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DEPARTAMENTO DE BIOLOGÍA MOLECULAR

Diseño de un dispositivo para la evaluación *in vitro* de daños celulares durante el transporte en el cérvix del semen ovino

Device design for *in vitro* evaluation of cell damage during ram semen transport through cervix

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CARMEN MARTÍNEZ RODRÍGUEZ

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El Dr. Paulino de Paz Cabello y la Dra. D. María Mercedes Álvarez García, como Directores de la Tesis Doctoral titulada "Diseño de un dispositivo para la evaluación *in vitro* del daño celular durante el transporte en el cérvix del semen ovino" realizada por Dña. Carmen Martínez Rodríguez, en el programa de Biología Molecular y Biotecnología, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

Lo que firman, en León a 25 de octubre de 2015.

Los Directores de la Tesis Doctoral

Fdo: Paulino de Paz Cabello **Fdo: María Mercedes Álvarez García**

A mi familia,

“Siempre es demasiado

pronto para rendirse”

Norman Vincent Peale

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RESUMEN

La inseminación artificial (IA) es la principal herramienta disponible para realizar la mejora genética animal. Para la mayoría de las especies domesticas el método de IA más utilizado es la aplicación de semen congelado-descongelado por vía cervical, debido a que la criopreservación del semen permite su almacenamiento a largo plazo y facilita la distribución geográfica de las dosis seminales. Sin embargo, el ganado ovino constituye una excepción en este campo debido a las bajas tasas de fertilidad obtenidas con esta técnica.

La fertilidad es una función biológica muy compleja que depende de varias propiedades de los espermatozoides que son necesarias para que estos puedan superar las distintas barreras presentes en el aparato reproductor femenino y alcanzar el sitio de fertilización. Sólo los espermatozoides con una morfología normal y una motilidad adecuada van a ser los que lleguen al punto de fecundación. La primera barrera que los espermatozoides se van a encontrar en su camino es el moco cervical. Éste está considerado como la principal barrera de selección de espermatozoides en muchas especies animales, y por ello, se han desarrollado varios métodos *in vitro* para estudiar la capacidad de los espermatozoides para atravesarlo. Estos métodos de análisis se denominan test de penetración en moco cervical *in vitro* y han sido aplicados en varias especies animales y en humanos. En la presente tesis doctoral se propone un nuevo modelo del test de penetración en moco cervical, ensayado específicamente en ganado ovino, para estudiar y determinar cuáles son las características específicas que las células han de tener para poder superar esta barrera cervical, y evaluar su capacidad como predictor de la fertilidad en campo.

El moco cervical es un fluido complejo cuyas características fisicoquímicas varían a lo largo del ciclo estral de la oveja, y además cada oveja produce solamente unos pocos mililitros del mismo. Por estas razones y para poder estandarizar el test de penetración es necesario desarrollar un sustituto artificial del moco cervical. Uno de los sustitutos más comunes son los geles de poliacrilamida y por ello lo probamos para ovino en una primera parte del estudio. Para intentar encontrar un moco sintético que tenga unas características reológicas lo más similares posibles al moco natural probamos 8 concentraciones distintas de acrilamida entre el 1 y el 2%. Además de estudiar la viscosidad de las distintas concentraciones de acrilamida para compararla con la del moco natural, realizamos también unos test de penetración utilizando moco natural y sintético para comprobar el comportamiento de ambos. En este primer ensayo hemos contado los espermatozoides que atraviesan el moco cervical en segmentos de 0,5 cm. La acrilamida de 1.6 % era la que mostraba una viscosidad más similar al moco natural en los estudios reológicos, y en el test de penetración los geles de 1.55% y 1.6% fueron los que mostraban un número de espermatozoides que migran más similar al moco natural. En una segunda fase del estudio se ha comprobado el comportamiento de estos 2 geles sintéticos y el moco natural como barrera para el test de penetración frente a muestras que han sido almacenadas a dos temperaturas distintas 5 y 15 °C, ya que la refrigeración del semen produce alteraciones en los patrones de movilidad de la muestra y queremos ver si se pueden detectar por medio del uso de test de penetración con moco sintético. La acrilamida 1.6% es la que mostró un comportamiento más similar al moco natural y se han visto también correlaciones entre el número de espermatozoides que atraviesa el test y varios parámetros de calidad seminal (positiva con la movilidad progresiva, y la velocidad en línea recta; y negativa con el porcentaje de células apoptóticas y con acrosomas dañados).

En este trabajo también se probaron otros sustitutos del moco natural como la metilcelulosa que ha sido aplicada en tests de penetración para semen humano. A diferencia de lo visto para humanos, la metilcelulosa resultó ser mucho menos permisiva que la

acrilamida para el paso de espermatozoides ovinos. Por lo tanto se sigue manteniendo el gel de poliacrilamida 1.6% como mejor alternativa sintética al moco natural ovino.

Una vez definido el moco sintético a usar procedemos a mejorar el dispositivo a emplear en el test de penetración para ovino. El objetivo es diseñar un sistema que permita aislar para su estudio la población celular que es capaz de atravesar la barrera de moco cervical en el test de penetración. Para ello se diseñó un dispositivo con 3 módulos (muestra, barrera de moco y unidad de recogida) y se pusieron a punto los siguientes parámetros de uso: longitud de la barrera de moco cervical, tiempo de incubación del test, concentración espermática de la muestra a analizar y posición del test durante la incubación. Una vez puesto a punto el test se realizaron unos ensayos usando como muestra de partida unos pool de semen de varios machos para testar la repetitividad del test, encontrando que esta es buena, ya que los resultados fueron muy similares para los diferentes ensayos realizados. Utilizando este dispositivo calculamos el número de espermatozoides que migran a través del test en distintas muestras seminales y su movilidad, así como la viabilidad, y el estatus acrosomal y mitocondrial de las 3 poblaciones espermáticas: muestra fresca, espermatozoides que no atraviesan el test y los que lo atraviesan. Observamos que el porcentaje de espermatozoides con alto potencial de membrana se ve reducido en la población de espermatozoides que migran a través del test.

En los últimos años se han realizado muchos estudios sobre la morfología de la cabeza de los espermatozoides, y varios autores muestran correlaciones entre la morfología de la cabeza y la movilidad, el índice de fragmentación del DNA y las tasas de fertilidad obtenidas en campo. Por ello, hemos utilizado el dispositivo que hemos diseñado para evaluar la relación entre la habilidad de los espermatozoides para superar la barrera de moco cervical en el test de penetración y la morfología de la cabeza de los espermatozoides que lo atraviesan. A la vez se analizaron también la movilidad, la viabilidad y el estatus acrosomal y mitocondrial de la muestra fresca y de la población que migra. El análisis de componentes principales de los valores obtenidos para los distintos parámetros morfológicos define tres subpoblaciones celulares. Una de estas subpoblaciones, formada por espermatozoides con cabeza pequeña y ancha no se encuentra entre la población de espermatozoides que migra a través del test, lo que indica que los espermatozoides con esa morfología en la cabeza tienen dificultades a la hora de atravesar el moco cervical. Este análisis fue corroborado por un estudio complementario en que la muestra seminal es sometida a un proceso de oxidación *in vitro* y posteriormente se aplica el test de progresión. Dado que ha habido autores que han relacionado las tasas de fertilidad con la morfología de la cabeza de los espermatozoides y con los resultados de los test de penetración realizamos unos ensayos con el nuevo dispositivo de tal manera que cada muestra seminal es testada en el test, se estudia la morfología de la cabeza de los espermatozoides de la muestra y de los espermatozoides que pasan el test y, finalmente, se usa parte de la muestra para inseminar varias ovejas cuyos celos han sido sincronizados previamente. No se observó ninguna correlación entre tasas de fertilidad y el número de espermatozoides que migra en el test, aunque sí aparece una correlación negativa entre la proporción de espermatozoides con cabezas cortas y anchas en la muestra fresca y la fertilidad. Todo esto indica que la morfología de la cabeza de los espermatozoides está asociada con su habilidad para superar la barrera de moco cervical en un test de migración, y que el tamaño relativo de la subpoblación de espermatozoides que no consigue pasar el test esta negativamente relacionada con la fertilidad del macho.

ABSTRACT

Artificial Insemination (AI) is the main tool available to develop a genetic improvement program in animals. The method of choice for artificial insemination in most animal species is the application of frozen-thawed sperm via cervix, as sperm cryopreservation allows long term sperm storage and facilitates the geographic distribution of the sperm doses. However, sheep is an exception due to the low fertility rates obtained when this methodology is applied.

Fertility is a very complex biological function that relies on several properties of the spermatozoa that are needed for these cells to be able to overcome the different barriers present in the female reproductive tract and reach the fertilization site. Only spermatozoa with normal morphology and vigorous motility will succeed to reach the fecundation point. The first barrier that spermatozoa need to face is the cervical mucus. In many animal species this has been considered the main selection barrier, and therefore different *in vitro* tests have been developed to study the ability of the spermatozoa to pass through it. These types of analysis methodology are known as *in vitro* penetration tests in cervical mucus and have been used in different animal species and humans. In this study we propose a new model of an *in vitro* cervical mucus penetration test, assessed specifically for sheep, to study and to identify the main spermatozoa features required to overcome the mucus barrier and evaluate it capacity as field fertility predictor.

The cervical mucus is a complex fluid and its physical-chemical characteristics change along the sheep oestrous cycle and each ewe produces just a few millilitres of cervical mucus fluid. Due to this, and to be able to standardize the *in vitro* penetration test device, the development of a cervical mucus synthetic surrogate is required. Polyacrylamide gels, being one of the most commonly used substances for this aim, have been evaluated as ewe cervical mucus surrogate in the first part of the study. To try to find a synthetic surrogate for the cervical mucus which rheological characteristics are as close as possible to the natural one, we have tested eight concentrations of acrylamide between 1% and 2%. We have assessed the viscosity of the different acrylamide gels and compared it to the natural mucus as well as performed some *in vitro* penetration tests using the synthetic and natural mucus as a barrier to see which ones had the most similar behaviour. In this first assay we have counted the number of spermatozoa that overcomes the mucus barrier at each 0.5 cm fragment of mucus. Acrylamide at 1.6% exhibited more similar viscosity parameters to the natural mucus at the rheological studies, and at the *in vitro* penetration test the 1.55% and 1.6% gels had the closest number of migrating spermatozoa to the natural mucus. On the second stage of the experiment, we applied fresh semen samples and semen stored at 15 °C and 5 °C to an *in vitro* penetration test with natural mucus and acrylamide 1.6% and 1.55% as the mucus barrier. Sperm refrigeration leads to alterations in the motility parameters of the semen sample and we wanted to assess if those alterations can be detected by using an *in vitro* penetration test. We observed that acrylamide at 1.6% produced more similar cell counts to the natural mucus, and we found significant correlations between the number of migrating spermatozoa and several sperm quality parameters (positive: progressive motility and velocity according to the straight path; negative: damaged acrosomes and apoptotic cells).

In this study we have also tried other cervical mucus surrogates like methylcellulose, as it has been previously used in human *in vitro* penetration tests. Opposite to what happens for humans, we have seen methylcellulose is less permissive than acrylamide for ram spermatozoa. Hence we selected 1.6% polyacrylamide gels as the best synthetic surrogate for ewe cervical mucus.

Once the synthetic ewe cervical mucus was obtained we proceeded to improve the device used for *in vitro* penetration test in sheep. The goal was to design a device that allowed the isolation of the population that passed through the mucus barrier in the *in vitro* test. We designed a device with 3 modules (sample unit, test unit and collection unit) and set its conditions of use: length of mucus, incubation time, sperm concentration and device position during incubation. When the device and method were set, we performed several *in vitro* penetration tests using ram sperm pools and we obtained similar sperm counts for the consecutive assays indicating an acceptable repeatability of the method. Using this device we ran some tests to determine the number of spermatozoa migrating, and the sperm motility, viability and the acrosomal and mitochondrial status of three sperm populations: fresh, not migrating and migrating spermatozoa. We observed that the percentage of high mitochondrial membrane potential spermatozoa was significantly reduced in the population of migrating spermatozoa.

The head morphology of sperm has been widely studied in the last few years, and there are various studies that have reported correlations between head morphology and motility, fertility rates and DNA fragmentation index of the sperm sample. Therefore, we have used our device to evaluate the relationship between the ability of ram spermatozoa to overcome the mucus barrier in an *in vitro* migration test and sperm head morphology of migrating spermatozoa. While performing the *in vitro* penetration test we assessed sperm motility and the acrosomal status, viability and mitochondrial status of the fresh sample and the migrating population of cells. Principal component analysis and clustering analysis of the values for the morphometric parameters assessed defined three cell subpopulations within the fresh sample. One of these subpopulations, defined by spermatozoa with a short and wide head was not found in the migrating spermatozoa population, indicating that the spermatozoa that contain this head morphology failed to pass through the mucus barrier. We performed a complementary study using seminal samples subjected to an *in vitro* oxidizing process in the *in vitro* penetration test and we found the same results as we mentioned before.

There have been some authors that have found a correlation between fertility rates and spermatozoa head morphology parameters, or fertility rates and *in vitro* penetration test results. For that reason, we used our new *in vitro* test to analyse if we could replicate the same results. We carried out the *in vitro* penetration test on seminal samples from different rams, analysed the head morphology parameters for the different samples and used the remaining amount to inseminate several ewes with synchronized oestrus. We did not find any correlation between fertility and the number of spermatozoa that migrated in the *in vitro* test, whereas there was a negative correlation between the proportion of spermatozoa with a short and wide head in the fresh sperm sample and fertility rate. We concluded that the head morphology of spermatozoa was associated with their ability to overcome a mucus barrier in a migration test, and the relative size of the non-migrating subpopulation was negatively related to male fertility.

INTRODUCCIÓN

1. La inseminación artificial ovina

La inseminación artificial (IA) es la principal herramienta disponible para realizar la mejora genética animal y conseguir así un incremento de la rentabilidad de las explotaciones ganaderas. Para la mayoría de las especies domesticas el método de IA más extendido es la aplicación de semen congelado-descongelado por vía cervical, ya que la criopreservación del semen permite su almacenamiento a largo plazo y una amplia distribución geográfica de las dosis seminales.

El ganado ovino constituye una excepción en este campo debido a las bajas tasas de fertilidad obtenidas con esta técnica, siendo la aplicación de semen fresco o refrigerado (15 °C) mantenido *in vitro* durante cortos periodos de tiempo y mediante inseminación cervical la metodología más extendida en el mundo (Alvarez *et al*. 1998). Esto se debe a la complejidad anatómica del cérvix que con sus anillos excéntricos impide una inseminación intrauterina rutinaria, y a la incapacidad del semen para atravesar o colonizar este segmento genital (Ayad *et al*. 2004). En caso de querer utilizar semen ovino congelado-descongelado la aplicación ha de hacerse por vía laparoscópica ya que permite salvar la barrera cervical para poder mantener así unas tasas de fertilidad aceptables (Maxwell and Salamon 1993, Anel *et al*. 2003, Anel *et al.* 2005). No obstante, esta última solución cuenta con el aspecto negativo del elevado coste que presenta y la especialización tecnológica que exige, lo que hace que sólo pueda ser considerado de interés en casos especiales, como animales de alto valor genético o de razas en peligro de extinción. Para intentar salvar estos inconvenientes también se ha estudiado como alternativa la inseminación por vía transcervical, pero los datos publicados hasta la fecha describen resultados de fertilidad muy dispares (Eppleston *et al*. 1994). Se cree que esto se debe a que el depósito transcervical de semen activa mecanismos relacionados con las defensas inmunitarias en el útero y desencadena un aumento de leucocitos en el lumen uterino que no se produce cuando lo que se deposita es el diluyente sin células (Wulster-Radcliff *et al*. 2004).

Todo esto indica que se necesita conocer las razones que explican la baja fertilidad alcanzada después de aplicar semen congelado/descongelado por vía cervical en el ganado ovino para poder desarrollar un procedimiento de IA que tenga un coste adecuado y produzca un rendimiento aceptable de partos (Anel *et al.* 2006).

2. Transporte espermático

El transporte del semen a lo largo del tracto reproductivo de la hembra está regulado de tal manera que permita maximizar las opciones de fertilización y para asegurar que sólo los espermatozoides con una morfología normal y una motilidad adecuada sean los que lleguen al punto de fecundación (Suarez *et al.* 2006).

El término transporte espermático engloba dos componentes principales: la actividad de los conductos femeninos (contracción muscular, corrientes de fluidos, movimiento ciliar...) y el movimiento intrínseco de los espermatozoides. Olds-Clarke (1986) demostró que el transporte espermático es dependiente de la motilidad espermática en algunos puntos del proceso y que éste está afectado por la actividad muscular de los diferentes segmentos del tracto reproductivo, así como por el microambiente definido por los diferentes fluidos que allí se encuentran. Además se ha visto que en su camino a través de las vías genitales de la hembra los espermatozoides deben superar diferentes barreras fisiológicas y anatómicas presentes, lo que lleva a una reducción notable del número de espermatozoides que van a ser

capaces de alcanzar el punto de fecundación. Hawk (1983) observó que tras depositar 500 x 10^6 espermatozoides en el orificio cervical externo en varias ovejas sólo recuperaba el 3% del total de estas células en el conjunto del tracto reproductivo, y esta cifra se reducía al 0.25% a las 12-24 horas después de la inseminación.

Algunos autores han recuperado espermatozoides que han alcanzado la región de la ampolla minutos después de la inseminación (Settlage *et al.* 1973, Hawk 1983) lo que demuestra la existencia de un transporte rápido de espermatozoides a través del tracto genital femenino. Sin embargo, cuando Overstreet and Cooper (1978) recuperaron en conejos estos espermatozoides que llegaban a la ampolla observaron que la mayoría estaban dañados y eran inmóviles. Por ello, propusieron que las olas de contracciones estimuladas tras la inseminación transportaban rápidamente algunos espermatozoides al sitio de fertilización, pero que estos estaban mortalmente dañados, no podían fertilizar y, en un momento posterior, los espermatozoides motiles pasarían gradualmente por el tracto femenino hasta la unión uterotubal, donde se formaría un reservorio de espermatozoides con capacidad de fertilizar.

Todo esto pone de manifiesto el importante papel que juegan las barreras del tracto genital femenino durante la fecundación y es la razón por la que se han realizado diferentes estudios *in vivo* para intentar determinar cuáles son los factores que afectan a la migración, el almacenamiento y la supervivencia espermática de los espermatozoides en las vías genitales de la hembra.

2.1. Cérvix y moco cervical

El cérvix y su moco son la primera barrera que los espermatozoides se van a encontrar en su camino hacia el oviducto. Hawk (1983) observó que si se utilizaban espermatozoides muertos en el proceso de inseminación solamente un pequeño número de ellos alcanzaba el lumen cervical y ninguno entraba en los pliegues cervicales perdiéndose la mayoría de las células por reflujo hacia el exterior.

El cérvix juega un papel muy importante en el tracto genital de la hembra previniendo el ascenso de patógenos de la vagina hacia el útero y permitiendo el ascenso del semen a los tubos de Falopio (Martyn *et al.* 2014). Presenta numerosos pliegues y criptas y el lumen está lleno de moco cervical. Este moco cervical está compuesto de agua (90-95%), iones, enzimas, proteínas bactericidas, proteínas plasmáticas y mucinas, que son proteínas glicosiladas responsables de dar la consistencia de gel del moco (Martyn *et al.* 2014). El moco cervical va a evitar que los espermatozoides que no presentan una morfología y movimiento óptimos atraviesen el cérvix (Suarez *et al.* 2006). Las propiedades físicoquímicas del moco cervical varían según la etapa del ciclo menstrual/estral (Martyn *et al.* 2014), y esto va a jugar un papel clave en el movimiento de los espermatozoides a lo largo del cérvix y en el establecimiento de la población espermática en sus pliegues y criptas. El comienzo del intervalo de fertilidad se corresponde con un aumento en los niveles de estrógenos previos a la ovulación y se produce una secreción de un moco cervical más acuoso (Flynn and Lynch 1976, Adamapoulus *et al.* 2000, Bigelow *et al.* 2004, Martyn *et al.* 2014) el cual facilita la migración de los espermatozoides hacia el útero (Bigelow *et al.* 2004). Tras la ovulación, cuando bajo la influencia del cuerpo lúteo el ovario comienza a segregar progesterona, se reduce la cantidad de moco cervical que es secretado y éste se vuelve más espeso y pegajoso bloqueando así la migración espermática (Ulcova-Gallova 2010).

Además de que las propiedades fisicoquímicas del moco sean favorables para poder pasar a través de la barrera del cérvix, los espermatozoides deben tener una movilidad vigorosa y una morfología normal para poder entrar en los pliegues cervicales y en las criptas del cérvix durante las primeras horas después de la inseminación, y desde allí poder participar en la etapa prolongada del transporte espermático (Katz *et al.* 1997).

2.2. Útero

Una vez atravesado el cérvix los espermatozoides alcanzan el útero. El transporte espermático en este tramo está ayudado por contracciones del miometrio. La realización de ultrasonografías en úteros humanos ha revelado la presencia de unas olas de contracciones en dirección craneal del musculo liso uterino que aumentan de intensidad durante la fase folicular tardía (Lyons *et al.* 1991, Kunz *et al.* 1996). Las contracciones uterinas que se observan en las mujeres durante el periodo preovulatorio están limitadas a la capa de miometrio justo debajo del endometrio, mientras que las contracciones que tienen lugar durante la menstruación se producen en todas las capas del miometrio (Lyons *et al.* 1991). Hawk (1983) realizó electromiografías en vacas y ovejas y observó que había una gran actividad contráctil durante el estro, mientras que durante la fase luteal estas contracciones eran más débiles y localizadas. Además de depender de la fase del ciclo menstrual/estral, los patrones de contracción de los cuernos uterinos presentan diferencias significativas entre ratas que no se habían apareado y las que sí (Crane and Marti, 1991). Estos autores vieron que cuando el apareamiento era con una macho vasectomizado la incidencia de contracciones uterinas fuertes descendía, lo que parece indicar que el plasma seminal puede tener un efecto estimulador sobre estas contracciones uterinas.

2.3. Unión uterotubal y oviducto

Después del útero los espermatozoides tienen que atravesar la unión uterotubal y continuar por el oviducto. En esta zona el transporte es un proceso más complejo y depende de la motilidad espermática, el batir de cilios de las células de la mucosa, las corrientes de fluido luminal y las contracciones musculares.

La unión uterotubal presenta barreras anatómicas, fisiológicas y mucosas para el paso de los espermatozoides en la mayoría de los mamíferos ya que el lumen es muy tortuoso y estrecho (Suarez 1987, Suarez 1997). En el lumen de la unión uterotubal hay muchos pliegues grandes y pequeños en la mucosa que parecen estar diseñados para atrapar los espermatozoides y evitar su ascenso por el oviducto (Suarez 2006). Cuando los espermatozoides pasan la unión uterotubal y entran en el itsmo son atrapados y conforman un reservorio celular (Overstreet *et al.* 1978, Hunter *et al.* 1983, Suarez *et al.* 1987). En este proceso de formación del reservorio también están involucradas varias proteínas de la membrana plasmática del espermatozoide que van a unirse a distintos ligandos en la mucosa y permanecer ahí atrapados; sólo las células que presenten estas proteínas superarán la barrera uterotubal (Suarez 2008). El que los espermatozoides sean atrapados y almacenados en el segmento inicial del tubo puede que sirva para prevenir una fertilización polispérmica al ir liberando solamente un pequeño número de células hacia el sitio de fertilización en cada momento (Suarez 2006).

Los espermatozoides sufren dos cambios importantes en su preparación para la fertilización: capacitación e hiperactivación. El proceso de capacitación conlleva cambios en la membrana plasmática, incluyendo movimiento de proteínas y colesterol, lo que prepara a los

espermatozoides para sufrir la reacción acrosomal y fertilizar los oocitos (De Jonge 2005). La pérdida o modificación de proteínas en la membrana plasmática de los espermatozoides podría reducir su afinidad con la mucosa del itsmo y facilitar su salida del reservorio hacia el punto de fertilidad. La hiperactivación, por otro lado, es un cambio en la batida del flagelo que normalmente conlleva un aumento de la amplitud de curvatura flagelar. Esto podría provocar la fuerza necesaria para superar la fuerza de atracción entre el espermatozoide y el epitelio en el itsmo (Ho and Suarez 2001). Puede que el epitelio del itsmo también haga que los espermatozoides activen los procesos de capacitación e hiperactivación al liberar factores que alteren los espermatozoides bajo control hormonal desde el ovario. La hiperactivación de los espermatozoides, aparte de tener un papel en la liberación de la célula del epitelio del itsmo, aumenta la habilidad de los espermatozoides de nadar a través de sustancias viscoelásticas como el mucus del lumen uterotubal y la matriz extracelular del cumulus ooforus y ayuda al espermatozoide a penetrar la zona pelúcida (Suarez 2006).

Una vez que los espermatozoides son liberados del reservorio del itsmo tienen aún que travesar una larga distancia hasta el punto de fertilización. Durante los últimos años se ha debatido bastante sobre la posible existencia de un sistema que guíe los espermatozoides hacia el oocito. Hay autores que demuestran la existencia un tres modelos de guía, uno termotáxico, uno de reotaxis y otro quimiotáxico (Cerezales *et al.* 2015). El modelo termotáxico se basa en la diferencia térmica de 2 °C observada entre el itsmo y la ampolla, siendo más alta la temperatura en la ampolla; en un estudio en conejos se observó que los espermatozoides capacitados tendían a nadar hacia zona más caliente (Bahat *et al.,* 2003). Otros estudios han encontrado indicios similares en varias especies diferentes (cerdo: Hunter *et al.,* 1986; humanos: Boryshpolets *et al.,* 2015) lo que sugiere que sea común en mamíferos. Miki and Clampham (2013) demuestraron también la existencia de un factor reotáxico que es capaz de guiar espermatozoides capacitados y no capacitados a través del tracto de la hembra tanto en ratones como en humanos, y por ello defienden que es el factor táxico más importante en mamíferos. En el tercer modelo, los agentes quimiotáxicos que sólo tienen efecto a distancias muy cortas atraerían al espermatozoide hacia el ovocito en el entorno de la ampolla. Hay varios autores que han apuntado a un gradiente de progesterona en concentraciones muy bajas como agente quimiotáxico liberado por el oocito (Armon et al. 2011, Gatica *et al*. 2013). Además se ha observado que el proceso de hiperactivación es reversible, pueden alternar entre una batida flagelar simétrica a la asimétrica propia de la hiperactivación, siendo este proceso dependiente dela presencia de agentes quimiotáxicos (Suarez 1987, Suarez 2006).

2.4. Transporte espermático ovino

En la oveja, durante el coito natural el semen es depositado en el fondo de la vagina y los espermatozoides son transportados hacia los oviductos en dos fases: una rápida poco significativa (2 horas) que afecta a un número reducido de espermatozoides, y otra lenta (24 horas o más) que asegura un número adecuado de espermatozoides en el oviducto para la fecundación. Este hecho se ha comprobado experimentalmente y se ha observado que el número total de espermatozoides en el cérvix es máximo a las 2 horas después de la inseminación y que este número se reduce significativamente 24 horas después (Hawk, 1983). Durante ese tiempo el número de espermatozoides presentes en útero y oviducto va incrementándose gradualmente, lo que indica que hay un movimiento continuo de espermatozoides desde el cérvix (Hunter & Nichol, 1993).

El cérvix de la oveja tiene una estructura anatómica muy complicada con una serie de anillos excéntricos cuya longitud y complejidad varía entre razas y con la edad de las ovejas (Kaabi *et al.* 2006). Esta elevada complejidad del cérvix es uno de los factores fundamentales implicados en las bajas tasas de fertilidad obtenidas cuando se realiza la IA utilizando semen congelado-descongelado. Mburu et al. (1997) observaron que el semen descongelado era menos capaz de penetrar en las criptas y pliegues del cérvix y de interactuar adecuadamente con el epitelio oviductal que el semen fresco, y postularon que podía deberse a la presencia de daños en la membrana plasmática de los espermatozoides producidos durante el proceso de congelación. Además si el semen congelado-descongelado es aplicado por vía intrauterina, saltando la barrera cervical y localizando el semen directamente en el útero, las tasas de fertilidad se recuperan hasta valores del 50-70%, lo que indica que el cérvix es un gran obstáculo en su progreso hacia la ampolla (Maxwell and Salamon 1993, Salamon and Maxwell 2000). Druart *et al.* (2009) han observado el paso de los espermatozoides ovinos a lo largo del tracto genital de la hembra *in vivo* utilizando un sistema de microscopía confocal. Estos autores observaron que el número de espermatozoides que alcanzan el útero 4 horas después de la inseminación era mucho menor para los espermatozoides almacenados durante 24 h en medio líquido que para la muestra fresca. También observaron que cuando el semen era depositado en la parte inferior del cuerno uterino por vía laparoscópica, la proporción de espermatozoides que alcanzaban el oviducto y la movilidad *in situ* de los espermatozoides era muy inferior para la muestra almacenada durante 24h en medio líquido que para la muestra fresca. Esta reducción de la fertilidad tras el almacenamiento líquido del semen puede deberse a que las células no son capaces de completar la migración a través del tracto genital o a que les requiera más tiempo alcanzar el oviducto cuando se aproxima el momento de ovulación en hembras con ciclos sincronizados (Druart 2012). Durante el estro natural el cérvix presenta una configuración específica que facilita el transporte espermático y el moco cervical es más acuoso lo que favorece el paso de los espermatozoides a través y, por tanto, permite obtener buenas tasas de fertilidad. Sin embargo, se ha visto que el moco cervical durante el estro en ovejas con ovulación inducida es más impermeable al paso de los espermatozoides lo que sugiere que la administración de progesterona o prostaglandinas provocan una inhibición de la función normal del cérvix (Cavaco-Goncalves *et al.* 2006).

Otra barrera importante para los espermatozoides ovinos es la unión uterotubal. Druart *et al.* (2009) vieron que la concentración de espermatozoides en el oviducto era claramente muy inferior a la presente en la unión uterotubal lo que prueba su rol como barrera. También observaron que las contracciones en la unión uterotubal eran mucho menos frecuentes que en el útero y que los espermatozoides que allí se encontraban presentaban una movilidad con trayectorias lineales con unos patrones de movilidad muy similares a los observados *in vitro*.

De los espermatozoides que alcanzan el istmo en el oviducto de la oveja sólo una pequeña fracción es capaz de adherirse al epitelio oviductal y de este modo prolongar su vida útil retrasando el proceso de capacitación hasta que señales específicas preovulatorias induzcan la liberación espermática. Se han propuesto varios mecanismos para explicar la acumulación de espermatozoides en el istmo: la inhibición de la motilidad espermática, la obstrucción de las vías por el moco, la adhesión de los espermatozoides a la mucosa y la barrera que representan los complejos pliegues epiteliales (Mburu *et al*, 1997). Este mecanismo de adhesión además de constituir la base para la formación de una reserva activa de espermatozoides representa un proceso de selección muy especializado, dado que solamente actúa sobre células de alta calidad. Las características particulares que debe

presentar el espermatozoide para fijarse al epitelio son: acrosoma intacto, estatus de no capacitación, bajo contenido en Ca^{2+} intracelular y una reducida fosforilación de proteínas quinasa presentes en la membrana plasmática (Gualtieri *et al*, 2005).

El proceso de refrigeración y el de congelación-descongelación del semen incrementa la proporción de espermatozoides capacitados antes de la inseminación cervical, lo cual reduce la viabilidad del espermatozoide en el tracto reproductor de la oveja (Maxwell y Watson, 1996). Comparado con el semen fresco, solamente una proporción relativamente pequeña de los espermatozoides refrigerados y depositados en el orificio cervical externo durante la inseminación de la oveja, es capaz de ascender hasta el lugar de la fecundación. Esto es debido a que los espermatozoides se ven afectados funcionalmente durante la refrigeración y congelación-descongelación, y que estas lesiones les impiden avanzar por las vías genitales o el avance es tan lento que reduce su supervivencia en el tracto genital femenino.

3. Evaluación de la calidad seminal

En el ganado ovino se considera que un eyaculado tiene calidad suficiente como para ser utilizado para IA si cumple las siguientes caracteristicas: color blanquecino normal, volumen mayor que 0,5 mL, motilidad masal mayor que 4 (se determina utilizando un microscopio a 40X, con una platina calefactable a 37 °C, y se asigna un valor de 0 si la muestra no se mueve, hasta 5 si se mueve vigorosamente) y una concentración espermática mayor de 3000 x 10^6 espermatozoides/mL. En el caso del semen fresco estas pruebas son suficientes para estimar la obtención de un buen resultado de fertilidad en campo. En el caso del semen humano es más complejo y existe un manual que describe todas las técnicas que deben realizarse para analizar la muestra seminal así como los valores que definen para cada prueba la calidad seminal (WHO, 2010).

Tras refrigerar o descongelar las muestras de ovino los parámetros que se estudian rutinariamente son: la movilidad (se suelen emplear sistemas de análisis computarizados que permiten un estudio más objetivo de los distintos parámetros de movilidad de la muestra), la viabilidad y la concentración celular. Sin embargo, estos parámetros no son suficientes para poder predecir los resultados de fertilidad que se obtengan tras la IA por vía cervical. En el caso del ganado ovino, las tasas de fertilidad que se obtienen inseminado con semen congelado-descongelado son muy bajas aunque se utilicen muestras que hayan obtenido muy buenos valores de movilidad y viabilidad (Alvarez *et al*. 1998).

En la presente tesis doctoral proponemos la optimización para semen ovino de una de las técnicas de valoración seminal que forman parte de la batería de pruebas aprobada por la OMS para humanos (WHO, 2010) para intentar aproximar los datos de calidad *in vitro* con las tasas de fertilidad *in vivo*.

3.1. Test de penetración en moco cervical

Debido a que el moco cervical está considerado como la principal barrera de selección de espermatozoides en muchas especies animales, se han desarrollado varios métodos *in vitro* para estudiar la capacidad de los espermatozoides para atravesarlo y predecir la fertilidad (Galli *et al.* 1991, Berberoglugil *et al.* 1993, Sharara *et al*. 1995, Anilkumar *et al.* 2001, Hirano *et al.* 2001, Ola *et al.* 2003, Aitken *et al.* 2006, Gillan *et al.* 2008, Robayo *et al.* 2008). Estos métodos de análisis denominados tests de penetración en moco cervical, han sido aplicadas en

varias especies animales y han sido aceptados también por la Organización Mundial de la Salud como método de análisis de semen humano (WHO, 2010).

El moco cervical es un fluido complejo cuyas características fisicoquímicas varían a lo largo del ciclo estral (Katz *et al.* 1997). Por esta razón y debido a que no se pueden obtener elevados volúmenes de moco cervical natural de cada animal y que las características fisicoquímicas del mismo varían mucho entre hembras ha sido necesario desarrollar sustitutos artificiales del moco, como por ejemplo geles de poliacrilamida (Lorton *et al.* 1981, Eggert-Kruse *et al.* 1996, Anilkumar *et al.* 2001, Tas *et al.* 2007a, Tas *et al.* 2007b), metilcelulosa (Ivic *et al.* 2002) o ácido hialurónico (Aitken *et al.* 1992), y poder definir un medio que facilite la estandarización de los test de penetración.

En un principio los test de penetración en moco cervical se basaban en la determinación de la distancia recorrida por el espermatozoide de vanguardia (el espermatozoide que recorría más espacio en el tiempo dado) en un capilar (Keel and Webster 1988, Mole and Fitzgerald 1990, Murase and Braun 1990, Galli *et al.* 1991, Verberckmoes *et al.* 2002, Cox *et al.* 2006, Robayo *et al.* 2008). Luego se desarrollaron otras estrategias que se basaron en el contaje del número total de espermatozoides que alcanzan una distancia determinada en un tiempo concreto utilizando capilares planos o pajuelas transparentes (Suttiyotin *et al.* 1992, Aitken *et al.* 1992, Clarke *et al.* 1998, Tang *et al.* 1999, Hamano *et al.* 2001, Ivic *et al.* 2002, Tas *et al.* 2007a, 2007b, Gillan *et al.* 2008, Al Naib *et al.* 2011). Tas *et al.* (2007a, 2007b) desarrollaron otro sistema que facilita el contaje de espermatozoides: utilizaban pajuelas de plástico transparentes y descargan el contenido de un segmento determinado de la pajuela en un portaobjetos para facilitar el contaje bajo el microscopio. En estos ensayos se observó una fuerte correlación positiva entre el número de espermatozoides de toro que alcanzaba una distancia predeterminada y la tasa de no retorno a 60-90 días.

Hay autores que han encontrado correlaciones entre los resultados obtenidos con algunos tipos de test de penetración y algunos parámetros de calidad seminal de la muestra original (Keel and Webster 1988, Galli *et al.* 1991, Aitken *et al.* 1992, Eggert-Kruse *et al.* 1996, Anilkumar *et al.* 2001, Ivic *et al.* 2002, Cox *et al.* 2006, Tas *et al.* 2007a, Tas *et al.* 2007b, Gillan *et al.* 2008, Robayo *et al.* 2008). También se ha observado que los resultados del test de penetración están correlacionados con la fertilidad en varias especies animales (Murase et al. 1990, Suttiyotin *et al.* 1992, Hamano *et al.* 2001, Aitken et al. 2006, Tas *et al.* 2007a, Tas *et al.* 2007b, Bacinoglu *et al.* 2008, Gillan *et al.* 2008, Al Naib *et al.* 2011), aunque otros autores han sugerido que esta relación no existe (Galli *et al.* 1991, Murase *et al.* 2001, Verberckmoes *et al.* 2002). Ola *et al.* (2003) hicieron una revisión sobre la precisión de los diferentes test de penetración en moco cervical como método para predecir la tasa de fertilidad. Demostraron que los test basados en la determinación de la distancia recorrida por el espermatozoide de vanguardia son menos precisos que los que se basan en el contaje del número de espermatozoides que recorre una distancia determinada en la barrera de moco cervical.

En la oveja se han realizado pocos estudios que relacionen los resultados del test de penetración en moco cervical y la calidad seminal de los eyaculados. Suttiyotin *et al.* (1992) observaron que la distancia recorrida por el espermatozoide de vanguardia en una solución Tris-Glucosa estaba relacionada con la tasa de no-retorno a 48 días y la de concepción a 60 días. Robayo *et al.* (2008) estudiaron la relación ente el resultado del test de penetración en moco cervical en rumiantes, basándose en la medida de la distancia recorrida por el espermatozoide de vanguardia, y los patrones de movilidad observados mediante un sistema computarizado de análisis de semen (CASA). Observaron que los únicos parámetros cinéticos

que se correlacionaban positivamente con el test de penetración eran la velocidad en línea recta y la velocidad media. O'Hara *et al.* (2010) utilizaron un sistema de test de penetración mejorado para determinar la habilidad de los espermatozoides ovinos de atravesar la barrera de moco cervical. Utilizaron capilares planos para realizar el test sobre muestras de semen que habían sido previamente teñidas con Hoechst 33342 facilitando de esta manera su visibilidad durante el contaje. Determinaron que la habilidad del semen ovino fresco para atravesar el moco cervical artificial está influida por los diluyentes empleados y el tiempo de almacenamiento de la muestra previo al desarrollo del test.

Hasta la fecha los test de penetración sólo se han empleado para determinar la distancia recorrida por el espermatozoide de vanguardia o para realizar el contaje del número de espermatozoides que atraviesan una distancia concreta en un tiempo determinado y ver si ese valor guarda relación con alguno de los parámetros de calidad seminal o con la fertilidad. Sin embargo podría ser interesante intentar recuperar estos espermatozoides que son capaces de atravesar la barrera de moco cervical ya que su estudio podría revelar o dar más información sobre cuáles son las características que estas células deben presentar para poder progresar a través del cérvix. Hasta la fecha se sabe que el moco cervical filtra los espermatozoides que presentan una motilidad adecuada (Suarez *et al.* 2006) pero dado el bajísimo número de espermatozoides que consigue atravesar esta barrera (Hawk 1983) probablemente haya más factores involucrados.

3.2. Análisis de la morfometría de la cabeza de los espermatozoides

El semen está constituido por una población de espermatozoides compleja y determinados estudios realizados sobre la morfología de la cabeza de estas células han revelado que dentro de esta población se pueden definir distintos subpoblaciones de espermatozoides (Thurston *et al.* 2001). Estos estudios de la morfometría de la cabeza de los espermatozoides se basan en la medida de diferentes parámetros básicos de la cabeza área (A), longitud (L), perímetro (P) y anchura (W) de al menos 200 células en la muestra seminal a evaluar. A partir de estos factores básicos se pueden calcular otros factores derivados como son la elongación ((L-W)/(L+W)), elipticidad (L/W), regularidad (π x LW/4A) y rugosidad (4π x A/P^2). Según los valores obtenidos para los distintos factores se pueden determinar distintas subpoblaciones de espermatozoides y calcular la proporción de cada una de ellas dentro de la muestra seminal. La proporción de espermatozoides de cada subpoblación presentes en la muestra de semen varía significativamente entre machos, y la abundancia de algunos de estos subtipos se ha correlacionado con la calidad de la muestra seminal.

La morfometría de la cabeza de los espermatozoides se ha estudiado como biomarcador de fertilidad en diversas especies animales incluyendo humanos (Kruger *et al.* 1993), cerdos (Hirai *et al.* 2001), caballos (Casey *et al.* 1997), cabras (Hidalgo *et al.* 2007), toros (Salier *et al.* 1996) y ovejas (de Paz *et al.* 2011). Además, algunos autores la han relacionado con la movilidad espermática y la migración a través del tracto reproductor femenino. Gomendio *et al.* (2007) observaron que la forma de la cabeza del espermatozoide y las dimensiones de los diferentes componentes del flagelo son los principales factores que determinan la velocidad a la que nadan los espermatozoides. Gillies *et al.* (2009) sugirieron que la dimensión de la cabeza es un factor importante que influye en la velocidad progresiva y la amplitud del movimiento lateral de la cabeza de los espermatozoides.

Hasta la fecha no hay ningún estudio de morfometría de la cabeza de los espermatozoides ovinos que se haya realizado sobre los espermatozoides que sean capaces de

atravesar la barrera de moco sintético tras un test de penetración *in vitro*. En la presente tesis doctoral proponemos un nuevo dispositivo de test de penetración en moco cervical para ovino que permite recuperar los espermatozoides que lo atraviesan en un diluyente libre moco. Esto nos va a permitir hacer el estudio morfológico y poder definir las características morfológicas de la cabeza de la subpoblación de espermatozoides dentro de la muestra seminal que es capaz de superar esta barrera, y que por tanto, tendría la capacidad de atravesar el cérvix de la oveja. También intentaremos determinar si hay alguna relación entre proporciones de distintas subpoblaciones definidas por sus características morfologías de la cabeza y las tasas de fertilidad obtenidas en campo.

Bibliografía

Adamopoulos, D.A., Kapolla, N., Abrahamian, A., Dessypris, A., Nicopoulou, S., Giannacodemos, G. (2000). Sex steroids in cervical mucus of spontaneous or induced ovulatory cycles. Steroids 65,1:1-7.

Aitken, R.J., Bowie, H., Buckingham, D., Harkiss, D., Richardson, D.W., West, K.M.. (1992). Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. J Androl 13:44-54.

Aitken, R. J. (2006). Sperm function tests and fertility. Int. J. Androl. 29, 69–75. doi:10.1111/ J.1365-2605.2005.00630.X

Álvarez, M., Anel, L., Anel, E., Boixo, J.C., Paz, P., Chamorro, C., Peña, F.J., Domínguez, J.C., Celorrio, I..(1998). Relation between the depth of cervical insemination and prolificacy in sheep. In Proc 50th ICAR: Gametes. Development and Function. Milan, Italy 504,

Al Naib, A., Hanrahan, J. P., Lonergan, P., and Fair, S. (2011). In vitro assessment of sperm from bulls of high and low field fertility. Theriogenology 76, 161–167. doi:10.1016/ J.THERIOGENOLOGY.2010.10.038

Anel, L., de Paz, P., Alvarez, M., Chamorro, C.A., Boixo, J.C., Manso, A., Gonzalez, M., Kaabi, M., Anel, E.. (2003). Field and in vitro assay of three methods for freezing ram semen. Theriogenology 60:1293-1308

Anel, L., Kaabi, M., Abroug, B., Alvarez, M., Anel, E., Boixo, J.C., de la Fuente, L.F. y Paz, P.: (2005) Factors influencing the fertility of vaginal and laparoscopic artificial insemination in churra ewes: a field assay. Theriogenology 63:1235-1247

Anel, L., Alvarez, M., Martinez-Pastor, F., Garcia-Macias, V., Anel, E. and Paz, P.. (2006). Improvement Strategies in Ovine Artificial Insemination. Reprod Dom Anim 41(2), 30–42.

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

Anilkumar, R., Devanathan, T.G., Pattabiraman, S.R., Edwin, M.J.. (2001). Correlation between the spermatozoal characteristics and sperm penetration distance in polyacrylamide gel and bovine cervical mucus. Theriogenology 55:685-91.

Ayad, V.J., Leung, S.T., Parkinson, T.J.,Wathes, D.C. (2004). Coincident increases in oxytocin receptor expression and EMG responsiveness to oxytocin in the ovine cervix at oestrus. Animal Reproduction Science 80:237–250

Bahat, A., Tur-Kaspa, I., Gakamsky, A., Giojalas, L.C., Breitbart, H., Eisenbach, M.(2003). Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nat Med. Feb;9(2):149-50.

Bacinoglu, S., Taş, M., Cirit, U., Ozdaş, O.B., Ak, K. (2008). The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. Anim Reprod Sci. Feb 1;104(1):38-46. Epub 2007 Jan 26.

Berberoglugil, P., Englert, Y., Van den Bergh, M., Rodesch, C., Bertrand, E., Biramane, J.. (1993). Abnormal sperm-mucus penetration test predicts low in vitro fertilization ability of apparently normal semen. Fertil Steril 59:1228-32.

Bigelow, J.L., Dunson, D.B., Stanford, J.B., Ecochard, R., Gnoth, C., Colombo, B.. (2004). Mucus observations in the fertile window: a better predictor of conception than timing of intercourse. Hum Reprod. 19,4:889-92.

Boryshpolets, S., Pérez-Cerezales, S., Eisenbach, M.. (2015). Behavioral mechanism of human sperm in thermotaxis: a role for hyperactivation. Hum Reprod. 30,4:884-92. doi: 10.1093/humrep/dev002. Epub 2015 Jan 21.

Casey, P.J., Gravance, C.G., Davis, R.O., Chabot, D.D., Liu, I.K. (1997). Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology. Jan 15;47(2):575-82.

Cavaco-Goncalves, S., Marques, C.C., Horta, A.E.M., Figueroa, J.P.. (2006). Increased cervical electrical activity during oestrus in progestagen treated ewes: Possible role in sperm transport. Animal Reproduction Science 93:360–365

Cerezales, S.P., Boryshpolets, S., Eisenbach, M.. (2015). Behavioral mechanisms of mammalian sperm guidance. Asian J Androl. doi: 10.4103/1008-682X.154308.

Clarke, G. N., Garrett, C., and Baker, H. W. (1998). Quantitative sperm mucus penetration: modified formulae for calculating penetration efficiency. Hum. Reprod. 13, 1255–1259. Doi: 10.1093/HUMREP/13.5. 1255

Cox, J. F., Alfaro, V., Montenegro, V., and Rodriguez-Martinez, H. (2006). Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus. Theriogenology 66, 860–867. doi:10.1016/J.THERIOGENOLOGY.2006.01.062

Crane, L.H., Martin, L.. (1991). Postcopulatory myometrial activity in the rat as seen by video-laparoscopy.Reprod Fertil Dev. 3,6:685-98.

De Jonge, C.. (2005). Biological basis for human capacitation. Hum Reprod Update. 11,3:205-14. Epub 2005 Apr 7.

de Paz, P., Mata-Campuzano, M., Tizado, E. J., Alvarez, M., Alvarez-Rodriguez, M.,Herraez, P., andAnel, L. (2011). The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. Theriogenology 76, 1313–1325. doi:10.1016/J.THERIOGENOLOGY.2011.05.038

Druart, X., Cognié, J., Baril, G., Clément, F., Dacheux, J.L., Gatti, J.L..(2009). In vivo imaging of in situ motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. Reproduction. 138,1:45-53. doi: 10.1530/REP-09-0108.

Druart, X..(2012). Sperm interaction with the female reproductive tract. Reprod Domest Anim. 47,4:348-52. doi: 10.1111/j.1439-0531.2012.02097.x.

Eggert-Kruse, W., Schwarz, H., Rohr, G., Demirakca, T., Tilgen, W., and Runnebaum, B. (1996). Sperm morphology assessment using strict criteria and male fertility under in vivo conditions of conception. Hum. Reprod. 11, 139–146. doi:10.1093/OXFORDJOURNALS .HUMREP. A019007

Eppleston, J., Salamon, S., Moore, N.W., Evans, G.. (1994). The depth of cervical insemination and site of intrauterine insemination and their relationship to the fertility of frozen-thawed ram semen. Anim. Reprod. Sci. 36:211-225

Flynn, A.M., Lynch, S.S.. (1976). Cervical mucus identification of the fertile phase of the menstrual cycle. Br J Obstet Gynaecol 83:656-659

Galli, A., Basetti, M., Balduzzi, D., Martignoni,M., Bornaghi, V., Maffii,M.. (1991). Frozen bovine semen quality and bovine cervical mucus penetration test. Theriogenology 35:837-44.

Gatica, L.V., Guidobaldi, H.A., Montesinos, M.M., Teves, M.E., Moreno, A.I., Uñates, D.R., Molina, R.I., Giojalas, L.C.. (2013). Picomolar gradients of progesterone select functional human sperm even in subfertile samples. Mol Hum Reprod.19,9:559-69. doi: 10.1093/ molehr/gat037.

Gillan, L., Kroetsch, T., Maxwell, W.M. C., Evans, G.. (2008). Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Animal Reproduction Science 103:201–214

Gillies, E., Cannon, R., Green, R., and Pacey, A. (2009). Hydrodynamic propulsion of human sperm. J. Fluid Mech. 625, 445–474. doi:10.1017/ S0022112008005685

Gomendio, M., Malo, A. F., Garde, J., and Roldan, E. R. (2007). Sperm traits and male fertility in natural populations. Reproduction 134, 19–29. doi:10.1530/REP-07-0143

Gualtieri, R., Boni, R., Tosti, E., Zagami, M., Talevi, R.. (2005). Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium in vitro. Reproduction 129:51–60

Hamano, K., Tanaka, S., Kawana, Y., Tsujii, H., Sasada, H., Sato, E., Takahashi, T., Miyawaki, K., and Arima, H. (2001). Evaluation of bull fertility by migration of frozen–thawed and washed sperm in medium containing cervical mucus. J. Reprod. Dev. 47, 393–398. doi:10.1262/ JRD.47.393

Hawk, H.W.. (1983). Sperm Survival and Transport in the Female Reproductive Tract. J Dairy Sci 66:2645-2660

Hidalgo, M., Rodriguez, I., and Dorado, J. M. (2007). The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Anim. Reprod. Sci. 100, 61–72. doi:10.1016/J.ANIREPROSCI.2006.07.003

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumuller, T. R., and Braun, J. (2001). Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. J. Androl. 22, 104–110.

Hirano, Y., Shibahara, H., Obara, H., Suzuki, T., Takamizawa, S., Yamaguchi, C.. (2001). Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. J Assist Reprod Genet 18:213-8.

Ho, H.C., Suarez, S.S..(2001). Hyperactivation of mammalian spermatozoa: function and regulation.Reproduction. 122,4:519-26.

Hossain, A.M., Barik, S., Rizk, B., Kulkarni, P.M., and Thorneycroft, I.H.. (1999). Analysis of in vitro migration patterns of human spermatozoa by a petri. dish-based horizontal column. Biol Reprod 61:406–410

Hunter, R.H., Nichol, R..(1983). Transport of spermatozoa in the sheep oviduct: preovulatory sequestering of cells in the caudal isthmus. J Exp Zool. 228,1:121-8.

Hunter, R.H., Nichol, R.. (1986). A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. J Reprod Fertil 77: 599-606.

Hunter, R.H., Nichol, R.. (1993). Rate of establishment of a fertilising population of spermatozoa in the sheep cervix after a single mating at the onset of oestrus. J Exp Zool 266(2):168-71

Ivic, A., Onyeaka, H., Girling, A., Brewis, I.A., Ola, B., Hammadieh, N..(2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. Hum Reprod 17:143 9

Kaabi, M., Alvarez, M., Anel, E., Chamorro, C.A., Boixo, J.C., de Paz, P., Anel, L.. (2006). Influence of breed and age on morphometry and depth of inseminating catheter penetration in the ewe cervix: a postmorten study. Theriogenology 66:1876-1883

Katz, D.F., Slade, D.A., Nakajima, S.T. (1997). Analysis of pre-ovulatory changes in cervical mucus hydration and sperm penetrability. Adv Contracept 13:143-51.

Katz, F., Bloom, T.D., Bondurant, R.H.. (1981). Movement of bull spermatozoa in cervical mucus. Biol Reprod 25,931-937

Keel, B. A., and Webster, B. W. (1988).Correlation of human sperm motility characteristics with an in vitro cervical mucus penetration test. Fertil. Steril. 49, 138–143.

Kruger, T. F., DuToit, T. C., Franken, D. R., Acosta, A. A., Oehninger, S. C.Menkveld, R., and Lombard, C. J. (1993). A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. Fertil. Steril. 59, 202–209.

Kunz, G., Beil, D., Deininger, H., Wildt, L., Leyendecker, G.. (1996).The dynamics of rapid sperm transport through the female genital tract: evidence from vaginal sonography of uterine peristalsis and hysterosalpingoscintigraphy.Hum Reprod. 11,3:627-32.

Lorton, S.P., Kummerfeld, H.L., Foote, R.H. (1981). Polyacrylamide as a substitute for cervical mucus in sperm migration tests. Fertil Steril 35:222-5.

Lyons, E.A., Taylor, P.J., Zheng, X.H., Ballard, G., Levi, C.S., Kredentser, J.V..(1991). Characterization of subendometrial myometrial contractions throughout the menstrual cycle in normal fertile women. Fertil Steril. 55,4:771-4.

Martyn, F., McAuliffe, F.M., Wingfield, M. (2014). The role of the cervix in fertility: is it time for a reappraisal?. Human Reproduction, 29:10,2092-2098.

Maxwell, W.M.C. & Watson, P.F.. (1996). Recent progress in the preservation of ram semen. Anim. Reprod. Sci. 42:55–65

Maxwell,W.M., Salamon, S..(1993). Liquid storage of ram semen: a review. Reprod Fertil Dev. 5,6:613-38.

Mburu, J.N., Rodriguez-Martinez, H., Einarsson, S.. (1997). Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. Animal Reproduction Science 47:137-148

Miki, K., Clapham, D.E..(2013). Rheotaxis guides mammalian sperm. Curr Biol 23: 443-52.

Mole, J. R., and Fitzgerald, J. A. (1990). Comparison of ram sperm interaction with bovine cervical mucus. Theriogenology 33, 1031–1043. doi:10.1016/0093-691X(90)90065-2

Murase, T., and Braun, J. W. (1990). Impact of methodological factors on sperm penetration into cervical mucus in cattle. Theriogenology 34, 73–80. doi:10.1016/0093-691X(90)90578-H

Murase, T., Mukohjima, K., Sakaguchi, S., Ohtani, T., Tsubota, T., and Kita, I. (2001). Characterization of frozen–thawed Japanese black bull spermatozoa by standard semen analysis, mucus-penetration test and the ability to undergo the acrosome reaction in response to calcium and the calcium ionophore A23187. J. Reprod. Dev. 47, 237–243. doi:10.1262/ JRD. 47.237

O'Hara, L., Hanrahan, J.P., Richardson, L., Donovan, A., Fair, S., Evans, A.C.. (2010). Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology 73:541-9.

Ola, B., Afnan, M., Papaioannou, S., Sharif, K., Björndahl, L., Coomarasamy, A..(2003). Accuracy of sperm-cervical mucus penetration tests in evaluating sperm motility in semen: a systematic quantitative review. Hum Reprod 18:1037-46.

Olds-Clarke, P..(1986). Motility characteristics of sperm from the uterus and oviducts of female mice after mating to congenic males differing in sperm transport and fertility. Biol Reprod 34:453-467

Overstreet , J.W., Cooper, G.W..(1978). Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. Biol Reprod. 19,1:101-14.

Robayo, I., Montenegro, V., Valdes, C., Cox, J.F.. (2008). CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. Reprod Domest Anim 43:393-9.

Sailer, B. L., Jost, L. K., and Evenson, D. P. (1996). Bull sperm head morphometry related to abnormal chromatin structure and fertility. Cytometry 24, 167–173. doi:10.1002/(SICI)1097-0320(19960601) 24:2,167::AID-CYTO9.3.0.CO;2-G

Salamon, S., Maxwell, W.M.. (2000). Storage of ram semen. Anim Reprod Sci. 62,1-3:77-111.

Settlage, D.S., Motoshima, M., Tredway, D.R.. (1973). Sperm transport from the external cervical os to the fallopian tubes in women: a time and quantitation study. Fertil Steril. 24,9:655-61.

Sharara, F.I., Illions, E.H., Coddington, C.C., Scott, R.T. Jr. (1995). Evaluation of the Tru-Trax cervical mucus penetration test in predicting fertilization and pregnancy rates in in-vitro fertilization. Hum Reprod 10:1481-5.

Suarez, S.S.. (1987).Sperm transport and motility in the mouse oviduct: observations in situ. Biol Reprod. 36,1:203-10.

Suarez, S.S., Brockman, K., Lefebvre, R.. (1997). Distribution of mucus and sperm in bovine oviducts after artificial insemination: the physical environment of the oviductal sperm reservoir. Biol Reprod 56, 447-453

Suarez, S.S., Pacey, A.A.. (2006). Sperm transport in the female reproductive tract. Hum Reprod Update 12:23-37.

Suarez, S.S.. (2008). Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol. 52,5-6:455-62. doi: 10.1387/ijdb.072527ss.

Suttiyotin, P., Thwaites, C.J., Baillie, N.D..(1992). Relationships between the results of a modified sperm penetration test and a swim-up technique and the fertility of ram semen. Theriogenology 37:851-7.

Tas, M., Bacinoglu, S., Cirit, U., Ozdas, O.B., Ak, K.. (2007a).Relationship between bovine fertility and the number of spermatozoa penetrating the cervical mucus within straws. Animal Reproduction Science 101:18–27

Tas, M., Bacinoglu, S., Cirit, U., Ozgumus, S., Kasgoz, H., Pabuccuoglu, S.. (2007b). Estimation of the potential fertility based upon non return rates of bulls: using polyacrylamide gel instead of cervical mucus in the sperm penetration test. Theriogenology 68:981-7.

Thurston, L. M., Watson, P. F., Mileham, A. J., and Holt, W. V. (2001). Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J. Androl. 22, 382–394. doi:10.1002/J.1939-4640.2001.TB02194.X

Ulcova-Gallova, Z..(2010). Immunological and physicochemical properties of cervical ovulatory mucus. J Reprod Immunol. 86,2:115-21. doi: 10.1016/j.jri.2010.07.002.

Verberckmoes, S., Van Soom, A., De Pauw, I., Dewulf, J., and de Kruif, A. (2002). Migration of bovine spermatozoa in a synthetic medium and its relation to in vivo bull fertility. Theriogenology 58, 1027–1037. doi:10.1016/S0093-691X(02)00931-7

Winet, H., Bernstein, G. S., and Head, J. (1984). Observations on the response of human spermatozoa to gravity, boundaries and fluid shear. J. Reprod. Fertil. 70, 511–523. doi:10.1530 /JRF.0.0700511

World Health Organization (WHO). (2010). 'WHO laboratory manual for the examination and processing of human semen.' (World Health Organization: Geneva.)

Wulster-Radcliffe, M.C., Wang, S., Lewis, G.S.. (2004). Transcervical artificial insemination in sheep: effects of a new transcervical artificial insemination instrument and traversing the cervix on pregnancy and lambing rates. Theriogenology 62:990–1002

OBJETIVOS

Los sistemas habituales de evaluación de la calidad seminal para el ganado ovino no presentan una buena correlación con los resultados de fertilidad obtenidos en campo. Aunque la calidad de la muestra utilizada para inseminación artificial por vía cervical tenga unos buenos resultados de calidad post descongelación, los resultados de fertilidad son muy bajos.

En la batería de pruebas de contrastación seminal para humano se incluye el test de penetración en moco cervical *in vitro* (WHO, 2010). Dado que el cérvix ovino es una de las principales barreras que los espermatozides se encuentran tras la inseminación artificial por vía cervical, en este trabajo evaluaremos si el desarrollo de un dispositivo de test de penetración específico para ganado ovino nos ayuda a acercar los resultados de análisis de calidad *in vitro* a los obtenidos en campo. Para ello vamos a desarrollar un sistema *in vitro* que permita simular el cérvix ovino con el objetivo de usar este dispositivo para el estudio de las características (morfología, movilidad, integridad de DNA…) que deben presentar los espermatozides para ser capaces de atravesar esta barrera del tracto reproductivo de la oveja.

En resumen, los objetivos principales de la presente tesis doctoral son:

1.- Obtener un moco cervical sintético que tenga las mismas características reológicas que le moco cervical ovino natural.

2.- Definir las condiciones óptimas de un test de penetración *in vitro* en ovino que simule el tránsito cervical ovino y desarrollar un sistema de contaje automatizado para determinar de una manera más objetiva el número de espermatozoides capaces de superar el test de penetración.

3.- Rediseñar el dispositivo de penetración *in vitro* para poder recoger los espermatozoides que atraviesan la barrera de moco cervical en un medio libre de este moco, con el objetivo de estudiar así diferentes características biológicas de estas células.

4.- Estudiar la morfometría de la cabeza de los espermatozoides ovinos que son capaces de atravesar el moco cervical sintético en los test de penetración *in vitro* y determinar las características morfológicas que deben presentar los espermatozoides para ser capaces de atravesar la barrera de moco cervical.

5.- Relacionar los resultados obtenidos para distintos parámetros de calidad seminal de los espermatozoides con los resultados obtenidos en el test de penetración *in vitro* y la fertilidad observada en campo.

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Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test

C. Martínez-Rodríguez[a,b], M. Alvarez[a,c], L. Ordás[a,c], C.A. Chamorro[a,d], F. Martinez-Pastor[a,b], L. Anel[a,c], P. de Paz[a,b,*]

[a] *ITRA-ULE, INDEGSAL, University of León, León, Spain*
[b] *Molecular Biology, University of León, León, Spain*
[c] *Animal Reproduction and Obstetrics, University of León, León, Spain*
[d] *Veterinary Anatomy, University of León, León, Spain*

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Abstract

Fertility is a very complex biological function that depends on several properties of the spermatozoa, including sperm motility. Two objectives are analyzed in this study: (1) Replace the cervical mucus by a synthetic medium in a sperm penetration test, and (2) evaluating the results of this test objectively analyzing the sperm number that migrates. In experiment 1, we have tested eight concentrations of acrylamide (1%–2%). Rheological properties of media were analyzed. The plastic straws, loaded with acrylamide, were placed vertically on the semen sample tube for 15 min at 39 °C. After, the acrylamides were placed, by segments of 5 mm, into wells of a 24-well plate, dyed with Hoechst 33342 and the number of spermatozoa were calculated by automated microscopy analysis. The 1.55% and 1.6% acrylamide gel showed a number of spermatozoa emigrating closer to that seen with natural mucus. In experiment 2, we applied the sperm penetration in acrylamide 1.6% and 1.55% using fresh semen and cooled semen at 15 °C and 5 °C. The spermatozoa counts were performed for each segment of 10 mm. Semen chilled at 15 °C presented intermediate values of sperm counts in comparison with fresh semen (higher) and 5 °C chilled semen. The sperm counts do not differ between acrylamides but the rheological properties of acrylamide 1.6% were more similar to those of the natural cervical mucus. In experiment 3, we have observed significant correlations between the number of spermatozoa and several sperm quality parameters (positive: progressive motility and velocity according to the straight path; negative: damaged acrosomes and apoptotic cells) in 1.6% acrylamide media. We conclude that the size of the cell subpopulation, objectively calculated, that migrate beyond 20 mm in 0.5-mL straws filled with acrylamide is a useful parameter in ram sperm quality assessment and further studies are needed to evaluate its relationship with field fertility.

Keywords: Ram; Sperm quality; Penetration test; Motility; Mucus

1. Introduction

The passage of sperm through the female reproductive tract is regulated to maximize the chance of fertilization and ensure that sperm with normal morphology and vigorous motility will be the ones to succeed [1]. Cervical mucus filters out sperm with poor morphology and motility and as such only a minority of ejaculated sperm actually enter the cervix [1]. Thus, mucus is considered a means of sperm selection in many species. Taking into account the effect of cervical mucus on sperm transport, the evaluation of the ability of spermatozoa to progress through natural mucus (cervical

* Corresponding author. Tel.: +0034987291320; fax: +0034987291322.
E-mail address: ppazc@unileon.es (P. de Paz).

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mucus penetration test [CMPT]) or mucus substitutes has been proposed as an analysis of sperm quality [2–9]. This penetration test has been applied in several animal species and is accepted by the World Health Organization [10] as a means of analyzing human semen.

Generally, the test is based on the visual assessment of the linear distance covered by the foremost sperm cell (vanguard spermatozoa) in the capillary tube. Another method using the number of spermatozoa accumulated in different segments of the capillary tube as a parameter of analysis. Visual sperm counts at certain distances (10, 20 mm, etc.) from the base of the tube in flat capillary tubes has been used for this kind of assessment [11,12]. Tas et al. [13] have developed a new CMPT technique in which transparent plastic straws are used instead of capillary tubes and the total number of spermatozoa penetrating to predetermined distances in cervical mucus are measured on slides. Ola et al. [7] reviewed the accuracy of in vitro sperm penetration into cervical mucus or substitutes in evaluating sperm motility in human semen, and they showed that vanguard distance as a diagnostic criterion has a low accuracy while sperm concentration is more accurate.

A number of diagnostic studies into the usefulness of the CMPT technique have been developed. Fertilizing capacity of spermatozoa has been shown to be strongly related to the parameters observed in the cervical mucus penetration test (human [14]; bull [13,15]). In other studies, the correlation between sperm migration capacity and fertility was not observed [2–4,16,17]. However, it is generally accepted that penetration of spermatozoa into cervical mucus in vitro provides important information predictive of sperm function [11].

The major problem with cervical mucus as a component of any test system is the difficulty encountered in standardizing the quality of this material. It is difficult to obtain large volumes of natural cervical mucus and the variation among lots of natural mucus is large, even between batches from the same female [18]. Thus, it is desirable to formulate a synthetic medium free of these problems, simple to prepare and with easily reproducible rheological properties. Acrylamide, methylcellulose, and hyaluronic acid have previously been used as a natural cervical mucus substitute for in vitro sperm penetration tests (human [11,12,19]; bull [20]; ram [21]).

In ram, few studies have been performed to analyze the relationship between the penetration test and sperm quality. A modified sperm penetration test was used by Suttiyotin et al. [22], noting that sperm penetration distance in Tris-glucose solution was correlated with a 48-day nonreturn rate and a 60-day conception rate. Robayo et al. [9] studied the relationship between sperm migration in ruminant cervical mucus (distance traveled by the vanguard spermatozoa) and motility patterns observed by computer assisted semen analysis (CASA). Continuous line velocity and average path velocity were the only kinematic parameters that presented significant positive correlations with the migration in sheep cervical mucus. O'Hara et al. [21] assessed the penetrating ability of fresh ram semen using flat capillary tubes and aiding visibility to cells with Hoechst 33342. These authors showed that the penetrating ability of fresh ram semen into artificial mucus was influenced by diluents and storage duration.

The aim of this study was to automate the quantitative analysis of the ram sperm population that migrates in a column of ovine cervical mucus or substitutes (acrylamide) into a plastic straw. We propose to evaluate the straw content by segments, placing each segment onto a slide or a plate, to stain spermatozoa with cell permeable nucleic acid stains and to analyze these samples automatically by a microscope to count the spermatozoa in each sample. This method is more objective than visually counting of the number of unstained spermatozoa in the straw and opens the possibility of assessing the physiological status of spermatozoa using other fluorescent probes.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: assessment of the suitability of eight synthetic media for in vitro evaluation of sperm progression by a mucus penetration test

To formulate a synthetic medium as an ovine cervical mucus substitute, eight concentrations of acrylamide (1%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.85%, and 2%) were compared in the sperm penetration test performed at 39 °C for 30 min. A test with ovine cervical mucus was used as a control assay. The sperm count of the migration assay was measured for each 5 mm of plastic straw (12 segments in total). The tests were conducted over 4 wk (in February and March). Ejaculates from four rams were collected twice a week by artificial vagina, and pooled. Two concentrations of acrylamide, the sperm count of which is more similar to that observed for the natural mucus, were selected for experiment 2.

2.1.2. Experiment 2: assessment of sperm progression in two synthetic media using semen samples stored in liquid state at a reduced temperature (5 °C or 15 °C)

The calculation of the population of sperm that migrate for each segment is instrumentally complex and thus we propose a more simple analysis. In this study we evaluated the sperm population that progresses for each 10 mm of plastic straw (6 segments in total) after 30 min at 39 °C. In a second analysis, these data are summarized in two variables: sperm count that progress beyond 20 mm or 30 mm (D20 or D30, respectively). This analysis was performed with four media: two acrylamides (1.55% and 1.6%), and two reference controls (sheep cervical mucus and 1.65% acrylamide). Ram semen was analyzed in three different physiological states: fresh semen, semen stored to 15 °C, and semen stored at 5 °C. The tests were conducted over 4 wk (during March and April). Ejaculates from four rams were collected twice a week by artificial vagina and pooled. The basic rheological properties of acrylamide gels prepared with four concentrations of reference were analyzed and these properties of natural cervical mucus were discussed.

2.1.3. Experiment 3: correlation between in vitro sperm progression and spermatozoa quality of fresh semen samples

The ability of the sperm penetration test to predict semen quality is evaluated by the relationship between quality parameters of ram spermatozoa and the sperm count observed in the sperm-mucus penetration test. This analysis was performed with acrylamide 1.6% and sheep cervical mucus, as reference control. We evaluated the sperm population of fresh ejaculates that progress beyond 20 or 30 mm after 30 min at 39 °C. The tests were conducted over 4 wk (during April and May). Ejaculates from four rams were collected twice a week by artificial vagina, and pooled.

2.2. Collection of cervical mucus

The ewes were synchronized using intravaginal sponges (Chronogest, Laboratorios Intervet SA, Madrid, Spain) impregnated with 20 mg of fluorogestone acetate. After 14 days, the sponges were removed, and the ewes received 500 IU of equine chorionic gonadotropin (Folligon, Laboratorios Intervet SA) intramuscularly. Cervical mucus was collected from 24 ewes during the induced estrus using plastic AI sheaths connected to a 20-mL syringe. Collected mucus samples were screened and only clear ones were used. Selected mucus samples were stored in 30-mL sterile tubes (pooling the mucus of several females) and were transported to the laboratory at 5 °C, where they were stored at −20 °C until use according to Memon and Gustafsson [23].

2.3. Preparation of synthetic media

Eight acrylamide gels (1%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.85%, and 2%) were prepared to test sperm progression. These synthetic media were obtained from 30% liquid acrylamide (30% ProtoGel, National Diagnostics, Atlanta, GA, USA), diluting it to the corresponding volume with 1.5 M Tris (pH 8.8) in water. Once mixed, a solution of 2% ammonium persulphate up to 1.5% of final volume and a 0.05% Tetramethylethylenediamine (TEMED, Sigma-Aldrich, Madrid, Spain) were added, to induce acrylamide polymerization [5]. The mixture (pH 7.2, 320 mOsm) was left at room temperature for 24 h and then stored at 4°C until use.

2.4. Evaluation of rheological properties of media

Rheological measurement of different media were made using a Rotovisco RV 12 viscometer (Haake Mess-Technik, GmbH, Co., Karlruhe, Germany) at 38 °C according to López-Gatius et al. [24].

2.5. Ram sperm collection

Semen from four Assaf rams was collected by means of an artificial vagina (40 °C) in the presence of a female decoy. The glass collection tube was placed in a thermoregulated bath at 34 °C, and a preliminary seminal evaluation was carried out (volume, mass motility, and concentration). Sperm concentration was assessed by Bürker hemocytometer (Marienfeld, GmbH, Marienfeld, Germany) using CASA (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). The ejaculates used in the experiment were those with a volume higher than 0.5 mL, mass motility ≥4 (determination by microscopy with warming stage at 37 °C, × 40; score: 0–5) and a sperm concentration greater than 3000×10^6 spermatozoa/mL. Ten min after collection, these ejaculates were diluted in Tris-Citric-Fructose diluent (TCF, 0.27 M Tris, 90 mM citric acid, 53 mM fructose) at 1600×10^6 spermatozoa/mL, obtaining a sperm pool.

2.6. Sperm refrigeration

A proportion of the diluted semen was used for immediate experiment (fresh sample), and the remainder was divided into two fractions of 200 μL which were cooled: (1) to 15 °C (R15 sample) in an incubator

(WTB Binder; REGO, Madrid, Spain); and (2) to 5 °C (R05 sample) in a refrigerator. The cooled semen samples were stored in the respective containers for 24 h and were then evaluated by the progression test.

2.7. Sperm motility evaluation

Sperm were diluted in TCF to 10 to 20 × 10^6 spermatozoa/mL and loaded into a Makler counting chamber (10-μm depth; Sefi Medical Instruments, Haifa, Israel) at 37 °C. The CASA system consisted of a triocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) using a 10 × negative phase-contrast objective, equipped with a warming stage at 37 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured and analyzed using a computer-assisted motility analyzer (ISAS; Proiser) with specific settings to ram spermatozoa. The software rendered the following parameters: (1) percentage of motile spermatozoa (PM), and (2) for each spermatozoon, three velocity parameters (VCL, velocity according to the actual path; VSL, velocity according to the straight path; and VAP, velocity according to the smoothed path), three track linearity parameters (LIN, linearity; STR, straightness; WOB, wobble), the amplitude of the lateral displacement of the sperm head (ALH), and the head beat-cross frequency (BCF).

2.8. Assessment of sperm quality by cytometry

2.8.1. Acrosomal status

Double stain with PNA-FITC (Sigma-Aldrich, Madrid, Spain) and propidium iodide (PI; Sigma-Aldrich) were used. The staining was performed by diluting the sperm sample (1–2 million spermatozoa/mL) in 300 μL of PBS with 1 μg/mL of PNA-FITC and 1.5 μM of PI. After 10 min at room temperature and darkness, samples were analyzed by flow cytometry. Spermatozoa were classified in four sperm subpopulations: red fluorescence (not viable), green fluorescence (viable with damaged acrosome), double fluorescence (not viable with damaged acrosome), and no staining (viable with intact acrosome).

2.8.2. Cell viability

The analysis was performed with a double staining SYBR-14 and PI using the Sperm Viability Kit (LIVE/DEAD, Invitrogen, Barcelona, Spain). The sperm sample was diluted in 300 μL of PBS (1–2 million spermatozoa/mL) with 500 nM of SYBR-14 and 0.8 mg/mL of PI. After 10 min at room temperature and darkness, samples were analyzed by flow cytometry. This double staining classified the spermatozoa in three different cell groups: sperm with red fluorescence in the nucleus (nonviable), sperm with green fluorescence in the nucleus (viable), and sperm cells with double fluorescence (nonviable).

2.8.3. Mitochondrial status

Sperm samples were diluted (1–2 million spermatozoa/mL) and 100 nM of Mitotracker Deep Red (Invitrogen) stock solution 1 mM in DMSO and 100 nM YO-PRO-1 (Iodide 491/509, Invitrogen) were added. Samples were incubated in the dark for 15 min at 38 °C and then analyzed by flow cytometry. Viable spermatozoa with high fluorescence for Mitotracker Deep Red (Invitrogen) were interpreted as having active mitochondria.

2.8.4. Apoptotic cells

Double stain with YO-PRO-1 (Invitrogen) and PI were used. The staining was performed by diluting the sperm sample (1–2 million spermatozoa/mL) in 300 μL of PBS with 100 nM YO-PRO-1 (Invitrogen) and 1.5 μM of PI. After 10 min at room temperature and in darkness, samples were analyzed by flow cytometry. This double staining allows us to differentiate three populations of spermatozoa: sperm with red fluorescence (dead cells), spermatozoa with green fluorescence by YO-PRO-1 (Invitrogen) (living apoptotic cells), and unstained spermatozoa (viable cells).

2.9. Flow cytometry analysis

Cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser (488 nm) and red diode laser (635 nm). SYBR-14, YO-PRO-1 (Invitrogen), PNA-FITC, and PI were excited at 488 nm and Mitotracker Deep Red (Invitrogen) was excited at 635 nm. The fluorescence emitted by SYBR-14, YO-PRO-1 (Invitrogen) and PNA-FITC was analyzed using the FL1 photodetector (530/28 band pass filter) and PI and Mitotracker Deep Red (Invitrogen) fluorescence using FL3 photodedector (670 long pass filter). The signals, forward scatter/side scatter (FSC/SSC), were used to discriminate the sperm population from other events. For each sample, we have acquired 10 000 spermatozoa using Cell Quest Pro v. 3.1 (BD Biosciences) software. The analysis of flow cytometry data were performed using Weasel v.2.6 (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

2.10. Sperm progression test

This test was carried out using 0.5-mL plastic straws (0.3 × 13 cm) filled with ovine cervical mucus or with each of the acrylamide gels to test (9 replicates). Semen samples were first diluted to 25 × 10^6 motile spermatozoa/mL in TCF. Diluted samples were distributed in 2-mL microtubes (180 μL/tube), and the straws were placed perpendicularly by a clamping device over the semen sample. Straw position was adjusted so that only 1 mm of mucus (natural or synthetic), protruding from the straw, stayed in contact with the sperm sample. This device remained for 15 minutes at 39 °C. After this time, straws were emptied in segments of 5 mm (experiment 1) or 10 mm (experiment 2), placing each segment in a well of a 24-well plate. The first 5 mm of the straw content was discarded, because of high sperm concentration due to direct sample contact, was irrelevant to real sperm progression. To automatically obtain the image of the sperm under a microscope, 100 μL of 10 μg/mL Hoechst 33342 was added to each well. The plates were left to dry on a plate at 39 °C.

The plates were analyzed on an inverted microscope (T2000 U; Nikon) equipped with epifluorescence and motorized stage. Each well was photographed automatically (ORCA digital camera, Hamamatsu, Tokyo, Japan), capturing 96 images (10× objective). The motorized plate and the digital camera were automatically controlled by Metamorph v.7 software (Molecular Devices, Inc., Downingtown, PA, USA). Subsequently, these images were analyzed with the image analysis software NIS Elements v.3 (Nikon), to objectively count the spermatozoa in each well. Specific adjustment to discriminate sperm with regard to debris, according to their shape and size, were applied in this analysis.

A negative control is performed to avoid an overestimation of sperm count in the cervical mucus, due to the presence of epithelial cells. In each trial, a straw filled only with ovine cervical mucus was emptied in segments of 5 mm or 10 mm (depending on the type of experiment), placing each segment in a well of a 24-well plate. As with the plates containing sperm, Hoechst 33342 was added to each well and finally the cells present are counted. This number was subtracted from the sperm counts that we obtained with the sperm progression test.

2.11. Statistical analysis

Results are presented as mean ± SEM (standard error of the mean). Data were analyzed with the Statistica v. 9 (StatSoft, Tulsa, OK, USA) program using general linear models (GLM) or, where appropriate, the Pearson correlation coefficient. In experiment 1, GLM was used to study the sperm progression in the straws by sperm counts in each straw segment (5 mm), and to compare the nine synthetic media with the cervical mucus control (post hoc comparison by Fisher's least-significant-difference test). The rheological data (consistency index) were fitted to an exponential function. In experiment 2, a GLM was carried out using as factors the four synthetic media, the three types of semen samples, and the two distances (20 or 30 mm), with the spermatozoa counts as the dependent variable. In experiment 3, Pearson correlation coefficients were carried out between the spermatozoa counts and the individual and progressive motility, the kinematic parameters, the acrosomal status, the sperm viability, the mitochondrial status and the proportion of apoptotic cells.

3. Results

3.1. Experiment 1: assessment of the suitability of eight synthetic media for in vitro evaluation of sperm progression

Sperm migration of fresh semen samples into straws containing each of the eight concentrations of acrylamide tested or ovine cervical mucus is presented in Table 1. Eleven 5-mm segments (from segment 2 -S02- to segment 12 -S12) were quantified. Ovine cervical mucus, used as control, showed higher sperm numbers than synthetic media. The data were compared for pairs of successive segments and we found significant differences between the first three segments (S02–S03 and S03–S04) both for the natural mucus as for the synthetic media with an acrylamide concentration between 1% and 1.6%. In the different media evaluated, the concentration of spermatozoa declined exponentially in relation to penetration depth.

The existence of two groups of synthetic media defined by the acrylamide concentration could be appreciated. One group was composed of synthetic media containing an acrylamide concentration of between 1% and 1.6%, with sperm count values which are closer to those of the cervical mucus (Table 1). In this group, the consistency index of synthetic media with acrylamide 1% and 1.5% (k = 0.01 and 0.11, respectively) was different from that of the cervical mucus (k = 0.17, Table 2). The fitting curve obtained with rheological values of the different media allows us to conclude that acrylamide 1.55% and 1.6% are more similar to the characteristics of cervical mucus. The second group, composed of the acrylamide media from 1.65% to 2%, showed significantly lower sperm count values and their rheological characteristics differ greatly from those of cervical mucus.

Table 1
Number of spermatozoa (mean ± standard error) observed in each segment of 5 mm (S02–S12)* in a sperm penetration test using fresh semen samples and testing eight different synthetic media (A 1% to A 2%).

Segment	A 1%	A 1.5%	A 1.55%	A 1.6%	A 1.65%	A 1.7%	A 1.85%	A 2%	Mucus
S02	3227 ± 522^{aA}	4171 ± 702^{aA}	3756 ± 622^{aA}	3452 ± 194^{aA}	1161 ± 422^{aB}	1022 ± 329^{aB}	350 ± 148^{aC}	287 ± 95^{aC}	5630 ± 337^{aD}
S03	2241 ± 363^{bA}	3299 ± 436^{bA}	3126 ± 475^{bB}	2520 ± 573^{bAB}	603 ± 229^{aC}	306 ± 114^{bC}	58 ± 18^{bD}	108 ± 36^{bD}	3863 ± 279^{bB}
S04	1450 ± 159^{cA}	1939 ± 265^{cA}	1865 ± 203^{cA}	1102 ± 135^{cA}	201 ± 87^{bB}	108 ± 48^{bB}	66 ± 36^{bC}	52 ± 19^{bC}	3624 ± 375^{bD}
S05	1159 ± 152^{cA}	1738 ± 530^{cA}	1388 ± 146^{bA}	1247 ± 147^{cA}	116 ± 37^{bB}	109 ± 31^{bB}	37 ± 11^{bC}	31 ± 10^{bC}	2994 ± 286^{bD}
S06	1068 ± 151^{cA}	1219 ± 207^{cA}	1140 ± 135^{bA}	1141 ± 137^{cA}	79 ± 27^{bB}	86 ± 28^{bB}	18 ± 11^{bC}	43 ± 19^{bC}	2429 ± 290^{bD}
S07	964 ± 109^{cA}	1021 ± 134^{cA}	1414 ± 304^{bA}	1145 ± 205^{cA}	130 ± 48^{bB}	53 ± 13^{bC}	36 ± 18^{bC}	41 ± 12^{bC}	2306 ± 287^{bD}
S08	824 ± 156^{cA}	613 ± 111^{cA}	1255 ± 296^{bB}	822 ± 191^{cA}	69 ± 31^{bC}	54 ± 17^{bC}	33 ± 12^{bC}	37 ± 8^{bC}	2066 ± 357^{bD}
S09	413 ± 93^{cA}	448 ± 74^{cA}	547 ± 90^{cA}	447 ± 97^{cA}	63 ± 37^{bB}	73 ± 16^{bB}	24 ± 8^{bB}	26 ± 8^{bB}	2234 ± 354^{bC}
S10	394 ± 80^{cA}	411 ± 62^{cA}	586 ± 68^{cA}	428 ± 90^{cA}	54 ± 18^{bB}	38 ± 14^{bB}	19 ± 6^{bB}	17 ± 6^{bB}	1692 ± 208^{bC}
S11	359 ± 83^{cA}	512 ± 100^{cA}	551 ± 83^{cA}	194 ± 80^{cB}	24 ± 8^{bC}	15 ± 5^{bC}	12 ± 5^{bC}	9 ± 3^{bC}	1588 ± 233^{bD}
S12	329 ± 55^{cA}	495 ± 91^{cA}	556 ± 95^{cA}	133 ± 32^{cB}	16 ± 3^{bC}	13 ± 4^{bC}	14 ± 4^{bC}	15 ± 3^{bC}	1410 ± 145^{bD}

Ovine cervical mucus (Mucus) was control media (9 replicates).
Different superscript letters (ab) in the same column indicate that pair of segments [S02–S03], [S03–S04], [S04–S05], [S05–S06], [S06–S07], [S07–S08], [S08–S09], [S09–S10] differ significantly within each media ($P < 0.05$). Different superscript letters (AB) in the same row indicate that media differ significantly within each segment ($P < 0.05$).
A, acrylamide gel.
* The first segment is not valued.

Table 2
Rheological properties (mean ± standard error) of ovine cervical mucus (Mucus) and four substitutes based on acrylamide (A) 1% to A 2.5% at 38°C (10 replicates).

Media	Index of consistency (K)*	Flow behavior index (n)	*r*
A 1%	0.01 ± 0.01	0.99 ± 0.08	0.99
A 1.5%	0.13 ± 0.02	0.79 ± 0.04	0.98
A 2%	0.87 ± 0.06	0.45 ± 0.02	0.94
A 2.5%	1.69 ± 0.1	0.48 ± 0.03	0.96
Mucus	0.17 ± 0.03	0.75 ± 0.05	0.98

* K in Pascals per second^n. Pearson moment-correlation coefficient (*r*) ($P < 0.05$).

3.2. Experiment 2: assessment of sperm progression in two synthetic media using semen samples with different types of preservation

The medium used as negative control (acrylamide 1.65%) showed the lowest cell density data in the three types of semen tested, whereas the highest values were observed for cervical mucus (Table 3). Semen chilled at 15 °C presented intermediate sperm count values with regard to those observed in fresh semen (higher) and 5 °C chilled semen. Segments S02 and S03 showed the highest sperm counts which are significantly different from the values observed in other analyzed segments, in both acrylamide 1.6% and acrylamide 1.55% media.

In order to evaluate the two synthetic media tested, we have obtained two single values: the sperm numbers that progress beyond 20 mm or 30 mm (D20 or D30, respectively; Fig. 1). We found that there were no significant differences between acrylamide 1.6% and acrylamide 1.55% media in any case. However, the acrylamide 1.6% medium showed a consistency index that was closer to the data presented in cervical mucus (Fig. 2).

3.3. Experiment 3: correlation between in vitro sperm progression of semen samples in different types of conservation and semen quality

Table 4 represents the correlation coefficients between the sperm count observed in a migration test with acrylamide 1.6% and ovine cervical mucus, in the two distances analyzed (D02 and D03), and the corresponding semen quality parameters. In both distances, we found that sperm count had a significant negative correlation with the percentage of apoptotic spermatozoa percentage (YO-PRO-1+/PI−) and the percentage of cells with damaged acrosome cells (PNA+), with both acrylamide 1.6% and mucus. However, for both media, we found no significant correlation with the percentage

Table 3
Number of spermatozoa (mean ± standard error) in each 1 cm segment (S02–S06)* observed in three different synthetic media (A 1.55% to 1.65%) and ovine cervical mucus (Mucus), for semen samples preserved by two procedures (refrigerated at 5°C [R05] or 15 °C [R15]) and fresh semen (12 replicates).

Medium	Semen	S02	S03	S04	S05	S06
A 1.55%	Fresh	$5326 \pm 356^{\alpha Aa}$	$3422 \pm 237^{\alpha Ab}$	$1734 \pm 155^{\alpha Ac}$	$1434 \pm 216^{\alpha Ac}$	$1145 \pm 246^{\alpha Acc}$
	R15	$4688 \pm 630^{\alpha Aa}$	$2743 \pm 429^{\alpha Ab}$	$1558 \pm 295^{\alpha Ac}$	$1336 \pm 199^{\alpha Ac}$	$1032 \pm 273^{\alpha Ac}$
	R05	$3003 \pm 425^{\beta Aa}$	$1844 \pm 233^{\beta Ab}$	$1290 \pm 185^{\alpha Ab}$	$945 \pm 156^{\alpha Ab}$	$646 \pm 132^{\alpha Ab}$
A 1.6%	Fresh	$5932 \pm 653^{\alpha Aa}$	$3918 \pm 458^{\alpha Ab}$	$1857 \pm 213^{\alpha Ac}$	$1240 \pm 181^{\alpha Ac}$	$873 \pm 60^{\alpha Ac}$
	R15	$4153 \pm 527^{\beta Aa}$	2522 ± 269^{cAb}	$1362 \pm 201^{\alpha\beta Ab}$	$1023 \pm 148^{\alpha\beta ABc}$	$818 \pm 109^{\alpha Ac}$
	R05	$3228 \pm 270^{\beta Ba}$	$1328 \pm 174^{\beta Ab}$	$846 \pm 185^{\beta Ac}$	$472 \pm 83^{\beta Bb}$	$601 \pm 82^{\alpha Ab}$
A 1.65%	Fresh	$1189 \pm 178^{\alpha Ba}$	$391 \pm 59^{\alpha Bb}$	$264 \pm 56^{\alpha Bc}$	$110 \pm 31^{\alpha Ab}$	$156 \pm 36^{\alpha Bb}$
	R15	$457 \pm 158^{\beta Ba}$	$386 \pm 38^{\alpha Ba}$	$90 \pm 23^{\alpha Ba}$	$134 \pm 95^{\alpha Aa}$	$124 \pm 41^{\alpha Ba}$
	R05	$352 \pm 51^{\beta Ca}$	$202 \pm 28^{\alpha Bb}$	$77 \pm 13^{\alpha Bb}$	$79 \pm 27^{\alpha Ab}$	$64 \pm 12^{\alpha Bb}$
Mucus	Fresh	$6882 \pm 574^{\alpha Ca}$	$4672 \pm 416^{\alpha Cb}$	$3665 \pm 439^{\alpha Cb}$	$2307 \pm 367^{\alpha Ac}$	$1418 \pm 629^{\alpha Ac}$
	R15	$4501 \pm 488^{\beta Aa}$	$2999 \pm 428^{\beta Ab}$	$2212 \pm 438^{\beta Cb}$	$1394 \pm 180^{\beta Bb}$	$707 \pm 60^{\beta Ab}$
	R05	$3820 \pm 499^{\beta Ba}$	$2410 \pm 411^{\beta Cb}$	$1224 \pm 178^{\beta Ab}$	$1034 \pm 132^{\beta Bb}$	$629 \pm 78^{\beta Ab}$

Different superscript letters (a,b) in the same row indicate that the pair of segments (S02–S03), (S03–S04), (S04–S05), and (S05–S06) differ significantly within each medium and preservation procedure ($P < 0.05$). Different superscript letters (A,B) in the same column indicate that media differ significantly within each segment and preservation procedure ($P < 0.05$). Different superscript letters (α,β) in the same column indicate that the preservation procedure differ significantly within each segment and media ($P < 0.05$).
A, acrylamide gel.
* The first segment was discarded.

of viable cells (SYBR+/PI−) or the potential of mitochondrial membrane. The number of spermatozoa showed a positive correlation with PM and velocity according to the straight path (VSL) for both media. In the case of acrylamide 1.6%, LIN and WOB also showed a significant correlation with the number of spermatozoa ($r = 0.59$, $P = 0.012$; $r = 0.54$, $P = 0.024$, respectively for D02). Straightness showed a significant correlation with sperm counts in cervical mucus. In this context, we must point out that, in general the correlation coefficients with the cervical mucus are higher.

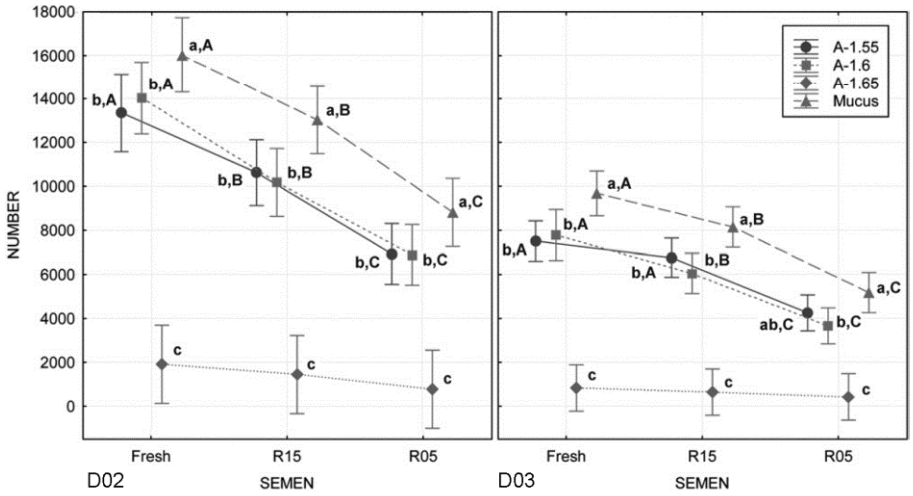

Fig. 1. Number of spermatozoa (least-squares means of 12 replicates ± 95% confidence interval) that progresses a distance beyond 20 (D02) or 30 mm (D03) in acrylamide gel or cervical mucus for three semen samples (fresh and refrigerated at 5 °C or 15 °C). Lowercase letters indicate differences between media in each semen sample and progression distance and capital letters indicate differences between semen samples in each media and distance.

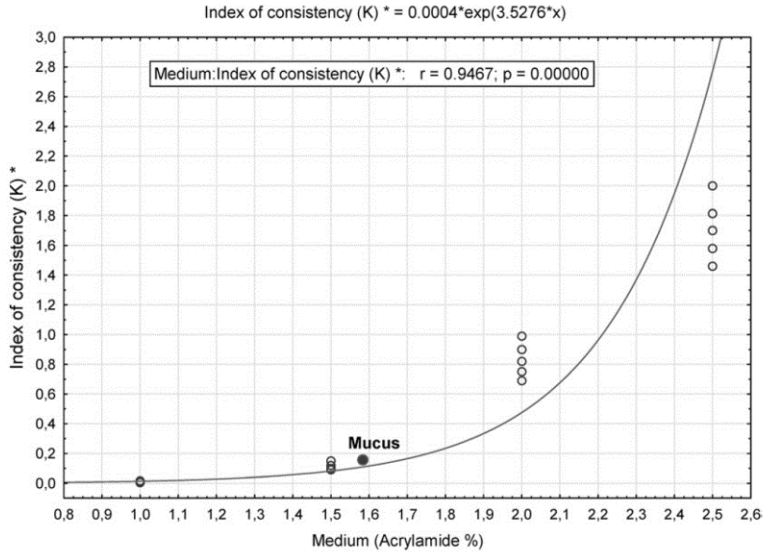

Fig. 2. Rheological properties of acrylamide gel: index of consistency fitted in an exponential manner in relation to proportion of acrylamide in medium (points indicate the values of five assays). Mean values of index for the ovine cervical mucus is shown.

4. Discussion

Fertility is a very complex biological function that depends on several properties of the spermatozoa, including sperm motility. The efficiency of sperm transport in the genital tract is an essential prerequisite for the reproduction success [25]. Given this fact, the evaluation of the capacity of spermatozoa to progress through natural mucus (CMPT) or a mucus substitute has been proposed to assess the sperm

Table 4
Correlation coefficients (test of significance) between the number of spermatozoa that progress a distance beyond 20 (D02) or 30 mm (D03) in acrylamide gel (A) 1.6% or ovine cervical mucus (Mucus) and the seminal quality parameters.

Parameter	D02				D03			
	Mucus		A 1.6%		Mucus		A 1.6%	
	r	P	r	P	r	P	r	P
TM	0.42	0.093	0.26	0.305	0.43	0.086	0.19	0.461
PM	0.62	0.008	0.55	0.021	0.62	0.006	0.49	0.043
VAP	0.41	0.060	0.40	0.090	0.43	0.068	0.42	0.086
VCL	0.36	0.152	0.21	0.413	0.38	0.137	0.26	0.319
VSL	0.49	0.045	0.48	0.049	0.52	0.034	0.44	0.046
LIN	0.42	0.092	0.59	0.012	0.44	0.080	0.50	0.041
STR	0.59	0.013	0.56	0.019	0.60	0.011	0.43	0.085
WOB	0.24	0.359	0.54	0.024	0.25	0.323	0.49	0.047
ALH	−0.28	0.283	−0.39	0.123	−0.32	0.215	−0.33	0.197
BCF	0.63	0.006	0.40	0.114	0.65	0.004	0.30	0.238
dACR	−0.40	0.002	−0.30	0.018	−0.41	0.001	−0.39	0.049
VIAB	0.45	0.052	0.32	0.062	0.47	0.057	0.35	0.068
MIT	0.37	0.180	0.36	0.187	0.35	0.196	0.32	0.246
APOP	−0.61	0.009	−0.62	0.008	−0.61	0.009	−0.59	0.013

ALH, amplitude of lateral head displacement (μm); APOP, living apoptotic cells, YO-PRO-1+ (%); BCF, head beat-cross frequency (%); dACR, damaged acrosomes, PNA+ (%); LIN, linearity index (%); MIT, active mitochondrial in live spermatozoa (%); PM, progressive motility (%); STR, straightnes. (%); TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, viability spermatozoa; VSL, straight-line velocity (μm/sec).

quality in vitro. The penetration test is one of the methods employed in seminal assessment using the distance traveled by the most advanced spermatozoa in the test device as diagnostic criterion. In this study, we propose an automatic analysis of the sperm numbers that migrates in a column of acrylamide to assess the semen quality in ram.

In our first experiment, a series of acrylamide concentrations were examined. The synthetic mucus have some advantages over natural mucus such as the ability to easily prepare large volumes and the ability to standardize environmental conditions, while the properties of cervical mucus are dependent on the estrus cycle [18] and only a small amount of it is produced by each ewe. Cervical mucus substitutes have been used in a number of studies [11,12,26]. Acrylamide has been previously used as a substitute of cervical mucus in other species (human [19,27]; bull [5,20]). Lorton et al. [27] noted that bull sperm migration in 1.8% acrylamide was similar to sperm migration in bovine cervical mucus. However, bull semen that varied widely in migration distances in bovine cervical mucus maintained similar relative migration distances in this synthetic medium. Eggert-Kruse et al. [19] evaluated polyacrylamide gel as a substitute for human cervical mucus in the sperm penetration test and among other results found that adequate sperm migration in polyacrylamide 1.8% was significantly more frequent in the fertile group.

We have observed a range of acrylamide concentrations (1.5%–1.6%) which allows the greatest number of spermatozoa to penetrate and these sperm counts were close to those observed in the test with cervical mucus. The differences among bull or ram regarding the characteristics of the synthetic medium most suitable for the progression test, can be interpreted according to the different characteristics of the cervical mucus from both species. Our rheological data of acrylamide gel fitted an exponential function, so that the 1.6% (suitable for ram semen) and 1.8% (suitable for bull semen) gels showed a noticeable difference in their consistency index. Moreover, the penetration of spermatozoa in synthetic media is highly dependent on the concentration and viscosity of media, as it has been documented by other authors [12].

In this study, the mean sperm count was always found to be higher in ovine cervical mucus than in any of the tested acrylamide concentrations. This greater difficulty of sperm to progress through the acrylamide has been observed also by Eggert-Kruse et al. [19]. According to these authors, human sperm ability to penetrate the acrylamide medium (concentrations 1.5%–1.8%) correlated significantly with the penetration of human cervical mucus, although polyacrylamide proved to be a stronger barrier so that sperm velocity and duration of progressive motility were markedly reduced in acrylamide.

Cervical mucus has a number of physicochemical properties at the time of ovulation, influenced by sex hormones, that facilitate easy sperm penetration through the uterine cervix [28]. Also, human cervical mucus creates channels in which the spermatozoa become oriented and distributed in a parallel direction to their long axis. Such alignment does not occur into hyaluronate polymer, in which the direction of sperm movement is essentially random [11]. These characteristics of cervical ovulatory mucus, which are not present in the acrylamide medium, might explain the differences observed by us between acrylamide and natural sheep mucus in the ability of progression of ram sperm. We bear in mind that freezing cervical mucus alters its functional properties and such mucus cannot be considered representative of what sperm encounter in in vivo insemination, but given the technical difficulties in handling fresh cervical mucus, we follow the method of Memon and Gustafsson [23] and we believe that the thawed mucus is acceptable control for acrylamide.

To assess the ability of the medium prepared with acrylamide to support sperm motility, we applied the progression test in three models of ovine semen conservation. We must highlight that the cooling-induced damage (cold shock) could explain the behavior of sperm in the test of progression. At present, the most useful method for ovine artificial insemination (vaginal via) is the application of semen cooled at 15 °C, which maintains the fertilizing capacity of sperm stored for 6 to 12 h [29,30], although motility is kept acceptably for up 24 to 48 h after ejaculation [31]. At 4 °C, sperm preservation can be prolonged, but storage time is significantly associated with the deterioration of motility parameters of ram sperm (total progressive motility, VAP, VSL, VCL, ALH, and straightness) [32]. Also, the lambing rates for ram spermatozoa after storage at 5 °C significantly decreased with 0-, 1-, 2-, or 3-day-old semen (60.0%, 34.3%, 33.8%, and 17.1%) [33]. Our results show that the population of sperm that moves beyond the 20 or 30 mm in the progression test is significantly affected by storage temperature. Some in vitro studies report that the spermatozoa stored at 15 °C or 5 °C for 2 days show a high motility (progressive

motility and kinetic parameters), so these parameters cannot explain the significant loss of sperm fertilizing capacity in this period. In our study, in which motility is evaluated as a complex variable by this stress test, the results show that the sperm damage is already present at 24 h. These results show the advantages of a functional test, such as the penetration test, which can integrate many cellular characteristics in a single assay.

In the present study, the number of ram spermatozoa that penetrate more than 20 or 30 mm into an acrylamide gel or ovine cervical mucus was significantly and positively correlated with progressive motility and one kinematics parameter (VSL); while this relationship is negative with acrosomal damage and injuries in the plasma membrane. Other specific correlations of sperm motility parameters, either for the cervical mucus (straightness, BCF) or acrylamide (straightness, LIN, WOB), were also observed. These results are not consistent with those described by Robayo et al. [9], who claim that continuous line velocity (VCL) and average path velocity (VAP) are the only sperm kinematic parameters that presented significant positive correlations with the ability to migrate in sheep cervical mucus. Procedural factors may explain these differences: Robayo et al. [9] analyzed the vanguard sperm distance and we were evaluating the sperm migration efficiency by the number of sperm reaching a specific segment. In this sense, we have to take into account the low or no association observed by Love et al. [34] between all velocity measures and total sperm motility, these authors suggests that the speed with which a sperm moves and how it moves is independent of the total percentage of moving sperm.

The significance of average-path velocity VAP in determining the success of cervical mucus penetration has been supported in several studies [9,11,35] although it has not been associated with fertility. However, differences in sperm migration through cervical mucus in vitro are related to the ability of spermatozoa to colonize the oviduct and to fertilize matured oocytes in vitro [36]. The importance of straight-line velocity VSL for the fertilizing capacity of the spermatozoa has been noted by different authors [8,37] and it has been speculated that a high VSL might be important in sperm transport through the female reproductive tract and penetration of the oocyte vestments [8]. The relationship between the number of cells that had penetrated in the cervical mucus test and fertility has been evaluated in cow [13]. When these authors compared bulls from the low fertility group with those from the high fertility group, the latter showed a higher number of spermatozoa at two defined penetration distances and a significant positive correlation was found between this number of spermatozoa and the nonreturn rates of the bulls.

Apart from findings derived from motility analyses, we found that the occurrence of apoptotic spermatozoa and spermatozoa with damaged acrosome were negatively correlated with the migration capacity of spermatozoa into mucus, while the mitochondrial membrane potential evaluated using a specific fluorophore showed no correlation with this migration. Anilkumar et al. [5] demonstrated that acrosome integrity was significantly and positively correlated with bull sperm penetration in mucus and acrylamide gel. Column filtration techniques have been suggested as useful ways of evaluating acrosome integrity, because they trap membrane-damaged or acrosome-reacted cells but allow motile, membrane-intact sperm to pass through [38]. The acrosome-reacted sperm are especially sticky, and reacted sperm can be seen to stick to glass, even in the presence of albumin [39]. This sticking could explain the negative correlation between acrosomal damage and the reduced ability of sperm to progress in the mucus and acrylamide gel observed in our study.

Garner et al. [40] noted that fluorometric measurement of mitochondrial function was highly correlated with the microscopic estimates of progressive forward motility. However, the importance of mitochondria for sperm motility has recently been reconsidered and it is believed that the mitochondrial activity is also important for maintaining ATP levels in the sperm head and midpiece required for housekeeping processes, such as membrane functionality [41]. As apoptotic spermatozoa, Martínez-Pastor et al. [42] observed that deer spermatozoa with "apoptotic-like" features would not be able to maintain motility for a long time. These authors found that this process was preceded by the loss of mitochondrial membrane potential, but that the loss of mitochondrial activity was not directly related to motility loss. These findings may explain the lack of correlation we have observed between the number of emigrating spermatozoa and mitochondrial status, whereas the proportion of apoptotic cells was negatively correlated with the sperm progression in mucus.

In conclusion, the results of this study suggest that the number of ram spermatozoa capable of going beyond of 20 mm into acrylamide gel, observed by automatic analysis in an optimized sperm migration test, is a useful parameter in the in vitro evaluation of sperm quality and further studies are needed to evaluate its relationship with field fertility.

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References

[1] Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod Update 2006;12:23–37.
[2] Galli A, Basetti M, Balduzzi D, Martignoni M, Bornaghi V, Maffii M. Frozen bovine semen quality and bovine cervical mucus penetration test. Theriogenology 1991;35:837–44.
[3] Berberoglugil P, Englert Y, Van den Bergh M, Rodesch C, Bertrand E, Biramane J. Abnormal sperm-mucus penetration test predicts low in vitro fertilization ability of apparently normal semen. Fertil Steril 1993;59:1228–32.
[4] Sharara FI, Illions EH, Coddington CC, Scott RT Jr. Evaluation of the Tru-Trax cervical mucus penetration test in predicting fertilization and pregnancy rates in in-vitro fertilization. Hum Reprod 1995;10:1481–5.
[5] Anilkumar R, Devanathan TG, Pattabiraman SR, Edwin MJ. Correlation between the spermatozoal characteristics and sperm penetration distance in polyacrylamide gel and bovine cervical mucus. Theriogenology 2001;55:685–91.
[6] Hirano Y, Shibahara H, Obara H, Suzuki T, Takamizawa S, Yamaguchi C, et al. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. J Assist Reprod Genet 2001;18:213–8.
[7] Ola B, Afnan M, Papaioannou S, Sharif K, Björndahl L, Coomarasamy A. Accuracy of sperm-cervical mucus penetration tests in evaluating sperm motility in semen: a systematic quantitative review. Hum Reprod 2003;18:1037–46.
[8] Gillan L, Kroetsch T, Maxwell WM, Evans G. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 2008;103:201–14.
[9] Robayo I, Montenegro V, Valdes C, Cox JF. CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. Reprod Domest Anim 2008;43:393–9.
[10] World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, Fifth Edition. WHO Press, 2010.
[11] Aitken RJ, Bowie H, Buckingham D, Harkiss D, Richardson DW, West KM. Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. J Androl 1992;13:44–54.
[12] Ivic A, Onyeaka H, Girling A, Brewis IA, Ola B, Hammadieh N, et al. Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. Hum Reprod 2002;17:143–9.
[13] Tas M, Bacinoglu S, Cirit U, Ozdas OB, Ak K. Relationship between bovine fertility and the number of spermatozoa penetrating the cervical mucus within straws. Anim Reprod Sci 2007;101:18–27.
[14] Alexander NJ. Evaluation of male infertility with an in vitro cervical mucus penetration test. Fertil Steril 1981;36:201–8.
[15] Bacinoglu S, Taş M, Cirit U, Ozdaş OB, Ak K. The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. Anim Reprod Sci 2008;104:38–46.
[16] Barratt CL, McLeod ID, Dunphy BC, Cooke ID. Prognostic value of two putative sperm function tests: hypo-osmotic swelling and bovine sperm mucus penetration test (Penetrak). Hum Reprod 1992;7:1240–4.
[17] Abu-Heija AT, Fleming R, Jamieson ME, Yates RW, Coutts JR. The effect of sperm-mucus interaction test on the outcome of in vitro fertilization and ovulation induction combined with intrauterin insemination. J Obstet Gynaecol Res 1996;22:229–33.
[18] Katz DF, Slade DA, Nakajima ST. Analysis of pre-ovulatory changes in cervical mucus hydration and sperm penetrability. Adv Contracept 1997;13:143–51.
[19] Eggert-Kruse W, Schwalbach B, Rohr G, Klinga K, Tilgen W, Runnebaum B. Evaluation of polyacrylamide gel as substitute for human cervical mucus in the sperm penetration test. Fertil Steril 1993;60:540–9.
[20] Tas M, Bacinoglu S, Cirit U, Ozgumus S, Kasgoz H, Pabuccuoglu S. Estimation of the potential fertility based upon non-return rates of bulls: using polyacrylamide gel instead of cervical mucus in the sperm penetration test. Theriogenology 2007; 68:981–7.
[21] O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans AC, et al. Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology 2010;73:541–9.
[22] Suttiyotin P, Thwaites CJ, Baillie ND. Relationships between the results of a modified sperm penetration test and a swim-up technique and the fertility of ram semen. Theriogenology 1992; 37:851–7.
[23] Memon MA, Gustafsson BK. Effects of various concentrations and combinations of prostaglandins on in vitro migration of frozen-thawed ram spermatozoa in cervical mucus of ewes. Theriogenology 1984;21:841–8.
[24] López-Gatius F, Miró J, Sebastián I, Ibarz A, Labèrnia J. Rheological properties of the anterior vaginal fluid from superovulated dairy heifers at estrus. Theriogenology 1993;40:167–80.
[25] Kölle S, Reese S, Kummer W. New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. Theriogenology 2010; 73:786–95.
[26] Engel S, Petzoldt R. Human sperm penetration in different media. Andrologia 1999;31:233–9.
[27] Lorton SP, Kummerfeld HL, Foote RH. Polyacrylamide as a substitute for cervical mucus in sperm migration tests. Fertil Steril 1981;35:222–5.
[28] Ulcova-Gallova Z. Immunological and physicochemical properties of cervical ovulatory mucus. J Reprod Immunol 2010;86: 115–21.
[29] Yániz J, Martí JI, Silvestre MA, Folch J, Santolaria P, Alabart JL, et al. Effects of solid storage of sheep spermatozoa at 15 degrees C on their survival and penetrating capacity. Theriogenology 2005;64:1844–51.

[30] Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, de Paz P. Improvement strategies in ovine artificial insemination. Reprod Domest Anim 2006;41 Suppl 2:30–42.
[31] López-Sáez A, Ortiz N, Gallego L, Garde JJ. Liquid storage (5 °C) of ram semen in different diluents. Arch Androl 2000;44: 155–64.
[32] Kasimanickam R, Kasimanickam V, Pelzer KD, Dascanio JJ. Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 degrees C. Anim Reprod Sci 2007; 101:60–73.
[33] Salamon S, Maxwell WMC, Firth JH. Fertility of ram semen after storage at 5°C. Anim Reprod Sci 1979;2:373–85.
[34] Love CC, Thompson JA, Brinsko SP, Rigby SL, Blanchard TL, Lowry VK, et al. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 2003;60:1127–38.
[35] Cox JF, Alfaro V, Montenegro V, Rodriguez-Martinez H. Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus. Theriogenology 2006;66:860–7.
[36] Cox JF, Zavala A, Saravia F, Rivas C, Gallardo P, Alfaro V. Differences in sperm migration through cervical mucus in vitro relates to sperm colonization of the oviduct and fertilizing ability in goats. Theriogenology 2002;58:9–18.
[37] Holt C, Holt WV, Moore HD, Reed HC, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J Androl 1997;18:312–23.
[38] Neild DN, Gadella BM, Agüero A, Stout TA, Colenbrander B. Capacitation, acrosome function and chromatin structure in stallion sperm. Anim Reprod Sci 2005;89:47–56.
[39] Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update 2008;14:647–57.
[40] Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57:1401–6.
[41] Silva PF, Gadella BM. Detection of damage in mammalian sperm cells. Theriogenology 2006;65:958–78.
[42] Martínez-Pastor F, Fernández-Santos MR, del Olmo E, Domínguez-Rebolledo AE, Esteso MC, Montoro V, et al. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod Fertil Dev 2008;20:547–56.

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Ram spermatozoa migrating through artificial mucus *in vitro* have reduced mitochondrial membrane potential but retain their viability

Carmen Martínez-Rodríguez[A,B], *Mercedes Alvarez*[A,C], *Elena López-Urueña*[A,C], *Susana Gomes-Alves*[A,C], *Luis Anel-López*[A,D], *Cesar A. Chamorro*[A,E], *Luis Anel*[A,C] *and Paulino de Paz*[A,B,F]

[A]ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain.
[B]Molecular Biology, University of León, 24071, León, Spain.
[C]Animal Reproduction and Obstetrics, University of León, 24071, León, Spain.
[D]SaBio IREC (CSIC-UCLM-JCCM), Campus Universitario s. n. 02071 Albacete, Spain.
[E]Veterinary Anatomy, University of León, 24071, León, Spain.
[F]Corresponding author. Email: ppazc@unileon.es

Abstract. Sperm motility *in vitro* is one of the most common predictors of fertility in male screening. We propose that a mucus-penetration assay can isolate a cellular subpopulation critical to reproductive success. To this end, a device was designed with three modules (sample, test and collection) and its conditions of use evaluated (length of mucus, incubation time, mucus medium, sperm concentration and position in relation to the horizontal). The number of spermatozoa migrating and the viability and acrosomal status of the spermatozoa not migrating were calculated. The second objective was to evaluate the qualitative parameters of the spermatozoa migrating in 1.6% polyacrylamide for 30 min. The number of spermatozoa migrating and the sperm motility, viability and the acrosomal and mitochondrial status of three sperm populations (fresh, not migrating and migrating) were determined. A higher number of migrating spermatozoa were observed after 60 min of incubation, but this situation adversely affected sperm quality. The methylcellulose-based test showed a significantly lower number of migrating spermatozoa than the polyacrylamide test. The position at an angle of 45° resulted in a higher number of migrating spermatozoa in the polyacrylamide-based test. The sperm counts for three consecutive assays indicated an acceptable repeatability of the method. The viability and acrosomal status of the migrating spermatozoa showed no significant changes with regard to the control when the device was placed at 45°, whereas these parameters showed lower values at 0°. The percentage of high mitochondrial membrane potential spermatozoa was significantly reduced in the population of migrating spermatozoa.

Additional keywords: acrosome status, motility, penetration test, synthetic mucus.

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Introduction

Genetic improvement programs are based on the application of artificial insemination (AI). Although frozen–thawed semen is the method of choice for AI in several species, fresh or cooled semen is usually applied in ewes (Anel *et al.* 2006) because the fertility rates obtained with frozen–thawed spermatozoa are very low. The development of semen conservation techniques aimed at improving fertility rates involves the *in vitro* evaluation of semen quality to determine which variables improve semen freezability. We should also highlight that, for the conservation of the spermatozoa of wild animals as a genetic resource in biobanks, this *in vitro* predictive evaluation is in most cases the only data available regarding the biological usability of the stored samples (Anel *et al.* 2008).

The fertilising potential of spermatozoa has been assessed on the basis of several descriptive criteria. Male fertility depends on a heterogeneous population of spermatozoa interacting at various levels with the female genital tract to ensure that a minority of spermatozoa with normal morphology and vigorous motility arrive at the fertilisation site. Presumably, sperm motility is not required for sperm transport through the uterus, but it may be necessary for transport through the cervix, uterotubal junction and oviduct (Suarez and Pacey 2006). Sperm transport through the female reproductive tract has been evaluated *in vivo* by some authors (Cox *et al.* 2002; Druart *et al.* 2011; Richardson *et al.* 2011), but an objective analysis is extremely difficult because of the many factors involved and the associated technical difficulty (Hossain *et al.* 1999; Defoin *et al.* 2008).

The evaluation of sperm motility *in vitro* is one of the most common predictors in the screening of males for fertility, and different techniques have been developed. The classical method is the subjective assessment of motility via a simple microscopic observation evaluating the proportion of motile sperm. Most recently, computer-aided sperm analysis (CASA) was developed to allow a more objective analysis of sperm motility based on measurements of individual spermatozoa, with the provision of a greater number of motility parameters. This analysis estimates parameters of cell velocity and also parameters related to the quality of cell movement (Defoin *et al.* 2008). In these methods of *in vitro* analysis, the motility of spermatozoa is evaluated by counting the cells that are in the field at a given moment in time; thus, the results do not necessarily reflect sperm behaviour over long periods of time (Hossain *et al.* 1999). Sperm motility is complex, and *in vitro* sperm-migration tests have been developed using cervical mucus or cervical mucus surrogates to mimic the ability of spermatozoa to migrate through the female reproductive tract. The ability of spermatozoa to penetrate a cervical mucus surrogate correlates extremely well with the outcome of cervical mucus penetration assays and shows the same dependence on sperm movement (Aitken 2006). These tests evaluate the total distance travelled by the vanguard spermatozoon into the mucus (Keel and Webster 1988; Mole and Fitzgerald 1990; Murase and Braun 1990; Galli *et al.* 1991; Verberckmoes *et al.* 2002; Cox *et al.* 2006; Robayo *et al.* 2008) or the number of spermatozoa that penetrate at a defined distance into the mucus (Suttiyotin *et al.* 1992; Clarke *et al.* 1998; Tang *et al.* 1999; Hamano *et al.* 2001; Taş *et al.* 2007*a*, 2007*b*; Gillan *et al.* 2008; Al Naib *et al.* 2011). Ola *et al.* (2003) performed a quantitative review of the available penetration tests and concluded that counting the spermatozoa that travel through a defined distance of cervical mucus is a more accurate diagnostic criterion than the distance of the vanguard spermatozoon and that the former has the potential to be a useful laboratory-based sperm-function test. The progression test has been applied in different positions: (1) test tube positioned vertically on the surface of semen sample (Taş *et al.* 2007*a*; Gillan *et al.* 2008; Al Naib *et al.* 2011), (2) test tube inclined at an angle of 45° (Aitken *et al.* 1992) or (3) using a horizontal column constructed on a Petri dish (Hamano *et al.* 2001). The influence of gravity and boundary effect on the migratory movements of spermatozoa has been studied (Winet *et al.* 1984) but no comparative study of the effect of gravity on the migration test was conducted. Therefore, we propose to analyse two angles in the position of the tube relative to the horizontal, to evaluate the possible effect of gravity.

Correlations between the results obtained with different types of penetration tests and some quality parameters of the original sperm samples have been shown (Keel and Webster 1988; Galli *et al.* 1991; Aitken *et al.* 1992; Eggert-Kruse *et al.* 1996; Anilkumar *et al.* 2001; Ivic *et al.* 2002; Cox *et al.* 2006; Taş *et al.* 2007*a*; Gillan *et al.* 2008; Robayo *et al.* 2008; Martínez-Rodríguez *et al.* 2012). The penetration of spermatozoa in mucus has been correlated with fertility in several species (Murase *et al.* 1990; Suttiyotin *et al.* 1992; Hamano *et al.* 2001; Taş *et al.* 2007*a*, 2007*b*; Bacinoglu *et al.* 2008; Gillan *et al.* 2008; Al Naib *et al.* 2011), although some authors have suggested that this relationship does not exist (Galli *et al.* 1991; Murase *et al.* 2001; Verberckmoes *et al.* 2002).

The stress defined by mucus-penetration assays imposes meaningful limitations on the physiological function of spermatozoa and should allow us to evaluate the effectiveness of sperm motility for progression in the female genital tract. We assessed the motility of spermatozoa using an *in vitro* test that simulates the physiological exercise of spermatozoa on their long journey through the female genital tract. The device creates a physiological test in which the spermatozoa are subjected to sustained stress over time in a controlled microenvironment, allowing the isolation of a dynamic cellular subpopulation. This sperm subpopulation that exhibits adequate motility such that the spermatozoa were able to progress in a mucus barrier is then analysed to determine several critical parameters for reproductive success (e.g. viability, mitochondrial status). The system must be modular and allow specific adjustments required by different semen samples. In this study, we designed and evaluated such an *in vitro* device to evaluate ram semen using some specific operating parameters already defined in a previous study (Martínez-Rodríguez *et al.* 2012) and included new variables that affect sperm motility (mucus substitute, migration distance, angle of migration). We quantified the population of migrating spermatozoa and analysed some of their quality parameters to allow an evaluation of the effects of migration stress on sperm physiology and to determine its value as a diagnostic tool of semen quality.

Materials and methods

Experimental design

Experiment 1: device design

The aim of this experiment was to design a device for an *in vitro* sperm-progression test that allows the collection of spermatozoa that have the physiological potential to overcome a defined barrier (Fig. 1). Once the device was designed, the next

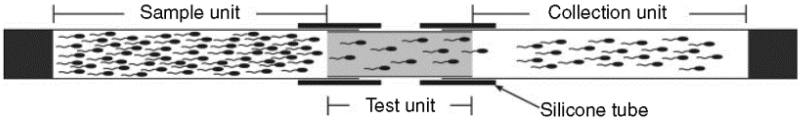

Fig. 1. Artificial mucus-penetration test device composed of three parts assembled by means of two silicone tubes (diameter 0.4 cm and length 1 cm): (1) a 0.5-mL plastic straw of 5 cm length (diameter 0.3 cm) filled with the sperm sample (sample unit), (2) a 0.25-mL plastic straw of 2 or 3 cm length (diameter 0.15 cm) filled with the cervical mucus surrogate (test unit) and (3) a 0.5-mL plastic straw of 5 cm length (diameter 0.3 cm) filled with TCF to collect the spermatozoa after migration (collection unit).

step was to define the working conditions of the device, taking into account conditions previously determined by us (25×10^6 spermatozoa mL^{-1}, incubation temperature of 39°C and 1.6% polyacrylamide as synthetic mucus; Martínez-Rodríguez *et al.* 2012). The parameters to be assayed in this first experiment were as follows: (1) two lengths of synthetic mucus (2 cm (S2) and 3 cm (S3)) and (2) three incubation times (15, 30 and 60 min (T15, T30 and T60)). We assessed (1) the number of cells traversing the synthetic mucus (2 or 3 cm, S2 or S3) and (2) the sperm quality (viability and acrosomal status) of the fresh sample and in those spermatozoa that were not able to traverse the device to evaluate variations in seminal quality due to the *in vitro* conditions during the assay. Semen from five rams collected on three days was used, and 10 technical replicates were performed for each experimental condition.

Experiment 2: establishment of different test variables

Based on the results obtained in Experiment 1, we set 30 min as the most convenient incubation time. We then tested (1) two synthetic mucus media (polyacrylamide gel (Acryl 1.6) and methylcellulose (MC 1.3)), (2) four sperm concentrations (12, 25, 50 and 100×10^6 spermatozoa mL^{-1}) and (3) two positions of the surface (0° and 45° to the horizontal) of the device during incubation. In this experiment, we examined (1) the number of spermatozoa overcoming the mucus barrier for every variable combination and (2) the sperm quality (viability and acrosomal status) of the sample before performing the test and in those spermatozoa that were not migrating to evaluate variations in seminal quality due to the *in vitro* conditions during the assay. Semen from five rams collected on four days was used, and 10 technical replicates were performed for each experimental condition.

Experiment 3: qualitative analysis of the spermatozoa population that travels through the synthetic cervical mucus

For this experiment, we used the device settings that showed the best results in Experiment 2: 30 min incubation at 39°C, a 2-cm segment of 1.6% polyacrylamide as the synthetic mucus barrier and a sperm concentration of 100×10^6 spermatozoa mL^{-1}. We again analysed two positions of the device during the incubation period (0° and 45°). We studied the following: (1) the number of cells traversing the mucus barrier, (2) the sperm quality (motility, viability and acrosomal and mitochondrial status) of the sample before performing the test and in those spermatozoa that were not able to traverse the device and (3) the sperm quality (viability and acrosomal and mitochondrial status) of the spermatozoa that passed through the mucus (it was not possible to analyse sperm motility). A post-migration qualitative analysis was exclusively performed in this experiment because of the small number of cells obtained (see the comment below). Semen from five rams collected on three days was used, and 10 technical replicates were performed for each experimental condition. The repeatability between the different measurements of the migration test was assessed in this experiment.

Animals

Ethics statement

Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were performed after obtaining approval from the Ethical Committee for Experimentation with Animals of León University (Spain).

Ram sperm collection

The semen from five adult Assaf rams (*Ovis aries*) of proven fertility was collected twice per week for five weeks by means of an artificial vagina (39°C) in the presence of a female decoy. The glass collection tube was placed in a thermoregulated bath at 39°C and a preliminary seminal evaluation was performed (volume, mass motility and concentration). The ejaculates used in the experiment were those with a volume higher than 0.5 mL, a mass motility ≥4 (determined by microscopy with warming stage at 39°C, 40× and 0–5 score) and a sperm concentration greater than 3000×10^6 spermatozoa mL^{-1}. The sperm concentration was assessed using a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) with CASA (Integrated Semen Analyzer System (ISAS); Proiser, Valencia, Spain). Ten minutes after collection, the ejaculates (five rams) were diluted in 0.27 M Tris, 90 mM citric acid and 53 mM fructose (TCF) at 1600×10^6 spermatozoa mL^{-1} and seminal plasma was removed; the same volume from each ejaculate was mixed to produce a pool. Lastly, the pool was diluted to a concentration of 12, 25, 50 or 100×10^6 spermatozoa mL^{-1} (depending on the experiment performed) in TCF. At this time, a semen quality analysis (motility, viability and acrosomal and mitochondrial status) was performed using the fresh sample.

Synthetic cervical mucus

Cervical mucus substitutes for *in vitro* sperm-penetration tests applied in the present study have been used in previous studies (review in Martínez-Rodríguez *et al.* 2012).

Polyacrylamide gel (1.6%) was prepared by diluting 30% liquid acrylamide (30% ProtoGel; National Diagnostics, Atlanta, GA, USA) with the corresponding volume of 1.5 M Tris (pH 8.8) in purified water. Once mixed, a solution of 2% ammonium persulfate (Sigma-Aldrich, Madrid, Spain) up to 1.5% of the final volume and 0.05% tetramethylethylenediamine (TEMED; Sigma-Aldrich) were added to induce polymerisation. The mixture (pH 7.2, 320 mOsm kg^{-1}) was left at room temperature for 24 h and then stored at 4°C until use.

After studying the rheological properties of the media of different methylcellulose concentrations following a previously described procedure (Martínez-Rodríguez *et al.* 2012; data not shown), we selected 1.33% methylcellulose (MC_1.33) as the most suitable methylcellulose concentration to be used as ovine synthetic cervical mucus. For the methylcellulose preparation, a volume of purified water equal to 1/3 of the final volume of the medium was heated to 80°C and the corresponding amount of methylcellulose powder (MC 1500; Sigma-Aldrich) was added. When the methylcellulose was dispersed in the water, another

1/3 of the final volume of cold 2× TCF was added, and the mixture was refrigerated at 4°C; another 1/3 of the final volume of cold TCF was added and the solution was agitated for 30 min. The prepared methylcellulose was stored at 4°C until use.

Sperm-progression test

The device designed for this *in vitro* migration test was a tubular structure composed of three parts assembled by means of two silicone tubes (1 × 0.04 cm) (Fig. 1): (1) a 0.5-mL plastic straw of 5 cm length (diameter 0.3 cm) filled with the sperm sample (sample unit), (2) a 0.25-mL plastic straw of 2 or 3 cm length (diameter 0.15 cm) filled with the cervical mucus surrogate (test unit) and (3) a 0.5-mL plastic straw of 5 cm length (diameter 0.3 cm) filled with TCF to collect the spermatozoa after migration (collection unit). The design of the device was influenced by the rheological properties of sheep cervical mucus and the surrogates, as previously described by our group (Martínez-Rodríguez *et al.* 2012). Preliminary tests in our laboratory showed that, if a mucus surrogate with high consistency index (equivalent to cow cervical mucus) was used, then the test device was assembled only with two parts: sample unit and a mixed unit (the test unit plus collection unit). The high fluidity of the mucus surrogate similar to sheep cervical mucus requires mounting the test unit as a separate piece because, when we attempted to use only one straw to contain the collection and test units, it was not possible to obtain the two phases in the straw due to the high diffusion of the synthetic mucus in the TFC during the straw-filling process.

The device was maintained at 39°C in all experiments, and 10 replicates were performed in Experiments 1 and 2. Once the migration test was completed, the contents of each collection unit were individually placed in a 1.5-mL tube (ten tubes) to count the number of migrating spermatozoa; 100 µL of 5 mg mL^{-1} Hoechst 33342 was added to each tube for 10 min and the tubes were then centrifuged at 600*g* for 5 min at room temperature (Alvarez *et al.* 2012*a*). The supernatant was removed, the pellet was suspended in 10 µL TCF and each cell suspension was transferred individually to a well of an 8-well slide. The contents of the 10 sample units (spermatozoa that did not overcome the mucus barrier) were also pooled in a tube, and the motility, viability and acrosomal and mitochondrial status of the spermatozoa were analysed.

In Experiment 3 we performed fourteen replicates each time the test was performed. After the migration test, six replicates were used to count the spermatozoa that migrated to the collection unit (as described for Experiments 1 and 2) and eight replicates were dedicated to evaluating sperm quality by flow cytometry. Due to the small number of spermatozoa available, to assess the postmigration semen quality the contents of four collection units were pooled in a 1.5-mL Eppendorf tube, centrifuged and resuspended in lectin PNA from Arachis hypogaea (peanut)-FITC conjugate/propidium iodide/Hoechst 33342 solution; the contents of the other four units were pooled, centrifuged and resuspended in Mitotracker Deep Red/YO-PRO-1/Hoechst 33342 solution. After 10 min, these samples were analysed by flow cytometry. The spermatozoa in the sample unit were analysed as in previous experiments.

The slides were analysed using an inverted microscope T2000 U (Nikon, Tokyo, Japan) equipped with a mercury-fibre illuminator (Intensilight C-HGFI; Nikon), motorised stage (PRIOR ProScan II; Prior Scientific, Cambridge, UK) and digital camera (ORCA; Hamamatsu, Tokyo, Japan). The motorised plate and digital camera were automatically controlled by Metamorph Version 7 software (Molecular Devices, Inc., Downingtown, PA, USA). Each well of different slides was automatically photographed at 10×, capturing 96 images at wavelengths of 435–485 nm. These images were analysed with the image analysis software NIS Elements Version 3 (Nikon) to count the spermatozoa in each well. Specific adjustments to discriminate spermatozoa with regard to debris, according to their shape and size, were applied in this analysis (Martínez-Rodríguez *et al.* 2012).

Sperm motility evaluation

The spermatozoa were diluted in TCF to 10 to 20 × 10^6 spermatozoa mL^{-1} and loaded into a Makler counting chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) at 39°C. The CASA system consisted of a triocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon) using a 10× negative phase-contrast objective equipped with a warming stage at 39°C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). The images were captured at 50 frames s^{-1} and analysed using a computer-assisted motility analyser (ISAS; Proiser) with settings specific for ram spermatozoa. The software rendered the following parameters: (1) percentage of motile spermatozoa (PM) and (2) for each spermatozoon, three velocity parameters (velocity according to the actual path, VCL; velocity according to the straight path, VSL and velocity according to the smoothed path, VAP), three track linearity parameters (linearity, LIN; straightness, STR; wobble, WOB), the amplitude of the lateral displacement of the sperm head (ALH) and the head beat-cross frequency (BCF).

Assessment of sperm quality by flow cytometry

We assessed sperm quality by flow cytometry at three different steps during the study: fresh sperm, spermatozoa that were not able to travel through the mucus and spermatozoa that traversed the cervical mucus. For this last point, we needed to pool together five test replicates because we otherwise would not have had enough cells to be able to perform the standard analysis.

Acrosomal status and viability

Triple staining with PNA-FITC, propidium iodide (PI) and Hoechst 33342 (all Sigma Aldrich) was used for this assessment. The staining was performed by diluting the sperm sample (1–2 million spermatozoa mL^{-1}) in 300 µL of PBS with 1 µg mL^{-1} PNA-FITC, 1.5 µM PI and 5 µM Hoechst 33342. The samples were analysed by flow cytometry after 10 min at room temperature in the dark. The spermatozoa (Hoechst+) were classified into three sperm subpopulations: PI+ (not viable), PI−/PNA+ (viable with damaged acrosome) and PI−/PNA− (viable with intact acrosome).

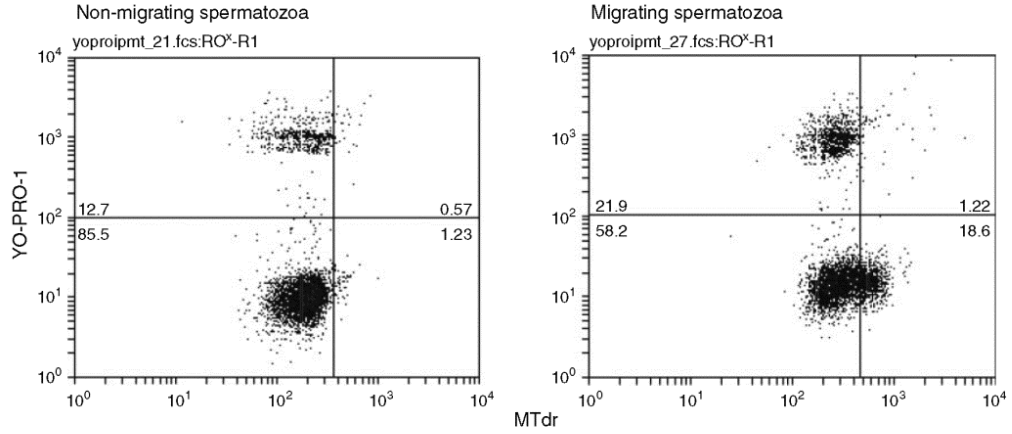

Fig. 2. Flow cytometric diagrams for Mitotracker versus YOPRO-1 labelling of non-migrating or migrating spermatozoa in 1.6% polyacrylamide after 30 min of incubation at 39°C with test tube at 45°C.

Mitochondrial status

Triple staining with YO-PRO-1, Mitotracker Deep Red (Invitrogen) and Hoechst 33342 was used to examine the mitochondrial status. The sperm samples were diluted (1–2 million spermatozoa mL^{-1}) in 300 μL of PBS, and 100 nM YO-PRO-1, 100 nM Mitotracker Deep Red and 5 μM Hoechst 33342 were added. The samples were analysed by flow cytometry after 10 min at room temperature in the dark. One population of spermatozoa (Hoechst+) was considered: Mitotracker+/YO-PRO-1− spermatozoa were classified as viable spermatozoa with active mitochondria (Fig. 2).

Flow cytometry analysis

Flow cytometry analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with 405-nm, 488-nm and 635-nm lasers. YO-PRO-1, PNA-FITC and PI were excited at 488 nm, Mitotracker Deep Red was excited at 635 nm and Hoechst 33342 was excited at 405 nm. The fluorescence emitted by YO-PRO-1 and PNA-FITC was analysed using the FL1 photodetector (530/40 band-pass filter), PI and Mitotracker Deep Red fluorescence was analysed using the FL3 photodetector (670 long-pass filter) and Hoechst 33342 fluorescence was analysed using the FL6 photodetector (450/50 band-pass filter). The signals, forward scatter/side scatter (FSC/SSC) and Hoechst 33342 fluorescence were used to discriminate the sperm population from other events. For each sample, we acquired 5000 spermatozoa using Summit Version 4.3 software (Beckman Coulter). Calibration was carried out periodically using standard beads (Calibrite; Becton Dickinson BioSciences, Madrid, Spain). The analysis of flow cytometry data was performed using the Weasel Version 2.6 program (the Walter and Eliza Hall Institute of Medical Research, Parkville, Vic., Australia).

Statistical analysis

The results are presented as the mean ± s.e.m. (standard error of the mean). The data were analysed with the STATISTICA Version 9 (StatSoft, Tulsa, OK, USA) program using the general linear models (GLM procedure) or, where appropriate, the Pearson correlation coefficient. GLM was used to study sperm progression in the device to examine the effect of different factors on the sperm count and sperm qualitative parameters (post-hoc comparison by Fisher l.s.d. test), as follows: Experiment 1, two lengths of synthetic cervical mucus (2 cm or 3 cm) and three incubation times (15, 30 and 60 min); Experiment 2, two synthetic mucus media (polyacrylamide and methylcellulose), four sperm concentrations (12, 25, 50 and 100×10^6 spermatozoa mL^{-1}) and two positions regarding the position of the surface (0° or 45°) and Experiment 3, three sampling sessions and two positions (0° or 45°). A significance level of $P < 0.05$ was used. A repeatability analysis of inter-session measurements was performed in Experiment 3 by calculating the coefficients of variation (CV) and the standard errors (s.e.m.) with the pooled semen of the same five males on three different days (D1, D2 and D3; Vyt *et al.* 2004).

Results

Experiment 1: device design

Fig. 3 shows the number of spermatozoa that migrated through the segment of mucus surrogate (2 or 3 cm) in the device after 15-, 30- and 60-min incubations at 39°C. We observed a significantly higher number of spermatozoa migrating through the device with a 2-cm synthetic mucus segment for every incubation time, and the migration through the artificial mucus was significantly higher after 60 min than 15 min using both segment lengths. The viability and acrosomal status of the spermatozoa that remained in the sample unit is shown in Table 1. A significant increase in acrosomal damage and a significant decrease in viability after the 60-min incubation at 39°C were consistently observed for both types of device (2 or 3 cm).

The samples incubated for 15 or 30 min displayed no significant differences in sperm quality (viability and acrosomal status) compared with the control sample, although differences

in terms of the number of spermatozoa that migrated were observed, being higher after 30 min.

Experiment 2: setting for progression test

Different parameters that determine the behaviour of the sperm cells during the test were analysed: two cervical mucus surrogates, two lengths of synthetic mucus segment, two incubation positions with respect to the horizontal plane and four sperm concentrations. The count of spermatozoa that travelled through the test unit is shown in Figs 3 and 4. As expected, the sperm concentration of the samples significantly influenced the number of migrating spermatozoa, being higher for concentrations C100 and C050 (12, 25, 50 and 100 $\times$ 10^6 spermatozoa mL^{-1}; C012, C025, C050 and C100 respectively) under all conditions analysed (Fig. 4). In general, the methylcellulose-based test showed a significantly lower number of migrating spermatozoa than the polyacrylamide-based test, and an increase in acrosomal damage was observed in some samples of the methylcellulose-based test. Additionally, the migration distance showed significant differences between the samples, with higher values for the shorter distance (2 cm).

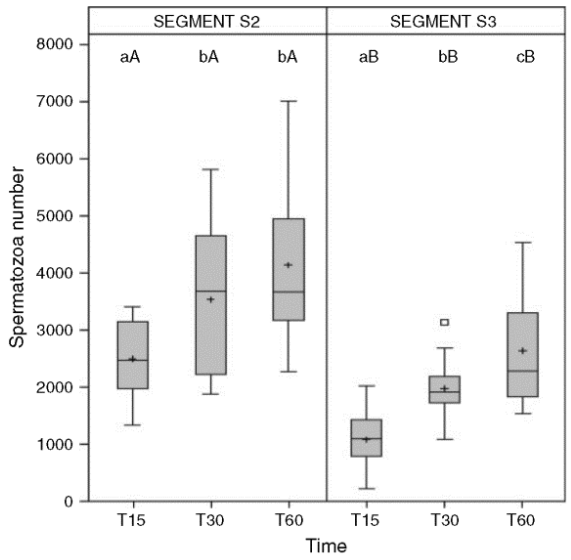

Fig. 3. Number of spermatozoa that progressed through a synthetic mucus segment 2 cm (S2) or 3 cm (S3) in length after incubation for 15 (T15), 30 (T30) and 60 min (T60) at 39°C (sample, 25 $\times$ 10^6 spermatozoa mL^{-1}; mucus, 1.6% polyacrylamide). The lowercase letters indicate significant differences between the different incubation times for the same length. The capital letters indicate significant differences between the synthetic mucus lengths for the same incubation time.

A dilution effect was also observed for the quality of the sperm cells that were collected from the sample unit after incubation: the C100 concentration sample revealed a viability and acrosomal status similar to the control sample, whereas the C012 and C025 samples had significantly lower values (Table 2).

The position of the test device with respect to the horizontal plane induced significant differences in the number of sperm cells that migrated in the polyacrylamide-based test, with higher values for the tests performed with the device positioned at an angle of 45°; however, these differences were not observed for the methylcellulose-based test (Fig. 5). In this experiment, the viability and acrosomal status of non-migrating spermatozoa was not affected by the conditions of the study (Table 3).

Experiment 3: qualitative analysis of the post-migration sperm population

The counts of spermatozoa that migrated through the polyacrylamide-based tests are summarised in Fig. 6. The sperm count for the three consecutive assays was not significantly different for each position analysed, though differences were observed between the values for each position. Overall, the CV were consistently lower that 30%, and the s.e.m. values were small (less that 10% of mean), indicating an acceptable repeatability of the methods.

Table 4 shows the sperm quality parameters (motility, viability, acrosomal status and mitochondrial membrane potential) for the control sample, the sperm sample that did not pass through the synthetic mucus (sample unit) and the sperm population that migrated (collection unit). The motility parameter of non-migrating spermatozoa showed significantly lower

Table 1. Percentage of spermatozoa with acrosomal damage and percentage of viable spermatozoa in the fresh sample (control) and in the sample of spermatozoa that remained in the sample unit of the device after incubation

The factors analysed were the incubation time (15, 30 and 60 min) of two devices (2 or 3 cm of synthetic mucus, S2 or S3). [a,b]Lowercase letters indicate significant differences between the incubation times for the same synthetic mucus length. *Significant difference with the fresh sample

Sample	Synthetic mucus length	Incubation time	Acrosomal damage (%)	Viability (%)
Fresh sample			8.7 ± 1.5	79.4 ± 1.3
Sample unit (after incubation)	S2	T15	10.8 ± 0.7[a]	76.8 ± 0.5[a]
		T30	11.6 ± 0.6[ab]	76.8 ± 2.1[a]
		T60	12.9 ± 0.5[b]*	70.6 ± 1.3[b]*
	S3	T15	12.4 ± 0.5[a]	72.9 ± 1.3[a]
		T30	11.9 ± 0.3[a]	69.2 ± 1.2[ab]
		T60	14.2 ± 0.5[b]*	65.9 ± 1.4[b]*

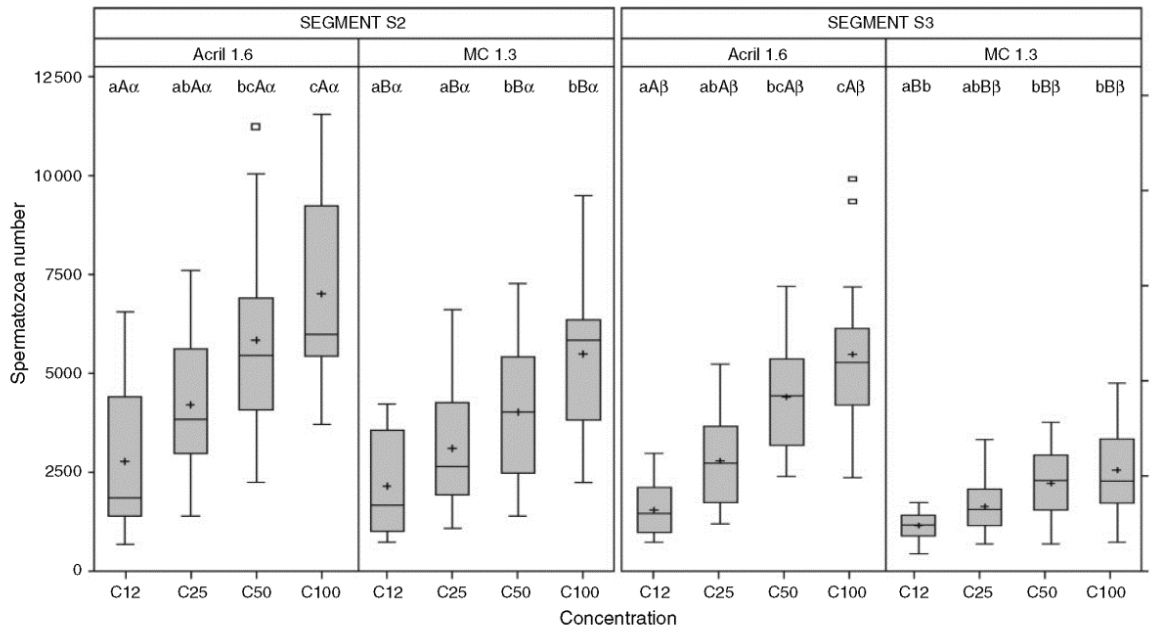

Fig. 4. Number of spermatozoa that progressed a distance of 2 cm (S2) or 3 cm (S3) through synthetic mucus (polyacrylamide (Acryl 1.6) or methylcellulose (MC 1.3)) using four different sperm concentrations (12, 25, 50 and 100×10^6 spermatozoa mL^{-1}; C012, C025, C050 and C100 respectively) after a 30-min incubation at 39°C in a horizontal position. The lowercase letters indicate significant differences between the different sperm concentration samples for the same distance and the same synthetic mucus. The capital letters indicate significant differences between the synthetic mucus for the same segment distance and sperm concentration. The Greek characters indicate significant differences between the distances for the same synthetic mucus and sperm concentration.

Table 2. Percentage of spermatozoa with acrosomal damage and percentage of viable spermatozoa in the fresh sample (control) and in the sample of spermatozoa that did not migrate (sample unit) after 30-min incubation

The factors analysed were the mucus surrogate (polyacrylamide (Acryl 1.6) or methylcellulose (MC 1.3)) and sperm concentration (12, 25, 50 or 100×10^6 spermatozoa mL^{-1}) with two devices (2 or 3 cm of synthetic mucus, S2 or S3). [a,b]Lowercase letters indicate significant differences between the different sperm concentration samples for the same distance and the same synthetic mucus. *Indicates a significant difference with the fresh sample

Segment	Mucus surrogate	Sperm concentration	Acrosomal damage (%)	Viability (%)
Fresh sample			8.1 ± 0.2	80.2 ± 0.6
S2	Acryl 1.6	C012	12.0 ± 0.7^{a}*	75.5 ± 1.1^{a}*
		C025	11.1 ± 0.6^{a}*	77.4 ± 1.5^{a}*
		C050	8.8 ± 0.5^{b}	83.1 ± 0.9^{b}
		C100	9.5 ± 0.9^{b}	81.1 ± 1.2^{b}
	MC 1.3	C012	11.0 ± 0.4^{a}*	75.2 ± 1.3^{a}*
		C025	10.2 ± 0.9^{ab}*	73.1 ± 1.3^{a}
		C050	9.4 ± 0.8^{ab}*	82.8 ± 1.1^{b}
		C100	9.2 ± 0.8^{b}	81.7 ± 0.9^{b}
S3	Acryl 1.6	C012	11.7 ± 0.5^{a}*	68.2 ± 1.0^{a}*
		C025	12.0 ± 0.5^{a}*	72.3 ± 1.3^{a}
		C050	9.8 ± 0.6^{ab}	73.6 ± 1.2^{a}
		C100	8.8 ± 0.3^{b}	74.8 ± 1.4^{a}
	MC 1.3	C012	16.0 ± 0.8^{a}*	64.1 ± 3.4^{a}*
		C025	14.4 ± 1.1^{a}*	67.6 ± 2.4^{a}*
		C050	10.8 ± 0.8^{b}	72.1 ± 1.9^{a}
		C100	9.9 ± 0.5^{b}	73.7 ± 1.0^{a}

values than the fresh sample for both incubation positions. The viability and acrosomal status of the migrating spermatozoa showed no significant changes from the control at the 45° position, but the samples placed at the 0° position showed lower values. The percentage of spermatozoa with a high mitochondrial membrane potential was significantly reduced in the population of cells that migrated through the synthetic mucus.

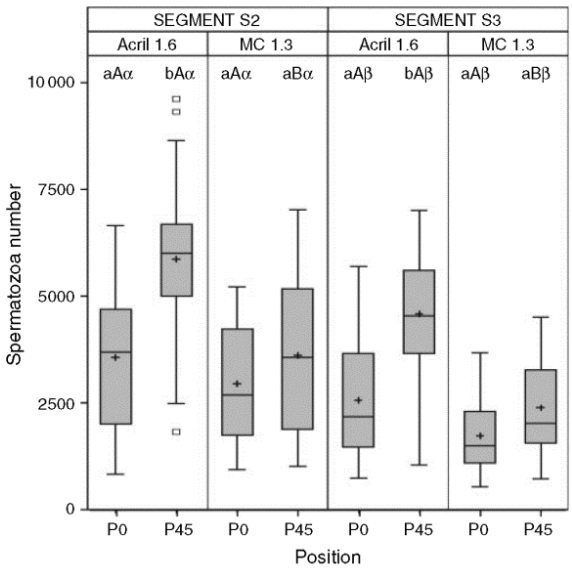

Fig. 5. Number of spermatozoa in 100×10^6 spermatozoa mL^{-1} samples that progressed a distance of 2 cm (S2) or 3 cm (S3) in two types of synthetic mucus (polyacrylamide (Acryl 1.6) or methylcellulose (MC 1.3)) after 30 min of incubation at 39°C in two positions with regard to the incubator surface (P0 = 0° (horizontal) and P45 = 45°). The lowercase letters indicate significant differences in the number of progressing spermatozoa between the incubation positions for the same synthetic mucus length and the same synthetic medium. The capital letters indicate significant differences in the number of progressing spermatozoa between synthetic media for the same synthetic mucus length and incubation position. The Greek characters indicate significant differences in the number of progressing spermatozoa between the segment lengths of synthetic mucus for the same synthetic medium and incubation position.

Discussion

The evaluation of sperm motility *in vitro* is one of the most common predictors in the screening of males for fertility, and different techniques have been developed. Sperm motility is

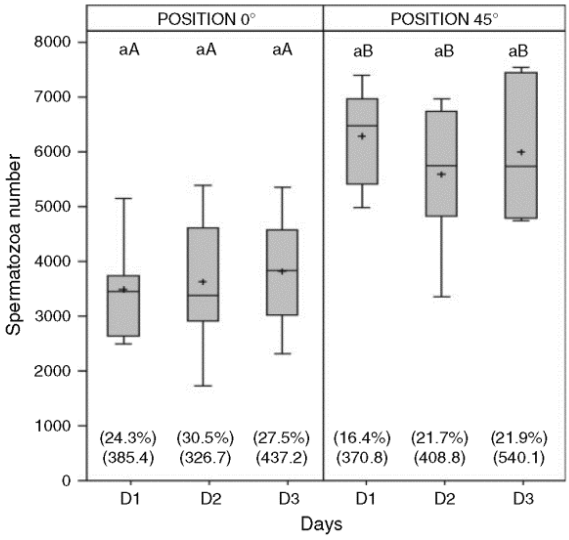

Fig. 6. Number of spermatozoa in a 100×10^6 spermatozoa mL^{-1} sample that progressed a distance of 2 cm in 1.6% polyacrylamide after 30 min of incubation at 39°C. The factors analysed were the incubation position (0° or 45°) and the sampling day (D1, D2 and D3). The CV (%) and s.e.m. values for the three consecutive days of analysis are shown in parentheses. The lowercase letters indicate significant differences in the number of spermatozoa between sampling day for the same incubation position. The capital letters indicate significant differences in the number of spermatozoa between incubation positions for the same sampling day.

Table 3. Percentage of spermatozoa with acrosomal damage and percentage of viable spermatozoa in the fresh sample (control) and in the sample of spermatozoa that did not migrate (sample unit) after 30 min incubation

The factors analysed were the mucus surrogate (polyacrylamide (Acryl 1.6) or methylcellulose (MC 1.3)) and the incubation position (0° or 45°) with two devices (2 or 3 cm of synthetic mucus, S2 or S3). [a,b]Lowercase letters indicate significant differences between the incubation positions for the same segment distance and the same synthetic mucus. *Indicates a significant difference with the fresh sample

Segment	Mucus surrogate	Position	Acrosomal damage (%)	Viability (%)
Fresh sample			9.8 + 0.2	79.2 + 0.5
S2	AC 1.6	P0	10.8 + 0.9[a]	81.5 + 3.2[a]
		P45	9.4 + 0.3[a]	80.9 + 4.1[a]
	MC 1.3	P0	8.1 + 1.3[a]	77.4 + 4.8[a]
		P45	8.4 + 1.2[a]	81.5 + 5.4[a]
S3	AC 1.6	P0	10.7 + 1.1[a]	71.9 + 3.6[a]
		P45	10.8 + 0.8[a]	71.5 + 2.3[a]
	MC 1.3	P0	8.5 + 1.5[a]	68.4 + 4.6[a]*
		P45	11.9 + 1.8[b]*	71.9 + 1.5[a]

complex, and *in vitro* sperm-migration tests have been developed using cervical mucus or cervical mucus surrogates to mimic the ability of spermatozoa to migrate through the female reproductive tract (Aitken 2006).

In a previous study with ram spermatozoa (Martínez-Rodríguez *et al.* 2012), we analysed a progression test based on polyacrylamide gel as a cervical mucus surrogate and demonstrated that 1.6% polyacrylamide is similar to ovine cervical mucus with regard to rheological properties and can be used to simulate ovine cervical mucus for *in vitro* sperm-progression tests. In this regard, we observed a significant correlation between the sperm count obtained in the polyacrylamide-based test and some sperm quality parameters obtained in the sample (positive: progressive motility and velocity according to the straight path; negative: damaged acrosomes and apoptotic cells). Indeed, the stress induced by mucus penetration assays imposes meaningful limitations on the physiological function of spermatozoa; therefore, we suggest that a dynamic cellular subpopulation can be isolated and analysed to determine several critical parameters for reproductive success.

The first aim of the present study was to evaluate the conditions of use of an *in vitro* test that allows the analysis of a large number of migrating spermatozoa through synthetic mucus. The device we designed for this experiment was determined by the characteristics of 1.6% polyacrylamide as a homogenous surrogate for sheep cervical mucus. The gel composed of polyacrylamide had a high fluidity for cervical mucus simulation, preventing the assessment of the device arranged vertically as it was not possible to fill a straw with TCF and synthetic mucus in two different phases. The number of migrating spermatozoa will be influenced by the duration of the trial, but this factor may also adversely affect the physiology of the spermatozoa themselves. Our results showed that a 60-min incubation time to perform the progression test significantly increased the number of spermatozoa that migrated but negatively affected their viability and acrosomal status. Considering this result, together with our goal of obtaining the greatest number of migrating spermatozoa to analyse the subpopulation that migrates through the synthetic mucus, we decided to discard the 60-min incubation time because of the cell damage found as well as the 15-min incubation time because of the small number of migrating cells.

The problems associated with the use of cervical mucus in sperm-migration tests (e.g. variable composition, availability difficulty) have led to the identification of surrogates. Polyacrylamide, hyaluronic acid and methylcellulose, among others, have been used (Aitken *et al.* 1992; Perry *et al.* 1996; Engel and Petzoldt 1999; Tang *et al.* 1999; Anilkumar *et al.* 2001; Ivic *et al.* 2002). Martínez-Rodríguez *et al.* (2012) showed that the rheological properties of 1.6% polyacrylamide were similar to those of ovine cervical mucus, and these authors suggested that polyacrylamide gel was a favourable medium for ram spermatozoa *in vitro* penetration tests. Polyacrylamide has been replaced by other substances in human *in vitro* tests due to its reduced efficiency in allowing sperm migration (Ivic *et al.* 2002). Based on this information, we evaluated methylcellulose as second substitute for cervical mucus. Our results showed that the number of migrating spermatozoa was highly dependent on the synthetic medium and that polyacrylamide gel was significantly more permissive than methylcellulose in allowing the migration of ram spermatozoa under the different conditions tested. These results contrast with those published for human semen (Ivic *et al.* 2002), which reported that polyacrylamide exhibits reduced efficiency in migration tests. Other studies have documented similar differences between hyaluronic acid and methylcellulose in human sperm-migration tests, with the

Table 4. Motility parameters, viable spermatozoa, spermatozoa with acrosomal damage (dACRO) and spermatozoa with a high mitochondrial membrane potential (High MITO) for the fresh sample (control), the spermatozoa that did not migrate (sample unit) and the spermatozoa migrating (collection unit) through 2 cm of 1.6% polyacrylamide gel after 30 min of incubation at 39°C in two positions (0° (P0) or 45° (P45))

TM, total motility (%); PM, % progressive motility; VAP, average path velocity (μm s^{-1}); VCL, curvilinear velocity (μm s^{-1}); VSL, straight-line velocity (μm s^{-1}); LIN, linearity index (%); STR, straightness (%); ALH, amplitude of lateral head displacement (μm); BCF, head beat-cross frequency (%). ^{a,b}Lowercase letters indicate significant differences between the incubation positions for the same sample (non-migrating or migrating). ^{A,B}Capital letters indicate significant differences between the sample (non-migrating or migrating) for the same incubation position. *Indicates a significant difference with the fresh sample (control)

Parameter	Fresh sample	Non-migrating spermatozoa		Migrating spermatozoa	
		P0	P45	P0	P45
TM	74.6 + 4.9	45.9 + 12.0^{a}*	34.7 + 11.3^{a}*		
PM	45.3 + 4.6	30.2 + 9.0^{a}*	19.2 + 2.7^{b}*		
VAP	87.9 + 5.7	32.6 + 3.2^{a}*	34.9 + 4.7^{a}*		
VCL	132.5 + 10.1	51.3 + 7.5^{a}*	53.4 + 9.3^{a}*		
VSL	63.0 + 5.6	18.7 + 3.1^{a}*	15.3 + 3.0^{a}*		
LIN	47.5 + 5.5	33.2 + 3.7^{a}*	31.3 + 2.7^{a}*		
STR	67.3 + 3.7	56.1 + 6.1^{a}	45.2 + 3.8^{a}*		
ALH	3.5 + 0.2	2.2 + 0.3^{a}*	1.7 + 0.2^{a}*		
BCF	11.8 + 1.8	7.8 + 0.7^{a}*	6.8 + 1.5^{a}*		
dACRO (%)	9.0 + 2.2	11.4 + 1.6^{aA}	9.9 + 1.3^{bA}	18.1 + 3.1^{aB}*	11.2 + 1.1^{bA}
Viability (%)	74.7 + 2.2	70.2 + 1.4^{aA}	71.6 + 5.3^{aA}	59.2 + 5.7^{aB}*	68.8 + 3.9^{bA}
High MITO (%)	39.4 + 6.1	41.1 + 5.3^{aA}	38.5 + 3.4^{aA}	13.9 + 1.7^{aB}*	12.9 + 1.5^{aB}*

sperm-migration count significantly higher when using methylcellulose (Ivic *et al.* 2002). These data highlight the specific differences between spermatozoa from different species in terms of their biophysical characteristics and indicate that an appropriate mucus substitute for sperm-migration tests should be chosen for each species. These mucus surrogates should possess the same sperm penetrability characteristics as cervical mucus; in our experiment, these features were confirmed via an evaluation of the index of medium consistency (Martínez-Rodríguez *et al.* 2012). However, other properties appear to be necessary to provide adequate support for sperm migration.

The number of spermatozoa that penetrate into a mucus substitute in a progression test is highly dependent on the concentration of spermatozoa in the sample, and we observed a linear relationship between these two parameters for the two media tested. The spermatozoa that remained in the sample unit during the test showed changes in the functional parameters that depend on the sperm concentration. In general, the sample with 12×10^6 or 25×10^6 spermatozoa mL^{-1} showed a significant reduction in viability and an increase in acrosomal damage, whereas the 100×10^6 spermatozoa mL^{-1} sample showed values similar to the control sample. The effects of sperm concentration on sperm quality parameters (Kasimanickam *et al.* 2007; Gundogan *et al.* 2010; López-Fernández *et al.* 2010) and fertility (Alvarez *et al.* 2012*b*) have been investigated during ram sperm storage. Overall, changes in sperm motility, morphology and sperm DNA integrity of ram spermatozoa extended to 100×10^6 spermatozoa mL^{-1}, and short-term preservation at 4°C was better than when the sample was diluted to 25×10^6 spermatozoa mL^{-1} (Gundogan *et al.* 2010). Our results show similarities with studies reporting that the quality of spermatozoa was better preserved at a higher concentration (Kasimanickam *et al.* 2007; Gundogan *et al.* 2010); however, López-Fernández *et al.* (2010) observed that a lower sperm concentration (6 and 12×10^6 spermatozoa mL^{-1}) resulted in a slower rate of DNA fragmentation. Kasimanickam *et al.* (2007) suggested that the possible physiological reasons for the decline of quality at low concentrations might be extracellular oxidative stress and that a higher volume of seminal plasma surrounding each spermatozoon in the sample at a higher concentration may be a reason for the better preservation of functional parameters.

Our study revealed that the ability of spermatozoa to travel through polyacrylamide was significantly stimulated when the device was inclined at an angle of 45° compared with what was observed with tubes at 0°; this effect was not observed in the methylcellulose assay, even though the mean values were always higher at the 45° position. Due to the technical difficulties discussed above, we did not perform this analysis with the test tubes at 90°. The influence of gravity and fluid flow on the migratory movements of spermatozoa has been studied (Winet *et al.* 1984; Makler *et al.* 1993; Katz and Pedrotti 1997; Miki and Clapham 2013), and motile spermatozoa undergo geotaxis *in vitro* through vertical columns containing various suspension media (Miki and Clapham 2013). The apparent taxic responses of human spermatozoa to gravity, fluid flow and rigid boundaries have been investigated by Winet *et al.* (1984) in semen samples drawn through a tube containing a culture medium. These authors showed that the most important factors for sperm distribution were negative rheotaxis and accumulation near the walls, whereas the response to gravity was relatively weak. In another study, a notable effect of gravitation on human spermatozoa was observed such that the number of motile spermatozoa that swam downward was 5–6 times more than those swimming upward (Miki and Clapham 2013). Although these data do not allow us to interpret the results we observed in the test at 45° position on the basis of a negative geotaxic effect, we must take into account the nature of artificial mucus with regard to moving spermatozoa. Indeed, cervical mucus is a fluid that changes in viscosity in response to shear and proteinases that may be secreted by the spermatozoa, thus creating a local boundary effect that alters the direction of spermatozoa (Winet *et al.* 1984). The polyacrylamide-based mucus in our experiment could present this configuration with a local boundary effect and a rheotaxis-like effect inducing the swim-up behaviour of spermatozoa. The polyacrylamide column is stable at the 45° position, but it is possible that the polyacrylamide is dissolving in the TCF medium at the TCF-polyacrylamide interface, which may cause microcurrents within the test unit that stimulate the ability of motile spermatozoa to orient themselves and migrate against the flow of the surrounding fluid. This phenomenon is consistent with the theory that rheotaxis is a major determinant of mammalian sperm guidance over long distances in the female reproductive tract (Miki and Clapham 2013).

The capacity of spermatozoa to overcome a cervical mucus barrier is an important determinant for that subpopulation of spermatozoa with normal morphology and vigorous motility to arrive at the fertilisation site (Suarez and Pacey 2006). *In vitro* migration tests have been developed to assess this ability of spermatozoa to migrate through cervical mucus, analysing, in most cases, the total distance travelled by the vanguard spermatozoon. In this study, we evaluated the number of spermatozoa that penetrated beyond 2 or 3 cm into the mucus surrogate and analysed the qualitative parameters of the spermatozoa migrating to this distance. This subpopulation showed a significant reduction of spermatozoa with a high mitochondrial membrane potential at the two positions tested. The spermatozoa migrating at the horizontal position underwent a significant reduction in viability and acrosome integrity, which was not observed in the cells migrating at 45°. The differences obtained between the two positions of migration tested suggest that the migrating subpopulations are not equivalent and that there must be physicochemical factors that differentially stimulate sperm motility. However, in both cases, the mitochondrial status was changed after the efforts of the sperm cells in the test while that energy source supplied was kept in the medium. Mammalian spermatozoa generate metabolic energy in the form of the ATP to swim in the female oviduct and fertilise the egg. The energy of spermatozoa may be obtained by oxidative phosphorylation in their mitochondria and/or glycolysis, and this pathway, which is different in the various mammalian species, must depend on the substrates provided in the oviduct (Storey 2008). The evaluation of active mitochondria by monitoring their membrane potential is a consistent indicator of sperm quality. Sousa *et al.* (2011) isolated specific subpopulations from an ejaculate using fluorescence-activated cell sorting after Mitotracker Green staining or the swim-up procedure and observed that the subpopulations

with active mitochondria or with migrated spermatozoa showed the best functional parameters. The comparison between these subpopulations (Mitotracker Green staining versus migration status) showed significant differences in various parameters of sperm quality (acrosome integrity and chromatin integrity).

Information on the quality parameters of migrating spermatozoa was low given the technical difficulties in obtaining it. Numerous studies have established correlations between the qualitative parameters of spermatozoa observed in the original sample and their migration in an *in vitro* test, as evaluated by the distance travelled by the vanguard spermatozoon (Takemoto *et al.* 1985; Hamano *et al.* 2001; Cox *et al.* 2006) or the number of spermatozoa that migrate to a specific distance (Barratt *et al.* 1992; Hamano *et al.* 2001; Gillan *et al.* 2008; Al Naib *et al.* 2011; Martínez-Rodríguez *et al.* 2012). However, we have found only a single study that evaluates bull sperm viability after migration in cervical mucus (Hamano *et al.* 2001). These authors confirmed that the viability of migrating spermatozoa varied with the different media used and had a positive correlation with bull fertility in a medium containing cervical mucus.

Another source of information on the quality of post-migration spermatozoa is the swim-up procedure, though there are important differences between this procedure and the *in vitro* migration test with regard to the physical effort made by the spermatozoa to pass the test. Swim-up tests have typically been used to isolate a population of spermatozoa with optimal kinetic, morphological and biochemical parameters (Younglai *et al.* 2001; Sousa *et al.* 2011; Shojaei *et al.* 2012). In contrast, in the synthetic mucus-penetration test at 45°C, the sperm subpopulation reaching the goal after an exhausting effort show a very different physiological profile; more spermatozoa with low mitochondrial membrane potential, slight increase in number of cells with damaged acrosomes and viability similar to the original sample in the test. Viable spermatozoa with low mitochondrial membrane potential may be associated with the first steps of spermatozoon death. However, Martin *et al.* (2007) have observed that the decrease in mitochondrial membrane potential can be partially reversible in some spermatozoa so that a small but significant increase of the proportion of spermatozoa with a normal mitochondrial membrane potential was obtained after returning to physiological conditions. Hence, it can be hypothesised in the present study that a proportion of viable migrated spermatozoa with low mitochondrial membrane potential may recover the normal mitochondrial membrane potential after a return to resting conditions.

Taking into consideration all these results, synthetic mucus penetration assays depend on intrinsic sperm motility and allow us to identify a subpopulation of spermatozoa that can be used to measure the ability of spermatozoa to reach the site of fertilisation. As it is easily performed in the laboratory and provides additional information to the usual quality tests that are performed on sperm samples, we suggest the use of an *in vitro* penetration test as an important tool to assess sperm quality in wild animal species for which fertility data cannot be obtained. Further studies are required to assess other parameters (e.g. morphology, DNA status) of spermatozoa migrating in an artificial mucus.

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References

Aitken, R. J. (2006). Sperm-function tests and fertility. *Int. J. Androl.* **29**, 69–75. doi:10.1111/J.1365-2605.2005.00630.X

Aitken, R. J., Bowie, H., Buckingham, D., Harkiss, D., Richardson, D. W., and West, K. M. (1992). Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. *J. Androl.* **13**, 44–54.

Al Naib, A., Hanrahan, J. P., Lonergan, P., and Fair, S. (2011). *In vitro* assessment of sperm from bulls of high and low field fertility. *Theriogenology* **76**, 161–167. doi:10.1016/J.THERIOGENOLOGY.2010.10.038

Alvarez, M., Nicolas, M., Borragan, S., Lopez-Uruena, E., Anel-Lopez, L., Martinez-Pastor, F., Tamayo-Canul, J., Anel, L., and de Paz, P. (2012*a*). The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. *Anim. Reprod. Sci.* **135**, 113–121. doi:10.1016/J.ANIREPROSCI.2012.09.009

Alvarez, M., Tamayo-Canul, J., Anel, E., Boixo, J. C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., and de Paz, P. (2012*b*). Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. *Theriogenology* **77**, 1111–1118. doi:10.1016/J.THERIOGENOLOGY.2011.10.013

Anel, L., Alvarez, M., Martinez-Pastor, F., Garcia-Macias, V., Anel, E., and de Paz, P. (2006). Improvement strategies in ovine artificial insemination. *Reprod. Domest. Anim.* **41**(Suppl 2), 30–42. doi:10.1111/J.1439-0531.2006.00767.X

Anel, L., Alvarez, M., Martinez-Pastor, F., Gomes, S., Nicolas, M., Mata, M., Martinez, A. F., Borragan, S., Anel, E., and de Paz, P. (2008). Sperm cryopreservation in brown bear (*Ursus arctos*): preliminary aspects. *Reprod. Domest. Anim.* **43**(Suppl 4), 9–17. doi:10.1111/J.1439-0531.2008.01248.X

Anilkumar, R., Devanathan, T. G., Pattabiraman, S. R., and Edwin, M. J. (2001). Correlation between the spermatozoal characteristics and sperm penetration distance in polyacrylamide gel and bovine cervical mucus. *Theriogenology* **55**, 685–691. doi:10.1016/S0093-691X(01)00436-8

Bacinoglu, S., Tas, M., Cirit, U., Ozdas, O. B., and Ak, K. (2008). The potential fertility estimation capacity of the hypo-osmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. *Anim. Reprod. Sci.* **104**, 38–46. doi:10.1016/J.ANIREPROSCI.2007.01.014

Barratt, C. L., McLeod, I. D., Dunphy, B. C., and Cooke, I. D. (1992). Prognostic value of two putative sperm function tests: hypo-osmotic swelling and bovine sperm mucus penetration test (Penetrak). *Hum. Reprod.* **7**, 1240–1244.

Clarke, G. N., Garrett, C., and Baker, H. W. (1998). Quantitative sperm mucus penetration: modified formulae for calculating penetration efficiency. *Hum. Reprod.* **13**, 1255–1259. doi:10.1093/HUMREP/13.5.1255

Cox, J. F., Zavala, A., Saravia, F., Rivas, C., Gallardo, P., and Alfaro, V. (2002). Differences in sperm migration through cervical mucus *in vitro* relates to sperm colonisation of the oviduct and fertilising ability in goats. *Theriogenology* **58**, 9–18. doi:10.1016/S0093-691X(02)00919-6

Cox, J. F., Alfaro, V., Montenegro, V., and Rodriguez-Martinez, H. (2006). Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus. *Theriogenology* **66**, 860–867. doi:10.1016/J.THERIOGENOLOGY.2006.01.062

Defoin, L., Granados, A., and Donnay, I. (2008). Analysing motility parameters of fresh bull semen could help to predict resistance to freezing: a preliminary study. *Reprod. Domest. Anim.* **43**, 606–611. doi:10.1111/J.1439-0531.2007.00964.X

Druart, X., Cognie, J., Baril, G., Clement, F., Dacheux, J. L., and Gatti, J. L. (2011). *In vivo* imaging of ram spermatozoa in the ewe genital tract using fibered confocal microscopy. *Gynecol. Obstet. Fertil.* **39**, 633–635. doi:10.1016/J.GYOBFE.2011.09.007

Eggert-Kruse, W., Schwarz, H., Rohr, G., Demirakca, T., Tilgen, W., and Runnebaum, B. (1996). Sperm morphology assessment using strict criteria and male fertility under *in vivo* conditions of conception. *Hum. Reprod.* **11**, 139–146. doi:10.1093/OXFORDJOURNALS.HUMREP.A019007

Engel, S., and Petzoldt, R. (1999). Human sperm penetration in different media. *Andrologia* **31**, 233–239. doi:10.1046/J.1439-0272.1999.00149.X

Galli, A., Basetti, M., Balduzzi, D., Martignoni, M., Bornaghi, V., and Maffii, M. (1991). Frozen bovine semen quality and bovine cervical mucus penetration test. *Theriogenology* **35**, 837–844. doi:10.1016/0093-691X(91)90424-C

Gillan, L., Kroetsch, T., Maxwell, W. M., and Evans, G. (2008). Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. *Anim. Reprod. Sci.* **103**, 201–214. doi:10.1016/J.ANIREPROSCI.2006.12.010

Gundogan, M., Yeni, D., Avdatek, F., and Fidan, A. F. (2010). Influence of sperm concentration on the motility, morphology, membrane and DNA integrity along with oxidative stress parameters of ram sperm during liquid storage. *Anim. Reprod. Sci.* **122**, 200–207. doi:10.1016/J.ANIREPROSCI.2010.08.012

Hamano, K., Tanaka, S., Kawana, Y., Tsujii, H., Sasada, H., Sato, E., Takahashi, T., Miyawaki, K., and Arima, H. (2001). Evaluation of bull fertility by migration of frozen–thawed and washed sperm in medium containing cervical mucus. *J. Reprod. Dev.* **47**, 393–398. doi:10.1262/JRD.47.393

Hossain, A. M., Barik, S., Rizk, B., Kulkarni, P. M., and Thorneycroft, I. H. (1999). Analysis of *in vitro* migration patterns of human spermatozoa by a petri dish-based horizontal column. *Biol. Reprod.* **61**, 406–410. doi:10.1095/BIOLREPROD61.2.406

Ivic, A., Onyeaka, H., Girling, A., Brewis, I. A., Ola, B., Hammadieh, N., Papaioannou, S., and Barratt, C. L. (2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Hum. Reprod.* **17**, 143–149. doi:10.1093/HUMREP/17.1.143

Kasimanickam, R., Kasimanickam, V., Pelzer, K. D., and Dascanio, J. J. (2007). Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 degrees C. *Anim. Reprod. Sci.* **101**, 60–73. doi:10.1016/J.ANIREPROSCI.2006.09.001

Katz, D. F., and Pedrotti, L. (1977). Geotaxis by motile spermatozoa: hydrodynamic reorientation. *J. Theor. Biol.* **67**, 723–732. doi:10.1016/0022-5193(77)90257-0

Keel, B. A., and Webster, B. W. (1988). Correlation of human sperm motility characteristics with an *in vitro* cervical mucus penetration test. *Fertil. Steril.* **49**, 138–143.

López-Fernández, C., Johnston, S. D., Fernández, J. L., Wilson, R. J., and Gosálvez, J. (2010). Fragmentation dynamics of frozen–thawed ram sperm DNA is modulated by sperm concentration. *Theriogenology* **74**, 1362–1370. doi:10.1016/J.THERIOGENOLOGY.2010.06.006

Makler, A., Stoller, J., Blumenfeld, Z., Feigin, P. D., and Brandes, J. M. (1993). Investigation in real time of the effect of gravitation on human spermatozoa and their tendency to swim-up and swim-down. *Int. J. Androl.* **16**, 251–257. doi:10.1111/J.1365-2605.1993.TB01188.X

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., and Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum. Reprod.* **22**, 380–388. doi:10.1093/HUMREP/DEL399

Martínez-Rodríguez, C., Alvarez, M., Ordás, L., Chamorro, C. A., Martinez-Pastor, F., Anel, L., and de Paz, P. (2012). Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm-penetration test. *Theriogenology* **77**, 1575–1586. doi:10.1016/J.THERIOGENOLOGY.2011.11.026

Miki, K., and Clapham, D. E. (2013). Rheotaxis guides mammalian sperm. *Curr. Biol.* **23**, 443–452. doi:10.1016/J.CUB.2013.02.007

Mole, J. R., and Fitzgerald, J. A. (1990). Comparison of ram sperm interaction with bovine cervical mucus. *Theriogenology* **33**, 1031–1043. doi:10.1016/0093-691X(90)90065-2

Murase, T., and Braun, J. W. (1990). Impact of methodological factors on sperm penetration into cervical mucus in cattle. *Theriogenology* **34**, 73–80. doi:10.1016/0093-691X(90)90578-H

Murase, T., Okuda, K., and Sato, K. (1990). Assessment of bull fertility using a mucus-penetration test and a human chorionic gonadotrophin stimulation test. *Theriogenology* **34**, 801–812. doi:10.1016/0093-691X(90)90552-5

Murase, T., Mukohjima, K., Sakaguchi, S., Ohtani, T., Tsubota, T., and Kita, I. (2001). Characterization of frozen–thawed Japanese black bull spermatozoa by standard semen analysis, mucus-penetration test and the ability to undergo the acrosome reaction in response to calcium and the calcium ionophore A23187. *J. Reprod. Dev.* **47**, 237–243. doi:10.1262/JRD.47.237

Ola, B., Afnan, M., Papaioannou, S., Sharif, K., Bjorndahl, L., and Coomarasamy, A. (2003). Accuracy of sperm cervical mucus-penetration tests in evaluating sperm motility in semen: a systematic quantitative review. *Hum. Reprod.* **18**, 1037–1046. doi:10.1093/HUMREP/DEG209

Perry, R. L., Barratt, C. L., Warren, M. A., and Cooke, I. D. (1996). Comparative study of the effect of human cervical mucus and a cervical mucus substitute, Healonid, on capacitation and the acrosome reaction of human spermatozoa *in vitro*. *Hum. Reprod.* **11**, 1055–1062. doi:10.1093/OXFORDJOURNALS.HUMREP.A019296

Richardson, L., Hanrahan, J. P., O'Hara, L., Donovan, A., Fair, S., O'Sullivan, M., Carrington, S. D., Lonergan, P., and Evans, A. C. (2011). Ewe breed differences in fertility after cervical AI with frozen–thawed semen and associated differences in sperm penetration and physicochemical properties of cervical mucus. *Anim. Reprod. Sci.* **129**, 37–43. doi:10.1016/J.ANIREPROSCI.2011.10.012

Robayo, I., Montenegro, V., Valdes, C., and Cox, J. F. (2008). CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. *Reprod. Domest. Anim.* **43**, 393–399. doi:10.1111/J.1439-0531.2007.00920.X

Shojaei, H., Kroetsch, T., Wilde, R., Blondin, P., Kastelic, J. P., and Thundathil, J. C. (2012). Moribund sperm in frozen–thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. *Theriogenology* **77**, 940–951. doi:10.1016/J.THERIOGENOLOGY.2011.09.026

Sousa, A. P., Amaral, A., Baptista, M., Tavares, R., Campo, P. C., Peregrin, P. C., Freitas, A., Paiva, A., Almeida-Santos, T., and Ramalho-Santos, J. (2011). Not all sperm are equal: functional mitochondria characterise a subpopulation of human sperm with better fertilisation potential. *PLoS ONE* **6**(3), e18112. doi:10.1371/JOURNAL.PONE.0018112

Storey, B. T. (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* **52**, 427–437. doi:10.1387/IJDB.072522BS

Suarez, S. S., and Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Hum. Reprod. Update* **12**, 23–37. doi:10.1093/HUMUPD/DMI047

Suttiyotin, P., Thwaites, C. J., and Baillie, N. D. (1992). Relationships between the results of a modified sperm-penetration test and a swim-up

technique and the fertility of ram semen. *Theriogenology* **37**, 851–857. doi:10.1016/0093-691X(92)90046-T

Takemoto, F. S., Rogers, B. J., Wiltbank, M. C., Soderdahl, D. W., Vaughn, W. K., and Hale, R. W. (1985). Comparison of the penetration ability of human spermatozoa into bovine cervical mucus and zona-free hamster eggs. *J. Androl.* **6**, 162–170.

Tang, S., Garrett, C., and Baker, H. W. (1999). Comparison of human cervical mucus and artificial sperm-penetration media. *Hum. Reprod.* **14**, 2812–2817. doi:10.1093/HUMREP/14.11.2812

Taş, M., Bacinoglu, S., Cirit, U., Özgumuş, S., Kaşgöz, H., and Pabuccuoğlu, S. (2007*a*). Estimation of the potential fertility based upon non-return rates of bulls: using polyacrylamide gel instead of cervical mucus in the sperm-penetration test. *Theriogenology* **68**, 981–987. doi:10.1016/J.THERIOGENOLOGY.2007.07.011

Taş, M., Bacinoglu, S., Cirit, U., Özdaş, O. B., and Ak, K. (2007*b*). Relationship between bovine fertility and the number of spermatozoa penetrating the cervical mucus within straws. *Anim. Reprod. Sci.* **101**, 18–27. doi:10.1016/J.ANIREPROSCI.2006.08.020

Verberckmoes, S., Van Soom, A., De Pauw, I., Dewulf, J., and de Kruif, A. (2002). Migration of bovine spermatozoa in a synthetic medium and its relation to *in vivo* bull fertility. *Theriogenology* **58**, 1027–1037. doi:10.1016/S0093-691X(02)00931-7

Vyt, P., Rijsselaere, T., Dejonckheere, E., Castryck, F., and Van Soom, A. (2004). Motility assessment of porcine spermatozoa: a comparison of methods. *Reprod. Domest. Anim.* **39**, 447–453. doi:10.1111/J.1439-0531.2004.00538.X

Winet, H., Bernstein, G. S., and Head, J. (1984). Observations on the response of human spermatozoa to gravity, boundaries and fluid shear. *J. Reprod. Fertil.* **70**, 511–523. doi:10.1530/JRF.0.0700511

Younglai, E. V., Holt, D., Brown, P., Jurisicova, A., and Casper, R. F. (2001). Sperm swim-up techniques and DNA fragmentation. *Hum. Reprod.* **16**, 1950–1953. doi:10.1093/HUMREP/16.9.1950

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Head morphology of ram spermatozoa is associated with their ability to migrate *in vitro* and correlates with fertility

Carmen Martínez-Rodríguez[A,C], *Mercedes Alvarez*[A,B], *Elena López-Urueña*[A,B], *Susana Gomes-Alves*[A,B], *Luis Anel-López*[A,E], *Jorge E. Tizado*[D], *Luis Anel*[A,B] *and Paulino de Paz*[A,C,F]

[A]ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain.
[B]Animal Reproduction and Obstetrics, University of León, 24071, León, Spain.
[C]Molecular Biology, University of León, 24071, León, Spain.
[D]Biodiversity and Environmental Management, University of León, 24071, León, Spain.
[E]SaBio IREC (CSIC-UCLM-JCCM), Campus Universitario s. n. 02071 Albacete, Spain.
[F]Corresponding author. Email: ppazc@unileon.es

Abstract. Fertility is a highly complex biological function that depends on several properties of spermatozoa that are necessary for them to overcome various barriers in the female reproductive tract to reach the fertilisation site. This ability has been evaluated *in vitro* using cervical mucus migration tests. Head morphology has been widely studied, and various studies have reported correlations between head morphology and motility, fertility and DNA fragmentation. In the present study, we first evaluated the relationship between the ability of ram spermatozoa to overcome the mucus surrogate barrier in an *in vitro* migration test and sperm head morphology. Sperm motility (determined by computer-aided sperm analysis) and the acrosomal status, viability and mitochondrial status (determined by flow cytometry) of control and migrating spermatozoa were assessed. Principal component analysis and clustering analysis of the values for the morphometric parameters assessed defined three cell subpopulations. One of these subpopulations, namely spermatozoa with a short and wide head, was absent from samples collected after conclusion of the migration test. Second, we evaluated relationships among head morphology characteristics, the ability to penetrate the artificial mucus and fertility. We did not find any correlation between fertility and the number of spermatozoa that migrated, whereas there was a negative correlation between the proportion of spermatozoa with a short and wide head in the fresh sperm sample and fertility. In conclusion, the head morphology of spermatozoa was associated with their ability to overcome a mucus barrier in a migration test, and the relative size of the non-migrating subpopulation was negatively related to male fertility.

Additional keywords: acrosome status, mitochondrial potential, motility, oxidative stress, penetration test, viability.

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Introduction

Male fertility depends on a heterogeneous population of spermatozoa interacting at various levels with the female reproductive tract to ensure that a minority of spermatozoa with normal morphology and vigorous motility arrive at the fertilisation site. Sperm motility may be necessary for transport through the cervix, uterotubal junction and oviduct (Suarez and Pacey 2006; Suarez 2008) but is presumably not required for sperm transport through the uterus. In recent years, sperm transport through the female reproductive tract has been evaluated *in vivo* (Cox *et al.* 2002; Druart *et al.* 2009, 2011; Richardson *et al.* 2011). The study of sperm transit in the female reproductive tract was limited by the lack of techniques to observe the behaviour of spermatozoa in the reproductive tract of the living female until Druart *et al.* (2009, 2011), using fibered confocal fluorescence microscopy (FCM), determined the *in situ* patterns of migration along the ewe reproductive tract and quantified the number of spermatozoa present in each part of the tract using both fresh and frozen–thawed semen.

Because of the technical difficulties involved in studying sperm migration *in vivo*, different *in vitro* sperm migration tests have been developed using cervical mucus or cervical mucus surrogates to mimic the environment of the female reproductive tract through which spermatozoa migrate. Aitken (2006) found that the ability of spermatozoa to penetrate a cervical mucus surrogate correlated extremely well with the outcome of cervical mucus penetration assays and showed the same dependence on sperm motility. Furthermore, the World Health Organization (WHO) included measurement of the ability of spermatozoa to penetrate a column of cervical mucus as a tool with which to evaluate the quality of human semen in the 5th edition of the WHO Laboratory Manual (WHO 2010).

Some authors have used systems based on capillary tubes in which, after a given incubation period, they locate the vanguard spermatozoon or count the number of spermatozoa that have travelled a defined distance into cervical mucus using phase contrast microscopy (for a review, see Martínez-Rodríguez *et al.* 2012). Others have used systems based on plastic straws in which the contents of different segments of the straw were loaded onto a slide or plate to achieve a more accurate count of the spermatozoa that had migrated a given distance (Taş *et al.* 2007*a*, 2007*b*; Bacinoglu *et al.* 2008). Ola *et al.* (2003) reviewed the accuracy of the different cervical mucus penetration tests available and concluded that counting the spermatozoa that have travelled a given distance through cervical mucus is a more accurate diagnostic tool than measuring the distance travelled by the vanguard spermatozoon. In this regard, Martínez-Rodríguez *et al.* (2012) described an automatic procedure to obtain maximum accuracy in evaluating the migrating sperm population. However, none of these *in vitro* sperm migration tests allowed qualitative analysis of the spermatozoa that were able to cross the mucus barrier because it was not possible to isolate them for such a study. In a previous study (Martínez-Rodríguez *et al.* 2014), we developed a model *in vitro* sperm migration test in which the spermatozoa that passed through the mucus barrier were collected in a diluent without the synthetic mucus. Thus, these spermatozoa could be counted and we had the opportunity to study their basic qualitative parameters.

One factor that has been studied intensively in recent years is sperm head morphology. Semen is a complex cellular population and morphometric studies of the sperm head have revealed specific biotypes defined by head morphology (Thurston *et al.* 2001). The proportion of spermatozoa in each sperm subpopulation varies significantly among males, and some of these subpopulations have been correlated with sperm quality. Sperm head morphology has been analysed as a fertility biomarker in various species, including humans (Kruger *et al.* 1993), boars (Hirai *et al.* 2001), stallions (Casey *et al.* 1997), goats (Hidalgo *et al.* 2007), bulls (Sailer *et al.* 1996) and sheep (de Paz *et al.* 2011). Some authors have related spermatozoa head morphology with sperm motility and migration through the female reproductive tract. Gomendio *et al.* (2007) reported that the shape of the sperm head and the dimensions of the components of the flagellum were the main determinants of sperm swimming velocity. Gillies *et al.* (2009) suggested that the head dimension is a factor in determining the progressive velocity and amplitude of the lateral head movement of spermatozoa.

Mammalian spermatozoa exhibit a large degree of variation in the size and shape of the sperm head. Different hypotheses as to the evolution of mammalian sperm heads have been published. Bedford (2014) suggested that the sperm head in eutherian mammals has evolved to a form that facilitates penetration of the zona pellucida, a process in which physical factors dominate. However, Firman and Simmons (2010) observed that selection history has had no influence on sperm morphology, but that environmental and/or maternal effects of the mating regimen do.

In the present study, we evaluated the correlation between sperm motility and sperm head morphology in ram spermatozoa by isolating the cellular subpopulation that migrated through the mucus barrier using the *in vitro* migration test described by Martínez-Rodríguez *et al.* (2014). Our aim was to assess whether the head morphology of the subpopulation of spermatozoa that migrated has specific features. To evaluate this relationship, we also subjected the semen samples to oxidative treatment to induce morphological changes in the sperm head and then assessed the effects of these changes using the migration test. In addition, the relationships among head morphometric characteristics, the ability to penetrate the cervical mucus surrogate and fertility were also investigated.

Materials and methods

Experimental design

Experiment 1: morphometry of the sperm heads of spermatozoa migrating through artificial mucus

In this experiment we used the pooled ejaculates of five rams at a concentration of 100×10^6 spermatozoa mL^{-1} in INRA 96 (IMV Technologies, L'Aigle, France). Three pools were obtained in three consecutive weeks and were evaluated in the migration test device for 30 min at 39°C at a 45° angle relative to the incubator surface (Martínez-Rodríguez *et al.* 2014). Three replicates were performed for each analysis.

The head morphology of the spermatozoa that were able to pass through a specific mucus barrier was evaluated to define the particular head morphology of this cell population. To this end, a multivariate analysis was conducted to identify the most suitable statistical procedure to categorise the different sperm subpopulations and to use in the subsequent experiments. In addition, we counted the number of spermatozoa in each sperm sample that were able to pass through the barrier.

Experiment 2: differences in sperm head morphology among individual males

The multivariate analysis method defined in Experiment 1 was used in this experiment to evaluate the sperm head morphology in samples from 12 individual male rams. Samples (100×10^6 spermatozoa mL^{-1} in INRA 96) were evaluated in the migration test device after 30 min at 39°C and at a 45° angle relative to the incubator surface. The assay was replicated three times.

Experiment 3: sperm subpopulations based on head morphology

The aim of this experiment was to determine whether changes in sperm head morphology affected the ability of the spermatozoa to migrate through the mucus barrier in the migration test device. The migration test was performed using semen samples (100×10^6 spermatozoa mL^{-1} in INRA 96; three semen pools from five different males each; three replicates) that had been incubated for 2 h at 39°C with an oxidant substance (100 μM Fe^{2+}–ascorbate) that induces oxidative stress in spermatozoa. Oxidative damage to spermatozoa, quantified by the DNA fragmentation index, is significantly correlated with sperm morphology (Song *et al.* 2006). Control samples were maintained for 2 h at 39°C in the absence of the oxidant substance. The number of cells in the oxidised and control (non-oxidised) samples that traversed the mucus barrier was counted.

Experiment 4: relationship between performance in the artificial mucus-penetration test, sperm head morphology and fertility

The aim of this experiment was to determine whether sperm head morphology and the number of spermatozoa that migrated in an artificial mucus test were good predictors of fertility rates attained in the field. Semen samples from seven different rams were used. Ten minutes after collection of the ejaculate, samples were diluted in INRA 96 to a final concentration of 1600×10^6 spermatozoa mL^{-1}. Then, 1 mL sample was sent to the laboratory for the sperm penetration test and morphological evaluation studies, whereas the rest of the sample was used to prepare semen doses that were refrigerated at 15°C and used for sheep insemination within 6 h of sample collection. Head morphology of fresh samples was analysed, as was the number of spermatozoa that migrated through the mucus barrier in each semen sample and the head morphology of the cells that traversed the artificial mucus barrier after 30 min at 39°C. Correlations among morphometric values, the number of spermatozoa that migrated and the fertility rates attained in the field were analysed.

Collection of ram spermatozoa

Semen from adult Assaf rams (*Ovis aries*) of proven fertility was collected using an artificial vagina (39°C) in the presence of a female decoy. The animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were approved by the Ethics Committee for Experimentation with Animals of Leon University (Spain; #21012008). A glass collection tube was placed in a thermoregulated bath at 35°C, and a preliminary seminal evaluation was performed (volume, mass motility and concentration). The ejaculates used in the experiments were those with a volume >0.5 mL, a mass motility ≥4 (determined by microscopy using a warming stage at 39°C and ×40 objective; score range 0–5, where 0 indicates non motility and 5 indicates total motility) and a sperm concentration greater $>3000 \times 10^6$ spermatozoa mL^{-1}. Sperm concentration was assessed using a haemocytometer (Bürker, Marienfeld, Germany) with a computer-aided sperm analysis (CASA) system (Integrated Semen Analyzer System (ISAS); Proiser, Valencia, Spain). Ten minutes after collection, the ejaculates were diluted in INRA 96 to a final concentration of 1600×10^6 spermatozoa mL^{-1}. To prepare the semen doses, 0.25-mL plastic straws were filled with ram spermatozoa at a concentration of 1600×10^6 spermatozoa mL^{-1} in INRA 96. To perform the penetration test, the sperm sample was diluted in INRA 96 to a final concentration of 100×10^6 spermatozoa mL^{-1} immediately before the test was conducted. At this time, 1 mL semen from each male was pooled (control sample) and semen quality was evaluated (based on morphology, motility, viability and acrosomal and mitochondrial status; see Methods in Supplementary Material). Each male contributed the same number of spermatozoa to the pool.

Synthetic cervical mucus

A 1.6% polyacrylamide gel was prepared by diluting 30% liquid acrylamide (30% ProtoGel; National Diagnostics, Atlanta, GA, USA), for 100 mL, 5.333 mL of 30% liquid acrylamide (30% ProtoGel; National Diagnostics, Atlanta, GA, USA) in 25 mL of TCF (0.27 M Tris, 90 mM citric acid and 53 mM fructose) and 68.867 mL of distilled water. Once mixed, 0.75 mL of 2% ammonium persulfate solution (Sigma-Aldrich, Madrid, Spain) was added and then 0.05 mL of tetramethylethylenediamine (TEMED; Sigma-Aldrich) was added to induce polymerisation. The mixture (pH 7.2, 320 mOsmol kg^{-1}) was maintained at room temperature for 24 h and then stored at 4°C until use.

Sperm progression test

The device used for the penetration test has been described previously (Martínez-Rodríguez *et al.* 2014). Briefly, the passage of spermatozoa through a 2-cm segment of mucus surrogate over a 30-min period of incubation at 39°C with the device at a 45° angle is evaluated. Eighteen replicates of each semen sample were tested. Once the test was performed, six replicates were used to count the number of spermatozoa that had migrated through the mucus. To that end, the contents of each of the six collection units were placed individually in a 1.5-mL tube (six tubes) and then 100 μL of 5 mg mL^{-1} Hoechst 33342 was added to each tube. After 10 min, the tubes were centrifuged at 600*g* for 5 min (Alvarez *et al.* 2012). Then, the supernatant was removed and the pellet was resuspended in 10 μL TCF and each cell suspension was transferred individually to a well of an eight-well microscope slide. Four replicates were used for the morphological studies. Because the number of spermatozoa available was very low, four collection units were pooled together in a 1.5-mL Eppendorf tube and centrifuged at 600*g* for 5 min. The pellets were resuspended in 20 μL prewarmed (39°C) 2% glutaraldehyde, smeared on a slide and air dried. The eight remaining replicates were used for evaluation of sperm quality by flow cytometry (migrated sample; see Methods in Supplementary Material).

Sperm counting

The spermatozoa on the slides were analysed under an inverted microscope (T2000U; Nikon, Tokyo, Japan) equipped with a mercury fibre illuminator (Intensilight C-HGFI; Nikon), a motorised stage (ProScan II; PRIOR, Cambridge, UK) and a digital camera (ORCA; Hamamatsu, Tokyo, Japan). The motorised stage and the digital camera were controlled automatically using Metamorph version 7 software (Molecular Devices, Downingtown, PA, USA). Each well was photographed automatically to capture 96 images (×10 objective) at wavelengths of 435–485 nm. These images were analysed using NIS Elements v.3 image analysis software (Nikon) to determine the number of spermatozoa in each well. Specific adjustments were made to discriminate spermatozoa from debris on the basis of their shape and size (Martínez-Rodríguez *et al.* 2012).

Head morphometry

The spermatozoa (fresh spermatozoa and spermatozoa that traversed the mucus barrier) were fixed in 2% glutaraldehyde (in BL-1 medium; composition: glucose 2.9 g, sodium citrate anhydrous 1.0 g, sodium bicarbonate 0.2 g and distilled water to 100 mL) and smeared on microscope slides, air dried and then stained using Diff-Quik (QCA, Tarragona, Spain; Eggert-Kruse *et al.* 1996). For staining, slides were immersed for 10 min

in Solution A, for 15 min in Solution B and then rinsed using distilled water, air dried and mounted with Entellan (Sigma-Aldrich). Samples were examined under a Nikon Eclipse E600 microscope equipped with a ×60 brightfield objective (×500). An average of 200 cells in each sample was photographed using a Nikon DF1200F digital camera and the pictures were processed using the NIS Elements v.3 image analysis system. The morphometric analysis was performed using a semi-automatic macro that allowed the operator to discard the sperm heads in the image that did not meet the technical requirements for the study (e.g. overlapping cells and the presence of staining artefacts). For each cell, the following four basic parameters were calculated: area (A; μm^2), length (L; μm), perimeter (P; μm) and width (W; μm). Another four derived parameters were calculated, namely elongation ((L − W)/(L+W)), ellipticity (L/W), regularity ($\pi \times LW/4A$) and rugosity ($4\pi \times A/P^2$).

Assessment of field fertility

Semen for insemination was diluted to 1600×10^6 spermatozoa mL^{-1} in INRA 96 solution 10 min after collection and was placed in French mini-straws (0.25 mL). The semen was chilled to 15°C and carried in a transportable refrigerator to the site of insemination (<6 h after ejaculation time).

Vaginal AI was performed using oestrus-synchronised ewes. Oestrus synchronisation was achieved using fluorogestone acetate sponges (Chronogest; 20 mg per ewe; MSD Animal Health, Huixquilucan, Mexico). The sponges were withdrawn 14 days after insertion and the ewes were then treated with 500 IU equine chorionic gonadotrophin (Folligon; MSD Animal Health) and inseminated 55 ± 1 h later. The AI was performed by experienced veterinarians using a speculum equipped with a light source and an ovine AI catheter (IMV™, L'Aigle, France). Chilled spermatozoa were deposited at the entrance of the cervix.

For each male, the semen was used on an average of 47 sheep (range 42–53). Fertility was deemed 'successful lambing' if parturition occurred between 139 and 153 days after insemination or 'negative' if parturition did not occur. The fertility percentage per male was calculated as the number of ewes that gave birth divided by the total number of ewes inseminated.

Statistical analysis

Data were analysed using different procedures in SAS/STAT v.9 (SAS Institute, Cary, NC, USA). Results are presented as the mean ± s.e.m. $P < 0.05$ for a two-sided test was considered significant. The main objective of the analysis was to differentiate sperm subpopulations using the morphometric data and a clustering procedure, and then to compare subpopulations. The Shapiro–Wilk normality test confirmed that the morphometric measurements were normally distributed.

Sperm count

The number of spermatozoa that migrated was analysed using mixed linear models (MIXED procedure). The significance of differences in the least-squares means was evaluated using Tukey's honestly significant difference (HSD) test. The relationship between fertility and the spermatozoa that migrated was analysed using the CORR procedure, and Spearman coefficients were calculated.

Identification of sperm subpopulations

We used the PRINCOMP procedure to perform a principal component analysis (PCA) of the morphometric data to reduce the number of original variables and to derive a small number of linear combinations (principal components) from these variables. A preliminary analysis was conducted to determine the number of morphometric parameters that should be used in the PCA. To determine the number of principal components to retain in our analysis, we considered the cumulative proportion of variance that was explained (≥90%). Using the selected principal components as variables, we performed a non-hierarchical cluster analysis using the FASTCLUS protocol, using Euclidean distances (k-means model) to calculate the cluster centres. The clusters obtained were analysed using the CLUSTER procedure, which performed a hierarchical clustering using the average linkage method (UPGMA) for joining the clusters, which finds the most significant solution possible by aiming to sort different objects into groups in such a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise. To determine the final number of clusters, we studied the following three statistics that were provided by the CLUSTER procedure: (1) the pseudo-t2; (2) the pseudo-F; and (3) the cubic clustering criterion (CCC). Three final clusters were established. After analysis, the number of spermatozoa in each cluster and their respective morphometric parameters were defined. We used the MIXED procedure for between-cluster comparisons. Tukey's HSD test was used for pairwise comparisons when the results were significantly different. The significance of differences in the proportion of spermatozoa in different clusters was evaluated using the χ^2 test. The relationship between fertility and the size of the morphometrically defined subpopulations, calculated as a proportion of the ejaculate, was analysed using the CORR procedure, and Spearman coefficients were calculated.

Results

Experiment 1: head morphology of migrating spermatozoa

Values for the head morphometric parameters for control and migrating spermatozoa are given in Table 1. The primary morphometric parameters did not reveal significant differences among pools of control samples, whereas some differences were found for the migrating spermatozoa.

The PCA of values for the morphometric parameters of the spermatozoa in the fresh semen samples provided the eigenvalues (variances) and eigenvectors given in Table 2. When four primary morphometric parameters were analysed, the first two components (Prin1 and Prin2) explained 91% of the sample variance; however, in a study of six morphometric parameters these two components (Prin1 and Prin2) explained only 78% of total variance. Therefore, we chose the four-parameter model for future analysis. For the fresh samples, the principal component Prin1 was defined by approximately equal contribution of the four variables, whereas Prin2 appeared to reflect a large width and a short length. Cluster 3 was the largest subpopulation

Table 1. Analysis of sperm head morphometric values in pooled samples of fresh semen (control) and spermatozoa that passed through the mucus surrogate (Migrating) over a 30-min incubation period at 39°C (three pools, three replicates)

Data are the mean ± s.e.m. Within rows, values with different superscript lowercase letters differ significantly. Different superscript uppercase letters indicate significant differences in parameters between the Control and Migrating groups. Primary parameters were area (A), length (L), perimeter (P) and width (W). Derived parameters were elongation (calculated as (L − W)/(L+W)), ellipticity (L/W), regularity ($\pi \times LW/4A$) and rugosity ($4\pi \times A/P^2$).

	Pool 1	Pool 2	Pool 3	Total
Control				
Area (μm^2)	35.3 ± 0.2^{aA}	35.8 ± 0.2^{aA}	35.1 ± 0.2^{aA}	35.4 ± 0.1^{A}
Perimeter (μm)	22.5 ± 0.1^{aA}	22.5 ± 0.1^{aA}	22.3 ± 0.1^{aA}	22.2 ± 0.1^{A}
Length (μm)	8.9 ± 0.1^{aA}	8.9 ± 0.1^{aA}	8.9 ± 0.1^{aA}	8.8 ± 0.1^{A}
Width (μm)	3.9 ± 0.1^{aA}	3.9 ± 0.1^{aA}	3.9 ± 0.1^{aA}	3.8 ± 0.1^{A}
Elongation	0.39 ± 0.01^{aA}	0.38 ± 0.01^{aA}	0.39 ± 0.01^{aA}	0.39 ± 0.01^{A}
Ellipticity	2.3 ± 0.1^{aA}	2.2 ± 0.1^{bA}	2.2 ± 0.1^{bA}	2.3 ± 0.1^{A}
Regularity	0.77 ± 0.01^{aA}	0.78 ± 0.01^{aA}	0.78 ± 0.01^{aA}	0.77 ± 0.01^{A}
Rugosity	0.87 ± 0.01^{aA}	0.88 ± 0.01^{bA}	0.88 ± 0.01^{bA}	0.87 ± 0.01^{A}
Migrating (5457 ± 608)				
Area (μm^2)	35.9 ± 0.1^{aB}	36.1 ± 0.1^{bA}	36.6 ± 0.1^{bB}	36.1 ± 0.1^{B}
Perimeter (μm)	22.8 ± 0.1^{aB}	22.7 ± 0.1^{aB}	22.9 ± 0.1^{bB}	22.7 ± 0.1^{B}
Length (μm)	9.1 ± 0.1^{aB}	8.9 ± 0.1^{bB}	9.1 ± 0.1^{aB}	9.0 ± 0.1^{B}
Width (μm)	3.9 ± 0.1^{aB}	4.1 ± 0.1^{bB}	4.1 ± 0.1^{bB}	4.0 ± 0.1^{B}
Elongation	0.39 ± 0.01^{aB}	0.38 ± 0.01^{aA}	0.38 ± 0.01^{aB}	0.38 ± 0.01^{B}
Ellipticity	2.2 ± 0.1^{aB}	2.2 ± 0.1^{aA}	2.2 ± 0.1^{aB}	2.3 ± 0.1^{A}
Regularity	0.79 ± 0.01^{aB}	0.79 ± 0.01^{aB}	0.79 ± 0.01^{aB}	0.79 ± 0.01^{B}
Rugosity	0.87 ± 0.01^{aB}	0.88 ± 0.01^{aA}	0.88 ± 0.01^{aB}	0.87 ± 0.01^{A}

Table 2. Analysis of pooled semen

Variances and eigenvectors are shown for the three main components (Prin1, Prin2, Prin3) obtained after principal component analysis (PCA) of four and six primary morphometric parameters obtained for fresh samples (three pools, three replicates). The eigenvectors are a measure of the association of the original parameters with the resulting principal components. The parameters (mean ± s.e.m.) are the morphometric descriptors of the sperm head for each cluster (subpopulations) found using PCA cluster analysis. The percentage of spermatozoa in the seminal sample belonging to each cluster is given in parentheses

Four morphometric parameters[A]					
Variance	Explained		Cumulative		
Prin1	0.73		0.73		
Prin2	0.18		0.91		
Prin3	0.06		0.97		
Eigenvectors	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	
Prin1	0.538	0.523	0.476	0.458	
Prin2	0.049	−0.147	−0.627	0.763	
Prin3	−0.237	−0.650	0.608	0.389	
Parameters	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (30.8%)	33.3 ± 1.9	21.8 ± 0.5	8.4 ± 0.3	3.8 ± 0.1	2.2
Cluster 2 (28.4%)	36.6 ± 1.8	23.1 ± 0.5	8.7 ± 0.2	4.1 ± 0.1	2.1
Cluster 3 (40.8%)	37.1 ± 1.7	22.4 ± 0.6	9.1 ± 0.3	3.8 ± 0.1	2.4
Six morphometric parameters[B]					
Variance	Explained		Cumulative		
Prin1	0.55		0.55		
Prin2	0.23		0.78		
Prin3	0.16		0.94		

[A]In the analysis of four morphometric parameters, the values for the morphometric parameters that defined each of the three clusters obtained using PCA and data clustering of the values for the fresh samples are shown. Note, the relative size of each cluster is given as a proportion.
[B]The variances, derived from the eigenvalues, indicate the proportion of the total variance explained by each principal component.

(including 40.8% of the total spermatozoa), and Clusters 1 and 2 were similar in size.

The PCA and data clustering results for the morphometric parameter data obtained from the migrating spermatozoa are given in Table 3. The first two components (Prin1 and Prin2) explained 92% of the sample variance. The definition of the principal components was similar to those observed in the statistical model described above for the fresh semen: Prin1

was defined by the four primary morphometric parameters, which had similar statistical contributions, and Prin2 had a greater contribution from the width data and a lower contribution from the length data. The size of the three clusters obtained and the values for their morphometric parameters are given in Table 3. Among the spermatozoa that migrated, Cluster 2, which was characterised by a short and wide head, comprised only a residual population (1.8%).

Experiment 2: differences among spermatozoa from individual males

The head morphometric parameters of the spermatozoa in the fresh sample and the spermatozoa that migrated were analysed using PCA, and the eigenvalues (variances) and eigenvectors that were obtained are given in Table 4 (fresh and migrating spermatozoa). As indicated in Table 4, the first two components

Table 3. Analysis of pooled semen

Variances and eigenvectors are shown for the three main components (Prin1, Prin2, Prin3) obtained after principal component analysis (PCA) of the values for the four primary morphometric parameters obtained from analysis of spermatozoa that migrated through the mucus surrogate over a 30-min incubation period (three pools, three replicates). The morphometric parameters (mean values ± s.e.m.) defining each of the three clusters obtained using PCA and data clustering of the values for the spermatozoa that migrated are shown. Note, the relative size of each cluster is given as a proportion. For further details, see Table 2

Variance		Explained		Cumulative	
Prin1		0.76		0.76	
Prin2		0.16		0.92	
Prin3		0.04		0.96	
Eigenvectors	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	
Prin1	0.556	0.542	0.448	0.444	
Prin2	0.061	−0.072	−0.695	0.713	
Prin3	0.298	−0.835	0.381	0.262	
Parameters	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (51.8%)	34.3 ± 1.6	22.2 ± 0.5	8.8 ± 0.2	3.8 ± 0.1	2.3
Cluster 2 (1.8%)	35.3 ± 2.7	22.3 ± 0.9	8.5 ± 0.4	4.1 ± 0.2	2.1
Cluster 3 (46.4%)	38.4 ± 1.8	23.4 ± 0.6	9.2 ± 0.2	4.0 ± 0.1	2.3

Table 4. Analysis of semen per ram for fresh samples and spermatozoa that migrated through the mucus surrogate over a 30-min incubation period

Variances and eigenvectors are shown for the three main components (Prin1, Prin2, Prin3) obtained after principal component analysis (PCA) of the values for the four primary morphometric parameters (12 males, three replicates). The morphometric parameters (mean ± s.e.m.) that defined each of the three clusters obtained using PCA and data clustering of the values are shown. Note, the relative size of each cluster is given as a proportion. For details, see Table 2

Fresh sample Variance		Explained		Cumulative	
Prin1		0.75		0.75	
Prin2		0.16		0.91	
Prin3		0.05		0.96	
Eigenvectors	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	
Prin1	0.539	0.531	0.474	0.449	
Prin2	0.084	−0.147	−0.640	0.749	
Prin3	0.346	−0.834	0.405	0.144	
Parameters	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (40.3%)	32.7 ± 1.6	21.7 ± 0.5	8.7 ± 0.2	3.7 ± 0.1	2.4
Cluster 2 (27.0%)	36.3 ± 2.2	22.7 ± 0.7	8.8 ± 0.3	4.1 ± 0.1	2.1
Cluster 3 (32.7%)	37.7 ± 2.2	23.5 ± 0.7	9.3 ± 0.3	3.9 ± 0.1	2.4
Migrating spermatozoa					
Variance		Explained		Cumulative	
Prin1		0.75		0.75	
Prin2		0.15		0.90	
Prin3		0.04		0.94	
Eigenvectors	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	
Prin1	0.551	0.542	0.458	0.440	
Prin2	0.076	−0.103	−0.671	0.730	
Prin3	0.382	−0.833	0.360	0.173	
Parameters	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (52.6%)	33.5 ± 1.6	21.9 ± 0.5	8.7 ± 0.2	3.8 ± 0.1	2.3
Cluster 2 (2.1%)	34.2 ± 5.8	22.5 ± 2.2	7.9 ± 0.8	4.2 ± 0.4	1.9
Cluster 3 (45.3%)	37.7 ± 1.7	23.2 ± 0.6	9.2 ± 0.2	4.1 ± 0.1	2.2

(Prin1 and Prin2) explained 91% of the sample variance. The principal component Prin1 was defined by approximately equal loadings of the four variables, whereas Prin2 appeared to reflect a large width and short length. The results of the clustering analysis indicated that Cluster 1 was the larger subpopulation (including 40.3% of the total spermatozoa).

Migrating spermatozoa showed results similar to those obtained using fresh samples. Components Prin1 and Prin2 explained 90% of the sample variance, and Prin 1 and Prin2 were defined in the same way as for the fresh samples. For the spermatozoa that migrated, Cluster 2, characterised by a short and wide head, was only a residual population (2.1%). A comparison of cluster sizes per individual male is shown in Fig. 1. Specific differences were seen in the distribution of the clusters among different males for both fresh semen and the spermatozoa that migrated.

Experiment 3: sperm subpopulations defined by head morphology

The number of spermatozoa in the oxidised and non-oxidised samples that migrated did not differ (Table 5). All the values for the primary morphometric parameters were modified by the oxidant treatment ($P < 0.05$); there was a linear increase in the values for these parameters comparing the spermatozoa in the oxidised sample that migrated with spermatozoa in the oxidised and control samples. The PCA and clustering analysis of the three different sample types (control sample, oxidised sample and spermatozoa in the oxidised sample that migrated)

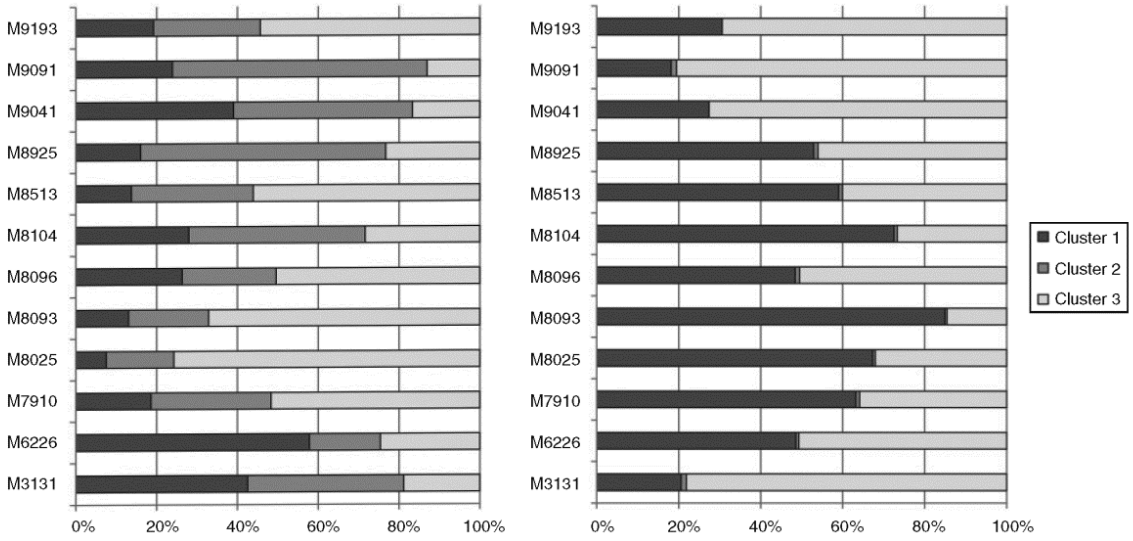

Fig. 1. Analysis of the semen from individual male rams. Distribution of the three clusters that were defined using principal component analysis and data clustering of the morphometric values for the semen from 12 males (three replicates) as fresh samples (left) or as the spermatozoa that were able to pass through the acrylamide barrier (right).

Table 5. Analysis of oxidised semen samples (three pools, three replicates)

Mean ± s.e.m. morphometric values of sperm heads from control, pre-migration oxidised (OXI_Damage) and post-migration oxidised (OXI_Migrating) samples. The number of spermatozoa that migrated is also given. Within rows, values with different superscript letters differ significantly. For details, see Table 1

	Control	OXI_Damage	OXI_Migrating
Number of migrated spermatozoa	4561 ± 156^{a}		3996 ± 298^{a}
Area (μm^2)	31.79 ± 0.15^{a}	32.42 ± 0.12^{b}	34.31 ± 0.15^{c}
Perimeter (μm)	21.53 ± 0.05^{a}	21.71 ± 0.04^{b}	22.27 ± 0.05^{c}
Length (μm)	8.56 ± 0.02^{a}	8.71 ± 0.02^{b}	8.88 ± 0.02^{c}
Width (μm)	3.68 ± 0.01^{a}	3.72 ± 0.01^{b}	3.86 ± 0.01^{c}
Ellipticity	2.33 ± 0.01^{ab}	2.35 ± 0.01^{a}	2.31 ± 0.01^{b}
Rugosity	0.86 ± 0.00^{a}	0.86 ± 0.00^{ab}	0.87 ± 0.00^{b}
Elongation	0.399 ± 0.001^{a}	0.401 ± 0.001^{a}	0.393 ± 0.001^{a}
Regularity	0.778 ± 0.001^{a}	0.786 ± 0.002^{b}	0.785 ± 0.002^{b}

Table 6. Analysis of oxidised semen samples (three pools, three replicates)

Variances and eigenvectors are shown for the three main components (Prin1, Prin2, Prin3) obtained after principal component analysis (PCA) of the values for the four primary morphometric parameters obtained in analyses of control, pre-migration oxidised (OXI_Damage) and post-migration oxidised (OXI_Migrating) samples. The morphometric parameters (mean ± s.e.m.) defining each of the three clusters obtained using PCA and data clustering of the three types of sample are shown. Note, the relative size of each cluster is given as a proportion. For details, see Table 2

Variance	Explained		Cumulative		
Prin1	0.77		0.77		
Prin2	0.14		0.91		
Prin3	0.03		0.94		
Eigenvectors	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	
Prin1	0529	0519	0474	0476	
Prin2	−0116	0119	0699	−0696	
Prin3	0156	−0831	0451	0285	
Control	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (41.4%)	30.5 ± 1.5	21.0 ± 0.5	8.5 ± 0.2	3.6 ± 0.1	2.4
Cluster 2 (20.1%)	32.2 ± 2.2	21.9 ± 0.8	8.4 ± 0.3	3.9 ± 0.2	2.2
Cluster 3 (38.5%)	34.6 ± 2.2	22.2 ± 0.7	8.9 ± 0.3	3.9 ± 0.1	2.3
OXI_Damage	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (33.6%)	32.2 ± 2.0	21.7 ± 0.6	8.9 ± 0.2	3.6 ± 0.1	2.5
Cluster 2 (37.9%)	33.9 ± 2.2	22.1 ± 0.8	8.7 ± 0.3	3.7 ± 0.2	2.4
Cluster 3 (28.5%)	36.5 ± 1.9	23.1 ± 0.6	9.3 ± 0.2	3.9 ± 0.2	2.4
OXI_Migrating	Area (μm^2)	Perimeter (μm)	Length (μm)	Width (μm)	Length/width
Cluster 1 (40.8%)	29.2 ± 1.4	20.7 ± 0.5	8.4 ± 0.2	3.7 ± 0.1	2.3
Cluster 2 (24.1%)	30.9 ± 1.7	21.3 ± 0.7	8.5 ± 0.2	3.9 ± 0.1	2.2
Cluster 3 (35.1%)	35.6 ± 1.6	22.7 ± 0.6	9.0 ± 0.2	4.0 ± 0.1	2.3

Table 7. Analysis of semen samples used for insemination (seven males, three replicates)

Data show the mean ± s.d. number of spermatozoa that migrated through the mucus surrogate over a 30-min incubation at an angle of 45°. Values with different superscript letters differ significantly.

Male ID	No. migrating spermatozoa
M131	4412.5 ± 639.2^{ab}
M190	4145.8 ± 125.3^{ab}
M239	5646.1 ± 559.6^{b}
M507	3325.6 ± 560.6^{ac}
M785	2950.3 ± 548.9^{c}
M919	2513.8 ± 314.1^{cd}
M920	1950.8 ± 201.8^{d}

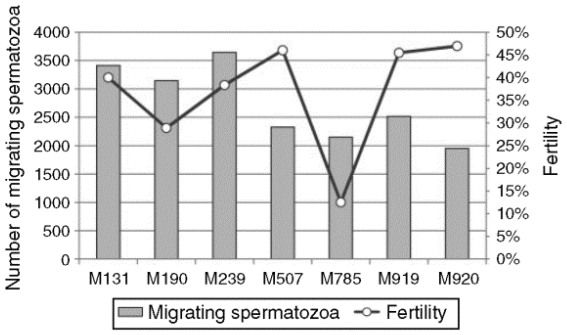

Fig. 2. Analysis of the correlation between the results of the migration and fertility tests (seven males, three replicates). Data show the number of cells that migrated through the mucus barrier in Experiment 4 and correlation with fertility rates observed in the field. The Spearman coefficient (no. spermatozoa that migrated vs fertility) = −0.35 ($P = 0.436$).

identified two principal components, which, as in the previous experiments, explained 91% of the total variance determined by the same combinations of primary morphometric parameters (Table 6). Three different clusters were found. An important difference with respect to the previous experiments was the presence of Cluster 2 in the migrating sample (24.1% of migrating spermatozoa). The proportion of spermatozoa present in each cluster differed among the three samples.

Experiment 4: relationships among performance in the artificial mucus-penetration test, head morphometric values and fertility

The number of spermatozoa that migrated in fresh sperm samples of seven males was determined (Table 7); significant differences were observed among the males. The relationship between the fertility rate and the number of spermatozoa that migrated (Fig. 2) for each male's semen was analysed and no correlation was found (Spearman coefficient = −0.35, $P = 0.43$). The results of PCA and clustering analysis are shown in Fig. 3. As observed in previous experiments, Cluster 2 spermatozoa did not migrate through the artificial mucus in the penetration test and thus only two subpopulations defined by morphometric parameters were observed in samples analysed after the *in vitro* test. The analysis also indicated that the relative

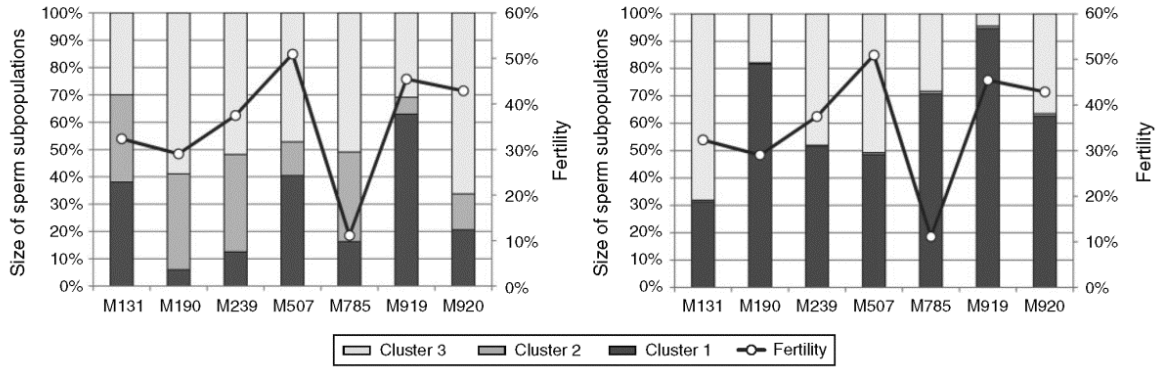

Fig. 3. Analysis of the correlation between the distribution of the sperm subpopulations and the fertility rate (seven males, three replicates). Data show sperm subpopulations defined on the basis of head morphometric parameters of the fresh samples (left) and the migrating samples (right) and their correlation with fertility. The Spearman coefficient of the fresh sample (Cluster 2 size vs fertility) = −0.75 ($P = 0.05$).

size of Cluster 2 was negatively correlated with male fertility (Spearman coefficient = −0.75, $P < 0.05$).

Discussion

Fertility is a very complex biological function that depends on several sperm properties necessary for the spermatozoa to overcome various barriers in the female reproductive tract to reach the fertilisation site (Hawk 1983; Suarez 2008; Kölle *et al.* 2010). This spermatozoa ability was previously evaluated *in vitro* using sperm migration tests, but only the number of cells that migrated was found to be an accurate tool for the prediction of field fertility (Ola *et al.* 2003). In a previous study, we developed a new mucus surrogate penetration test that allowed the collection and counting of spermatozoa able to overcome the mucus barrier in a mucus surrogate-free diluent, which makes it possible to analyse the qualities of those migrating cells (Martínez-Rodríguez *et al.* 2014).

It is known that a sperm sample is not a homogeneous cell population and that different sperm subpopulations can be detected (Maroto-Morales *et al.* 2010, 2012; Martí *et al.* 2011, 2012). Morphometric analysis of the spermatozoa has proven to have high relevance as a predictor of fertility (Gomendio *et al.* 2007; Maree *et al.* 2010). Considering all data together, we believe that it is important to determine the head morphology of the cells that pass the *in vitro* penetration test to define the optimal head morphology of the spermatozoa that migrated, a process that is essential for them to reach the fertilisation site *in vivo*.

Evaluation of subpopulations defined by sperm head morphology

The aim of Experiment 1 in the present study was to conduct a morphometric study and to select the best method for the statistical analysis of the morphometric data obtained using different tests. We first used fresh pooled semen to assess the eight morphometric parameters most commonly considered in sperm head morphometric analyses (Rubio-Guillén *et al.* 2007; Esteso *et al.* 2009; de Paz *et al.* 2011; Martí *et al.* 2011; Maroto-Morales *et al.* 2012). We observed very small differences in morphometric values among control samples from different pools, which indicated the reliability of the morphometric measurements. When the head morphology of the spermatozoa that migrated was analysed, it was observed that in all cases the values for the morphometric parameters were higher in those samples than in the control samples.

To describe the head morphology of the different sperm subpopulations, a PCA was performed to identify the principal factors that explained the sample variance. In a previous PCA study (de Paz *et al.* 2011), we observed that the variation in the values for almost all the parameters was fully explained by the first two principal components, which were defined by eight morphometric parameters. Similar results were reported by Martí *et al.* (2011, 2012), who found that the PCA of the values for seven initial morphometric parameters of different samples rendered two principal components with eigenvalues that explained more than 94% of the cumulative variance. In other studies, three or more principal components derived from the morphometric data were used to describe sperm subpopulations (Peña *et al.* 2005; Maroto-Morales *et al.* 2012). In the aforementioned studies, seven or eight parameters were used to define the principal components. In contrast, in the present study, after conducting several PCAs, we found that four primary parameters were sufficient to define two principal components that achieved an optimal explanation of the sample variance (≥90%). In the same way, Peña *et al.* (2005) conducted PCA and clustering analysis of the 12-parameter morphometric data collected for boar ejaculates to identify different sperm subpopulations present in the samples and found that subpopulations could be characterised using only three parameters, namely head length, width and area.

After the clustering analysis of the data for the fresh samples, three sperm subpopulations were defined. The area, length and width of the head were the parameters that best differentiated these three subpopulations, as follows: Subpopulation 1 included spermatozoa with the smallest head area and for which the head width and length defined an elongated cell; Subpopulation 2 was characterised by a medium head area and relatively short and wide

spermatozoa; and Subpopulation 3 contained long and narrow spermatozoa with the largest heads. Others have also identified three subpopulations within fresh or frozen–thawed sperm samples (Thurston *et al.* 2001; Rubio-Guillén *et al.* 2007; Esteso *et al.* 2009; Martí *et al.* 2012), although some studies described four subpopulations (Peña *et al.* 2005; Maroto-Morales *et al.* 2012). The differences depend on the analytical method used.

The presence of the three sperm subpopulations defined in the control samples was evaluated in spermatozoa that overcame the mucus barrier in the penetration test. We observed that Subpopulation 2 was absent; thus, spermatozoa with a short and wide head failed to overcome the cervical mucus barrier in the *in vitro* migration test. This observation indicates that sperm motility *in vitro* is determined, in part, by the morphology of the sperm head. In this regard, the relationship between sperm hydrodynamics and shape was analysed previously by Dresdner and Katz (1981), who found a highly non-linear correlation between these parameters, so that small differences in the geometric parameters of cell morphology can produce large differences in hydrodynamic behaviour. The results of the present study indicated that width and length are the factors that partly determine the capacity of the cells to migrate through the mucus. Several authors have performed morphometric studies in different animal species and have found correlations between head morphometric characteristics and various physiological features. Martí *et al.* (2011, 2012) described the effect of ram age on the distribution of the different morphometrically defined sperm subpopulations. They also observed that the survival rate of spermatozoa after cryopreservation is higher for the subpopulation characterised by smaller, more elongated and elliptical heads and is compromised for the subpopulation with larger, round heads. Gravance *et al.* (1997) studied stallion spermatozoa and obtained higher fertility rates when the semen sample was rich in small-headed spermatozoa. Peña *et al.* (2005) reported that the presence of a high percentage of small-headed spermatozoa in fresh samples of boar semen was positively correlated with better sperm quality after thawing. Oliveira *et al.* (2013) observed a positive correlation between bull fertility and certain morphological variables (width/length ratio, Fourier 2 descriptor) of the sperm in the semen sample. Together, these results indicate that the presence of a high proportion of small-headed spermatozoa clustered in a semen sample would lead to better fertility results, because this subpopulation has a high potential to overcome the cervical barrier and to be of good quality after thawing. Thurston *et al.* (2001) reported three morphologically distinct subpopulations of spermatozoa defined by Fourier descriptors in boar ejaculates and observed that the percentage of spermatozoa in two subpopulations was correlated with the percentage of motile spermatozoa. These subpopulations are not comparable to those observed in the present study because of the method used in their identification.

In the present study, we focused on evaluation of sperm motility using the *in vitro* penetration test based on sperm head morphometry. However, sperm motility has also been correlated with sperm length based on the assumption that there is a link between morphology and speed. In this regard, Humphries *et al.* (2008) suggest that the ratio between flagellum and head length may provide a reasonable predictor of sperm swimming speed, but its use is not appropriate for internal fertilisers. Further studies can be done in this field in the future by using our *in vitro* penetration test device.

Oxidative stress and head sperm morphometry

The susceptibility of spermatozoa to oxidative damage is a problem during sperm storage because it leads to important physiological changes in the spermatozoa (Aitken and Krausz 2001; Storey 2008; Aitken *et al.* 2010; Guthrie and Welch 2012) that lead to decreased pregnancy rates after AI. A small number of studies has been performed to assess the possible effect of oxidant treatments on sperm motility. The reagents most commonly used to induce oxidative stress in spermatozoa produce various reactive oxygen species (ROS), which affect sperm motility in different ways (Armstrong *et al.* 1999). Thus, despite increasing intracellular ROS, Fe^{2+}–ascorbate had little effect on the motility of red deer spermatozoa, whereas the addition of a medium concentration of H_2O_2 (100 μM) greatly decreased sperm motility (Martínez-Pastor *et al.* 2009). To evaluate the relationship between sperm head morphology and the ability of spermatozoa to overcome the mucus barrier in the *in vitro* penetration test, we applied an oxidative treatment to semen samples to induce changes in sperm morphology and then assessed differences in the results of the migration test.

We observed that the values for almost all the morphometric parameters were significantly higher in the oxidised than control samples, but the number of migrating spermatozoa was similar. In previous studies, several authors observed a significant correlation between sperm chromatin status and sperm morphology (Liu and Baker 1992; Ostermeier *et al.* 2001). We also observed a significant correlation between head morphometric values and the sperm chromatin stability assessment (SCSA) parameters in bear spermatozoa and thus deduced that subtle changes in the shape of the bear sperm head detected using the CASMA (Computer-Assisted Sperm Morphology Assessment) system could be related to changes in chromatin structure (Álvarez *et al.* 2008).

The proportion of spermatozoa present in each morphometric subpopulation defined by the PCA and clustering analysis differed between the control and oxidised (OXI-damaged) samples. The control sample had a significantly higher proportion of small-headed spermatozoa than the OXI-damaged sample. The spermatozoa in the oxidised sample that migrated showed the highest head elongation values for all subpopulations in the three samples tested and, again, the relationship between morphology and motility become apparent.

Relationship between fertility and the head morphology of spermatozoa that migrated in vitro

The relationship between fertility and the results of the penetration tests measured as the distance travelled by the vanguard spermatozoon or the number of spermatozoa that travelled a given distance through cervical mucus has been reported by some authors (Suttiyotin *et al.* 1992; Taş *et al.* 2007*a*, 2007*b*; Bacinoglu *et al.* 2008; Gillan *et al.* 2008). In the present study, the number of spermatozoa that migrated through the mucus in the penetration test showed no correlation with fertility rates

following AI. These differences can be explained by methodological differences between the studies.

The subpopulations defined by the morphometric evaluation of the spermatozoa that migrated showed no relationship with fertility rates, although the relative size of Cluster 2 in the fresh samples was negatively correlated with fertility. Thus, the proportion of spermatozoa with a short and wide head present in the fresh semen sample may be a predictor of fertility with regard to it possible affect on the motility of the cells. Eggert-Kruse *et al.* (1996) confirmed the significance of human sperm morphological properties for *in vitro* mucus migratory ability when semen was tested using a standardised cervical mucus penetration test. Eggert-Kruse *et al.* (1996) found a positive correlation among fertility rate, the number of spermatozoa that migrated through a penetration test device and standard sperm morphology. The experimental methods used by Eggert-Kruse *et al.* (1996) differed from those used in the present study, although in both cases sperm morphological assessment using objective criteria provided valuable information about male fertility. In bulls, the correlation between sperm morphology and fertility after AI has been studied (Phillips *et al.* 2004; Al-Makhzoomi *et al.* 2008) and it was found that the percentage of morphologically normal spermatozoa was positively related to fertility. Al-Makhzoomi *et al.* (2008) reported that the proportion of morphologically deviant sperm head shapes was negatively correlated with fertility, particularly with regard to pear-shaped sperm heads. The morphometric dimensions of the bull sperm head have been related to the post-thaw fertility, and the non-return rates were positively correlated with changes in the width/length ratio after cryopreservation (Gravance *et al.* 2009), which is consistent with the results of the present study.

In conclusion, the migration of spermatozoa through a mucus surrogate in the *in vitro* penetration test was associated with the morphology of the sperm head. PCA and clustering analysis of the values for the morphometric parameters of the sperm head defined three cell subpopulations, among which is a subpopulation defined by spermatozoa with short and wide heads that fail to migrate through the mucus surrogate in the penetration test. Oxidative stress induced changes in the morphology of the sperm head and modified the motility patterns of the treated sample in the mucus penetration test. The number of spermatozoa that migrated is not related to fertility, but the proportion of spermatozoa with a short and wide head in the fresh sperm sample may be used as a predictor of male fertility.

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References

Aitken, R. J. (2006). Sperm function tests and fertility. *Int. J. Androl.* **29**, 69–75. doi:10.1111/J.1365-2605.2005.00630.X

Aitken, R. J., and Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122**, 497–506. doi:10.1530/REP.0.1220497

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., and McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum. Reprod.* **25**, 2415–2426. doi:10.1093/HUMREP/DEQ214

Al-Makhzoomi, A., Lundeheim, N., Haard, M., and Rodriguez-Martinez, H. (2008). Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology* **70**, 682–691. doi:10.1016/J.THERIOGENOLOGY.2008.04.049

Álvarez, M., García-Macías, V., Martínez-Pastor, F., Martínez, F., Borragán, S., Mata, M., Garde, J., Anel, L., and De Paz, P. (2008). Effects of cryopreservation on head morphometry and its relation with chromatin status in brown bear (*Ursus arctos*) spermatozoa. *Theriogenology* **70**, 1498–1506. doi:10.1016/J.THERIOGENOLOGY.2008.06.097

Alvarez, M., Nicolas, M., Borragan, S., Lopez-Uruena, E., Anel-Lopez, L., Martinez-Pastor, F., Tamayo-Canul, J., Anel, L., and de Paz, P. (2012). The percentage of spermatozoa lost during the centrifugation of brown bear (*Ursus arctos*) ejaculates is associated with some spermatozoa quality and seminal plasma characteristics. *Anim. Reprod. Sci.* **135**, 113–121. doi:10.1016/J.ANIREPROSCI.2012.09.009

Armstrong, J. S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W. J., and Sikka, S. C. (1999). Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic. Biol. Med.* **26**, 869–880. doi:10.1016/S0891-5849(98)00275-5

Bacinoglu, S., Tas, M., Cirit, U., Ozdas, O. B., and Ak, K. (2008). The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. *Anim. Reprod. Sci.* **104**, 38–46. doi:10.1016/J.ANIREPROSCI.2007.01.014

Bedford, J. M. (2014). Singular features of fertilization and their impact on the male reproductive system in eutherian mammals. *Reproduction* **147**, R43–R52. doi:10.1530/REP-13-0436

Casey, P. J., Gravance, C. G., Davis, R. O., Chabot, D. D., and Liu, I. K. (1997). Morphometric differences in sperm head dimensions of fertile and subfertile stallions. *Theriogenology* **47**, 575–582. doi:10.1016/S0093-691X(97)00015-0

Cox, J. F., Zavala, A., Saravia, F., Rivas, C., Gallardo, P., and Alfaro, V. (2002). Differences in sperm migration through cervical mucus *in vitro* relates to sperm colonization of the oviduct and fertilizing ability in goats. *Theriogenology* **58**, 9–18. doi:10.1016/S0093-691X(02)00919-6

de Paz, P., Mata-Campuzano, M., Tizado, E. J., Alvarez, M., Alvarez-Rodriguez, M., Herraez, P., and Anel, L. (2011). The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. *Theriogenology* **76**, 1313–1325. doi:10.1016/J.THERIOGENOLOGY.2011.05.038

Dresdner, R. D., and Katz, D. F. (1981). Relationships of mammalian sperm motility and morphology to hydrodynamic aspects of cell function. *Biol. Reprod.* **25**, 920–930. doi:10.1095/BIOLREPROD25.5.920

Druart, X., Cognie, J., Baril, G., Clement, F., Dacheux, J. L., and Gatti, J. L. (2009). *In vivo* imaging of *in situ* motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. *Reproduction* **138**, 45–53. doi:10.1530/REP-09-0108

Druart, X., Cognie, J., Baril, G., Clement, F., Dacheux, J. L., and Gatti, J. L. (2011). *In vivo* imaging of ram spermatozoa in the ewe genital tract using fibered confocal microscopy. *Gynecol. Obstet. Fertil.* **39**, 633–635. doi:10.1016/J.GYOBFE.2011.09.007

Eggert-Kruse, W., Schwarz, H., Rohr, G., Demirakca, T., Tilgen, W., and Runnebaum, B. (1996). Sperm morphology assessment using strict criteria and male fertility under *in-vivo* conditions of conception. *Hum. Reprod.* **11**, 139–146. doi:10.1093/OXFORDJOURNALS.HUMREP.A019007

Esteso, M. C., Fernandez-Santos, M. R., Soler, A. J., Montoro, V., Martinez-Pastor, F., and Garde, J. J. (2009). Identification of sperm-head

morphometric subpopulations in iberian red deer epididymal sperm samples. *Reprod. Domest. Anim.* **44**, 206–211. doi:10.1111/J.1439-0531.2007.01029.X

Firman, R. C., and Simmons, L. W. (2010). Experimental evolution of sperm quality via postcopulatory sexual selection in house mice. *Evolution* **64**, 1245–1256.

Gillan, L., Kroetsch, T., Maxwell, W. M., and Evans, G. (2008). Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. *Anim. Reprod. Sci.* **103**, 201–214. doi:10.1016/J.ANIREPROSCI.2006.12.010

Gillies, E., Cannon, R., Green, R., and Pacey, A. (2009). Hydrodynamic propulsion of human sperm. *J. Fluid Mech.* **625**, 445–474. doi:10.1017/S0022112008005685

Gomendio, M., Malo, A. F., Garde, J., and Roldan, E. R. (2007). Sperm traits and male fertility in natural populations. *Reproduction* **134**, 19–29. doi:10.1530/REP-07-0143

Gravance, C. G., Champion, Z., Liu, I. K., and Casey, P. J. (1997). Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. *Anim. Reprod. Sci.* **47**, 149–155. doi:10.1016/S0378-4320(96)01634-X

Gravance, C. G., Casey, M. E., and Casey, P. J. (2009). Pre-freeze bull sperm head morphometry related to post-thaw fertility. *Anim. Reprod. Sci.* **114**, 81–88. doi:10.1016/J.ANIREPROSCI.2008.09.014

Guthrie, H. D., and Welch, G. R. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology* **78**, 1700–1708. doi:10.1016/J.THERIOGENOLOGY.2012.05.002

Hawk, H. W. (1983). Sperm survival and transport in the female reproductive tract. *J. Dairy Sci.* **66**, 2645–2660. doi:10.3168/jds.S0022-0302(83)82138-9

Hidalgo, M., Rodriguez, I., and Dorado, J. M. (2007). The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. *Anim. Reprod. Sci.* **100**, 61–72. doi:10.1016/J.ANIREPROSCI.2006.07.003

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumuller, T. R., and Braun, J. (2001). Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): relation to fertility and seminal plasma growth factors. *J. Androl.* **22**, 104–110.

Humphries, S., Evans, J. P., and Simmons, L. W. (2008). Sperm competition: linking form to function. *BMC Evol. Biol.* **8**, 319–330. doi:10.1186/1471-2148-8-319

Kölle, S., Reese, S., and Kummer, W. (2010). New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. *Theriogenology* **73**, 786–795. doi:10.1016/J.THERIOGENOLOGY.2009.11.002

Kruger, T. F., DuToit, T. C., Franken, D. R., Acosta, A. A., Oehninger, S. C., Menkveld, R., and Lombard, C. J. (1993). A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. *Fertil. Steril.* **59**, 202–209.

Liu, D. Y., and Baker, H. W. (1992). Sperm nuclear chromatin normality: relationship with sperm morphology, sperm–zona pellucida binding, and fertilization rates *in vitro*. *Fertil. Steril.* **58**, 1178–1184.

Maree, L., du Plessis, S. S., Menkveld, R., and van der Horst, G. (2010). Morphometric dimensions of the human sperm head depend on the staining method used. *Hum. Reprod.* **25**, 1369–1382. doi:10.1093/HUMREP/DEQ075

Maroto-Morales, A., Ramon, M., Garcia-Alvarez, O., Soler, A. J., Esteso, M. C., Martinez-Pastor, F., Perez-Guzman, M. D., and Garde, J. J. (2010). Characterization of ram (*Ovis aries*) sperm head morphometry using the Sperm-Class Analyzer. *Theriogenology* **73**, 437–448. doi:10.1016/J.THERIOGENOLOGY.2009.10.003

Maroto-Morales, A., Ramon, M., Garcia-Alvarez, O., Soler, A. J., Fernandez-Santos, M. R., Roldan, E. R., Gomendio, M., Perez-Guzman, M. D., and Garde, J. J. (2012). Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in ram ejaculates: intra- and interindividual variation. *Theriogenology* **77**, 1529–1539. doi:10.1016/J.THERIOGENOLOGY.2011.11.020

Martí, J. I., Aparicio, I. M., and Garcia-Herreros, M. (2011). Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. *Theriogenology* **76**, 97–109. doi:10.1016/J.THERIOGENOLOGY.2011.01.022

Martí, J. I., Aparicio, I. M., Leal, C. L., and Garcia-Herreros, M. (2012). Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. *Theriogenology* **78**, 528–541. doi:10.1016/J.THERIOGENOLOGY.2012.02.035

Martínez-Pastor, F., Aisen, E., Fernández-Santos, M. R., Esteso, M. C., Maroto-Morales, A., García-Álvarez, O., and Garde, J. J. (2009). Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction* **137**, 225–235. doi:10.1530/REP-08-0357

Martínez-Rodríguez, C., Alvarez, M., Ordas, L., Chamorro, C. A., Martinez-Pastor, F., Anel, L., and de Paz, P. (2012). Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test. *Theriogenology* **77**, 1575–1586. doi:10.1016/J.THERIOGENOLOGY.2011.11.026

Martínez-Rodríguez, C., Alvarez, M., López-Urueña, E., Gomes-Alves, S., Anel-López, L. A., Chamorro, C., Anel, L., and de Paz, P. (2014). Ram spermatozoa migrating through artificial mucus *in vitro* have reduced mitochondrial membrane potential but retain their viability. *Reprod. Fertil. Dev.* doi:10.1071/RD13377

Ola, B., Afnan, M., Papaioannou, S., Sharif, K., Bjorndahl, L., and Coomarasamy, A. (2003). Accuracy of sperm-cervical mucus penetration tests in evaluating sperm motility in semen: a systematic quantitative review. *Hum. Reprod.* **18**, 1037–1046. doi:10.1093/HUMREP/DEG209

Oliveira, L. Z., de Arruda, R. P., de Andrade, A. F., Celeghini, E. C., Reeb, P. D., Martins, J. P., dos Santos, R. M., Beletti, M. E., Peres, R. F., Monteiro, F. M., and Hossepian de Lima, V. F. (2013). Assessment of *in vitro* sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. *Anim. Reprod. Sci.* **137**, 145–155. doi:10.1016/J.ANIREPROSCI.2013.01.010

Ostermeier, G. C., Sargeant, G. A., Yandell, B. S., Evenson, D. P., and Parrish, J. J. (2001). Relationship of bull fertility to sperm nuclear shape. *J. Androl.* **22**, 595–603.

Peña, F. J., Saravia, F., García-Herreros, M., Núñez-Martinez, I., Tapia, J. A., Johannisson, A., Wallgren, M., and Rodríguez-Martínez, H. (2005). Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. *J. Androl.* **26**, 716–723. doi:10.2164/JANDROL.05030

Phillips, N. J., Mcgowan, M. R., Johnston, S. D., and Mayer, D. G. (2004). Relationship between thirty post-thaw spermatozoal characteristics and the field fertility of 11 high-use Australian dairy AI sires. *Anim. Reprod. Sci.* **81**, 47–61. doi:10.1016/J.ANIREPROSCI.2003.10.003

Richardson, L., Hanrahan, J. P., O'Hara, L., Donovan, A., Fair, S., O'Sullivan, M., Carrington, S. D., Lonergan, P., and Evans, A. C. (2011). Ewe breed differences in fertility after cervical AI with frozen–thawed semen and associated differences in sperm penetration and physicochemical properties of cervical mucus. *Anim. Reprod. Sci.* **129**, 37–43. doi:10.1016/J.ANIREPROSCI.2011.10.012

Rubio-Guillén, J., González, D., Garde, J. J., Esteso, M. C., Fernández-Santos, M. R., Rodríguez-Gil, J. E., Madrid-Bury, N., and Quintero-Moreno, A. (2007). Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. *Reprod. Domest. Anim.* **42**, 354–357. doi:10.1111/J.1439-0531.2006.00788.X

Sailer, B. L., Jost, L. K., and Evenson, D. P. (1996). Bull sperm head morphometry related to abnormal chromatin structure and fertility.

Cytometry **24**, 167–173. doi:10.1002/(SICI)1097-0320(19960601)24:2<167::AID-CYTO9>3.0.CO;2-G

Song, G. J., Norkus, E. P., and Lewis, V. (2006). Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. *Int. J. Androl.* **29**, 569–575. doi:10.1111/J.1365-2605.2006.00700.X

Storey, B. T. (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* **52**, 427–437. doi:10.1387/IJDB.072522BS

Suarez, S. S. (2008). Regulation of sperm storage and movement in the mammalian oviduct. *Int. J. Dev. Biol.* **52**, 455–462. doi:10.1387/IJDB.072527SS

Suarez, S. S., and Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Hum. Reprod. Update* **12**, 23–37. doi:10.1093/HUMUPD/DMI047

Suttiyotin, P., Thwaites, C. J., and Baillie, N. D. (1992). Relationships between the results of a modified sperm penetration test and a swim-up technique and the fertility of ram semen. *Theriogenology* **37**, 851–857. doi:10.1016/0093-691X(92)90046-T

Taş, M., Bacinoglu, S., Cirit, U., Özdaş, O. B., and Ak, K. (2007*a*). Relationship between bovine fertility and the number of spermatozoa penetrating the cervical mucus within straws. *Anim. Reprod. Sci.* **101**, 18–27. doi:10.1016/J.ANIREPROSCI.2006.08.020

Taş, M., Bacinoglu, S., Cirit, U., Ozgümüs, S., Kaşgöz, H., and Pabuccuoğlu, S. (2007*b*). Estimation of the potential fertility based upon non-return rates of bulls: using polyacrylamide gel instead of cervical mucus in the sperm penetration test. *Theriogenology* **68**, 981–987. doi:10.1016/J.THERIOGENOLOGY.2007.07.011

Thurston, L. M., Watson, P. F., Mileham, A. J., and Holt, W. V. (2001). Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J. Androl.* **22**, 382–394. doi:10.1002/J.1939-4640.2001.TB02194.X

World Health Organization (WHO). (2010) 'WHO laboratory manual for the examination and processing of human semen.' (World Health Organization: Geneva.)

Suplementary material

Materials and methods

S1. Sperm motility evaluation

The sperm were diluted in INRA 96 solution to 10 to 20 x 10^6 spermatozoa/mL and loaded into a Makler counting chamber (10-µm depth; Sefi Medical Instruments, Haifa, Israel) at 39 °C. The CASA system consisted of a trinocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) with a 10 X negative phase-contrast objective, equipped with a warming stage set to 39 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured at 50 frames/sec and were analyzed using a Computer-Assisted Semen Analysis (CASA: ISAS; Proiser, Valencia, Spain) with specific settings for ram spermatozoa. The software rendered the following parameters: (1) percentage of motile spermatozoa (PM), and (2) for each spermatozoon, three velocity parameters (VCL, velocity according to the actual path; VSL, velocity according to the straight path; and VAP, velocity according to the smoothed path), three track-linearity parameters (LIN, linearity; STR, straightness; and WOB, wobble), the amplitude of the lateral displacement of the sperm head (ALH), and the head beat-cross frequency (BCF).

S2. Assessment of sperm quality using flow cytometry

We used flow cytometry to assess the quality of fresh sperm and of the spermatozoa that traversed the cervical mucus barrier. The non-sperm particles and debris were excluded using viability staining (Petrunkina & Harrison, 2011).

S2.1. Acrosomal status and viability

Triple staining with PNA-FITC (Sigma-Aldrich, Madrid, Spain), propidium iodide (PI; Sigma-Aldrich, Madrid, Spain), Hoechst 33342 (Sigma Aldrich, Madrid, Spain) was conducted. The staining was performed by diluting the sperm sample (1-2 x 10^6 spermatozoa/ml) with 300 µL of PBS containing 1 µg/ml of PNA-FITC, 1.5 µM PI and 5 µM Hoechst 33342. After 10 min at room temperature in the dark, the samples were analyzed using flow cytometry. The spermatozoa (Hoechst+) were classified into three sperm subpopulations, as follows: PI+ (not viable), PI-/PNA+ (viable with a damaged acrosome, dACRO), and PI-/PNA- (viable with an intact acrosome, VIAB).

S2.2. Mitochondrial status

Triple staining with YO-PRO-1, Mitotracker Deep Red (Invitrogen) and Hoechst 33342 was conducted. The sperm samples were diluted (1-2 x 10^6 spermatozoa/ml) in 300 µL of PBS and 100 nM YO-PRO-1, 100 nM Mitotracker Deep Red and 5 µM Hoechst 33342 were added. After 10 min at room temperature in the dark, the samples were analyzed using flow cytometry. One population of spermatozoa (Hoechst+) was considered, as follows: Mitotracker+/YO-PRO-1-, which were classified as viable spermatozoa with active mitochondria.

S2.3. Flow cytometric analysis

Flow cytometric analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, Miami, FL, USA) equipped with 405 nm, 488 nm and 635 nm lasers. YO-PRO-1, PNA-FITC and PI were excited at 488 nm, Mitotracker Deep Red was excited at 635 nm and Hoechst 33342 was excited at 405 nm. The fluorescence emitted by YO-PRO-1 and PNA-FITC was analyzed using the FL1 photodetector (530/40-nm band pass filter), the PI and Mitotracker Deep Red fluorescence was analyzed using the FL3 photodetector (670-nm long-wavelength pass filter) and Hoechst 33342 fluorescence was analyzed using FL6 photodetector (450/50-nm band pass filter). The signals of the forward scatter/side scatter (FSC/SSC) and the Hoechst 33342 fluorescence were used to discriminate the sperm population from other events and debris. For each sample, we acquired 5000 spermatozoa using Summit v 4.3 software (Beckman Coulter). The analysis of the flow cytometric data were performed using the Weasel v.2.6 program (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

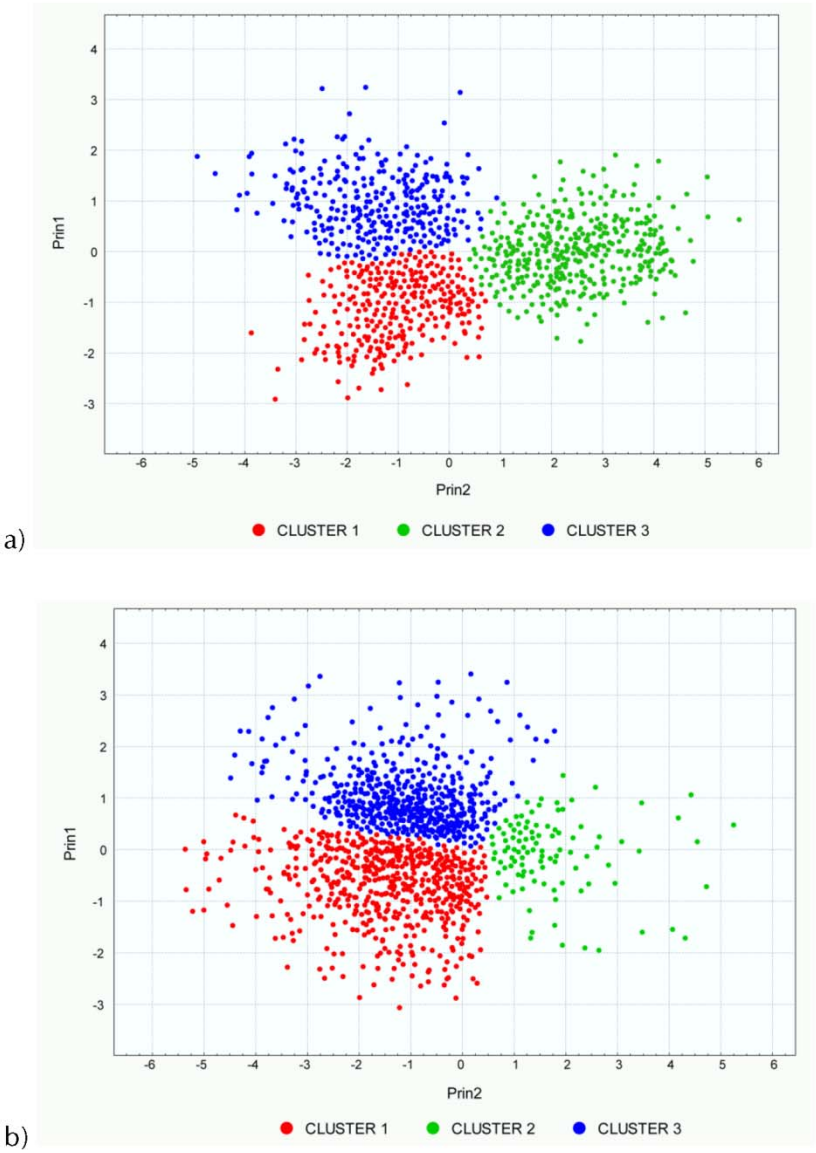

Figure S1. Plots of PC1 and PC2 (3 Pools, 3 replicates) showing the three clusters defined in the analysis for the fresh (a) and migrating (b) samples. The plots were obtained with STATISTICA 9 (StatSoft Inc. Tulsa, OK, USA) and merged using Illustrator CS6 (Adobe, San José, CA, USA)

Results

S1. Experiment 1: Qualitative analysis of the spermatozoa that migrated through the artificial mucus penetration test, evaluated using pooled semen.

The motility, viability (VIAB), acrosomal damage (dACRO) and mitochondrial activity (hMITO) of the fresh sperm sample and the spermatozoa that migrated (except motility) were evaluated (Table S1). The spermatozoa that migrated presented a higher level of acrosomal damage and lower mitochondrial activity and viability than did the control sample ($P<0.05$). The main components obtained after PCA of the four primary morphometric parameters obtained using all the spermatozoa (pre-and post-migrating samples) in the same analysis (3 Pools, 3 replicates) are shown in Table S2.

S2. Experiment 3: Evaluation of the sperm subpopulations defined by head morphology after performing the penetration test using semen samples damaged by oxidation

The quality of the sperm of the fresh samples, the samples after 2-h incubation with the oxidant substance and the spermatozoa from the oxidized sample that migrated through the mucus surrogate in the penetration test are shown in Table S3. The motility, viability, acrosomal status and mitochondrial-membrane potential were negatively affected by the oxidant treatment. The spermatozoa in the oxidized sample that migrated showed a lower level of viability and a higher mitochondrial membrane potential than those of the control and oxidized samples ($P<0.05$), but the percentage of spermatozoa with damaged acrosomes differed only from that of the control sample.

S3. Experiment 4: Relationship among performance in the artificial mucus-penetration test, the head morphometric values and fertility.

The motility, viability, acrosomal damage and mitochondrial activity of spermatozoa that migrated in the fresh sperm of 7 males were evaluated (Table S4) and significant differences were observed among the males.

	Control		**Migrating**	
TM	87.6 ± 2.2			
PM	66.5 ± 2.1			
VAP	140.9 ± 4.7			
VCL	215.4 ± 6.7			
VSL	111.1 ± 4.3			
LIN	51.9 ± 1.4			
STR	76.1 ± 0.8			
WOB	66.4 ± 1.2			
ALH	3.8 ± 0.1			
BCF	26.3 ± 0.6			
VIAB	76.4 ± 1.14	A	68.9 ± 0.8	B
dACRO	8.4 ± 0.6	A	13.6 ± 1.1	B
hMITO	70.8 ± 2.9	A	47.5 ± 1.2	B

Table S1. Analysis of pooled semen (mean values ± SEM). Motility parameters of the viable spermatozoa (VIAB), spermatozoa with acrosomal damage (dACRO) and spermatozoa with a high mitochondrial-membrane potential (hMITO) in the fresh sample (Control) and of the spermatozoa that migrated through the mucus surrogate (Migrating) during a 30-min incubation at 39°C (3 pools, 3 replicates).TM, total motility (%); PM, progressive motility (%); VAP, average path velocity (μm s^{-1}); VCL, curvilinear velocity (μm s^{-1}); VSL, straight-line velocity (μm s^{-1}); LIN, linearity index (%); STR, straightness (%); ALH, amplitude of lateral head displacement (μm); BCF, head beat-cross frequency (%); VIAB, viable spermatozoa (IP-/PNA-); dACRO, acrosomal damage (IP-/PNA+); hMITO, spermatozoa with a high mitochondrial-membrane potential (Mitotracker+/YO-PRO-1-). The letters indicate significant differences between the samples (Control and Migrating).

Discussion

In experiment 1, we observed that the spermatozoa that migrated through the synthetic mucus showed a modest reduction in viability (10%) and a significant reduction in the percentage of spermatozoa with a high mitochondrial membrane potential (33%), as in a previous study (Martínez-Rodríguez *et al.* 2014). Windsor & White (1995) observed that the mitochondrial activity in ram spermatozoa decreased during procedures associated with frozen storage due to the effects of various stresses and suggested that this could be a factor in the reduced fertility of frozen-thawed ram semen. This decrease in the mitochondrial membrane potential was also observed by Martin *et al.* (2007) during the cryopreservation of bovine spermatozoa, although they found it to be a reversible process because a significant proportion of the spermatozoa showed a normal mitochondrial membrane potential when returned to physiological conditions.

The energy required for mammalian sperm motility is generated from intracellular ATP. This energy can be provided either through mitochondrial oxidative phosphorylation or glycolysis,

independent of one another (Storey 2008). Most of the energy needed for mouse sperm motility is generated by glycolysis (Miki *et al.* 2004), although in bull sperm, oxidative phosphorylation is the predominant pathway for energy generation; therefore, the performance of similar studies using sperm from different mammals might reveal species-specific differences (Storey 2008).

A) 4-PARAMETER					
VARIANCES	**EXPLAINED**		**CUMULATIVE**		
Prin1	0.78		0.78		
Prin2	0.17		0.96		
Prin3	0.03		0.99		
EIGENVECTORS	**AREA**	**PERIMETER**	**LENGTH**	**WIDTH**	
Prin1	0.552	0.540	0.460	0.437	
Prin2	0.070	-0.107	-0.661	0.739	
Prin3	0.155	-0.809	0.481	0.297	
PARAMETERS	**AREA**	**PERIMETER**	**LENGTH**	**WIDTH**	**L/W**
CLUSTER 1 (40.8%)	33.6 ± 1.7	22.1 ± 0.6	8.6 ± 0.3	3.7 ± 0.1	2.4
CLUSTER 2 (17.1%)	36.8 ± 1.8	23.3 ± 0.6	8.9 ± 0.3	4.1 ± 0.1	2.2
CLUSTER 3 (42.1%)	36.5 ± 2.1	22.8 ± 0.6	8.9 ± 0.3	3.9 ± 0.1	2.3
B) 6-PARAMETER					
VARIANCES	**EXPLAINED**		**CUMULATIVE**		
Prin1	0.53		0.53		
Prin2	0.28		0.81		
Prin3	0.17		0.98		

Table S2. Analysis of pooled semen. A) Variances (eigenvalues) and eigenvectors for the three main components obtained after PCA of the four primary morphometric parameters obtained for the fresh and migrating samples (3 Pools, 3 replicates). The values for the morphometric parameters (mean values ± SEM) that defined each of the three clusters obtained using PCA and data clustering of the values for the fresh samples (the relative size of each cluster is noted as the proportion) are shown. B) Variances of the three main components obtained using PCA of the values for six morphometric parameters of the fresh and migrating samples (3 Pools, 3 replicates). The variances, which were derived from the eigenvalues, indicate the proportion of the total variance explained by each principal component (Prin). The eigenvectors are a measure of the association of the original parameters with the resulting principal components. The parameters (mean values and SEM) are the morphometric descriptors of the sperm head for each cluster (subpopulations) found using PCA-cluster analysis (L/W is the Length/Width ratio). The relative number of spermatozoa in the seminal sample belonging to each cluster is indicated in parentheses as a percentage.

	Control		**OXI_Damage**		**OXI_Migrating**	
TM	90.9 ± 0.6	a	82.6 ±7.2	b		
PM	71.1 ± 1.2		71.1 ± 3.1			
VAP	140.5 ± 3.8		136.5 ± 10.1			
VCL	219.3 ± 6.7		216.2 ± 8.9			
VSL	107.1 ± 3.3		113.6 ± 10.7			
LIN	49.6 ± 1.2		52.6 ± 3.9			
STR	74.7 ± 0.9	a	81.8 ± 2.2	b		
WOB	65.1 ± 1.2		63.6 ± 3.5			
ALH	3.8 ± 0.1		3.5 ± 0.2			
BCF	27.5 ± 0.4		32.1 ± 1.9			
VIAB	81.2 ± 2.5	a	70.5 ± 1.4	b	62.8 ± 1.6	c
dACRO	6.3 ± 0.4	a	18.7 ± 1.7	b	17.7 ± 1.3	b
hMITO	66.2 ± 5.1	a	51.8 ± 5.2	b	43.6 ± 2.6	c

Table S3. Analysis of oxidized semen samples (3 Pools, 3 replicates). The values for the motility parameters, VIAB, dACRO and hMITO for the three following types of samples (mean value ± SEM): 1) fresh semen (Control); 2) semen sample after a 2-h incubation with an oxidizing substance (OXI_Damage), 3) spermatozoa from the oxidized sample that migrated through the mucus surrogate during a 30-min incubation at 39 °C (OXI_Migrating) are shown. See description for Table S1. The letters indicate significant differences between the samples (Control, OXI_Damage and OXI_Migrating).

Bull spermatozoa are dependent upon respiratory metabolism during passage through the cervix (Storey 2008) and consequently, if ram sperm have the same behaviour, that fact would explain

the reduction in mitochondrial activity observed in the present study. The synthetic mucus barrier present in the *in-vitro* test incurred physiological stress on the spermatozoa, which may explain the decrease in the mitochondrial activity in the spermatozoa that migrated. Therefore, as described for bull semen (Martin e*t al*. 2007), it can be hypothesized that a proportion of the viable spermatozoa with low mitochondrial membrane potential that migrated in the present study might recover a normal mitochondrial membrane potential when returned to the resting conditions.

2. Oxidative stress and head sperm morphometry

Oxidative stress negatively affected the total motility, viability, and the acrosomal and mitochondrial membrane status of the spermatozoa. Despite this, the number of spermatozoa that migrated was similar in the oxidized semen and the control sample, which is consistent with the similarities in the progressive motility and kinetic parameters observed in both samples.

MALE	TM		PM		VIAB		dACRO		hMITO	
M131	93.9 ± 1.7	a	74.5 ± 2.6	a	90.7 ± 1.1	A	7.8 ± 0.8	a	73.9 ± 9.1	ab
M190	80.9 ± 7.3	b	60.9 ± 6.7	a	87.8 ± 1.8	ab	8.4 ± 1.5	a	79.9 ± 4.3	ab
M239	94.1 ± 2.2	a	73.8 ± 1.1	a	90.6 ± 2.1	ab	7.5 ± 1.6	a	70.6 ± 9.3	a
M507	85.3 ± 1.5	ab	63.6 ± 5.7	a	84.5 ± 1.9	ab	13.3 ± 1.6	b	75.2 ± 8.6	ab
M785	76.2 ± 16.2	b	60.8 ± 12.1	a	81.5 ± 5.2	B	14.6 ± 3.1	ab	70.1 ± 8.3	b
M919	85.5 ± 9.6	ab	58.2 ± 6.5	a	89.9 ± 1.1	ab	7.2 ± 0.8	a	75.4 ± 9.9	ab
M920	74.1 ± 11.1	b	58.8 ± 10.1	a	84.7 ± 4.1	B	10.8 ± 2.1	ab	80.1 ± 5.9	b

Table S4. Analysis of the semen samples used for insemination (7 males, 3 replicates) as to the motility, viability, damaged acrosomes and a high mitochondrial-membrane potential of the spermatozoa (mean values ± SEM). Different letters indicate significant differences among the males' specimens.

References

Martin G, Cagnon N, Sabido O, Sion B, Grizard G, Durand P, Levy R 2007 Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum Reprod* 22 380-388.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, A Chamorro C, Anel L, de Paz P 2014 Ram spermatozoa migrating through artificial mucusin vitrohave reduced mitochondrial membrane potential but retain their viability. *Reprod Fertil Dev* 2014; http://dx.doi.org/10.1071/RD13377

Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA 2004 Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc Natl Acad Sci USA* 101 16501-16506.

Petrunkina AM, Harrison RAP 2011 Mathematical analysis of mis-estimation of cell subsets in flow cytometry: Viability staining revisited. *Journal of Immunological Methods* 368 71–79.

Storey BT 2008 Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* 52 427-437.

Windsor D, White I 1995 Mitochondrial injury to ram sperm during procedures associated with artificial insemination or frozen storage. *Animal Reproduction Science* 40 43-58

DISCUSIÓN

La fertilidad es una función biológica muy complicada que depende de varias propiedades de los espermatozoides, como la movilidad, la morfología, presencia de ciertas proteínas en la superficie de la membrana plasmática... La presencia de un transporte espermático eficaz es un requisito indispensable para el éxito reproductivo (Hawk 1983, Suarez 2008, Kölle *et al.* 2010) y la capacidad de los espermatozoides de superar la barrera de moco cervical es un factor determinante para que la subpoblación de espermatozoides con morfología normal y movilidad vigorosa llegue al sitio de fertilización (Suarez y Pacey 2006). Debido a este hecho, en varias especies animales se ha propuesto la evaluación de la capacidad de los espermatozoides para atravesar una barrera de moco natural o artificial (test de penetración) *in vitro* como método de determinación de la calidad seminal.

Los test de penetración utilizados hasta la fecha se basaban en la determinación de la distancia recorrida por el espermatozoide de vanguardia (el espermatozoide que recorría mayor distancia en un tiempo dado) en un capilar (Keel and Webster 1988, Mole and Fitzgerald 1990, Murase and Braun 1990, Galli *et al.* 1991, Verberckmoes *et al.* 2002, Cox *et al.* 2006, Robayo *et al.* 2008); o en el contaje del número total de espermatozoides que alcanzan una distancia determinada en un tiempo concreto utilizando capilares planos o pajuelas transparentes (Suttiyotin *et al.* 1992, Aitken *et al.* 1992, Clarke *et al.* 1998, Tang *et al.* 1999, Hamano *et al.* 2001, Ivic *et al.* 2002, Tas *et al.* 2007a, 2007b, Gillan *et al.* 2008, Al Naib *et al.* 2011). Ola *et al.* (2003) hicieron una revisión sobre la precisión de los diferentes test de penetración en moco cervical como método para predecir la tasa de fertilidad. Demostraron que los test basados en la determinación de la distancia recorrida por el espermatozoide de vanguardia son menos precisos que los que se basan en el contaje del número de espermatozoides que recorre una distancia determinada en la barrera de moco cervical. En esta tesis doctoral hemos propuesto un nuevo dispositivo para el test de penetración que permite el contaje automático de los espermatozoides que atraviesan una distancia determinada en moco cervical sintético y la evaluación de la calidad de los espermatozoides que atraviesan la barrera de moco.

1. Moco cervical sintético ovino para test de penetración *in vitro*

La primera aproximación para desarrollar el nuevo test de penetración para ovino requiere el desarrollo de un moco cervical sintético lo más parecido posible al moco natural. El moco sintético tiene varias ventajas sobre el natural como son el permitir preparar grandes volúmenes y estandarizar el test ya que las propiedades fisicoquímicas del moco sintético, a diferencia de moco natural, no van a ser dependientes del ciclo estral (Katz *et al.* 1997). Ha habido muchos estudios anteriores en los que se han utilizado diversos sustitutos del moco cervical en los test de penetración *in vitro* (Aitken *et al.* 1992, Perry *et al.* 1996, Engel et al.1999, Tang *et al.* 1999, Anilkumer *et al.* 2001, Ivic *et al.* 2002). Para desarrollar un moco sintético de características reológicas similares al moco cervical de la oveja hemos probado distintas sustancias como base: acrilamida, ácido hialurónico y metilcelulosa.

Los geles de poliacrilamida han sido utilizados previamente como sustitutos de moco cervical en varias especies (hombre: Eggert-Kruse *et al.* 1993, Lorton *et al.* 1981; toro: Anilkumar *et al.* 2001, Tas *et al.* 2007b). Lorton *et al.* (1981) vieron que la migración del semen de toro en geles de acrilamida del 1,8 % era similar a la obtenida en moco cervical bovino natural y Eggert-Kruse *et al.* (1993) observaron que la migración correcta de las muestras de semen humano en gel de poliacrilamida del 1,8 % era mucho más frecuente en el caso del grupo de individuos fértiles. Para probar el gel de poliacrilamida como sustituto del moco natural ovino se prepararon diferentes concentraciones de la misma y se realizaron estudios

reológicos sobre estos mocos sintéticos y el natural. Se vio que el gel de acrilamida de concentración 1.6 % era el más similar al moco natural. Además de estos estudios reológicos se realizaron test de penetración *in vitro* utilizando como moco cervical sintético las distintas concentraciones de acrilamida, y otros utilizando moco natural sobre las mismas muestras de partida. Observamos un correlación positiva entre el número de espermatozoides capaces de atravesar el moco sintético para las concentraciones de acrilamida de 1,5 % a 1,8 % y los capaces de atravesar el moco natural, siendo esta similitud máxima en el caso de la acrilamida 1,6 %.

En humanos, los geles de poliacrilamida se han remplazado por otras sustancias en los test de penetración *in vitro* debido a su baja eficiencia para la migración espermática (Ivic *et al.* 2002). Debido a esto estudiamos el uso de la metilcelulosa como alternativa a la acrilamida para los test en ovino y nuestros resultados mostraron que el número de espermatozoides que migran en el test era altamente dependiente del moco sintético utilizado pero, a diferencia de Ivic *et al.* (2002), observamos que el gel de poliacrilamida era significativamente más permisivo que la metilcelulosa para los espermatozoides ovinos. Estos datos ponen de manifiesto las diferencias en los espermatozoides de distintas especies respecto a sus características biofísicas e indica que para cada especie hay que poner a punto y elegir un moco sintético apropiado para los test de penetración *in vitro*. Teniendo en cuenta todos los resultados anteriores elegimos la acrilamida 1,6 % como mejor medio para sustituir el moco natural ovino en los test de penetración *in vitro*.

Para evaluar la capacidad del gel de acrilamida 1.6 % como moco sintético en test de penetración *in vitro* para discriminar entre espermatozoides con distintos patrones de movilidad aplicamos este test en 3 modelos de conservación de semen ovino: semen fresco y semen refrigerado a 5 o 15 °C durante 24 h. Kasimanckam *et al.* (2007) demostraron que el tiempo de almacenamiento está significativamente asociado con el deterioro de los parámetros de movilidad en semen ovino (motilidad progresiva total, VAP, VSL, VCL, ALH y rectitud). Además, Salamon *et al.* (1979) observaron que el número de partos obtenidos con semen refrigerado a 5 °C disminuía significativamente tras almacenar el semen durante 0, 1, 2, o 3 días siendo los porcentajes de fertilidad 60,0%, 34,3%, 33,8% y 17,1% respectivamente. Nuestro trabajo muestra que el tamaño de la población que atraviesa 2 o 3 cm en el test de penetración está significativamente afectado por la temperatura de almacenamiento. También hemos observado que el número de espermatozoides que atraviesa 2 o 3 cm en la barrera de moco cervical natural o sintético está positivamente relacionado con la movilidad progresiva (PM) y con la velocidad en línea recta (VSL), la frecuencia de batida de la cabeza (BCF), linealidad (LIN), oscilación (WOB) y rectitud (STR), mientras que esta relación es negativa para el daño acrosomal y daños en la membrana plasmática de la muestra de partida. Nuestros resultados no concuerdan con los descritos por Robayo *et al.* (2008), que defendían que la velocidad continua en línea recta (VCL) y la velocidad media (VAP) son los únicos parámetros cinéticos que presentaban una correlación positiva significativa con la habilidad para migrar en moco cervical ovino. Estas diferencias pueden ser explicadas debido a que se utilizaron diferentes procedimientos de evaluación: Robayo *et al.* (2008) analizaron la distancia recorrida por el espermatozoide de vanguardia y nosotros evaluamos la eficiencia de migración espermática como el número de espermatozoides que alcanza una determinada distancia dentro de la columna de moco cervical. En este sentido hay que tener en cuenta también que Love *et al.* (2003) encontraron una asociación muy baja o nula entre todas las medidas de velocidad y la motilidad espermática total, estos autores sugieren que la velocidad a la que un

espermatozoide se mueve y como se mueve es independiente del porcentaje total de espermatozoides móviles.

Hay varios estudios que defienden la importancia de la VAP para determinar el éxito de la muestra en los test de penetración en moco cervical *in vitro* (Robayo *et al.* 2008, Aitken *et al.* 1992, Cox *et al.* 2002) aunque no ha sido asociado con la fertilidad. Sin embargo, las diferencias en la capacidad de los espermatozoides para migrar a través de moco cervical *in vitro* están relacionadas con la habilidad de los espermatozoides para colonizar el oviducto y para fertilizar oocitos madurados *in vitro* (Cox et al 2002). De la misma manera, la importancia de la VSL para determinar la capacidad de fertilización de los espermatozoides también ha sido defendida por diferentes autores (Gillan et al. 2008, Holt *et al.* 1997) y se ha especulado que una elevada VSL puede ser importante durante el transporte espermático a través del tracto reproductor femenino y para la penetración de la cubierta del ovocito (Gillan *et al.* 2008).

Además de las correlaciones con los parámetros de movilidad, encontramos que la presencia de espermatozoides apoptóticos y con daños en el acrosoma está negativamente correlacionada con la capacidad de la muestra para migrar en el moco cervical, mientras que el potencial de membrana no mostraba correlación con la habilidad de migrar de estas células. Anilkumar *et al.* (2001) también demostraron que la integridad acrosomal estaba significativa y positivamente correlacionada con la capacidad del semen de toro de penetrar a través del gel de poliacrilamida en el test de penetración *in vitro*. Neild *et al.* (2005) sugirieron el uso de técnicas basadas en filtración por columnas como una herramienta útil para evaluar la integridad acrosomal ya que estas columnas atrapaban células con daños en la membrana o con acrosomas reaccionados pero permitían el paso de los espermatozoides móviles con membrana intacta. Los espermatozoides con el acrosoma reaccionado son especialmente adherentes y se pueden adherir al vidrio incluso en presencia de albúmina (Suarez 2008). Esta adherencia podría explicar la correlación negativa que hemos encontrado en nuestro estudio entre el daño acrosomal y la reducida habilidad de los espermatozoides para atravesar el moco natural y sintético.

Garner *et al.* (1997) vieron que la medición fluorométrica de la función mitocondrial estaba altamente correlacionada con la estimación microscópica de la movilidad progresiva individual. Sin embargo, la importancia de las mitocondrias en la movilidad espermática se ha reconsiderado últimamente y se cree que la actividad mitocondrial es importante también para mantener los niveles de ATP en la cabeza del espermatozoide, y que la pieza intermedia participa en este proceso de mantenimiento (Silva and Gadella 2006). Respecto a los espermatozoides apoptóticos Martínez-Pastor *et al.* (2008) observaron que los espermatozoides de ciervo que presentaban características típicas de células apoptóticas no son capaces de mantener la movilidad a largo del tiempo. Estos autores vieron que este proceso estaba precedido por la pérdida de potencial de membrana mitocondrial, pero que la pérdida de actividad mitocondrial no estaba directamente relaciona con la pérdida de movilidad. Este hecho podría explicar la ausencia de correlación que hemos observado entre el número de espermatozoides que migran y el estado mitocondrial, mientras que la proporción de célula apoptóticas en la muestra estaba negativamente correlacionada con la progresión espermática en el moco cervical.

2. Diseño de un dispositivo de test de penetración *in vitro* que permita aislar los espermatozoides que pasan el test

Una vez elegido el gel de poliacrilamida al 1.6% como mejor sustituto del moco cervical ovino pasamos a mejorar el dispositivo de test de penetración con el objetivo de conseguir aislar la población de espermatozoides en un diluyente libre de moco sintético, para poder proceder al estudio y caracterización de esta población. Debido a que el moco sintético es muy fluido no fue posible mantener las 2 fases (moco sintético y medio de recogida) en la misma pajuela, teniendo que idear un sistema en 3 fases. El nuevo dispositivo incluía una primera fase donde se sitúa la muestra a testar (pajuela de 0.5 mL de 5 cm de largo), una segunda donde se sitúa el moco sintético (pajuela de 0.25 mL de 2 o 3 cm de largo) y una tercera donde se sitúa el diluyente TFC de recogida (pajuela de 0.5 ml de 5 cm de largo). Estas 3 fases están conectadas y fijadas por medio de unos adaptadores de silicona para que permanezcan unidas durante todo el desarrollo del test. Ya que le número de espermatozoides que migran a través del test va a estar influido por el tiempo de duración del mismo, uno de los primeros pasos fue fijar este tiempo. Probamos los tiempos: 15, 30 y 60 minutos de incubación a 39 °C, ya que es la temperatura del tracto reproductor de la oveja. Los resultados mostraron que tras una incubación de 60 min para desarrollar el test de penetración el número de espermatozoides que atravesaba el test aumentaba significativamente, pero se observó que este tiempo de incubación tan largo afectaba negativamente a la viabilidad y al estado acrosomal de la muestra, y que tras 15 minutos de incubación el número de espermatozoides que atravesaba el test era muy pequeño. Teniendo todos los resultados anteriores en cuenta elegimos 30 min como tiempo de incubación a 39 °C para realizar el test en ovino, ya que permitía obtener un buen número de espermatozoides que migraban a través del test sin afectar mucho la viabilidad y estado acrosomal de la muestra.

El número de espermatozoides que penetra a través del moco cervical sintético en los test de penetración también es altamente dependiente de la concentración de espermatozoides en la muestra seminal, observándose una relación lineal entre estos dos parámetros en los experimentos que hemos realizado. Las muestras con 12 x 10^6 y 25 x 10^6 espermatozoides por mililitro mostraron una significativa reducción en la viabilidad y un aumento en el daño acrosomal tras la incubación del dispositivo durante 30 min a 39 °C, mientras que la muestra de 100 x 10^6 espermatozoides presentaba valores próximos al control de muestra fresca. Diversos autores han estudiado los efectos de la concentración espermática, durante el almacenamiento del semen ovino, sobre los parámetros de calidad espermática (Kasimanickam *et al.* 2007, Gundogan *et al.* 2010, López-Fernández *et al.* 2010) y fertilidad (Alvarez *et al.* 2012). Estos autores vieron que los cambios en la movilidad espermática, morfología e integridad del ADN en las muestras diluidas a 100 x 10^6 espermatozoides por mililitro durante la preservación a corto plazo a 4 °C eran menores que cuando la muestra se diluía a 25 x 10^6 espermatozoides por mililitro (Gundogan *et al.* 2010). Nuestros resultados muestran similitudes con estos estudios encontrando que la calidad de los espermatozoides se mantiene mejor en muestras de altas concentraciones (Kasimanickam *et al.* 2007, Gundogan *et al.* 2010), sin embargo, López-Fernández et al. (2010) observaron que a bajas concentraciones (6 y 12 x 10^6 espermatozoides por mililitro) se obtenían menores ratios de fragmentación del DNA tras el almacenamiento de los espermatozoides. Kasimanickam *et al.* (2007) sugirieron que las posibles razones fisiológicas para la reducción de la calidad a bajas concentraciones podría ser el estrés oxidativo extracelular, y que la razón por la que se produce una mejor preservación en las muestras más concentradas es porque hay un mayor volumen de plasma seminal rodeando a cada espermatozoide.

En la puesta a punto del dispositivo hemos visto que la habilidad de los espermatozoides para atravesar el gel de poliacrilamida estaba significativamente estimulada cuando el dispositivo se incubaba con una inclinación de 45 grados respecto a la superficie del incubador en vez de en horizontal. La influencia de la gravedad y el flujo de los fluidos en los movimientos migratorios de los espermatozoides ha sido estudiado por diversos autores (Katz y Pedrotti 1977, Winet *et al.* 1984, Makler *et al.* 1993, Miki y Clapham 2013) y Miki and Clampham (2013) demostraron que los espermatozoides móviles experimentan geotaxis *in vitro* en columnas verticales que contienen diferentes medios, y defienden que es el factor táxico más importante en mamíferos. La aparente respuesta táxica de los espermatozoides a la gravedad ha sido investigada por Winet *et al.* (1984) en muestras de semen humano. Estos autores mostraron que los factores más importantes para la distribución de los espermatozoides eran la reotaxis negativa y la acumulación cerca de las paredes, mientras que la respuesta a la gravedad era relativamente débil. En otro estudio, se encontró que el efecto de la fuerza de la gravedad sobre los espermatozoides móviles era notable, ya que el número de espermatozoides que nadaban hacia abajo era 5-6 veces mayor de los que nadaban hacia arriba (Miki and Clapham 2013). La barrera de moco sintético de poliacrilamida de nuestro experimento es estable en la posición de 45 grados respecto a la horizontal del incubador pero sería posible que una pequeña parte de la poliacrilamida se estuviera disolviendo en el medio TFC en la superficie de contacto entre e TCF y la acrilamida, lo que podría causar microcorrientes en el dispositivo que estimularan la habilidad de los espermatozoides para orientarse y migrar en contra del flujo del líquido circundante. Este fenómeno concordaría con la teoría de que la reotaxis es el principal mecanismo de guía de los espermatozoides de mamíferos a larga distancia en el tracto reproductor femenino (Miki y Clapham 2013).

Una vez puesto a punto el test se ha determinado el número de espermatozoides que atraviesan una distancia de 2 o 3 cm de moco cervical sintético en el mismo y se han analizado varios parámetros de calidad en los espermatozoides que migran. Si el dispositivo se coloca en horizontal durante la incubación se observa una reducción significativa en la viabilidad y la integridad acrosomal de los espermatozoides que no se observa cuando la incubación se realiza a 45 grados. Además se ha observado una reducción significativa en el número de espermatozoides con alto potencial de membrana mitocondrial para ambas posiciones de incubación. Las diferencias observadas entre las dos posiciones en el test de penetración sugieren que las subpoblaciones que migran no son equivalentes y que deben existir factores físico-químicos que estimulan la migración de los espermatozoides de manera diferencial, probablemente debido a factores reotáxicos como hemos descrito anteriormente. El cambio en el potencial de membrana mitocondrial puede deberse al esfuerzo que deben realizar las células en su paso a través del moco. Los espermatozoides de mamíferos generan energía metabólica en forma de ATP para nadar en el tracto genital femenino y fertilizar el oocito. Esta energía puede ser obtenida por fosforilación oxidativa y/o glicólisis, y esta ruta, que es diferente entre especies, debe depender de los sustratos de los que los espermatozoides dispongan en el oviducto (Storey 2008). La presencia de espermatozoides viables con bajo potencial de membrana puede estar asociada con las primeras señales de muerte celular. Sin embargo, Martin *et al.* (2007) han observado que la disminución en el potencial de membrana mitocondrial puede ser parcialmente reversible en algunos espermatozoides por lo que se puede llegar a obtener un aumento en la proporción de espermatozoides con un potencial de membrana normal una vez se ha vuelto a condiciones fisiológicas. Los espermatozoides de toro dependen de la fosforilación oxidativa en su paso por el cérvix (Storey 2008) y consecuentemente, si los espermatozoides de carnero tienen el mismo comportamiento, este

hecho podría explicar la reducción en el potencial de membrana observado en los espermatozoides tras pasar el test de penetración. La barrera de mocos sintético presente en este test *in vitro* produce un estrés fisiológico a los espermatozoides, lo que puede explicar la reducción en la actividad mitocondrial de estos espermatozoides que migran. De la misma manera, y como se ha descrito para semen de toro (Martin e*t al.* 2007), podría ser que la proporción de espermatozoides con bajo potencial de membrana que hay entre los espermatozoides que pasan el test se reduzca una vez estas células hayan alcanzado un estado de reposo.

3. Análisis morfométrico de la cabeza de los espermatozoides que migran a través de la barrera de moco cervical

Se sabe que una muestra espermática no es una población homogénea de células, si no que se pueden encontrar distintas subpoblaciones celulares dentro de la misma (Maroto-Morales *et al.* 2010, 2012, Marti *et al.* 2011, 2012). El análisis morfométrico de los espermatozoides ha demostrado tener una gran relevancia como predictor de la fertilidad (Gomendio et al. 2007, Maree *et al*. 2010) y está siendo muy estudiado en los últimos años. Utilizando el nuevo dispositivo de test de penetración *in vitro* para ganado ovino que hemos diseñado, hemos estudiado la morfología de la cabeza de los espermatozoides que atraviesan el test para intentar definir cuál es la morfología óptima que deben presentar para atravesar el moco sintético, proceso que es esencial para que puedan alcanzar el sitio de fertilización *in vivo*. El primer paso en el estudio de la morfología de la cabeza de los espermatozoides es la selección del mejor método de análisis para el estudio de los diferentes datos morfométricos recogidos tras el test. Para describir la morfología de la cabeza de las diferentes subpoblaciones en la muestra espermática, se realizó un análisis de componentes principales (PCA) para identificar los principales factores que expliquen la varianza de la muestra. En un estudio PCA que habíamos realizado en nuestro grupo con anterioridad (de Paz *et al.* 2011) observamos que la variación en los valores para casi todos los parámetros quedaba explicada completamente por los 2 componentes principales que estaban definidos por 8 parámetros morfométricos. Martí *et al.* (2011,2012) describieron resultados similares al encontrar que el PCA de los valores de 7 parámetros morfométricos iniciales de diferentes muestras daba como resultado 2 componentes principales con 8 eigenvalores que explicaban más del 94% de la varianza acumulada. En otros estudios se utilizaban 3 o más componentes principales derivados de los datos morfométricos para describir las subpoblaciones espermáticas (Peña *et al.* 2005; Maroto-Morales *et al.* 2012). En los estudios mencionados anteriormente se utilizaron 7 o 8 parámetros para definir los componentes principales. Sin embargo, en el presente estudio, tras llevar a cabo varios PCAs, encontramos que eran necesarios solamente 4 componentes principales primarios para definir dos componentes principales y conseguir una explicación óptima de la varianza de la muestra (≥ 90%). De la misma manera, Peña *et al.* (2005) llevaron a cabo un PCA y un análisis de los datos de 12 parámetros morfométricos de semen de cerdo para identificar diferentes subpoblaciones espermáticas presentes en la muestra y encontraron que las subpoblaciones podían ser caracterizadas utilizando sólo 3 parámetros: longitud, anchura y área de la cabeza.

En nuestros experimentos tras el análisis de los datos de la muestra fresca se definieron 3 subpoblaciones espermáticas. El área, la longitud y la anchura de la cabeza fueron los parámetros que mejor diferenciaban estas 3 subpoblaciones como se describe a continuación: subpoblación 1 que incluye los espermatozoides con la cabeza más alargada y de menor área; la subpoblación 2 está caracterizada por un área de cabeza área mediana siendo

ésta corta y ancha; y la subpoblación 3 que está formada por espermatozoides con cabezas grandes, largas y anchas. Ha habido otros autores que también han identificado 3 subpoblaciones espermáticas dentro de las muestras de semen (Thurston *et al.* 2001; Rubio-Guillén *et al.* 2007; Esteso *et al.* 2009; Martí *et al.* 2012), aunque algunos estudios describen 4 subpoblaciones (Peña *et al.* 2005; Maroto-Morales *et al.* 2012). Las diferencias dependen del método analítico utilizado.

La presencia de las 3 subpoblaciones espermáticas definidas en las muestras control fue evaluada en los espermatozoides que atraviesan la barrera de moco en el test de penetración. Observamos que la subpoblación 2 no estaba presente; por lo tanto, los espermatozoides con cabeza corta y ancha no consiguen atravesar la barrera de moco cervical en los test de migración *in vitro*. Este hecho indica que la movilidad espermática *in vitro* está determinada, en parte, por la morfología de la cabeza del espermatozoide. En este aspecto, la relación entre la hidrodinámica de los espermatozoides y su forma había sido descrita anteriormente por Dresdner and Katz (1981), ya que encontraron una alta correlación no lineal entre estos parámetros, por lo que pequeñas diferencias en los parámetros geométricos de la morfología celular pueden producir grandes variaciones en el comportamiento hidrodinámico. Los resultados de nuestros estudios indican que la anchura y longitud de la cabeza son los factores que definen parcialmente la capacidad de las células de migrar a través del moco. Varios autores han realizado estudios morfométricos en muestras seminales de diferentes especies animales y se han encontrado correlaciones entre las características morfométricas de la cabeza de los espermatozoides y varias características fisiológicas. Martí *et al.* (2011, 2012) describieron el efecto de la edad del carnero en la distribución de las diferentes subpoblaciones espermáticas definidas morfológicamente. También observaron que la tasa de supervivencia de los espermatozoides tras la criopreservación era mayor para la subpoblación que estaba caracterizada por una cabeza elíptica más pequeña y más alargada, mientras que estaba más comprometida para la subpoblación con cabezas más grandes y redondeadas. Gravance *et al.* (1997) estudiaron espermatozoides de caballo y vieron que se obtenían mayores tasas de fertilidad cuando la muestra era rica en espermatozoides de cabeza pequeña. Peña *et al.* (2005) encontraron que la presencia de un gran porcentaje de espermatozoides con cabeza pequeña en muestras frescas de semen de cerdo estaba positivamente correlacionada con una mayor calidad seminal post-descongelación. Oliveira *et al.* (2013) observaron una correlación positiva entre los datos de fertilidad en toro y algunas variables morfológicas (ratio anchura/longitud, descriptor Fourier 2) para los espermatozoides en la muestra seminal. Todos esos resultados juntos indican que la presencia de una elevada proporción de espermatozoides de cabeza pequeña en la muestra seminal podría llevar a mejores resultados de fertilidad, porque esta subpoblación tiene un elevado potencial para atravesar la barrera cervical y para tener una buena calidad post-congelación. Thurston *et al.* (2001) describieron 3 subpoblaciones morfológicamente diferentes definidas por los descriptores de Fourier en eyaculados de cerdo y observaron que le porcentaje de espermatozoides en 2 de las subpoblaciones estaba correlacionado con el porcentaje de espermatozoides móviles. Estas subpoblaciones no se pueden comparar con las de nuestro estudio porque el método usado en su identificación es distinto.

4. Detección de daño por estrés oxidativo en la muestra seminal usando el test de penetración *in vitro*

La susceptibilidad de los espermatozoides al daño oxidativo es un problema durante el almacenamiento del semen porque produce importantes cambios fisiológicos en los

espermatozoides (Aitken and Krausz 2001; Storey 2008; Aitken *et al.* 2010; Guthrie and Welch 2012) que llevan a una disminución de las tasas de fertilidad tras IA. Se ha realizado un pequeño número de estudios para evaluar el posible efecto de tratamientos oxidantes sobre la motilidad espermática. Los agentes más comúnmente utilizados para inducir estrés oxidativo en los espermatozoides producen diversas especies reactivas del oxígeno (ROS), que afectan la movilidad espermática de distintas maneras (Armstrong *et al.* 1999). De esta manera, el Fe^{2+}-ascorbato a pesar de aumentar el nivel de ROS intracelular, tiene escaso efecto en la movilidad de los espermatozoides de ciervo rojo, mientras que la adición de una concentración media de H_2O_2 (100 μM) produce un gran descenso en la movilidad espermática (Martínez-Pastor *et al.* 2009).

Para evaluar la relación entre morfología de la cabeza y la habilidad de los espermatozoides de superar la barrera de moco en los test de penetración *in vitro*, hemos aplicado un tratamiento oxidativo a las muestras seminales para inducir cambios en la morfología espermática y ver si se obtienen así diferencias en los resultados del test *in vitro*. Hemos observado que los valores para casi todos los parámetros morfométricos eran significativamente mayores en las muestras oxidadas que en los controles, pero que el número de espermatozoides que migraba era similar. En estudios anteriores ha habido diversos autores que han observado una correlación significativa entre el estado de la cromatina y la morfología espermática (Liu and Baker 1992; Ostermeier *et al.* 2001). Nosotros también hemos observado una correlación significativa entre los parámetros morfológicos de la cabeza y los resultados del análisis del estatus de la cromatina (SCSA) en espermatozoides de oso de lo que se deduce que cambios significativos en la forma de la cabeza de los espermatozoides de oso utilizando el sistema CASMA (Computer-Assisted Sperm Morphology Assessment) podrían estar relacionados con cambios en la estructura de la cromatina (Álvarez *et al.* 2008).

La proporción de espermatozoides presentes en cada una de las subpoblaciones morfométricas definidas por los análisis PCA son diferentes para la muestra control y la oxidada (OXI-damaged). La muestra control tiene una proporción significativamente mayor de espermatozoides con cabeza pequeña que la muestra oxidada. Los espermatozoides que migraban de la muestra oxidada mostraban valores mayores de elongación de la cabeza para todas las subpoblaciones en todas las muestras testadas y, de nuevo, la relación entre morfología de la cabeza y movilidad vuelve a ser aparente.

5. Relación entre fertilidad y la morfología de la cabeza de los espermatozoides que migran *in vitro*

Hay varios autores que han descrito la relación entre fertilidad y los resultados del test de penetración medidos bien la distancia recorrida por los espermatozoides de vanguardia o por el número de espermatozoides que viajan a lo largo de una distancia determinada a través de la barrera de moco sintético (Suttiyotin *et al.* 1992; Taş *et al.* 2007*a*, 2007*b*; Bacinoglu *et al.* 2008; Gillan *et al.* 2008). En nuestro estudio, el número de espermatozoides que atraviesan la barrera de moco cervical no muestra una correlación con la fertilidad tras IA. Estas diferencias pueden explicarse por las diferencias metodológicas entre estos estudios.

Las subpoblaciones definidas por la evaluación morfométrica de los espermatozoides que migran no mostraba relación con las tasas de fertilidad, pero el tamaño relativo de la subpoblación 2 en la muestra fresca se ha visto que sí está relacionado negativamente con la fertilidad. Por lo tanto, la proporción de espermatozoides con cabezas pequeñas, cortas y anchas presentes en la muestra fresca podría ser un factor importante para predecir la

fertilidad debido al posible efecto de esta morfología sobre el movimiento espermático. Egger-Kruse *et al.* (1996) encontraron una correlación positiva entre la tasa de fertilidad, el número de espermatozoides que migraban en el test de penetración *in vitro* y la morfología espermática. Los métodos experimentales utilizados por Egger-Kruse *et al.* (1996) son diferentes a los utilizados en nuestro estudio, lo que podría explicar las diferencias encontradas en los resultados. La relación entre morfología espermática y fertilidad tras IA también ha sido estudiada en toros (Phillips *et al.* 2004; Al-Makhzoomi *et al.* 2008) y se vio que el porcentaje de espermatozoides morfológicamente normales dentro de la muestra seminal estaba positivamente relacionado con la fertilidad. Al-Makhzoomi *et al.* (2008) describieron que la proporcion de espermatozoides con una morfología de la cabeza anormal estaba negativamente correlacionado con la fertilidad; siendo particularmente llamativa esta correlación negativa para los espermatozoides con cabeza en forma de pera. Las dimensiones morfométricas de las cabezas de los espermatozoides de toros han sido relacionadas con las tasas de fertilidad post-congelación, observándose que las tasas de no retorno estaban positivamente correlacionadas con cambios en el ratio anchura/longitud de la cabeza de los espermatozoides tras la criopreservación (Gravance *et al.* 2009), lo que concuerda con los resultados que hemos obtenido en nuestro estudio.

Bibliografia

Aitken, R. J., Bowie, H., Buckingham, D., Harkiss, D., Richardson, D. W., and West, K. M. (1992). Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. J. Androl. 13, 44–54.

Aitken, R. J., and Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 122, 497–506. doi:10.1530/REP.0. 1220497

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., and McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum. Reprod.* 25, 2415–2426. doi:10.1093/HUM REP/DEQ214

Al-Makhzoomi, A., Lundeheim, N., Haard, M., and Rodriguez-Martinez, H. (2008). Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology* 70, 682–691. doi:10.1016/J.THERIOGEN OLOGY.2008.04.049

Al Naib, A., Hanrahan, J. P., Lonergan, P., and Fair, S. (2011). In vitro assessment of sperm from bulls of high and low field fertility. Theriogenology 76, 161–167. doi:10.1016/ J.THERIOGENOLOGY.2010.10.038

Álvarez, M., García-Macías, V., Martínez-Pastor, F., Martínez, F., Borragán, S., Mata, M., Garde, J., Anel, L., and De Paz, P. (2008). Effects of cryopreservation on head morphometry and its relation with chromatin status in brown bear (*Ursus arctos*) spermatozoa. *Theriogenology* 70, 1498–1506. doi:10.1016/J.THERIOGENOLOGY.2008.06.097

Alvarez, M.,Tamayo-Canul, J., Anel, E., Boixo, J. C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., and de Paz, P. (2012). Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. Theriogenology 77, 1111–1118. doi:10.1016/J.THERIOGENOLOGY. 2011.10.013

Armstrong, J. S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W. J., and Sikka, S. C. (1999). Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic. Biol. Med.* 26, 869–880. doi:10.1016/S0891- 5849(98)00275-5

Anilkumar, R., Devanathan, T.G., Pattabiraman, S.R., Edwin, M.J..(2001). Correlation between the spermatozoal characteristics and sperm penetration distance in polyacrylamide gel and bovine cervical mucus. Theriogenology 55:685-91.

Bacinoglu, S., Tas, M., Cirit, U., Ozdas, O. B., and Ak, K. (2008). The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. *Anim. Reprod. Sci.* 104, 38–46. doi:10.1016/J.ANIRE PROSCI.2007.01.014

Clarke, G. N., Garrett, C., and Baker, H. W. (1998). Quantitative sperm mucus penetration: modified formulae for calculating penetration efficiency. Hum. Reprod. 13, 1255–1259. Doi: 10.1093/HUMREP/13.5. 1255

Cox, J. F., Alfaro, V., Montenegro, V., and Rodriguez-Martinez, H. (2006). Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus. Theriogenology 66, 860–867. doi:10.1016/J.THERIOGENOLOGY.2006.01.062

Cox, J.F., Zavala, A., Saravia, F., Rivas, C., Gallardo, P., Alfaro, V..(2002). Differences in sperm migration through cervical mucus in vitro relates to sperm colonization of the oviduct and fertilizing ability in goats. Theriogenology 58:9-18.

de Paz, P., Mata-Campuzano, M., Tizado, E. J., Alvarez, M., Alvarez-Rodriguez, M.,Herraez, P., andAnel, L. (2011). The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. *Theriogenology* 76, 1313–1325. doi:10.1016/J.THERIOGENOLOGY.2011.05.038

Dresdner, R. D., and Katz, D. F. (1981). Relationships of mammalian sperm motility and morphology to hydrodynamic aspects of cell function. *Biol. Reprod.* 25, 920–930. doi:10.1095/BIOLREPROD25.5.920

Eggert-Kruse, W., Schwalbach, B., Rohr, G., Klinga, K., Tilgen, W., Runnebaum, B..(1993). Evaluation of polyacrylamide gel as substitute for human cervical mucus in the sperm penetration test. Fertil Steril 60:540-9.

Engel, S., Petzoldt R. (1999). Human sperm penetration in different media. Andrologia 31:233-9.

Esteso, M. C., Fernandez-Santos, M. R., Soler, A. J., Montoro, V., Martinez-Pastor, F., and Garde, J. J. (2009). Identification of sperm-head morphometric subpopulations in iberian red deer epididymal sperm samples. *Reprod. Domest. Anim.* 44, 206–211. doi:10.1111/J.1439- 0531.2007.01029.X

Galli, A., Basetti, M., Balduzzi, D., Martignoni, M., Bornaghi, V., and Maffii, M. (1991). Frozen bovine semen quality and bovine cervical mucus penetration test. Theriogenology 35, 837–844. doi:10.1016/0093- 691X(91)90424-C

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M., Marshall, C.E.. (1997). Fluorometric assessments of mitochondrial function viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57:1401-6

Gillan, L., Kroetsch, T., Maxwell, W. M., and Evans, G. (2008). Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. *Anim. Reprod. Sci.* 103, 201–214. doi:10.1016/J.ANIREPROSCI.2006. 12.010

Gomendio, M., Malo, A. F., Garde, J., and Roldan, E. R. (2007). Sperm traits and male fertility in natural populations. *Reproduction* 134, 19–29. doi:10.1530/REP-07-0143

Gravance, C. G., Champion, Z., Liu, I. K., and Casey, P. J. (1997). Sperm head morphometry analysis of ejaculate and dismount stallion semen samples. *Anim. Reprod. Sci.* 47, 149–155. doi:10.1016/S0378-4320(96) 01634-X

Gundogan, M., Yeni, D., Avdatek, F., and Fidan, A. F. (2010). Influence of sperm concentration on the motility, morphology, membrane and DNA integrity along with oxidative stress parameters of ram sperm during liquid storage. Anim. Reprod. Sci. 122, 200–207. doi:10.1016 /J.ANIR EPROSCI.2010.08.012

Guthrie, H. D., and Welch, G. R. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology* 78, 1700–1708. doi:10.1016/ J.THERIOGENOLOGY.2012.05.002

Hamano, K., Tanaka, S., Kawana, Y., Tsujii, H., Sasada, H., Sato, E., Takahashi, T., Miyawaki, K., and Arima, H. (2001). Evaluation of bull fertility by migration of frozen–thawed and washed sperm in medium containing cervical mucus. J. Reprod. Dev. 47, 393–398. doi:10.1262/ JRD.47.393

Hawk, H. W. (1983). Sperm survival and transport in the female reproductive tract. *J. Dairy Sci.* 66, 2645–2660. doi:10.3168/jds.S0022-0302(83) 82138-9

Holt, C., Holt, W.V., Moore, H.D., Reed, H.C., Curnock, R.M..(1997). Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J Androl 18:312-23

Ivic, A., Onyeaka, H., Girling, A., Brewis, I. A., Ola, B., Hammadieh, N., Papaioannou, S., and Barratt, C. L. (2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. Hum. Reprod. 17, 143–149. doi:10.1093/HUMREP/17.1.143

Kasimanickam, R., Kasimanickam, V., Pelzer, K.D., Dascanio, J.J.. (2007). Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 degrees C. Anim Reprod Sci 101:60-73.

Katz, D. F., and Pedrotti, L. (1977). Geotaxis by motile spermatozoa: hydrodynamic reorientation. J. Theor. Biol. 67, 723–732. doi:10.1016/ 0022-5193(77)90257-0

Katz DF, Slade DA, Nakajima ST. (1997). Analysis of pre-ovulatory changes in cervical mucus hydration and sperm penetrability. Adv Contracept;13:143-51.

Keel, B. A., and Webster, B. W. (1988). Correlation of human sperm motility characteristics with an in vitro cervical mucus penetration test. Fertil. Steril. 49, 138–143.

Kölle, S., Reese, S, Kummer, W.(2010). New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. Theriogenology 73:786 -95.

Liu, D. Y., and Baker, H. W. (1992). Sperm nuclear chromatin normality: relationship with sperm morphology, sperm–zona pellucida binding, and fertilization rates *in vitro*. *Fertil. Steril.* 58, 1178–1184.

Lopez-Fernandez, C., Johnston, S. D., Fernandez, J. L., Wilson, R. J., and Gosalvez, J. (2010). Fragmentation dynamics of frozen–thawed ram sperm DNA is modulated by sperm concentration. Theriogenology 74, 1362–1370. doi:10.1016/J.THERIOGENOLOGY.2010.06.006

Lorton, S.P., Kummerfeld, H.L., Foote, R.H..(1981). Polyacrylamide as a substitute for cervical mucus in sperm migration tests. Fertil Steril 35:222-5.

Love, C.C., Thompson, J.A., Brinsko, S.P., Rigby, S.L., Blanchard, T.L., Lowry, V.K.. (2003). Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 60:1127-38.

Makler, A., Stoller, J., Blumenfeld, Z., Feigin, P. D., and Brandes, J. M. (1993). Investigation in real time of the effect of gravitation on human spermatozoa and their tendency to swim-up and swim-down. Int. J. Androl. 16, 251–257. doi:10.1111/J.1365-2605.1993.TB01188.X

Maree, L., du Plessis, S. S., Menkveld, R., and van der Horst, G. (2010). Morphometric dimensions of the human sperm head depend on the staining method used. *Hum. Reprod.* 25, 1369–1382. doi:10.1093/ HUMREP/DEQ075

Maroto-Morales, A., Ramon, M., Garcia-Alvarez, O., Soler, A. J., Esteso, M. C., Martinez-Pastor, F., Perez-Guzman, M. D., and Garde, J. J. (2010). Characterization of ram (*Ovis aries*) sperm head morphometry using the Sperm-Class Analyzer. *Theriogenology* 73, 437–448. doi:10.1016/ J.THERIOGENOLOGY.2009.10.003

Maroto-Morales, A., Ramon, M., Garcia-Alvarez, O., Soler, A. J., Fernandez- Santos, M. R., Roldan, E. R., Gomendio, M., Perez-Guzman, M. D., and Garde, J. J. (2012). Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in ram ejaculates: intra- and interindividual variation. *Theriogenology* 77, 1529–1539. doi:10.1016/ J.THERIOGENOLOGY.2011.11.020

Martí, J. I., Aparicio, I. M., and García-Herreros, M. (2011). Sperm morphometric subpopulations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. *Theriogenology* 76, 97–109. doi:10.1016/J.THERIOGENOLOGY. 2011.01.022

Martí, J. I., Aparicio, I. M., Leal, C. L., and Garcia-Herreros, M. (2012). Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. *Theriogenology* 78, 528–541. doi:10.1016/J.THERIOGENOLOGY. 2012.02.035

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., Levy, R.. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum Reprod* 22 380-388.

Martínez-Pastor, F., Fernández-Santos, M.R., del Olmo, E., Domínguez-Rebolledo, A.E., Esteso, M.C., Montoro, V.. (2008). Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod Fertil Dev 20:547-56.

Martínez-Pastor, F., Aisen, E., Fernández-Santos, M. R., Esteso, M. C., Maroto-Morales, A., García-Álvarez, O., and Garde, J. J. (2009). Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction* 137, 225–235. doi:10.1530/REP-08-0357

Miki, K., and Clapham, D. E. (2013). Rheotaxis guides mammalian sperm. Curr. Biol. 23, 443–452. doi:10.1016/J.CUB.2013.02.007

Mole, J. R., and Fitzgerald, J. A. (1990). Comparison of ram sperm interaction with bovine cervical mucus. Theriogenology 33, 1031–1043. doi:10.1016/0093-691X(90)90065-2

Murase, T., and Braun, J. W. (1990). Impact of methodological factors on sperm penetration into cervical mucus in cattle. Theriogenology 34, 73–80. doi:10.1016/0093-691X(90)90578-H

Neild, D.N., Gadella, B.M., Agüero, A., Stout, T.A., Colenbrander, B.. (2005). Capacitation, acrosome function and chromatin structure in stallion sperm. Anim Reprod Sci. Oct;89(1-4):47-56.

Ola, B., Afnan, M., Papaioannou, S., Sharif, K., Bjorndahl, L., and Coomarasamy, A. (2003). Accuracy of sperm cervical mucuspenetration tests in evaluating sperm motility in semen: a systematic quantitative review. Hum. Reprod. 18, 1037–1046. doi:10.1093/ HUMREP/DEG209

Oliveira, L.Z., de Arruda, R.P., de Andrade, A.F., Celeghini, E.C., Reeb, P.D., Martins, J.P., dos Santos, R.M., Beletti, M.E., Peres, R.F., Monteiro, F.M., Hossepian de Lima, V.F..(2013). Assessment of in vitro sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Anim Reprod Sci.Mar;137(3-4):145-55. doi:10.1016/j.anireprosci.2013.01.010.Epub 2013 Feb 8.

Ostermeier, G. C., Sargeant, G. A., Yandell, B. S., Evenson, D. P., and Parrish, J. J. (2001). Relationship of bull fertility to sperm nuclear shape. *J. Androl.* 22, 595–603.

Peña, F. J., Saravia, F., García-Herreros, M., Núñez-Martínez, I., Tapia, J. A., Johannisson, A., Wallgren, M., and Rodríguez-Martínez, H. (2005). Identification of sperm morphometric subpopulations in two

different portions of the boar ejaculate and its relation to postthaw quality. *J. Androl.* 26, 716–723. doi:10.2164/JANDROL.05030

Perry, R. L., Barratt, C. L., Warren, M. A., and Cooke, I. D. (1996). Comparative study of the effect of human cervical mucus and a cervical mucus substitute, Healonid, on capacitation and the acrosome reaction of human spermatozoa in vitro. Hum. Reprod. 11, 1055–1062. doi:10.1093/ OXFORDJOURNALS.HUMREP.A019296

Phillips, N. J., Mcgowan, M. R., Johnston, S. D., and Mayer, D. G. (2004). Relationship between thirty post-thaw spermatozoal characteristics and the field fertility of 11 high-use Australian dairy AI sires. *Anim. Reprod. Sci.* 81, 47–61. doi:10.1016/J.ANIREPROSCI.2003.10.003

Robayo, I., Montenegro, V., Valdes, C., and Cox, J. F. (2008). CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. Reprod. Domest. Anim. 43, 393–399. doi:10.1111/J.1439-0531.2007.00920.X

Rubio-Guillén, J., González, D., Garde, J. J., Esteso, M. C., Fernández-Santos, M. R., Rodríguez-Gil, J. E., Madrid-Bury, N., and Quintero-Moreno, A. (2007). Effects of cryopreservation on bull spermatozoa distribution in morphometrically distinct subpopulations. *Reprod. Domest. Anim.* 42, 354–357. doi:10.1111/J.1439-0531.2006.00788.X

Salamon, S., Maxwell, W.M.C., Firth, J.H..(1979). Fertility of ram semen after storage at 5°C. Anim Reprod Sci 2:373-85.

Silva, P.F., Gadella, B.M..(2006). Detection of damage in mammalian sperm cells. Theriogenology 65:958-78.

Storey, B. T. (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int. J. Dev. Biol. 52, 427–437. doi:10.1387/IJDB. 072522BS

Suarez, S. S., and Pacey, A. A. (2006). Sperm transport in the female reproductive tract. Hum. Reprod. Update 12, 23–37. doi:10.1093/ HUMUPD/DMI047

Suarez, S.S.. (2008). Control of hyperactivation in sperm. Hum Reprod Update 14:647-57.

Suttiyotin, P., Thwaites, C. J., and Baillie, N. D. (1992). Relationships between the results of a modified sperm penetration test and a swim-up technique and the fertility of ram semen. *Theriogenology* 37, 851–857. doi:10.1016/0093-691X(92)90046-T

Tang, S., Garrett, C., and Baker, H. W. (1999). Comparison of human cervical mucus and artificial sperm-penetration media. Hum. Reprod. 14, 2812–2817. doi:10.1093/HUMREP/ 14.11.2812

Taş, M., Bacinoglu, S., Cirit, U., Ozdas-, O. B., and Ak, K. (2007*a*). Relationship between bovine fertility and the number of spermatozoa penetrating the cervical mucus within straws. *Anim. Reprod. Sci.* 101, 18–27. doi:10.1016/J.ANIREPROSCI.2006.08.020

Taş, M., Bacinoglu, S., Cirit, U., Ozgumus, S., Kas-goz, H., and Pabuccuoglu, S. (2007*b*). Estimation of the potential fertility based upon non-return rates of bulls: using polyacrylamide gel instead of cervical mucus in the sperm penetration test. *Theriogenology* 68, 981–987. doi:10.1016/ J.THERIOGENOLOGY.2007.07.011

Thurston, L. M., Watson, P. F., Mileham, A. J., and Holt, W. V. (2001). Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J. Androl.* 22, 382–394. doi:10.1002/J.1939-4640.2001.TB02194.X

Verberckmoes, S., Van Soom, A., De Pauw, I., Dewulf, J., and de Kruif, A. (2002). Migration of bovine spermatozoa in a synthetic medium and its relation to in vivo bull fertility. Theriogenology 58, 1027–1037. doi:10.1016/S0093-691X(02)00931-7

Winet, H., Bernstein, G. S., and Head, J. (1984). Observations on the response of human spermatozoa to gravity, boundaries and fluid shear. J. Reprod. Fertil. 70, 511–523. doi:10.1530 /JRF.0.0700511

CONCLUSIONES

1.- El gel de poliacrilamida 1,6 % es el medio adecuado para sustituir al moco cervical ovino en los test de penetración *in vitro* de espermatozoides ovinos.

2.- El número de los espermatozoides de carnero que penetran 20 o 30 mm en gel de poliacrilamida 1,6 % o en moco cervical ovino está significativa y positivamente correlacionado con la movilidad progresiva y con los parámetros cinéticos VSL, BCF, LIN, WOB y linealidad; al mismo tiempo esta correlación es negativa respecto al daño acrosomal y las lesiones de la membrana plasmática.

3.- Un dispositivo de test *in vitro* de espermatozoides ovinos configurado por tres cámaras consecutivas: una de carga de muestra, otra con gel sintético para la penetración y la de recolección de espermatozoides y operando en unas condiciones definidas, permite el análisis de calidad específico de los espermatozoides que emigran.

4.-Los espermatozoides que emigran en el dispositivo para el test penetración *in vitro* no modifican su viabilidad pero se reduce significativamente el porcentaje de espermatozoides con alto potencial de membrana.

5.-El análisis mediante componentes principales y de cluster de los parámetros morfométricos de la cabeza de los espermatozoides define tres subpoblaciones celulares en la muestra de semen fresco del morueco. La subpoblación definida por espermatozoides con cabeza corta y ancha no emigra a través del moco sintético en los test de penetración.

6.- El estrés oxidativo induce cambios en la morfología de la cabeza de los espermatozoides y modifica los parámetros de movilidad de la muestra tratada en los test de penetración in vitro.

7.- El número de espermatozoides que migra en el test de penetración no está relacionado con la fertilidad, pero la proporción de espermatozoides con cabeza corta y ancha en la muestra fresca podría ser utilizada como predictor de la fertilidad del macho.

CONCLUSIONS

1.- The polyacrylamide gel at 1.6 % is the best mucus subsitute of ewe cervical mucus to perform in vitro penetration test in sheep.

2.- The number of ram spermatozoa that penetrated 20 or 30 mm into an acrylamide gel or ovine cervical mucus was significantly and positively correlated with progressive motility, and the kinematics parameters VSL, BCF, LIN, WOB and linearity; at the same time this relationship was negative with acrosomal damage and injuries in the plasma membrane.

3.- An in vitro test device for ram spermatozoa consisting on three adjoining units: sample loading unit, synthetic mucus barrier unit and spermatozoa collection unit, working under defined settings, allows the quality assessment of the spermatozoa that overcome the mucus barrier.

4.- Spermatozoa that migrates through the in vitro penetration test device did not show changes on their viability parameters but the percentage of spermatozoa with high membrane potential was significantly reduced.

5.-The principal components and clustering analysis of the morphometric parameters of the sperm head, in fresh semen samples, defined three cell subpopulations. One subpopulation defined by spermatozoa with short and wide heads failed to migrate through the mucus surrogate in the penetration test.

5.- Oxidative stress induced changes in the morphology of the sperm head and modified the motility patterns of the treated sample in the mucus penetration test.

6.- The number of spermatozoa that migrated through the penetration test is not related to fertility, but the proportion of spermatozoa with a short and wide head in the fresh sperm sample may be used as a predictor of male fertility.

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LISTA DE ABREVIATURAS

A	Area
Acryl 1.6/ A 1.6	1.6% polyacrylamide gel
AI	Artificial Insemination
ALH	Lateral displacement of the sperm Head
BCF	Head beat-cross frequency
CASA	Computer-Aided Sperm Analysis
CASMA	Computer-Assisted Sperm Morphology Assessment
CCC	Cubic Clustering Criterion
CMPT	Cervical Mucus Penetration Test
CV	Coefficients of Variation
C012, C025, C050 and C100	12, 25, 50 and 100 x 10^6 spermatozoa mL^{-1} Concentrations
dACRO	damaged acrosome
D20/D30	Sperm progression beyon 20 or 30 mm of mucus
FCM	Fibered Confocal fluorescence Microscopy
FSC	Forward Scatter
GLM	General Linear Models
hMITO	High Mitochondrial activity
HSD	Honestly Significant Difference
IA	Inseminación Artificial
ISAS	Integrated Semen Analyzer System
L	Length/ Longitud
LIN	Linearity
MC_1.33	1.33% methylcellulose
MIXED	Mixed linear models
OXI_Damage	pre-migration oxidised
OXI_Migrating	post-migration oxidised
P	Perimeter/ Perimetro
PCA	Principal Component Analysis
PI	Propidium Iodide
PM	Ppercentage of Motile spermatozoa
PNA-FITC	*Arachis hypogaea* (Peanut) lectin -FITC conjugate
ROS	Reactive Oxygen Species
R05/R15	Samples refrigerated at 5/15 °C
SCSA	Sperm Chromatin Stability Assessment
S.E.M.	Standard Error of the Mean

SSC	Side Scatter
STR	Straightness
S2/S3	2cm/3cm of synthetic mucus
TCF	Tris, Citric acid and Fructose diluent
TEMED	TetraMethylEthyleneDiamine
T15, T30, T60	15, 30 or 60 minutes incubation time
VAP	Velocity according to the smoothed path
VCL	Velocity according to the actual path
VIAB	Viable with an intact acrosome
VSL	Velocity according to the straight path
W	Width/ Anchura
WHO	World Health Organization
WOB	Wobble

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Apellidos, nombre: **Martínez Rodríguez Carmen** NIF/NIE: **12406616W**
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ORGANISMO: Universidad de Sheffield
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becasfpi@mec.es

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SUBDIRECCION GENERAL DE RECURSOS HUMANOS PARA LA INVESTIGACIÓN

CERTIFICADO DE REALIZACIÓN DE ESTANCIA BREVE

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Referencia de la ayuda: EEBB-	**BES-2009-026339**	Referencia del proyecto:	**AGL2008-03087**

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HARAS NATIONAUX
Physiologie de la Reproduction
et des Comportements - UMR 6175
37380 NOUZILLY - France

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The undersigned certifies that the scholar has remained in this centre from (day) of (month) of 201- until (day) of (month) of 201-

CORREO ELECTRONICO

becasfpi@mineco.es

MINISTERIO
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COMPETITIVIDAD

SECRETARÍA DE ESTADO DE INVESTIGACIÓN
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SUBDIRECCIÓN GENERAL DE RECURSOS
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ORGANISMO: Universidad de Murcia
CENTRO: Facultad de Veterinaria
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Nombre y apellidos del firmante: Emilio Martínez García

Cargo: Catedrático de Universidad

Fecha: 30/09/2013

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(*) TO BE COMPLETED BY THE HOST RESEARCH DIRECTOR
The undersigned certifies that the scholar has remained in this centre from (day) of (month) of 201- until (day) of (month) of 201-

becasfpi@mineco.es

Subprograma de Formación de Personal Investigador
C/ Albacete, 5, Planta 6ª Sur
28027 Madrid

INFORMES INTERNACIONALES

Nouzilly le samedi 17 octobre 2015

Pascal Mermillod, PhD, HDR
Responsable Equipe « Interactions Cellulaires et Fertilité »

Tél : +33 2 47 42 79 20
GSM : +33 6 08 27 97 59
Mail : Pascal.Mermillod@tours.inra.fr

Student: ***Carmen Martinez Rodriguez,*** PhD student at University of Leon (Spain)

Title: Device design for in vitro evaluation of cell damage during ram semen transport through cervix

Directors: Dr. Paulino de Paz Cabello and Dr. D. María Mercedes Álvarez García

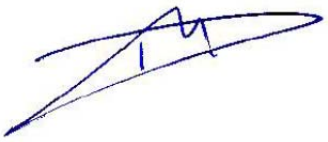
Comments: Artificial insemination is still problematic in ovine species since the results obtained with frozen semen remain very low. Insemination with fresh semen requires a very heavy and strict logistic to distribute the collected semen within a short period of time. Improving this system will require a better knowledge of the regulation of sperm cells survival and fertilization ability through the female genital tract and to have more precise markers of sperm cell function.

In this view, the aims of the present PhD thesis were to set up an original system of migration of sperm cells through a polyacrylamide gel mimicking cervical mucus and to use this system to evaluate sperm quality and find out new and reliable markers of this quality. Indeed, the proportion of short-headed spermatozoa in the sample was shown to reflect the in vivo fertility of the donor male. This research work leaded to the publication of three original papers as first author in two of the highest ranked journals in that field. In addition, the student developed several international collaborations, translating into short term experimental visits in other laboratories in Spain (Murcia) or abroad (Tours and Sheffield). These collaborations, in addition to enlarge the skills of the student, resulted in several co-authoring of collaborative publications.

.../...

Finally, the candidate showed a high motivation to share her results in national and international conferences. To summarize, I think that this PhD work was successful in terms of scientific results, as well as in preparing a fruitful research career.

Conclusion: Given the quality, quantity and originality of the scientific work done, the quality of the publications and the level of personal investment of the candidate in collaborative research at international level, I warmly recommend the defense of this thesis.



Dr Pascal Mermillod

UMR 6175 Physiologie de la Reproduction et des Comportements

Centre INRA de Tours, 37380 Nouzilly, France

Marta F. Riesco, Ph.D.
Centre of Marine Sciences (CCMAR), edf 7
University of Algarve
Campus de Gambelas
8005-139 Faro (Portugal)
Tel.: +351 289 800 900 ext. 7258
Skype name: MARTA F RIESCO

21st-October-2015

To Whom It My Concern

Report on the thesis entitled: "Device design for in vitro evaluation of cell damage during ram semen transport through cervix"

By the PhD student: Carmen Martinez Rodríguez, ITRA-ULE INDEGSAL, University of León, Spain.

Directors: Dr. Paulino de Paz Cabello and Dr. D. María Mercedes Álvarez García

Currently sheep has been characterized as problematic species in terms of artificial insemination successful reporting low fertility rates when this methodology is applied. Fertility is a complex biological process that could be influenced by several properties of the spermatozoa that allow these cells overcome the different barriers in the female reproductive tract to reach fertilization success. In this respect, the main problematic barrier in sheep is cervical mucus composition, variable along the sheep oestrous cycle. Attending to this fact, *in vitro* penetration test device standardization in this species could be a very useful tool for describe the cervical mucus behaviour. One of the major goals of this PhD thesis were to determine a synthetic mucus composition (methylcellulose, acrylamide...). Authors described that acrylamide at 1.6% produced more similar cell counts to the natural mucus, and they found significant correlations between the number of migrating spermatozoa and several sperm quality parameters.

The second goal consisted on design an *in vitro* penetration device to collect the spermatozoa and study its biological features. This objective allowed them the isolation of the population that passed through the mucus barrier in the *in vitro* test. This population was characterized in terms of the number of spermatozoa migrating, motility, viability and the acrosomal and mitochondrial status. Moreover, authors demonstrated that the head morphology parameter was involved in the success to overcome a mucus barrier in a migration test.

Authors interpreted all these results and discussed critically about them providing very interesting and original findings that could help to solve this sheep problematic. Moreover, different analysis and techniques provide a very solid work. As consequence of this, three scientific articles were published demonstrating the high relevance of these studies as definitive evidence. This scientific production evidences the candidate ability to perform an excellent and

Marta F. Riesco, Ph.D.
Centre of Marine Sciences (CCMAR), edf 7
University of Algarve
Campus de Gambelas
8005-139 Faro (Portugal)
Tel.: +351 289 800 900 ext. 7258
Skype name: MARTA F RIESCO

rigorous technical work, as well as, to carry out result interpretation and scientific writing in order to conduct and perform her own research.

Furthermore, the PhD candidate, as shown in her CV, she is a co-author on eight other scientific articles and she has several contributions to international scientific meetings, remarking her hard and rigorous work in conducing her experiments and result analyses. She has a wide experience in teaching activities in the University of León and performed different stays in several groups demonstrating her ability to collaborate with other groups.

For all these reasons, I considerer and it is a real pleasure for me to recommend her for the PhD degree.

Sincerely yours and with kind regards,

Marta F Riesco, PhD

The University Of Sheffield.

The Medical School.

Dr Paulino de Paz Cabello
Edificio LTI planta baja
Servicio de Microscopia
Universidad de León
Campus de Vegazana S/N
24071 León
Spain

Professor William Holt
Academic Unit of Reproductive and Developmental
Medicine
Department of Human Metabolism
Level 4, The Jessop Wing, Tree Root Walk
Sheffield S10 2SF
United Kingdom

Telephone: +44 114 2268345
Fax: +44 114 2261074
Email: bill.holt@sheffield.ac.uk
fazelilab.group.shef.ac.uk

19th October 2015

Dear Dr de Paz Cabello,

Re: PhD thesis by Carmen Martínez-Rodríguez, entitled:
Device design for in vitro evaluation of cell damage during ram semen transport through cervix.

I am writing to express my congratulations to you and your student for the research that has done into her PhD thesis on ram sperm function in relation to semen transport through the cervix and cervical mucus. This is an important topic that has been the subject of considerable interest for many years, mainly because of the need to find techniques that predict ram fertility after artificial insemination. Breeding sheep by the use of artificial insemination represents an important international industry, and it is evident from this and other studies that the assessment of semen quality in sheep has advanced very little over recent decades. The analysis presented in this thesis therefore represent a major contribution to this field.

The research presented here demonstrates that the candidate was able to use a variety of techniques for her research, and was also able to set them in context.

Yours sincerely,

W. V. Holt.

William V. Holt

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El desarrollo de esta tesis doctoral ha sido un camino largo y duro que no podría haber recorrido sin el apoyo y la ayuda de muchas personas, y que volvería a recorrer sólo por encontrarme con ellas. Por ello, me gustaría expresar mi agradecimiento, sólo esperando no olvidarme de nadie:

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CURRICULUM VITAE

Publicaciones

López-Urueña E, Alvarez M, Gomes-Alves S, Anel-López L, **Martínez-Rodríguez C,** Manrique P, Borragan S, Anel L, de Paz P. Optimization of conditions for long-term prefreezing storage of brown bear sperm before cryopreservation. Theriogenology. 2015 Oct 15;84(7):1161-71. doi: 10.1016/j.theriogenology.2015.06.017. Epub 2015 Jun 30.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, Tizado JE, Anel L, de Paz P. Head morphology of ram spermatozoa is associated with their ability to migrate in vitro and correlates with fertility. Reprod Fertil Dev. 2015 May 26. doi: 10.1071/RD15022.

Anel-López L, **Martínez-Rodríguez C**, Soler AJ, Fernández-Santos MR, Garde JJ, Morrell JM. Use of Androcoll-S after thawing improves the quality of electroejaculated and epididymal sperm samples from red deer. Anim Reprod Sci. 2015 Jul;158:68-74. doi: 10.1016/j.anireprosci.2015.04.009. Epub 2015 May 7.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, Chamorro CA, Anel L, de Paz P. Ram spermatozoa migrating through artificial mucus in vitro have reduced mitochondrial membrane potential but retain their viability. *Reproduction, Fertility and Devolopment.* 2014

Gomes-Alves S, Alvarez M, Nicolas M, **Martínez-Rodríguez C**, Borragán S, Chamorro CA, Anel L, de Paz P. Salvaging urospermic ejaculates from brown bear (Ursus arctos). Anim Reprod Sci. 2014 Nov 30;150(3-4):148-57. doi: 10.1016/j.anireprosci.2014.09.007. Epub 2014 Sep 22.

Gomes-Alves S, Alvarez M, Nicolas M, Lopez-Urueña E, **Martínez-Rodríguez C**, Borragan S, de Paz P, Anel L. Use of commercial extenders and alternatives to prevent sperm agglutination for cryopreservation of brown bear semen. Theriogenology. 2014 Aug;82(3):469-74

López-Urueña E, Alvarez M, Gomes-Alves S, **Martínez-Rodríguez C**, Borragan S, Anel-López L, de Paz P, Anel L. Tolerance of brown bear spermatozoa to conditions of pre-freezing cooling rate and equilibration time. *Theriogenology*. 2014

Alvarez-Rodríguez M, Alvarez M, López-Urueña E, **Martínez-Rodríguez C**, Borragan S, Anel-López L, de Paz P, Anel L. Brown bear sperm double freezing: Effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles. *Cryobiology*. 2013 Oct 14

Alvarez-Rodríguez M, Alvarez M, Anel-López L, **Martínez-Rodríguez C**, Martínez-Pastor F, Borragan S, Anel L, de Paz P. The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (Ursus arctos) spermatozoa. *Reprod Fertil Dev*. 2013 Oct;25(8):1185-93

Alvarez M, Tamayo-Canul J, **Martínez-Rodríguez C**, López-Urueña E, Gomes-Alves S, Anel L, Martínez-Pastor F, de Paz P. Specificity of the extender used for freezing ram sperm depends of thespermatozoa source (ejaculate, electroejaculate or epididymis). Anim Reprod Sci 2012 Jun;132(3-4):145-54

Martínez-Rodríguez C, Alvarez M, Ordás L, Chamorro CA, Martinez-Pastor F, Anel L. Paz PD. 2012. Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test. Theriogenology 2012 May;77(8):1575-86

Comunicaciones presentadas en congresos

Lopez-Urueña E, Alvarez M, Gomes-Alves S, **Martínez-Rodríguez C**, Anel-Lopez L, de Paz P, Martinez-Pastor F, Boixo J, Anel L (2012) Evaluation of motility patterns from ram sperm long-term solid storage at 5 °C up to 24 h. 16th ESDAR (European Society for Domestic Animal Reproduction) symposium.

Alvarez M, Lopez-Urueña E, Nicolas M, **Martínez-Rodríguez C**, de Paz P, Martinez-Pastor F, Anel L (2012). Use of viscous media for ram sperm long-term storage at 5°C. 11th AERA International Conference (Spanish Association for Animal Reproduction).

Ordás L, **Martínez-Rodríguez C**, Mata-Campuzano M, Nicolás M, Álvarez M, Anel L, de Paz P, Martínez-Pastor F (2012). DMSO as a vehicle for delivering drugs to sperm suspensions: effects on viability and acrosomal integrity during incubation. 11th AERA International Conference (Spanish Association for Animal Reproduction).

Álvarez-Rodríguez M, Alvarez M, Borragán S, **Martínez-Rodríguez C**, Tamayo Canul J, Martinez-Pastor F, Anel L, de Paz P (2012). Detrimental effects of the elimination of supernatant fraction on non-commercial LDL extenders on sperm of brown bear (*Ursus arctos*) cryopreservation. 11th AERA International Conference (Spanish Association for Animal Reproduction).

Almiñana C, Evans C, **Martínez-Rodríguez C**, Gil MA, Parrilla I, Cuello C, Sanchez-Osorio J, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Wright P and Fazeli A (2011). Embryo alters uterine proteomic profile. 4th COST Action GEMINI General Conference Maternal Interactions with Gametes and Embryos.

López-Urueña E, Alvarez M, Mata-Campuzano M, Gomes-Alves S, **Martínez-Rodríguez C**, de Paz P, Martínez-Pastor F, Anel L. (2011) Preliminary approach of viscous media use for ram sperm freezing. 14th ESDAR (European Society for Domestic Animal Reproduction) symposium.

Martínez-Rodríguez C, Ordás L, Pérez Cereales S, Pérez-Sanchiz R, Herráez P, Anel L, de Paz P, Martínez-Pastor F. (2010). Optimization of the digestion step for isolating genomic DNA from ram spermatozoa. 14th ESDAR (European Society for Domestic Animal Reproduction) symposium.

Ordás L, Mata-Campuzano M, **Martínez-Rodríguez C**, Caspio A, Anel L, de Paz P and Martínez-Pastor F. (2010). Suitability of ISAS sperm counting chambers for motility assessment. 10th AERA International Conference (Spanish Association for Animal Reproduction).

Colaboración en docencia

Asignatura Biología Celular en el grado de Biología (curso 2012/2013)

Asignatura Biotecnología de la Reproducción en la licenciatura de Biotecnología (curso 2012/2013)

Asignatura Citología e Histología Animal y Vegetal en el grado de Biología (curso 2012/2013)

Asignatura Técnicas en Biología Celular en la licenciatura de Biotecnología (curso 2012/2013)

Asignatura Biología Celular en la licenciatura de Biología (curso 2011/2012)

Asignatura Fundamentos en Biología Celular Aplicada en la licenciatura de Biología (curso 2011/2012)

Asignatura Técnicas en Biología Celular en la licenciatura de Biotecnología (curso 2011/2012)

Participación en proyectos de investigación

Proyecto: Inseminación artificial en el oso pardo. Financiación: CANTUR SA. Duración: 2004-2014

Proyecto: Mejora de la rentabilidad de las explotaciones ovinas mediante la optimización de la técnica de la inseminación artificial. Financiación: Sociedad Cooperativa ASOVINO. Duración: 2013-2016

Proyecto: Desarrollo de un programa de inseminación artificial en el oso pardo (Ursus arctos) en el Parque de la Naturaleza de Cabárceno. Financiación: CANTUR SA. Duración: 2012

Proyecto: Desarrollo de un programa de inseminación artificial en el oso pardo (Ursus arctos) en el Parque de la Naturaleza de Cabárceno. Financiación: CANTUR SA. Duración: 2011

Proyecto: Diseño de un dispositivo para la evaluación in vitro de daños celulares durante el transporte en el cervix de semen ovino. Financiación: CICYT (AGL2008-03087GAN). Duración: 2009-2011