Marini PE (2011). Porcine oviduct sperm binding glycoprotein and its deleterious effect on sperm: a mechanism for negative selection of sperm? *Biological research.* 44: 329-337.

Tienthai P, Johannisson A & Rodriguez-Martinez H (2004). Sperm capacitation in the porcine oviduct. *Animal Reproduction Science.* 80: 131-146. (doi: 10.1016/S0378-4320(03)00134-9).

Töpfer-Petersen E, Wagner A, Friedrich J, Petrunkina AM, Ekhlasi-Hundrieser M, Waberski D & Drommer W (2002). Function of the mammalian oviductal sperm reservoir. *Journal of Experimental Zoology.* 292: 210-215. (doi: 10.1002/jez.1157).

Vermes I, Haanen C, Steffens-Nakken H & Reutellingsperger C (1995). A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *Journal of immunological methods.* 184: 39-51. (doi: 10.1016/0022-1759(95)00072-I).

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS (1995). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development.* 121: 1129-1137.

Waberski D, Magnus F, Ferreira FM, Petrunkina AM, Weitze K & Töpfer-Petersen E (2005). Importance of sperm-binding assays for fertility prognosis of porcine spermatozoa. *Theriogenology.* 63: 470-484. (doi: 10.1016/j.theriogenology.2004.09.025).

Waberski D, Magnus F, Ardon F, Petrunkina AM, Weitze K & Töpfer-Petersen E (2006). Binding of boar spermatozoa to oviductal epithelium *in vitro* in relation to sperm morphology and storage time. *Reproduction.* 131: 311-318. (doi: 10.1530/rep.1.00814).

Watson P (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, fertility and development.* 7: 871-891. (doi: 10.1071/RD9950871).

Watson P (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science.* 60: 481-492. (doi: 10.1016/S0378-4320(00)00099-3).

Zhu J, Barratt C, Lippes J, Pacey A & Cooke I (1994). The sequential effects of human cervical mucus, oviductal fluid, and follicular fluid on sperm function. *Fertility and sterility.* 61: 1129-1135.

Zumoffen C, Caille A, Munuce M, Cabada M & Ghersevich S (2010). Proteins from human oviductal tissue-conditioned medium modulate sperm capacitation. *Human reproduction. 25:* 1504-1512. (doi: 10.1093/humrep/deq063).

Conclusions/
Conclusiones

CONCLUSIONS

CHAPTER 1

1. Different Percoll gradients can be used to separate sperm subpopulation based on their functional parameters.
2. The spermatozoa selected through a 45/90% Percoll gradient exhibit higher penetration levels when they are used for *in vitro* fertilization, due to the acquisition of a greater degree of capacitation compared with 45/60 and 60/75 gradients.

CHAPTER 2

3. Sperm-oviductal epithelial cell binding can be considered a mechanism for selecting sperm populations with a low degree of protein tyrosine phosphorylation, as demonstrated in *in vivo*, *ex vivo*, and *in vitro* conditions.

CHAPTER 3

4. The sperm selected by oviductal epithelial cells led to better output when used in *in vitro* fertilization systems.
5. Oviductal epithelial cells exert a high degree of sperm selection, binding spermatozoa of better quality based on different functional parameters.

GENERAL CONCLUSION

Boar semen is composed of different sperm populations, of which those with greater functionality can be selected *in vitro* by Percoll gradients or through a more rigorous selection carried out by the epithelial cells of the oviduct, which are able to distinguish between a higher sperm fertilizing capacity.

CONCLUSIONES

CAPÍTULO 1

1. El uso de diferentes gradientes de Percoll nos permiten separar subpoblaciones espermáticas en base a sus características funcionales.

2. Los espermatozoides seleccionados a través del gradiente de Percoll 45/90% presentan mayores niveles de penetración cuando son utilizados en fecundación *in vitro* debido a la adquisición de un mayor grado de capacitación comparado con los gradientes 45/60 y 60/75.

CAPÍTULO 2

3. Las células epiteliales del oviducto, bajo condiciones *in vivo*, *ex vivo* e *in vitro*, ejercen un mecanismo de selección espermática permitiendo la unión de aquellos espermatozoides que presentan un bajo grado de fosforilación en tirosina.

CAPÍTULO 3

4. Los espermatozoides seleccionados mediante células epiteliales del oviducto dan lugar a mejores resultados cuando son empleados en sistemas de fecundación *in vitro.*

5. Las células epiteliales del oviducto ejercen un alto grado de selección espermática, uniendo aquellos espermatozoides de mayor calidad en base a diferentes parámetros funcionales.

CONCLUSIÓN GENERAL

El semen porcino se encuentra compuesto por diferentes poblaciones espermáticas, de las cuales, aquellas con una mayor funcionalidad pueden ser seleccionadas *in vitro* mediante gradientes de Percoll o bien a través de una selección más rigurosa llevada a cabo por las células epiteliales de oviducto, siendo capaces de distinguir entre espermatozoides con una capacidad fecundante superior.

Appendix

APPENDIX

Publications derived from the Doctoral Thesis

The results obtained in the present Doctoral Thesis have been included in the following publications:

1. ARTICLES IN JOURNALS INCLUDED IN THE SCIENCE CITATION INDEX (SCI) OF THE INSTITUTE FOR SCIENTIFIC INFORMATION (ISI):

- **López-Úbeda R**, García-Vázquez FA, Gadea J, Matás C. (2015). Sperm selection by oviductal epithelial cells is not merely a morphological-based phenomenon. *Under review*.

- Luño V, **López-Úbeda R**, García-Vázquez FA, Gil L, 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. (doi: 10.1530/REP-13-0159).

- Matás C, Vieira L, García-Vázquez FA, Avilés-López K, **López-Úbeda R**, CarvajaL JA, Gadea J. (2011). Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. *Animal Reproduction Science*. 127 (1-2): 62-72. (doi: 10.1016/j.anireprosci.2011.06.009).

2. OTHER ARTICLES:

- **López-Úbeda R**, Matás C. (2015). An approach to the factors related to sperm capacitation process. *Andrology-Open Access*. 4: 128. (doi: 10.4172/2167-0250.1000128).

3. ABSTRACTS IN JOURNALS INCLUDED IN THE SCIENCE CITATION INDEX (SCI):

- Luño V, **López-Úbeda R**, Lefievre L, Matás C. (2012). Protein tyrosine phosphorylation pattern of sperm bound to porcine oviduct during periovulation stage. 39th Annual conference of International Embryo Transfer Society (IETS), Hannover, Germany. January 19-23, 2013. *Reproduction, Fertility and Development*. 25 (1): 270. (doi: 10.1071/RDv25n1Ab244).

- **López-Úbeda R**, Matás C. (2012). Effect of incubation in oviductal epithelial cells and co-culture with oocytes in the protein tyrosine phosphorylation pattern in boar sperm. 39th Annual conference of International Embryo

Transfer Society (IETS), Hannover, Germany. January 19-23, 2013. *Reproduction, Fertility and Development*. 25 (1): 270. (doi: 10.1071/RDv25n1Ab245).

- Luño V, **López-Úbeda R**, Gadea J, Matás C. (2011). Protein tyrosine phosphorylation in boar sperm during co-culture with oviductal epithelial cells. 37th Annual conference of International Embryo Transfer Society (IETS), Orlando, Florida, EEUU, January 9-12, 2011. *Reproduction, Fertility and Development*. 23 (1): 216. (doi: 10.1071/RDv23n1Ab236).

- **López-Úbeda R**, Luño V, Vieira L, Gadea J, Matás C. (2011). Protein-tyrosine phosphorylation and calcium uptake in boar sperm subpopulations after different discontinuous Percoll gradient centrifugations. 37th Annual conference of International Embryo Transfer Society (IETS), Orlando, Florida, EEUU, January 9-12, 2011. *Reproduction, Fertility and Development*. 23 (1): 217. (doi: 10.1071/RDv23n1Ab237).

4. COMMUNICATIONS TO CONGRESS

- **López-Úbeda R**, Diego R, Matás C. (2015). Bajo condiciones *in vitro*, la adhesión de los espermatozoides a las células del oviducto se ve facilitada si no tienen exteriorizada la fosfatidilserina. *XVI Jornadas Sobre Producción Animal. Zaragoza*. 19 y 20 de Mayo de 2015. Tomo II. P: 364-366. (ISBN: 978-84-606-7971-4).

- **López-Úbeda R**, Matás C. (2013). The sperm bound to oviductal cells change their proteins phosphorylated. *1st COST Action FA1201 EPICONCEPT, General Conference "Epigenetics and Periconception Environment"*, Antalya (Turkey), 24 y 25 de April de 2013. P: 34. 2013. (ISBN: 978-975-7636-95-3).

- **López-Úbeda R**, Matás C. (2013). La selección de espermatozoides por las células oviductales modifica los parámetros de fecundación *in vitro*. *XV Jornadas sobre Producción Animal*, Zaragoza 14 y 15 de Mayo 2013. Tomo I. P: 383-385. (ISBN: 978-84-695-7684-7).

- Luño V, **López-Úbeda R**, García-Vázquez F.A, Matás C. (2013). Fosforilación de tirosina en espermatozoides incubados en explantes oviductales en la especie porcina: efecto del lavado espermático. *XV Jornadas sobre Producción Animal*, Zaragoza 14 y 15 de Mayo 2013. Tomo I. P: 386-388. (ISBN: 978-84-695-7684-7).