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Departamento de Ciencia y Tecnología Agroforestal y Genética

Empleo de la citometría de flujo como herramienta para el estudio de las características espermáticas de ovino manchego durante procesos de fecundación *in vitro*: Relación con fertilidad *in vivo*

Use of flow cytometry as a tool for the study of manchego sheep sperm characteristics during *in vitro* fertilization processes: relationship with *in vivo* fertility

Por

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TESIS DOCTORAL

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Que la Tesis Doctoral titulada: "Uso de la citometría de flujo como herramienta para el estudio de las características del semen de ganado ovino manchego durante los procesos de fecundación *in vitro*: relación con la fertilidad *in vivo*" ha sido realizada por D. Enrique del Olmo de Medina, con DNI 51448692-S, Licenciado en Biología, bajo nuestra dirección y que tras su revisión, consideramos que tiene la debida calidad para su presentación y defensa, así como para optar a la mención internacional.

Albacete, 10 de Noviembre de 2015

D. José Julián Garde López-Brea

Dña. María del Rocío Fernández Santos

A Justo, Mercedes y Alejandro

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Capítulo 1

Sumario

Abstract

In artificial insemination centers sperm samples are routinely evaluated by different seminal assessment tests in order to discriminate samples without a minimum required seminal quality. Some of these tests show a relationship with fertility, however, often correlations founded between these tests and contrasted male fertility is minimal. This loss of correlation may be due to several reasons. One is that the number of spermatozoa used for artificial insemination is high, it is difficult to discriminate subfertile semen samples since there will always be a large number spermatozoa to fertilize the eggs. Another problem is that there are variations in fertility depending on the operation, management and even the inseminator becoming difficult establish a direct relation between semen quality and fertility. Finally, many of the tests used, such as motility, are subjective tests therefore exist differences in assessments. Not determine fertility in an early form results in a economic cost for stallions centers since it is possible that animals are kept with low fertility rates. Therefore, determining the fertility of a male from early assessments seminal efficient economic performance improved since those males with fertility problems could retire and not be used for artificial insemination.

This work was aimed towards the search of new sperm parameters from thawed ram samples, which *in vitro* evaluation would yield liable *in vitro* and *in vivo* fertility patterns. Firstly, flow cytometry and motility evaluation using image analysis automatic systems were used for spermatic analysis. Those techniques allow impartial and adequate sample analysis in a short time due to the high number of spermatozoa examined.

The initial studies performed in this thesis pointed out to a direct relationship of two sperm speed parameters, i.e. VCL and VAP, and *in vivo* fertility in thawed ram samples after 2h of incubation at 37 °C. Nevertheless, a correlation could not be determined for sperm parameters measured by flow cytometry [sperm viability and apoptosis-like changes (YO-PRO-1/PI), mitochondrial membrane potential (JC-1), acrosome integrity (FITC-PNA) and intracellular calcium concentration (Fluo-3)] and fertility.

Those results prompted us to a new experiment to set up new flow cytometry techniques for direct association with fertility. Hence, we studied whether sperm reactive oxygen species (ROS) production would harm sperm physiology and DNA. This study required fluorescent probes such as fluorescein (CM-H_2DCFDA) for the detection of ROS production and different tests for the evaluation of DNA integrity (SCSA®, TUNEL y Chromomycin A3). Those results let us to conclude that there was a link that connect ROS production and *in vivo* fertility; showing that there was lower *in vitro* ROS production when

the males were more fertile, under simulated female reproductive track conditions (SOF medium, 38 °C, 5% CO_2).

The second set of experiments included in this thesis were based on the previous results from our research group indicating that *in vitro* fertilization (IVF) was one of the *in vitro* techniques with the highest *in vivo* fertility correlation, after artificial insemination with thawed ram sperm. Therefore, we decided to go into detail about the *in vitro* capacitation process and its influence on the *in vivo* fertility results.

The study of the capacitation process was included in the following investigations by simulating the *in vitro* capacitation conditions (SOF, 38 °C, 5% CO_2, 10% estrous sheep serum (ESS). These experiments aimed to determine whether estrous sheep serum really triggers *in vitro* capacitation or its effects were related to apoptosis process. In order to achieve this aim we subsequently analyzed sperm caspase expression of the samples. Caspases are the main transducers and effectors of the apoptosis signal leading to programmed cell death. The results suggested that *in vitro* induction of capacitation in thawed ram spermatozoa by using ESS suggests a downregulation I apoptotic pathways. Besides, semen samples with a higher amount of activated caspases were related to lower fertility.

In line with the sperm capacitation analysis, we evaluated the role of ROS production in the relationship between apoptosis and capacitation as several authors reported previously. The presence of ROS is required for capacitation but in high number they induce apoptosis and hence sperm death. Our results showed that in capacitation conditions (SOF, 38 °C, 5% CO_2, 10% ESS), ESS maintains adequate ROS levels for capacitation and avoids ROS excess that might induce apoptosis.

All the knowledge gained through the previous experiments led us to the determination of different sperm speed parameters after 2h of incubation at 37 °C by image analysis automatic systems, as well as ROS determination in simulated female reproductive track conditions. The aim of ROS determination is to identify those subfertile samples that most likely would yield low fertilization levels after artificial insemination. Moreover, we propose that during *in vitro* capacitation, ESS helps to avoid apoptosis by lowering caspase activity and maintaining ROS levels high enough to allow capacitation but avoiding an excess that would lead to apoptosis.

Resumen

En los centros de Inseminación Artificial se vienen evaluando de forma rutinaria diferentes pruebas de evaluación seminal con el objetivo de discriminar aquellas muestras que no tengan una calidad seminal mínima exigida. Algunas de estas pruebas muestran una relación con la fertilidad, sin embargo, muchas veces las correlaciones encontradas entre estas pruebas de contrastación seminal y la fertilidad de los machos son mínimas. Esta pérdida de correlación puede deberse a varios motivos. Uno es que el número de espermatozoides utilizados en la inseminación artificial es elevado siendo difícil discriminar las muestras seminales subfértiles puesto que siempre habrá espermatozoides en un número elevado para fecundar los ovocitos. Otro problema, es que existen variaciones en la fertilidad dependiendo de la explotación, del manejo e incluso del inseminador siendo complicado establecer una relación directa entre la calidad seminal y la fertilidad. Finalmente, muchas de las pruebas utilizadas, como por ejemplo la motilidad, son pruebas subjetivas existiendo por tanto diferencias en las evaluaciones. No determinar de forma precoz la fertilidad supone un coste económico para los centros de sementales puesto que es posible que se mantengan animales con bajas tasas de fertilidad. Por lo tanto, determinar la fertilidad de un macho de forma temprana a partir de evaluaciones seminales eficientes mejoría el rendimiento económico puesto que aquellos machos con problemas de fertilidad podrían retirarse y no ser utilizados para inseminación artificial.

El objetivo general de la presente tesis se orientó a la búsqueda de nuevos parámetros espermáticos, en muestras descongeladas de ovino, que evaluados *in vitro* pudieran correlacionarse con fertilidad tanto *in vivo* como *in vitro*. En primer lugar, decidimos utilizar la citometría de flujo y la evaluación de la motilidad mediante sistemas automáticos de análisis de imagen como herramientas para el análisis espermático debido al alto número de espermatozoides que estas técnicas son capaces de analizar en un breve espacio de tiempo y debido a su objetividad, y buscamos relaciones con fertilidad *in vivo*.

En la primera experiencia experimental de la presente tesis encontramos una relación directa entre dos parámetros de velocidad espermática, VCL y VAP, y la fertilidad *in vivo*, tras 2h de incubación a 37 °C de las muestras descongeladas de ovino. Sin embargo, no obtuvimos el mismo éxito en nuestro intento de encontrar relaciones entre parámetros espermáticos evaluados mediante citometría de flujo [viabilidad y apoptosis (YO-PRO-1/PI), evaluación del potencial de membrana mitocondrial (JC-1), integridad del acrosoma (FITC-PNA) y determinación de la concentración de calcio intracelular (Fluo-3)] y fertilidad.

Los resultados obtenidos nos llevaron a diseñar un nuevo experimento, con el fin de buscar nuevas técnicas de citometría de flujo que pudieran presentar una relación directa con la fertilidad. Así, decidimos incluir el estudio de la producción de especies reactivas del oxígeno (ROS) por parte del espermatozoide y el posible daño que estos ROS podrían causar a la fisiología espermática y a su ADN. Para ello, utilizamos nuevas sondas como la fluoresceína (CM-H_2DCFDA) para la detección de la producción de especies reactivas del oxígeno (ROS) y diferentes test para la evaluación de la integridad del ADN (SCSA®, TUNEL y Cromomicina A3). Tras la evaluación de los resultados, observamos que existía una relación directa entre la producción de ROS y los resultados de fertilidad *in vivo*, de tal modo, que cuanto más fértil es un macho, menor es su producción de ROS *in vitro*, en condiciones que simulan el tracto reproductor femenino (medio SOF, 38 °C, 5% CO_2).

En una segunda fase de la presente tesis y basándonos en resultados previos de nuestro grupo de investigación que apuntaban a que la FIV era una de las técnicas evaluadas *in vitro* que mayor correlación presentaba con la fertilidad *in vivo*, obtenida por inseminación artificial intrauterina con semen descongelado de ovino, decidimos estudiar en profundidad el proceso de capacitación *in vitro* y ver la relación que tenía este proceso con los resultados de fertilidad *in vivo.*

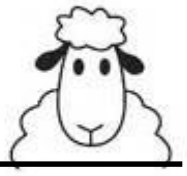
En el tercer experimento de la presente tesis doctoral, simulamos *in vitro* las condiciones de capacitación (medio SOF, 38 °C, 5% CO_2, 10% de suero de oveja en celo (SOC)). Nos preguntamos si verdaderamente durante este proceso de "capacitación *in vitro*" los espermatozoides se capacitaban o de si su respuesta estaba más relacionada con procesos de apoptosis. En esta ocasión nuestros análisis se centraron en la expresión por parte de los espermatozoides de actividad caspasa. Siendo las caspasas unas proteínas de expresión constitutiva en su forma de zimógeno en la mayoría de las células optamos por añadir un tratamiento inhibidor con el fin de poder comparar su efecto con el del suero de oveja en celo. Los resultados de esta experiencia mostraron la capacidad inhibitoria del SOC sobre la actividad caspasa. El suero de oveja en celo, durante la inducción *in vitro* de la capacitación de los espermatozoides ovino descongelado, parece regular a la baja las vías de apoptosis. Este mecanismo podría asegurar que los espermatozoides mantienen su competencia fisiológica en este proceso crítico previo a la fecundación. Además, tras la evaluación de diferentes parámetros espermáticos, se constató que el suero de oveja en celo no solo favorece la capacitación sino que además previene el deterioro de los espermatozoides. Nuestros resultados mostraron, además, una relación directa entre la actividad caspasa y la fertilidad *in*

vivo, siendo los machos más fértiles los que presentaban una menor actividad caspasa durante la capacitación *in vitro*.

Siguiendo con nuestro estudio en profundidad de la capacitación espermática, en un cuarto experimento, decidimos evaluar si, como otros autores habían indicado, el proceso de capacitación estaba directamente relacionado con la apoptosis a través de la producción de ROS, en el sentido de que ciertos ROS son necesarios para la capacitación pero si se sobrepasan ciertos límites, entonces lo que se provoca es la apoptosis y por tanto la muerte espermática. Nuestros resultados mostraron, que en condiciones capacitantes (medio SOF, 38 °C, 5% CO_2, 10% de suero de oveja en celo), el SOC mantiene niveles suficientes de ROS para que se produzca la capacitación y previene de un exceso de los mismos que pudiera desencadenar la apoptosis.

Para finalizar, y teniendo en cuenta los resultados obtenidos en la presente tesis doctoral, proponemos la determinación de los diferentes parámetros de velocidad espermática, tras 2h de incubación a 37 °C, mediante sistemas automáticos de análisis de imagen, así como la determinación de ROS en condiciones que simulan en tracto reproductor femenino, para la identificación de muestras subfértiles que tendrán un bajo éxito reproductivo tras inseminación artificial. Además, sugerimos que el suero de oveja en celo, durante la capacitación *in vitro*, previne de la apoptosis, disminuyendo la actividad caspasa y manteniendo los niveles de ROS suficientemente altos para que se produzca la capacitación pero impidiendo un aumento excesivo que llevaría a apoptosis.

Capítulo 2



Introducción general

La Biología de la Reproducción es la ciencia dedicada al estudio de los procesos reproductivos de los animales desde un punto de vista fisiológico. Su desarrollo se ha visto incrementado estas últimas décadas por la necesidad de obtener mayores rendimientos en el ganado doméstico. Desde hace años los investigadores dedicados a la Biología de la Reproducción han estado buscando un test o prueba que pueda determinar el potencial fértil de una muestra seminal (Amann 1989). En la reproducción sexual se ven implicados tanto los genomas como la correcta fisiología de dos individuos de la misma especie pero de distinto género. Generalmente el foco en la investigación ha estado centrado en los gametos masculinos por diversas razones: La fácil obtención y manipulación de los mismos, el alto número de gametos obtenido, su rápida y duradera conservación mediante procesos de criopreservación y el alto rendimiento que, en ganadería, se puede obtener de un único individuo. Estas características intrínsecas de la naturaleza del individuo de sexo masculino han determinado que la mayor parte de los esfuerzos se dirijan a mejorar la calidad y capacidad fecundante de los espermatozoides.

FISIOLOGÍA DEL ESPERMATOZOIDE

El espermatozoide es una célula muy especializada. En mamíferos la célula espermática cuenta con dos estructuras principales, cabeza y cola, que conforman compartimentos independientes (Roldan y Vazquez 1996). La cabeza, que puede ser oval o alargada, contiene un núcleo con cromatina altamente condensada gracias a la sustitución de las histonas por protaminas, proteínas con mayor capacidad de compactar el ADN. La cabeza presenta además en su parte apical, entre el núcleo y la membrana plasmática, una vesícula denominada acrosoma. El acrosoma deriva del aparato de Golgi y acumula enzimas, como la hialorounidasa, que serán secretadas en el momento en que el espermatozoide contacte con la zona pelúcida del ovocito. Estas enzimas debilitaran dicha zona mediante hidrólisis y permitirán el paso del espermatozoide.

El espermatozoide es una célula altamente diferenciada y compartimentada, cuya morfología está estrictamente relacionada con su funcionalidad. Esta estructura es el resultado de una compleja metamorfosis que ocurre en el epitelio de los túbulos seminíferos, y durante la cual una espermátida redonda se transforma en espermátida elongada ya madura (espermiogénesis) que finalmente es liberada a la luz de dichos túbulos (espermiación). El proceso se completa durante el tránsito a través de la luz de los túbulos seminíferos, conductos

eferentes y epidídimo, y la espermátida alargada se convierte en un espermatozoide maduro que será liberado en el eyaculado.

La célula espermática consta de dos estructuras principales: cabeza y flagelo, que conforman compartimentos independientes (Roldan y Vazquez 1996). La cabeza posee el núcleo haploide de cromatina altamente condensada, que contiene la información genética, y un único gránulo secretor (el acrosoma), necesario para la penetración del ovocito, que se encuentra en la región apical entre en núcleo y la membrana plasmática. La cola es un flagelo complejo y uno de los factores importantes que determinan la velocidad de propulsión del espermatozoide y por lo tanto el potencial que la célula tiene que tener para conseguir la fertilización (Noorafshan y Karbalay-Doust 2010). La cola se divide en pieza intermedia, pieza principal y pieza terminal (Zaneveld 1978). La pieza intermedia contiene las mitocondrias que producen la energía necesaria para la motilidad (Yanagimachi 1994). La pieza principal es la única parte donde se produce el batido flagelar (Noorafshan y Karbalay-Doust 2010). La pieza terminal es la última porción del flagelo posterior a la terminación de la vaina fibrosa y sólo contiene el axonema central que está cubierto por la membrana plasmática (Hafez 2000).

Capacitación espermática

En cuanto a la fisiología del espermatozoide propiamente dicha, un espermatozoide recién eyaculado es incapaz de fecundar, es necesario que sufra un proceso denominado *capacitación* espermática por el cual se vuelva competente para reconocer las diversas señales que le indiquen que ha llegado el momento de sufrir los cambios irreversibles que conlleva el proceso de la fecundación. Una capacitación temprana o tardía conllevarán por lo tanto a un fallo en la fecundación y un fracaso en la fertilidad.

La *capacitación* lleva a la "hiperactivación" de la motilidad, la cual se cree que facilita el transporte de los espermatozoides durante los últimos pasos de la fertilización (Florman y Ducibella 2006). Además, solo los espermatozoides capacitados son capaces de llevar a cabo la reacción acrosómica en respuesta a las señales emitidas por el óvulo (Florman y Ducibella 2006). Durante la reacción acrosómica, las enzimas acumuladas en el acrosoma se liberan y es espermatozoides puede entonces penetrar las capas que recubren al ovocito (Cummins y Yanagimachi 1986) y fusionarse a la membrana plasmática (Florman y Ducibella 2006). La reacción acrosómica necesita estar totalmente sincronizada con la ovulación; los espermatozoides que llevan a cabo la reacción acrosómica de manera espontánea y no en respuesta a las señales del ovocito no podrán ser capaces de fertilizarlo (Cummins y Yanagimachi 1986; Florman y Ducibella 2006).

La capacitación espermática comprende una serie de cambios que incluyen reacciones enzimáticas complejas y la reorganización de la composición de los fosfolípidos y el colesterol de la membrana (Harrison, 1996; Visconti et al. 2002, 2011; Brewis et al. 2005; Florman y Ducibella 2006). Hay tres procesos celulares importantes en la capacitación; el incremento de la concentración intracelular de $Ca2+$ y los niveles de pH, así como una disminución de los niveles intracelulares de $Na+$. La albúmina externa y el bicarbonato son esenciales para el inicio de la capacitación (Brewis et al. 2005; Visconti et al. 2011). La entrada de bicarbonato en el espermatozoide resulta en un incremento de los niveles de cAMP, que culmina con la fosforilación de tirosinas mediadas por la proteína kinasa A (PKA) (Visconti et al. 2002, 2011). Esta fosforilación y la presencia de albúmina están involucradas en la retirada del colesterol de la membrana espermática, lo que incrementa su fluidez (Visconti et al. 1999). La entrada de $Ca2+$ y bicarbonato al espermatozoide resulta en la activación de las fosfolipasas durante la capacitación (Roldan y Shi 2007). La hidrólisis de fosfolípidos mediante la fosfolipasa A2 contribuye a la generación de ácidos grasos poliinsaturados y lisofosfolípidos que hacen que la membrana sea más inestable para prepararse para la exocitosis del contenido del acrosoma en respuesta a las señales del ovocito (Roldan y Shi 2007).

El proceso de la capacitación culmina para muchos autores en el reconocimiento de la zona pelúcida del ovocito, desencadenándose la reacción acrosómica mediante la cual el acrosoma se fusiona con la membrana plasmática liberando al exterior su contenido rico en enzimas ,principalmente hialorounidasa, que debilitan la zona pelúcida. Con la zona pelúcida debilitada el espermatozoide es capaz de alcanzar la membrana del ovocito produciéndose el reconocimiento intercelular y la singamia o unión de ambos gametos (Visconti et al. 1995; Florman y Ducibella 2006).

El tracto genital femenino está altamente diferenciado, siendo por lo tanto necesario realizar cada paso de la capacitación espermática en el momento y la zona adecuada. Generalmente los espermatozoides tras alcanzar el oviducto forman reservorios donde permanecen aletargados durante cierto tiempo. Los espermatozoides en estos reservorios están unidos de forma reversible a la pared del tracto genital donde experimentan por oleadas un estímulo que les fuerza a desarrollar el siguiente paso de la capacitación, librándose de la pared del tracto genital femenino y ascendiendo a través de él mientras los procesos capacitantes actúan sobre ellos.

Apoptosis espermática

La fisiología de toda célula está controlada por procesos pro-apoptoticos y pro-mitóticos que se inhiben mutuamente. La apoptosis es un proceso de suicidio celular programado que termina con la degradación del núcleo y expulsión fragmentos de este mediante vesículas que son fagocitadas por las células adyacentes (Bredesen 2000). La principal característica de este proceso es la falta de reacción inflamatoria ya que en ningún momento el contenido intracelular pasa al espacio extracelular. Los espermatozoides, debido a su especialización, pierden la capacidad de dividirse una vez termina la meiosis y comienza la espermiación, el proceso por el cual pasan de ser células redondas a células aerodinámicas con un largo flagelo. Pero la capacidad apoptótica no se pierde del todo (Aitken *et al.* 1992). Gracias a la compartimentalización del espermatozoide las enzimas responsables de este proceso pueden seguir siendo funcionales sin que esto afecte al ADN del espermatozoide (Marchiani *et al.* 2007). Durante la capacitación, la producción de ROS se incrementa y puede llegar a sobrepasar la capacidad del sistema antioxidante de los espermatozoides (Dominguez-Rebolledo *et al.* 2009). Si dicha producción sigue aumentando la membrana plasmática comienza a oxidarse y producirse eventos que conducen a la apoptosis y posterior muerte celular, la cual dispara una respuesta inmune silenciosa, sin que curse inflamación, mediante la cual el espermatozoide será fagocitado y eliminado (Aitken *et al.* 1992).

ESTRÉS OXIDATIVO Y ESPERMATOZOIDE

El término de estrés oxidativo se refiere a un desequilibrio entre la producción excesiva o acumulación de especies reactivas de oxígeno (ROS) y el efecto protector del sistema antioxidante responsable de su neutralización y eliminación. Entre las ROS se incluyen radicales altamente oxidantes tales como el radical hidroxilo (OH·), el anión superóxido (O_2^-) o el peróxido de hidrógeno (H_2O_2). También se incluyen especies reactivas de nitrógeno (Doshi *et al.* 2012).

Los ROS sirven como coordinadores de la señal del movimiento al flagelo lo largo de la cola (Parodi 2014) y son además son responsables de la oxidación del colesterol. Esta oxidación produce oxiesteroles inductores de la perdida de colesterol en la membrana plasmática (Boerke *et al.* 2013) las cuales mediante reacciones redox inactivan o activan secuencialmente determinadas enzimas como la tirosín fosfatasa o la adenilato ciclasa, claves en el proceso de la capacitación (Aitken 2013). La generación de ROS pese a ser fisiológica entraña un riesgo para los espermatozoides ya que sus membranas plasmáticas, ricas en

fosfolípidos insaturados son especialmente vulnerables a la peroxidación lipídica la cual sucede de forma espontánea en presencia de los ROS (Dominguez-Rebolledo *et al.* 2009). Un alto contenido en lípidos peroxidados conducen a la senescencia del espermatozoide, dejándolo inútil para la fecundación.

El estrés oxidativo no sólo altera la integridad del ADN del espermatozoide sino que también limita el potencial fertilizante de estas células como resultado de un daño colateral a las proteínas y lípidos de la membrana plasmática del espermatozoide. La relación negativa entre las especies reactivas de oxígeno y la función espermática involucra una cascada de peroxidación lipídica que culmina en la generación de aldehídos citotóxicos de pequeña masa molecular tales como acroleína, malondialdehído y 4-hidroxinonenal (4HNE) (Lord *et al.* 2015).

Los espermatozoides son particularmente vulnerables a la peroxidación lipídica debido a que contienen altas concentraciones de ácidos grasos poliinsaturados (AGPI). En los AGPI2 la energía de disociación del hidrógeno de carbono es menor en la posición del metileno bis alílico, por lo que se promueve la abstracción del hidrógeno (que da inicio a la peroxidación lipídica), formándose un radical alquilo (L). Una vez que se forma este radical, los grupos adyacentes le proveen estabilización por resonancia. Si está presente el oxígeno molecular reaccionará rápidamente con el radical alquilo para formar radicales peroxilo (ROO·) y alcoxilo (RO·). Estos radicales son importantes intermediarios en la cadena de propagación de la peroxidación porque una vez formados, continuarán la cadena de reacciones oxidativas abstrayendo un átomo de hidrógeno de otros grupos alquilo cercanos, a fin de estabilizarse. Este ciclo de reacciones propagadoras se repite siempre que se encuentran disponibles suficientes moléculas de O_2 y sustratos lipídicos insaturados. La peroxidación lipídica es extremadamente perjudicial para los espermatozoides, teniendo un efecto negativo tanto en el movimiento de estos como en su competencia en la fertilización (Alvarez *et al.* 1987; Aitken y Curry 2011).

El proceso de peroxidación lipídica conduce a una pérdida de integridad de la membrana y a un aumento de su permeabilidad, a la inactivación celular de enzimas, daño en la estructura del ADN, y apoptosis celular.

Aunque niveles altos de ROS son perjudiciales para los espermatozoides, las especies reactivas del oxígeno desempeñan un papel fundamental en la fisiología del espermatozoide (Vernet *et al.* 2004; de Lamirande y O'Flaherty 2008), ya que promueven las vías de transducción de señales asociadas con la capacitación, hiperactivación (de Lamirande y Gagnon 1994), reacción acrosómica (Griveau *et al.* 1995) y fusión espermatozoide-oocito.

La principal fuente celular de ROS en el semen son los espermatozoides inmaduros y la infiltración leucocitaria. El aumento en el número de leucocitos puede ser debido a una infección o una inflamación, pero también puede ser secundaria a factores nocivos del medio ambiente, un período de abstinencia sexual largo, o al varicocele (Agarwal *et al.* 2015). Incluso un pequeño número de leucocitos pueden generar una cantidad significativa de ROS, dañando irrevocablemente a los espermatozoides (Aitken y West 1990) causando fragmentación de ADN además de un aumento de superóxido y peróxido de hidrógeno (Lobascio *et al.* 2015).

En la mayoría de los casos, el estímulo desencadenante de esta cascada apoptótica puede ser la acumulación gradual de productos del metabolismo finales citotóxicos (4-HNE, acroleína) generada como resultado de la peroxidación lípidica. Estos aldehídos inducen la generación mitocondrial de superóxido mediante la unión a flavoproteínas en la cadena de transporte electrónico mitocondrial, induciendo a un aumento importante en la generación de ROS lo cual inducirá más peroxidación lipídica, más acumulación de aldehído, y a su vez, más generación de ROS en un ciclo de autopropagación que culmina en apoptosis (activación de caspasas y exteriorización de fosfatidilserina) (Koppers *et al.* 2011).

Debido a la estructura del espermatozoide, el único producto generado durante la cascada apoptótica intrínseca que puede inducir daño en el ADN en el núcleo del espermatozoide es el peróxido de hidrógeno liberado de las mitocondrias espermáticas, que, debido a su pequeño tamaño y falta de carga, se puede mover fácilmente desde la pieza intermedia a la cabeza del espermatozoide y penetrar en el núcleo.

El daño en el ADN de los espermatozoides se ha relacionado con tasas de fecundación reducidas, deterioro en la preimplantación, aborto involuntario, y morbilidad en la descendencia (Avendano y Oehninger 2011). Existen dos mecanismos principales por el cual se puede inducir una rotura en las cadenas del ADN en espermatozoides humanos: a través de la acción de nucleasas o como un subproducto del estrés oxidativo. Este último implica la oxidación de las bases, en particular, las guaninas, en las que se genera 8-hidroxi, 2'-desoxiguanosina (8OHdG). La presencia de tales compuestos desestabiliza el enlace glicosílico (que conecta la base a la unidad de ribosa adyacente) conduciendo a la pérdida de la base afectada y a la generación de un sitio abásico. Los sitios abásicos se sabe que tienen un fuerte efecto desestabilizador en la estructura del ADN, lo que resulta en la rotura localizada de una hebra (Aitken *et al.* 2015).

Un posible indicador inicial de daño en el ADN, parece ser la generación del anión superóxido por las mitocondrias espermáticas (Koppers *et al.* 2008; Koppers *et al.* 2011). Las

causas por las que comienza la producción de ROS en las mitocondrias son muy variadas, desde la transición de metales (Kiziler *et al.* 2007) hasta moléculas que pueden intercalarse en las membranas de la mitocondria e interrumpir el intercambio de electrones (Aitken y Baker 2006; Koppers *et al.* 2010). Esta producción puede acelerarse por factores como estrés oxidativo, congelación (Kim *et al.* 2011) o deficiencias en la protección antioxidante (Aitken y Curry 2011).

Además, la presencia de radicales libres generados por los leucocitos en el tracto reproductor masculino puede crear suficiente estrés oxidativo como para desencadenar una respuesta apoptótica en espermatozoides humanos, incluyendo daños en el ADN, disfunción mitocondrial, y pérdida de la motilidad (Erenpreiss et al. 2002; Zorn et al. 2010; Shi et al. 2012).

ESTUDIO DE LA FERTILIDAD

Si definimos fertilidad como el número de nacimientos de crías vivas a partir de un número determinado de cruces entre machos y hembras podemos determinar que la fertilidad de un macho determinado se calcula mediante el porcentaje de hembras que se han apareado con él y que han llevado a buen término la gestación.

Este proceso se inicia con el coito o la inseminación en el caso del uso de técnicas de reproducción asistida (TRA) y la posterior fecundación del óvulo a partir de un espermatozoide del macho y la posterior embriogénesis y gestación. La fertilidad no es un valor fijo, varía a lo largo de la vida de un individuo ya sea por su edad (Purdy *et al.* 2010), condición física o stress ambiental (Guerrero-Bosagna *et al.* 2013); hay que tener en cuenta además que las muestras seminales utilizadas para las TRA pueden verse afectadas por los tratamientos que sufran en el laboratorio. El *origen de la muestra* también tiene muchas implicaciones en la fertilidad final de la muestra, dependiendo de si los espermatozoides son de origen epididimarios o eyaculados, si la eyaculación de produjo mediante estimulación o electroeyaculación o han sufrido un proceso de congelación-descongelación (Bordson *et al.* 1986).

Origen de la muestra

Con objeto de su uso en las TRA el semen se puede obtener de diversas formas, generalmente por *vagina artificial* si el animal ha sido entrenado desde pequeño para ello o mediante electroeyaculación. El método de recolección mediante vagina artificial es el más

empleado por la similitud de la muestra obtenida con el eyaculado natural (Wulster-Radcliffe *et al.* 2001).

La ***electroeyaculación*** es el método escogido para utilizar animales de alto valor reproductivo sin entrenamiento o para fauna salvaje. La muestra obtenida suele presentar diferencias respecto a un eyaculado normal e incluso pueden presentarse problemas de contaminación por orina. La recolección del eyaculado en diversas fracciones para evaluar la calidad de cada una de ellas y controlar la presencia o no de orina mediante análisis de urea suelen solventar estos problemas pero el resultado final difiere de forma significativa del semen obtenido mediante vagina artificial (Austin *et al.* 1968; Memon *et al.* 1986; Marco-Jimenez *et al.* 2008).

Las muestras también pueden obtenerse ***post-mortem***, mediante la retirada de los testículos en el momento de la muerte del animal. Las muestras seminales epididimarias difieren principalmente de las obtenidas mediante vagina artificial o electroeyaculación por no haber entrado en contacto con el plasma seminal (Chen *et al.* 2003).

Tras obtener una muestra seminal, si se desea obtener descendencia de la misma, se necesita una hembra que se encuentre cerca del momento de la ovulación, en celo. Sincronizar el momento de la extracción con el celo de las hembras puede presentar complicaciones logísticas: distancia, tiempo, localización.

Los espermatozoides en el epidídimo están en un ambiente inhibitorio, esperando el momento de ser eyaculados (Dacheux y Dacheux 2014). El ***plasma seminal*** es una colección de secreciones procedentes del epidídimo, testículos y glándulas accesorias (vesículas seminales, próstata y glándulas bulbouretrales) que se van uniendo a los espermatozoides según salen del epidídimo y atraviesan el conducto deferente y la uretra durante la eyaculación.

Su función ha sido discutida por numerosos investigadores, estando compuesto por sales, antioxidantes, ácidos grasos, azucares, enzimas, hormonas, factores de crecimiento, prostaglandinas y proteínas alterando la funcionalidad de los espermatozoides (Juyena y Stelletta 2012).

En ganado porcino, la adición de plasma seminal de un individuo con alta fertilidad a espermatozoides procedentes de individuos con baja fertilidad incrementa el éxito reproductor de estos últimos demostrando que la composición del plasma seminal influye notablemente sobre la fertilidad (Flowers 1997). Se han identificado varios tipos de proteínas en el plasma seminal, atendiendo a su actividad pueden clasificarse en: i) Proteinas cobertoras de la membrana espermática; ii) Proteinas inhibidoras del movimiento; iii) factores decapacitantes. Se han identificado como proteínas cobertoras de la membrana plasmática o

espermadhesinas AWN, AQN-1, AQN-3, PSP-I, PSP-II. Estas proteínas cubren el espermatozoide durante su tránsito en el epidídimo y el conducto deferente durante la eyaculación y ayudan a la membrana plasmática a mantener su estabilidad y su posterior unión con el epitelio del oviducto (Liberda *et al.* 2006; Manaskova *et al.* 2003). Estas proteínas también tienen funciones inmuno-inhibitorias (Yang *et al.* 1998) o quimiotácticas (Assreuy *et al.* 2003) y pueden estar involucradas en los primeros estadios del reconocimiento espermatozoide-ovocito y la reacción acrosómica (Topfer-Petersen *et al.* 1998; Jonakova *et al.* 1998).

Los factores decapacitantes presentes en el plasma seminal suelen prevenir los procesos iniciales de la capacitación e impedir la reacción acrosómica temprana (Shivaji 1988). Los inhibidores de la motilidad espermática han sido descritos por Iwamoto *et al.* (1992) y mostraron cómo su eliminación provoca la recuperación de la motilidad. Kumar *et al.* (2009) analizaron un tipo muy común de proteínas presentes en el plasma seminal, las proteínas con capacidad de unir heparina, y encontró que el 38 % pertenecía a la categoría enzimas, el 20% estuvieron involucradas en el procesamiento del ARN y la transcripción, el 18% en la estructura y función de transporte, y el 16% en el reconocimiento celular y la transducción de señales. Este ejemplo de una sola familia de proteínas presentes en el plasma seminal da una idea de la variedad de funciones que realiza y su influencia respecto a la fisiología del espermatozoide.

Las técnicas de *criopreservacion espermática* permiten dilatar el uso de la muestra seminal en el tiempo. Los procesos de criopreservacion suelen incluir dilución en un medio con crioprotectores intra y extracelulares y un enfriamiento y equilibración previos a la congelacíon (Curry 2007). Pese a que tras la descongelación la muestra seminal sufre una disminución en todos los parámetros cualitativos la mejora de los protocolos de congelación ha propiciado que su utilidad práctica se imponga sobre la pérdida de calidad.

PREDICCIÓN DE LA FERTILIDAD

La predicción fehaciente de la fertilidad de una muestra seminal puede tener un alto impacto en diversos sectores de la sociedad: al aumentar la eficiencia en los laboratorios de reproducción humana permitiría utilizar la técnica de reproducción asistida más idónea para cada pareja de pacientes, determinando desde el primer momento si es mejor realizar una inseminación artificial (IA), una fecundación *in vitro* (FIV) o una Inyección Intracitoplasmática (ICSI). Para la biología de la conservación, determinar el potencial fértil

de cada individuo permitiría preparar dosis a menor concentración de los individuos más fértiles para así poder disponer de más dosis aumentando la eficiencia de muestras generalmente muy valiosas debido al bajo número de individuos que conforman las poblaciones amenazadas. En ganadería, para las granjas en las que se trabaja con IA determinar qué individuos son más fértiles permitiría mejorar la producción evitando un alto número de hembras sin preñar e identificando a su vez qué hembras no son idóneas para la producción animal (Curry 2007). En el caso de la industria farmacéutica poder determinar de forma prematura los efectos de nuevos medicamentos en la fertilidad reduciría los costes necesarios para comprobar cómo pueden afectar nuevos fármacos a la capacidad reproductiva (Holt y Van Look 2004).

Sin embargo, no parece haber una sola técnica capaz de identificar correctamente el potencial fértil de una muestra (Rodriguez-Martinez 2007). Los esfuerzos se centran en analizar, en la muestra seminal, todos los atributos relevantes para la fertilización y el desarrollo embrionario (Rodriguez-Martinez 2006). Estos atributos se clasifican en dos tipos: compensables y no compensables.

Saacke *et al.* (2000) y Braundmeier y Miller (2001) sugirieron que la relación entre las características del semen y la fertilidad mediante IA de un macho toma forma de una curva asintótica con una pendiente positiva. En la parte alta de la curva se forma una meseta que representa la máxima fertilidad de esa muestra y viene determinada por los factores no compensables. La parte creciente de la curva está influenciada por los factores compensables, por lo que se puede inferir la concentración idónea a partir de la cual la baja calidad en alguno de los atributos espermáticos para la fecundación no se puede compensar más.

Los ***atributos o variables compensables*** son todos aquellos que pueden ser mejorados aumentando la concentración espermática de la muestra o utilizando más volumen. Estos valores pueden ser valores objetivos como la motilidad (Mortimer 1997), morfometría (Maroto-Morales *et al.* 2010), morfología (Morrell *et al.* 2011), estado acrosomal (Yanagimachi y Noda 1972), viabilidad (De Geyter *et al.* 1988) o funcionales como la capacidad de desarrollar la capacitación (Rodriguez-Martinez 2006), atravesar el mucus cervical (De Geyter *et al.* 1988), experimentar la reacción acrosómica (Rodriguez-Martinez y Barth 2007), expresar el movimiento de hiperactivación (Okada *et al.* 1985) o la capacidad de fertilizar *in vitro* (Garcia-Alvarez *et al.* 2009).

Los ***atributos o variables no compensables*** son aquellos que son independientes de la cantidad de muestra que se añada: la integridad del ADN (Cortes-Gutierrez *et al.* 2014) o la presencia o no de determinadas proteínas de membrana responsables del reconocimiento

interespecífico entre espermatozoide y ovocito (Clark 2011), el comienzo de la reacción acrosómica (Franken y Oehninger 2012) o el desarrollo de la espermatogénesis de forma exitosa son algunos de estos atributos (Shukla *et al.* 2012).

Los análisis de los valores físicos son técnicas sencillas pero proporcionan información parcial sobre la calidad espermática; los análisis de los valores funcionales proporcionan información de gran calidad sobre los espermatozoides pero suelen ser muy complejos y difíciles de incorporar a un análisis rutinario.

La *predicción del potencial fértil de una muestra espermática* mediante el uso de las técnicas convencionales ha resultado ser ineficiente debido a la gran cantidad de tiempo que requieren para ser realizadas (Holt and Van Look 2004). El espermiograma clásico ha dado paso en la última década al uso de tecnologías computerizadas como el análisis computerizado de imágenes (CASA) y la citometría de flujo (Hallap *et al.* 2006).

Motilidad y fertilidad

Los sistemas CASA constan de un microscopio al que se acopla una cámara conectada a un ordenador. El análisis informático de cortas secuencias de imágenes permite discriminar cantidad y calidad del movimiento de cada espermatozoide y calcular sus trayectorias. Del cálculo de dichas trayectorias se obtienen diversos parámetros cinéticos: la velocidad curvilínea (VCL), la velocidad media (VAP), la velocidad rectilínea (VSL), la amplitud del desplazamiento lateral de la cabeza (ALH), la frecuencia de cruce de los espermatozoides (BCF) y la linealidad de la trayectoria curvilínea (LIN) (Mortimer 2000).

Malo et al. (2005) encontraron relaciones entre la fertilidad de los machos de ciervo rojo con los parámetros de velocidad de la natación del esperma (VCL, VSL y VAP) y esta relación también ha sido demostrada en un gran número de taxones (Gage *et al.* 2004; Holt *et al.* 1989). En concreto, esta relación ha sido ampliamente estudiada en especies ganaderas (toro:(Amann *et al.* 2000);(Farrell *et al.* 1998);(Kathiravan *et al.* 2008); cabra:(Fernandez-Santos *et al.* 2011), mostrando que parámetros cinemáticos como VCL, VSL y VAP se relacionan frecuentemente con la fertilidad. Otros investigadores han encontrado correlaciones positivas entre diferentes parámetros de velocidad y la fertilidad de los espermatozoides humanos (Fetterolf y Rogers 1990).

Se han encontrado relaciones de la fertilidad con multitud de variables espermáticas, pero las correlaciones tienden a ser bajas y nos encontramos con estudios contradictorios. Se han encontrado relaciones con fertilidad y motilidad en caballo (Kjaestad *et al.* 1993), toro (Correa *et al.* 1997), ciervo (Malo *et al.* 2005), pero no en oveja (O'Meara *et al.* 2008). Gran

parte de la variabilidad en los resultados puede deberse a la diferente concentración de las muestras espermáticas las cuales pueden enmascarar la baja motilidad de una muestra con un incremento en el número de espermatozoides utilizados, manteniendo la proporción de espermatozoides motiles pero incrementando su número real.

En la presente tesis doctoral hemos estudiado la posible relación entre fertilidad y motilidad espermática. Los resultados se mostrarán en el capítulo 4.

Morfometría y fertilidad

Los sistemas CASA también pueden analizar la forma y dimensiones de la cabeza del espermatozoide de una forma objetiva mediante medidas estandarizadas (ancho, largo, perímetro, área y factor forma p2a de la cabeza del espermatozoide, el diámetro de la pieza intermedia y la longitud del flagelo). La relación entre la morfología del espermatozoide y la calidad espermática comenzó a estudiarse con los trabajos de MacLeud y Gold (1951) . El análisis de la morfometría arroja diferentes resultados según la especie estudiada. Existen estudios que correlacionan la morfometría de los espermatozoides de toro con su fertilidad (Einarsson *et al.* 2009) mientras que otros autores no han encontrado dicha relación (Gravance *et al.* 2009). También se han encontrado relaciones entre morfometría y fertilidad en ciervo (Malo *et al.* 2005) y cerdo (Hirai *et al.* 2001) mientras que Popwell y Flowers (2004) no han encontrado con esta última especie ninguna relación entre estas dos variables. Maroto-Morales *et al.* (2015) no encontraron ninguna relación directa entre la morfometria de la cabeza del espermatozoide de morueco y la fertilidad, pero la proporción de la subpoblación de espermatozoides cortos y elongados en el eyaculado explicaban las diferencias entre la fertilidad de los moruecos estudiados.

Parámetros espermáticos evaluados mediante citometría de flujo y fertilidad.

La *citometría de flujo* es una técnica analítica basada en la difracción de la luz láser. Mediante la suspensión e inyección de células en un flujo laminar dicha suspensión es comprimida hasta que se consigue que las células queden alineadas de forma individual en la dirección del flujo. Dicho flujo es hecho pasar por una cámara donde incide un láser que golpea de forma perpendicular y directa a dichas células a una alta velocidad, generalmente entre 200 y 300 células por segundo. La difracción y modificación de la longitud de onda del láser es recogida por varios sensores que envían la señal a un ordenador que analiza dicha información de forma individual. Se suelen utilizar sondas fluorescentes: moléculas con la capacidad de unirse específicamente a determinados sustratos celulares. Dichas moléculas

absorben la luz del láser y emiten una luz con una longitud de onda mayor. Este fenómeno, conocido como fluorescencia, fue descrito por Stokes en 1852. Las sondas fluorescentes nos permiten distinguir entre distintos eventos o tipos celulares. Prácticamente existe una sonda para cada evento celular que se desee estudiar y cada día los laboratorios descubren, mejoran y perfeccionan nuevas sondas.

Actividad mitocondrial

La actividad mitocondrial ha sido evaluada con diversas sondas fluorescentes, Kasai *et al.* (2002) han encontrado relación entre fertilidad y actividad mitocondrial en el hombre, pero Hallap *et al.* (2005) no han encontrado el mismo resultado. La mitocondria como tal suele tener una importancia relativa en el espermatozoide, siendo responsable del anabolismo en diversas especies mientras que en otras su importancia en el cómputo global de la producción energética suele ser mínimo (Marin *et al.* 2003). La mitocondria es la piedra angular de los procesos de producción de radicales libres y/o apoptosis (Aitken y Baker 2013) por lo que el análisis de su actividad suele incluirse frecuentemente en los análisis rutinarios de espermatologia mediante citometría (Martinez-Pastor *et al.* 2010). Recientemente se han encontrado correlaciones entre la fertilidad y el estado mitocondrial analizando semen de toro, pero esta relación fue muy baja y solo en combinación con otros parámetros se ha llegado a una correlación de suficiente entidad (Sellem *et al.* 2015).

Integridad del acrosoma

Saacke and White (1972) encontraron una relación positiva entre el porcentaje de espermatozoides con el acrosoma intacto y la fertilidad. El estudio del estado acrosomal ha evolucionado según han ido apareciendo técnicas más complejas: desde la valoración mediante microscopía óptica al uso de marcadores fluorescentes unidos a diversas lecitinas (PNA o PSA), las cuales tienen una afinidad específica por la hemimembrana interna del acrosoma (Martinez-Pastor *et al.* 2010), su estudio mediante microscopia óptica o citometría de flujo pasando por tinciones con Clortetraciclina (Kato *et al.* 2011), el estudio de la proteína ZP y su receptor ZPr o la respuesta del acrosoma a los flujos de calcio intracelular (Petit *et al.* 2013).

Capacitación espermática

La sonda fluorescente Merocianina 540 mide el desorden de los fosfolípidos de la membrana plasmática, típico de espermatozoides capacitados, y en los espermatozoides vivos ha sido relacionada con la fertilidad (Garcia-Alvarez *et al.* 2010). Mediante análisis con Clortetraciclina (Thundathil *et al.* 1999) en semen bovino descongelado han encontrado

relación entre el porcentaje de espermatozoides vivos sin capacitar y la fertilidad pero Gillan *et al.* (2008) no encontraron relación en esa misma especie.

DNA y fertilidad

Tras la fecundación, el ADN del espermatozoide se descondensa y evidencia como pronúcleo antes de la primera división del zigoto. La expresión de su material genético durante el desarrollo puede inducir el bloqueo de la división celular si dicho ADN está dañado. El estudio del estado del ADN ha sido relacionado con la fertilidad mediante la técnica del SCSA® (Evenson *et al.* 2002). Esta técnica está basada en la capacidad metacromatica de la naranja de acridina, la cual al unirse al ADN monocatenario emite fluorescencia roja y fluorescencia verde cuando se une a ADN bicatenario. El origen del ADN monocatenario se aduce a roturas en la cromatina. Otras técnicas como el Terminal UTP Nick End Labeling (TUNEL) o el estudio del metabolito resultante de la oxidación del ADN 8-hidroxi-2'-deoxiguanosina pese a ser interesantes desde el punto de vista fisiológico no han mostrado relación alguna con la fertilidad (Lewis *et al.* 2013).

Producción de ROS

Para ponderar la producción de ROS actualmente se utiliza la citometría de flujo en detrimento de las técnicas de luminometria y/o espectrometría, más usadas en décadas pasadas. El uso del lumiol y la lucigenina ha dado paso a sondas fluorescentes como como CM-H_2DCFDA (5-(and-6)-chloromethyl-20,70-dichlorodihydrofluorescein acetyl ester) y DHE (Dihydroetidium). Lamentablemente ninguna sonda es capaz de detectar todas las distintas especies de radicales libres a la vez (Hossain *et al.* 2011). El exceso de ROS en el medio de cultivo y su efecto sobre los embriones ha sido comprobado *in vitro* (Silva *et al.* 2007). No obstante la producción de radicales libres no puede ser totalmente eliminada ya que un exceso de antioxidantes también puede tener efectos nocivos sobre la fertilidad (Menezo *et al.* 2007; Walczak-Jedrzejowska *et al.* 2013).

Tests funcionales y fertilidad

La capacidad de los espermatozoides de realizar determinados hitos que ocurren en el tracto femenino ha sido estudiada *in vitro* por diversos investigadores y se ha intentado relacionar con la fertilidad. La técnica HOST (**Hypo-osmotic swelling test**) es una prueba que se realiza en un breve periodo de tiempo (30 min.) y permite comprobar tanto la viabilidad como la competencia bioqúimica a la hora de reajustar el equilibrio hídrico de la célula cuando ésta se sumerge en un medio con una concentración de solutos inferior al espacio intracitoplásmático. Numerosos estudios han demostrado que las pruebas de vitalidad convencionales o fluorescentes no correlacionan con ningún parámetro de viabilidad (Brito

et al. 2003; Hammoud *et al.* 2009). La técnica HOST, al analizar, además de la viabilidad celular, la capacidad de regular a través de su membrana plasmática el flujo de electrolitos y no-electrolitos sí ha mostrado relaciones con la fertilidad *in vivo* e *in vitro* (Hauser *et al.* 1992). Sin embargo esta técnica aqueja de los inconvenientes de las técnicas utilizadas en microscopia óptica: la gran cantidad de tiempo que consume evaluar cada muestra y la necesidad de tener varios técnicos especializados que realicen dicha evaluación con los mismos criterios.

El *test de penetración del moco cervical* es una prueba rudimentaria que evalúa la capacidad del espermatozoide de atravesar una barrera de concentración creciente de moco cervical para evaluar la motilidad de los espermatozoides (Hafez 1979). El *test de unión al oviducto* mide la capacidad de los espermatozoides de unirse al epitelio germinal del oviducto (Lefebvre y Suarez 1996). Entre los múltiples pasos importantes en el proceso de fertilización se ha demostrado que la capacidad de unión zona pelúcida de una población de espermatozoides es un acontecimiento crucial. (Codde y Berger 1995). Originalmente, la interacción espermatozoide-ovocito humano se definió en un ensayo descrito por Overstreet and Hembree (1976). Durante este ensayo *in vitro* se comparó la tasa de penetración de los espermatozoides en la zona pelúcida humana. Aunque este ensayo fue desarrollado para evaluar la penetración de zona, la metodología constituyó la piedra angular de las futuras pruebas de interacción-esperma ovocito: el test de hemizona (Burkman *et al.* 1988) y el test de unión a zona pelúcida intacta (Liu y Baker 1992). Ambos ensayos tienen la ventaja de proporcionar una prueba homóloga funcional para la unión del esperma a la zona pelúcida, y la comparación de las poblaciones de espermatozoides fértiles y estériles en el mismo ensayo. Collins *et al.* (2008) han encontrado una relación de estos ensayos con la fertilidad en cerdo, pero es poco frecuente ya que estos ensayos implican que el resto de barreras fisiológicas y anatómicas han funcionado de forma correcta permitiendo durante este ensayo que espermatozoides que en ningún caso hubiesen podido superar dichas barreras entren en contacto con la zona pelúcida. Además, este tipo de ensayos es costoso tanto en tiempo como en material ya que requiere el uso de zonas pelucidas de difícil obtención.

Los *test de fertilización in vitro (FIV)* son los que imitan de manera más exhaustiva las condiciones que rodean a la fertilización *in vivo*. Esta técnica permite analizar la capacidad fecundante de una muestra seminal expuesta a diversos tratamientos o comparar la fertilidad de diversos machos.

En el desarrollo de una FIV los espermatozoides deben ser previamente capacitados y posteriormente puestos en contacto con uno o más ovocitos en un medio definido químicamente. En estos test la temperatura es crítica debido a su efecto sobre los microtúbulos

del ovocito (Magistrini y Szollosi 1980). También es de vital importancia una baja tensión de oxígeno (Peng *et al.* 2015). Para atajar estos inconvenientes técnicos la FIV se desarrolla en incubadores con capacidad para regular la atmosfera que se desarrolla en su interior. Mediante la inyección de CO_2 o N_2 se consiguen presiones parciales de oxigeno cercanas al 5%. La presencia de CO_2 en una tensión cercana al 5% permite el uso de un tampón extracelular fisiológico en los medios de cultivo, el tampón bicarbonato. La capacitación del espermatozoide depende de la especie que estemos estudiando, estando definidos químicamente los medios de capacitación para determinadas especies. Así, los espermatozoides de toro requieren la presencia de heparina para capacitarse (Parrish 2014), los medios de capacitación para el semen humano requieren albúmina sérica humana (HSA), calcio y bicarbonato sódico (Bailey 2010). En el caso del ganado ovino el medio de capacitación aún no se ha definido químicamente, utilizando suero de oveja en celo (SOC). Este suero se obtiene mediante el sangrado de ovejas hembra en celo, a las cuales se les retiran 500 ml de sangre. Una vez producida la coagulación se retira el suero y se inactivan las proteínas remanentes. Esta técnica presenta múltiples problemas ya que los resultados pueden variar notablemente si se utilizan distintos sueros obtenidos de distintos individuos (Karami Shabankareh *et al.* 2011).

El mayor obstáculo para la realización de estos test es el alto número de ovocitos que requiere para obtener resultados estadísticamente significativos. Normalmente los ovocitos son obtenidos de animales sacrificados en mataderos. La FIV puede ser homóloga si se utilizan ovocitos de la misma especie o heteróloga (Rodriguez-Martinez y Barth 2007), con ovocitos de especies distintas. La FIV heteróloga se utiliza si la posibilidad de conseguir ovocitos de la especie a estudiar es complicada, generalmente en estudios de fauna salvaje utilizando ovocitos de animales domesticados filogenéticamente próximos.

Genómica, Proteómica y Fertilidad

En estos últimos años se han incorporado las técnicas de genómica y proteomica al estudio y análisis de la fertilidad. Thurston *et al.* (2002) aisló genes que determinaban la capacidad de resistir un proceso de congelabilidad en cerdo. Moura (2006) detectó proteínas procedentes de la cabeza el epidídimo en el plasma seminal de toros que se comportaban como marcadores de fertilidad. Kwon *et al.* (2015) han descubierto que la presencia de la subunidad 1 del complejo citocromo b-c1 (UQCRC2) está relacionado con mayores camadas en cerdo. Novak *et al.* (2010b) encontraron que la abundancia de las proteínas kallikreina-1E2 (KLK2), clusterina, y las proteínas SP1 y SP2 en el plasma seminal funcionaban como marcadores negativos de fertilidad en caballo mientras que en otra publicación, los mismos

autores encontraron que la enzima antioxidante glutatión peroxidasa (GPX5) se revelaba como un potencial marcador de fertilidad en cerdo (Novak *et al.* 2010a; Novak *et al.* 2010b).

ESTRÉS OXIDATIVO, CAPACITACIÓN Y APOPTOSIS

Como se ha comentado anteriormente, los ROS tienen un papel fundamental en la fisiología espermática, pero también pueden afectar la capacidad fecundante de los espermatozoides. El estrés oxidativo puede ser definido como el resultado del desequilibrio entre las especies reactivas de oxígeno y la capacidad de producción de antioxidantes para eliminarlos. Este estrés oxidativo es motivo de gran preocupación ya que los altos niveles de ROS tienen efectos negativos sobre la funcionalidad de los espermatozoides (Gil-Guzman *et al.* 2001).

La producción de ROS se considera como uno de los mediadores de los daños causados por la crioconservación (Alvarez y Storey 1992; O'Flaherty *et al.* 1997; Chatterjee y Gagnon 2001; Fernandez-Santos *et al.* 2007) ya que puede inducir daño en el ADN (Lopes *et al.* 1998), alteraciones del citoesqueleto (Hinshaw *et al.* 1986), la inhibición de la fusión espermatozoide con el ovocito (Aitken *et al.* 1989) y provocar daños sobre el axonema del espermatozoide, seguido por la pérdida de la motilidad (de Lamirande y Gagnon 1992). Por lo tanto el estrés oxidativo puede estar relacionado con la muerte celular y la pérdida de la función celular (O'Flaherty *et al.* 1997; Bailey *et al.* 2000).

Durante la capacitación los espermatozoides culminan el ensamblaje y presentación de un complejo número de receptores que reconocerán la zona pelucida del ovocito. McPartlin *et al.* (2009) demostraron que el cambio del patrón de un movimiento lineal a uno oscilante y con fuerte cabeceo denominado "hiperactivacion" en caballo estaba relacionado con la fertilidad. Durante su ascenso por el tracto genital femenino el movimiento del espermatozoide variará de rectilíneo a "hiperactivado", dicho movimiento genera la fuerza propulsora necesaria para desligarse del epitelio oviductal y penetrar a través de las diversas capas que rodean al ovocito.

Como se ha comentado anteriormente, la base química de la capacitación espermática involucra la oxidación y pérdida de colesterol de la membrana plasmática y un incremento de la fosforilación de las tirosinas de determinadas proteínas, sobretodo en la cola, bajo la influencia del cAMP. Todo este proceso está mediado por ROS, los cuales son producidos principalmente en las mitocondrias y las NADPH oxidasas de la membrana plasmática (McPartlin *et al.* 2009).

Los procesos de congelación-descongelación seminal que se producen de forma rutinaria en los laboratorios de reproducción asistida generan un efecto sobre los espermatozoides denominado criocapacitación (Amaral *et al.* 2013). Dicha criocapacitación puede engañar a un técnico inexperto ya que no es debido a un proceso fisiológico sino a un daño producido por el proceso de congelación. Los espermatozoides criocapacitados suelen tener una esperanza de vida corta y presentar una motilidad hiperactivada. Los espermatozoides prematuramente capacitados tienen una influencia negativa en la fertilidad ya que su capacidad de ser transportados a través del tracto genital femenino se ve mermada. La capacitación prematura de los espermatozoides además es responsable de su incapacidad para colonizar el oviducto y formar un reservorio de espermatozoides (Lymberopoulos *et al.* 2010), lo cual es necesario para proporcionar un suministro continuo de esperma a la espera de la aparición de los ovocitos (Gillan *et al.* 2000).

Durante la espermatogénesis muchas espermátidas presentan errores en la meiosis o la maduración, las cuales degeneran mediante el proceso de la apoptosis siendo reabsorbidas por las células de Sertoli circundantes. Un error en dicha reabsorción o apoptosis puede producir que dichas espermátidas aberrantes sean expulsadas a la luz de los túbulos seminíferos pudiendo llegar incluso a ser eyaculadas produciendo efectos adversos en la fertilidad (Suarez 2007).

Recientemente, la relación entre los procesos apoptoticos y capacitantes que sufre el espermatozoide y el papel que realizan los ROS ha comenzado a esclarecerse (Aitken *et al.* 2015). El espermatozoide sufre a lo largo de su vida toda una serie de estímulos que le inducen a capacitarse o inducir la apoptosis. Durante la espermatogénesis cualquier fallo en la meiosis provoca la apoptosis y la reabsorción de las espermátidas aberrantes por parte de las células de Sertoli (Ramm *et al.* 2014). Posteriormente, en el epidídimo, el espermatozoide está sometido a un ambiente que inhibe la capacitación (Dacheux y Dacheux 2014). Tras la eyaculación los espermatozoides en contacto con el plasma seminal comienzan a sufrir estimulos que en una primera instancia, gracias al alto contenido en antioxidantes, inhiben el proceso de capacitación hasta que los espermatozoides comienzan a ascender por el oviducto. Una vez ahí los ROS, principalmente el peroxinitrito, oxidan el colesterol de la membrana, produciendo oxisterol el cual fluye fuera de la membrana plasmática y se desorganizan los lípidos de membrana. Los ROS activan la enzima adenilato ciclasa la cual activa las tyrosin kinasas. A la vez, inhiben la tirosin fosfatasa produciendo una fosforilacion generalizada de los residuos tirosina de proteínas clave en el proceso de capacitación (Aitken *et al.* 2015).

Según realizan su función, los ROS comienzan también a inducir peroxidación lipídica, provocando la externalización de fosfatidil serina (Aitken *et al.* 2015), indicador de la apoptosis y promotor de los procesos de fagocitosis por parte de los leucocitos presentes en el ambiente (Mupfiga *et al.* 2013).

Si mientras se capacita el espermatozoide no encuentra el ovocito, la producción de ROS acaba venciendo los sistemas antioxidantes, sobrepasando la capacidad del espermatozoide para mantenerlos en niveles fisiológicos, lo cual desemboca en daño celular y senescencia del espermatozoide (Gallardo Bolanos *et al.* 2014).

La producción continua de ROS provoca la oxidación de los esteroles de membrana en la mitocondria, produciendo oxisteroles (Boerke *et al.* 2013). Recientes estudios sugieren que la producción de oxisteroles en la mitocondria puede inducir la activación de la via intrínseca de la apoptosis, provocando la activación de las caspasas (Aitken 2011; Aitken *et al.* 2015). Por tanto un desequilibrio en la producción de ROS, podría forzar el paso de los espermatozoides hasta apoptosis, impidiendo el desarrollo de la capacitación y por tanto la posible fecundación del ovocito.

Finalmente, podemos determinar por tanto que en un espermatozoide deben de converger diversos eventos para que sea capaz de fecundar un ovocito: su espermatogénesis debe de ser correcta, sin cargar con efectos pre-apoptoticos, debe de haberse librado de los elementos inhibidores del plasma seminal, ser capaz de desarrollar un movimiento lineal al principio e hiperactivado tras la capacitación, su producción de ROS debe de estar dentro de límites fisiológicos, debe de haber capacitado en el momento preciso para no verse afectado por procesos apoptoticos posteriores, ser capaz de reconocer la zona pelucida , desarrollar la reacción acrosómica y reconocer el ovocito mediante sus receptores de membrana. De los millones de espermatozoides que constituyen un eyaculado solo unos cientos son capaces de reunir todas estas condiciones y llegar a través del oviducto hasta el ovocito en tiempo y forma adecuados.

El objetivo de la presente tesis doctoral es el de la búsqueda de nuevos parámetros espermáticos evaluados *in vitro* que se correlacionen con fertilidad *in vivo*. Este análisis puede revelar qué técnicas analíticas pueden ayudarnos a determinar mejor la fertilidad de una muestra seminal descongelada.

Así, en el primer y segundo trabajo de la presente tesis hemos intentado analizar la relación entre diferentes parámetros espermáticos analizados por citometría de flujo y su posible relación con la fertilidad *in vivo*. Además, también se han buscado relaciones entre la motilidad analizada mediante SCA® y la fertilidad.

En una segunda fase de esta tesis y basándonos en resultados de nuestro propio grupo de investigación que apuntaban a la FIV como la técnica con mayor correlación con fertilidad, decidimos estudiar más en profundidad cómo se comportaban los espermatozoides durante este proceso, concretamente durante la capacitación espermática. Este estudio del comportamiento espermático durante la capacitación *in vitro* nos permitiría conocer el comportamiento de los mismos durante la fecundación *in vivo*, y poder mejorar así los resultados de fertilidad. Así, en el tercer trabajo de la presente tesis decidimos estudiar la relación entre capacitación y apoptosis, dado que podría tratarse dos procesos relacionados, con comportamientos similares, aunque final completamente diferente. Finalmente, en el último trabajo de la presente tesis, decimos dar un paso más y comprobar si como apuntan otros autores la capacitación y la apoptosis son procesos opuestos provocados por el estrés oxidativo producido por los radicales libres.

Como ya hemos comentado anteriormente la relación de dichos parámetros con la fertilidad varía según la especie a estudiar, el origen de la muestra y los tratamientos que haya sufrido dicha muestra. Debido a que la mayor parte de las muestras seminales se utilizan tras un protocolo de congelación-descongelación, enfocar el estudio en muestras que han sufrido dicho protocolo se revela como la opción con mayor utilidad práctica. La elección del ganado ovino para la realización de esta tesis se realizó en base a varios factores: la facilidad para obtener muestras seminales, para sincronizar los celos, obtener datos y diseñar experimentos de fertilidad. Además, la raza manchega además es una raza de producción lechera, por lo que su selección no se ha dirigido hacia una alta fertilidad o prolificidad mostrando los moruecos gran variedad de características espermáticas.

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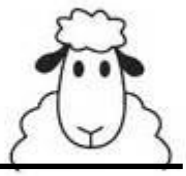
Capítulo 3



Objetivos

En el presente trabajo nos hemos planteado los siguientes objetivos:

1. Evaluar la relación entre la motilidad espermática, de muestras espermáticas de ovino descongeladas, analizada mediante sistemas automáticos de análisis de imagen y la fertilidad *in vivo*.

2. Estudiar las posibles características seminales de muestras espermáticas de ovino descongeladas, que analizadas mediante citometría de flujo nos puedan ofrecer una relación con la fertilidad *in vivo.*

3. Estudiar el impacto que el proceso de apoptosis tiene sobre los espermatozoides de ovino descongelados durante el proceso de capacitación espermática *in vitro.*

4. Evaluar el efecto de la producción de los radicales libres de oxígeno por parte de los espermatozoides descongelados de ovino sobre el proceso de capacitación espermática.

Capítulo 4

Fertility of cryopreserved ovine semen is determined by sperm velocity

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Fertility of cryopreserved ovine semen is determined by sperm velocity

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ABSTRACT

The present study aims to examine the predictive value of some sperm parameters on male fertility. Semen samples from six Manchega rams were collected and cryopreserved. Sperm quality was assessed after thawing and after 2 h of incubation, either in the freezing extender (37 °C) or after dilution in Synthetic Oviductal Fluid (SOF) (38 °C, 5% CO_2), attempting to mimic the physiological conditions of the female reproductive tract. The following sperm parameters were evaluated: motility and kinetic parameters by computer-assisted semen analyzer (CASA), and sperm viability (propidium iodide), mitochondrial membrane potential (JC-1), apoptotic-like membrane changes (YO-PRO-1), acrosomal status (PNA-FITC), and intracellular calcium (fluo-3) by flow cytometry. Results showed no significant differences between incubation media neither after thawing nor after incubation. There were no significant correlations between fertility and sperm parameters assessed by flow cytometry. However, after incubation in the freezing extender, sperm samples from males with poor fertility yielded less linearity and velocity ($P < 0.05$) as indicated by motility parameters analyzed by CASA. These results indicate that kinematic sperm motility parameters evaluation by CASA might be useful to identify samples with poor fertility.

1. Introduction

The assessment of the fertility potential of a semen sample has been the paramount objective of semen analysis, to predict the outcome of a future artificial insemination. Many studies have aimed at uncovering this relationship between sperm quality parameters and *in vivo* fertility, with different outcomes (Papadopoulos et al., 2005; Rodríguez-Martínez, 2003; Schneider et al., 1999; Zhang et al., 1998). Conventional semen assessment using light microscopy has been increasingly replaced by fluorescent staining techniques, flow cytometry and computer-assisted sperm analysis (CASA) (Hallap et al., 2006). Moreover, an increasing number of techniques for *in vitro* semen evaluation have aimed at evaluating more precisely characteristics of the sperm that are essential for fertility. However, any study has yielded a conclusive link among sperm quality and fertility.

Thus, the objective in the present study was to explore laboratory techniques that would allow to quickly and effectively evaluate the potential fertility of a sperm sample. The present study follows the previous study by García-Alvarez et al. (2009a), which showed that heterologous *in vitro* fertilization assays were related to ram sperm fertility. In the present study, it was decided to study the behavior of sperm samples after incubation in the same medium used for *in vitro* fertilization, to determine if after

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"physiological-like" incubation the relation of sperm quality tests with fertility results would be improved.

To perform the present study, we have focused in flow cytometry as a tool with great statistical power because of its ability to analyze thousands of cells in a few seconds, improving existing analyses of fluorescence microscopy and allowing for new multi-parametric analyses (Martínez-Pastor et al., 2010). There have been many attempts to correlate flow cytometry results and fertility (Gillan et al., 2003). For instance, mitochondrial function and membrane integrity with fertility after artificial insemination with ram (Soler et al., 2008) or bull (Gualtieri et al., 2005) semen. However, García-Alvarez et al. (2009a) did not find any relation to fertility with the following sperm parameters: viability (PI membrane exclusion), membrane stability (YOPRO1), membrane phospholipid disorder (M540), and mitochondrial membrane potential (Mitotracker Deep Red), all assessed by flow cytometry. This study is a further step, testing additional sperm parameters in a modified experimental setting, to clarify the relation of several sperm variables to fertility.

Thus, with the present study it was decided to evaluate different sperm parameters and their relation to *in vivo* fertility, to study whether these assays have the attributes to determine the potential fertility of a sperm sample. Acrosomal status (peanut agglutinin – PNA – conjugated with fluorescein), viability and apoptosis (using the fluorochromes propidium iodide (PI) and YOPRO1, respectively) were evaluated because the activation of apoptotic pathways could be responsible for poor fertility resulting from use of a sperm sample for artificial insemination. Moreover, the presence of active mitochondria is important because they participate in many regulatory and maintenance processes, and could also be linked to sperm death (Aitken et al., 2007), so mitochondrial activity (JC-1) was evaluated.

Calcium is an intracellular messenger that has a key role in sperm capacitation. Recently, Marquez and Suarez (2007) established the relationship among capacitation status and intracellular calcium concentration in frozen-thawed sperm. In this regard, intracellular calcium concentrations were analyzed as a factor related to sperm capacitation (fluo-3) (Maxwell and Watson, 1996).

To fully address this topic, sperm motility was evaluated using a computer-assisted semen analyzer (CASA) which provides precise and accurate information on sperm kinematic parameters (Gravance and Davis, 1995), allowing a more accurate prediction of fertility than the parameters assessed by the routine microscopic semen evaluation (Farrell et al., 1998; Malo et al., 2005). The objective assessment of sperm function could increase the chances of predicting the fertilizing capacity of a frozen-thawed semen sample or diagnosing infertility problems.

2. Materials and methods

2.1. Reagents and media

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The remaining of the chemicals (Reagent grade or higher) and propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes were purchased from Invitrogen (Barcelona, Spain). Stock solutions of the fluorescence probes were: 7.5 mM PI in water; 50 μm YOPRO1 in DMSO; 100 μg/mL FITC-PNA in water; 0.7 mM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) in DMSO; 5 μM fluo-3 in DMSO.

All fluorescent stocks were kept at −20 °C, in the dark until needed. The freezing extender was prepared using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA). The fluorochrome acridine orange was of electrophoretic grade and purchased from Polysciences Inc. (Warrington, PA, USA).

Syntetic Oviductal Fluid (SOF) was composed of: NaCl 107 mM, KCl 7.17 mM, KH_2PO_4 1.19 mM, $Ca_2Cl{\cdot}2H_2O$ 1.71 mM, $MgCl_2{\cdot}6H_2O$ 0.49 mM, $NaHCO_3$ 25.07 mM, Na lactate 3.3 mM, Na pyruvate 0.3 mM and glutamine 200 mM.

2.2. Animals and semen collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. Adult males belong to the Regional Center for Animal Selection and Reproduction in Valdepeñas (CERSYRA). Six males of white Manchega sheep breed (age > 3 years) were used. Males were selected based on average fertility by artificial insemination. Thus, three males were selected with an average fertility over 50% and three males with an average fertility under 50%. After males were chosen, semen collection, and the intrauterine insemination and the assessment of sperm quality was performed. Semen collection was performed with an artificial vagina. The volume, concentration, mass motility (0: no movement to 5: strong movement) and motility (%) immediately after collection were evaluated. Only the ejaculates with mass movement greater than 4 and individual motility greater than 80% after 10 min in a warm bath at 37 °C were used in the present study.

2.3. Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. The extender used was prepared as described by Fiser et al. (1987). Fraction 1 was added 3:2 to semen and the sample was slowly cooled from 30 °C to 5 °C for 2 h. Then, the samples were further diluted (3:1) with Fraction 2 at 5 °C, reaching a final concentration of 200×10^6 sperm/mL, and held at this temperature for equilibration for 2 h (total refrigeration time at 5 °C was 4 h). At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-ml plastic straws and frozen. The straws were frozen in a programmable biofreezer (Planner) at −20 °C/min to −100 °C, and at −10 °C/min from −100 °C to −140 °C and then plunged into liquid nitrogen. Thawing was performed by putting the straws in a water bath with saline at 37 °C for 30 s, and the contents were transferred into a glass tube.

2.4. Artificial insemination trials

Thawed sperm samples from the six males were used to inseminate 551 ewes in eight farms. Sperm samples from each male were used to inseminate between 11 and 262 females. The ewes were synchronized using progestagen pessaries (30 mg fluorogestone acetate, FGA; Chronogest, Intervet, The Netherlands) for 13 d followed by 500 IU equine chorionic gonadotrophin (eCG) at pessary removal. Ewes were inseminated intrauterine by laparoscopy at 55–58 h after pessary removal. Two technicians performed all intrauterine inseminations in different dates.

A male was considered to have contributed to a successful fertilization when the female lambed. Fertility rate for each male was calculated as follows: number of lambed ewes/number of ewes inseminated × 100. This rate was called male fertility.

2.5. Assessment of frozen-thawed sperm

Thawed samples were incubated for 2 h (37 °C) without dilution (*i.e.*, in the freezing extender) or after dilution 1:25 in SOF medium, at 5% CO_2 (38 °C). Samples were analyzed after this incubation time by CASA and flow cytometry. Sperm motility (subjective) was assessed for each sample after thawing. Percentage of individual motile sperm (motility) was noted.

2.6. Sperm motility assessed by CASA

Semen were diluted down to 10–20 × 10^6 sperm/mL and loaded into a Makler counting chamber (10 μm depth) at 37 °C. The CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan), equipped with a warming stage at 37 °C and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the Sperm Class Analyzer (SCA2002) software (Microptic S.L.; Barcelona, Spain). Sampling was conducted using a × 10 negative phase contrast objective (no intermediate magnification). Image sequences were saved and analyzed afterwards. Software settings were adjusted to ram sperm. The standard parameter settings were as follows: 25 frames/s; 20–90 μm^2 for head area; VCL > 10 μm/s to classify a spermatozoon as motile. For each sperm, the software rendered the percentage of motile sperm (TM), the percentage of progressive motile sperm (PM) three velocity parameters (VCL: velocity according to the actual path (μm/s); VSL: velocity according to the straight path (μm/s); VAP: velocity according to the smoothed path (μm/s), LIN: linearity (%); ALH: amplitude of the lateral displacement of the sperm head (μm); and BCF: head beat-cross frequency, (Hz). These parameters have been defined elsewhere (Mortimer, 1997).

2.7. Flow cytometry analyses

Sperm samples were analyzed using a Cytometer, Cytomics FC500 (Beckman Coulter, Brea, CA, USA). Excitation was provided by a 488 nm Argon-Ion laser. The FSC (forward – scattered light) and SSC (side-scattered light) signals were used to gate out debris (non-sperm events). FL1 photodetector (530/28BP filter) was used for YOPRO1, FITC-PNA, and JC-1; FL2 (575/26BP filter) for JC-1; FL3 (620SP filter) for PI events. The acquisition was controlled using the MXP software. All the parameters were read using logarithmic amplification. About 5000 sperm cells were acquired from each sample.

Staining solutions were prepared using SOF-HEPES (10 mL of SOF medium supplemented with 23.5 mg of HEPES). Sperm were diluted in 0.5 mL of the different staining solutions in polypropylene tubes for flow cytometry (final concentration 5 × 10^6 sperm/mL).

2.7.1. Sperm viability and apoptosis-like changes

State of plasma membrane (apoptosis-like changes) with YOPRO1 and the viability with propidium iodide (PI) were assessed (García-Alvarez et al., 2009b). A staining solution using SOF-HEPES was prepared by adding 50 nM YOPRO1, and 15 μM PI. Amounts of 20 μL of sample were diluted in 0.5 mL of staining solution in polypropylene tubes for flow cytometry. The tubes were allowed to equilibrate for 15 min in the dark and then analyzed by low cytometry. The PI stains the nucleus of sperm with damaged plasma membranes. YOPRO1 stains the nucleus when the membrane permeability increases, a phenomenon associated to apoptosis in other cell types (Martínez-Pastor et al., 2009). YOPRO1−/PI− were considered viable sperm (indicating live sperm with intact plasmalemma), whereas YOPRO1+/PI− were considered as sperm with continuous plasmalemma, but with apoptotic-like disorders.

2.7.2. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)

The lipophilic cationic probe JC-1 was used to assess the mitochondrial status of the sperm. According to the manufacturer (Molecular Probes, Invitrogen Life Sciences, Fullerton, CA, USA.) and as described Robles and Martínez-Pastor (2013), JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when the mitochondrial membrane potential is great. Sperm samples were diluted with SOF-HEPES to a concentration of 5 × 10^6 sperm/mL, 300 mL of each sample were transferred to a polypropylene tube, and 1.2 mL of JC-1 stock solution (0.7 mM JC-1 in DMSO) was added. The tubes were incubated at 37 °C for 30 min in the dark. The stained sperm samples were then analyzed by flow cytometry, which identified cells with great mitochondrial membrane potential (hMMP; orange-stained cells).

2.7.3. Assessment of acrosomal integrity

Acrosomal status was assessed in a 12 μM PI and FITC-PNA 1 μg/mL staining solution. The PNA (peanut agluttinin) binds specifically to the internal side of the external membrane of the acrosome, labeling acrosome-damaged sperm. The fluorescent technique allows distinguishing among four sperm populations: PI−/PNA− were considered as living cells with intact acrosomes, PI+/PNA− as dead cells with intact acrosomes, PI+/PNA+ as dead cells with

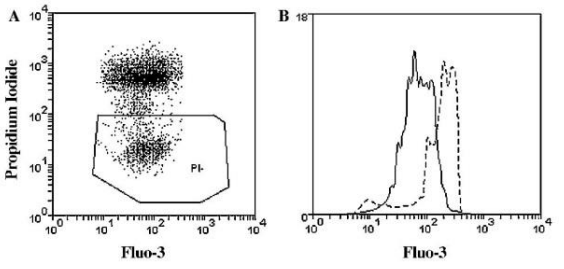

Fig. 1. Representative cytogram and histogram obtained by flow cytometry analysis of a ram sample after loading the sperm with Fluo-3, a specific stain for intracytoplasmatic calcium, and counterstained with propidium iodide (PI), a non-permeable membrane stain, for assessing viability. (A): Fluo-3/PI dot plot, showing a gate to discard PI positive sperm (membrane damaged). (B): Fluo-3/PI negative (Non-damaged membrane) histogram, showing only fluorescence from viable sperm (gated). The histogram shows fluorescence results from an untreated sample (solid line) and after incubating with 1 μM of calcium ionophore (dashed line). The mean fluorescence was obtained from each histogram, and a rate was obtained by dividing the non-treated mean by the ionophore-incubated mean, resulting in an estimation of the intracellular calcium concentration. Data were obtained from Cytomics FC500 Cytometer (Beckman Coulter, Brea, CA, USA).

67 damaged acrosomes and PI−/PNA+ as live cells with damaged acrosomes.

2.7.4. Detection of intracelular calcium concentration

To assess the amount of intracellular calcium existing in the cytoplasm and reserves held by the sperm, samples were stained in a 5 μM fluo-3 and 12 μM PI in SWB (Sucrose Wash Buffer) as described by Harrison et al. (1993). The fluo-3 has affinity for Ca^{2+} and to a lesser extent by Mg^{2+}; when it binds to these cations, it emits green fluorescence. Mean of green fluorescence of living cells (PI−) was evaluated (Fig. 1). A replicate was performed adding to a second tube 1 μM of calcium ionophore (A23187) and incubating 10 min. The average calcium content of viable sperm in each sample was estimated by the ratio of untreated and ionophore-treated tubes.

2.8. Statistical analysis

All statistical analysis was performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). All variables that were not normal were transformed using the arc sine (percentages) or decimal logarithm. Statistical signification was considered when $P < 0.05$.

A preliminary study was conducted to estimate the effects of some environmental factors on fertility outcomes. These factors were: year and season of insemination, farm in which females were managed, technicians who perform the inseminations and the number of ewes inseminated per male. All factors showed a significant effect with the exception of number of ewes inseminated per male. Therefore, prior to examining the relationships among male fertility and sperm traits, fertility outcome was corrected by all these significant factors as a way to reduce the variability due to other factors than the sperm characteristics. The study of the relationships among male fertility by intrauterine insemination and sperm features were performed by using a uni-variate linear regression.

3. Results

3.1. Semen evaluation

The effects of sperm incubation either in the freezing extender or in the SOF medium are summarized in Tables 1 and 2. Table 1 shows several motility variables as yielded by the CASA system. No significant differences ($P > 0.05$) were detected between the two media after thawing and after 2 h of incubation. Motility parameters PM, VAP, VCL, VSL and ALH decreased during incubation, regardless of the medium used for incubation. Total motility, however, decreased ($P < 0.05$) only in those samples diluted with the freezing extender, while no significant differences were observed when a SOF medium (38 °C, 5% CO_2) was used.

Table 2 presents the effect of dilution and incubation during 2 h in the freezing extender (37 °C) and in the SOF (38 °C, 5% CO_2) on flow cytometry variables. Results showed an overall decrease in sperm quality as assessed by flow cytometry after incubation, whereas no significant differences were observed between the two media ($P > 0.05$).

3.2. Correlations between sperm parameters and in vivo fertility

The sperm samples used in this study were selected based on heterogeneity regarding its *in vivo* fertility. Three males were selected with an average fertility above 50% and three males with an average fertility below 50%. Male fertility by intrauterine artificial insemination ranged from 22%

Table 1
Sperm motility parameters assessed by the CASA system Sperm Class Analyzer (SCA®). Values are expressed as Mean ± S.E.M. Sperm analyses were conducted immediately after thawing or after dilution in Synthetic Oviductal Fluid (SOF), and after 2 h of incubation in the freezing medium (37 °C) or in SOF (38 °C, 5% CO_2).

Time (h)	Treatment	TM	PM	VAP	VCL	VSL	LIN	ALH	BCF
0	Freezing extender	83.8 ± 3.8^{a}	24.8 ± 4.2^{a}	70.1 ± 3.9^{a}	89.5 ± 2.5^{a}	49.7 ± 6.5^{a}	47.0 ± 3.9^{a}	2.9 ± 0.2^{a}	4.9 ± 0.2^{a}
	SOF	79.8 ± 4.0^{a}	21.6 ± 3.9^{a}	64.8 ± 7.0^{a}	83.5 ± 6.0^{a}	43.9 ± 6.7^{a}	45.4 ± 3.2^{a}	2.8 ± 0.2^{a}	4.69 ± 0.3^{a}
2	Freezing extender	43.8 ± 11.9^{b}	9.2 ± 2.6^{b}	37.0 ± 7.4^{*b}	49.9 ± 7.3^{*b}	25.6 ± 5.7^{*b}	44.2 ± 3.6^{a}	2.1 ± 0.1^{b}	4.1 ± 0.6^{***b}
	SOF	63.1 ± 10.0^{a}	8.8 ± 1.2^{b}	29.4 ± 2.3^{b}	43.1 ± 1.9^{b}	19.3 ± 2.12^{b}	38.4 ± 1.6^{a}	2.1 ± 0.1^{b}	3.3 ± 0.2^{b}

Motility parameters: TM: % total motile sperm, PM: % sperm with a progressive movement; VAP: velocity according to the smoothed path (μm/s), VCL: velocity according to the actual path (μm/s); VSL: velocity according to the straight path; LIN: linearity (%); ALH: amplitude of the lateral displacement of the sperm head; μm); the BCF (head beat-cross frequency, Hz). ^{ab}Different letters indicate differences between incubation times ($P < 0.05$). * correlation with fertility *in vivo* ($P < 0.05$). *** correlation with fertility *in vivo* ($P < 0.001$).

Table 2
Results of sperm parameters evaluated by flow cytometry and its relation to *in vivo* fertility assessed by artificial insemination. Values are expressed as (Mean ± S.E.M.) provided by cytometry and their relation to fertility. Sperm analyses were conducted immediately after thawing or dilution in SOF and after 2 h of incubation, either in the freezing medium (37 °C) or after dilution in Synthetic Oviductal Fluid (SOF) medium (38 °C, 5% CO_2).

Time (h)	Treatment	Viability (%)	hMMP (%)	Apoptotic-like membrane changes (%)	Acrosomal integrity (%)	Intracellular calcium rate (%) (viable cells)
0	Freezing extender	26.5 ± 4.7[a]	29.5 ± 4.3[a]	3.9 ± 0.4[a]	19.4 ± 3.0[a]	46.0 ± 9.0[a]
	SOF	32.7 ± 3.3[a]	29.8 ± 5.1[a]	5.7 ± 0.8[a]	22.7 ± 2.8[a]	39.7 ± 9.0[a]
2	Freezing extender	15.3 ± 2.4[a]	14.6 ± 2.7[b]	5.0 ± 1.1[a]	9.8 ± 1.8[a]	55.9 ± 9.0[b]
	SOF	24.1 ± 3.4[a]	16.3 ± 3.8[a]	6.7 ± 1.6[a]	12.4 ± 3.2[a]	54.4 ± 9.0[a]

Cytometry parameters: Positive sign (+) indicates cell staining, negative sign (−) indicate the lack of staining for each flourochrome. PI: propidium iodide. Viability: % of YO-PRO−/PI− (membrane intact) sperm; hMMP: % of sperm with high mitochondrial membrane potential (JC-1 +); Apoptotic-like membrane changes: % of YO-PRO+/PI− sperm; acrosomal integrity: % of PNA+/PI− sperm; Intracellular calcium rate (viable cells): result of dividing the average fluo-3 mean fluorescence by the average fluo-3 mean fluorescence after incubating with 1 μM calcium ionophore, as percentage. [a,b]Different letters indicate differences between incubation times ($P < 0.05$).

to 83%, with a mean value of 44%. There were differences in fertility among males ($P = 0.003$).

The possible relation between kinematic and flow cytometry variables with *in vivo* fertility was studied using a lineal regression analysis. There were relationships between some kinematic parameters and field fertility only after 2 h of incubation in the freezing extender. In this regard, average-path velocity (VAP), the curvilinear velocity (VCL) and the head beat-cross frequency (BCF), showed a high positive correlation with fertility ($P = 0.044$ ($R^2 = 0.678$), $P = 0.027$ ($R^2 = 0.745$) and $P = 0.006$ ($R^2 = 0.852$), respectively; Fig. 2; Table 1).

4. Discussion

Recently, García-Alvarez et al. (2009a) showed that heterologous *in vitro* fertilization was a good procedure to predict the fertility of ram semen, unlike sperm evaluation by flow cytometry. However, methods based in IVF are costly in time needed to conduct the procedure and from a financial perspective. Therefore, in the present study two objectives were proposed. First, the objective was to study the quality of cryopreserved sperm using an incubation model in freezing extender or IVF media to determine if these stressful conditions could improve the relation between sperm quality variables (as assessed after incubation) with field fertility. Therefore, it could be concluded from these results whether these incubation models were practical, as a standard method for improving sperm quality assessment. Complementary with the first objective, the second objective aimed to identify laboratory techniques that would be most appropriate for, in the experimental approach defined by the first objective, to quickly and effectively evaluate the fertility potential of a sperm sample.

Motility has been considered one of the most important characteristics associated with the fertilizing ability of sperm (Saacke and White, 1972). In the present study, a decrease in motility variables was detected after the incubation, reflecting the stressful situation that sperm incur in these conditions. Interestingly, a positive relationship was detected among several kinematic variables (VCL, VAP, VSL, BCF) measured after the incubation in the extender and field fertility. To our knowledge, this is the first time a relationship was demonstrated between motility assessed by CASA and fertility of cryopreserved ram sperm. The difference in the present study with previous studies is the inclusion of a post-thawing incubation prior to the assessment. Thus, O'Meara et al. (2008) and García-Alvarez et al. (2010) did not detect a significant relationship between fertility and sperm quality (functional parameters or CASA, respectively) in thawed ram sperm. Results of the present study show the importance of pre-treating sperm before assessing the quality (in this case, submitting them to incubation at 37 °C). Malo et al. (2005) found differences in fertility between red deer stags related strongly to sperm swimming velocity parameters (VCL, VSL and VAP), and results of the present study support the hypothesis that sperm velocity is one of the key features in the process of fertilization, which has been also demonstrated in a large number of taxa (Gage et al., 2004; Holt et al., 1989). Greater motility is a result of a physiologically functional sperm, and sperm with decreasing motility are indicative of decreased sperm metabolism or failing organelles. This might indicate not only a decreased ability to reach the oocyte (of lesser importance in laparoscopic insemination), but reflects a lesser ability to undergo capacitation, the production of an excess of free radicals or to execute key steps in egg fertilization (Aitken et al., 2012; Martínez-Pastor et al., 2009). Results of the present study agree with studies in other species (bull: Amann et al., 2000; Farrell et al., 1998; Kathiravan et al., 2008; goat: Fernandez-Santos et al., 2011), which show that kinematic parameters as VCL, VSL and VAP are related to fertility. Other researchers have found positive correlations between different velocity parameters and fertility in human sperm (Fetterolf and Rogers, 1990).

Flow cytometry has been successful as a tool for the study of several physiological features of sperm (Petrunkina et al., 2007), and many tests have been related with *in vivo* fertility (Gillan et al., 2003; Januskauskas et al., 2000; Wilhelm et al., 1996). However, the study of ram sperm by flow cytometry has not been related to field fertility. For instance, O'Meara et al. (2008) did not found that viability or acrosomal status of ram sperm studied by flow cytometry were related to *in vivo* fertility. In 2007, Rodríguez-Martínez and Barth indicated that both modern sperm evaluation techniques and conventional techniques have been related to fertility, but these relationships are modest and quite variable between laboratories.

It must be pointed out that these studies did not make use of a pre-treatment of thawed sperm prior to the study

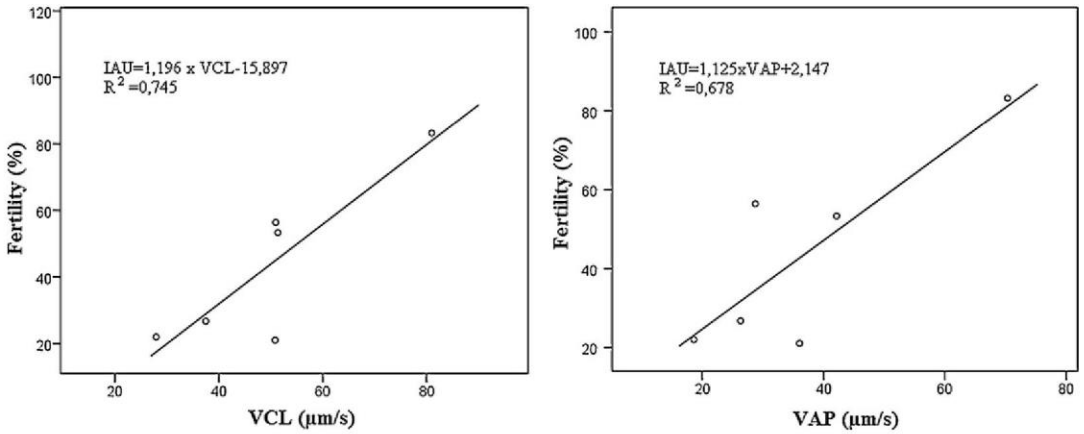

Fig. 2. Relationship between *in vivo* male fertility and VCL (velocity according to the actual path) and VAP (velocity according to the smoothed path). Regression lines and their equations are showed ($P < 0.05$). Data were obtained using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, SCA2002, Microptic S.L.: Barcelona, Spain).

of sperm quality. The incubation of sperm at physiological of above physiological temperatures is a challenge that may help to uncover the authentic fertility potential of the sample. This kind of treatment can be helpful for other kind of experiments. For instance, in a recent study the post-thawing quality of red deer sperm cryopreserved with different antioxidants were not very differentfrom the control, but after incubating the samples at 39 °C many differences were detected (Anel-López et al., 2012). Because in the present study it was found that there was a direct relationship between motion parameters and fertility, and mitochondria have been considered a fundamental organelle to sperm physiology (Mukai and Okuno, 2004; Peña et al., 2009), it would be logical to find a relationship between mitochondrial status and fertility. There, however, was not any relation between mitochondrial activity as measured using JC-1, and fertility in the present study. In this regard, Volpe et al. (2009) and Cheuquemán et al. (2011) indicated that JC-1 is suitable for detection of inner mitochondrial membrane potential changes in canine sperm, but it should always be associated with an objective motility analysis to avoid an incorrect evaluation of potential sperm fertility. García-Alvarez et al. (2009a) also showed that mitochondrial membrane potential of ram sperm, analyzed with MitoTracker deep red, was unrelated to fertility. Indeed, it has been considered that the main role of the sperm mitochondria was the production of energy for sperm motility, but this concept is under assessment and the roles of mitochondria will likely be broadened as more research assessing these roles is conducted (Marin et al., 2003; Miki et al., 2004; Mukai and Okuno, 2004). Mitochondria also have a crucial role in diverse cellular functions apart from energy production, such as modulation of the redox balance, osmotic regulation and Ca^{++} homeostasis (Peña et al., 2009). Therefore, the assessment of mitochondrial status could eventually offer important information relative to the sperm fertilizing ability, but this might require other experimental approaches.

Different researchers have demonstrated that intracytoplasmatic calcium efflux is a necessary component for capacitacion (Gualtieri et al., 2005). Although in the present study greater calcium concentrations were detected after incubation, there was not any relation between fertility and the relative intra-cellular calcium concentration with any treatment. Thus, the concentration of Ca^{2+} in the sperm after thawing or after incubation did not reflect the fertility of the sperm samples. That does not mean that Ca^{2+} concentrations are irrelevant. Measurement of the Ca^{2+} profile after treating sperm with progesterone or other physiological signals could offer relevant information related to sperm fertility (Arienti et al., 2010). The primary conclusion from the present research is that thawed semen samples with a greater sperm velocity is related to a greater field fertility, but only when measurements were conducted after 2 h of incubation in the freezing extender. Interestingly, incubation in SOF in conditions seeking to mimic the female oviductal environment did not yield any significant relationship with fertility. To our knowledge, no other researchers have found this relationship between sperm motility parameters and *in vivo* fertility of rams following intrauterine artificial insemination of ewes with frozen-thawed semen. Results cannot be generalized with conclusions of the present study, because of the limited number of males involved. Nevertheless, the experimental approach in the present study evaluating the semen samples after a 2 h incubation at 37 °C in the freezing medium merits further research, and it could be the basis of protocols for predicting the *in vivo* fertility of frozen-thawed ram sperm samples.

Acknowledgements

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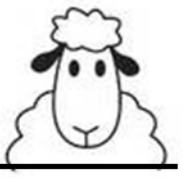
Capítulo 5



Free-radical production after post-thaw incubation of ram spermatozoa is related to decreased in vivo fertility

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Free-radical production after post-thaw incubation of ram spermatozoa is related to decreased *in vivo* fertility

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Abstract. The aim of the present study was to evaluate the effect of sperm reactive oxygen species (ROS) production and DNA changes on male fertility. For that purpose, six rams with significantly different pregnancy rates were used; these were classified as having high fertility, i.e. 59.4% average pregnancy rate, or low fertility, i.e. 23.1% average pregnancy rate. Sperm quality was assessed after a two-step process of sample thawing followed by an incubation of 2 h, either in the freezing extender (37°C) or after dilution in synthetic oviductal fluid (SOF; 38°C, 5%CO_2). Sperm viability (YO-PRO-1), ROS production (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein acetyl ester (CM-H_2DCFDA)) and undamaged chromatin (sperm chromatin structure assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, chromomycin A3) were evaluated by flow cytometry. Although no significant differences in sperm viability were observed, our results showed increased ROS production during incubation in the freezing extender as well as in SOF medium. Comparison between fertility groups showed significant differences in ROS production after 2 h of incubation for the two treatments. Regarding DNA integrity, our results showed no significant differences either between treatments and incubation times or fertility groups. Linear regression analysis showed that ROS production determined by CM-H_2DCFDA was a good indicator parameter for *in vivo* male fertility of SOF-incubated samples, yielding a fair correlation between both parameters ($r = -0.92$). These results indicate that detection of ROS production by CM-H_2DCFDA and flow cytometry after 2 h of incubation in SOF could be a useful procedure for predicting fertility of ram spermatozoa.

Additional keywords: artificial insemination, flow cytometry, fluorescein (CM-H_2DCFDA), frozen semen.

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Introduction

Oxidant species have a crucial role on sperm physiology, but they may also impair the fertilising ability of spermatozoa. Oxidative stress may be defined as the outcome of the imbalance between reactive oxygen species (ROS) production and the ability of antioxidants to scavenge them. This oxidative stress is then considered of high concern as high levels of ROS have negative effects on sperm functionality (Gil-Guzman *et al.* 2001).

Furthermore, ROS production is considered as one of the mediators of damage caused by cryopreservation (Alvarez and Storey 1992; O'Flaherty *et al.* 1997; Chatterjee and Gagnon 2001; Fernández-Santos *et al.* 2007) as it may lead to DNA damage (Lopes *et al.* 1998), cytoskeletal alterations (Hinshaw *et al.* 1986), inhibition of spermatozoon–oocyte fusion (Aitken *et al.* 1989) and effects on the sperm axoneme followed by loss of motility (de Lamirande and Gagnon 1992). Thus, it could be feasible that oxidative stress may be related to cell death and loss of cell function during or following a freeze–thaw cycle (O'Flaherty *et al.* 1997; Bailey *et al.* 2000).

Therefore, the study of oxidative stress of thawed sperm samples and its relationship to fertility is of great interest, as these samples could be used for artificial insemination. Many studies have aimed to elucidate the relationship between sperm-quality parameters and *in vivo* fertility, with different outcomes (Schneider *et al.* 1999; Rodríguez-Martínez 2003; Papadopoulos *et al.* 2005; Petrunkina *et al.* 2007).

The mechanisms by which oxidative stress limits the functional competence of mammalian spermatozoa involve the peroxidation of lipids, the induction of oxidative DNA damage and the formation of protein adducts (Aitken *et al.* 2012). Thus, the effect of oxidative stress on spermatozoa can also be monitored by assessing the levels of DNA damage (Lewis and Aitken 2005). The sperm chromatin structure assay (SCSA) technique and the terminal deoxynucleotidyl

transferase-mediated dUTP nick-end labelling (TUNEL) assay have been proposed for the evaluation of sperm DNA fragmentation. By using SCSA, a high DNA fragmentation index (DFI) has been related to reduced fertility, longer times to pregnancy and higher spontaneous miscarriage rates in humans (Virro *et al.* 2004; Evenson and Wixon 2006). It has also shown a correlation with fertility and prolificacy in domestic animals (bull, Waterhouse *et al.* (2006); García-Macías *et al.* (2007); boar, Boe-Hansen *et al.* (2008)). Similarly, the TUNEL assay is considered to be a promising technique in accordance with SCSA and fertility results (Waterhouse *et al.* 2006; Benchaib *et al.* 2007). The results of the chromomycin A3 (CMA3) test, a fluorochrome that detects protamine deficiency in loosely packed chromatin, are correlated with the extent of nicked DNA (Gillan *et al.* 2005). Therefore, the aim of this work was to evaluate the effect of ROS production on post-thaw sperm characteristics, in order to gain knowledge about the underlying mechanisms of differences in ram fertility. To this end, ROS production and DNA fragmentation levels of cryopreserved sperm samples were assessed, and an *in vivo* fertilisation test was conducted. Both the study of oxidative stress and DNA analysis could be useful tools for quality assessment of thawed semen doses from domestic animals, before artificial insemination, and potential fertility determination of given semen samples.

Materials and methods

Experimental design

Two straws per male were thawed. Sperm samples were previously analysed to ensure that the two straws had similar characteristics and there were no differences between them. Standardised insemination doses were used. Thawed sperm samples were incubated for 2 h (37°C) without dilution (in the freezing diluent) or after dilution 1 : 25 in synthetic oviductal fluid (SOF) medium, at 5% CO_2 (38°C). SOF medium is an *in vitro* fertilisation medium that tries to mimic the female reproductive tract, commonly used as a test of endurance. Samples were analysed just after thawing and after dilution in SOF. After 2 h of incubation, sperm samples were also analysed.

Reagents and media

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probe propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes were purchased from Invitrogen (Barcelona, Spain) unless otherwise stated. Stock solutions of the fluorescent probes were: PI, 1.5 mM in Milli-Q water; YO-PRO-1, 50 μM in DMSO (dimethyl sulfoxide); CM-H_2DFCDA, 0.5 mM in DMSO; CMA3, 0.25 mg mL^{-1} in DMSO. All fluorescent stocks were kept at −20°C in the dark until needed. The freezing extender was prepared in our laboratory as described by Fiser *et al.* (1987), using reagent-grade chemicals purchased from Panreac Química SA (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA). The fluorochrome acridine orange was of electrophoretic grade and purchased from Polysciences Inc. (Warrington, PA, USA).

SOF was composed of 107 mM NaCl, 7.17 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM $Ca_2Cl{\cdot}2H_2O$, 0.49 mM $MgCl_2{\cdot}6H_2O$, 25.07 mM $NaHCO_3$, 3.3 mM Na lactate, 0.3 mM Na pyruvate and 200 mM glutamine. Osmolarity was 270–280 mOsm kg^{-1} and pH (room temperature) 7.2–7.3 (Gardner *et al.* 1994).

Animals and sperm collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. Adult males belonged to the Regional Center for Animal Selection and Reproduction in Valdepeñas (CERSYRA). A total of six males of Manchega sheep breed (age >3 years) were used. Semen collection was performed using an artificial vagina. Volume, concentration, wave motion (0 no movement to 5 strong wave movement) and sperm motility were assessed shortly after collection. Only ejaculates with wave-motion values of 4 or 5 and sperm motility higher than 80% were frozen.

Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. Initially, before freezing, the ejaculates were diluted to a final concentration of 200×10^6 spermatozoa mL^{-1}. First, sperm samples were diluted in fraction 1 of the diluent to a concentration of 400×10^6 spermatozoa mL^{-1} and slowly cooled from 30 to 5°C in 2 h. Then, sperm samples were further diluted (2 : 1) with fraction 2 (5°C) and held for equilibration at 5°C for 2 h (total refrigeration time at 5°C was 4 h). At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen. The straws were frozen in a programmable biofreezer (IceCube 14S-Ver. 1.30; SY-Laboratory Geräte GmbH, Minitüb®, Tiefenbach, Germany) at −20°C min^{-1} to −100°C, and at −10°C min^{-1} from −100°C to −140°C and then plunged into liquid nitrogen. Two straws per male were thawed for 30 s at 37°C in a warm bath and aliquots were used to assess sperm quality and concentration.

Artificial insemination trials

Thawed sperm samples from the six males were used to inseminate 351 ewes in eight farms. Sperm samples from each male were used to inseminate between 27 and 147 females. The number of inseminated ewes per male was 49, 29, 27, 147, 42 and 57 for Males 1, 2, 3, 4, 5 and 6, respectively. The insemination dose was 100×10^6 spermatozoa and contained more than 55% motile spermatozoa. Only one ejaculate per male was used in this study. Sperm samples sourced from selected rams of a reproductive selection centre. All samples had a minimum quality that includes low morphological abnormalities.

The ewes were synchronised using progestagen pessaries (30 mg fluorogestone acetate, (FGA); Chronogest, Intervet, Booxmer, The Netherlands) for 13 days followed by 500 IU equine chorionic gonadotrophin (eCG) at pessary removal. Ewes were inseminated intrauterine by laparoscopy at 55–58 h after pessary removal. Two technicians carried out all intrauterine inseminations on different dates.

We considered that a male scored a successful fertilisation when the female lambed. The fertility rate for each male was calculated as follows: number of lambed ewes/number of ewes inseminated × 100. This rate was called male fertility.

The males were classified according to fertility in two groups: high fertility, those with fertility above mean (male fertility 55%) and, low fertility, those with fertility below mean (male fertility <55%).

Flow cytometric analysis

After appropriate treatment with fluorescent probes (see below), sperm suspensions preincubated with or without SOF were analysed on a Cytomics FC500 flow cytometer (Beckman Coulter) controlled by WEASEL software Ver. 3 (WEHI, Melbourne, Vic., Australia). Cells were passed through the instrument at 150–300 cells s^{-1}, and data were collected for 10 000 cells. The cells were excited at 488 nm using an argon laser. Fluorescein isothiocyanate (FITC), CM-H_2DCFDA, CMA3 and acridine orange (AO) green fluorescence was detected with a 530/28 band-pass filter (FL-1) while propidium iodide and AO red fluorescence was detected with a 620/40 band-pass filter (FL-3), both on logarithmic scales. The control settings were adjusted for measuring the fluorescence in FL-1 and FL-3 channels essentially as described in the Supplementary Material available in the online version of this paper.

Sperm viability

Sperm viability was checked by means of propidium iodide staining (PI, 15 μM; stock, 50 μM in DMSO) and YO-PRO-1 (50 nM; stock, 7.5 mM in milli-Q water) in combination with flow cytometry as described by Martínez-Pastor *et al.* (2008). The staining solution was prepared using SOF-HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) (~$1–2 \times 10^6$ spermatozoa mL^{-1}). After 15 min of incubation in the dark, sperm samples were analysed by flow cytometry. Propidium iodide binds to DNA in membrane-defective spermatozoa and allows the identification of viability. YO-PRO-1 is a probe capable of staining early apoptotic cells (with intact plasmalemma, not stained by PI, but showing increased permeability; Martínez-Pastor *et al.* 2009). The control settings were adjusted for measuring the fluorescence in FL-1 and FL-3 channels essentially as described in the Supplementary Material. The percentage of YO-PRO-1-positive non-DNA particles ranged from 15.7% (freezing extender) to 17.9% (SOF).

Production of reactive oxygen species

Reactive oxygen species production was recorded using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein acetyl ester (CM-H_2DCFDA) as described by Domínguez-Rebolledo *et al.* (2010). CM-H_2DCFDA penetrates the plasma membrane and is retained after intracellular esterases cleave the acetate groups and emits green fluorescent (504 nm) upon oxidisation. The intensity of fluorescence of CM-H_2DCFDA increases as ROS production increases. Sperm suspensions (1×10^8 cells mL^{-1}) were loaded at 37°C, 20 min with CM-H_2DCFDA in SOF-HEPES (final concentration 0.5 μM; stock solution, 500 μM). Main population at 0 h was set under the first decade of the logarithmic scale of the 530/28 BP photodetector as reference value in a histogram. With these settings, samples were analysed at 0 h and 2 h of incubation and the mean value was noted (Fig. 1).

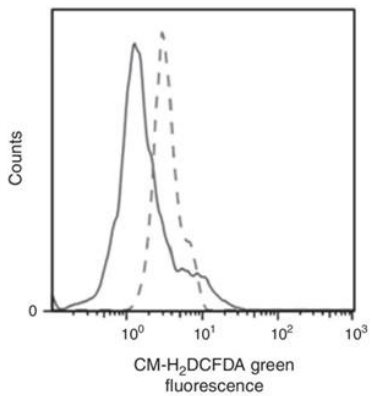

Fig. 1. Free-radical production assessed by CM-H_2DCFDA histogram for incubated sperm samples in SOF. Continuous line indicates free-radical production at 0 h. Semi-continuous line indicates free-radical production at 2 h of incubation at 37°C. Displacement of the mean was analysed to assess the increase in radical production.

Sperm chromatin structure assay (SCSA)

Chromatin stability was assessed following the sperm chromatin structure assay (SCSA), based on the susceptibility of sperm DNA to acid-induced denaturation *in situ* and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson *et al.* 2002) Acridine orange (AO) fluorescence shifts from green (double strand (dsDNA)) to red (single strand (ssDNA)). Samples were diluted in TNE buffer (0.01 M TRIS-HCl, 0.15 M NaCl, 1 mM EDTA; pH 7.4) to a final sperm concentration of 2×10^6 cells mL^{-1}. Samples were frozen (−80°C) until needed. For analysis, the samples were thawed in crushed ice. Acid-induced denaturation of DNA *in situ* was achieved by adding 0.4 mL of an acid–detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl; pH 1.4) to 200 μL of sample. After 30 s, the cells were stained by adding 1.2 mL of an acridine orange solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 6 μg mL^{-1} acridine orange; pH 6.0). The stained samples were analysed by flow cytometry exactly 3 min after adding the acridine orange solution.

A tube with 0.4 mL of acid–detergent solution and 1.2 mL of acridine orange solution was run through the system before running any samples and between samples. At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0–1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence versus total intensity of the fluorescence (red/[red+green] × 100), called the DNA fragmentation index (DFI; formerly called αt) for each spermatozoon, representing the shift from

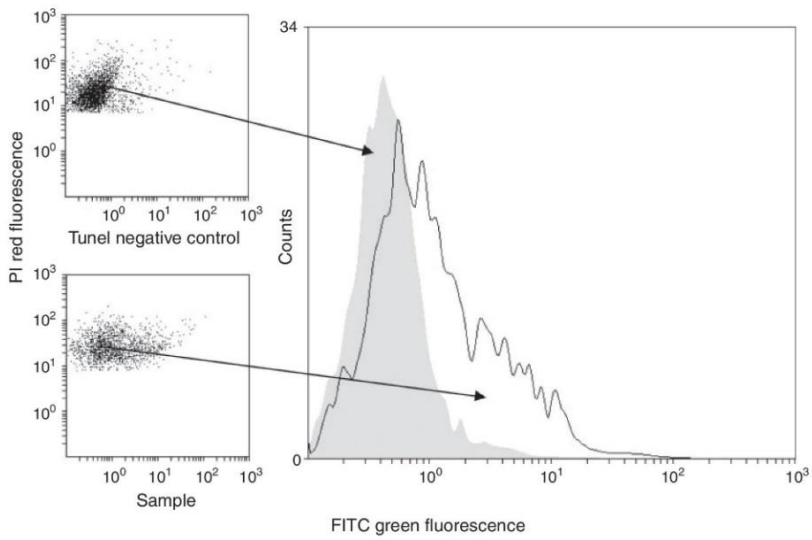

Fig. 2. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. PI is used to discriminate fixed cells from debris. Grey histogram represents basal fluorescence (negative control) from no-antibody FITC-conjugated treated cells. White histogram represents sample fluorescence. Sample cells exhibiting a greater green fluorescence than the basal level are considered TUNEL positive and are expressed as a percentage (%).

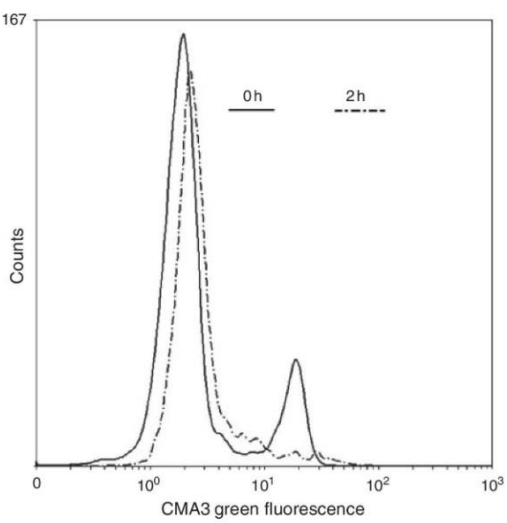

Fig. 3. Sperm histone re-emplacement measured by CMA3. Dotted line indicates chromatin compaction at 2 h of incubation. Changes after 2 h and displacement of the mean were compared.

green to red fluorescence. High values of DFI indicate chromatin abnormalities. Flow cytometry data was processed to obtain %DFI (percentage of spermatozoa with DFI >25) and high DNA stainability (HDS; percentage of spermatozoa with green fluorescence higher than channel 600 of 1024 channels).

TUNEL assay

DNA damage was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) assay, following the manufacturer's instructions (ApoTarget APO-BRDU Kit; Life Technologies). Briefly, spermatozoa diluted in phosphate-buffered saline (PBS; 10^6 cells mL^{-1}) were fixed for 1 h in 2% paraformaldehyde. The cells were washed twice with PBS and resuspended in 70% ethanol at 0°C. The samples were left at −20°C overnight. Then, the cells were washed twice using the wash buffer provided with the kit, adding the DNA labelling mixture after removing the wash buffer. After 60 min at 37°C (with agitation), the cells were washed twice using the rinse buffer. Finally, the cells were resuspended in the antibody solution (FITC–anti-BrdUTP mAb) and incubated for 30 min at room temperature in the dark. Samples were resuspended in a PI–RNase A solution and analysed by flow cytometry within 2 h. Positive and negative controls (incubation of fixed cells with DNase A and substituting water for the DNA labelling mixture, respectively) were used to standardise the assay. DNA damage was measured using FL1 for the detection of the emission of FITC in the green spectrum and the FL3 photodetector for the emission of PI in the red spectrum. Non-red events (PI–) were discarded as debris. Setting non-DNA-labelled spermatozoa as control their basal green fluorescence was established as a threshold; all events above that limit were considered TUNEL+ (Fig. 2).

Chromomycin A3 (CMA3) determination

The CMA3 penetrability test was used to assess the extent of sperm nuclear protamination and as an indicator of chromatin

condensation. CMA3 labelling and analysis by flow cytometry were performed as described previously by Zubkova *et al.* (2005). CMA3 has affinity for DNA but is unable to access it when histones have been replaced by protamines or when the disulfide bridges are intact. Sperm samples were stained for 20 min in McIlvaine's buffer with CMA3 antibiotic at a concentration 0.25 mg mL^{-1}. CMA3 emits green fluorescence when bound to DNA. The mean of the green fluorescence was taken for each sample, once adjusted with a control (green emission at 0 h incubation was adjusted at first decade of the logarithmic scale). Variations between 0 h and 2 h were compared (Fig. 3).

Statistical analysis

Three statistical analyses were performed in this study. First, a multiple regression analysis was carried out to determine which variables significantly affected *in vivo* fertility. Four explicative variables were considered: date of insemination, farm and male as the factors of interest, with the number of ewes inseminated included as a weighing factor. Second, the effect of incubation times and extenders was compared with ANOVA, carrying out a pairwise comparison (Bonferroni's correction) when any of the effects was significant.

Table 1. GLM (generalised linear model) of male fertility on number of inseminated ewes, insemination date, farm and male (model: $R^2 = 0.95$; $P = 0.02$)

Dependent variable	Independent variable	*P* value
Male fertility	Number of inseminated ewes	0.441
	Insemination date	0.100
	Farm	0.560
	Male	0.006

Finally, the two fertility groups were compared for all the sperm parameters assessed on thawed samples using a *t*-test. This allowed us to examine if sperm characteristics differed between the two groups, and therefore could be responsible for the fertility differences. The relationship between ROS production and *in vivo* fertility was examined by using linear regression analysis.

All statistical analyses were performed using SPSS for Windows version 20.0 (IBM SPSS Statistics Inc., Chicago, IL, USA). Differences were considered to be statistically significant at $P < 0.05$.

Results

Male fertility rates ranged from 22% to 64% with a mean value of 40.5%. Differences in fertility rates among males were significant ($P = 0.006$). Of all effects we considered to affect the fertility success, only male showed a significant effect. Thus, the insemination date, the farm in which insemination took place and the number of inseminated ewes per male did not affect male fertility in a significant way (Table 1).

Sperm viability results of each male and for the two treatments are shown in Fig. 4. There were no significant differences in viability between the two treatments (freezing extender vs SOF) either after thawing ($P = 0.30$) or after 2 h of incubation ($P = 0.06$).

Values for the sperm ROS production after thawing and after 2 h of incubation in the two different media are shown in Fig. 5. ROS production increased significantly after incubation both in the freezing extender ($P = 0.02$) and in the SOF medium ($P = 0.01$).

As can be observed in Table 2, there were no significant differences in viability between high-fertility males and low-fertility ones, at any time of evaluation, either in the freezing extender or in SOF. After 2 h of incubation, males classified as

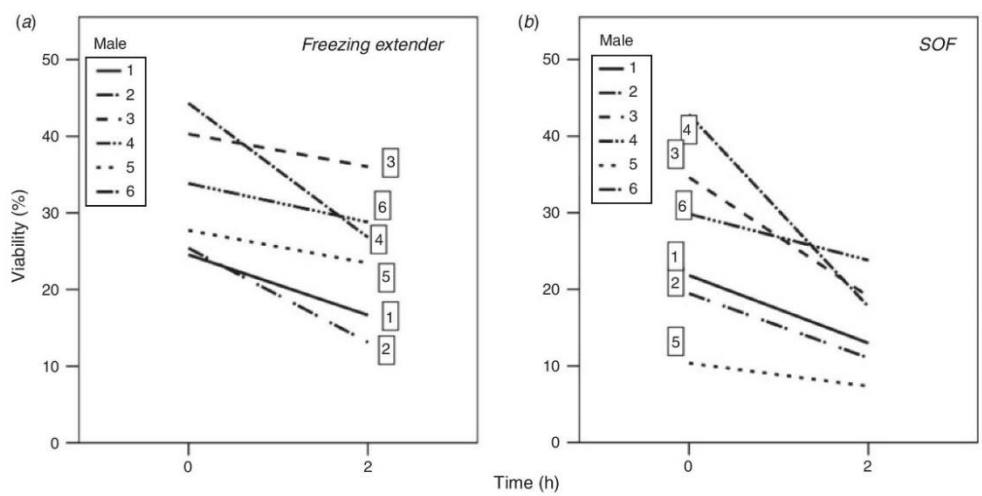

Fig. 4. These plots show the effect of the individual males (1–6) on sperm viability (YO-PRO-1−/PI−; live spermatozoa with intact plasmalemma) after 2 h of incubation in (*a*) freezing extender or (*b*) SOF medium (5% CO_2, 38°C).

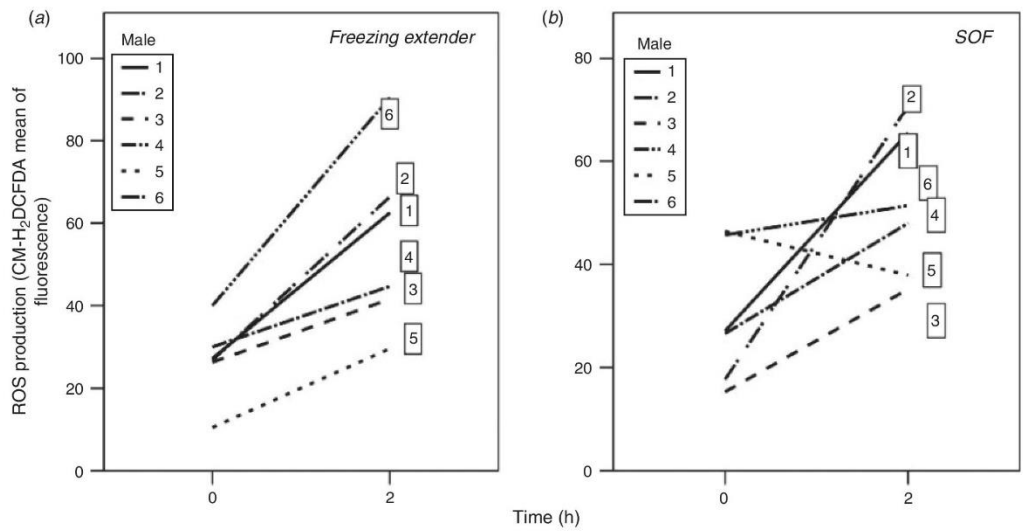

Fig. 5. These plots show the effect of the individual males (1–6) on ROS production assessed by CM-H_2DCFDA (mean of the fluorescence) after 2 h of incubation in (*a*) freezing extender or (*b*) SOF medium (5% CO_2, 38°C).

Table 2. Values (least squares mean ± s.e.m.) for the sperm parameters in the groups of high and low fertility

FE, freezing extender; SOF, synthetic oviductal fluid; viability, percentage of YO-PRO-1−/PI− spermatozoa (live with intact plasmalemma); ROS production, mean fluorescence intensity for CM-H_2DCFDA; tDFI, DNA fragmentation index (SCSA; % of DNA fragmentation); TUNEL, percentage of TUNEL+ spermatozoa; CMA3, chromatin compaction status (mean fluorescence intensity for CMA3). *$P < 0.05$

Time of evaluation	Fertility group	Viability (%)	ROS production (mean)	tDFI (%)	TUNEL (%)	CMA3 (mean)
After thawing	High	29.2 ± 7.2	29.4 ± 8.6	0.6 ± 0.04	7.1 ± 0.9	32.4 ± 7.4
	Low	23.7 ± 7.2	30.1 ± 8.6	0.7 ± 0.04	8.5 ± 0.9	42.3 ± 7.4
After dilution in SOF	High	37.4 ± 4.1	22.2 ± 5.2	0.3 ± 0.04	7.4 ± 2.7	46.6 ± 10.0
	Low	27.9 ± 4.1	31.3 ± 5.2	0.4 ± 0.04	9.7 ± 2.7	55.3 ± 10.0
2 h incubation in FE	High	15.9 ± 3.8	40.3 ± 6.9	0.8 ± 0.15	12.1 ± 2.1	32.3 ± 2.1
	Low	14.7 ± 3.8	62.5 ± 4.9*	0.7 ± 0.15	9.8 ± 2.1	37.9 ± 2.1
2 h incubation in SOF	High	28.7 ± 4.2	38.6 ± 6.9	0.3 ± 0.03	20.7 ± 5.4	30.2 ± 2.4
	Low	19.5 ± 4.2	73.1 ± 6.9*	0.5 ± 0.03	11.7 ± 5.4	36.2 ± 2.4

of high fertility had significantly lower ROS production than those of low fertility, both in the freezing extender (FE; 40.36 ± 6.9 vs 62.53 ± 6.9; $P = 0.03$) and SOF (38.63 ± 6.9 vs 73.13 ± 6.9; $P = 0.02$). Once differences in ROS production between fertility groups were identified, we decided to investigate whether DNA sperm parameters were also modified between both groups. Nevertheless, there were no significant differences for DNA fragmentation either after thawing or after incubation, as detected by both the SCSA and TUNEL techniques. Furthermore, we did not find any significant difference for packaging quality as assessed by CMA3 (Table 2).

Finally, we examined the relationship between ROS production levels and *in vivo* male fertility. Although initially we did not find a relationship between ROS production and *in vivo* male fertility, after 2 h of incubation in SOF, ROS production levels showed a negative correlation ($r = -0.92$; $P < 0.01$) with male fertility (Fig. 6). However, this correlation was not found after 2 h of incubation in the freezing extender.

Discussion

In the present study, despite finding no significant differences in sperm viability, our results showed increasing ROS production during incubation both for freezing extender and SOF medium. Comparison between fertility groups showed significant differences in ROS production after 2 h of incubation for the two treatments. After 2 h of incubation in SOF, ROS production levels were negatively correlated with *in vivo* male fertility.

Oxidative stress has an important role in sperm physiology. In this context, oxidative stress is increasingly recognised as a major cause in the aetiology of human male infertility (Aitken and Krausz 2001; Tremellen 2008). Moreover, cryopreservation not only reduces sperm quality, but also induces oxidative stress and decreases the antioxidants in semen (Aisen *et al.* 2005; Peris *et al.* 2007). The mechanisms of oxidative stress that limit the functional competence of mammalian spermatozoa involve the peroxidation of lipids, the induction of oxidative DNA

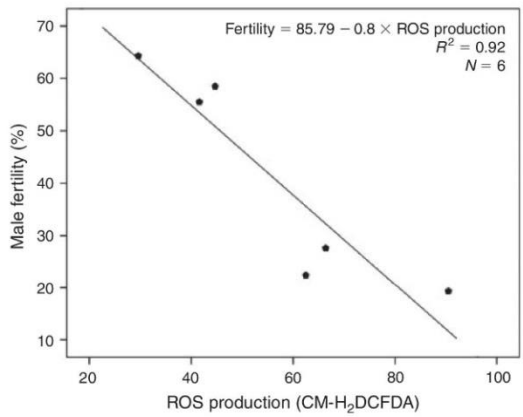

Fig. 6. Relationship between pregnancy rate and ROS production assessed by CM-H_2DCFDA after 2 h of incubation in SOF medium (5% CO_2, 38°C).

damage and the formation of protein adducts (Aitken *et al.* 2012). It is clear that ROS are important contributors to the regulation of sperm function from a positive and a negative point of view. The beneficial and detrimental effects of ROS probably represent a continuum (Aitken 2011). Thus, following insemination, these cells generate low levels of ROS in order to promote capacitation and the functional evolution of sperm behaviours needed for fertilisation, including hyperactivation and the presentation of zona-recognition molecules on their surface (Aitken *et al.* 1989; de Lamirande and Gagnon 1993). If fertilisation does not occur, as is usually the case for an individual spermatozoon, then the continued ROS generation overwhelms the meagre intrinsic antioxidant defences of these cells and activates the intrinsic apoptotic cascade. In light of the foregoing, an excessive production of ROS could be related to early development of capacitation and hyperactivation processes, preventing spermatozoa from developing properly. Thus, as our results show, incubation in IVF media may also result in an overproduction of ROS, which impairs sperm function and fertility. The increase of ROS could not result in negative effects by itself, but could indicate a subjacent cause (mitochondrial failure, pathway deregulation.etc), which produces both increased ROS and lower fertility. Thus, the increased ROS would not be the cause of the lower fertility, but a signal that something is not being regulated correctly in the spermatozoa.

Indeed, it seems that the information provided by the CM-H_2DCFDA test combined with SOF incubation is useful for improving sperm-quality assessment, and both methods could be considered as a standard tool for sperm-quality determination. In fact, other results indicate that spermatozoa with higher levels of ROS, measured by CM-H_2DCFDA, produced embryos that could not progress beyond the first division (Silva *et al.* 2007).

The assessment of the fertility potential of a semen sample has been the paramount objective of semen analysis. Thus, our objective is to develop laboratory techniques that allow us, in a quick and effective manner, to evaluate the potential fertility of a given sperm sample. However, the predictive power of these tests is generally low and very variable among different authors (Rodríguez-Martínez 2007). The objective assessment of sperm function could increase the chances of predicting the fertilising capacity of a frozen–thawed semen sample or diagnosing infertility problems. Taking into account the importance of ROS production in thawed sperm samples, we have addressed this issue evaluating its effect on fertility. It is remarkable that García-Álvarez *et al.* (2009) showed that heterologous *in vitro* fertilisation was a good procedure to predict the fertility of ram semen. According to our results, differences in ROS production could be responsible for different *in vitro* fertilisation results. However, methods based on IVF are costly and time-consuming. There are many *in vitro* techniques that have attempted to correlate with *in vivo* fertility (Schneider *et al.* 1999; O'Meara *et al.* 2008; García-Álvarez *et al.* 2009). In this context, significant positive correlation between different velocity parameters and fertilisation percentage has been reported (Amann *et al.* 2000; Kathiravan *et al.* 2008; Fernández-Santos *et al.* 2011). Recently, Del Olmo *et al.* (2013) showed that evaluation of kinematic ram sperm motility parameters by computer assisted perm analyser (CASA) might be useful to identify samples with poor fertility. Specifically, O'Meara *et al.* (2008) did not find any relationship between *in vitro* sperm functional test results and *in vivo* fertility of rams following artificial insemination of ewes with frozen–thawed semen. In this context, it is remarkable that designing a method that could predict *in vivo* fertility is of great interest as assessing individual male fertility by artificial insemination (AI) is an expensive and laborious procedure. Hence, there is a clear need for alternative techniques capable of evaluating *in vitro* sperm characteristics with good correlation to *in vivo* fertility.

Knowing that an excessive accumulation of ROS could cause damage not only to the plasma membrane but also the sperm DNA (Bennetts and Aitken 2005), we decided to evaluate DNA integrity and its relationship to *in vivo* fertility. DNA damage in spermatozoa has been linked to reduced rates of fertilisation, impaired preimplantation development, miscarriage and morbidity in the offspring (Zini and Sigman 2009; Aitken and De Iuliis 2010; Avendaño and Oehninger 2011). In general, significant DNA damage is rarely found in a proven fertile male, and the incidence of DNA damage is higher in infertile males. Although most researchers would intuitively agree that DNA damage is probably detrimental, the clinical impact of DNA damage has been more challenging to prove, perhaps because the correct functional end points have not been identified. Some studies suggest that sperm DNA damage levels can predict success using assisted reproductive techniques (ART) (Bungum *et al.* 2007) and they may also predict the likelihood of recurrent pregnancy loss (Carrell *et al.* 2003). Zini *et al.* (2005) suggested that sperm DNA damage may provide a useful correction biomarker for detrimental fertility impairing conditions. Since our results showed that free-radical production after post-thawing incubation of ram spermatozoa is related to decreased *in vivo* fertility, it is possible that DNA damage could be related to oxidation processes. In this regard, Thomson *et al.* (2011) have shown that 8-hydroxy-2́-deoxyguanosine, a biomarker of oxidative DNA damage, is a marker with high predictive values of

human semen fertility after artificial insemination. Although some authors have found a negative relationship of DNA damage with fertility (Evenson *et al.* 2002) we could not find this relationship in our study either analysing DNA with SCSA or with TUNEL assay and CMA3. We had no fragmentation, and thus we could say that our fertility results have not been affected by the chromatin status of samples. This effect is more commonly observed for human spermatozoa than for ruminants, maybe due to the high degree of DNA packaging of ruminants (Januskauskas *et al.* 2001). Thus, our results showed that the possible mechanism responsible for fertility differences is not due to DNA damage.

Having discovered a significant connection between ROS production and field fertility, we suggest that the addition of antioxidant to the freezing extender could have positive effects on the freezability of ram spermatozoa and therefore on the results of fertility after artificial insemination with thawed ram sperm samples. In this regard, many researchers have demonstrated the benefits of adding antioxidants to the freezing extender (Breininger *et al.* 2005; Roca *et al.* 2005; Fernández-Santos *et al.* 2007) or after thawing (Fernández-Santos *et al.* 2009; Bucak *et al.* 2010; Domínguez-Rebolledo *et al.* 2010). However, it should be determined which antioxidants are suitable for the preservation of ram sperm samples, because depending on the origin of samples and conservation, antioxidants may be beneficial or not (Foote *et al.* 2002; Fernández-Santos *et al.* 2007; Fernández-Santos *et al.* 2009; Domínguez-Rebolledo *et al.* 2010). In any case, benefits of antioxidant addition on *in vivo* fertility in sheep have thus far not been shown.

In conclusion, we have found that the information provided by the CM-H_2DCFDA test combined with the SOF incubation is useful and could be considered a standard method for improving sperm-quality assessment; however, the limited number of males used in this study suggests a cautious interpretation. This combination seems to be a good procedure to identify the signal that something is not being regulated correctly in the spermatozoa and that directly affects fertility. Nevertheless, the experimental approach in the present study evaluating the semen samples after a 2-h incubation at 38.0°C (5% CO_2) in the SOF medium merits further research, and it could be the basis of protocols for predicting the *in vivo* fertility of frozen–thawed ram sperm samples.

Acknowledgements

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Capítulo 6

Estrous sheep serum enables in vitro capacitation of ram spermatozoa while preventing caspase activation

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ABSTRACT

Estrous sheep serum (ESS) is considered the most efficient agent for *in vitro* capacitation of ram spermatozoa. We have explored the relationship between caspase activation and capacitation in ram. Semen samples from 17 rams were cryopreserved. *In vivo* fertility was evaluated after intrauterine artificial insemination. Samples were submitted to four treatments: control, ESS (10%), caspase inhibitor (Z-VAD-FMK), and estrous ewe serum plus caspase inhibitor (I + E). Sperm samples were incubated for 30 minutes at 38.5 °C and 5% CO_2 and analyzed with flow cytometry for mitochondrial membrane potential (Mito-Tracker deep red), sperm viability and apoptosis-like changes (YO-PRO-1/propidium iodide), acrosomal status (peanut agglutinin–fluorescein isothiocyanate), membrane fluidity (merocyanine 540), and caspase activity (Vybrant FAM kits for polycaspases, caspase-8, and caspases 3–7). Estrous sheep serum induced changes compatible with capacitation, doubling the proportion of viable spermatozoa with increased merocyanine 540 and increasing YO-PRO-1$^+$ and acrosome-reacted spermatozoa ($P < 0.05$). Incubation increased the proportion of spermatozoa with activated caspases ($P < 0.05$), which was abolished by the treatments. We detected a simultaneous decrease in the proportion of the viable and caspase$^-$ spermatozoa after the incubation, which was prevented by the presence of estrous ewe serum ($P < 0.05$). The analysis of caspases 3/7 and 8 resulted in less marked differences. Fertility was positively related to viability and inactivated caspases and negatively to viable-capacitated spermatozoa and active caspases. *In vitro* induction of capacitation in thawed ram spermatozoa by using ESS suggests a downregulation in apoptotic pathways. However, males with the lowest fertility showed parameters similar to high-fertility males, suggesting that other factors were involved apart from capacitation and/or caspase activation.

1. Introduction

Mammalian spermatozoa are not able to fertilize immediately after ejaculation, but they must undergo a set of physiological changes, collectively called "capacitation," to become fertilization competent [1]. These changes are

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the result of interactions with different factors of the seminal plasma, the fluids in the female genital tract, and the intimate association with epithelial cells in the oviduct. These result in profound changes in the composition and distribution of membrane components, loss of cholesterol, intracellular pH changes, activation of intracellular signaling pathways and, ultimately, phosphorylation of a wide range of proteins. These changes allow the spermatozoa to change their motility (hyperactivation) [2,3], release from the oviductal epithelium, interact with the ZP, develop the acrosomal reaction, and fuse with the oocyte membrane [4,5].

Capacitation can be induced *in vitro*, and in some species, it has been achieved in chemically defined media [6–9]. However, there are huge differences between species, and it is necessary to tune-up protocols to achieve acceptable induction of capacitation and, therefore, good IVF results [10]. In fact, attempts to capacitate ram spermatozoa in chemically defined media have failed until relatively recently [11,12], and in some species, nondefined additives must be used. In sheep, estrous sheep serum (ESS) is still considered the most efficient agent for *in vitro* capacitation and fertilization [13,14], although success with defined media has been reported [9]. Incubation of ram sperm samples in medium with ESS leads to changes that are consistent with an increase in the proportion of viable spermatozoa with high membrane fluidity and an increase in the sperm subpopulation characterized by a hyperactivated-like motility pattern [14].

In contrast, apoptosis is a basic biological principle of programmed cell death that occurs in almost every cell type [15]. In spermatozoa, apoptosis is not strictly a physiological event but rather a spontaneous consequence of the changes underwent in the female genital tract and the engagement in the process of fertilization [16]. In humans, presence of apoptotic markers has been associated with low fertilization and implantation rates in assisted reproductive techniques [17]. Caspases (cysteinyl-aspartate-specific proteases) are the main transducers and effectors of the apoptosis signal leading to programmed cell death [18]. They comprise a family of cysteine proteases that cleave proteins after aspartic acid residues. In mammals, caspases are divided into two groups: initiator caspases, such as caspases 2, 8, 9, and 10, and effector or executioner caspases, such as caspases 3, 6 and 7. Reproductive cells can undergo apoptosis during their development and even in their mature state [16].

Spermatozoa are highly specialized cells and are considered transcriptionally inactive, which conditions the kind of apoptotic events taking place and their consequences [16]. Apoptosis has been proposed as a default position for spermatozoa because their fate is to undergo apoptotic death if they fail to fertilize the oocyte [16]. Thus, the key question that several authors invite to answer is not "what induces apoptosis in spermatozoa?" but rather "what prevents this process from occurring?" Interestingly, it seems that a balance between the capacitation and the apoptosis signaling systems could be taking place in spermatozoa because the activation of capacitation in human spermatozoa seems to prevent apoptosis [18].

Therefore, our objective in this study is to test if this balance is taking place in ram spermatozoa. We hypothesized that the effect of ESS *in vitro* would be not only to induce capacitation but also to block apoptosis. We aim to provide new insights into the effects of ESS on physiological changes induced in ram spermatozoa during *in vitro* capacitation.

2. Materials and methods

2.1. Reagents and media

Flow cytometry equipment, software, and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probe propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes and caspase analysis kits were purchased from Life Technologies (Barcelona, Spain). Stock solutions of the fluorescent probes were PI, 1.5 mM in Milli-Q water; YO-PRO-1, 50 μM in DMSO; merocyanine 540 (M540), 3.4 mM in DMSO; MitoTracker deep red, 1 mM in DMSO; peanut agglutinin–fluorescein isothiocyanate (PNA-FITC) 1 mg/mL. All fluorescent stocks were kept at −20 °C in the dark until needed. The freezing extender was prepared in our laboratory as described by Fiser et al. [19] using reagent-grade chemicals purchased from Panreac Química SA (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA).

Synthetic oviduct flui0d (SOF) was composed of 107-mM NaCl, 7.17-mM KCl, 1.19-mM KH_2PO_4, 1.71-mM Ca_2Cl, 0.49-mM $MgCl_2$, 25.07-mM $NaHCO_3$, 3.3-mM Na lactate, 0.3-mM Na pyruvate, and 200-mM glutamine. Osmolality was 270 to 280 mOsm/kg and pH 7.2 to 7.3 [20].

2.2. Animals and sperm collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD 53/2013 which conforms to European Union Regulation 2010/63/UE. Adult males belonged to the Regional Center of Animal Selection and Reproduction in Valdepeñas (CERSYRA, Spain). In total, 17 rams (Manchega breed, age: >3 years) were used. Semen collection was performed using an artificial vagina. Volume, concentration, wave motion (0: no movement to 5: strong wave movement), and sperm motility were assessed shortly after collection. Only ejaculates with wave-motion values of 4 or 5 and sperm motility higher than 80% were frozen.

2.3. Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. First, sperm samples were diluted in fraction 1 of the diluent down to a concentration of 400×10^6 spermatozoa/mL and slowly cooled from 30 °C to 5 °C for 2 hours. Then, sperm samples were further diluted with the same volume of fraction 2 at 5 °C and held for equilibration for 2 hours more. At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen with a

programmable biofreezer (IceCube 14S-Ver. 1.30; SY-Laboratory Geräte GmbH, Minitüb Tiefenbach, Germany) at −20 °C/min to −100 °C and at −10 °C/min from −100 °C to −140 °C and then plunged into liquid nitrogen.

2.4. Experimental design

A total of 17 rams were used in this experiment. Two straws per male were thawed for 30 seconds at 37 °C in a warm bath and aliquots. Sperm quality and concentration were checked, ensuring that the two straws had similar characteristics and there were no differences between them. Then, they were pooled. Sperm samples were deposited in the upper side of a discontinuous Percoll gradient [21] (upper layer: 45% of Percoll in SOF-HEPES [10-mL SOF, 9.8-mM HEPES, and 0.04 mg/mL of gentamicin]; lower layer: 90% of Percoll and 10% of a 9% wt/vol NaCl solution). The tubes were centrifuged for 15 minutes at 700 × g, the supernatant was removed, and the pellet concentration was calculated after addition of 500 μL of SOF + BSA 0.3%. Concentration was adjusted to 10^7 spermatozoa/mL. Samples were divided in four aliquots and submitted to different treatments: control, estrous sheep serum (estrous ewe serum [EES], 10% as used for sheep IVF) [22,23], caspase inhibitor (INH; 100-μM Z-VAD-FMK [24]), and EES plus caspase inhibitor (I + S). Each tube was then incubated for 30 minutes at 38.5 °C and 5% CO_2. Flow cytometry analysis was carried out in the untreated sample after selection (0 minutes) and in the four tubes after the 30 minutes of incubation.

2.5. Artificial insemination trials

Thawed sperm samples from the 17 males were used to inseminate 1012 ewes in eight farms. Doses from each male were used to inseminate between 21 and 262 females. Each insemination dose consisted of 100 × 10^6 spermatozoa, with at least 55% motile spermatozoa. Only one ejaculate per male was used in this study.

The ewes were synchronized using progestagen pessaries (30-mg fluorogestone acetate, Chronogest; Intervet, Booxmeer, The Netherlands) for 13 days followed by 500-IU eCG at pessary removal. Ewes were inseminated by laparoscopy (intrauterine deposition) at 55 to 58 hours after pessary removal. Two technicians carried out all intrauterine inseminations on different dates.

We considered that a male scored a successful fertilization when the female lambed. The fertility rate for each male was calculated as follows: number of lambed ewes/number of ewes inseminated × 100. This ratio was called male fertility.

2.6. Sperm evaluation

2.6.1. Caspase activity evaluation

Caspase activity detection was evaluated using the Vybrant FAM kits. The Poly Caspases Assay kit was used to detect the overall activation of caspases. To evaluate the activation of initiator caspases in the extrinsic pathway, we used the Vybrant FAM Caspase-8 Assay kit for caspase 8, and for the executioner caspases 3 and 7, we used the Vybrant FAM Caspase-3 and -7 Assay kit. Assays were performed as indicated by the manufacturer: aliquots of sperm suspensions of each treatment were diluted to 10^6 spermatozoa/mL in PBS and 300 μL was transferred to cytometer tubes. Ten microliters of the reactive (FLICA working solution) was added directly to the tubes, mixed, and incubated for 30 minutes at 37 °C. Samples were washed twice with 2 mL of washing solution and centrifuged at 300 × g for 5 minutes. The pellet was resuspended with 300 μL of washing solution, adding PI at 12 μM and incubating for 5 minutes before analysis.

2.6.2. Sperm viability

Sperm viability was checked by means of PI staining (15 μM; stock: 50 μM in DMSO) and YO-PRO-1 (50 nM; stock: 7.5 mM in Milli-Q water) in combination with flow cytometry as described by Martínez-Pastor et al. [25]. The staining solution was prepared using SOF-HEPES (~1–2 × 10^6 spermtazoa/mL). After 15 minutes of incubation in the dark, sperm samples were analyzed by flow cytometry. This PI binds to DNA in membrane-defective sperm and allows the identification of viability. YO-PRO-1 is a probe capable of staining early apoptotic cells (with intact plasmalemma, not stained by PI, but showing increased permeability) [26].

2.6.3. Assessment of mitochondrial membrane potential

MitoTracker deep red is a probe that accumulates in mitochondria, becoming fluorescent when membrane potential is high. Staining solution was prepared using SOF-HEPES, with 100-nM MitoTracker deep red and 50-mM YO-PRO-1, adding spermatozoa up to 10^6/mL. MitoTracker$^+$/YOPRO-1$^-$ spermatozoa were considered viable with active mitochondria, and MitoTracker$^-$/YO-PRO-1$^-$ spermatozoa were considered viable with low mitochondrial membrane potential. YOPRO-1$^+$ spermatozoa were considered nonviable.

2.6.4. Assessment of membrane fluidity

The lipophilic probe M540 was used to assess the membrane fluidity. This probe intercalates becoming more fluorescent when membrane fluidity increases. Staining solution was prepared using SOF-HEPES with 1-μM M540 and 50-nM YOPRO-1, adding spermatozoa up to 10^6/mL. M540$^-$/YOPRO-1$^-$ spermatozoa were considered viable with low membrane fluidity, and M540$^+$/YOPRO-1$^-$ spermatozoa were considered viable with high membrane fluidity. YOPRO-1$^+$ spermatozoa were considered nonviable.

2.6.5. Assessment of acrosome integrity

Peanut agglutinin binds specifically to the internal side of the external membrane of the acrosome, labeling acrosome-damaged spermatozoa. Staining solution was prepared using SOF-HEPES with 12-μM PI and 1 μg/mL of FITC-PNA 1, adding spermatozoa up to 10^6/mL. This fluorescent technique allows distinguishing among four sperm populations: PI$^-$/PNA$^-$ were considered alive with intact acrosomes; PI$^+$/PNA$^-$ as dead with intact acrosomes; PI$^+$/PNA$^+$ as dead with damaged acrosomes; and PI$^-$/PNA$^+$ as alive with damaged acrosomes.

2.7. Flow cytometry analysis

After 15 minutes of incubation with the fluorescent probes (except caspase probes), sperm suspensions were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) controlled with the MXP software (ver. 1). Cells were run through the instrument at 150 to 300 cells/s, collecting data from 10,000 cells. Excitation was carried out with a 488-nm argon-ion laser, except for MitoTracker deep red, which was excited with a 633-nm He-Ne laser. FAM, YO-PRO-1, and FITC green fluorescence was detected with a 530/28BP filter, M540 orange fluorescence with a 575/26BP filter, PI red fluorescence with a 615 DSP filter, and MitoTracker deep red with a 675/40BP filter. Flow cytometry data were analyzed with WEASEL software Ver. 3 (WEHI, Melbourne, Australia).

2.8. Statistical analysis

Statistical analysis was performed using the R statistical environment (ver. 3.1.1). Flow cytometry parameters were analyzed by linear mixed-effects models, using treatment as fixed effects, incorporating the male into the random block structure. The relationships between fertility and flow cytometry parameters were assessed by carrying out regression analyses. Fertility was previously adjusted by linear mixed-effects models for taking into account farm, technician, and date of artificial insemination.

3. Results

The spermatozoa capacitation status, as assessed by the M540 assay, was modified by the treatments. In all cases, the viability of the sperm samples decreased (according to YO-PRO-1 exclusion; Fig. 1A), with an ~20 percent-point difference after ~30 minutes of incubation. Viability was slightly lower when the caspase inhibitor was included (INH and I + S). Considering the proportion of YO-PRO-1$^-$ spermatozoa which presented increased M540 fluorescence (considered "capacitated", Fig. 1B), it did not significantly change if the samples were simply incubated at 37 °C. In the presence of EES, this proportion doubled. The effect sizes were not affected by the presence of the inhibitor, despite the concomitant decrease in viability.

The polycaspase assay showed that the incubation increased the proportion of spermatozoa with activated caspases (Fig. 2A), but both EES and the inhibitor moderated this effect. No synergic effect of EES and the inhibitor was detected. We also detected a simultaneous decrease in the proportion of the viable/caspase$^-$ spermatozoa after the incubation (Fig. 2B), which was partly prevented by EES (But this effect was not evident in the presence of inhibitor.) The proportion of caspase$^+$ spermatozoa within the PI$^-$ population was slightly increased by the incubation ($P < 0.05$) with no differences between treatments (Fig. 2C). The analysis of caspase-3/7 resulted in similar but less marked differences. The proportion of spermatozoa with increased caspase-3 activity was significantly higher after the incubation (Fig. 2D). The addition of neither ESS nor the caspase inhibitor caused a significant change of this variable, and only the combination of both treatments showed a decrease ($P < 0.05$). Similarly, the proportion of viable and caspase$^-$spermatozoa decreased after incubation ($P < 0.001$; Fig. 2E), and the proportion of caspase$^+$ spermatozoa within the PI$^-$ population increased after the incubation with $P < 0.001$ (Fig. 2F), although the combination of ESS and the inhibitor caused a small but significant increase with respect to the control (Fig. 2F).

Caspase-8 activity also showed small changes because of incubation or treatment. The incubation did not significantly increase the proportion of caspase-8$^+$ spermatozoa, but the combination of the inhibitor and ESS decreased it with respect to the control with $P < 0.05$ (Fig. 2G). The proportion of viable and caspase$^-$ spermatozoa decreased

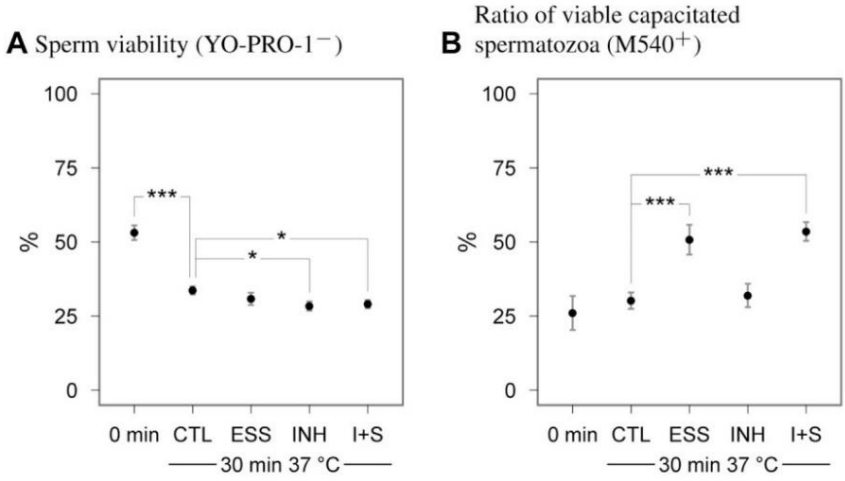

Fig. 1. Viability and capacitation status of ram spermatozoa submitted to the different treatments (YO-PRO-1/merocyanine 540 (M540) assay; mean ± standard error of the mean). Sperm viability (A) is shown as the percentage of YO-PRO-1 unstained events, and capacitation (B) is shown as the percentage of M540$^+$ events with respect to YO-PRO-1$^-$ spermatozoa. Lines join groups with significant differences: before and after the incubation (initial [0 minutes] vs. control [CTL]), and after the incubation, (CTL vs. additives; $^*P < 0.05$; $^{***}P < 0.001$). ESS, estrous sheep serum; INH, caspase inhibitor; I + S, inhibitor and ESS.

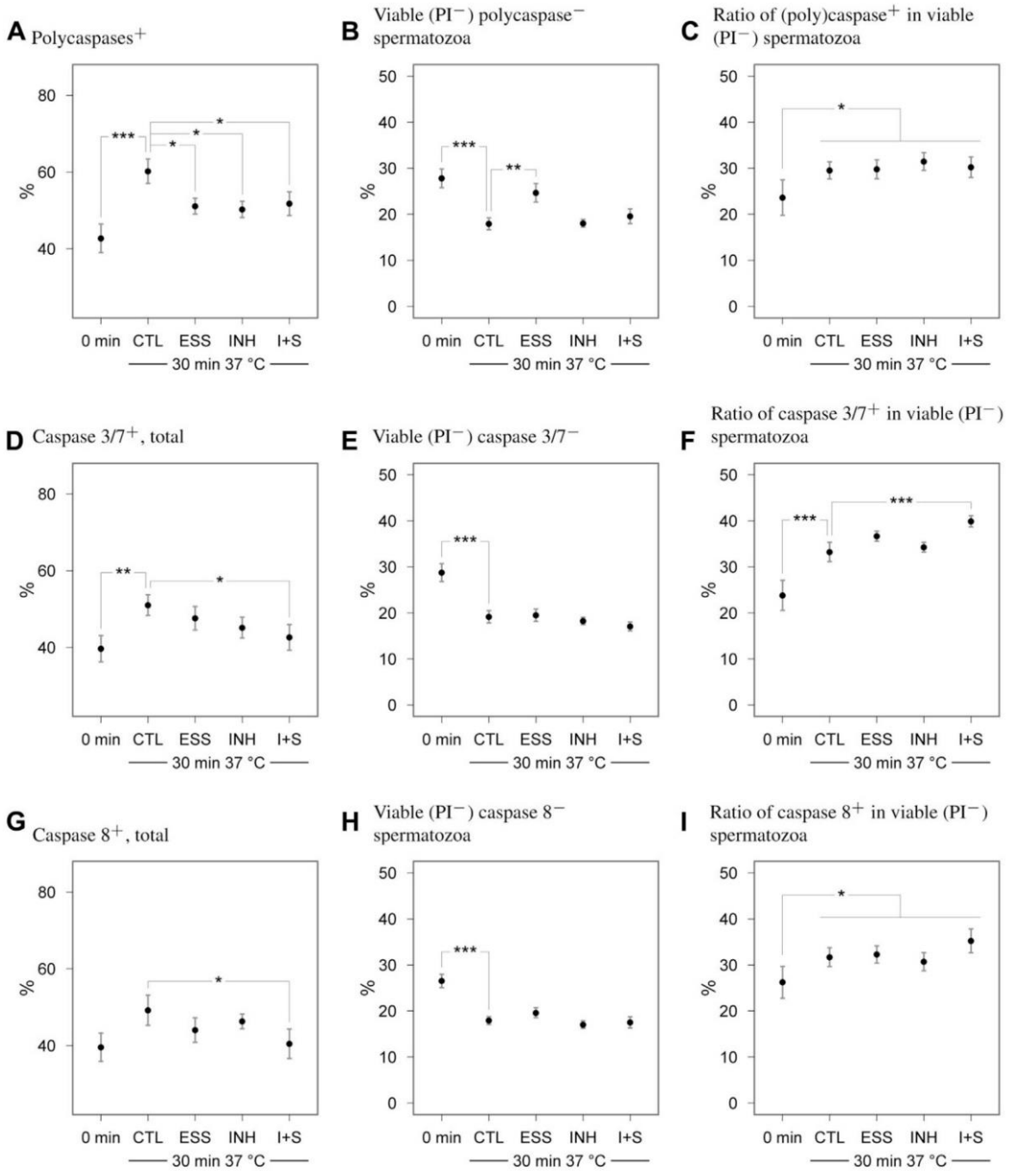

Fig. 2. Results of the caspase activity assays on ram spermatozoa submitted to the different treatments (mean ± standard error of the mean). Plots show the proportion of caspase$^+$ events (A, D, G), percentage of viable and caspase$^-$ spermatozoa (B, E, H), and percentage of caspase$^+$ events with respect to the PI$^-$ sperm subpopulation (C, F, I). Lines join groups with significant differences: before and after the incubation (initial [0 minutes] vs. control [CTL]) and, after the incubation (CTL vs. additives; $^*P < 0.05$; $^{***}P < 0.001$). ESS, estrous sheep serum; INH, caspase inhibitor; I + S, inhibitor and ESS; PI, propidium iodide.

after the incubation but it was not significantly affected by any treatment (Fig. 2H). The proportion of caspase$^+$ spermatozoa within the PI$^-$ population increased with the incubation ($P < 0.05$ considering all postincubation treatments together), but no differences were detected between treatments (Fig. 2I).

Figure 3 shows the effects of incubation and ESS supplementation on apoptosis, acrosomes, and mitochondrial

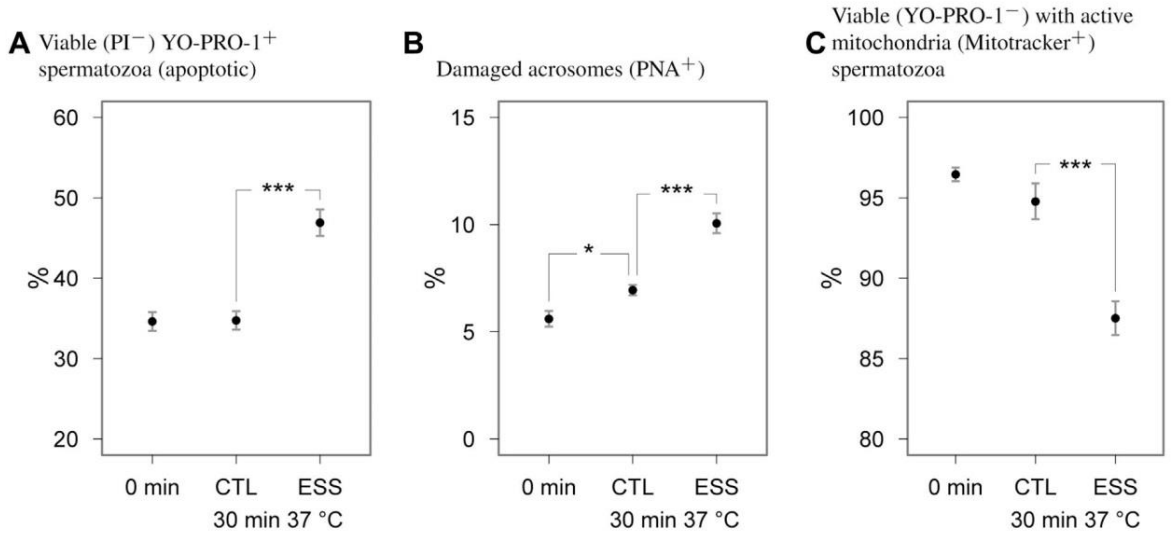

Fig. 3. Some physiological analyses of ram spermatozoa submitted to the different treatments (mean ± standard error of the mean). Apoptotic spermatozoa (A) are shown as the percentage of YO-PRO-1$^+$ spermatozoa within the subset of PI$^-$ spermatozoa (continuous plasmalemma) from the YO-PRO-1/PI assay. Acrosomal damage is represented as the total percentage of PNA$^+$ spermatozoa from the fluorescein isothyocyanate–peanut agglutinin (PNA)/PI assay (B). Mitochondrial activity shows the proportion of MitoTracker$^+$ spermatozoa within the subset of viable spermatozoa (YO-PRO-1$^-$) from the YO-PRO-1/MitoTracker deep red assay (C). Lines join groups with significant differences: before and after the incubation (initial [0 minutes] vs. control [CTL]), and after the incubation (CTL vs. ESS; $^*P < 0.05$; $^{***}P < 0.001$). ESS, estrous sheep serum; PI, propidium iodide.

activity. Incubation did not affect the proportion of viable spermatozoa (PI$^-$) with apoptotic changes in the membrane (YO-PRO-1$^+$), but ESS supplementation increased it with $P < 0.001$ (Fig. 3A). We also detected a slight increase of reacted acrosomes after the incubation ($P < 0.05$), which increased with EES to nearly doubling the initial proportion ($P < 0.001$; Fig. 3B). The study of mitochondrial activity determined that a high proportion of viable (YO-PRO-1$^-$) spermatozoa (around 95%) had active mitochondria initially and after the incubation, which decreased slightly when ESS was present ($P < 0.001$; Fig. 3C).

The fertility data distribution is showed in Figure 4. These data were used for carrying out a regression analysis with the variables obtained by flow cytometry (testing results from the initial, control, and ESS treatments). Using data from the 17 males resulted in very low R^2 values and no significant results. Only the FAM$^-$/PI$^-$ subpopulation in the caspase-3/7 analysis and the control treatment yielded an acceptable model, with $R^2 = 0.307$ and $P = 0.015$ ($F_{1,14} = 7.63$, slope: 0.636). Since we noticed that males with the lowest motility tended to present quality parameters similar to the group with the highest fertility, in a second analysis, males were grouped on the basis of quartiles, separating the males in "low," "low to medium", "medium to high," and "high" fertility (Fig. 4). The analysis of these groups showed that several variables tended to vary with fertility, but indeed, the low-fertility group broke this trend, and in many cases, the parameters for this group were similar to those of the "high-fertility" group (Fig. 5). Therefore, we excluded the "low-fertility" group from the regression, which resulted in many significant regression models, several of them with acceptable R^2 values (Table 1). We took the adjusted R^2 value, which is lower than the typical R^2 but prevents against spurious increases of its value. Almost all the models corresponded to data from the control treatment, whereas data from the initial or ESS group yielded very poor models in general. Fertility was positively related to sperm viability and to the presence of spermatozoa without activated caspases, whereas it was negatively related to the proportion of viable spermatozoa showing capacitation and to caspase activation (Fig. 5). The more explicative models, in terms of R^2 and F values, were those including the proportion of viable/caspase$^-$ spermatozoa (FAM$^-$/PI$^-$) from the polycaspase and caspase-8 assessments of the control treatment.

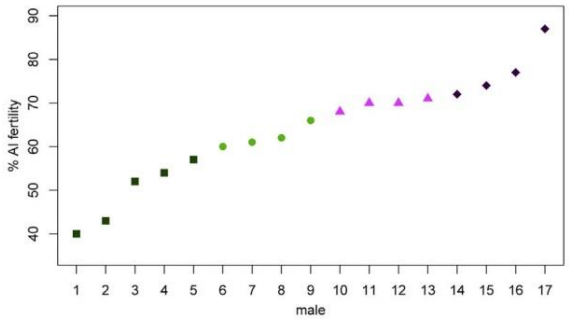

Fig. 4. Distribution of the fertility results (Intrauterine artificial insemination) used in the study. Each fertility group is shown with different shapes (square: low fertility; round: low to medium fertility; triangle: medium to high fertility; lozenge: high fertility).

4. Discussion

The supplementation with ESS is standard in small ruminants to achieve good results in IVF [13,14,27,28]. To

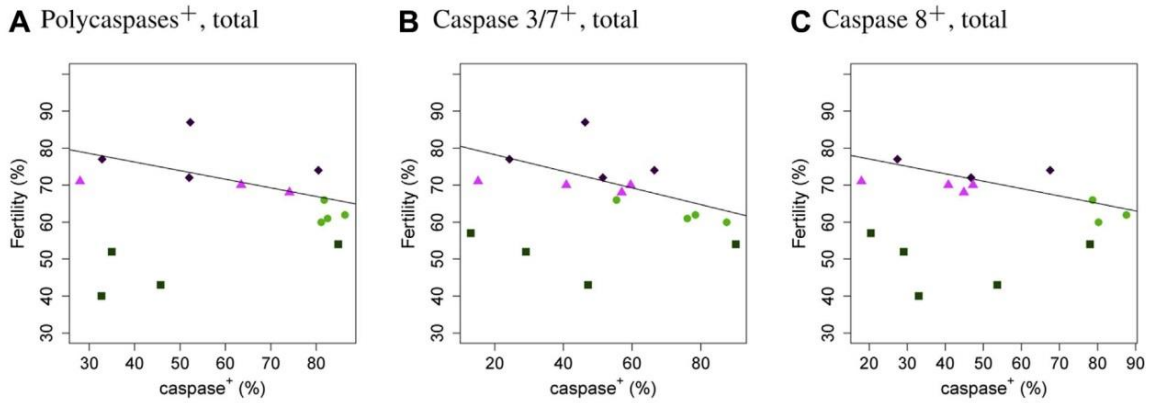

Fig. 5. Relationship of the proportion of caspase-positive events in the control samples (incubated 30 minutes at 37 °C) with fertility. Symbols indicate different fertility groups (square: low; round: low to medium; triangle: medium to high; lozenge: high). Most males in the low-fertility group showed values similar to those of the high and medium-to-high-fertility groups. After leaving out this group, significant regression models could be obtained (Table 1). The lines show the regression excluding the low-fertility group.

provide new insights into the mechanisms through which ESS is able to capacitate spermatozoa, we aimed to examine the balance between *in vitro* capacitation and apoptosis.

Our results confirm that ESS is an effective agent for capacitating ram spermatozoa, considering the increase of membrane fluidity (M540). In a previous study, we confirmed the association between our IVF protocol using ESS, M540 results, and the increase of spermatozoa presenting a hyperactivated-like motility pattern [14]. In fact, the concomitant increase of spontaneous acrosome reaction in a small subset of spermatozoa supports that the media is effective. It is known that ESS enhances cholesterol efflux from the plasma membrane in ram spermatozoa [26], which causes an increase of membrane fluidity and permeability. These membrane changes cause an increase of Ca^{2+} [28], which might induce an early acrosome reaction in sensitive cells. Other physiological events documented in ram capacitated spermatozoa are the increase in tyrosine phosphorylation [29,30] and changes in motility pattern [31,32].

The impact of capacitation on the apoptosis-related signal transduction pathways is still little known in spermatozoa, and most studies have been carried out in humans. Some authors reported the externalization of phosphatidylserine under capacitation conditions [33,34], which was not confirmed in later studies. Recently, Grunewald et al. [7] suggested that apoptosis and capacitation are balanced processes in human spermatozoa. Apoptotic spermatozoa would loss their ability to undergo capacitation, whereas capacitated sperm would inactivate proapoptotic signaling. Our results support that similar processes take place in ram spermatozoa too. Likewise, Martí et al. [22] indicated that caspase activities decreased after incubation of ram semen in capacitating conditions. Lately, Aitken et al. [35] have explored the possibility that capacitation and apoptosis were linked processes joined by their common dependence on the continued generation of reactive oxygen species (ROS). According to this model, capacitation is initiated in spermatozoa as a consequence of intracellular ROS generation, but the continued generation

Table 1
Summary of the regression models between the variables obtained from the flow cytometry analyses and the artificial insemination fertility (after removing data from low-fertility males and discarding $P < 0.05$).

Assay	Variable	Treatment	Slope	*F* value[a]	P value	R^2
Capacitation (YO-PRO-1/M540)	Viability	Control	0.607	7.86	0.019	0.384
	% Capacitated within viable	Control	−0.191	8.41	0.016	0.322
Polycaspase activity (FLICA/PI)	FAM^-/PI^-	Control	0.736	32.78	<0.001	0.743
	$Caspase^+$ (total)	Control	−0.243	9.41	0.012	0.433
	% $Caspase^+$ within viable	Control	−0.708	7.93	0.018	0.387
Caspase-3/7 activity (FLICA/PI)	FAM^-/PI^-	Initial	0.434	8.39	0.018	0.425
		Control	0.679	15.24	0.003	0.564
	$Caspase^+$ (total)	Initial	−0.299	18.52	0.002	0.637
		Control	−0.233	7.73	0.019	0.379
	% $Caspase^+$ within viable	Control	−0.391	8.76	0.014	0.414
Caspase-8 activity (FLICA/PI)	FAM^-/PI^-	Control	0.557	28.6	<0.001	0.754
		ESS	0.436	8.41	0.016	0.402
	$Caspase^+$ (total)	Control	−0.203	19.26	0.002	0.670
	% $Caspase^+$ within viable	Control	−0.282	20.99	0.002	0.690

Models result from transformed data (arc sine transformation).
Abbreviations: ESS, estrous sheep serum; M540, merocyanine 540; PI, propidium iodide.
[a] Degrees of freedom were (1, 10), except for the caspase-8 assays in the initial (1, 9) and control (1, 8) treatments, and for the caspase-9 assays in the initial (1, 9) treatment.

of ROS by capacitating populations of spermatozoa could eventually overwhelm the limited capacity of these cells to protect themselves from oxidative stress. As a result of overcapacitation, the apoptotic cascades activate, leading to sperm death. Considering our results at the light of this hypothesis, ESS may be modulating sperm physiology, facilitating sperm capacitation while slowing or blocking the entry into apoptosis. Preliminary results from our own research group suggest that the addition of ESS during *in vitro* capacitation significantly decreased ROS production (unpublished data), which supports the initial idea of Aitken et al. [35]. This decrease of ROS could be due to a direct antioxidant effect (due to antioxidants present in ESS) and/or to the effect of ESS on physiological pathways related to ROS generation or removal.

Either directly or by the modulation of ROS concentration, ESS was able to prevent the activation of caspase cascades. The polycaspase assay (including caspases 1, 3, 4, 5, 6, 7, 8, and 9) detected a decrease in the proportion of the viable and caspase$^-$ spermatozoa when samples were incubated, which was partly prevented by the presence of EES. In fact, EES was as effective as the caspase inhibitor in maintaining the proportion of caspase$^+$ spermatozoa low. However, the analysis of specific caspases (8 and 3/7) yielded less marked effects. Grunewald et al. [7] showed that initiator caspases (1 and 9) were inhibited by capacitation in a larger amount than effector caspases (such as caspase-3/7), which might explain our results. Moreover, results might be modulated by the different roles of these caspases in the cell and their relative amounts. The activation of caspase-3 marks a "point of no return" in the apoptosis signaling cascade, as it has been shown in somatic cells [36], whereas activation of initiator caspases is a reversible process. Caspase-8 is part of the extrinsic pathway of activation and has been detected in ram spermatozoa [36]. However, its role in this species is not clearly defined because it did not seem to activate when typical proapototic stimuli were applied [37]. Our results suggest that caspase-8 could play a minor role in the changes occurring during incubation and capacitation.

It is noteworthy that ESS caused an increase in events that could be considered as apoptotic harbingers: YO-PRO-1 and loss of mitochondrial activity within the viable population of spermatozoa. These results seem to be contradictory because mitochondria are one of the main hubs for apoptosis regulation [38], and their inactivation has been proposed as a prelude for apoptosis and sperm death [25]. However, it is possible that the changes detected by these probes were a result of the membrane changes caused by capacitation under the influence of ESS, rather than being related to apoptosis. Indeed, the exposure of aminophospholipids in the external leaflet of the plasma membrane, another marker for apoptosis which is also related to YO-PRO-1 labeling, has been found to be independent from apoptosis in capacitating spermatozoa [9,33,34]. Regarding the increase of a subpopulation of viable spermatozoa with low mitochondrial activity, a study on boar spermatozoa found that capacitation induces changes in mitochondrial physiology, and the authors suggested that subpopulations of spermatozoa could coexist in the semen sample while varying on the mitochondrial response to capacitation [39]. Thus, we might be witnessing the emergence of these subpopulations while viable spermatozoa undergo capacitation, as subsets of spermatozoa might fail to respond adequately to these stimuli, losing mitochondrial activity [25].

Early stages of sperm capacitation can be measured by loading spermatozoa with the lipid dye M540. Recently, Steckler et al. [40] have proposed YO-PRO-1/M540 stain combination as a more sensitive indicator of viability, identifying membrane destabilization with sperm capacitation in dogs. Previous studies have reported that YO-PRO-1 identifies spermatozoa early in the process of cell deterioration and death. In subviable cells in which the plasma membrane has been destabilized, the cells lack appropriate amounts of ATP to transport YO-PRO-1 back out of the cell. In this respect, it appears that YO-PRO penetrates the plasmalemma of sperm committed to cell death via specific pannexin channels and stains sperm DNA before complete membrane disruption would allow entrance of other classic membrane-impermeant DNA stains [41–44]. However, because Aitken et al. [35] suggested that apoptosis in spermatozoa is significantly different from the phenomenon observed in somatic cells and that our results show that a highest subpopulation of merocyanin-responsive cells remains negative to YO-PRO-1 stain, it is possible that YO-PRO-1 entry is not related to apoptosis but to other phenomena linked to capacitation.

Until now, the roles attributed to ESS during *in vitro* ram sperm capacitation were related to induce cholesterol efflux from the plasma membrane in ram spermatozoa [27], increase tyrosine phosphorylation [29,30], and change the motility pattern [31,32]. To our knowledge, this is the first time that the interactions between parts of the apoptotic signal transduction pathway and *in vitro* capacitation by EES have been studied in ram spermatozoa. One important consequence from this study is the possibility that this information could be used to improve capacitation media, providing an efficient chemically defined replacement of ESS. It is possible that the capacitation induced by ESS may differ from the physiological process, being successful in the IVF setting, though. In our study, we have found that ESS has also a role in preventing sperm deterioration.

The sperm doses used in this study were selected because they were also used in fertility trials, therefore allowing to associate *in vitro* results with lambing outcomes. The most outstanding finding in the fertility study was the identification of the "low-fertility" group, in which most samples resembled the "high-fertility" group in terms of sperm quality, both after collection in the farm and in the *in vitro* analyses carried out in this study. Most interestingly, four of these five males showed the lowest levels of activated caspases. A subsequent investigation could not determine the cause of the bad fertility of these animals, although three of the males shared the same father. Whereas being beyond the objectives of this study, this opens an interesting line of research, to determine whether

specific genetic markers could be influencing these males' fertility. This follows other studies in which single nucleotide polymorphisms and genome-wide methylation in bulls were associated to embryonic development success [45,46]. We have to highlight that these males were mostly undetected by the *in vitro* tests, therefore suggesting that we might combine our laboratory tests with molecular analyses, to obtain a complete picture of semen fertility. After we removed that subset of males, we obtained a few models that related some variables with fertility. Apart from sperm viability and capacitated spermatozoa, several variables derived from caspase analyses were related with fertility (we rejected significant regressions, which yielded low R^2, because the large number of analyses increased the risk of considering spurious regressions as true ones). Agreeing with other authors [16,47,48], we propose that samples with relatively high levels of activated caspases (either intrinsically or resulting from the inability for preventing its activation) tend to have a lower fertility, showing the importance for the spermatozoon to keep apoptotic pathways inactivated to fulfill its mission. Therefore, caspase probes could be useful to identify samples with potentially lower fertility. However, at the light of our findings, we must insist in the need of using molecular and genetic tools to characterize the males for other variables that could be lowering sperm fertility.

It is interesting that most significant regressions emerged when using data from the control treatment. Incubating sperm samples at different temperatures is a typical endurance test [49,50] and allows testing sperm quality more thoroughly. The lack of association of ESS-treated samples with fertility might appear with a bit of surprise, but in fact, it was not unexpected. In the context of artificial insemination, it might be more important for spermatozoa to maintain a good endurance after thawing, rather than to respond to a capacitating medium. Indeed, *in vitro* capacitation is very different from *in vivo* capacitation, especially considering the environment and the cell-to-cell interactions taking place in the latter [51]. This supports our hypothesis that the incubation with ESS might alter sperm parameters (at least those measured in the present study) in a way that differs from the physiological process and therefore losing the relationship with the events occurring after artificial insemination. More complex tests (for instance, combining an endurance period with a subsequent capacitation step) might increase the predictive power of these analyses.

In conclusion, we have found that *in vitro* induction of capacitation of thawed ram spermatozoa, by using ESS, seems to downregulate apoptotic pathways. This mechanism might assure that spermatozoa maintain their physiological competence in this critical process previous to fertilization. Thus, the protective role of ESS in preventing sperm deterioration is also an important finding of this study. In fact, semen samples with a higher amount of activated caspases (incubating in noncapacitating conditions) were related to lower fertility. Additionally, the presence of a subset of males with low levels of activated caspases and good sperm quality, but the lowest fertility, highlights the need of other molecular and genetic analysis tools, to achieve a complete evaluation of semen samples.

Acknowledgments

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Competing Interests

There is no conflict of interest of any kind whatsoever in this work.

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Capítulo 7

Estrous sheep serum balances the levels of ROS to supply in vitro capacitation of ram spermatozoa.

Estrous sheep serum balances the levels of ROS to supply *in vitro* capacitation of ram spermatozoa.

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Abstract

Reactive oxygen species (ROS) are fundamental for intracellular signaling. In spermatozoa, they are involved both to apoptosis and to capacitation, and changes in ROS can alter the balance between these two processes. Estrous sheep serum (ESS) is considered an efficient agent for *in vitro* capacitation of ram spermatozoa. We have explored the effects of ESS on ram sperm physiology, especially on ROS production, during *in vitro* capacitation. Semen samples from 15 rams were cryopreserved. Samples were submitted to four treatments: control (CTL), ESS (10%), caspase inhibitor (INH, Z-VAD-FMK), and EES plus caspase inhibitor (I+E). Sperm samples were incubated for 30 min at 38.5 °C and 5% CO_2 and evaluated: motility and kinetic parameters by computer-assisted semen analysis (CASA), and viability (propidium iodide), apoptotic-like membrane changes (YO-PRO-1), acrosomal status (PNA-FITC), intracellular calcium (FLUO-3), membrane fluidity (M540) and ROS production (CM-H_2DCFDA) by flow cytometry. ESS induced changes in kinetic parameters compatible with capacitation, with a decrease in the percentage of progressive motility and linearity, and an increase in the amplitude of the lateral displacement of the sperm head ($P<0.05$). Moreover, ESS increased the proportion of M540+ viable spermatozoa, YO-PRO-1+ and acrosome reacted spermatozoa ($P<0.05$). After incubation, ESS and I+E achieved lower ROS levels ($P < 0.05$). Ca^{2+} levels did not change with the incubation, but were slightly higher ($P<0.05$) when both ESS and the inhibitor were present. We suggest that ESS may modulate ROS levels, allowing intracellular signalling for capacitation to occur while preventing higher levels that could trigger apoptosis.

Keywords: ram, oxidative stress, apoptosis, capacitation, estrous sheep serum

Introduction

Oxidant species have a crucial role on sperm physiology, but they may also impair the fertilising ability of spermatozoa. Reactive oxygen species (ROS) are major byproducts of sperm metabolism that play critical roles in the physiological regulation of sperm capacitation, but an excess can drive to the induction of DNA damage and to impaired fertility [1, 2]. ROS are implicated in tyrosine phosphorylation events associated with the attainment of a capacitated state, as a consequence of their ability to inhibit tyrosine phosphatase activity and induce cAMP generation [3-7]. In addition, ROS are thought to play key roles in inducing cholesterol oxidation during capacitation, facilitating sterol efflux from the sperm plasma membrane and enhancing plasma membrane fluidity [8, 9].The continued generation of ROS by capacitating spermatozoa eventually leads to a state of cell senescence characterized by high levels of oxidative damage to the spermatozoa and is accompanied by accelerated mitocondrial ROS generation, as these cells enter a truncated version of the intrinsic apoptotic cascade [10].

Capacitation can be induced *in vitro*, and in some species it has been achieved in chemically defined media [11-14]. In sheep, estrous sheep serum (ESS) is still considered an efficient agent for *in vitro* capacitation and fertilization [15, 16], although success with defined media have been reported [14]. However, a main drawback of using ESS is that it is not easily attainable, given that it requires extraction and purification from blood samples and, therefore, its composition can vary from batch to batch. Moreover, the presence of undefined compounds in IVF media is not desirable, as it complicates any standardization of this technique. Therefore, understanding how ESS affects sperm physiology serum is of great interest in order to establish defined media conditions supporting *in vitro* ram sperm capacitation.

We have recently found that the *in vitro* induction of capacitation of thawed ram spermatozoa by using EES, seems to downregulate apoptotic pathways [17]. This mechanism might assure that spermatozoa maintain its physiological competence in this critical process previous to fertilization. Thus, ESS seems to enable *in vitro* capacitation of ram spermatozoa while preventing caspase activation. Besides being a capacitating agent, ESS showed a protective role by preventing sperm deterioration [17]. Recently, [18] have explored the possibility that capacitation and apoptosis are linked processes joined by their common dependence on the continued generation of ROS. In this regard, we aim to provide new insights into the effects of ESS on physiological changes induced in ram spermatozoa during *in vitro* capacitation. Thus, our objective is testing if the role of ESS in preventing sperm deterioration is related to ROS production.

Materials and methods

1.1. Reagents and media

Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA). The rest of the chemicals (reagent grade or higher) and the fluorescence probe propidium iodide (PI) were acquired from Sigma (Madrid, Spain). Other fluorescent probes and caspase analysis kits were purchased from Life Technologies (Barcelona, Spain). Stock solutions of the fluorescent probes were: PI, 1.5 mM in Milli-Q water; YO-PRO-1, 50 μM in DMSO; Merocyanine 540 (M540), 3.4 mM in DMSO; PNA-FITC 1 mg mL^{-1} in water. All fluorescent stocks were kept at –20 °C in the dark. The freezing extender was prepared in our laboratory using reagent-grade chemicals purchased from Panreac Química SA (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, MO, USA) [19]. SOF was composed of 107 mM NaCl, 7.17 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM Ca_2Cl, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 3.3 mM Na lactate, 0.3 mM Na pyruvate and 200 mM glutamine. Osmolality was 270–280 mOsm/kg and pH 7.2–7.3 [20].

1.2. Animals and sperm collection

All animal procedures were performed in accordance with Spanish Animal Protection Regulation RD53/2013 which conforms to European Union Regulation 2010/63/UE. Adult males belonged to the Regional Center for Animal Selection and Reproduction in Valdepeñas (CERSYRA, Spain). Seventeen rams (Manchega breed, age >3 years) were used. Semen collection was performed using an artificial vagina. Volume, concentration, wave motion (0 no movement to 5 strong wave movement) and sperm motility were assessed shortly after collection. Only ejaculates with wave-motion values of 4 or 5 and sperm motility higher than 80% were frozen.

1.3. Cryopreservation of semen

After initial semen evaluation, each ejaculate was diluted with the freezing extender. First, sperm samples were diluted in fraction 1 of the diluent down to a concentration of 400×10^6 spermatozoa mL^{-1} and slowly cooled from 30 °C to 5 °C for 2 h. Then, sperm samples were further diluted with the same volume of fraction 2 at 5 °C and held for equilibration for 2 h more. At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen with a programmable biofreezer (IceCube 14S-Ver. 1.30; SY-Laboratory Geräte GmbH, Minitüb® Tiefenbach, Germany) at –20 °C min^{-1} to –100 °C, and at –10 °C min^{-1} from –100 °C to –140 °C, and then plunged into liquid nitrogen.

1.4. Experimental design

A total of 15 rams were used in this experiment. Two straws per male were thawed 30 s at 37 °C in a warm bath and aliquots. Sperm quality and concentration were checked, ensuring that the two straws had similar characteristics and there were no differences between them. Then they were pooled. Sperm samples were deposited in the upper side of a discontinuous Percoll gradient [19] (Upper layer: 45% of Percoll in SOF-HEPES (10 ml SOF, HEPES 9.8 mM and Gentamicin 0.04 mg mL^{-1}; Lower layer: 90% of Percoll and 10% of a 9% w/v NaCl solution). The tubes were centrifuged 15 min at 700×g, the supernatant was removed and the pellet concentration was calculated after addition of 500 µL of SOF+BSA 0.3%. Concentration was adjusted to 10^7 spermatozoa mL^{-1}. Samples were divided in 4 aliquots and submitted to different treatments: control (CTL), estrous sheep serum (EES, 10% as used for sheep IVF [21, 22], caspase inhibitor (INH, 100 µM Z-VAD-FMK [23]], and EES plus caspase inhibitor (I+S). Each tube was then incubated for 30 min at 38.5°C and 5% CO_2. Flow cytometry analysis were carried out in the untreated sample after selection (0 min) and in the four tubes after the 30 min of incubation.

1.5. Sperm motility assessed by CASA

Sperm were diluted down to 10–20×106 spermatozoa/mL and loaded into a Makler counting chamber (10 µm depth) at 37 ºC. The CASA system consisted of a triocular optical phase contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan), equipped with a warming stage at 37 ºC and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the Sperm Class Analyzer (SCA2002) software (Microptic S.L.; Barcelona, Spain). Sampling was carried out using a ×10 negative phase contrast objective (no intermediate magnification). Image sequences were saved and analyzed afterwards. Software settings were adjusted to ram spermatozoa. The standard parameter settings were as follows: 25 frames/s; 20 to 90 µm2 for head area; VCL > 10 µm/s to classify a spermatozoon as motile. For each spermatozoa, the software yielded the percentage of motile spermatozoa (TM), the percentage of progressively motile spermatozoa (PM), VCL (velocity according to the actual path, µm/s), VSL (velocity according to the straight path, µm/s), VAP (velocity according to the smoothed path, µm/s), LIN (linearity, %),ALH (amplitude of the lateral displacement of the sperm head, µm), and BCF (head beat-cross frequency, Hz).

1.6. Flow cytometry analysis

Spermatozoa were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) controlled with the MXP software (v. 1). Cells were run through the instrument

at 150–300 cells s^{-1}, collecting data from 10 000 cells. Excitation was carried out with a 488-nm Ion-Ar laser. YO-PRO-1 and FITC green fluorescence was detected with a 530/28BP filter, M540 orange fluorescence with a 575/26BP filter and PI red fluorescence with a 615DSP filter. Flow cytometry data was analyzed with WEASEL software Ver. 3 (WEHI, Melbourne, Australia)

1.6.1. Sperm viability

Sperm viability was checked by means of propidium iodide staining (PI, 15 μM, stock: 50 μM in DMSO) and YOPRO-1 (50 nM; stock: 7.5 mM in milli-Q water) in combination with flow cytometry as described by Martínez-Pastor et al. (2008) [22]. The staining solution was prepared using SOF-HEPES (~1-2 x 10^6 spermtazoa/mL). After 15 min of incubation in the dark, sperm samples were analyzed by flow cytometry. PI binds to DNA in membrane-defective sperm and allows the identification of viability, whereas YO-PRO-1 stains early apoptotic cells (but showing increased permeability, not stained by PI yet) [24].

1.6.2. Assessment of membrane fluidity

The lipophilic probe M540 was used to assess the membrane fluidity. This probe intercalates in the membrane, becoming more fluorescent when membrane fluidity increases. The staining solution was prepared using SOF-HEPES with 1 μM M540 and 50 nM YOPRO-1, adding spermatozoa up to 10^6 mL^{-1}. $M540^-$/$YOPRO\text{-}1^-$ spermatozoa were considered viable with low membrane fluidity $M540^+$/$YOPRO\text{-}1^-$ spermatozoa were considered viable with high membrane fluidity. $YOPRO\text{-}1^+$ spermatozoa were considered non-viable.

1.6.3. Assessment of acrosome integrity

PNA (peanut agluttinin) binds specifically to the internal side of the external membrane of the acrosome, labeling acrosome-damaged spermatozoa. The staining solution was prepared using SOF-HEPES with 12 μM PI and 1 μg mL^{-1} FITC-PNA, adding spermatozoa to 10^6 mL^{-1}. This fluorescent technique allows distinguishing among four sperm populations: PI^-PNA^- were considered alive with intact acrosomes; PI^-PNA^- as dead with intact acrosomes: PI^+PNA^+ as dead with damaged acrosomes: and PI^-PNA^+ as alive with damaged acrosomes.

1.6.4. Measuring Intracelular Calcium Concentration.

Calcium is an intracellular messenger that plays a key role in sperm capacitation. [25] established the relationship among capacitation status and intracellular calcium concentration in frozen-thawed spermatozoa. In this regard, we analyzed the intracellular calcium levels as a fact related to sperm capacitation. To assess the amount of intracellular calcium existing in the cytoplasm and reserves held by the sperm, samples were stained with 5 μM FLUO-3 and 12 μM PI in SWB (Sucrose Wash Buffer) as described by [26]. The FLUO-3 has affinity for Ca^{2+} and

to a lesser extent by Mg^{2+}; when it binds to these cations, it emits green fluorescence. Mean of green fluorescence of living cells (PI-) was evaluated as described by [27]. A replicate was performed adding 1 μM of calcium ionophore (A23187) to a second tube and incubating 10 min to evaluate the increase of cytoplasmatic calcium when triggering the entrance of this cation. A measurement of "free Ca^{2+}" was estimated as the ratio of the two [Ca^{2+}] measurements.

1.6.5. Production of reactive oxygen species

Reactive oxygen species production was assessed by using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein acetyl ester (CM-H_2DCFDA) as described by Domínguez-Rebolledo *et al.* (2010) [27] . CM-H_2DCFDA penetrates the plasma membrane and is retained after intracellular esterases cleave the acetate groups, emitting green fluorescent (504 nm) upon oxidisation. The intensity of fluorescence of CM-H_2DCFDA increases as ROS production increases. Sperm suspensions (10^8 cells mL^{-1}) were loaded at 37°C with CM-H_2DCFDA in SOF-HEPES (final concentration 0.5 μM; stock solution, 500 μM), and analyzed after 20 min.

1.7. Statistical analysis

Statistical analysis was performed using the R statistical environment (v. 3.1.1). Flow cytometry parameters were analysed by linear mixed-effects models (LME), using treatment as fixed effects, incorporating the male into the random block structure.

Results

The kinematic variables (Table 1) behaved differently in the experiment. The proportion of actively motile spermatozoa decreased overall after the incubation (63.1%±5.4 vs. 45.4%±1.9, $P<0.001$), and motility was still lower after the incubation when ESS or inhibitor were added (Table 1). Progressivity followed a similar trend, but whereas total motility followed ESS > Inhibitor > ESS+Inh, progressivity loss was higher in presence of ESS irrespective of the inhibitor presence.

Sperm velocity, while not differing significantly before and after incubation overall, was significantly higher in the Control after 30 min ($P=0.016$), with the inhibitor preventing any increase. Linearity followed an opposite pattern, decreasing with incubation and even more when ESS was applied ($P=0.003$ comparing to the Control), although the combination with inhibitor abolished this effect. ALH increased with incubation and especially in presence of ESS alone. The inhibitor prevented the increase of ALH with the incubation, whereas ESS+inhibitor nullified one another.

Table 1. Motility changes during the experiment (mean±SEM). Asterisks (P<0.05, ** P<0.01, *** P<0.001) next to post-thaw values indicate significant differences with overall incubation values. Asterisks next to incubation values for treatments (ESS, Inhibitor, ESS+Inhibitor) indicate significant differences with Control values.*

Treatment		MOT (%)	PROG (%)	VAP (μm/s)	LIN (%)	ALH (μm)
Post-thaw		63±4.3***	41.1±2.5***	139.6±5.8	73.9±1.5***	2.7±0.2*
Incubation	Control	54.1±2.6	30.7±1.3	159.5±4.6	67.8±0.9	3.1±0.1
	ESS	44.6±3.2*	21.5±2***	148.1±6.7	62.1±1.7***	4±0.2***
	Inhibitor	42.8±2.6**	25±1.9*	140.4±5.1*	68.9±1.4	2.6±0.1*
	ESS+Inh.	39.5±2.7***	21.3±1.8***	133.2±5.4*	66.5±1	3.2±0.1

MOT: Total motility, PROG: progressive motility, VAP: velocity according to the average path, LIN: linearity, ALH: amplitude of the lateral movement of the head.

Table 2. Physiological status of the spermatozoa assessed by flow cytometry (mean±SEM), regarding viability (membrane integrity, PI), acrosomal status (PNA^+, only for PI and for total cells), capacitation (membrane disorder, $MC540^+$ as % of $YO\text{-}PRO\text{-}1^-$ cells) and apoptotic changes in the membrane ($YO\text{-}PRO\text{-}1^+$ as % of PI cells). Comparisons described in Table 1.

Treatment		Viability (%)	Reacted acrosomes, viable (%)	Reacted acrosomes, total (%)	Capacitated (% of viable)	Apoptotic (% of viable)
Post-thaw		50.5±2.6***	0.3±0.1***	26.1±1.2***	54.5±4.5**	9±1.5***
Incubation	Control	28.8±2	0.7±0.1	35±1.4	56.8±2.3	16.3±1.9
	ESS	21.1±1.4**	0.9±0.1*	42.1±1.5***	70±2.9***	25.5±1.1***
	Inhibitor	28.7±1.7	0.5±0.1	36.9±1.3	62.2±1.9	15.1±1.4
	ESS+Inh.	19.4±1.7***	0.8±0.1	46.8±1***	67.8±2***	20.8±1.9

Tables 2 and 3 show the results of the flow cytometry analysis. Plasma membrane integrity (Table 2) almost halved after incubation, and the presence of ESS (irrespective of the inhibitor)

augmented this decrease. The proportion of viable spermatozoa with reacted acrosomes was very low (average<1%), but nevertheless higher after the incubation and significantly higher when samples were incubated with ESS alone. Considering the total acrosome-reacted population, we found a similar pattern, with both ESS treatments showing a significantly higher proportion of PNA^+ spermatozoa than the Control.

The proportion of $M540^+$ (considered capacitated) and YO-PRO-1^+ (membrane with apoptotic features) showed similar variations (studying only PI^- spermatozoa). The proportion increased with the incubation overall, and ESS had the effect of increasing it more than the Control treatment. In the case of YO-PRO-1^+ spermatozoa, adding the inhibitor to ESS partially cancelled the increase of YO-PRO-1^+ spermatozoa, although the inhibitor alone did not change the average comparing to the Control.

The levels of reactive oxygen species (ROS) detected by H_2DCFDA were statistically different, ESS decrease significantly the production of ROS ($P < 0.05$) (Table 3). Ca^{2+} levels did not change with the incubation, but were slightly higher when both ESS and the inhibitor were present. When we measured it after treating the spermatozoa with the calcium ionophore, we noticed that the levels also significantly increased with the inhibitor alone. However, "free Ca^{2+}" was unchanged.

Table 3. Physiological status of the spermatozoa assessed by flow cytometry (mean±SEM), regarding oxidative stress (ROS levels) and intracellular [Ca^{2+}] (untreated, after adding ionophore and a estimation of free Ca^{2+} as the ratio of both values). All measurements were carried out after excluding PI^- spermatozoa (only viable spermatozoa). Comparisons described in Table 1.

Treatment		ROS (MFI)	[Ca^{2+}] (MFI)	[Ca^{2+}]after ionophore challenge (MFI)	Free Ca^{2+} (%)
Post-thaw		3.9±0.3	10.4±0.3	20.7±1.4	48.2±3
Incubation	Control	4.0±0.2	9.6±0.3	20.9±1	45.9±1.8
	ESS	3.3±0.1*	9.9±0.2	21.3±0.9	44.3±1.4
	Inhibitor	4.6±0.2	9.6±0.4	23.3±0.8*	42.3±2.2
	ESS+Inh.	3.4±0.1*	10.9±0.4***	22.3±1.6**	47.6±2.9

MFI: Mean fluorescence intensity.

Discussion

Oxidative stress has an important role in sperm physiology. It is evident that ROS are important contributors to sperm function, both positively and a negatively. The beneficial and detrimental effects of ROS probably represent a continuum [8].

Apoptosis in spermatozoa is still a poorly understood process, significantly different from the phenomenon observed in somatic cells. One of the earliest events observed during the intrinsic apoptotic cascade in spermatozoa is a sudden increase in the generation of mitochondrial ROS and a concomitant loss of sperm motility [10]. The next events observed during the intrinsic apoptotic cascade are the activation of caspases in the sperm cytosol and the expression of phosphatidylserine in the external layer of the plasmalemma [28-33]. Our results showed that ESS caused an increase in the proportion of spermatozoa showing events that could be considered as harbingers of apoptosis (YO-PRO-1$^+$). However, adding the caspase inhibitor had no effect on these events (comparing to the control). In a previous study, we confirmed that ESS caused and increase in the proportion of YO-PRO-1$^+$ spermatozoa and a loss of mitochondrial activity. However, we posed that that the changes detected by YO-PRO-1 were a result of membrane changes caused by capacitation under the influence of ESS, rather than being actually related to apoptosis [17]. Indeed, we found in the same study that ESS prevented caspase activation. Therefore, ESS may be modulating sperm physiology, inducing sperm capacitation while slowing or blocking the entry into apoptosis [17].

Reactive oxygen metabolites are not only fundamental to the apoptotic process, they are also involved in the capacitation of spermatozoa [34]. ROS are thought to exert a positive influence on tyrosine phosphorylation in capacitating spermatozoa through their ability to enhance intracellular levels of cAMP while inhibiting tyrosine phosphatase activity [2]. Recently, Aitken et al. (2015) [18] speculated that, since capacitation and apoptosis are redox-regulated, both processes may be part of the same continuum. Thus, we have tried to find out how ROS and other variables are affected by *in vitro* capacitation by ESS, if both capacitation and apoptosis are processes linked by their common dependence on the continued generation of ROS. The dependence of ROS generation for capacitation is a risky strategy for spermatozoa, because of the sensitivity of this cell type to oxidative stress, due to their relative lack of antioxidant protection and abundance of polyunsaturated fatty acids. That means that, unless this process is carefully regulated, the intrinsic generation of ROS during capacitation could overwhelm the spermatozoa's antioxidant system [18]

ESS enhances cholesterol efflux from plasma membrane in ram spermatozoa [27], which causes an increase of membrane fluidity and permeability. Increased membrane permeability

contributes to higher intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[28], which advances capacitation and triggers hyperactivation [35]. However, our results did not show any significant differences in the intracellular Ca^{2+} concentration between ESS and control. Only the combination of ESS and the caspase inhibitor increased $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ changes may occur earlier in a small subpopulation in ESS-treated samples, or either the method used did not have enough sensitivity. Adding the caspase inhibitor could have any effect lengthening or enhancing the $[Ca^{2+}]_i$ increase, thus allowing to detect it.

Therefore, our results confirm that ESS is an effective agent for capacitating ram spermatozoa, considering the increase of membrane fluidity (M540) and sperm hyperactivated-like motility pattern (increased velocity and ALH, and decreased linearity). Moreover, ESS seems to modulate ROS production during *in vitro* capacitation, reducing its concentration in the cytoplasm. This modulation appears to be upstream to the activation of caspases, since the presence of the caspase inhibitor did not have the ability to reduce free radicals, but we previously demonstrated that ESS reduced the proportion of spermatozoa with activated caspases [17]. This supports the hypothesis that ESS may allow ROS production for promoting capacitation, while preventing excessive levels that could trigger apoptosis. This mechanism might assure that spermatozoa maintain its physiological competence in this critical process previous to fertilization.

To date, several media additives, both synthetic and animal origin, have been successfully used to support *in vitro* capacitation of mammalian spermatozoa. For instance, several hormones such as progesterone or strogens, heparin and BSA [36-39]. Recently, García-álvarez et al. (2015) [40] have shown that heparin–hypotaurine, progesterone (P4), estrogens (E_2), β-cyclodextrin, or BSA are not suitable for replacing ESS in capacitation and fertilization media for ram spermatozoa. However, a cocktail of several substances, including phosphatase inhibitors, has proved highly successful [41]. Thus, the knowledge of the mechanism of action of serum is of great interest in order to establish defined media conditions supporting *in vitro* ram sperm capacitation. Our results suggest that modulators of ROS production, such as some antioxidants, could be considered in combination with other substances for the development of a chemically defined medium to replace the ESS in capacitating media for ram spermatozoa. Nevertheless, we should consider that antioxidants could be also detrimental for sperm function in some circumstances, possibly by excess removal of ROS [42]

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Competing interests

There is no conflict of interest of any kind whatsoever in this work.

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Capítulo 8

Discusión General

La estimación del valor de fertilidad de un eyaculado o de un macho es de gran interés debido a la repercusión que podría suponer el conocimiento previo de esta información. Determinar el nivel exacto de fertilidad de un macho adquiere mayor importancia en las especies domésticas que cuentan con programas de mejora genética debido al hecho de que un solo macho, vía inseminación artificial (IA), es utilizado para cubrir un elevado número de hembras. Sin embargo, aunque durante muchos años se han estudiado multitud de características espermáticas con esta finalidad, todavía no se ha encontrado ningún parámetro que estime el nivel exacto de fertilidad de una muestra seminal.

Los objetivos de la presente tesis se han orientado hacia un mayor conocimiento de la fisiología de los espermatozoides descongelados de ovino que nos permitan establecer relaciones entre la evaluación espermática *in vitro* y la fertilidad *in vivo*. Hemos realizado diversos experimentos, con los que hemos pretendido realizar una serie de aportaciones que nos permitan predecir la fertilidad de una determinada muestra espermática. Además, dado que la FIV había sido apuntada como la técnica que mayor correlación presentaba con la fertilidad *in vivo*, decidimos estudiar más en profundidad los procesos de capacitación espermática *in vitro* para poder conocer mejor el comportamiento de los espermatozoides ante el proceso de fecundación. Así, aunque el objeto del estudio hayan sido muestras de ovino, posiblemente gran parte de las conclusiones derivadas de nuestro estudio en cuanto a fisiología espermática puedan adaptarse a los espermatozoides de otras especies.

Hace unos años, en un trabajo previo de nuestro grupo de investigación (Garcia-Alvarez *et al.* 2009) se demostró que la fecundación *in vitro* heteróloga es un procedimiento adecuado para predecir la fertilidad del semen ovino. Sin embargo, los métodos basados en la FIV son costosos tanto por el tiempo necesario para llevar a cabo el procedimiento como desde una perspectiva económica. Por lo tanto, nuestro objetivo será el de encontrar parámetros seminales evaluados *in vitro* que nos permitan "predecir fertilidad". En primer lugar, decidimos evaluar la motilidad espermática mediante sistemas automáticos de análisis de imagen (CASA) y diversos parámetros espermáticos mediante citometría de flujo (viabilidad, apoptosis, potencial de membrana mitocondrial, integridad acrosómica y niveles de Ca^{2+} intracelulares). Además, incorporamos en nuestro estudio un modelo de incubación de las muestras en condiciones que simulan el tracto reproductor femenino, para determinar si estas condiciones estresantes podrían mejorar la relación entre las variables de calidad de esperma (según la evaluación después de la incubación) con los resultados de fertilidad *in vivo*.

En base a los resultados obtenidos en los experimentos presentados en el capítulo 4 de esta tesis, se propuso como una herramienta útil para la predicción de la fertilidad *in vivo* de una determinada muestra seminal de ovino, el análisis de la motilidad analizada mediante sistemas CASA, concretamente los parámetros de velocidad VCL y VAP, combinada con la incubación de las muestras descongeladas durante 2h a 37 °C.

La motilidad espermática se ha considerado como una de las características más importantes asociadas con la capacidad de fertilización de los espermatozoides (Saacke y White 1972). En el presente estudio, se detectó una disminución en la motilidad así como en los parámetros cinemáticos tras la incubación, situación que refleja el estrés que los espermatozoides sufren en estas condiciones. Curiosamente, se detectó una relación positiva entre varias variables cinemáticas (VCL, VAP, VSL, BCF) medidas después de la incubación a 37 °C y la fertilidad *in vivo*. A nuestro entender, esta es la primera vez que se demuestra la existencia de una relación directa entre la motilidad evaluado por CASA y la fertilidad de los espermatozoides criopreservados de morueco.

Nuestro trabajo presenta un aspecto novedoso, la inclusión de una incubación de post-descongelación antes de la evaluación, así O' Meara *et al.* (2008) y Garcia-Alvarez *et al.* (2010) no detectaron una relación significativa entre la fertilidad y la calidad del esperma (parámetros funcionales o CASA, respectivamente) en el esperma ovino recién descongelado. Los resultados del presente estudio muestran la importancia del pre-tratamiento de los espermatozoides antes de su evaluación (en este caso, someterlos a la incubación a 37 °C durante 2h). Malo *et al.* (2005) encontraron diferencias en la fertilidad entre machos de ciervo rojo relacionadas fuertemente con los parámetros de velocidad de natación de esperma (VCL, VSL y VAP), así, nuestros resultados apoyan la hipótesis de que la velocidad de los espermatozoides es una de las características clave en el proceso de la fecundación, idea que ha sido también demostrada en un gran número de taxones (Gage *et al.* 2004; Holt *et al.* 1989). Una mayor motilidad es el resultado de un espermatozoide fisiológicamente funcional y un espermatozoide con una motilidad disminuida es indicativo de un metabolismo reducido o con orgánulos que fallan. Esta idea podría indicar no sólo una disminución de la capacidad para alcanzar el ovocito (de menor importancia en la inseminación laparoscópica), sino también reflejar una menor capacidad para desarrollar la capacitación, una tendencia hacia un exceso en la producción de radicales libres o dificultades para llevar a cabo pasos clave en la fertilización del óvulo (Aitken *et al.* 2012; Martinez-Pastor *et al.* 2009). Los resultados de nuestro primer estudio coinciden con los resultados encontrados por otros autores en otras especies (toro: (Farrell *et al.* 1998; Amann *et al.* 2000; Presicce et al. 1998; Kathiravan *et al.* 2008; cabra: Fernandez-Santos *et al.* 2011), que

muestran que parámetros cinemáticos como VCL, VSL y VAP se relacionan directamente con la fertilidad. También se han encontrado correlaciones positivas entre los diferentes parámetros de velocidad y la fertilidad en semen de hombre (Fetterolf y Rogers 1990).

En los últimos años, la citometría de flujo se ha mostrado como una herramienta para el estudio de varias características fisiológicas de los espermatozoides de bastante éxito, y muchos parámetros espermáticos analizados por citometría de flujo se han relacionado con la fertilidad *in vivo* (Gillan *et al.* 2005; Petrunkina *et al.* 2007; Januskauskas *et al.* 2000; Wilhelm *et al.* 1996) en distintas especies, no obteniéndose éxito para las muestras de semen ovinas. O'Meara *et al.* (2005) no encontraron ninguna relación entre los espermatozoides utilizados en pruebas funcionales *in vitro* y de la fertilidad *in vivo* de moruecos después de la inseminación artificial de las ovejas con el semen descongelado. En 2007, Rodriguez-Martinez y Barth (2007) indicaron que tanto en las técnicas de evaluación del esperma más modernas y como en las más convencionales que se han relacionado con la fertilidad, se muestran relaciones muy modestas y con resultados muy variables entre los laboratorios.

Hay que señalar que estos estudios no hacen uso de un pretratamiento de los espermatozoides descongelados antes del estudio de la calidad del esperma. La incubación de los espermatozoides en temperaturas fisiológicas es un desafío que puede ayudar a descubrir el potencial de la auténtica fertilidad de una muestra. Este tipo de tratamiento puede ser útil para otro tipo de experimentos. Por ejemplo, en un estudio de Anel-Lopez *et al.* (2012), la calidad post-descongelación del esperma de ciervo criopreservado con diferentes antioxidantes no era muy diferente del control, pero después de la incubación de las muestras a 39 °C se detectaron diferencias importantes. Debido a que en la primera experiencia de esta tesis se encontró que existía una relación directa entre los parámetros de movimiento y la fertilidad *in vivo*, y que las mitocondrias se han considerado un orgánulo fundamental para la fisiología espermática (Mukai y Okuno 2004; Rodríguez Martínez et al. 2009), sería lógico encontrar una relación entre el estado de la mitocondria y la fertilidad. Sin embargo, no encontramos ninguna relación entre la actividad mitocondrial utilizando JC-1, y la fertilidad. En este sentido, Volpe et al. (2009) y Cheuquemán et al. (2011) indicaron que JC-1 es una sonda adecuada para la detección de posibles cambios internos de la membrana mitocondrial en el esperma canino, pero siempre debe estar asociado con un análisis de la motilidad con el objetivo de evitar una evaluación incorrecta del potencial de fertilidad de los espermatozoides. García-Álvarez et al. (2009a) también mostraron que el potencial de membrana mitocondrial de los espermatozoides de carnero, analizados con MitoTracker Deep Red, no estaba relacionado con la fertilidad. Además de la producción de energía, las mitocondrias están involucradas en la modulación del equilibrio

redox, la regulación osmótica y la homeostasis del Ca^{2+} (Peña et al. 2009). Por lo tanto, la evaluación del estado mitocondrial eventualmente podría ofrecer información importante relativa a la capacidad fecundante de espermatozoides, pero podría requerir otros enfoques experimentales.

Diferentes investigadores han demostrado que el flujo de calcio intracitoplasmático es un componente necesario para la capacitación (Gualtieri et al. 2005). Aunque en el primer experimento se han detectado mayores concentraciones de calcio después de la incubación, no había ninguna relación entre la fertilidad y la concentración de calcio intracelular. Eso no quiere decir que las concentraciones de Ca^{2+} sean irrelevantes. La medición de la concentración de Ca^{2+} después de tratar el esperma con progesterona u otras señales fisiológicas podrían ofrecer información relevante relacionada con la fertilidad del semen (Arienti et al. 2010). La conclusión principal del primer estudio de esta tesis doctoral es que el semen descongelado con una mayor velocidad está relacionada con una mayor fertilidad *in vivo*, pero sólo cuando se llevan a cabo las mediciones después de 2h de incubación en el medio de congelación. Curiosamente, la incubación en SOF en condiciones que tratan de imitar el ambiente del oviducto femenino no mostró ninguna relación significativa con la fertilidad. Hasta donde sabemos, nunca antes se había encontrado esta relación entre los parámetros de motilidad de espermatozoides de los moruecos y la fertilidad *in vivo* mediante inseminación artificial intrauterina con semen congelado-descongelado. Un aspecto a tener en cuenta es el reducido número de machos en este estudio, sin embargo, este enfoque experimental de evaluación de las muestras de semen después de 2 h de incubación a 37 °C merece un mayor desarrollo ya que podría ser la base de los protocolos para la predicción de la fertilidad *in vivo* de las muestras de espermatozoides de morueco descongeladas.

Dado que en el trabajo anterior no encontramos relaciones significativas entre los parámetros espermáticos analizados mediante citometría de flujo, decidimos seguir buscando, incorporando nuevas técnicas de análisis espermático mediante esta técnica. Así, en el segundo trabajo de esta tesis doctoral, nuestros resultados mostraron un aumento de la producción de ROS, evaluada mediante CM-H_2DCFDA, tanto para la incubación en medio de congelación (37 °C) como en medio SOF (38 °C, 5% CO_2) Concretamente, se encontró una correlación negativa entre la fertilidad *in vivo* y la producción de ROS tras 2h de incubación en medio SOF (38°C, 5% CO_2).

El estrés oxidativo tiene un papel importante en la fisiología de esperma. En este contexto, el estrés oxidativo se reconoce cada vez más como una de las principales causas en la etiología de la infertilidad masculina humana (Aitken y Krausz 2001; Tremellen 2008). Por otra parte, la

criopreservación no sólo reduce la calidad del esperma, también induce estrés oxidativo y disminuye la actividad antioxidante en el semen (Quintana et al. 2005; Peris, Bilodeau et al. 2007). Los mecanismos de estrés oxidativo que limitan la competencia funcional de los espermatozoides de los mamíferos implican la peroxidación de los lípidos y la inducción de la oxidación del ADN (Rodriguez-Martinez y Barth 2007). Teniendo en cuenta la importancia de la producción de ROS en muestras de semen descongelado, abordamos esta cuestión para evaluar su efecto sobre la fertilidad. De acuerdo con nuestros resultados, las diferencias en la producción de ROS podrían ser responsables de los diferentes resultados de fecundación *in vitro*.

Está claro que los ROS contribuyen de forma importante a la regulación de la función de los espermatozoides tanto positiva como negativamente (Aitken y Curry 2011). Así, después de la inseminación, los espermatozoides generan bajos niveles de ROS con el fin de promover la capacitación y las funciones espermáticas necesarias para la fecundación, incluyendo la hiperactivación y la presentación de moléculas de reconocimiento en su superficie (Aitken 1989;de Lamirande y Gagnon 1993). Una excesiva producción de ROS podría estar relacionada con el desarrollo temprano de los procesos de capacitación y hiperactivación, e impedir al espermatozoide el llevar a cabo su función fisiológica en el momento adecuado. Por lo tanto, como muestran nuestros resultados, la incubación en medios de FIV también puede dar lugar a una sobreproducción de ROS, que altera la función del esperma y la fertilidad. El aumento de ROS podría no provocar efectos negativos por sí mismo, pero podría indicar una causa subyacente (fallo mitocondrial, desregulación, etc...), responsable tanto del aumento de ROS como de la baja fertilidad. El aumento de ROS no sería la causa de la baja fertilidad, sino una señal de que algo no se está regulando correctamente en los espermatozoides.

En base a los resultados obtenidos en este estudio, la información proporcionada por la sonda CM-H_2DCFDA combinada con la incubación en SOF (38 °C, 5% CO_2) sería útil para mejorar la evaluación de la calidad del esperma, y ambos métodos se podría considerar como una herramienta estándar para la determinación de la calidad del esperma. De hecho, otros resultados indican que los embriones producidos con los espermatozoides con mayores niveles de ROS, medidos con CM-H_2DCFDA, no podían avanzar más allá de la primera división (Silva *et al.* 2007).

La evaluación del potencial de fertilidad de una muestra de semen ha sido el objetivo primordial del análisis de semen. Por lo tanto, nuestro objetivo es desarrollar técnicas de laboratorio que nos permitan, de manera rápida y eficaz, evaluar la fertilidad potencial de una muestra seminal.

Sabiendo que una acumulación excesiva de ROS podría causar daños no sólo a la membrana plasmática, sino también el ADN de esperma (Bennetts y Aitken 2005), decidimos evaluar la integridad del ADN y su relación con la fertilidad *in vivo*. El daño del ADN en los espermatozoides se ha vinculado a porcentajes reducidos de fertilización, el desarrollo de una preimplantación deteriorada, aborto involuntario y morbilidad en la descendencia ((Zini y Sigman 2009; Avendano y Oehninger 2011). En general, el daño en el ADN raramente se encuentra de forma significativa en un macho de fertilidad probada, y la incidencia de daño en el ADN es mayor en varones infértiles. Aunque la mayoría de los investigadores están de acuerdo que intuitivamente el daño del ADN es probablemente perjudicial, el impacto clínico de daño en el ADN ha sido más difícil de demostrar, tal vez porque los puntos finales funcionales correctos no han sido identificados. Algunos estudios sugieren que los niveles de daño de ADN en los espermatozoides pueden predecir el éxito cuando se usan técnicas de reproducción asistida (ART) (Bungum *et al.* 2008) así como predecir la probabilidad de aborto recurrente (Carrell *et al.* 2003). Zini *et al.* (2005) sugirieron que los daños del ADN espermático puede proporcionar un biomarcador útil para la corrección de las condiciones capaces de influir negativamente la fertilidad. Dado que nuestros resultados mostraron que la producción de radicales libres después de la incubación post-descongelación de espermatozoides de morueco está relacionada con la disminución de la fertilidad *in vivo*, es posible que el daño del ADN podría estar relacionado con los procesos de oxidación. En este sentido, Thomson *et al.* (2011) demostraron que el 8-hidroxi-2'-deoxiguanosina, un biomarcador de daño oxidativo del ADN, es un marcador con altos valores predictivos de la fertilidad del semen humano, tras una inseminación artificial. Aunque algunos autores han encontrado una relación negativa de daño en el ADN con la fertilidad (Evenson *et al.* 2002) no encontramos esta relación en nuestro estudio, ya sea el análisis de ADN con SCSA, TUNEL o CMA3. Este efecto se observa más comúnmente para los espermatozoides humanos que para los rumiantes, tal vez debido al alto grado de empaquetamiento del ADN de los rumiantes (Januskauskas *et al.* 2003). Por lo tanto, nuestros resultados mostraron que el posible mecanismo responsable de diferencias de fertilidad no era debido al daño en el ADN.

La adición con suero de oveja en celo (SOC) se utiliza de forma rutinaria en los pequeños rumiantes para lograr buenos resultados en la FIV (Cognie *et al.* 2003, Garcia-Alvarez *et al.* 2011; Garcia-Alvarez *et al.* 2014, Huneau *et al.* 1994). Nuestros resultados confirman que el SOC es un agente eficaz para la capacitación de los espermatozoides de carnero, teniendo en cuenta el aumento de la fluidez de la membrana (M540). En un estudio anterior, se confirmó la asociación entre nuestro protocolo de FIV usando SOC, y la capacitación espermática mediante un aumento

en los resultados de M540, así como un aumento en el patrón de hiperactivación en la motilidad (Garcia-Alvarez *et al.* 2014). Se sabe que el SOC aumenta la perdida de colesterol de la membrana plasmática en los espermatozoides ovinos (Martinez-Pastor *et al.* 2009), lo que provoca un aumento de la fluidez de la membrana y la permeabilidad. Estos cambios de la membrana causan un aumento de Ca^{2+} (Garcia-Alvarez *et al.* 2011) lo que podría inducir una reacción acrosomal temprana en células sensibles. Otros eventos fisiológicos documentados en semen ovino capacitado son el aumento de la fosforilación de la tirosina (Perez-Pe *et al.* 2002; Sidhu *et al.* 2004) y cambios en el patrón de motilidad (Aitken y Nixon 2013;Vulcano *et al.* 1998).

El impacto de la capacitación en las vías de transducción de señales relacionadas con la apoptosis es todavía poco conocido en los espermatozoides, y la mayoría de estudios se han llevado a cabo en los seres humanos. Recientemente, Grunewald *et al.* (2009a) han sugerido que la apoptosis y capacitación son procesos equilibrados en espermatozoides humanos. La apoptosis en los espermatozoides conllevaría la pérdida de su capacidad para someterse a la capacitación, mientras que los espermatozoides capacitados podrían inactivar la señalización proapoptótica. Nuestros resultados apoyan que procesos similares ocurren también en los espermatozoides de morueco. Del mismo modo, Marti *et al.* (2008) indicaron que las actividad caspasa disminuía tras la incubación del semen de carnero en condiciones capacitantes. Recientemente, Aitken *et al.* (2015) han estudiado la posibilidad de que los procesos de capacitación y la apoptosis estén vinculados, unidos por su dependencia común de la continua generación de especies reactivas de oxígeno. Según este modelo, la capacitación se inicia en los espermatozoides como consecuencia de la generación de ROS intracelular, pero la continua generación de ROS por la eventual capacitación de poblaciones de espermatozoides podría sobrepasar la limitada capacidad de estas células para protegerse a sí mismas del estrés oxidativo. Como resultado de sobre-capacitacion, las cascadas apoptóticas se activan, lo que lleva a la muerte espermática. Teniendo en cuenta nuestros resultados, el SOC podría modular la fisiología espermática, facilitando la capacitación espermática mientras que promueve la desaceleración o el bloqueo de la entrada en apoptosis.

El tratamiento con SOC fue capaz de prevenir la activación de la cascada de caspasas. El ensayo polycaspase (incluyendo las caspasas 1, 3, 4,5, 6, 7, 8 y 9) detectó una disminución en la proporción de los espermatozoides viables y caspasa negativos cuando se incubaron las muestras, dicha disminución fue impedida en parte por la presencia de SOC. De hecho, el SOC fue tan eficaz como el tratamiento inhibidor de las caspasas en el mantenimiento de la proporción de espermatozoides caspasa 9 en un nivel bajo. Sin embargo, el análisis de las caspasas específicas (8 y 3/7) produjo efectos menos marcados. Grunewald *et al.* (2009b) mostraron que las caspasas

iniciadoras de la apoptosis (1 y 9) son inhibidas por la capacitación en una cantidad mayor que las caspasas efectoras (tales como la caspasa 3 y 7), lo que podría explicar nuestros resultados. Por otra parte, los resultados pueden estar modulados por las diferentes funciones de estas caspasas en la célula y sus cantidades relativas. La activación de la caspasa-3 marca un "punto de no retorno" en la cascada de señalización de la apoptosis, como se ha demostrado en las células somáticas (Green y Amarante-Mendes 1998), mientras que la activación de las caspasas iniciadoras es un proceso reversible. La caspasa-8 es parte de la vía extrínseca de la activación y se ha detectado en espermatozoides de morueco. Sin embargo, su papel en esta especie no es claramente definido, ya que no parecía activarse cuando se aplicaron estímulos típicos proapototicos (Pena *et al.* 2009). Nuestros resultados sugieren que la caspasa-8 podría desempeñar un papel menor en los cambios que se producen durante la incubación y capacitación de muestras espermáticas de ovino descongeladas.

Es de destacar que el tratamiento con SOC provocó un aumento en lo que podrían ser considerados como precursores de apoptosis: YO-PRO-1 y la pérdida de la actividad mitocondrial en la población viable de espermatozoides. Estos resultados parecen ser contradictorios, ya que las mitocondrias son uno de los principales centros para la regulación de la apoptosis (Pena *et al.* 2009) y su inactivación se ha propuesto como un preludio para la apoptosis y la muerte de los espermatozoides (Martinez-Pastor *et al.* 2008). Sin embargo, es posible que los cambios detectados por estas sondas sean resultado de los cambios de la membrana causadas por la capacitación bajo la influencia de SOC, en lugar de estar relacionados con la apoptosis. De hecho, la exposición de los aminofosfolípidos en la hemimembrana externa de la membrana plasmática, otro marcador de la apoptosis, que también está relacionado con la tinción YO-PRO-1, es independiente de la apoptosis en espermatozoides capacitados (de Vries *et al.* 2003; Grasa *et al.* 2006; Gadella y Harrison 2002). Con respecto al aumento de una subpoblación de espermatozoides viables con baja actividad mitocondrial, un estudio sobre espermatozoides de cerdo encontró que la capacitación induce cambios en la fisiología mitocondrial, y los autores sugirieron que las subpoblaciones de espermatozoides podrían coexistir en la muestra de semen mientras varían su respuesta mitocondrial a la capacitación (Ramio-Lluch *et al.* 2011). Por lo tanto, podría haber espermatozoides viables capacitados, a la vez que subconjuntos de espermatozoides que dejan de responder adecuadamente a estos estímulos, perdiendo actividad mitocondrial (Martinez-Pastor *et al.* 2008).

Las primeras etapas de la capacitación espermática se pueden medir mediante la sonda M540. Steckler *et al.* (2015) han propuesto la combinación YO-PRO-1/M540 como un indicador más sensible de la viabilidad, identificando la desestabilización de la membrana con la

capacitación espermática en el perro. Anteriormente, se encontró que los espermatozoides YO-PRO-1 positivos se identifican tempranamente en el proceso de deterioro y muerte celular. En las células subviables en las que la membrana plasmática se ha desestabilizado, las células carecen de cantidades adecuadas de ATP para transportar YO-PRO-1 de nuevo fuera de la célula. A este respecto, parece que YO-PRO penetra en el plasmalema de los espermatozoides a través de canales de pannanexina específicos del espermatozoide tiñiendo el ADN antes de la obliteración completa de la membrana, la cual permite la entrada de otras tinciones de ADN impermeables a la membrana (Idziorek *et al.* 1995; Wronski *et al.* 2002; Pena *et al.* 2005; Gallardo Bolanos *et al.* 2014). El que Aitken *et al.* (2015) sugieran que la apoptosis en los espermatozoides es significativamente diferente del fenómeno observado en las células somáticas y el que nuestros resultados muestren una mayor subpoblación de células M540 que sigue siendo negativa a YO-PRO-1, podría explicar que la entrada de YO-PRO-1 no esté relacionada con la apoptosis, sino a otros fenómenos vinculados a la capacitación.

Una contribución importante de este estudio es la posibilidad de que esta información pueda ser utilizada para mejorar los medios de capacitación *in vitro* de espermatozoides de morueco, pudiendo remplazar el SOC por un medio definido. Es posible que la capacitación inducida por SOC pueda diferir del proceso fisiológico, pero tiene éxito en el entorno de la FIV. Además, teniendo en cuenta el resto de parámetros evaluados, el SOC tendría también un papel de prevención del deterioro del esperma. La importancia del estudio recogido en el capítulo 6 reside en la constatación por primera vez que durante la capacitación *in vitro* del semen de morueco, el suero de oveja en celo permite el proceso de capacitación *in vitro* mediante la prevención de la actividad caspasa.

Otro punto a destacar del capítulo 6, es la identificación de un grupo de moruecos de "baja fertilidad" que en la que la mayoría de las muestras se parecían al grupo de "alta fertilidad" en términos de la calidad del esperma, tanto después de la recogida en la granja como en el análisis *in vitro*. Lo más interesante es que cuatro de estos cinco machos mostraron los niveles más bajos de las caspasas activadas. Una investigación posterior no pudo determinar la causa de la mala fertilidad de estos animales, aunque tres de los machos comparten el mismo padre. Aunque la búsqueda de una explicación a este hecho va más allá de nuestro objetivo inicial, esto abre una interesante línea de investigación, para determinar si marcadores genéticos específicos podrían influir en la fertilidad de estos machos. Así, otros autores han encontrado que los polimorfismos de nucleótido único y la metilación en todo el genoma en toros se asocian al éxito en el desarrollo embrionario (Verma *et al.* 2014; Cochran *et al.* 2013). Tras eliminar ese subconjunto de machos de fertilidad baja, hemos obtenido unos modelos que relacionan algunas

variables con la fertilidad. Además de la viabilidad del esperma y espermatozoides capacitados, varias variables derivadas de los análisis de la actividad caspasa estaban relacionados con la fertilidad (rechazamos regresiones significativas, con bajo R2, ya que el gran número de análisis aumentó el riesgo de considerar regresiones espurias como verdaderas). Coincidiendo con otros autores (Espino *et al.* 2011; Gallardo Bolanos *et al.* 2012; Aitken y Baker 2013), encontramos que las muestras con niveles relativamente altos de caspasas activadas (ya sea intrínsecamente o resultantes de la incapacidad de prevenir su activación) tienden a tener una menor fertilidad *in vivo*, que muestra la importancia que tiene para el espermatozoide mantener las vías de apoptosis inactivadas.

Por lo tanto, las pruebas de actividad caspasa podrían ser útiles para identificar las muestras con una fertilidad potencialmente menor. Sin embargo, hay que insistir en la necesidad de utilizar herramientas moleculares y genéticas para caracterizar los machos de baja fertilidad.

Es interesante que la mayoría de las regresiones significativas se hayan encontrado cuando se utilizan datos del tratamiento de control. La incubación de muestras espermáticas a diferentes temperaturas es una prueba de resistencia típica (Dominguez-Rebolledo *et al.* 2010, Mata-Campuzano *et al.* 2012;) y permite pruebas de calidad de esperma más a fondo. La falta de asociación de muestras tratadas con SOC con la fertilidad podría parecer sorprendente, pero en realidad, no es inesperado. En el contexto de la inseminación artificial, podría ser más importante para los espermatozoides mantener una buena resistencia después de la descongelación, en lugar de responder a un medio capacitante. De hecho, la capacitación *in vitro* es muy diferente de la capacitación *in vivo*, especialmente teniendo en cuenta el medio ambiente y las interacciones célula a célula que tienen lugar en este (Hunter y Gadea 2014). Esto apoya nuestra hipótesis de que la incubación con SOC podría alterar los parámetros del esperma (por lo menos los medidos en el presente estudio) de una manera que difiere del proceso fisiológico y por lo tanto perder la relación con los procesos que tienen lugar tras la inseminación artificial. Pruebas más complejas (por ejemplo, la combinación de un período de resistencia con un paso capacitación posterior) podrían aumentar el poder predictivo de estos análisis.

Finalmente, en el capítulo 7 de la presente tesis decidimos comprobar si, como Aitken *et al.* (2015) proponen, la capacitación y la apoptosis son procesos relacionados mediante la producción de ROS. Así, como consecuencia de la producción de ROS, inicialmente se produciría la apoptosis y cuando los niveles de ROS sobrepasaran la capacidad de los espermatozoides para protegerse de los ROS, entonces se iniciaría la cascada apoptótica.

Durante la cascada apoptótica via intrínseca, lo que inicialmente se observa es un aumento repentino en la generación de ROS mitocondrial y una pérdida de la motilidad del esperma

(Koppers *et al.* 2011). Después se observa la activación de caspasas en el citosol del espermatozoide y la expresión en superficie de fosfatidilserina, como se detecta por la unión con Anexina V (Leclerc *et al.* 1997; Johannisson *et al.* 2009; Kostro *et al.* 2014; Mohan y Atreja 2014; Yu 2014; Zeng *et al.* 2014; Zhao *et al.* 2014).

Las especies reactivas del oxígeno no sólo son fundamentales para el proceso de apoptosis, sino que también están involucradas en la capacitación de los espermatozoides (Harayama 2013). Se cree que los ROS ejercen una influencia positiva sobre la fosforilación de tirosinas durante la capacitación de los espermatozoides a través de su capacidad para mejorar los niveles intracelulares de AMPc e inhibir la actividad de la tirosina fosfatasa (Aitken *et al.* 1998). La generación de ROS es crítica para que la capacitación se produzca, sin embargo representa una estrategia relativamente arriesgada para este tipo de células, porque los espermatozoides son especialmente susceptibles al estrés oxidativo debido a su relativa falta de protección antioxidante y la abundancia de sustratos oxidables en forma de ácidos grasos poliinsaturados, las proteínas y el ADN. Por lo tanto, es probable que surja un punto de inflexión durante la capacitación cuando la generación intrínseca de ROS sobrepasa la capacidad espermática para defenderse contra el ataque oxidativo (Aitken *et al.* 2015).

Como ya se ha comentado anteriormente, se sabe que el SOC aumenta la salida de colesterol de la membrana plasmática en los espermatozoides (Harrison *et al.* 1993), lo que provoca un aumento de la fluidez de la membrana y la permeabilidad. Estos cambios de la membrana causan un aumento de Ca^{2+} (Mohan y Atreja 2014), lo que podría inducir una reacción acrosomal temprana en células sensibles. Sin embargo, nuestros resultados no mostraron diferencias significativas en la concentración intracelular de Ca^{2+} entre SOC y control; sólo la combinación de SOC y el inhibidor de caspasa aumentaron la concentración intracelular de Ca^{2+}. Los cambios en el Ca^{2+} intracelular normalmente se producen debido a la capacitación como un proceso continuo, formando un patrón en el tiempo, por lo que, quizás, el método utilizado en nuestro estudio para el cálculo del Ca^{2+} intracelular no tenga suficiente sensibilidad.

Nuestros resultados mostraron que SOC además de ser un agente eficaz para capacitar espermatozoides de morueco, aumenta la fluidez de la membrana (M540) y el establece el patrón de motilidad hiperactivado (aumento de la VCL y ALH, y la disminución de linealidad). El que el SOC disminuya la producción de ROS, nos indica que el SOC parece pudiera actuar como un antioxidante, reduciendo la producción de ROS durante la capacitación *in vitro*. Sin embargo, esta acción de protección no parece estar relacionada con la actividad caspasa debido a que el inhibidor de la caspasa no tiene la capacidad de reducir los radicales libres. El suero fue capaz de mantener bajos niveles de ROS después de la incubación e incluso disminuir ligeramente el

control (tiempo 0h de incubación). La combinación de suero e inhibidor de caspasas también redujo significativamente la producción de ROS. Podríamos concluir que el tratamiento con SOC podría mantener niveles suficientes de ROS para que se produzca la capacitación y previniendo un que desencadenara la apoptosis.

Hasta la fecha, se han utilizado con éxito diversos aditivos a los medios de cultivo, tanto de origen sintético como animal, para favorecer la capacitación de los espermatozoides *in vitro* de los mamíferos. Por ejemplo, se han utilizado como agentes capacitantes varias hormonas como la progesterona o estrógenos, heparina y BSA (Therien *et al.* 1995; Galantino-Homer *et al.* 1997; Goncalves *et al.* 2014; Parrish 2014). Recientemente, Garcia-Alvarez *et al.* (2015) han demostrado que la heparina-hipotaurina, la progesterona (P4), estrógenos (E2), β-ciclodextrina, o BSA no son adecuados para la sustitución del SOC en medios de capacitación *in vitro* y FIV para espermatozoides de morueco. Por lo tanto, el conocimiento de los mecanismos de acción del suero es de gran interés ya que nos permitirá establecer las condiciones de los medios de cultivos que favorezcan la capacitación espermática *in vitro*. Nuestros resultados sugieren que los antioxidantes en combinación con otras sustancias podrían ser considerados para el desarrollo de un medio químicamente definido capaz de reemplazar el SOC en los medio de capacitación *in vitro*.

Consideraciones finales

Los resultados de la presente tesis doctoral proporcionan información útil y potencialmente aplicable a los laboratorios de reproducción. Nuestros resultados sugieren que la determinación de la velocidad (VCL o VAP) tras 2h de incubación a 37 °C es un parámetro altamente relacionado con el resultado de fertilidad *in vivo* de una determinada muestra, pudiendo ser considerado este procedimiento para la predicción de la fertilidad de una determinada muestra de semen descongelado de morueco. De igual modo, nuestros resultados indican que la determinación de la producción de radicales libres mediante la sonda fluoresceína (CM-H_2DCFDA) en espermatozoides descongelados de morueco tras 2h de incubación en condiciones que simulan el tracto reproductor femenino (en SOF, a 38 °C y 5% CO_2) nos permitiría identificar muestras "subfértiles", pudiendo ser retiradas éstas de los procedimientos de inseminación artificial.

Uno de los hallazgos más importantes de este estudio es la constatación por primera vez que durante la capacitación *in vitro* del semen de morueco, el suero de oveja en celo permite el proceso de capacitación *in vitro* mediante la prevención de la actividad caspasa. Además, el suero

de oveja en celo podría mantener niveles suficientes de ROS para que se produzca la capacitación pero previniendo una producción excesiva que desencadenara la apoptosis.

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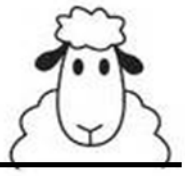
Capítulo 9



Conclusions

1. Thawed semen samples with a greater sperm velocity (VCL and VAP) are related to a greater field fertility, when measurements are conducted after 2h of incubation after thawing. Therefore, sperm motility parameters evaluation by CASA is useful to identify samples with poor fertility.

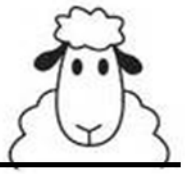
2. ROS production determined by CM-H_2DCFDA and flow cytometry, after 2h of incubation (SOF, 38 °C, 5% CO_2), of frozen-thawed ovine sperm samples is a good indicator parameter for *in vivo* male fertility. The detection of ROS and flow cytometry after 2h of incubation in conditions that mimic the female reproductive tract, could be a useful procedure for predicting fertility of ram spermatozoa.

3. *In vitro* induction of capacitation in thawed ram spermatozoa by using estrous sheep serum suggests a downregulation in apoptotic pathways, preventing caspase activation.

4. Estrous sheep serum, besides its "capacitation" activity have a protective role in preventing sperm deterioration of thawed sperm samples of ram during *in vitro* capacitation.

5. Estrous sheep serum may modulate ROS levels during *in vitro* capacitation of ram spermatozoa, allowing intracellular signaling for capacitation to occur while preventing higher levels that could trigger apoptosis.

Anexo



Los trabajos llevados a cabo en la presente tesis doctoral que componen los capítulos del 3 al 7 han dado lugar a 4 artículos científicos:

1. Del Olmo, E., Bisbal, A., Maroto-Morales, A., Garcia-Alvarez, O., Ramon, M., Jimenez-Rabadan, P., Martinez-Pastor, F., Soler, A.J., Garde, J.J., y Fernandez-Santos, M.R. (2013). Fertility of cryopreserved ovine semen is determined by sperm velocity. *Anim Reprod Sci* 138(1-2), 102-9.

2. Del Olmo, E., Bisbal, A., Garcia-Alvarez, O., Maroto-Morales, A., Ramon, M., Jimenez-Rabadan, P., Anel-Lopez, L., Soler, A.J., Garde, J.J., y Fernandez-Santos, M.R. (2014). Free-radical production after post-thaw incubation of ram spermatozoa is related to decreased *in vivo* fertility. *Reprod Fertil Dev* doi: 10.1071/RD14043.

3. Del Olmo, E., Garcia-Alvarez, O., Maroto-Morales, A., Ramon, M., Jimenez-Rabadan, P., Iniesta-Cuerda, M., Anel-Lopez, L., Martinez-Pastor, F., Soler, A.J., Garde, J.J., y Fernandez-Santos, M.R. (2015). Estrous sheep serum enables *in vitro* capacitation of ram spermatozoa while preventing caspase activation. *Theriogenology*. doi: 10.1016/j.theriogenology.2015.09.005.

4. Del Olmo, E., García-Álvarez, O., Maroto-Morales, A., Iniesta-Cuerda, M., Anel-Lopez, L., Martinez-Pastor, F., Soler,A.J., J.J. Garde, J.J., y Fernández-Santos, M.R. (2016). Estrous sheep serum balances the levels of ROS to supply *in vitro* capacitation of ram spermatozoa. Enviado a la revista *Theriogenology*.