UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Medicina y Cirugía Animal

TESIS DOCTORAL

Optimización de la criopreservación (refrigeración y congelación) de espermatozoides de morueco para su aplicación en inseminación artificial a tiempo fijo (IATF) transcervical.

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADO POR

Diego Andrés Galarza Lucero

Directores

Julián Santiago Moreno PhD

Antonio López Sebastián PhD

Madrid

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Optimización de la criopreservación (refrigeración y congelación) de espermatozoides de morueco para su aplicación en inseminación artificial a tiempo fijo (IATF) transcervical

y dirigida por: Dr. Julián Santiago Moreno PhD y Dr. Antonio López Sebastián PhD ,
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"OPTIMIZACIÓN DE LA CRIOPRESERVACIÓN (REFRIGERACIÓN Y CONGELACIÓN) DE ESPERMATOZOIDES DE MORUECO PARA SU APLICACIÓN EN INSEMINACIÓN ARTIFICIAL A TIEMPO FIJO (IATF) TRANSCERVICAL"

DIEGO ANDRÉS GALARZA LUCERO

MADRID, 2019

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Esta tesis fue desarrollada en el Departamento de Reproducción Animal, del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, INIA, Madrid, España.

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Memoria presentada por

Diego Andrés Galarza Lucero

Para optar al grado de Doctor

por la Universidad Complutense de Madrid

VºBº de los Directores

Dr. Julián Santiago Moreno **Dr. Antonio López Sebastián**

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RESUMEN

La presente tesis doctoral fue diseñada para optimizar los procesos de criopreservación de espermatozoides de morueco y conseguir una mejor respuesta celular basada en el incremento del vigor cinético, supervivencia, funcionalidad e integridad morfológica. Los espermatozoides preservados deben ser capaces de atravesar la barrera cervical luego de una inseminación artificial (IA) cervical o transcervical. Las técnicas de reproducción asistida (TRA) usadas en los actuales programas de selección y mejora genética ovina están orientadas a mejorar la respuesta espermática para incrementar la fertilidad. En este sentido, la presente tesis está enfocada en la elección de un método de purificación celular más eficaz, diluyentes y nuevos aditivos que preserven exitosamente los espermatozoides tanto en refrigeración como en congelación, así como a la optimización de rampas de congelación que minimicen el daño a la células espermática. En el Capítulo 1 se evaluó la eficacia de los métodos de selección de espermatozoides por centrifugación de gradientes de densidad (DGC) usando BoviPure®, Percoll® y Accudenz® y un sistema de filtración usando columnas de Sephadex G-15® en semen fresco y refrigerado durante 24 h. Los parámetros cinéticos de motilidad y el estado de las membranas del espermatozoide (plasma y acrosoma) fueron analizados mediante sistema CASA y fluorescencia (*PI / PNA-FITC)*. En semen fresco, la filtración con Sephadex ofreció mejores resultados de motilidad progresiva (%PSM), velocidad rectilínea (VSL) y amplitud del desplazamiento lateral de la cabeza (ALH) ($P < 0,05$) respecto a la selección de espermatozoides con BoviPure, Accudenz y Percoll, respectivamente. Asimismo, en semen refrigerado, el %PSM después de la filtración con Sephadex fue mayor ($P < 0,05$) que el control (semen sin filtrar) y todos los métodos DGC. Los resultados demostraron que la filtración en columnas de Sephadex seleccionó eficientemente los espermatozoides de morueco tanto

en fresco como refrigerado, debido a una mayor recuperación de espermatozoides con progresividad, rectitud y menor desplazamiento de cabeza, comparado con los métodos de DGC.

En el Capítulo 2, fueron evaluados el efecto de la filtración mediante columnas de Sephadex, y la suplementación con glicerol al 2% a diluyentes a base de leche desnatada (UHT) o Tris-Tes-Glucosa (TEST), sobre los parámetros cinéticos, integridad de la membrana plasmática, acrosomal y mitocondrial (*PI / PNA-FITC / Mitotracker*) y capacidad de fertilización, después del almacenamiento del semen a largo plazo. En el semen sin filtrar, el %PSM, VSL, y el porcentaje de espermatozoides con una membrana plasmática intacta / acrosoma intacto / membrana mitocondrial intacta (%IPIAIM) fueron superiores ($P < 0,01$) cuando se usó el diluyente UHT, comparado con TEST durante 96 h, independientemente de la adición de glicerol. Sin embargo, cuando el semen se filtró previamente con Sephadex, la adición de glicerol al diluyente UHT mejoró la motilidad total (%TM), %PSM y VSL en comparación con todos los demás diluyentes ($P < 0,01$). La fecundación *in vitro* (FIV) heteróloga con ovocitos bovinos con zona pelúcida intacta y semen de morueco sin filtrar, diluido con UHT, fresco o refrigerado por 24 o 48 h, demostró que la capacidad fecundante (unión espermática, penetración, formación pronuclear y división celular) fue mantenida ($P > 0,05$) hasta las 48 h. A pesar de que la filtración con Sephadex y la adición de glicerol no proporcionaron beneficios extra, el diluyente UHT preservó mejor la integridad, funcionalidad e incluso la capacidad fecundante del esperma de morueco almacenado en frio que el diluyente TEST.

Consecuentemente, en el Capítulo 3, se evaluó el efecto antioxidante de la L-carnitina (LC) suplementada al diluyente UHT, en concentraciones de 0 (control), 1 (LC1), 2,5 (LC2,5), 5 (LC5), 7,5 (LC7,5) y 10 mM (LC10) para preservar semen ovino refrigerado a largo plazo. Los resultados mostraron que a las 96 h de refrigeración, los %SM y %PSM

fueron superiores con el grupo LC5 comparado con el grupo control, la VCL, VSL y %IPIAIM fueron mejoradas (P < 0,001) en todos los grupos de LC, en comparación con el grupo de control hasta las 96 h. Las tasas de gestación de tres rebaños de ovejas inseminadas vía cervical usando semen ovino diluido con LC5 fresco o refrigerado durante 24 h no evidenciaron diferencias significativas (52,4% vs 42,8%, P > 0,05) en un rebaño, sin embargo, en los otros dos rebaños la fertilidad disminuyó notablemente (P < 0,001). Por lo tanto, la LC mejora las variables cinéticas, integridad de las membranas espermáticas y la fertilidad de semen de morueco refrigerado, no obstante, la fertilidad in vivo después de IATF a las 24 h de conservación necesita ser más investigada.

Finalmente, en el Capítulo 4 se compararon tres protocolos de congelación; Protocolo 1 (que simula la congelación con vapores de NL_2 estático): de +5 ° C a −35 ° C (40 °C/min), de - 35 °C a −65 °C (17 °C/min), y finalmente de −65 °C a −85 °C (3 °C/min); Protocolo 2 (enfriamiento acelerado de tres pasos): de +5 °C a −5 °C (4 °C/min), de −5 °C a −110 °C (25 °C/min), y finalmente de −110 °C a −140 °C (35 °C/min); y Protocolo 3 (enfriamiento acelerado en dos pasos), de +5 °C a −10 °C (5 °C/min), y después de −10 °C a −130 °C (60 °C/min). La calidad del esperma después de la descongelación se redujo en todos los protocolos (P < 0,05) en comparación con el semen fresco. Los %SM, %PSM y %IPIAIM a la descongelación fueron superiores utilizando el Protocolo 3 que el Protocolo 2 (P < 0,05) y el Protocolo 1 (P < 0,01). Asimismo, el porcentaje de espermatozoides con ADN fragmentado después de la descongelación fue menor (P < 0,05) al utilizar el Protocolo 3 que el Protocolo 1. Estos resultados sugieren que una velocidad de enfriamiento de 60 °C/min alrededor y después del punto de tiempo de la nucleación de hielo, proporciona una mejor supervivencia y funcionalidad del esperma de morueco después de la descongelación que las velocidades de enfriamiento más bajas (y/o por deceleración).

SUMMARY

The present thesis was designed to optimize the processes of cryopreservation of ram sperm to achieve a successful cellular response based on an increase in kinetic vigor, survival, functionality and morphological integrity. The preserved sperm must be able to cross the cervical barrier after either cervical or transcervical artificial insemination (AI). Assisted reproductive techniques (ART) used in the current ovine crossbreeding programs are aimed to improve sperm response to increasing fertility. In this sense, this thesis was focused on the choice of most effective sperm selection method, extenders and new additives that successfully preserve sperm both in cooling or freezing using controlled cooling rates. In Chapter 1, the effectiveness of sperm selection methods was evaluated by density gradient centrifugation (DGC) using either BoviPure®, Percoll® or Accudenz® and filtration using Sephadex G-15® columns in fresh-extended or cold-stored (24 h) ram semen. Kinetic parameters and the status of sperm membranes (plasma and acrosome) were analyzed by CASA system and fluorescence test (*PI / PNA-FITC*), respectively. In fresh semen, after Sephadex filtration, progressive motility (%PSM), straight line velocity (VSL) and the amplitude of lateral head displacement (ALH) were higher ($P < 0.05$) than after treatment with BoviPure, Accudenz, and Percoll, respectively. Likewise, in cold-stored semen, the %PSM value after Sephadex filtration was greater than the control (non-filtered semen) ($P < 0.05$) and all DGC methods. Hence, the results indicated that Sephadex filtration columns successfully selected both fresh-extended or cold-stored ram semen, due to a higher recovery of spermatozoa with progressive motility, straightness, and lower head displacement compared with DGC methods. In Chapter 2, the effect of Sephadex filtration and addition of 2% glycerol to UHT skimmed milk- or TEST (Tris-Tes-Glucose)-based extenders were evaluated on kinetics parameters, integrity of plasma membrane, acrosome, and mitochondrial membrane (*PI /*

PNA-FITC / Mitotracker), and fertilizing ability after cold-storage for long-term. In non-filtered semen, the %PSM, VSL, and the percentage of spermatozoa with an intact plasma membrane / intact acrosome / intact mitochondrial membrane index (%IPIAIM) were higher ($P < 0.01$) with UHT-extender than with TEST-extender during 96 h, irrespective of presence of glycerol. However, when semen was previously filtered with Sephadex, the addition of glycerol to UHT-extender improved total motility (%TM), %PSM and VSL compared to all other extenders ($P < 0.01$). A heterologous *in vitro* fertilization (IVF) using zona-intact bovine oocytes and non-filtered semen, diluted with UHT, either fresh or cold-stored for 24 or 48 h showed that the fertilizing capacity (sperm bound, penetration, pronuclear formation, and cleavage rate) did not decrease ($P > 0.05$) after 48 h of chilling. Although Sephadex filtration and addition of glycerol did not provide extra benefits, the UHT-extender preserved better integrity, functionality and even fertilizing ability of cold-stored ram sperm than TEST-extender. Consequently, in Chapter 3, the antioxidant effect of L-carnitine (LC) supplemented to UHT-extender was evaluated either at a concentration of 0 (control), 1 (LC1), 2.5 (LC2.5), 5 (LC5), 7.5 (LC7.5) and 10 mM (LC10) to preserve long-term chilled ram sperm. The results showed that at 96 h, the %SM and %PSM were greater with LC5 group than the control group. Surprisingly, VCL, VSL, and %IPIAIM values were improved ($P < 0.001$) with all LC groups compared to the control group up to 96 h of chilling. Pregnancy rate of three flocks of ewe inseminated via cervically using ram semen diluted with LC5 in fresh or cold-stored for 24 h showed no significant differences ($P > 0.05$) (52.4% vs 42.8%) in one flock, nevertheless, in the other two flocks, the pregnancy rate decreased strongly ($P < 0.001$). Thus, LC improves the kinetic variables, the integrity of sperm membranes, and the fertility of chilled ram semen, nevertheless, the fertility at 24 h needs to be more investigated.

Finally, Chapter 4 compared three freezing protocols: Protocol 1 (that mimic to liquid nitrogen static vapors method): from +5 °C to −35 °C (40 °C/min), from −35 °C to −65 °C (17 °C/min), and then −65 °C to −85 °C (3 °C/min); Protocol 2 (three-step accelerating cooling): from +5 ° C to −5 °C (4 °C/min), from −5 °C to −110 °C (25 °C/min), and then from −110 °C to −140 °C (35 °C/min); and Protocol 3 (three-step accelerating cooling), from +5 °C to −10 °C (5 °C/min), and then from −10 °C to −130 °C (60 °C/min). Sperm quality after thawing was reduced in all protocols ($P < 0.05$) compared to fresh semen. The motilities and %IPIAIM were greater using Protocol 3 than Protocol 2 ($P < 0.05$) and Protocol 1 ($P < 0.01$) after thawing. Likewise, the percentage of sperm with fragmented DNA after thawing was lower ($P < 0.05$) when using Protocol 3 than Protocol 1. Therefore, these results suggest that a cooling rate of 60 °C/min around and after the time point of ice nucleation provided better post thaw survival and function of ram sperm than lower (and/or decelerating) cooling rates.

INTRODUCCIÓN GENERAL

Antecedentes

Una de las principales limitantes del rendimiento productivo de los pequeños rumiantes es su baja eficacia reproductiva, como consecuencia en parte de las marcadas variaciones estacionales de su reproducción (Colas, 1979). Esto repercute en el uso extensivo de la inseminación artificial (IA) y su aplicación en los programas de mejora genética, también limitada por las dificultades de manejo, que han hecho necesaria la aplicación de métodos de inducción y sincronización de ciclo y ovulación para la aplicación de la IA sistemática, sin detección previa de celos, conocida como inseminación artificial a tiempo fijo (IATF) (Maxwell and Watson, 1996).

A pesar de los avances y esfuerzos realizados para mejorar de las tasas de fertilidad con espermatozoides congelados-descongelados aplicados mediante IA en ovejas, la congelación de espermatozoides sigue suponiendo una limitación tecnológica importante, debido a las variables y, frecuentemente, bajas tasas de fertilidad que se obtienen (Santiago-Moreno and Galarza, 2019). Además, en el caso del ganado ovino, la baja fertilidad con el uso de semen congelado-descongelado por vía cervical, en hembras con celos sincronizados mediante tratamientos en cualquier época de año, limita aún más la utilización de la IA como instrumento fundamental de mejora genética.

La reducida fertilidad obtenida con el uso de la IATF cervical en ovejas, ha estimulado el desarrollo de métodos para optimizar la criopreservación del semen de morueco. Los métodos de criopreservación que actualmente son objeto de investigación incluyen la mejora de los procedimientos de almacenamiento líquido de semen de

morueco a bajas temperaturas (5 a 15 °C), y la congelación con diferentes rampas de enfriamiento: lentas, rápidas, ultrarrápidas y vitrificación cinética.

La criopreservación de semen a temperaturas muy bajas (−196 °C), provoca que las reacciones metabólicas de los espermatozoides estén completamente detenidas. La capacidad fecundante de los espermatozoides después del proceso de congelación-descongelación, no obstante, disminuye debido a la lesión criogénica sufrida por el proceso en si. Las principales lesiones criogénicas de los espermatozoides descongelados incluyen el daño de la membrana plasmática (ej. rotura de la membrana, desnaturalización y desplazamiento de las proteínas de membrana), daño del acrosoma (reacción acrosómica), ruptura de la membrana mitocondrial con disminución significativa de la motilidad, y choque osmótico entre otros. Además, el proceso de congelación-descongelación determina una desestructuración de los microtúbulos que conforman el flagelo del espermatozoide. Así, las alteraciones de la membrana y de los microtúbulos están determinadas por el daño osmótico que se produce durante el proceso de congelación-descongelación. Por otro lado, la producción de especies de oxígeno reactivo (ROS), generados principalmente a nivel mitocondrial durante la criopreservación, va a determinar la peroxidación de los fosfolípidos (LPO) de la membrana del espermatozoide. Estos radicales libres son responsables del desencadenamiento de procesos apoptóticos que se reflejan en la alteración del potencial de membrana mitocondrial interna, fragmentación del ADN, inversión en la membrana de la fosfatidilserina y activación de caspasas (Santiago-Moreno et al., 2013). La optimización de los procesos de criopreservación, intenta reducir estas alteraciones del espermatozoide y se enfoca en varias líneas de investigación tales como rampas de enfriamiento, crioprotectores permeables, aditivos, temperaturas de descongelación, y otros métodos alternativos (ej. vitrificación espermática).

Inseminación artificial en ovejas

La IA en ovejas se ve afectado por varios factores intrínsecos y extrínsecos relacionados con la hembra (estacionalidad, estatus reproductivo y condición corporal, estructura cervical, respuesta al tratamiento hormonal de sincronización de celos-ovulaciones, etc), con el macho (estacionalidad, calidad del esperma, conservación del esperma, etc.), tipo de explotación (condiciones ambientales, estado sanitario, manejo, etc.) y la técnica propiamente dicha (vía de aplicación, calidad de los espermatozoides, dosis, inseminador, etc) (Donovan et al., 2004). Investigaciones previas han demostrado diferencias en la fertilidad entre la IA vaginal y cervicales (esperma refrigerado) y la IA laparoscópica (esperma descongelado) en ovejas de raza Churra (Anel et al., 2006). Además, las bajas tasas de fertilidad en ovejas inseminadas vía cervical con semen de morueco congelado-descongelado ha limitado su uso en los programas de selección ovina (Faigl et al., 2012).

La fertilidad del semen congelado-descongelado después de la IA cervical en ovejas ha sido examinada por varios investigadores, que van desde 3,8–20,5% (Valente et al., 2010) y 20,0–22,0% (Masoudi et al., 2017). Tasas de partos más altas han sido obtenidas bajo condiciones experimentales muy controladas. Donovan et al. (2004) reportaron una tasa de preñez en ovejas de 34–52% después de una IA cervical con congelado-descongelado independientemente del tipo de celo (natural o sincronizado). En todos los casos, sin embargo, siempre están muy por debajo de los obtenidos después de usar semen fresco sin diluir o diluido. Por ejemplo, tasas de preñez del 65–75% (Evans and Maxwell, 1987) y 70–82% (Donovan et al., 2004) fueron obtenidas luego de una IA cervical usando semen fresco de morueco. Por otro lado, la IATF laparoscópica con semen ovino

congelado-descongelado ha evidenciado tasas de fertilidad aproximadamente del 55,2–64,5% (Fukui et al., 2008). No obstante, la dificultad técnica, profesional y de bienestar animal que involucra esta técnica también ha limitado su uso en la mayoría de programas de cruzamiento y mejora genética en el ganado ovino (Salamon and Maxwell, 2000).

Actualmente, en los países europeos (ej. España y Francia) con un mayor número de hembras inseminadas en los programas selección y mejora genética, el procedimiento más común es la IA cervical con semen de morueco almacenado en líquido a bajas temperaturas (5 °C o 15 °C). El periodo de almacenamiento con semen refrigerado afecta a la fertilidad, manteniendo un intervalo óptimo de uso hasta las 6–8 h (Maxwell and Stojanov, 1996). Otras investigaciones han demostrado que la fertilidad disminuye drásticamente cuando la inseminación por vía cervical se realiza con semen conservado durante más de 24 h (Anel et al., 2006). De hecho, reportes previos revelaron una tasa fertilidad de 45–50%, 25–30%, y 15–20% después de la IA cervical con semen almacenado a 5 °C durante 24, 48 y 72 h, respectivamente, en contraste con una tasa de fertilidad de 65–75% usando semen fresco (Maxwell and Salamon, 1993). Independientemente del diluyente, la velocidad de dilución, la temperatura o las condiciones de almacenamiento, los espermatozoides se deterioran a medida que aumenta el periodo de conservación. Asimismo, otros estudios concuerdan con esta afirmación, obteniendo una tasa de fertilidad del 60,2% luego de una IATF cervical usando semen fresco, sin embargo, este valor fue disminuyendo según se incrementó el tiempo de almacenamiento en refrigeración a 5 °C durante 24 h (52,2%), 48 h (30,4%) y 72 h (18,3%) (O'Hara et al., 2010).

A pesar de muchos años de innovación tecnológica y reproductiva en el ganado ovino, la baja fertilidad después de la IA cervical con semen refrigerado, todavía sigue sin resolverse. El aumento de la profundidad de la inseminación cervical puede mejorar la fertilidad, pero hasta el momento ha sido problemático lograr una penetración suficiente debido a la gran variabilidad de las características morfológicas y estructurales del cérvix de la oveja (Kershaw et al., 2005). Se han desarrollado nuevos dispositivos para conseguir una mayor penetración en el canal cervical adaptándose a la forma del cérvix y conseguir depositar la dosis de esperma a mayor profundidad (Anel et al., 2006), sin embargo, las tasas de fertilidad no han alcanzado incrementos significativos en la aplicación práctica de estas tecnologías.

Otros intentos para mejorar la fertilidad de la IA cervical han sido los relacionados con el incremento del número de espermatozoides por dosis de semen refrigerado, incrementado la concentración espermática desde 200 a 400 $\times$ 10^6 espermatozoides / oveja).

Otro factor de variabilidad en los resultados de fertilidad de la IA son las diferencias entre hembras inseminadas durante el celo natural respecto de las hembras con celos sincronizados mediante tratamiento hormonal (progestágeno y eCG) (Rhodes and Nathanielsz, 1988; Evans and Robinson, 1980). Utilizando la IA con celo natural (celo detectado por un macho recela) se consiguen los mejores resultados de fertilidad después de los índices reproductivos conseguidos por monta natural. No obstante, esta técnica se ve limitada por las dificultades de manejo para conseguir en la práctica lotes adecuados de inseminación.

La IATF permite la inseminación de un elevado número de hembras previamente sincronizadas durante cualquier época del año. Los protocolos habituales de sincronización de celo incluyen la aplicación de progesterona (DICO® o CIDR®) (Casali

et al., 2017), o progestágenos como acetato de medroxiprogesterona (MAP), o acetato de fluorogestona (FGA), en dispositivos intravaginales mantenidos durante 12–14 días (Robinson, 1965; Evans and Robinson, 1980) seguidas de la aplicación de gonadotropina coriónica equina (eCG) en el momento de retirada del progestágeno, realizándose la IA por vía cervical con semen refrigerado a las 52–56 h después de la retirada del dispositivo intravaginal (Menchaca and Rubianes, 2004).

Unido a estos tratamientos de sincronización el uso del semen ovino almacenado en líquido bajo condiciones de refrigeración a 15 °C ha sido utilizado extensamente en programas de selección ovina (Maxwell and Salamon, 1993) y se mantiene hasta la actualidad. En España, la mayoría de los programas de inseminación utilizan en la práctica diluyentes comerciales a una concentración de 400×10^6 espermatozoides / oveja, almacenados a 5 °C o 15 °C durante periodos no superiores a las 6–8 h.

Métodos de selección espermática en moruecos

Las características cinéticas, integridad de membranas espermáticas y la capacidad de fertilización del semen de morueco pueden mejorarse aplicando métodos de selección espermática antes de la IA. Los espermatozoides muertos y no funcionales, los desechos celulares y todas las células muertas pueden afectar a los espermatozoides vivos, debido probablemente a un incremento de la producción ROS que se acumulan en concentraciones nocivas, que se han descrito como inductores de apoptosis (Wang et al., 2003).

Los métodos de selección espermática desarrollados en semen ovino incluyen el procedimiento swim-up, la centrifugación de gradientes de densidad (DCG) de dos o más capas y la filtración de espermatozoides, ya sea en columnas de Sephadex o en filtros de

vidrio (glass wool). Los métodos de selección espermática son empleados para obtener suspensiones libres de plasma seminal, con espermatozoides mótiles y con presencia reducida de espermatozoides anormales, inmaduros y muertos, células epiteliales, residuos celulares, linfocitos, etc (Valcárcel et al., 1996), permitiendo potencialmente una mayor capacidad de fertilización (Graham and Graham, 1990). Sin embargo, los métodos de selección espermática pueden tener efectos "iatrogénicos" adversos relacionados con el daño mecánico debido a los procesos tanto de centrifugación como de re-suspensión, lo que estimula la LPO e incrementa la producción de ROS (Sbracia et al., 1996). En los métodos de DGC, los espermatozoides se seleccionan de acuerdo con la densidad, lo que permite el aislamiento de espermatozoides funcionales y morfológicamente normales. Los espermatozoides mótiles con integridad y funcionalidad de las membranas espermáticas son más densos que los espermatozoides degenerados, muertos y restos celulares.

Soluciones como BoviPure®, Percoll®, y Accudenz® han sido utilizadas para seleccionar espermatozoides de individuos humanos así como animales domésticos y silvestres, mediante el método DGC de dos o más capas (Batista et al., 2011; Samardzija et al., 2006). Otra técnica de separación de espermatozoides es la filtración a través de columnas de Sephadex. El Sephadex® es un gel de perlas de dextrano que proporciona diferentes tamaños de poros. Su eficacia ha sido demostrada al seleccionar esperma de humanos (Drobnis et al., 1991) y animales domésticos (Bussalleu et al., 2008; Januskauskas et al., 2005; Sieme et al., 2003). El mecanismo para la captura de espermatozoides en las columnas de Sephadex sigue sin estar claro. Sin embargo, se cree que la filtración se basa en el hecho de que los espermatozoides no viables tienden a adherirse a la matriz de Sephadex en mayor medida que los espermatozoides mótiles y

aparentemente funcionales, y estos últimos son capaces de cruzar la barrera de filtración sin modificación de sus características funcionales (Bussalleu et al., 2008).

Varios métodos de selección de espermatozoides han sido usados en el morueco, incluida la filtración con Sephadex, métodos de inmersión (García-López et al., 1996), lavado con sacarosa y filtración Millipore (Marti et al., 2006), así como la DGC de Percoll (Valcárcel et al., 1996). Aunque se han producido grandes avances en los métodos de DGC para la selección de espermatozoides con diferentes especies (Santiago-Moreno et al., 2016), estos procedimientos incluyen fuerzas centrífugas que pueden ser dañinas, especialmente para esperma ovino (García-López et al., 1996). En consecuencia, es requerida la necesidad de investigar un método de selección para espermatozoides de morueco que mejore las características cinéticas e integridad de las membranas espermáticas.

Almacenamiento líquido de semen de morueco

El almacenamiento de semen en estado líquido bajo condiciones de refrigeración se puede lograr mediante métodos que reducen el metabolismo de los espermatozoides y prolongan su vida fértil. El almacenamiento de semen en estado líquido incluye el enfriamiento a temperaturas reducidas entre 0–5 °C o 10–15 °C y a temperatura ambiente (Maxwell and Salamon, 1993). En la década de 1940, se sugirió una temperatura óptima de 10–15 °C para el almacenamiento de semen en estado líquido (Chang and Walton, 1940), sin embargo, posteriormente otros estudios demostraron que la supervivencia de los espermatozoides de morueco y toro fueron mejores después del almacenamiento a 0–5 °C (Salamon and Maxwell, 2000). Los efectos perjudiciales del choque de frío se superaron mediante el enfriamiento gradual del semen, desde la temperatura ambiente y

mediante la adición de lípidos al diluyente. Se ha demostrado que, la yema de huevo y más probablemente su fracción de lipoproteínas de baja densidad y alto peso molecular, además de proporcionar protección contra el choque por frío, reduce la pérdida de enzimas acrosómicas y previene cambios degenerativos en el acrosoma, (Forouzanfar et al., 2010).

Varios diluyentes de base sintética y no sintética han sido usados para el almacenamiento del semen de morueco en estado líquido. Existe evidencia científica de que el diluyente a base de glucosa-citrato-yema de huevo protege al espermatozoide ovino contra el choque de frío y previene cambios degenerativos en el acrosoma cuando el semen es refrigerado (Maxwell and Salamon, 1993). Otros azúcares, como la sacarosa y lactosa agregados al diluyente base, solo pueden actuar extracelularmente para mantener la presión osmótica del diluyente y la integridad de la membrana de los espermatozoides durante el almacenamiento (Salamon and Maxwell, 2000).

Los diluyentes sintéticos a base de Tris (*hidroximetil-aminometano*) han sido valorados para el almacenamiento de semen del toro, jabalí y morueco (Visser and Salamon, 1973). En el semen ovino, concentraciones entre de 10 a 50 mM de Tris tienen poco o ningún efecto sobre la motilidad y el metabolismo celular. De hecho, se ha sugerido que las concentraciones más altas de Tris en los diluyentes son más ventajosas para preservar la integridad y funcionalidad de espermatozoides almacenados en refrigeración (Salamon and Maxwell, 1995a). Otros diluyentes sintéticos a base de Zwitterion (tampones anfóteros) proporcionan una capacidad eficiente de amortiguación de iones de hidrógeno en el rango de "tolerantes a los espermatozoides" (pH 6,5 a 7,5). Los tampones anfóteros como el Tes [N-Tris (*hydroxymethyl)-methylaminoethane-sulfonic acid*], Hepes [*N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid*], Mops [*3-(N-morpholino)propane sulfonic acid*], Mes [*2(N-morpholino)ethane sulfonic acid*], and

Piepes [(*piperazine-N,N-bis)(2-ethane sulfonic acid*)] necesitan ser ampliamente estudiados. Los diluyentes sintéticos a base de Tris o de la combinación Tris-Tes (diluyentes TEST) han sido utilizados para preservar el semen ovino bajo condiciones de refrigeración (Paulenz et al., 2002; Quan et al., 2016).

Los diluyentes no sintéticos, a base de leche desnatada en polvo reconstituida o tratada con calor (UHT), han sido ampliamente utilizados para la dilución y conservación de semen refrigerado, consiguiendo altos índices de supervivencia y fertilidad (Paulenz et al., 2003; Salamon and Maxwell, 2000). Asimismo, la suplementación con aditivos tales como la yema de huevo, ha supuesto mejoras en los rendimientos tanto de los diluyentes sintéticos (Hollinshead et al., 2004; Kasimanickam et al., 2011) como no sintéticos (O'Hara et al., 2010), dada la protección que brinda a la membrana plasmática contra el choque por frío.

Aditivos y antioxidantes

La incorporación de nuevos aditivos a diluyentes de base sintética o no sintética, son necesarios para mitigar el estrés oxidativo de las células espermáticas, la producción de ROS y LPO, y lograr una mayor supervivencia y funcionalidad de las membranas espermáticas (Budai et al., 2014; Maxwell and Stojanov, 1996).

El estrés oxidativo provoca un desequilibrio entre la producción de ROS y las defensas antioxidantes en el organismo. La producción de ROS en cantidades fisiológicas desempeña un papel fundamental en las funciones de los espermatozoides durante su capacitación, la reacción acrosómica y la adhesión a la zona pelúcida del ovocito (Agarwal and Said, 2004; Bansal and Bilaspuri, 2011). El incremento de la producción de ROS como el peróxido de hidrógeno (H_2O_2), los aniones superóxido (O_2–) y los

radicales hidroxilos (OH–) pueden inducir apoptosis, peroxidación de lípidos de membrana, alteración de las mitocondrias y daño al ADN en el esperma ovino (Kasimanickam et al., 2006), que conduce a una reducción de la motilidad y viabilidad del esperma (Amidi et al., 2016). Los espermatozoides de morueco son muy vulnerables al ataque de los radicales libres, ya que son ricos en ácidos grasos poliinsaturados (Alvarez et al., 1987).

Los antioxidantes son el principal factor de defensa contra el choque frío y el estrés oxidativo inducido por los radicales libres proporcionando un efecto crioprotector durante el proceso de congelación-descongelación de espermatozoides (Silva et al., 2011). Varios antioxidantes han sido evaluados en un intento de minimizar la LPO. Estos incluyen quelantes de cationes, como el ácido etilen-diamino tetraacético (EDTA), y eliminadores directos de ROS como el hidroxitolueno butilado (BHT) (Hammerstedt et al., 1976) y la vitamina E (tocoferol) (Azawi and Hussein, 2013). Estudios anteriores evidenciaron un efecto positivo de los antioxidantes superóxido-dismutasa (SOD), catalasa (CAT), citocromo C (CHc), y glutatión peroxidasa (GP) en semen de morueco almacenado en líquido a 5 °C (Maxwell and Stojanov, 1996). Estos antioxidantes fueron capaces de mejorar tanto la supervivencia como la integridad acrosómica de los espermatozoides, incluso la fertilidad (33–53%) utilizando la IA por vía laparoscópica usando antioxidantes SOD (800 U / ml) y CAT (200 U/ml) respectivamente, suplementadas al diluyente tris-glucosa-yema de huevo. En este sentido, la suplementación de aditivos antioxidantes a los diluyentes sintéticos y no sintéticos, resultaría ser una alternativa exitosa para incrementar la calidad espermática y fertilidad usando semen ovino refrigerado y almacenado a corto y largo plazo. Actualmente, se intenta identificar el efecto de nuevos antioxidantes, que permitan minimizar los daños celulares del esperma ovino que se producen durante el almacenamiento a largo plazo.

La *levocarnitina* o L-carnitina (LC), amina sintetizada a partir de los aminoácidos esenciales lisina y metionina, desempeña un papel importante en el transporte de ácidos grasos de cadena corta, media y larga hacia la mitocondria para la oxidación β, que produce energía fácilmente disponible (ATP) para su uso en el espermatozoide (Agarwal and Said, 2004; Jeulin et al., 1987). En efecto, la LC afecta positivamente la motilidad del esperma, así como el proceso de maduración y espermatogénesis (Ramsay et al., 2001). Dentro del tracto genital masculino, la LC se concentra en el epidídimo y el espermatozoide, mientras que en el líquido seminal eyaculado, la mayoría de la LC y la acetil-L-carnitina (ALC) se encuentran en el plasma seminal y muy poco en el propio espermatozoide (Brooks, 1984). La propiedad antioxidante de la LC se produce debido a un mecanismo de reparación mediante el cual se elimina el acetil-coenzima A (acetil-CoA) tóxica intracelular y/o se reemplazan los ácidos grasos en los fosfolípidos de membrana (Vicari and Calogero, 2001). Se ha demostrado que la LC aumenta la motilidad del esperma humano y también puede tener un efecto crioprotector (Agarwal and Said, 2004). Además, la suplementación con LC a diluyentes a base de Tris, mejoró significativamente sus rendimientos debido a una mayor protección de la membrana plasmática y la integridad funcional mitocondrial (El-Raey et al., 2016). No obstante, un estudio reciente demostró que la suplementación de LC a diluyentes sintéticos a base de Tris no tuvo un efecto beneficioso en el esperma ovino congelado-descongelado (de Souza et al., 2019 Hasta el momento no se han reportado estudios que evalúen el efecto de la LC sobre el esperma ovino bajo condiciones de refrigeración a largo plazo.

Almacenamiento congelado de semen de morueco

La congelación es el procedimiento más usado para criopreservar espermatozoides y ovocitos de animales domésticos y silvestres. Los procedimientos de criopreservación se

orientan a intentar minimizar los daños celulares que se producen durante el proceso de congelación-descongelación celular, principalmente daños ultraestructurales, bioquímicos y funcionales (Maxwell et al., 1993) que incluyen el choque térmico, la formación de cristales de hielo, la deshidratación, el aumento de la concentración de sales y el choque osmótico (Stanic et al., 2000). Además, los cambios de temperatura producidos durante el proceso de crioconservación inducen daños a los orgánulos y membranas de los espermatozoides (ej. la capacitación de los espermatozoides y la reacción acrosómica) de la población sobreviviente (Bailey et al., 2000). Los espermatozoides de morueco (especialmente la membrana plasmática), son susceptibles a diversas tensiones mecánicas durante el proceso de congelación (Anel et al., 2006). De hecho, se han realizado muchos avances en la crioconservación, pero son solo un 50% las células que sobreviven a la descongelación y esto implica una reducida tasa de fertilidad cuando se aplican mediante IA (Watson, 1995, 2000; Grötter et al., 2019).

En los protocolos de congelación de esperma de morueco se ha valorado el tipo de diluyente base (Holt et al., 2005; Johnson et al., 2000; Salamon and Maxwell, 2000), método de adición y concentración de agentes crioprotectores (De Leeuw et al., 1993; Holt, 2000; Watson, 1995), velocidades de enfriamiento y congelación (Ashrafi et al., 2011; Dalal et al., 2016; Demir et al., 2015; Kumar et al., 2003), así como métodos de descongelación para lograr esperma de alta calidad (Salamon and Maxwell, 1995b).

La congelación de espermatozoides mediante vapores de nitrógeno liquido (NL_2) estático, es el procedimiento convencional más usado para congelar semen de morueco. No obstante, la supervivencia e integridad, así como funcionalidad de las membranas espermáticas disminuyen durante el proceso de congelación y descongelación. La congelación tradicional de esperma ovino con vapor de NL_2 estático, proporciona una velocidad de enfriamiento inicial muy rápida, seguida de una velocidad más lenta

(deceleración) (Anel et al., 2003). Esta velocidad de enfriamiento por deceleración, estimula la rápida formación de cristales de hielo intracelular y choque de frío (Hammadeh et al., 2001). Velocidades de enfriamiento altas durante la etapa de nucleación de hielo, y formación de hielo intracelular, inducen que la congelación con vapores de NL_2 estático no sea la más idónea, debido al daño celular y lesiones criogénicas (Mazur, 1984). Por esta razón, tomando como modelo diferentes especies de pequeños rumiantes se han establecido otros métodos alternativos de criopreservación, tales como la congelación mediante biocongelador en dos o tres fases con rampas de enfriamiento a velocidades controladas y crecientes (Esteso et al., 2018), congelación ultrarrápida (Pradieé et al., 2015) y la vitrificación de espermatozoides (Jiménez-Rabadán et al., 2015; Pradieé et al., 2017). Varios protocolos de congelación de semen de morueco han sido propuestos en biocongeladores usando velocidades controladas lineales y en dos fases. Sin embargo, la velocidad óptima de enfriamiento que produce una mayor supervivencia en espermatozoides de morueco aún no ha sido establecida (Dalal et al., 2018). El uso de velocidades bajas durante la disipación del calor latente de fusión y nucleación de hielo, para evitar el choque de frío y formación de cristales de hielo intracelular, seguida por altas velocidades durante la etapa de transición vítrea, podría ser una alternativa exitosa a los métodos de congelación convencionales.

La velocidad de enfriamiento tiene un gran impacto en el volumen de flujo de agua en las células y la tasa de supervivencia. Velocidades de enfriamiento demasiado altas o bajas son perjudiciales para la célula, mientras que una mayor velocidad de enfriamiento induce la formación de hielo intracelular (Holt and Penfold, 2014; Woods et al., 2004), una velocidad de enfriamiento muy baja provoca una deshidratación excesiva y las membranas de las células están expuestas durante períodos más prolongados a las bolsas de soluciones hipertónicas, con posibles efectos perjudiciales, como la extracción de

proteínas y lípidos y la generación ROS (Katkov and Bolu, 2012). La velocidad a la que se forma el hielo durante la crioconservación de espermatozoides depende en gran medida del protocolo de enfriamiento. El hielo extracelular es la causa principal del daño a los espermatozoides durante el proceso de crioconservación (Said et al., 2010), y su tamaño y forma dependen de la velocidad de enfriamiento (Bóveda et al., 2018). En efecto, la velocidad de enfriamiento óptima debe considerarse como un compromiso entre estos efectos opuestos mencionados anteriormente (Mazur, 1984), para lograr minimizar el daño criogénico, así como la viabilidad y fertilidad de las muestras de esperma después de la descongelación (Pegg, 2002). En este sentido, el uso de velocidades de enfriamiento por aceleración, usando velocidades iniciales lentas para evitar el choque de frío y deshidratación celular severa, seguidas de velocidades de enfriamiento más rápidas puede producir menos daño a las células espermáticas y una mejor calidad post-descongelación, como ha sido demostrada en la congelación de espermatozoides de cabra montés (*Capra pyrenaica*) (Esteso et al., 2018).

La presente investigación tiene como objetivo mejorar la respuesta espermática a procesos de selección y criopreservación de espermatozoides de morueco. Dado que la preservación de espermatozoides de morueco supone la reducción de la supervivencia, integridad y funcionalidad celular tanto en condiciones de refrigeración (5 °C) como de congelación (196 °C), esta investigación examina la selección del diluyente (sintético o no sintético) que permita recuperar las mejores características celulares, cuando el semen es almacenado a corto y largo plazo bajo condiciones de refrigeración. Las rampas de congelación suponen uno de los elementos claves parara conseguir una criopreservación exitosa de los espermatozoides, por lo tanto, el presente trabajo aborda la optimización de la congelación de espermatozoides de morueco, evaluando diferentes rampas de congelación, teniendo en consideración los periodos de desarrollo del choque de frío y

los de fusión y nucleación del hielo. Los resultados generados proporcionarán un pilar de conocimiento en la criobiología espermática del morueco para su aplicación en programas de selección y mejora genética ovina mediante el uso práctico de la IA.

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OBJETIVOS

El objetivo general de la presente tesis doctoral fue optimizar la criopreservación de espermatozoides de morueco tanto en condiciones de refrigeración como de congelación para su aplicación en inseminación artificial transcervical a tiempo fijo (IATF).

Los objetivos específicos fueron:

1. Evaluar métodos de selección espermática por centrifugación con gradientes de densidad y columnas de filtración en semen fresco y refrigerado y su influencia sobre la calidad espermática *in vitro*.
2. Estudiar el efecto de la filtración espermática y adición de crioprotectores permeables en diluyentes sintéticos y no sintéticos, en las variables espermáticas y fertilidad en condiciones de refrigeración.
3. Investigar la influencia de la L-carnitina en las variables espermáticas y capacidad fecundante en condiciones de refrigeración a medio plazo.
4. Evaluar técnicas de criopreservación utilizando rampas de enfriamiento lentas en 2–3 fases.

CAPÍTULO 1

Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics.

D. A. Galarza, A. Lopez-Sebastian, H Woelders, E. Blesbois, J. Santiago-Moreno. **Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics**. 2018. *Animal Reproduction Science, 192,* 261-270. https://doi.org/10.1016/j.anireprosci.2018.03.022. Artículo Original.

Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics.

RESUMEN

La centrifugación de gradientes de densidad (DGC) y las columnas de filtración (FC) se utilizan para separar los espermatozoides, desechos y otras células en formación o muertas que pueden afectar negativamente la capacidad de fertilización de los espermatozoides en semen fresco, refrigerado y congelado-descongelado. El presente estudio se realizó para evaluar la eficacia de los métodos de selección espermática usando DGC (BoviPure®, Percoll® y Accudenz®) y FC (Sephadex G-15®) para el semen de morueco fresco y refrigerado por 24 h, mediante la evaluación de las variables de calidad espermática posterior al tratamiento. Se utilizaron veinte eyaculados normospérmicos de diez moruecos Merinos adultos. Los resultaos indicaron que la concentración de espermatozoides de las células recuperadas fue superior ($P < 0,001$) después del tratamiento con BoviPure que otros procedimientos tanto en semen fresco y refrigerado. La filtración mediante columnas de Sephadex produjo valores más deseables que con el uso de procedimientos de DGC en diferentes variables de motilidad espermática medidas mediante el uso del sistema computarizado CASA. En muestras de semen no refrigeradas, el porcentaje de motilidad espermática progresiva (%PSM) después de la filtración con Sephadex fue mayor ($P < 0,05$) que después del tratamiento con BoviPure; el valor de la velocidad en línea recta (VSL) después de la filtración con Sephadex fue mayor ($P < 0,01$) que después del tratamiento con Accudenz; la amplitud del desplazamiento lateral de la cabeza (ALH) después del tratamiento con Sephadex y Accudenz fue menor que el semen no filtrado ($P < 0,001$) y después de los tratamientos con Percoll ($P < 0,01$) y BoviPure ($P < 0,05$). En muestras de semen refrigeradas, el %PSM después de la filtración con

Sephadex fue mayor que el semen no filtrado ($P < 0,05$), BoviPure ($P < 0,05$), Percoll ($P < 0,05$) y Accudenz ($P < 0,001$). Se concluye que la filtración en columnas de Sephadex se puede usar para seleccionar espermatozoides de morueco, ya sea en semen no refrigerado o almacenado en frío por 24 h, debido a que el porcentaje de espermatozoides móviles progresivamente y algunas otras características de motilidad espermática son mayores con el uso de estas técnicas en comparación con el uso de métodos de DGC.

***Palabras clave**:* Esperma ovino; Selección; Filtración; Centrifugación; Gradientes

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Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics

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ABSTRACT

Density-gradients centrifugation (DGC) and filtration columns (FC) are used to separate deformed or dead sperm, debris, and other cells that may negatively affect the fertilizing capacity of sperm in fresh, chilled and frozen/thawed semen. The present study was conducted to evaluate the suitability of DGC (BoviPure®, Percoll® and Accudenz®) and FC (Sephadex G-15®) sperm selection procedures for fresh-extended and cold-stored ram semen by assessment of post-treatment sperm quality variables. Twenty normospermic ejaculates from ten adult Merino rams were used. Sperm concentration of recovered cells was greater ($P < 0.001$) after BoviPure treatment than other procedures in both fresh and cold semen. With the Sephadex method, there were more desirable values than with use of DGC procedures in several sperm motility variables measured by using the CASA system. In non-refrigerated semen samples, the percentage of progressive sperm motility (%PSM) after Sephadex filtration was greater ($P < 0.05$) than after BoviPure treatment; the straightline velocity (VSL) value after Sephadex filtration was greater ($P < 0.01$) than after Accudenz treatment; the amplitude of lateral head displacement (ALH) after Sephadex and Accudenz treatment was less than non-filtered semen ($P < 0.001$) and after Percoll ($P < 0.01$) and BoviPure ($P < 0.05$) treatments. In cold-stored semen samples, the %PSM after Sephadex filtration was greater than non-filtered ($P < 0.05$) semen and after BoviPure ($P < 0.05$), Percoll ($P < 0.05$) and Accudenz ($P < 0.001$) treatments. It is concluded that Sephadex column filtration can be used to select ram sperm in non-refrigerated and cooled semen, because percentage progressively motile sperm and some other sperm motility characteristics are greater with use of this techniques as compared with use of DGC methods.

1. Introduction

Artificial insemination has an important role in sheep breeding but its use is limited because the poor fertility achieved when stored semen is used for vaginal insemination (Gil et al., 2003). The success of this procedure in sheep is limited by the anatomic characteristics of the ewe's cervix and the short time that ram sperm can be stored as a liquid.

The sperm characteristics and fertilizing capacity of extended ram semen may be improved by applying methods for sperm selection prior to artificial insemination. Dead and non-functional sperm, debris and all dead cells could affect live sperm probably as

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a production of reactive oxygen species (ROS) that accumulates in deleterious concentrations, which have been reported as inductors of apoptosis (Wang et al., 2003). Sperm selection methods are used with the aim of gaining seminal plasma-free suspensions of predominantly motile sperm with reduced presence of abnormal, immature, and dead spermatozoa, epithelial cells, cell debris, lymphocytes, etc. (Valcárcel et al., 1996; Phillips et al., 2012), potentially allowing for an enhanced fertilizing capacity (Graham and Graham, 1990). Sperm selection methods, however, may have adverse "iatrogenic" effects related to mechanical damage due to centrifugation-resuspension procedures, and the promotion of lipid peroxidation during sperm pelleting due to the close vicinity of leukocytes and damaged sperm that may generate reactive oxygen species (ROS)(Sbracia et al., 1996).

With selective washing techniques based on density-gradient centrifugation (DGC), the sperm are selected according to the density, allowing for the isolation of motile and morphologically normal sperm. Currently, there are several commercial solutions available for sperm separation by DGC´s procedures. BoviPure® is an iso-osmotic salt solution containing colloidal silica particles coated with silane used to select bull sperm for use with artificial reproductive techniques (Samardzija et al., 2006). Silane-coated silica media are have also been proposed to be used in a single layer centrifugation (Martinez-Alborcia et al., 2013). Percoll® is a medium composed of colloidal silica particles (15–30 nm in diameter), coated with polyvinylpyrrolidone, and can be used for sperm selection in several mammalian species (Batista et al., 2011). Accudenz® (formerly called Nycodenz) is a non-ionic tri-iodinated derivative of benzoic acid with three aliphatic hydrophilic side chains of high density (2.1 g/ml) due to the presence of a substituted ring, which is linked to hydrophilic groups to enhance water solubility, and has been used in sperm selection of humans (Gellert-Mortimer et al., 1988; Sbracia et al., 1996), and cheetahs (Crosier et al., 2009), among other species. To our knowledge, no studies have been reported on the use of Accudenz® for selecting ram sperm.

Another technique of sperm separation is filtration through Sephadex columns. Sephadex® is a dextran gel available in different pore (filtration) sizes (G-10 to G-120). The post-thaw quality of sperm recovered after filtration through Sephadex was assessed to be highly acceptable when Sephadex was used for human (Drobnis et al., 1991), bull (Januskauskas et al., 2005; Lee et al., 2009), buffalo (Ahmad et al., 2003), stallion (Sieme et al., 2003), and boar (Bussalleu et al., 2008) semen, and was promising when used for filtering ram sperm (Landa et al., 1980; Valcárcel et al., 1996). In addition, with use of semen from bulls with lesser fertility for insemination that had been filtered through Sephadex there was an improved 60–90 day non-return rate (Graham and Graham, 1990). The mechanism for the trapping of sperm in Sephadex columns remains unclear. The filtration, however, is believed to be based on the fact that non-viable sperm tend to adhere to the Sephadex matrix to a greater extent than motile and seemingly functional sperm, and the latter are able to cross the filtration barrier without modification of functional characteristics (Bussalleu et al., 2008).

For ram semen, a number of sperm selection methods have been tried, including Sephadex filtration (see above), swim-up methods (García-López et al., 1996), sucrose washing, and Millipore filtration (Marti et al., 2006), as well as Percoll density gradient centrifugation (DGC) (Valcárcel et al., 1996). Even though there have been great advances in DGC procedures for sperm selection with different species (Santiago-Moreno et al., 2014, 2016), these procedures include centrifugal forces that may be harmful, especially for ram sperm (García-López et al., 1996). Hence, it was hypothesized that methods that involve little or no centrifugation, such as Sephadex filtration methods, may have advantages as compared with DGC for use in sperm selection in this species. In the present research, the effectiveness of Sephadex G-15® was compared with different DGC procedures (BoviPure®, Percoll®, Accudenz®) in fresh-extended semen and cold-stored ram semen.

2. Material and methods

Percoll® (Sigma P1644) and Sephadex G-15® (Sigma G15120-50 g) were obtained from Sigma Chemical Co., (St. Louis, Missouri, USA); Accudenz® (AN7050) was obtained from Accurate Chemical and Scientific Corporation (Westbury, New York, USA); and BoviPure® (BP-100) was obtained from Nicadon Laboratory (Nidacon, Mölndal, Sweden). All diluents and media were prepared in the INIA Department of Animal Reproduction Research Laboratory using reagent-grade chemicals purchased from Panreac Chemistry S.A. (Barcelona, Spain) and Sigma Chemical Co.

2.1. Animals, semen collection and initial evaluation

Ten adult Merino rams (2–7 years of age) that were assessed to be clinically healthy were used. All animals were housed at INIA Department of Animal Reproduction (Madrid, Spain, 40°25´N) using the same management conditions for all animals and rams were fed a basal diet that consisted of: grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available *ad libitum*. All animals were handled according to procedures approved by the INIA Ethics Committee, and the research was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Twenty semen ejaculates (two ejaculates per ram, collected with a 7 day interval) were collected using an artificial vagina (42–43 °C). The rams were previously trained with an intact oestrus induced-ewe and then an ovariectomized female was used as a "teaser" to stimulate ram sexual behaviors. The volume of each ejaculate was measured in a graduated conical glass tube in millilitres (ml). Briefly, after collection, the semen was diluted 1:1 with TTG solution (210.59 mM Tes, 95.75 mM Tris, 10.09 mM Glucose, 0.54 mM Streptomycin, and 2.14 mM Penicillin; 324 mOsm/kg, pH 7.1). All the materials, including the artificial vagina and glass collecting tube were maintained at 37 °C before collections. This fresh-extended semen samples were transported to the research laboratory immediately after collection at 37 °C and initial motility was evaluated prior to processing. Sperm concentration was determined using a photometer (SDM 1, Minitube, Germany).

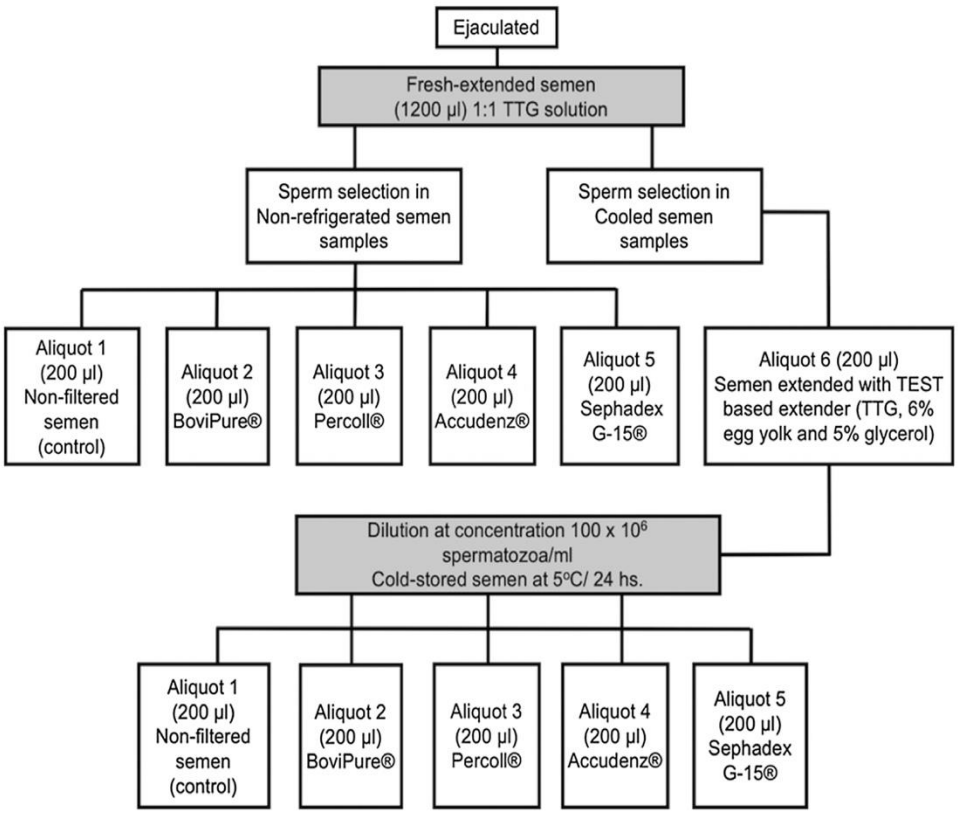

Fig. 1. Experimental design.
TTG solution: Tes, Tris and glucose.

2.2. Experimental design

A total of 1200 µl of fresh-extended semen from each ejaculate was used and divided into six 200 µl-aliquots. The sperm selection procedures were performed with both non-refrigerated and cooled semen samples (Fig. 1).

2.2.1. Sperm selection in non-refrigerated semen samples

Five aliquots of 200 µl each of fresh-extended semen of each ejaculate were maintained and used at room temperature. One aliquot was considered as the control (non-filtered semen samples) and the four other aliquots were used to purify the sperm with BoviPure, Percoll, Accudenz and Sephadex G-15 procedures. These five samples per ejaculate are referred to as "non-refrigerated semen".

2.2.2. Sperm selection in cooled semen samples

The remaining (sixth) aliquot of 200 µl fresh-extended semen of each ejaculate was diluted with TEST based extender (TTG solution, egg yolk 6% (v/v), and glycerol 5% (v/v); 320 mOsm/kg water, pH 7.2) to a concentration of 100×10^6 sperm/ml. These diluted sperm samples were chilled at 5 °C and stored for 24 h, then five aliquots of 200 µl each were taken and processed as described above for non-refrigerated semen samples. One aliquot was considered as the control (non-filtered cooled semen) and the remaining four aliquots were processed with the four sperm selection procedures. These chilled samples are referred to as "cold-stored semen".

2.3. Sperm selection procedures

Sperm selection was performed in fresh-extended and cold-stored semen samples. The entire sperm selection process for both DGC and filtration column (FC) methods were performed at room temperature (i.e. cold stored semen samples were allowed to rewarm to room temperature prior to being used).

2.3.1. BoviPure® DGC

Following the protocol described by Santiago-Moreno et al. (2017), BoviPure® solution was diluted with BoviDilute® solution to obtain BoviPure® Bottom layer medium and BoviPure upper layer of medium, at 80% and 40% concentration, respectively. The

BoviPure DGC columns were prepared in 15-ml Falcon® tubes: equal volumes of BoviPure® Bottom layer- and Top layer medium were successively layered in the tubes, using a total column volume of 1 ml per 333 million sperm present in the 200 µl sperm sample. The fresh-extended or cold-stored semen samples, which had been initially allowed to reach room temperature, were gently layered on top of the BoviPure Top medium. The columns were centrifuged at 300 *g* for 20 min. After centrifugation, the fluid above the sperm pellet was carefully removed. The final pellets were re-suspended, each in 100 µl TEST based extender at 37 °C, and sperm quality variables were evaluated.

2.3.2. Percoll® DGC

The 90% isotonic density solution was prepared by diluting 900 µl commercial Percoll® with 100 µl TAPL10 X medium (992.30 mM NaCl, 248 mM NaHCO3, 99.87 mM HEPES sodium, 0.03 mM KH2PO4 H2O, 0.15 mM MgCl 6H2O, 0.26 mM CaCl 2H2O, and 0.1 mM Na-Lactate); the 60% isotonic density solution was prepared by diluting 335 µl of 90% isotonic density solution and 165 µl of TALP Stock medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH2PO4 H2O, 1 mM Na-Lactate, 1.97 mM CaCl 2H2O, 0.50 mM MgCl 6H2O, 10 mM HEPES sodium, and 25 mM NaHCO3; 320 mOsm/Kg, pH 7.3); and the 30% isotonic density solution was prepared by diluting 165 µl of 90% working density solution and 335 µl of TALP Stock medium (Parrish et al., 1995). The Percoll density gradients were made by layering sequentially 400 µl of 90% Percoll solution, 400 µl of 60% solution, and 400 µl of 30% solution in 15 ml Falcon® tubes. Subsequently, 200 µl of the fresh-extended or cold-stored semen samples, which had been allowed first to reach room temperature, were layered on top of the prepared Percoll layers and then tubes were centrifuged at 600 *g* for 10 min. The pellets were re-suspended in 2.5 ml Sperm TALP medium (TALP Stock medium, 6 mg/ml BSA (Sigma-Aldrich A-9647), 0.11 mg/ml Na Pyruvate, and 5 µl/ml gentamycin; 326 mOsm/Kg, pH 7,6) at 37 °C and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.3.3. Accudenz® DGC

An iso-osmotic Accudenz® solution was prepared according to the manufacturer's directions. The Accudenz Stock medium was prepared by mixing 2.76 g Accudenz® (density = 1.15 g/ml) with a buffer solution (7.5 mg/ml NaCl dissolved in a solution of 100 ml of 5 mM Tris-HCl (pH 7.5), 3 mM KCl, and 0.3 mM CaNa2 EDTA). A series of isotonic Accudenz media were prepared with 35%, 40%, 50%, 65%, 70%, 80% and 100% of the Accudenz stock medium mixed with the appropriate amount of TALP stock medium. Centrifugation times and centrifugal (*g*) forces were used according to Gellert-Mortimer et al. (1988) and Sbracia et al. (1996) to test which of these combinations resulted in greater sperm motility after semen processing (subjective sperm motility was used as a reference sperm quality variable). These working conditions were established in previous experiments (data not shown). The discontinuous gradients that gave the optimal motile sperm selection consisted of 400 µl of 100%, 400 µl of 65%, 800 µl of 50%, and 400 µl of 35% Accudenz media layered on top of each other in 15-ml Falcon® tubes. The fresh-extended and cold-stored semen samples, which had been allowed first to reach room temperature, were gently placed on top of the top layer (35% solution) and the tubes were centrifuged at 300 *g* for 12 min. The pellets were re-suspended in 2.5 ml of sperm TALP medium at 37 °C and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.3.4. Sephadex® filtration column

A Sephadex suspension was prepared by hydrating Sephadex G-15® for at least 24 h in sodium citrate 3% (v/v), according to Valcárcel et al. (1996). Filtration columns of three cm height were prepared in a 2.5-ml glass chromatography syringes (Sigma-2099820,998. Hamilton® syringe, 1000 series GASTIGHT®, PTFE luer lock. 1002 TL L, volume 2.5 mL), with needle size 22 G, L 51 mm-2 in (Sigma-21746. Hamilton® needles). The columns were prepared immediately before filtration and kept in a vertical position at room temperature. The fresh-extended and cold-stored semen samples, which had been initially been allowed to reach room temperature, were placed on top of the Sephadex columns and filtered for 15 min. The filtration process was achieved by lightly pressing the syringe plunger. Subsequently, the filtrate liquid was diluted in 2.5 ml of sperm TALP medium at room temperature and centrifuged at 200 *g* for 10 min. The final pellets were re-suspended each in 100 µl TEST based extender at 37 °C and sperm quality variables were evaluated.

2.4. Sperm analysis

The sperm concentration post-selection was estimated using a Neubauer chamber (Marienfeld, Lauda- Königshofen, Germany). The motility analysis was objectively assessed using a CASA system (Sperm Class Analyzer, SCA® 1999, v.4.0, software. Microptic S.L., Barcelona, Spain) coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast). The sperm samples were loaded into a warmed (37 °C) 20 µm Leja® 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). A minimum of three fields and 200 sperm tracks were evaluated at 100 X for each sample chamber (image acquisition rate 25 frames/s). The following sperm kinetic variables were assessed, as previously described by Dorado et al. (2007): percentage motile sperm (%SM), percentage progressive sperm (%PSM), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), wobble (%WOB), linearity (%LIN) straightness (%STR), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, µm). Sperm viability and acrosomal membrane status were analyzed by fluorescence microscopy (counting 200 cells), using a Nikon Eclipse E200 epifluorescence microscope (D-FL epifluorescence, C-SHG1 super high-pressure mercury power supply; Nikon Instruments Inc., New York, USA), using propidium iodide (PI, Sigma P-4170) and fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC, Sigma L7381), as previously described by Santiago-Moreno et al. (2014).

The FITC-PNA/PI fluorescence test provides percentages of four sub-categories: sperm with intact plasma and acrosome membranes (%PIAI), with intact plasma membrane and damaged acrosome (%PIAD); with damaged plasma membrane and intact acrosome (% PDAI); and with damaged plasma and acrosome membranes (%PDAD). Sperm morphological abnormalities were evaluated placing a 5 µl of the semen samples in 100 µl of glutaraldehyde 2% (v/v) solution. A drop of 5 µl of this mixture was examined using a phase contrast microscope at magnification 400×. The morphology of 200 sperm was assessed to score the percentage of abnormal heads, loose normal heads, mid-piece defects, abnormal tails and coiled tails and cytoplasmic droplets (Frank, 1950).

2.5. Statistical analysis

The results are presented as mean ± SEM. The sperm variable values were normally distributed as determined by Shapiro-Wilk´s test. These percentage variables (kinetic variables of CASA analysis, plasma and acrosome membrane integrity and morphological abnormalities), therefore, were arcsine transformed and the non-percentages variables (sperm concentration of cells recovered, velocities, BCF and ALH) were log-transformed before analysis. One-way ANOVA and Bonferroni's multiple comparisons test was used to examine the effect of sperm selection procedures in the non-refrigerated and cooled semen samples on sperm kinetic variables, plasma and acrosomal membrane integrity and sperm concentration post-selection. In addition, data were compared for non-refrigerated vs cooled semen samples in each sperm selection methods by one-way ANOVA. For the assessment of sperm morphological abnormalities, a factorial analysis of 2 × 5 was used that included semen type (fresh-extended and cold-stored semen samples) and sperm selection method (non-filtered semen and filtered semen with BoviPure, Percoll, Accudenz and Sephadex). A multifactorial ANOVA and Bonferroni´s test was used to assess the interaction between factors and the effect of the sperm selection procedures.

All calculations were made using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA).

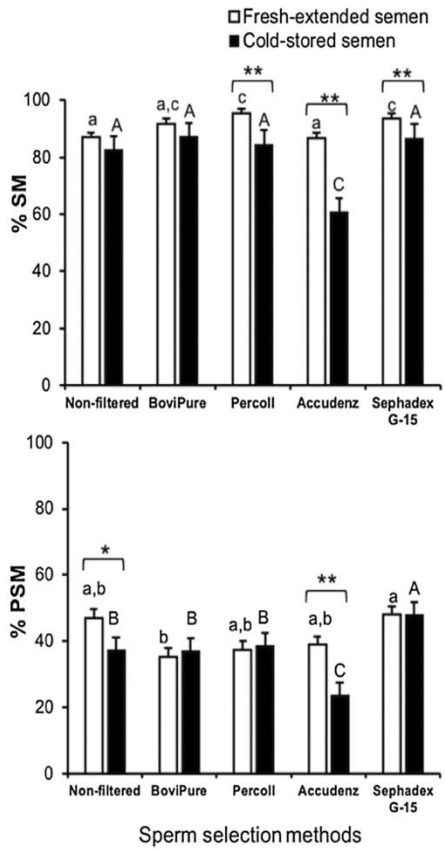

Fig. 2. Sperm motility (%SM) and progressive sperm motility (%SPM) in non-filtered samples and after sperm selection using different procedures with fresh-extended and cold-stored semen.

[a,b,c]Different superscripts for the fresh – extended semen data indicate differences between selection procedures ($^{a,b}P < 0.05$ and $^{a,c}P < 0.001$).

[A,B,C]Different superscripts for the cold – stored semen data indicate differences between selection procedures ($^{A,B}P < 0.05$; $^{B,C}P < 0.01$, and $^{A,C}P < 0.001$).

**Asterisks indicate differences between values for fresh-extended and cold-stored semen within each selection procedure ($^{*}P < 0.05$, $^{**}P < 0.001$).

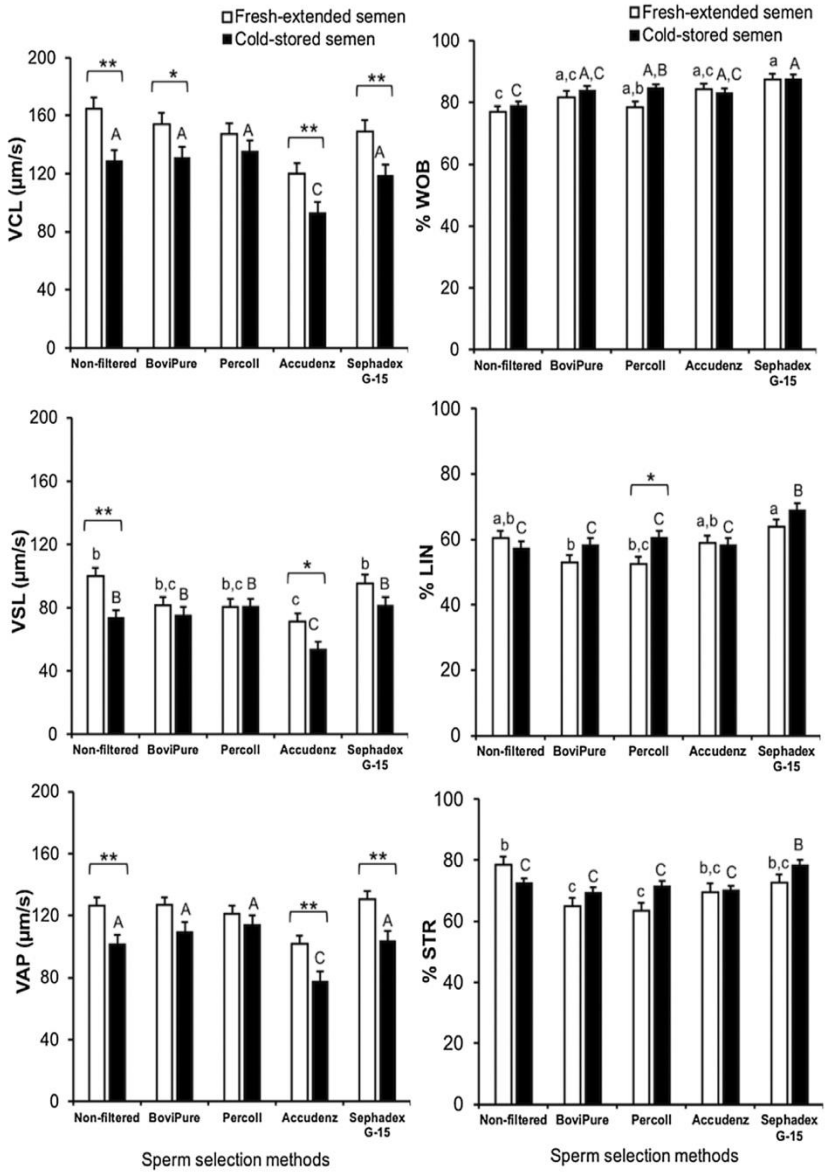

Fig. 3. Sperm motility characteristics (curvilinear velocity: VCL; straight-line velocity: VSL; average path velocity: VAP; linearity: LIN; straightness: STR; Wobble: WOB) for values of non-filtered samples and after sperm selection with different procedures, in fresh-extended and cold-stored semen.

[a,b,c]Different superscripts associated with values for fresh−extended semen indicate differences between selection procedures ($^{a,b}P < 0.05$, $^{b,c}P < 0.01$ and $^{a,c}P < 0.001$).

[A,B,C]Different superscripts associateD with values for cold−stored semen indicate differences with use of the different selection procedures ($^{B,C}P < 0.01$, and $^{A,C}P < 0.001$).

**Asterisks indicate differences in values between fresh-extended and cold-stored semen for each selection procedure ($^{*}P < 0.05$, $^{**}P < 0.001$).

3. Results

Differences in sperm motility variables between sperm selection methods, and between fresh-extended and cold-stored samples are depicted in Figs. 2–4. Data for membrane and acrosome integrity, and morphological abnormalities are included in Tables 1 and 2.

3.1. Sperm selection in non-refrigerated semen samples

Sperm concentration (x 10^6 sperm/ml) in non-filtered fresh-extended semen samples was 2238.1 ± 28. The recovered sperm

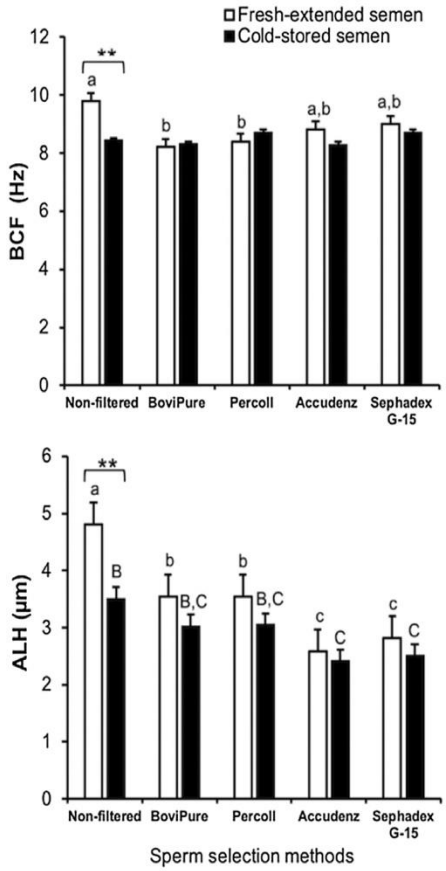

Fig. 4. Flagellar beat frequency (BCF) and amplitude of lateral head displacement (ALH) in non-filtered samples and after sperm selection using different procedures for fresh-extended and cold-stored semen.

[a,b,c]Different superscripts for values of the fresh – extended semen indicate differences with use of different selection procedures ([a,b]$P < 0.05$, [b,c]$P < 0.01$ and [a,c]$P < 0.001$).

[B,C] Different superscripts associated with values for the cold – stored semen indicate differences between selection procedures ([B,C]$P < 0.01$).

Asterisks indicate differences in values between fresh-extended and cold-stored semen within each selection procedure ($P < 0.001$).

Table 1
Plasma and acrosomal membrane integrity in fresh-extended and cold-stored semen (mean ± SEM).

Fluorescence parameters	Non-filtered semen		Sperm selection procedures			
			BoviPure®	Percoll®	Accudenz®	Sephadex G-15®
%PIAI	1	71.6 ± 3.4	74.7 ± 2.4	74.5 ± 3.3	71.2 ± 3.7	78.6 ± 3.6
	2	66.9 ± 2.7	77.8 ± 2.5	71.5 ± 3.1	73,8 ± 3.1	75.3 ± 2.9
%PIAD	1	2.3 ± 0.8	1.5 ± 0.8	1.0 ± 0.6	1.6 ± 0.6	1.3 ± 0.5
	2	1.9 ± 0.5[a,b]	1.6 ± 0.5[a,b]	2.1 ± 0.7[a]	1.7 ± 0.4[a,b]	1.5 ± 0.4[b]
%PDAI	1	20.4 ± 2.8	21.6 ± 2.0	22.3 ± 2.9	24.9 ± 3.2	16.5 ± 3.0
	2	24,2 ± 2.2	17.2 ± 2.0	23.2 ± 2.5	21.7 ± 2.5	18.7 ± 2.3
%PDAD	1	5.8 ± 0.9[A]	2.7 ± 0.6[B]	2.3 ± 0.7[B]	2.3 ± 0.5[B]	3.6 ± 0.9[A,B]
	2	7.1 ± 1.4[a]	3.5 ± 0.9[a,b]	3.8 ± 0.7[a,b]	2.9 ± 0.8[b]	4.0 ± 0.9[a,b]

[1] Fresh-extended semen samples; [2] Cold-stored semen samples; %PIAI, spermatozoa percentage with intact plasma and acrosome membranes; %PIAD, spermatozoa percentage with intact plasma membrane and damaged acrosome; %PDAI, spermatozoa percentage with damaged plasma membrane and intact acrosome; and % PDAD, spermatozoa percentage with damaged plasma and acrosome membranes; [a,b,A,B] Values within a row in both fresh – extended and cold – stored semen samples with different superscripts, differ significantly ([a,b] $P < 0.05$; [A,B] $P < 0.01$).

concentration was greater ($P < 0.001$) with BoviPure® method (838.0 ± 72) than with Percoll®, Accudenz® and Sephadex® (701.0 ± 82, 643.5 ± 42 and 523.5 ± 43, respectively).

The %SM after Sephadex and Percoll treatment was greater ($P < 0.001$) than in the non-filtered semen and after Accudenz

Table 2
Sperm morphological abnormalities (mean ± SEM) in fresh-extended and cold-stored semen.

Morphological abnormalities	Non-filtered semen		Sperm selection methods			
			BoviPure®	Percoll®	Accudenz®	Sephadex G-15®
Abnormal heads (%)	1	1.4 ± 0.3	0.4 ± 0.1	1.1 ± 0.4	0.4 ± 0.1	1.1 ± 0.3
	2	1.4 ± 0.3^{a}	1.2 ± $0.4^{a,b}$	0.6 ± $0.2^{a,b}$	0.6 ± $0.2^{a,b}$	0.3 ± 0.1^{b}
Loose heads (%)	1	1.9 ± 0.4^{a}	1.0 ± 0.3^{a}	1.1 ± 0.4^{a}	0.5 ± 0.2^{b}	1.6 ± 0.4^{a}
	2	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	0.6 ± 0.3	0.4 ± 0.1
Abnormal midpieces (%)	1	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
	2	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.9 ± 0.4	0.3 ± 0.2
Abnormal tails (%)***	1	2.6 ± 0.8^{B}	1.4 ± 0.2^{B}	1.0 ± 0.3^{B}	4.9 ± 0.7^{A}	1.5 ± 1.4^{B}
	2	1.8 ± 0.4^{B}	2.7 ± 0.9^{B}	7.3 ± 1.8^{A}	6.0 ± 0.6^{A}	2.2 ± 0.6^{B}
Coiled tails (%)	1	6.6 ± 0.8	4.8 ± 0.7	3.9 ± 0.9	5.5 ± 1.1	6.0 ± 1.3
	2	18.9 ± 3.0	17.8 ± 3.4	10.5 ± 2.1	16.7 ± 2.3	21.7 ± 3.4
Cytoplasmic droplets (%)	1	1.2 ± 0.4	1.4 ± 0.5	0.4 ± 0.4	0.7 ± 0.5	0.6 ± 0.4
	2	1.7 ± 0.5	1.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.4	0.6 ± 0.3
Total abnormalities (%)	1	1 4.1 ± $1,2^{A}$	9.1 ± 1.1^{A}	7.5 ± 1.1^{B}	12.0 ± 1.5^{A}	10.9 ± 1.4^{A}
	2	24.9 ± 2.8	24.2 ± 3.3	19.8 ± 2.8	25.3 ± 2.8	25.4 ± 3.7

[1] Fresh-extended semen samples; [2] Cold-stored semen samples; ***Interaction between factors (fresh-extended and cold-stored semen and type of selection procedures) at P < 0.001; ^{a,b,A,B} Values within a row with different superscripts show difference significantly between values of sperm selection methods (^{a,b} P < 0.05; ^{A,B} P < 0.01).

treatment, respectively. The %PSM after Sephadex filtration was greater ($P < 0.05$) than after BoviPure treatment (Fig. 2). The VSL value of Sephadex filtered sperm and of non-filtered sperm were both greater ($P < 0.01$) than after Accudenz treatment. The %WOB after Sephadex filtration ($P < 0.001$) and after Percoll ($P < 0.05$) treatment were greater than in non-filtered semen. The %LIN after Sephadex filtration was greater than after BoviPure ($P < 0.05$) and Percoll ($P < 0.01$) treatment (Fig. 3). The ALH after Sephadex and Accudenz treatment was less than in non-filtered semen ($P < 0.001$) and after Percoll ($P < 0.01$) and BoviPure ($P < 0.01$) treatments (Fig. 4).

In non-refrigerated semen, the percentage of sperm with a damaged plasma membrane and acrosome was greater in non-filtered semen compared with cells after all DGC treatments, except after Sephadex filtration ($P < 0.05$) (Table 1).

There was an interaction between factors (non-refrigerated/cooled semen and type of sperm selection procedure) for the percentage of abnormal sperm tails ($P < 0.001$). The percentage of sperm with loose heads was less ($P < 0.05$) after Accudenz treatment compared with the others sperm selection procedures. The percentage of sperm with abnormal tails after Accudenz treatment, however, was greater ($P < 0.001$) than when the other selection procedures were used. The percentage of total abnormalities after Percoll treatment was less ($P < 0.01$) than in non-filtered semen and after the other sperm selection methods (Table 2).

3.2. Sperm selection in cold-stored semen samples

Similar to non-refrigerated semen samples, the concentration of sperm (x 10^6 sperm/ml) recovered in cooled samples was greater ($P < 0.001$) after BoviPure treatment than after Accudenz, Sephadex and Percoll treatments (67.0 ± 6.1 compared with 55.5 ± 6.6, 52.5 ± 5.8 and 43.0 ± 7.2, respectively).

The %SM after Accudenz treatment was less ($P < 0.001$) than in non-filtered semen and after all other selection methods treatments. The %PSM after Sephadex filtration was greater than in non-filtered ($P < 0.05$) semen and after BoviPure ($P < 0.05$), Percoll ($P < 0.05$) and Accudenz ($P < 0.001$) treatments (Fig. 2). The VCL ($P < 0.001$), VSL ($P < 0.01$) and VAP ($P < 0.001$) values after Accudenz treatment were less than in non-filtered semen and after all other sperm selection methods treatments (Fig. 3). The %WOB after Sephadex filtration ($P < 0.001$) and Percoll ($P < 0.01$) treatment were greater than in non-filtered semen. Furthermore, the %LIN and %STR after Sephadex filtration were greater ($P < 0.01$) than in non-filtered semen and after all DGC treatments (Fig. 3). The ALH after Sephadex and Accudenz treatments was less ($P < 0.01$) than in non-filtered semen (Fig. 4).

For the cold-stored semen, the percentage of sperm with an intact plasma membrane and damaged acrosome after Sephadex filtration was less ($P < 0.05$) than with the Percoll treatment. Furthermore, the percentage of sperm with a damaged plasma and acrosome membranes after Accudenz treatment was less (P < 0.05) than non-filtered semen (Table 1).

The percentage of abnormal heads after Sephadex filtration was less ($P < 0.05$) than non-filtered semen. The percentage of abnormal tails after Sephadex and BoviPure treatments and non-filtered semen were also less ($P < 0.01$) than after Percoll and Accudenz treatments (Table 2).

4. Discussion

In non-refrigerated semen, the use of all sperm selection treatments appeared to reduce the percentage abnormalities and percentage of sperm cells with a damaged plasma membrane and/or acrosome membrane, although the effects were small and not all pairwise comparisons with non-filtered sperm were significant, and the use of Accudenz had a negative effect on the % abnormal

sperm tails. Regarding motility, only the Percoll and Sephadex treatments resulted in a greater percentage of motile sperm. The BoviPure and Accudenz treatments had negative effects on percentage of motile sperm in non-refrigerated and in cold-stored semen, respectively. The percentage progressively motile sperm was only greater with use of Sephadex filtration and not with use of the DGC methods. Also, several of the CASA motility characteristics were greater with Sephadex filtration (e.g., progression ratio values) than after using DGC selection procedures (with a number of comparisons beings significant). This suggests that Sephadex filtration results in a more desirable ram sperm quality than with use of the DGC procedures. For many of the measured variables, Accudenz centrifugation had a negative effect on sperm quality.

For ram semen, it has been reported that centrifugation of semen is very harmful to sperm (García-López et al., 1996). Sperm selection procedures without centrifugation, therefore, could be beneficial for sperm quality of the treated semen. For this reason, alterative techniques, such as the dextran-swim up method, have been developed in rams (García-López et al., 1996; Pérez-Pé et al., 2001; Grasa et al., 2004). It, therefore, was hypothesized that filtration methods, such as Sephadex G-15® filtration, may be a useful technique for successfully selecting sperm in rams. Marti et al. (2006) found that there was no satisfactory selection of relatively greater quality sperm by filtration of ram semen when using a 5 μm pore Millipore filter. But Sephadex filtration appears to involve a different mechanism of trapping sperm with relatively lesser fertilizing capacity. Graham and Graham (1990) reported that Sephadex columns essentially trap non-motile sperm, while also more specific binding may be involved (e.g., capacitated sperm, see references in Ahmad et al., 2003) or of sperm with a damaged acrosomal membrane (Maurya and Tuli, 2003). The results obtained in the present study are consistent with the results of other studies where Sephadex filtration was used (Graham and Graham, 1990; Bollendorf et al., 1994; Ahmad et al., 2003; Januskauskas et al., 2005; Bussalleu et al., 2008; Lee et al., 2009) and also consistent with the finding in the present study where there were lesser percentages of (i.e. removal of part of the) immotile sperm with Sephadex filtration.

Cooling of the semen (cold shock) or cooled storage (chilling injury) leads to reduced sperm quality in non-filtered semen (e.g., regarding %MS and %PSM). In addition, Accudenz centrifugation of cooled semen had a marked negative effect on sperm quality (e.g., %SM and %PSM).

The advantage of the Sephadex filtration method as compared with DGC methods used in the present study is that the use of Sephadex more effectively traps immotile sperm than with use of the DGC methods, or the DGC methods cause more damage to sperm cells (e.g., because of centrifugation). With the Sephadex method in the present study, a relatively lesser force (200 *g* during 10 min) centrifugation was used for recovering sperm in the pellet after filtration. For specific CASA sperm motility characteristics, ALH values were less after use of all sperm selection treatments in the present study, but most clearly after Accudenz centrifugation and Sephadex filtration. It is believed that greater values of ALH are not compatible with effective progressive movement of sperm cells (Santiago-Moreno et al., 2017). Effective progressive movement of the sperm is required to overcome the anatomical and physiological barriers in the female genital tract, such as folds of the cervix, and the cervical mucus, among others (Aitken et al., 1985). The value of ALH increases during sperm capacitation (Santiago-Moreno et al., 2017). Thus, a lesser ALH of the selected sperm populations would be consistent with trapping of prematurely capacitated sperm, as has been suggested as a mechanism of Sephadex filtration by Januskauskas et al. (2005). The greater values of %SM and %PSM together with lesser values of ALH in the present study for the Sephadex-filtrated semen suggest that Sephadex filtration selects non-capacitated ram sperm with greater progressive motility and enhanced capacity to cross the cervical barriers of ewes.

Use of the DGC methods in non-refrigerated semen resulted in lesser percentages of sperm with a damaged plasma membrane acrosome, compared with non-filtered semen. In cooled samples, this reduction was numerically greater, however, was only significant for the Accudenz-treated semen. There appeared to be greater detrimental effects on Accudenz-treated semen that resulted in a decrease in a majority of motility variables, and this was especially so for cooled semen. The use of Accudenz, therefore, is not a recommended method for ram sperm, at least according to the findings in the present study, unlike results obtained with use of Accudenz for human sperm centrifugation (Sbracia et al., 1996).

Sperm morphology is one of the most commonly assessed variables for characterizing sperm, because the percentage of normal sperm morphology is positively associated with fertility outcomes (Kot and Handel, 1987; Chenoweth, 2005). Most selection methods studied in the present study were effective for reducing the percentage of at least one type of sperm abnormality: Sephadex decreased the percentage of abnormal heads in cold-stored semen, Accudenz decreased the percentage of loose heads in fresh-extended semen, and Percoll decreased the percentage of total abnormalities in fresh-extended semen. Also for bull semen, the use of Sephadex filtration has been reported to reduce the percentages of abnormal sperm (Graham and Graham, 1990; Januskauskas et al., 2005). It, however, is important to consider that the frequency of such abnormalities was already less in the samples before filtration. In the present study, there were no significant differences between non-filtered semen and after BoviPure treatment, regarding the types of morphological abnormalities. This finding is consistent with a previous report in wild sheep where DGC was also used with silane-coated colloidal silica particles (Santiago-Moreno et al., 2014).

It is concluded that Sephadex filtration can be used to select ram sperm in fresh-extended and cold-stored semen, with an advantage compared with the tested DGC methods as to percentage progressively motile sperm and sperm motility characteristics. The rationale of selecting normal, motile sperm is that abnormal spermatozoa may increase ROS production, which could lead to a greater damage of normal sperm in semen (Henkel, 2011) during cold storage, freezing, and after insemination. In addition, after insemination, the presence of damaged or abnormal sperm may increase recruitment of phagocytes in the female genital tract. This occurred to a limited extent in pigs (Matthijs et al., 2000). In humans, Eisenbach (2003) suggested, and Oren-Benaroya et al. (2007) reported that sperm with enhanced maturity ('post-capacitated')were preferentially phagocytosed. In addition, sperm purification would allow adjusting the concentration of sperm doses to a more desirable number of normal and functional sperm cells. Future research must be conducted to evaluate whether fertility with fresh, cold-stored, or frozen-thawed ram semen can be improved by

using sperm selection prior to vaginal artificial insemination.

Conflict of interest

None of the authors have any conflict of interest to declare.

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CAPÍTULO 2

Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm.

D. A. Galarza, Ladrón de Guevara, P. Beltrán-Breña, MJ Sánchez-Calabuig*, D. Rizos, A. López-Sebastián, J. Santiago-Moreno. **Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm.** 2019. *Theriogenology, 133*, 29-37. https://doi.org/10.1016/j.theriogenology.2019.04.027. Artículo Original.

Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm

RESUMEN

La baja fertilidad del semen del morueco almacenado en frío durante largos períodos ha fomentado el desarrollo de protocolos diseñados para mejorar el vigor cinético de los espermatozoides y la capacidad para atravesar la barrera cervical. El presente trabajo evaluó el efecto de la selección de espermatozoides mediante la filtración de columnas de Sephadex y la suplementación de glicerol al 2% (GLY) a los diluyentes a base de leche desnatada tratada con ultra calor (UHT) o Tris-Tes-Glucosa (TEST) en parámetros cinéticos de esperma de morueco, integridad de la membrana plasmática, integridad del acrosoma, función mitocondrial y capacidad de fertilización, durante largos tiempos de enfriamiento. Los resultados mostraron que, para el semen no filtrado, los valores de motilidad espermática progresiva (%PSM), la velocidad rectilínea (VSL, μm/s) y el porcentaje de espermatozoides con una membrana plasmática intacta / acrosoma intacto / un índice de función mitocondrial alto (%IPIAHM) en todo momento hasta 96 h de enfriamiento fue mayor cuando se usó el diluyente UHT ($P < 0,01$) en comparación con el diluyente TEST, independientemente de la presencia de GLY. Cuando el semen se filtró previamente con Sephadex, la adición de GLY al diluyente UHT mejoró la motilidad total (%TM), la %PSM y la VSL a las 96 h en comparación con todos los demás tratamientos ($P < 0,01$). Los mejores resultados de todos se obtuvieron con semen no filtrado y UHT con o sin GLY. Se utilizó una Fertilización in vitro (FIV) heteróloga utilizando ovocitos bovinos de zona intacta para evaluar la capacidad de fertilización de semen de morueco fresco no filtrado (FS0), refrigerado durante 24 h (CS24) o refrigerado durante 48 h (CS48) diluido en UHT extensor (sin GLY). La FIV heteróloga mostró que los espermatozoides de morueco, FS0, CS24 o CS48, eran igualmente capaces de penetrar

la zona pelúcida en los ovocitos bovinos intactos, lo que llevó a la formación de pronúcleos y la división de los embriones híbridos (46,3 ± 3,2; 48,8 ± 3,2; y 43,3 ± 3,5%, respectivamente). No se observaron diferencias con respecto a los espermatozoides frescos en términos de unión de esperma, penetración, polispermia, formación de pronúcleos o tasas de división (P > 0,05). En conclusión, ni la filtración con Sephadex ni la adición de glicerol proporcionaron beneficios adicionales al esperma de morueco enfriado hasta 96 h. Los espermatozoides no filtrados y enfriados diluidos con UHT sin GLY mostraron una mejor funcionalidad de los espermatozoides que los espermatozoides similares diluidos con TEST. De hecho, los espermatozoides diluidos con UHT mantuvieron la capacidad de fertilización hasta 48 h.

***Palabras clave**:* Leche desnatada, TEST, Almacenamiento en frío, Esperma, Filtración

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Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm

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Filtration

ABSTRACT

The poor fertility of ram semen stored chilled for long periods has encouraged the development of protocols designed to improve the kinetic vigour and cervical barrier-crossing capacity of sperm. The present work evaluated the effect of sperm selection with Sephadex filtration and the supplementation of 2% glycerol (GLY) to extenders based on ultra-heat-treated skimmed milk (UHT) or Tris-Tes-Glucose (TEST) on ram sperm kinetic parameters, plasma membrane integrity, acrosome integrity, mitochondrial function and fertilizing ability, over long chilling times. The results showed that for non-filtered semen, values for progressive sperm motility (%PSM), straight line velocity (VSL, μm/s) and the percentage of sperm with an intact plasma membrane/intact acrosome/a high mitochondrial function index (%IPIAHM) at all times up to 96 h of chilling were higher when the UHT extender ($P < 0.01$) was used compared to TEST extender irrespective of the presence of GLY. When semen was previously filtered with Sephadex, the addition of GLY to the UHT extender improved total motility (%TM), the %PSM and the VSL at 96 h compared to all other treatments ($P < 0.01$). The best results of all were obtained with non-filtered semen and UHT either with or without GLY. Heterologous IVF using zona-intact bovine oocytes was used to assess the fertilizing capacity of non-filtered fresh (FS0), chilled-for-24 h (CS24) or chilled-for-48 h (CS48) ram semen diluted in UHT extender (GLY-free). Heterologous IVF showed that ram sperm, either FS0, CS24 or CS48, were equally capable of penetrating zona pellucida intact bovine oocytes, leading to pronuclear formation and hybrid embryo cleavage (46.3 ± 3.2; 48.8 ± 3.2; and 43.3 ± 3.5, respectively). No differences were seen with respect to fresh sperm in terms of sperm binding, penetration, polyspermy, pronucleus formation or cleavage rates ($P > 0.05$). In conclusion, neither Sephadex filtration nor addition of glycerol provided extra benefits to ram sperm chilled up to 96 h. Chilled, non-filtered sperm extended with UHT without GLY showed better sperm functionality than did similar sperm extended with TEST extenders. Indeed, sperm diluted in UHT extender, maintained fertilizing ability up to 48 h.

1. Introduction

Artificial insemination (AI) is an essential technique to the running of sheep selection programs. Success is limited by the short length of time that ram sperm can be stored as a liquid if it is to maintain its fertility capacity [1]. The majority of sheep that undergo AI do so against a backdrop of hormone-induced oestrus synchronization, which allows the procedure to be performed at desired times [2]. Cervical AI with frozen-thawed sperm is associated with low fertility rates (e.g. 3.8–20.5% or 20.0–22.0% reported by Valente et al. [3] or Masoudi et al. [4], respectively), and has only been employed on a limited scale. Laparoscopic AI with frozen-thawed sperm returns better results [5]. However, a number of technical, handling and animal welfare issues determine that cervical AI using chilled sperm is the most common procedure

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followed for genetic improvement programs in sheep [6].

The survival of ram sperm over extended periods inversely correlates with the cells' metabolic activity and motility, morphological integrity and fertility [7]. Synthetic TEST and Tris-based extenders have both been used to preserve ram sperm under chilled conditions [8–10], but non-synthetic skimmed milk powder-based extender is associated with better survival and fertility when using either chilled [11] or frozen-thawed [12] sperm. The use of ultra-heat-treated "long-life" skimmed milk has been shown a satisfactory extender for use with chilled ram sperm [13]. Egg yolk (EY) has been added to both synthetic-based [14,15] and non-synthetic-based extenders [6] given the protection it affords the plasma membrane against cold shock.

Glycerol (GLY) is the main penetrating cryoprotectant agent used when freezing ram sperm [16], but many of its effects remain unclear. Early studies reported GLY to reduce the quality of fresh and frozen-thawed semen in some species, including sheep [17]. It has also been reported harmful when added to extenders at over 30 °C, as well as at concentrations over 6% or below 2% [12]. Certainly, high concentrations of GLY have a negative effect on sperm membrane integrity [18]. In addition, GLY is osmotically active, changing the water content of sperm cells [19]; any osmotic stress induced could reduce sperm longevity and accelerate sperm capacitation. Only a few studies examining the addition of GLY to extenders for chilling [20,21] have been performed; its effects on sperm functionality, especially kinetic variables and mitochondrial integrity, need to be further investigated. Unlike that reported by Colas [12], Morrier et al. [22] indicated that the addition of 7% GLY did not significantly reduce the motility or viability of ram sperm maintained for 24 h at 5 °C, suggesting this agent might be used in cryopreservation protocols. Reducing the percentage of GLY below 7% under similar chilling conditions might allow its cryoprotective advantages to be maintained while reducing its cytotoxic effects; it could also provide an extra source of energy [23], and promote membrane stabilization [24].

Sperm selection by density-gradient centrifugation (DGC) has been used to select ram sperm prior to *in vitro* fertilization (IVF) [25]. In goats, the advantages of DGC become clear with chilled sperm, with live and motile sperm numbers reported to remain constant for up to 96 h [26]. This suggests that DGC removes sperm with sub-optimal functionality along with most of the apoptotic cells [27]. Galarza et al. [28] who compared different sperm selection procedures, found Sephadex filtration to be even better than DGC, improving both the motility and viability of ram sperm. Considering the aforementioned, it should be expected that the addition of GLY in low concentration to extenders following sperm selection through the use of Sephadex columns, would improve the functionality and fertilizing capacity of ram sperm chilled and stored for long times.

Alongside the basic parameters evaluating sperm fertilizing ability, IVF is a competent indicator providing information on gametes interaction, sperm penetration, pronuclear formation and early embryo development [29]. The fertilizing capacity of frozen-thawed ram semen has been successfully assessed via homologous IVF using either ewe oocytes [30], or heterologous IVF involving either zona-free hamster ova [31], and or zona-intact bovine oocytes [32]. However, chilled ram semen has been successfully used in either homologous [6] or heterologous [33] IVF in limited scale involving only zona-free oocytes. Furthermore, mainly in sheep, the accessibility to homologous adequate oocytes is limited. Therefore, the use of heterologous IVF bypasses this limitation.

The aim of this study was to evaluate the effect of addition of GLY in low concentration to extenders, following sperm selection through the use of Sephadex G-15® columns, on sperm functionality of ram sperm chilled to 5 °C and stored up to 96 h. The fertilizing capacity of the sperm returning the best results was examined by heterologous IVF involving mature zona-intact bovine oocytes.

2. Materials and methods

All extenders and media were prepared at the Department of Animal Reproduction Research Laboratory (INIA, Madrid, Spain) using reagent-grade chemicals purchased from Panreac Chemistry S.A. (Barcelona, Spain), Sigma Chemical (St. Louis, MO, USA) or Invitrogen (Eugene, OR, USA).

2.1. Extenders

Two synthetic-based extenders for the liquid storage of ram semen were made with TTG solution (210.59 mM Tes, 95,75 mM Tris, 10.09 mM glucose, 0.54 mM streptomycin, and 2.14 mM penicillin; 324 mOsm/kg, pH 7.1) and 6% (v:v) EY: (1) TEST, and (2) TEST plus 2% (v:v) glycerol (TEST-GLY). In addition, two non-synthetic-based extenders were made according Gil et al. [20] with some modifications: (1) ultra-heat-treated (UHT) made from commercial skimmed milk plus antibiotics (100000 IU penicillin sodium and 100 mg dihydrostreptomycin/100 mL) plus 6% (v:v) EY, and (2) UHT plus 2% (v:v) glycerol (UHT-GLY). All extenders were centrifuged for 30 min at 4000×*g* to remove any large particles, and the supernatant filtered through a sterile Minisart® NML Syringe Filter 16555 (pore size 0.45 μm) (Sartorius, Germany). The osmolarity of all extenders was 298–310 mOsm/kg. The pH was adjusted to 7.2. All extenders were stored at −20 °C until use.

2.2. Animals and semen collection

The animals used in this work were eight healthy Merino rams aged 2–7 years. All were handled according to procedures approved by the INIA Ethics Committee, and all work was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. The rams were housed at the INIA Department of Animal Reproduction. All were fed a diet of grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available *ad libitum*.

Using an artificial vagina (pre-warmed at 42–43 °C), a total of 21 semen ejaculates were collected from the experimental animals during the September–December 2017 rutting season. Immediately after collection, each ejaculate was diluted with TTG (1:1 v:v) solution at 37 °C and transported to the laboratory for initial assessment. Those ejaculates with a volume of 0.75–2 mL, a sperm motility value of >70%, a score of >3 on a mass motility scale of 0–5, and a sperm concentration of $>3.5 \times 10^9$ sperm/mL were used in the subsequent experimental work.

2.3. Sperm filtration

Sperm selection by Sephadex filtration columns was performed according to Galarza et al. [28]. For this purpose, each semen ejaculate diluted 1:1 (v:v) with TTG solution was divided into two aliquots. The first aliquot (400 μL) was used for non-filtered semen and then was subdivided and extended at room temperature with each diluent type to a concentration of 800×10^6 sperm/mL. The second aliquot (1200 μL) was filtered through Sephadex columns. The filtered samples were then re-suspended in 400 μL TTG solution at 37 °C, subdivided and diluted with each extender type under same conditions as non-filtered semen samples (temperature and

concentration). All these samples were then slowly cooled to 5 °C and stored for 0, 24, 48, 72 or 96 h. Sperm variables were then assessed.

2.4. Sperm analysis

Sperm motility analysis was assessed using a CASA system (Sperm Class Analyzer, SCA® 1999, v.4.0 software. Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) in accordance with Santiago-Moreno et al. [26]. The sperm samples were loaded into a warmed (37 °C) 20 μm Leja® 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). A minimum of three fields and 200 sperm tracks were evaluated at 100 X for each sample chamber (image acquisition rate 25 frames/s). Values were recorded for the percentage of motile sperm (%SM), percentage of progressive sperm (%PSM), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), and the amplitude of lateral head displacement (ALH, μm).

Plasma and acrosome membrane status and mitochondrial function, were assessed using a triple association of fluorescent probes - propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut *(Arachis hypogaea)* agglutinin (PNA-FITC, Sigma L7381), and Mitotracker Green *FM*® (MITO, Invitrogen M7514) - according to Forero-Gonzalez et al. [34] with some modifications. For this, samples of 150 μL of semen diluted in TALP Stock medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH2PO4 H2O, 1 mM Na-Lactate, 1.97 mM CaCl2 2H2O, 0.50 mM MgCl2 6H2O, 10 mM HEPES sodium, and 25 mM NaHCO3; 320 mOsm/Kg, pH 7.3) to a concentration of 25×10^6 sperm/mL were mixed with 2 μL of PI (2 mg/mL), 2 μL of MITO (1 mM) and 50 μL of PNA-FITC (100 μg/mL) and co-incubated in the dark at 38.5 °C for 8 min (preliminary studies were performed to examine the intensity of staining of each fluorescent probe and its association with different incubation times [3, 5, 8, 10, 12 and 15 min]; data not shown). After incubation, the samples were transferred to a slide, covered with a cover slip, and examined immediately using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (excitation: 450 nm, emission: 490 nm). A total of 200 sperm cells per slide were examined and eight subpopulations of cells quantified, i.e., those showing: (1) intact plasma membrane/intact acrosome/high mitochondrial function (%IPIAHM); (2) intact plasma membrane/intact acrosome/low mitochondrial function (%IPIALM); (3) intact plasma membrane/damaged acrosome/high mitochondrial function (%IPDAHM); (4) intact plasma membrane/damaged acrosome/low mitochondrial function (%IPDALM); (5) damaged plasma membrane/intact acrosome/high mitochondrial function (%DPIAHM); (6) damaged plasma membrane/intact acrosome/low mitochondrial function (%DPIALM); (7) damaged plasma membrane/damaged acrosome/high mitochondrial function (%DPDAHM); and (8) damaged plasma membrane/damaged acrosome/low mitochondrial function (%DPDALM).

Sperm morphological abnormalities (abnormal heads, loose heads, coiled tails, abnormal tails, cytoplasmic droplets) were assessed according to Galarza et al. [28] using 5 μL sperm samples in 100 μL of 2% (v:v) glutaraldehyde solution, and counting 200 sperm cells.

2.5. Heterologous in vitro fertilization (Bos taurus oocytes x Ovis aries sperm)

The fertilizing capacity of non-filtered sperm extended with UHT, GLY-free, stored for 0 (FS0), 24 h (CS24) or 48 h (CS48) was tested by heterologous IVF involving zona-intact bovine oocytes according to Pradieé et al. [35] with some modifications. Briefly, oocyte *in vitro* maturation was performed in 60 μL drops (30 COCs per drop) of maturation medium (TCM-199) supplemented with 10-ng/mL EGF and 10% (v:v) fetal calf serum (FCS) for 24 h at 38.5 °C under an atmosphere of 5% CO_2 in air at maximum humidity. After 24 h, the matured oocytes were washed twice in FERT medium (FERT-TALP media supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 10 μg/mL heparin) and transferred to 30 μL drops of FERT medium (30 COCs per drop).

For heterologous IVF, a pool of semen samples from three individual rams was performed in each replicate for all experimental groups (FS0, CS24 and CS48). In addition, for each sperm group, a control was prepared using frozen-thawed (in a water bath at 37 °C for 50 s) sperm from a single Asturian Valley bull (Asturgen, Gijon, Spain) of proven fertility. Semen samples from FS0, CS24 and CS48 groups were maintained at 37 °C for 30 min before sperm selection. Motile sperm from each group were selected by density gradient centrifugation (BoviPure, Nidacon International, Sweden) prior to attempting heterologous IVF. Sperm were diluted in FERT medium and 30 μL of this suspension was added to each fertilization drop obtaining a final concentration of 1×10^6 sperm/mL.

A heterologous group using ram sperm, a homologous control group using bovine sperm and a parthenogenetic control group were tested in each IVF replicate. A total of fifteen IVF replicates were performed in the three chilling time point groups: FS0 (n = 5), CS24 (n = 5), and CS48 (n = 5). Gametes were co-incubated at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

Sperm-oocyte interaction was assessed by sperm-zona pellucida binding assay at 2.5 h post-insemination (hpi). For that purpose, oocytes were vortexed for 3 min, fixed and stained with Hoechst 33342 to count the number of sperm that remained bound to the zona pellucida by a Nikon Eclipse E200 epifluorescence microscope (UV-2E/C, excitation: 340–380 nm, emission: 435–485 nm). Sperm penetration and polyspermy was evaluated at 12 hpi, while pronucleus formation was evaluated at 18, 20, 22, 24 and 26 hpi for heterologous and at 18 h for homologous IVF. For this, presumptive zygotes were treated and examined as explained above for sperm-zona pellucida binding. The cleavage rate was evaluated at 48 hpi in all groups.

2.6. Experimental design

The effects of the different extenders, cold storage time, and sperm selection on different sperm variables and sperm fertilization ability were examined. Eight experimental groups (samples of 300 μL at a concentration of 800×10^6 sperm/mL) of sperm were established: (1) non-filtered sperm extended with TEST; (2) non-filtered sperm extended with TEST-GLY; (3) non-filtered sperm extended with UHT; (4) non-filtered sperm extended with UHT-GLY; (5) filtered sperm extended with TEST; (6) filtered sperm extended with TEST-GLY; (7) filtered sperm extended with UHT; and (8) filtered sperm extended with UHT-GLY. All groups were maintained in chilled conditions and sperm parameters were assessed at 0, 24, 48, 72 or 96 h. The freshly collected semen samples (n = 21) extended simply with TTG [1:1 (v:v), at concentration of $2178.5 \pm 221.5 \times 10^6$ sperm/mL] provided controls.

The fertilizing capacity of non-filtered sperm extended with UHT without GLY (selected based on the optimal sperm quality parameters measured in the previous analysis), stored for 0, 24 and 48 h, was tested by heterologous IVF with zona intact bovine oocytes. Homologous fertilization and parthenogenesis were used as control. A total of 3840 cumulus oocyte complexes (COCs) obtained from ovaries from heifers and cows at slaughter, in 15 individual

replicates were used for heterologous [FS0 (n = 707), CS24 (n = 832) or CS48 (n = 611)] and homologous IVF (n = 1356), or parthenogenesis (non-fertilized oocytes, n = 334). Sperm zona binding (2.5 hpi), penetration and polyspermy (12 hpi) pronuclei formation (18, 20, 22, 24 and 26 hpi) and cleavage rate (48 hpi) were evaluated in all groups.

2.7. Statistical analysis

Data were expressed as means ± S.E.M. Values for sperm variables showing a skewed distribution (as determined by the Shapiro-Wilks test) were arcsine- (for percentages values) or Log10- (for numeric values) transformed. Factorial ANOVA and Bonferroni post hoc multiple comparison tests were used to examine the effects of the interactions between extender type with and without GLY, Sephadex filtration, and cold storage up to 96 h, on sperm kinetic variables, plasma membrane and acrosome status, mitochondrial function, and morphological integrity. For heterologous IVF, the percentage of bound sperm, oocyte penetration, polyspermy, male pronucleus formation and the cleavage rate were analyzed by parametric one-way ANOVA and Tukey's post hoc multiple comparison tests. All calculations were made using Statistica for Windows v.12.0 software (StatSoft, Tusla, OK, USA).

3. Results

The interaction *Sephadex filtration x extender type x chilling time* had no significant effect on the sperm variables examined. However, the interaction *Sephadex filtration x extender type* did have an effect on %PSM ($P < 0.001$) and AHL ($P < 0.001$), and the interaction *Sephadex filtration x chilling time* significantly influenced VCL ($P < 0.05$) and VSL ($P < 0.01$). It should be noted that the results for ALH obtained with the non-filtered sperm and TEST extenders (with or without GLY) were better than those obtained for filtered sperm (see Fig. 1).

3.1. Effect of extenders and storage time on non-filtered semen

The motility values (%SM and %PSM) of sperm diluted with either UHT extender were greater ($P < 0.01$) than those recorded for either TEST extender (i.e., with or without GLY) up to 96 h of cold storage. However, when diluted with either UHT extender (i.e., with or without GLY), a significant reduction ($P < 0.05$) was seen in %PSM and VSL between 48 and 72 h of storage (Fig. 1). The VSL value was greater ($P < 0.01$) with either UHT extender than either TEST extender up to 72 h. The ALH value was lower ($P < 0.001$) in the samples diluted with either UHT extender (either type) than either TEST extender at all times. The addition of GLY to either the TEST or UHT extender had no significant effect ($P > 0.05$) on these kinetic variables compared to the same extenders without GLY.

Similarly, the %IPIAHM value was greater ($P < 0.05$) with either UHT extender than either TEST extender at all times. However, the values fell with increasing storage time for all extenders. No differences were seen in the %IPDAHM, %DPIAHM and %DPDALM values at 96 h between the UHT and TEST extenders with or without GLY, despite both the UHT extenders returning high values at 24 h. The %DPDAHM value was lower ($P < 0.05$) when either UHT extender was used compared to either TEST extender at all times (Fig. 2).

No differences were recorded between any of the extenders in terms of morphological abnormalities (percentage of abnormal heads, loose heads, abnormal tails, cytoplasmic droplets or total abnormalities) at any storage time. At 24 h, however, the percentage of sperms with coiled tails was higher ($P < 0.05$) with the UHT-GLY extender than with any other.

3.2. Effect of extenders and storage time on filtered semen

The values for %SM and %PSM and VSL were higher ($P < 0.01$) with the UHT-GLY extender than with any other extender after 96 h of cold storage - although these values dropped between 48 h and 72 h ($P < 0.05$). The ALH value was lower ($P < 0.001$) in samples diluted with either UHT extender than either TEST extender (Fig. 1).

The %IPIAHM value was greater ($P < 0.05$) with either UHT extender than either TEST extender from 24 to 96 h. The %DPDAHM value was lower ($P < 0.05$) with either UHT extender than either TEST extender at all times. Finally, the %DPDALM value was lower with UHT diluents than using TEST diluents at 96 h ($P < 0.05$).

The percentage of abnormal heads was higher ($P < 0.05$) with the UHT extender than with UHT-GLY at 48 h of storage. The percentage of coiled tails at 0 h was greater ($P < 0.05$) with UHT-GLY than with TEST-GLY; from 24 to 48 h the value was higher with UHT-GLY than any other extender; and at 72 h the values were higher with UHT diluents than TEST diluents. In addition, the percentage of coiled tails increased ($P < 0.05$) at 96 h of storage with the TEST-GLY and UHT (without GLY) extenders.

3.3. Heterologous in vitro fertilization

Results of heterologous IVF are depicted in Table 1. Heterologous IVF showed that ram sperm, either fresh or cold-stored (24 or 48 h), were equally capable of penetrating bovine oocytes, leading to pronuclear formation and hybrid embryo cleavage. No differences were seen in sperm binding, penetration, polyspermy, pronuclear formation or cleavage rate between fresh semen and semen chilled for 24 h or 48 h ($P > 0.05$).

As expected, homologous IVF was associated with higher percentages of penetration compared to heterologous IVF for the FS0 sperm ($P < 0.01$). Polyspermy was higher in the CS24 heterologous IVF group than in the CS24 and CS48 homologous IVF group ($P < 0.05$). As expected, and for all groups, homologous IVF group returned a higher percentage of pronucleus formation at 18 hpi than did heterologous IVF ($P < 0.001$). Nevertheless, no differences were seen in pro-nuclei formation between the heterologous IVF groups at different time points (18–26 hpi) ($P > 0.05$). Similarly, for all groups, the cleavage rate was higher ($P < 0.001$) in homologous than heterologous IVF.

4. Discussion

The results showed that chilling non-filtered ram semen diluted with UHT extender without GLY preserves sperm motility, plasma membrane, acrosome integrity, mitochondrial function and morphological integrity, better than TEST extenders of either type at all times, maintaining a fertilizing capacity similar to that of fresh semen for up to 48 h of cold storage. Interestingly, the results also showed that neither sperm selection via the use of Sephadex columns, nor the addition of GLY, improves the functionality of stored sperm.

The use of GLY in semen refrigeration has returned variable results across different studies. Low GLY concentrations have been successfully used with ram sperm diluted with skimmed milk-based extenders [20], and with goat sperm diluted with a Tris-fructose-citric acid-soybean lecithin-based extender [18]. The present results indicate that 2% GLY provided no extra benefit to unfiltered ram sperm cold-stored for up to 96 h; however, when the semen sample was previously filtered with Sephadex, GLY improved total motility, the percentage of sperm showing progressive motility, and the VSL values. These findings suggest that Sephadex filtration might select sperm cells subpopulations capable of metabolizing GLY, rendering it an extra source of energy

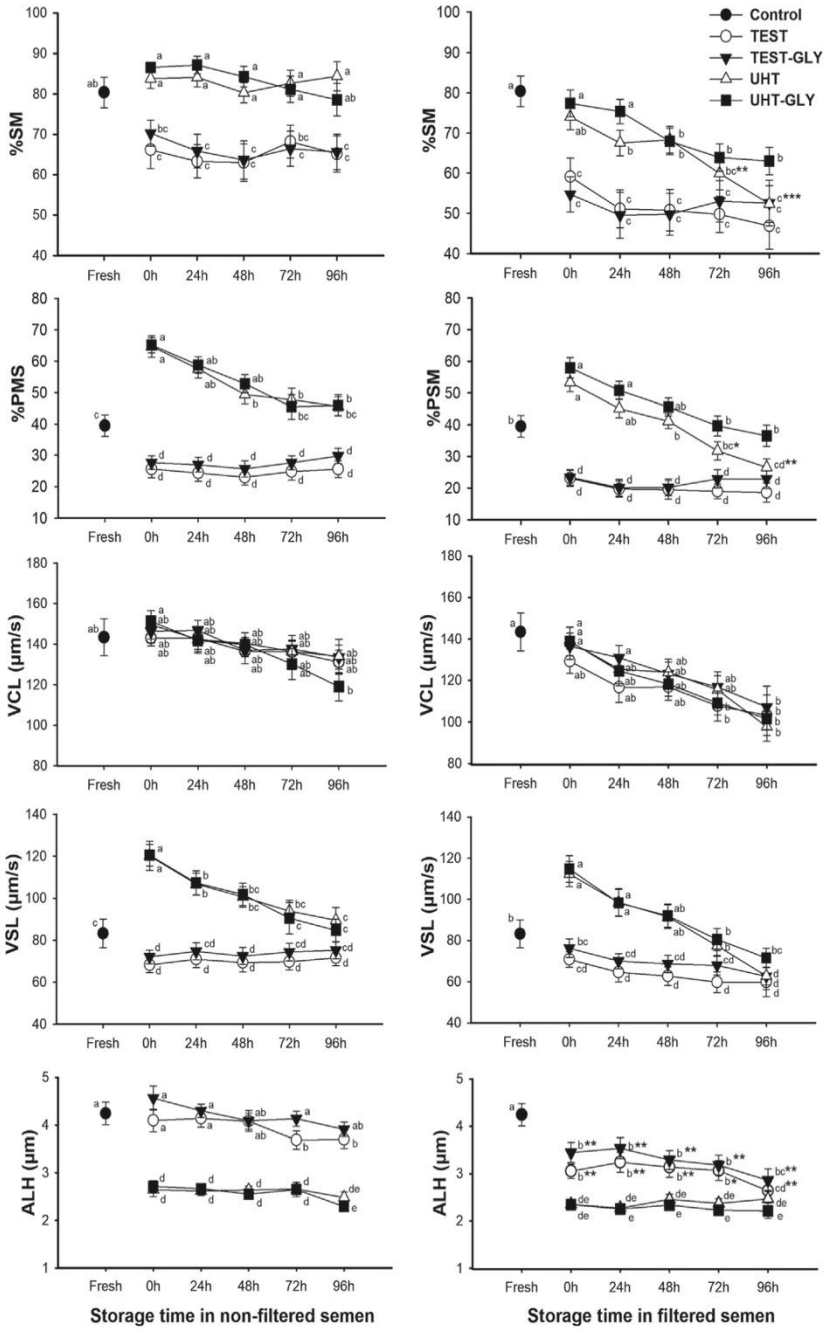

Fig. 1. Kinetic variables of non-filtered and filtered ram semen samples, diluted with different extenders - Control, TEST, TEST-GLY, UHT, UHT-GLY - and cold-stored at 5 °C. %SM, sperm motility; %PSM, progressive sperm motility; VCL, curvilinear velocity; VSL, straight line velocity; and ALH, amplitude of lateral head displacement. Different letters in superscript (a–e) at each evaluation time indicate significant differences between values for extenders and cold storage times ($P < 0.05$ for a – b, and b - c; $P < 0.01$ for a – c, c – d, and c - e; and $P < 0.001$ for a – d, a – e, b – d, and b - e). * Significant reduction between non-filtered and filtered semen samples at the same evaluation-time and for the same extender (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

[23]. A similar improvement in VCL and in the fertility rate was reported for bull sperm when using 7% GLY [21].

Glycerol has been indicated to accelerate the acrosome reaction in ram sperm stored at 5 °C [36,37] which might reduce the reproductive lifespan of cells [38]. However, the triple association of fluorescent probes showed a lack of difference in %IPDAHM at

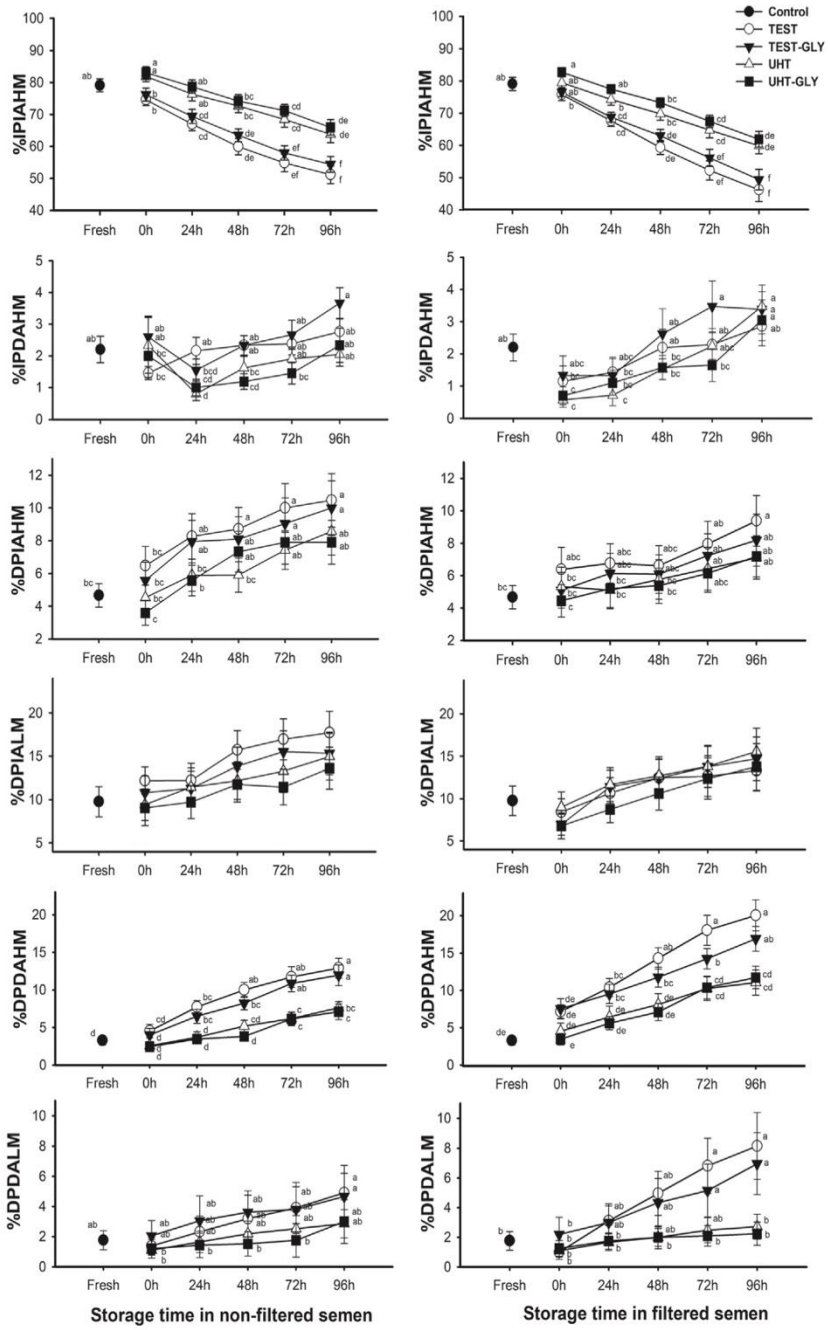

Fig. 2. Status of sperm, plasma-, acrosome- and mitochondrial membranes (association of PI/PNA-FITC/Mitotracker probes) in non-filtered and filtered ram semen samples, diluted with different extenders - Control, TEST, TEST-GLY, UHT, UHT-GLY - and cold-stored at 5 °C. %IPIAHM, intact plasma membrane/intact acrosome/high mitochondrial function; %IPDAHM, intact plasma membrane/damage acrosome/high mitochondrial function; %DPIAHM, intact plasma membrane/intact acrosome/high mitochondrial function; %DPIALM, damaged plasma membrane/intact acrosome/low mitochondrial function; %DPDAHM, damaged plasma membrane/damage acrosome/high mitochondrial function; %DPDALM, damaged plasma membrane/damage acrosome/low mitochondrial function. Different letters in superscript (a–f) at each evaluation time indicate significant differences between values for extenders and cold storage times ($P < 0.05$ for a - b, b - c, c - d, d - e, and e – f; $P < 0.01$ for a - c, b - d, and c - f; and $P < 0.001$ for a - d, a – e, a - f, b – e, b – f, and c - f).

96 h when any of the four types of extender were used.

Sperm selection via Sephadex filtration improves the quality of both normospermic and asthenospermic ram sperm [39]. We previously reported that filtration with Sephadex columns improves the kinetic characteristics of fresh and cold-stored ram semen outside of the reproductive season [28]. The fact that the

Table 1
Rates of penetration, polyspermy, pronucleus formation and cleavage after homologous and heterologous co-incubation involving fresh ram sperm (FS0), ram sperm cold-stored for 24 h (CS24), and ram sperm cold-stored for 48 h (CS48), at different times post-insemination (h). Values are expressed as (mean ± SEM) of five replicates (total number of oocytes or presumptive zygotes examined = 3840).

Ram semen	Groups	Bound sperm	Penetration (%)	Polyspermy (%)	Pronuclear formation (%)					Cleavage rate (%)
		2.5 h	12 h	12 h	18 h	20 h	22 h	24 h	26 h	48 h
FS0	Ho-IVF (n = 477)	64 (0.25 ± 0.1)	54 (44.4 ± 6.8)^{a}	54 (5.6 ± 3.1)^{ab}	52 (67.3 ± 5.8)^{a}					253 (78.3 ± 2.6)^{a}
	He-IVF (n = 707)	61 (0.31 ± 0.1)	56 (12.5 ± 4.5)^{b}	57 (7.0 ± 3.4)^{ab}	54 (35.2 ± 5.6)^{b}	53 (32.1 ± 6.5)	49 (34.7 ± 6.9)	58 (48.3 ± 6.6)	69 (44.9 ± 6.0)	177 (46.3 ± 3.2)^{b}
	Parthenogenic (n = 86)									86 (7.0 ± 2.3)^{c}
CS24	Ho-IVF (n = 534)	75 (0.29 ± 0.1)	78 (42.3 ± 5.6)^{a}	78 (3.8 ± 2.2)^{b}	48 (72.1 ± 4.5)^{a}					255 (78.4 ± 2.6)^{a}
	He-IFV (n = 832)	72 (0.22 ± 0.0)	70 (30.0 ± 5.5)^{ab}	70 (11.4 ± 3.4)^{a}	73 (37.0 ± 5.7)^{b}	72 (29.2 ± 5.4)	66 (37.9 ± 6.0)	78 (42.3 ± 5.6)	81 (45.7 ± 5.6)	252 (48.8 ± 3.2)^{b}
	Parthenogenic (n = 109)									109 (4.9 ± 2.0)^{c}
CS48	Ho-IVF (n = 345)	65 (0.32 ± 0.1)	62 (40.3 ± 6.3)^{a}	62 (1.6 ± 1.6)^{b}	54 (63.0 ± 6.0)^{a}					102 (78.4 ± 3.3)^{a}
	He-IFV (n = 611)	59 (0.29 ± 0.1)	57 (29.8 ± 6.1)^{ab}	57 (7.0 ± 3.4)^{ab}	63 (27.0 ± 5.6)^{b}	63 (34.9 ± 6.1)	58 (46.6 ± 6.6)	52 (44.2 ± 7.0)	59 (47.5 ± 6.6)	143 (43.3 ± 3.5)^{b}
	Parthenogenic (n = 139)									139 (4.3 ± 1.2)^{c}

FS0, fresh semen; CS24, semen cold-stored for 24 h; CS48, cold-stored up to 48 h; Ho-IVF, Homologous *in vitro* fertilization; He-IVF, Heterologous *in vitro* fertilization. $^{a-c}$ Different superscripts in the same column indicate significant differences. $^{a-b;\ b-c}$ $P < 0.05$; $^{a-c}$ $P < 0.001$.

ejaculates in the present study were collected during the rutting season, and were already of good quality, might explain why Sephadex filtration had no effect; it may be that such filtration is only worthwhile outside of the rutting season. Nevertheless, if barriers such as that imposed by cervical mucus are to be overcome, and the oocyte cumulus cell layers are to be penetrated, sperm ALH values need to be appropriate [40], and for the filtered semen diluted with either the TEST or UHT extender (either with or without GLY), the ALH values obtained were lower than for the non-filtered semen. This agrees with previous studies on goat semen [26], in which sperm selection by density gradient centrifugation determined a lower ALH value than that achieved with non-washed or classically washed sperm. An increased ALH value greatly raises the number of hyperactive sperm cells undergoing capacitation; Sephadex filtration therefore seems to facilitate the selection of non-capacitated ram sperm.

To prove the fertilizing capacity of ram sperm fresh or chilled-diluted in UHT extender we used *in vitro* fertilization. However, as mentioned before, the accessibility to homologous adequate oocytes (i.e: from adult ewes) may be limited, as it was in our case. Therefore, the use of heterologous IVF with bovine oocytes was used as a tool to bypass this limitation. Heterologous IVF using ram sperm and bovine oocytes has been described as an effective method for predicting the fertilization capacity of frozen-thawed [25] or chilled [33] ram sperm. To the best of our knowledge this is the first report of the fertilizing capacity of cold-stored ram semen using heterologous IVF and zona-intact bovine oocytes, providing specific information regarding sperm binding, penetration, polyspermy, pronuclei formation (e.i. 18 to 26 hpi) and cleavage rate. The present study revealed the ability of both fresh and chilled sperm to equally bind zona-intact bovine oocytes, penetrate and fertilize them. A similar oocyte-sperm interaction was found between heterologous groups and its control groups. Controversially, previous studies evidenced a greater interaction between zona-intact bovine oocyte and bottlenose dolphin sperm [41,42]. Furthermore, it has been suggested that sperm interaction and penetration could be improved by low levels of sperm DNA fragmentation along with a stable chromatin, as demonstrated in heterologous IVF using Iberian ibex sperm and zona-intact bovine oocytes [35].

We demonstrated that fresh (FS0) or chilled (CS24 or CS48) sperm were able to penetrate zona-intact bovine oocytes leading to pronuclear formation. This indicates that ram sperm were capable to undergo acrosome reaction and bovine oocytes were capable to recognize ram sperm, that is in agreement with a previous study suggesting that the acrosome lytic system is conserved among mammals [43]. The influence of chilling for 48 h on fertilizing capacity was examined since, at this time, the fertility rate is strongly reduced when chilled sperm is used for AI [7]; indeed it is usually recommended that semen be used within 12 h [44]. Interestingly, the present results showed no differences in terms of sperm binding, penetration, polyspermy, pronucleus formation or cleavage rate in heterologous IVF, between sperm (diluted using the UHT extender without GLY) chilled for 24 h or 48 h. The present data indicate that ram sperm refrigerated in a UHT medium for 48 h maintain an *in vivo* fertilizing capacity similar to that of fresh sperm.

On the other hand, previous studies have reported *in vivo* fertility rates of 29.0–39.0% or 47.0–49.0% in ewes after cervical AI using ram chilled sperm diluted with Tris–Citric–Fructose [45] or UHT [2] extenders, respectively. Thus, the IVF values obtained in the present study suggest that ram sperm preserved in UHT extender up to 48 h, maintain its fertilizing ability. In addition, our findings provide useful information for future cervical AI trials in sheep.

5. Conclusion

In conclusion, sperm filtration with Sephadex within the rutting season did not improve sperm quality during chilled storage. Neither did the addition of 2% GLY, provide any benefits for non-filtered semen. The UHT extender with or without GLY returned better sperm quality results than the TEST extenders with or without GLY for storage at 5 °C over 96 h, irrespective of sperm filtration. Long-term chilled sperm with intact plasma-, acrosome- and mitochondrial membranes should have enough kinetic vigour to cross the cervical barrier following AI if an UHT diluent is used. Moreover, ram sperm stored chilled for up to 48 h is able to penetrate and fertilize zona-intact oocytes under *in vitro*

conditions.

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CAPÍTULO 3

L-carnitine supplementation to UHT skimmed milk-based extender improves motility, membranes integrity and fertility of chilled ram sperm.

Diego A Galarza*, Antonio López-Sebastián, J. Santiago-Moreno. **L-carnitine supplementation to UHT skimmed milk-based extender improves motility, membranes integrity and fertility of chilled ram sperm.** 2019. Research article to be sent to the journal Reproduction in Domestic Animals

L-carnitine supplementation to UHT skimmed milk-based extender improves motility, membranes integrity and fertility of chilled ram sperm.

RESUMEN

La presente investigación fue se diseñada para evaluar el efecto de la L-carnitina (LC) en el semen líquido de morueco almacenado a 5 °C a largo plazo. Las muestras de semen se recolectaron, se agruparon y se mezclaron con un diluyente de leche desnatada (UHT) + 6% de yema de huevo y suplementado con 0 (control), 1 (LC1), 2,5 (LC2,5), 5 (LC5), 7,5 (LC7,5) y 10 mM (LC10) de LC. Los parámetros cinéticos de motilidad y el estado de las membranas de esperma (plasma, acrosoma y mitocondrial) fueron analizados a las 0, 48 y 96 h mediante sistema CASA y un test de triple marcadores de fluorescencia (*PI / PNA-FITC / MITO*). Además, se evaluó la fertilidad de semen de morueco diluido con LC5 usando semen fresco conservado a 15 ºC (LC5-fresco) o refrigerado a 5 ºC durante 24 h (LC5-24h) en tres rebaños de ovejas por inseminación artificial de tiempo fijo (IATF). A las 96 h, la motilidad del esperma fue superior ($P < 0,001$) con los grupos LC5 y LC10 que con el grupo LC7.5 y el grupo control y la motilidad progresiva fue mayor ($P < 0,05$) con el grupo LC5 que con el grupo control. La velocidad curvilínea y rectilínea, así como el porcentaje total de espermatozoides con membranas intactas (plasma / acrosoma / mitocondria) fueron mejoradas ($P < 0,001$) en todos los grupos de LC en comparación con el grupo control hasta las 96 h. Los resultados de la tasa de preñez indicaron que en un rebaño no hubo diferencias significativas ($P > 0,05$) entre los grupos de semen LC5-fresco y LC5-24h (52,4% vs 42,8%), sin embargo, en otros dos rebaños la tasa de preñez disminuyó ($P < 0,001$) notablemente a las 24 h (12,5% y 20,0%). En conclusión, la LC mejoró las variables de motilidad e integridad de las membranas espermáticas. Además, la fertilidad del semen fresco, diluido y conservado a 15 ºC fue

satisfactoria, no obstante los resultados de fertilidad con semen conservado a 5 ºC durante 24 h indican que los efectos de este antioxidante deben ser sujetos a mayores investigaciones.

Palabras clave: L-carnitina, semen ovino, enfriado, leche desnatada, fertilidad.

ABSTRACT

The current study was designed to evaluate the effect of L-carnitine (LC) on liquid ram semen stored at 5 °C for long-term. Semen samples were collected, pooled and diluted with extender based on skimmed milk (UHT) + 6% egg yolk and supplemented with LC to either 0 (control), 1 (LC1), 2.5 (LC2.5), 5 (LC5), 7.5 (LC7.5) and 10 mM (LC10). Computer assisted sperm analysis (*CASA*) and triple fluorescence markers test (*PI/ PNA-FITC/ MITO*) were used to evaluate spermatozoa kinematics and status of sperm membranes (plasma, acrosome and mitochondrial) at 0, 48, and 96 h. Also, fertility of ram semen diluted with LC5 using fresh preserved at 15 ºC (LC5-fresh) or stored at 5 ºC for 24 h (LC5-24h) was evaluated in three ewe flocks by fixed-time artificial insemination. At 96 h, the sperm motility was higher ($P < 0.001$) with both LC5 and LC10 groups than both LC7.5 and control group; and progressive motility was higher ($P < 0.05$) with LC5 group than control group. Surprisingly, curvilinear and straight-line velocity and percentage of sperm with intact membranes (plasma / acrosome / mitochondria) were improved with all LC groups compared with control group at all times ($P < 0.001$). The results of pregnancy rate indicated that only a flock there was no significant difference ($P > 0.05$) between LC5-fresh and LC5-24h semen groups (52.4% vs 42.8%), nevertheless, in other two flocks, the pregnancy rate decreased ($P < 0.001$) notably at 24 h (12.5% and 20.0%). In conclusion, LC improves chilling-stored ram sperm motility and membranes integrity. Also, the fresh ram semen supplemented with LC indicated a satisfactory fertility response, nevertheless, the controversial results of this semen preserved at 5 ºC for 24 h indicated that fertility must be more investigated.

Keywords: L-carnitine, ram sperm, chilled, skimmed milk, fertility.

INTRODUCTION

Fixed-time artificial insemination (FTAI) via cervically in ewe using liquid ram sperm diluted with skim milk extender (e.g., INRA-96®) and maintained at 15 °C for short-term (1–6 h), is the reproductive biotechnology most useful in genetic European improvements programs so far. Moreover, liquid ram sperm stored at 5 °C are capable of decreasing its metabolic activity and preserve successfully sperm with intact plasma membrane and fertilizing ability for a longer time, even high *in vitro* fertility rate has been yielded with semen preserved up to 10 days (Maxwell and Salamon, 1993). Nevertheless, while increasing the time of cold storage for long-term, the kinetic vigor, the integrity of sperm membranes decreases (Gil et al., 2011). The pregnancy rate of ewe cervically inseminated to synchronized estrus (O'Hara et al., 2010) or spontaneous estrus (Olivera-Muzante et al., 2011) with ram semen diluted in skimmed milk-based extender decrease after 24 h at 5 °C.

Two principal factors affect sperm preserved in liquid-chilled conditions. The first is the cold shock produced by reduction of temperature that causes irreversible changes e.g., capacitation, apoptosis, and DNA hypomethylation when semen is stored at low temperature (Budai et al., 2014). Damage of plasma membrane, induction of caspase cascade producing apoptosis, DNA fragmentation and low fertilizing capacity of sperm are caused by cold shock (Salamon and Maxwell, 2000). Furthermore, during storage ram semen under chilled conditions for long-term, the unsaturated fatty acids of the sperm membrane bind oxygen and evolve numerous peroxide bonds (Budai et al., 2014). The second factor contributing to poor quality semen has been reported to be oxidative stress, which involves lipid peroxidation (LPO). Consequently, LPO leads to unbalanced oxidative stress that causes different impairments of sperm cells, and acrosome damage. The high concentration of polyunsaturated fatty acids (PUFA) in membranes of small

ruminants' spermatozoa lead to the sperm highly vulnerable to oxidative damage, resulting from the production of ROS (Bucak et al., 2010). Ram sperm cells are vulnerable to free radical (e.g., H_2O_2, O_2-, and OH-) attack since are rich in PUFA, therefore ROS can combine readily with them, directly causing LPO (Alvarez et al., 1987). An increase in ROS causes detrimental effect on ram sperm such as disruption of mitochondrial and plasma membranes, impaired cell function, along with impaired sperm motility (Amidi et al., 2016), chromosomal and DNA fragmentation (Kasimanickam et al., 2006), and apoptosis induction.

The antioxidants are the main defense factors against both cold shock and oxidative stress induced by free radicals. Antioxidants, thus ameliorate the detrimental effect of ROS, and improving the sperm quality either post-thaw process (Silva et al., 2011) or cold-storage process (Bucak and Tekin, 2007). Various enzymatic antioxidants e.g., reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) has demonstrated a protective effect against the LPO of ram semen, maintaining sperm motility and integrity of the plasma membrane after the frozen-thawed process (Câmara et al., 2011a; Câmara et al., 2011b). Likewise, other antioxidants such as resveratrol, vitamin E, or its combination showed a beneficial effect on motility of cold-stored ram semen up to 10 days than the control group (Sarlós et al., 2002). This antioxidant capacity of semen may, however, be insufficient in preventing LPO during chilling-stored of ram semen for long-term. In addition, fertility remains low when liquid stored ram semen and cervical AI is used (Maxwell and Salamon, 1993). Supplementation of new antioxidants to diluents could be a promising solution to prevent or reserve detrimental membrane changes during the chilling process.

Levocarnitine or L-carnitine (LC) is an amine biosynthesized from two essential aminoacids, lysine and methionine, that plays a powerful role in transporting short-,

medium-, and long-chain fatty acids into the mitochondria for β-oxidation, which produces readily available energy (ATP) for use by spermatozoa (Jeulin et al., 1987). Thus, LC positively affects sperm motility, maturation and the spermatogenic process (Ramsay et al., 2001). The LC has also antioxidant properties against ROS production. In vitro studies have documented that carnitines enhance human sperm motility when added in vitro and may also have a cryoprotectant effect (Agarwal and Said, 2004). LC supplementation to semen extender has been previously tested in bull (Hufana-Duran et al., 2017), buffalo (El-Raey et al., 2016), goat (Bucak et al., 2010), rabbit (Sariözkan et al., 2014), among others; but, in these studies, they were neither evaluated in cold-stored condition nor supplemented to a non-synthetic-based extender. However, a recent study has demonstrated that LC added to synthetic-based extender did not provide consistent beneficial to frozen-thawed ram semen (de Souza et al., 2019). To our best knowledge, the effect of LC on sperm variables and fertility of liquid ram sperm under chilled conditions still remained unclear. Previous reports indicated that UHT-based extender provide better protection and survival to ram sperm when those is cold-stored up to 72 h (Gil et al., 2011) or 96 h (Galarza et al., 2019). In this sense, we hypothesized that LC supplemented to UHT-based extender could be ameliorated sperm survival and functionality for long-term as well as to prolong fertility of ram sperm chilled up to 24 h after cervical AI. This research was therefore conducted to evaluate both antioxidant and locomotion enhancer effects of LC on motility variables, membranes integrity (plasma, acrosome and mitochondria) and fertility of chilled ram sperm.

MATERIALS AND METHODS

L-Carnitine® for synthesis (Sigma 8400920025) was obtained from Sigma Chemical Co., (St. Louis, Missouri). The diluent and media were prepared in the INIA Department

of Animal Reproduction Research Laboratory using reagent-grade chemicals purchased from Panreac Chemistry S.A. (Barcelona, Spain) and Sigma Chemical Co.

Extender and L-carnitine supplementation

A non-synthetic-based extender were performed according Galarza et al. (2019). In brief, ultra-heat-treated made from commercial skimmed milk (UHT) plus antibiotics (100000 IU penicillin sodium and 100 mg dihydrostreptomycin/100 ml) and supplemented with 6% (v/v) egg yolk was used. The osmolarity was 298–310 mOsm/kg and the pH was adjusted to 7.2. UHT-based extender was centrifuged for 30 min at 4000 × g to remove any large particles, and the supernatant filtered through a sterile Minisart® NML Syringe Filter (16555, Sartorius, Germany) (pore size 0.45 μm). Five concentrations of LC were supplemented to UHT: 1 (LC1), 2.5 (LC2.5), 5 (LC5), 7.5 (LC7.5), and 10 mM (LC10). Those extenders were stored at -20 °C until use.

Animal and semen collection

Semen ejaculates were collected during non-reproductive season (June–August) from 4–6 years-old twelve adult Merino rams housed at INIA Department of Animal Reproduction (Madrid, 40º 25´N). All animals were handled according to procedures approved by the INIA Ethics Committee, and all work was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. All were fed a diet of grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available ad libitum.

Thirty-six semen ejaculates (three/ram) were collected weekly using an artificial vagina (pre-warmed at 42–43 °C) in twelve collection sessions. The volume of each semen ejaculate was measured in a graduated conical glass tube in milliliters. Briefly, after collection, each ejaculate was diluted (1:1) with UHT-based extender at 37 °C and transported to the laboratory for initial evaluation. Those ejaculates with a volume of 0.75–2 ml, a sperm motility value of >70%, a score of >3 on a mass motility scale of 0–5, and a sperm concentration of $>3.5 \times 10^9$ sperm/ml were included in the subsequent experimental work. Thereafter, twelve pools of semen from three different semen ejaculates randomized were formed. Each pool was divided into six aliquots and then diluted each with either LC1, LC2.5, LC5, LC7.5, and LC10. A control group that containing UHT-based extender without LC supplementation was included. A total of six groups were processed at room temperature and with a final concentration of 200×10^6 sperm/ml. Then, each group was placed into Falcon tube of 15 ml, assessed its sperm quality (see below), and holding at 5 °C in refrigerator up to 96 h.

Analysis of sperm quality parameters

Sperm kinematics

The motility analysis was objectively assessed using a CASA system (Sperm Class Analyzer, SCA® 1999, v.4.0, software. Microptic S.L., Barcelona, Spain) coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast). The sperm samples of 5-µl were placed over slide warmed at 37 °C and covered by a coverslip. A minimum of three fields and 200 sperm tracks were evaluated at 100× for each sample chamber (image acquisition rate 25 frames/s). The following sperm kinetic variables were assessed, as previously described by Galarza et al. (2018): percentage total motile sperm

(SM, %), percentage progressive sperm (PSM, %), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, μm).

Status of sperm membranes

Plasma, acrosome and mitochondrial membrane status were assessed using a triple association of fluorescent probes - propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381), and Mitotracker Green FM® (MITO, Invitrogen M7514) - according to Galarza et al. (2019). A total of 200 sperm cells per slide were examined and eight subpopulations of cells quantified, i.e., those showing in percentages: (1) intact plasma membrane / intact acrosome / intact mitochondrial membrane (IPIAIM); (2) intact plasma membrane / intact acrosome / damaged mitochondrial membrane (IPIADM); (3) intact plasma membrane / damaged acrosome / damaged mitochondrial membrane (IPDAIM); (4) intact plasma membrane / damaged acrosome / damaged mitochondrial membrane (IPDADM); (5) damaged plasma membrane / intact acrosome / intact mitochondrial membrane (DPIAIM); (6) damaged plasma membrane / intact acrosome / damaged mitochondrial membrane (DPIADM); (7) damaged plasma membrane / damaged acrosome / intact mitochondrial membrane (DPDAIM); and (8) damaged plasma membrane / damaged acrosome / damaged mitochondrial membrane (DPDADM).

In vivo fertility

The fertility test was performed at Center of Selection And Genetic Improvement of Sheep and Goats (Ovigen), located in Toro, Spain (Zamora, 41º 29’42.6” N, 5º33’5.2”

W). Before starting the experiment, the fertility of fresh ram semen diluted with either INRA-96® or LC5-extenders was assessed, without determining significant differences (data not shown). The fertility of liquid ram semen diluted with UHT supplemented with 5 mM LC, using either fresh-extended stored at 15 °C (LC5-fresh) or cold-stored for 24 h at 5 °C (LC5-24h) was assessed. For this purpose, semen ejaculates from five Assaf rams were collected in two consecutive times, at 24 hr and at 6 hr before AI, repeated in three sessions. The semen ejaculates were assessed in its initial sperm quality (volume, concentration and motility mass of 1–5) and then diluted with UHT-based extender supplemented with 5 mM of LC at 28 °C to reach final concentration of 400×10^6 sperm/ewe. The semen samples collected 24 h before AI were cooled up to 5 °C (at cooling rate of 0.3 °C/min) and then stored at 5 °C; and the semen collected the next day were cooled up to 15ºC (at cooling rate of 0.3 °C /min). Both LC5-fresh and LC5-24h semen types where then loaded into 0.25 ml French straws (IMV, L'Aigle, France) pre-cooled at 15°C or 5°C, respectively. Multiparous ewes of Assaf breed in three flocks (A, B, C) were cervically inseminated to a synchronized estrus with LC5-fresh (A, n = 27; B, n = 25; and C, n = 25) and LC5-24h (A, n = 21; B, n = 24; and C, n = 25) semen types. Synchronization was achieved via insertion of an intravaginal FGA sponge (Chronogest® CR, Intervet, Boxmeer, The Netherlands, 20 mg) for 14 days followed by eCG (Follogin®, Intervet, Boxmeer, The Netherlands, 480 IU i.m.) at pessary removal. Ewes were cervically inseminated 53–54 h after pessary removal by using an universal insemination syringe (Ref: 007071, IMV Technology, France). Ultrasonography pregnancy diagnosis in ewes was applied by transabdominal way using the convex (sectorial) probe at 35 days post AI. It was considered as pregnant the ewe containing amniotic sac at ultrasound examination.

Statistics analysis

The results are presented as mean ± SEM. All statistical analyses were performed using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by Shapiro-Wilk test, were transformed to *arcsine* (percentages values) or *log10* (numeric values) before analysis. The effects of six LC concentrations and cold storage time on in vitro sperm quality were compared by one-way ANOVA and Bonferroni´s multiple range test, using the General Linear Model procedure. Furthermore, the fertility rate of both LC5-fresh and LC5-24h ram semen groups was analyzed by Chi-square test. Significance was set at $P < 0.05$.

RESULTS

Differences in sperm motility variables between experimental groups of LC, and between cold storage times are depicted in Fig. 1. Data for status of sperm membranes are included in Table 1. Data of pregnancy rate either fresh semen or cold-stored for 24 h are depicted in Fig. 2.

Overall, the results showed that kinetic variables such as values of PSM, VCL, VSL, and the percentage of sperm with intact plasma, acrosome and mitochondrial membranes (IPIAIM) decreased ($P < 0.05$) as cold storage time increased in all experimental groups.

The results revealed a higher ($P < 0.01$) percentage of SM in all LC groups than control group at 48 hr. However, at 96 h, both LC5 and LC10 groups showed a SM higher ($P < 0.001$) than both LC7.5 and control groups. The percentage of PSM was higher ($p < 0.05$) only with LC5 group than control group at 48 and 96 h. Moreover, the values of both VCL and VSL were improved with all LC groups compared with control group at 0h (P

< 0.01), 48 h (P < 0.001) and 96 h (P < 0.001). In addition, at 96 h, the value of VSL resulted higher with LC7.5 group than all LC groups (P < 0.05) and control (P < 0.001). Likewise, at 96 h of cold storage, the value of ALH resulted lower (P < 0.05) with both LC5 and LC7.5 groups than other groups.

After 48 and 96 h of cold storage ram semen, the percentage IPIAIM was greater (P < 0.001) with all LC groups than control group. Lower percentages of IPDAIM were observed after cold storage at 96 h with LC10 group than other LC groups (P < 0.01) and control group (P < 0.001). Likewise, lower (P < 0.05) percentages of DPIAIM were observed after 48 and 96 h of cold storage with both LC7.5 and LC10 groups than control group.

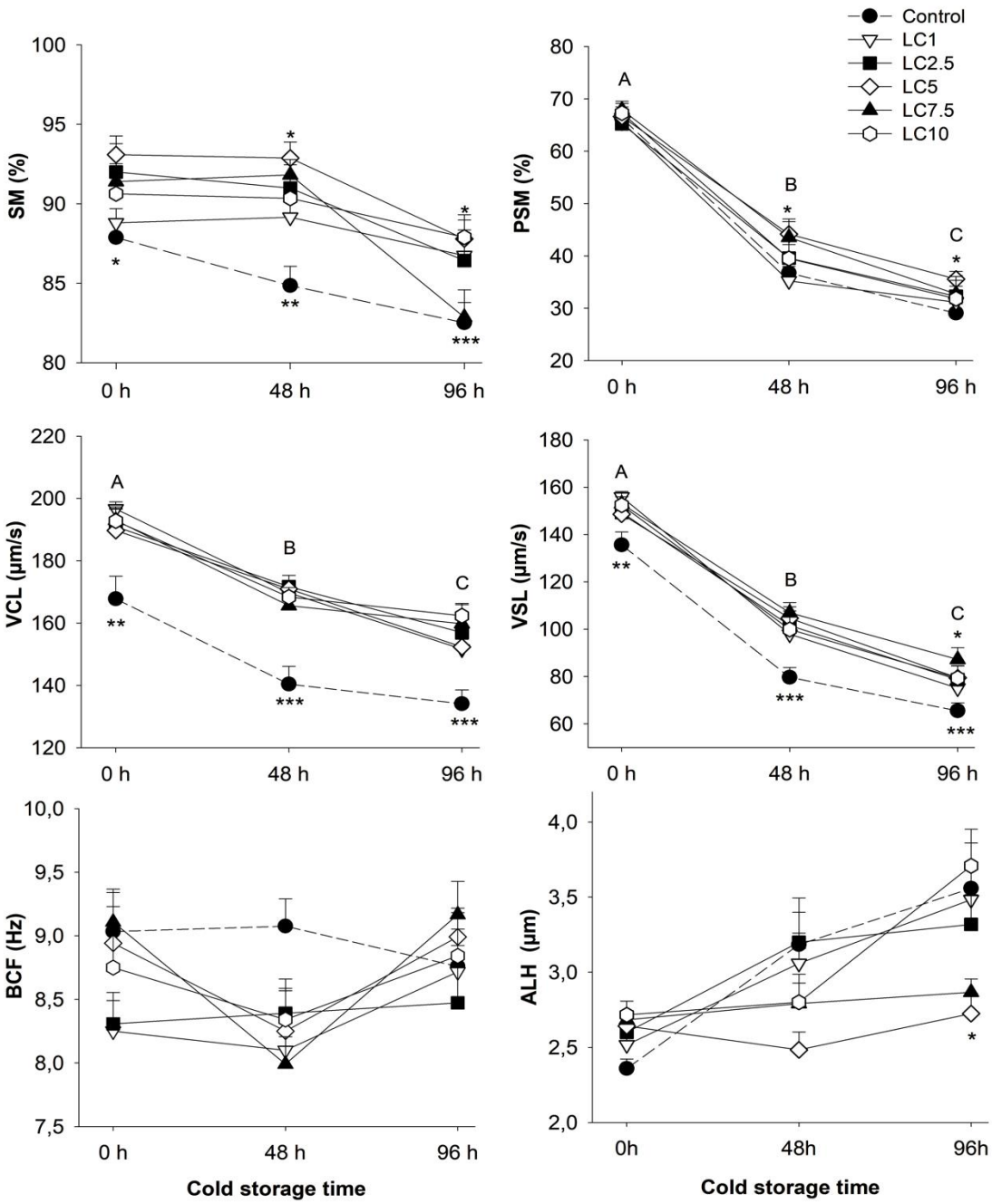

Fig. 1. Kinetic variables of CASA system of cold-stored ram semen, diluted with UHT skimmed milk-based extender supplemented either 0 (control), 1 (LC1), 2.5 (LC2.5), 5 (LC5), 7.5 (LC7.5), and 10 mM (LC19) of LC and cold-stored at 5 °C up to 96 h. SM (%), sperm motility; PSM (%), progressive sperm motility; VCL (µm/s), curvilinear velocity; VSL (µm/s), straight line velocity; BCF (Hz), beat-cross frequency; and ALH (µm), amplitude of lateral head displacement. Significant reductions between evaluation-time for all semen groups. ($P < 0.05$ for $^{A–B}$, and $^{B–C}$; and $P < 0.01$ for $^{A–C}$). * Significant differences between different semen groups at different evaluation-times (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 1. Percentages of sperm (mean ± SEM) in the various categories of membrane integrity (assessed with fluorescent markers *PI / PNA-FITC / MITO*) in ram semen diluted with UHT-based extender supplemented with different LC concentrations under chilled conditions.

Fluorescence parameters	Time (h)	Control	L-carnitine groups				
			LC1	LC2.5	LC5	LC7.5	LC10
IPIAIM (%)	0	74.8 ± 2.7 ^{A}	80.5 ± 1.4 ^{A}	82.1 ± 2.0 ^{A}	83.2 ± 1.4 ^{A}	82.8 ± 1.6 ^{A}	84.0 ± 1.4 ^{A}
	48	58.8 ± 3.2 $^{d\,B}$	69.3 ± 2.0 $^{a\,B}$	70.5 ± 2.0 $^{a\,B}$	73.8 ± 1.3 $^{a\,B}$	74.0 ± 1.4 $^{a\,B}$	73.3 ± 1.7 $^{a\,B}$
	96	49.2 ± 3.0 $^{d\,C}$	62.3 ± 2.0 $^{a\,C}$	63.3 ± 1.7 $^{a\,C}$	63.3 ± 2.8$^{a\,C}$	66.5 ± 2.4 $^{a\,C}$	66.3 ± 1.9 $^{a\,C}$
IPDAIM (%)	0	0.7 ± 0.2 ^{A}	0.5 ± 0.3	0	0.2 ± 0.1	0.3 ± 0.2	0.8 ± 0.4
	48	2,7 ± 0.7 ^{AB}	1.4 ± 0.4	1.9 ± 0.6 ^{A}	1.3 ± 0.5	1.3 ± 0.5	1.1 ± 0.4
	96	4.3 ± 1.0 $^{a\,B}$	1.7 ± 0.4 ^{c}	2.5 ± 0.7 $^{ac\,B}$	2.1 ± 0.5 ^{ac}	1.6 ± 0.5 ^{c}	0.9 ± 0.4 ^{d}
DPIAIM (%)	0	10.8 ± 1.8 ^{A}	9.5 ± 0.7 ^{A}	10.0 ± 1.2	9.8 ± 1.1 ^{A}	10.2 ± 0.9	8.8 ± 0.7
	48	18.3 ± 2.2 $^{a\,AB}$	13.8 ± 1.2 $^{ab\,AB}$	12.8 ± 1.1 ^{ab}	12.2 ± 1.0 $^{ab\,AB}$	11.9 ± 0.8 ^{b}	12.5 ± 1.2 ^{b}
	96	20.0 ± 1.7 $^{a\,C}$	16.7 ± 1.2 $^{ab\,B}$	15.0 ± 1.0 ^{ab}	16.9 ± 1.3 $^{ab\,B}$	13.7 ± 1.2 ^{b}	12.6 ± 1.4 ^{b}
DPIADM (%)	0	4.9 ± 1.3	4.4 ± 1.1	2.9 ± 0.8	2.7 ± 0.6	2.5 ± 0.9	2.3 ± 0.8
	48	6.9 ± 0.9	5.0 ± 1.0	5.3 ± 1.1	4.5 ± 0.9	3.9 ± 0.6	4.3 ± 0.6
	96	9.3 ± 1.0	4.8 ± 1.1	6.6 ± 1.7	6.5 ± 1.2	6.5 ± 1.0	6.4 ± 0.9
DPDAIM (%)	0	8.2 ± 1.4 ^{A}	4.8 ± 0.7 ^{A}	4.7 ± 1.0 ^{A}	4.3 ± 0.8 ^{A}	3.8 ± 0.6 ^{A}	4.2 ± 0.8 ^{A}
	48	12.0 ± 0.9 ^{AB}	10.0 ± 1.1 ^{AB}	8.8 ± 1.1 ^{AB}	7.2 ± 1.2 ^{AB}	9.5 ± 0.9 ^{AB}	8.3 ± 0.8 ^{AB}
	96	15.7 ± 1.9 ^{B}	14.2 ± 1.5 ^{B}	11.9 ± 1.6 ^{B}	11.0 ± 1.8 ^{B}	11.3 ± 1.8 ^{B}	12.6 ± 1.3 ^{B}
DPDADM (%)	0	0.6 ± 0.4	0.3 ± 0.2	0.3 ± 0.3	0	0.5 ± 0.3	0
	48	1.3 ± 0.6	0.4 ± 0.3	0.7 ± 0.4	0.9 ± 0.6	0.1 ± 0.1	0.5 ± 0.3
	96	1.1 ± 0.6	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	1.1 ± 0.5

Different superscripts within a same row differ significantly between experimental groups ($P < .05$ for $^{a–b}$, and $P < .01$ for $^{a–d}$). Different superscript within a same column and fluorescence parameter differ significantly between cold storage time ($P < 0.05$ for $^{A–B}$, and $P < 0.001$ for $^{A–C}$). IPIAIM: intact plasma membrane / intact acrosome / intact mitochondrial membrane; IPDAIM: intact plasma membrane / damage acrosome / intact mitochondrial membrane; DPIAIM: damage plasma membrane / intact acrosome / intact mitochondrial membrane; DPIADM: damaged plasma membrane / intact acrosome / damaged mitochondrial membrane; DPDAIM: damaged plasma membrane / damage acrosome / intact mitochondrial membrane ; DPDADM: damaged plasma membrane / damaged acrosome / damaged mitochondrial membrane.

The results of pregnancy rate indicated that flock A, there was no significant difference ($P > 0.05$) between LC5-fresh and LC5-24h semen (52.4% vs 42.8%) after cervical AI of ewes. Nevertheless, the pregnancy rate in flock B ($P < 0.001$) and flock C ($P < 0.01$) decreased after ewe insemination with LC5-24h semen compared with LC5-fresh semen (12.5% vs 56.0%, and 20.0 vs 52.0% respectively).

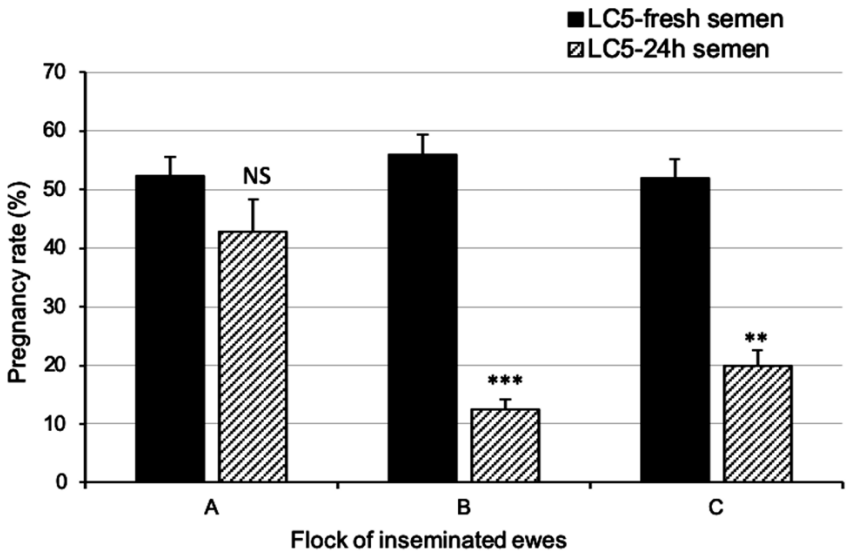

Fig. 2. Pregnancy rate of ram semen diluted with UHT-LC5 extender either fresh (LC5-fresh) or cold-stored for 24 h on three flocks of ewe inseminated cervically. Significant differences between different semen groups (**$P < 0.01$; ***$P < 0.001$). NS, no significant differences ($P > 0.05$).

DISCUSSION

The results of the current study indicated that LC supplementation to UHT (with 6% egg yolk) diluent, would be able to protect ram spermatozoa from deleterious effects of LPO, and enhance motility with improved kinetics during low-temperature liquid storage. In addition, UHT-based extender supplemented with 5 mM LC showed that despite the pregnancy rate did no decreased after storing the semen for 24 h at 5 °C with regard to fresh semen in an only flock (A), in others flocks (B and C) of ewes the fertility decreased. Anyway, pregnancy rates with fresh semen were high after cervical FTAI. To our best

knowledge, the present study is the first report on the influence of LC supplementation to liquid-stored ram semen under chilled conditions on sperm quality parameters and fertility.

Previous reports have revealed that ram semen supplemented with antioxidants such as CAT, GSH, taurine, trehalose, quercetin, palmitoleate, and, butylated hydroxytoluene, kinetin, and idebenone, enhances motility characteristics and integrity of sperm membranes (plasma and acrosome) when stored as liquid at 4–5 °C (Banday et al., 2017; Bucak and Tekin, 2007; Câmara et al., 2011a; Eslami et al., 2017; Eslami et al., 2019; Rather et al., 2016). The results of the present study indicated that LC at 1–10 mM enhanced motility, velocities, and protected the integrity of sperm membranes (IPIAIM) of ram semen stored as liquid at 5°C for 48 and 96 h. Moreover, more desirable values of PSM yielded with 5mM of LC than control group. Surprisingly both velocities were improved with all LC concentrations at all times. Progressive sperm motility, and velocities (VCL and VSL) and the proportion of large and long spermatozoa assessed by CASA system has previously been identified as the most consistent indicators of fertility (Yániz et al., 2015). In addition, the ALH is an important motility variable, and adequate values are required if barriers, such as that imposed by cervical mucus, are to be overcome, and the oocyte barriers to fertilization (e.g., the cumulus cell layers and the zona pellucida) are to be penetrated (Aitken et al., 1985). Previous reports suggested that lower values of ALH are compatible with effective progressive movement of cold-stored ram sperm (Galarza et al., 2018; 2019). Indeed, the ALH increases greatly in hyperactive sperm undergoing capacitation (Santiago-Moreno et al., 2017). The ALH values of current study are in agreement with the studies previously cited showing lower ALH with both LC5 and LC7.5 compared with other LC groups and control at 96 h.

The LC supplementation to synthetic-based extenders for sperm cryopreservation enhanced post-thawing sperm motility and membranes integrity (plasma and acrosome) in human (Banihani et al., 2014) and ruminant (bull, buffalo and goat) (Bucak et al., 2010; Bucak et al., 2010; El-Raey et al., 2016; Hufana-Duran et al., 2017). In addition, this motility enhancer effect has been demonstrated in mice sperm with Ham's F10 medium supplemented with LC compared with Pentoxifylline (Aliabadi et al., 2012). Gibb et al. (2015) has obtained a better total and progressive motilities in stallion sperm maintained in skimmed milk extender supplemented with pyruvate and LC after 72 h stored at 20 °C. The data presented by this research also confirm previous studies. Conversely, there is only a first recent report, nevertheless, that has demonstrated LC supplementation to Tris-based extenders (Tris or optiXcell™) had no consistently beneficial effect on freezing-thawed ram sperm (de Souza et al., 2019).

During liquid storage of semen, LPO occurs and increases in a time dependency manner (Eslami et al., 2017). High production of ROS increases the sensitivity of the sperm, resulting in damage to the sperm structure, morphology, and function – accompanied by decreased sperm motility, viability and fertilizing ability (Bucak et al., 2010). Free radicals oxygen species into base-diluents can disturb sperm motility via interruption with ATP production or flagellar axoneme phosphorylation (Aitken & Baker, 2006), thereby, mitochondrial energy availability decrease (Lamirande and Gagnon, 1992). Then, LC supplementation enhanced locomotion sperm, due to its function as energy supplier and being a facilitator for activated fatty acid transport into the mitochondrial matrix for β-oxidation (Agarwal and Said, 2004). Furthermore, LC, as antioxidant, may protect sperm plasma membrane with high level of unsaturated fatty acid content (Aitken and Clarkson, 1987; Aliabadi et al., 2012). Antioxidant properties of LC have previously been tested against cold-shock and ROS production in

cryopreserved sperm. This occurs as a result of a repairing mechanism by which elevated intracellular toxic acetyl-coenzyme A (A-CoA) is removed and/or fatty acids in membrane phospholipids are replaced (Kalaiselvi and Panneerselvam, 1998; Vicari and Calogero, 2001). We believe that the interaction of skim milk-egg yolk-LC can be supporting mitochondrial ATP production while minimizing both ATP depletion and the damaging effects of metabolic by products such as free radicals. Therefore, *in vitro* results of the present study suggest that supplementation with LC to UHT-based extender provides a protective effect against cold-shock due to that preserve better the integrity and functionality of sperm membranes.

Fertility test after cervical FTAI using fresh liquid ram semen supplemented with LC (LC5-fresh) was 52–56%. Moreover, one flocks of ewe inseminated did not decrease pregnancy rate when using liquid ram cold-stored for 24 h (LC5-24h) compared with fresh semen (42.8% vs 52,4%, respectively). This trend was not observed in the other two flocks despite in a previous study we demonstrated that the in vitro fertility of ram semen diluted in UHT-extender and cold-stored up to 48 h, did not diminish (43.3–48.8%) (Galarza et al., 2019). Current FTAI protocols in sheep in many European countries include the use of liquid ram semen diluted with INRA-96® extender (egg yolk free) at high concentration (400×10^6 sperm/ewe) and maintained at 15 °C until the insemination moment (1–6 h). The FTAI in a context of hormone-induced estrus synchronization allows the procedure to be performed at the desired moments (Olivera-Muzante et al., 2011). Nevertheless, the effect of seasonality and FTAI with previous treatment of estrus-induction and synchronization can have negative effects on fertility (Anel et al., 2006). Hence, the pregnancy rate in ewe inseminated via cervically under these conditions reaches 40% in the best case under very controlled conditions. Indeed, the pregnancy rate prior to the experiment on the farm was 38–40% using INRA-96® extender under same

conditions that current AI protocols of sheep. In a report presented in a meeting of Ovigen (center of AI of sheep and goats), it indicated that the average fertility of Assaf sheep in Spain was 37% in 2017 and 35% in 2018 (Mantecón, 2019). A recent study reported that, using ram semen diluted with skim milk, the pregnancy rate was 36.0, 42.3 and 50.2% in flocks of ewe after either cervical, transcervical, and intrauterine FTIA, respectively (Casali et al., 2017). Therefore, according to results obtained in the present study with fresh semen and despite that fertility of cold-stored semen for 24 h was variable among flocks, LC supplemented to UHT-extender seems to have a positive effect on fertility after cervical AI in sheep.

CONCLUSION

The supplementation of L-carnitine to UHT skimmed milk diluent allows preserving chilling-stored ram semen for long-term, enhancing motility variables, protecting successfully plasma, acrosome, and mitochondrial membranes. Also, the fresh ram semen supplemented with LC indicated a satisfactory fertility response, nevertheless, the controversial results of this semen preserved at 5 ºC for 24 h indicated that fertility must be more investigated. Therefore, the use of L-carnitine can be a promising alternative in sheep breeding programs using cervical FTAI with chilled sperm.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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CAPÍTULO 4

Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols

Diego A. Galarza*, Antonio López-Sebastián, Henri Woelders, Elizabeth Blesbois, Julián Santiago-Moreno. **Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols.** 2019 (Article in press). Research article accepted in Cryobiology journal. https://doi.org/10.1016/j.cryobiol.2019.10.007. Artículo original.

Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols

RESUMEN

El presente estudio compara un protocolo que imita la congelación del semen de morueco en vapores de nitrógeno líquido estático con dos protocolos que incluyen una velocidad de enfriamiento inicial baja en la primera fase, seguida de velocidades de enfriamiento más altas durante el período de la nucleación del hielo. Los eyaculados de semen, obtenidos de doce carneros adultos, se diluyeron con un diluyente TEST y se congelaron con el Protocolo 1 (enfriamiento por desaceleración de tres pasos): de +5 °C a –35 °C (40 °C/min), de –35 °C a –65 °C (17 °C/min), y luego de –65 °C a –85 °C (3 °C/min); Protocolo 2 (enfriamiento acelerado de tres pasos): de +5 °C a –5 °C (4 °C/min), de –5 °C a –110 °C (25 °C/min), y luego de –110 °C a –140 °C (35 °C/min); o Protocolo 3 (enfriamiento acelerado en dos pasos), de +5 °C a –10 °C (5 °C/min), y luego de –10 °C a –130 °C (60 °C/min). La calidad del esperma después de la descongelación se redujo en todos los protocolos ($P < 0,05$) en comparación con el semen fresco. Los porcentajes las variables de motilidad y de los espermatozoides con membrana plasmática intacta, acrosoma intacto y membrana mitocondrial intacta fueron mayores utilizando el Protocolo 3 que el Protocolo 2 ($P < 0,05$) y el Protocolo 1 ($P < 0,01$) después de la descongelación. Además, el porcentaje de espermatozoides con ADN fragmentado después de la descongelación fue menor ($P < 0,05$) al utilizar el Protocolo 3 en comparación con el Protocolo 1. Los resultados actuales indican que una velocidad de enfriamiento de 60 °C/min alrededor y después del punto de tiempo de la nucleación de hielo proporcionó una mejor supervivencia y funcionalidad del esperma de morueco

después de la descongelación que las velocidades de enfriamiento más bajas(y/o desaceleradas).

***Palabras clave**:* Morueco, criopreservación de semen, enfriamiento, calidad seminal, Integridad de ADN.

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Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols

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ABSTRACT

The present study compares a protocol that mimics freezing of ram semen in static nitrogen vapor with two protocols with an initial low cooling rate in the first step, followed by higher cooling rates where ice nucleation occurs. Semen ejaculates, obtained from twelve adults rams, were diluted with TEST-based extender and frozen with either Protocol 1 (three-step decelerating cooling): from +5 °C to −35 °C (40 °C/min), from −35 °C to −65 °C (17 °C/min), and then from −65 °C to −85 °C (3 °C/min); or Protocol 2 (three-step accelerating cooling): from +5 °C to −5 °C (4 °C/min), from −5 °C to −110 °C (25 °C/min), and then from −110 °C to −140 °C (35 °C/min); or Protocol 3 (two-step accelerating cooling), from +5 °C to −10 °C (5 °C/min), and then from −10 °C to −130 °C (60 °C/min). Post-thaw sperm quality was reduced for all protocols ($p < .05$) compared with fresh semen. Post-thaw percentages of sperm motility characteristics and sperm with intact plasma membrane, intact acrosome, and intact mitochondrial membrane were greater using Protocol 3 than Protocol 2 ($p < .05$) and Protocol 1 ($p < .01$). In addition, the post-thaw percentage of sperm with fragmented DNA was lower ($p < .05$) using Protocol 3 compared with Protocol 1. The present results indicate that a cooling rate of 60 °C/min around and after the time point of ice nucleation provided better post thaw survival and function of ram sperm than lower (and/or decelerating) cooling rates.

1. Introduction

Cryopreservation of semen is used in order to facilitate long-term storage and transportation. This is of special importance for use in artificial insemination of domestic animals, including sheep. Ram sperm cells are susceptible to various stresses during cryopreservation [1], and ultrastructural, biochemical and functional damage occurs in many ram sperm cells during the freezing-thawing process [28]. Usually no more than 50% of sperm cells survive cryopreservation, providing low fertility rates [39,40]. In addition to cell death, the remaining survived sperm cells may have damaged sperm organelles and membranes and the biological efficiency reduced (e.g., sperm capacitation and acrosome reaction) [4]. Cryoinjury during freeze-thawing, can be caused by factors such as thermal shock, ice formation, dehydration, increased salt concentration and osmotic shock [29,42].

Studies on freezing methods for ram semen have considered factors such as the base-diluents [22,24,36], methods of addition and concentration of cryoprotectant agents [10,21,40], and freezing rates [3,8,11,26] to achieve high quality sperm after thawing [35].

The cooling rate is important for sperm survival. As extracellular ice formation takes place, the cells and the dissolved salts are excluded from the ice and become concentrated in the 'unfrozen fraction' remaining between the growing ice masses. Therefore, the osmotic strength of the unfrozen fraction increases, causing an efflux of water from the cells, resulting in cell shrinkage. Both high and low cooling rates are detrimental to the cell [30]. Higher cooling rate induces intracellular ice formation [23,31,44], while too low cooling rate may cause excessive dehydration of the cell and the cells membranes are exposed for longer

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periods to the pockets of hypertonic solutions, with possible deleterious effects such as protein and lipid extraction and the generation of reactive oxygen species (ROS) [25]. The velocity at which ice forms during sperm cryopreservation is strongly dependent on the freezing protocol. Extracellular ice or changes in the composition of the external solution brought about by the conversion of water into the ice are believed to be a cause of sperm damage during the cryopreservation process [29]. Also, the size and shape of ice crystals and ice masses depend on the cooling rate [5]. Thus, the optimal cooling rate has to be regarded as a compromise between these opposing effects mentioned above to achieve minimal cryoinjury and maximize viability and fertility of sperm samples after thawing. Fiser and Fairfull (1984) [17] reported a comprehensive study on the effect of cooling rates and the interaction of cooling rate and glycerol concentration on survival and intactness of ram spermatozoa. More recent studies on the optimal cooling rate for ram semen were reviewed by Dalal et al. (2018) [9]. However, these studies have not addressed different cooling rates in the separate phases of the freezing program, most notably the phases prior to and after ice nucleation.

The cooling rate inside the sample that is frozen is not constant due to the release of latent heat of fusion. Thus, the freezing rate inside the straw may be different than expected from the programmable biological freezer, due to the heat generated by ice formation. Indeed, the cooling rate right after the dissipation of heat of fusion may be much higher than the programmed cooling rate [43].

In static liquid nitrogen (LN_2) vapor freezing methods, quite high cooling rate in the freezing phase after ice nucleation can be perfectly obtained, if required, by choosing a small distance between the straws and the surface of LN_2 [12]. However, the cooling rate of the cooling phase from the holding temperature (+5 °C) until ice nucleation (<−20 °C) will then also be high, which could cause damage due to 'cold shock'. Indeed, traditional freezing of ram semen in static LN_2 vapor provides a relatively high initial cooling rate prior to ice nucleation, while the cooling rate after ice nucleation decreases at lower temperatures [2,15]. Previous reports suggested that the use of static LN_2 vapors to freezing ram sperm was more harmful to sperm integrity and functionality than controlled freezing methods with the low initial cooling rate (e.g., 0.5–5 °C/min) in the temperature range where the ice nucleation occurs [3,38]. In contrast, many protocols provide a low initial cooling rate in the phase prior to ice nucleation (e.g., Refs. [15,32]), which may help prevent cold shock. The study by Esteso et al. (2018) [15] indeed showed better post thaw sperm quality of Iberian ibex semen frozen with a controlled rate freezer with a low cooling rate between +5 an −5 °C, followed by a higher cooling rate, than semen frozen in static LN_2 vapor at similar overall cooling rates.

Therefore, the aim of the present study was to compare three different freezing protocols in a controlled-rate freezer, of which Protocol 1 mimics the static LN_2 vapor freezing method [15], featuring a relatively high cooling rate in the phase prior to ice nucleation. In contrast, protocols 2 and 3 had a low cooling rate in the first cooling phase, in order to minimize cold chock, followed by higher programmed cooling rates in the temperature-range in which ice nucleation and ice growth occur (−25 and −60 °C/min in Protocols 2 and 3, respectively).

2. Materials and methods

2.1. Animal, semen collection and initial evaluation

Semen was collected over October to November from 4 to 6 year-old twelve adult Merino ram males housed at INIA Department of Animal Reproduction (Madrid, 40° 25′N). All animals were fed with grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available ad libitum. All animals were handled according to procedures approved by the INIA Ethics Committee, and the research was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

A total of ninety-eight semen ejaculates were collected in nine collection session carried out weekly using an artificial vagina. The rams were previously trained with an intact oestrus-induced ewe and then an ovariectomized female was used as a "teaser" to stimulate ram sexual behaviors. The volume of each ejaculate was measured in a graduated conical glass tube in milliliters (mL). Briefly, after collection, the raw semen was diluted 1:1 (v/v) with Tris/Tes/Glucose (TTG) solution (210.6 mM Tes, 95.75 mM Tris, 10.09 mM Glucose, 0.54 mM Streptomycin, and 2.14 mM Penicillin; 324 mOsm/kg, pH 7.1) pre-warmed at 37 °C. All the materials, including the artificial vagina and glass collecting tube were maintained at 37 °C before collections. These fresh-extended semen samples with TTG solution were transported to the research laboratory immediately after collection at 37 °C and initial motility and status of sperm membranes were evaluated prior to processing (see below). Sperm concentration was determined using a photometer (SDM 1, Minitube, Germany). Those ejaculates with a volume of 0.75–2 mL, a sperm motility higher than 70%, a score higher than 3 (mass motility scale of 0–5), and a sperm concentration higher than 3.5×10^9 sperm/mL were used in the subsequent experimental work.

2.2. Processing and freezing procedures

The fresh-extended semen samples of each semen ejaculate were mixed with TEST-based extender [freezing medium composed of TTG solution, 6% egg yolk (v/v), and 5% glycerol (v/v)] at room temperature, to reach final concentration of 100×10^6 sperm/mL (4.8% glycerol final concentration). Semen samples were then placed in a beaker with 30 mL of water at room temperature, and maintained at this temperature for 5 min before transfer to a refrigerator at 5 °C where they were maintained for a further 3 h (1 h of cooling time plus a further 2 h of holding time). Aliquots of these samples where then loaded into 0.25 mL French straws (IMV, L'Aigle, France). All straws were filled with a volume of approximately 220 μL and a small air bubble of approximately 10 μL inside and finally sealed. Thereafter, the straws were frozen using a programmable biological freezer (Computer Freezer-IceCube 1810, Minitüb, Tiefenbach, Germany). A total of 236 straws were frozen using the following three freezing protocols (Fig. 1):

Protocol 1 (n = 78 straws from 36 semen ejaculates of 12 males): Three-step decelerating cooling rate (similar to conventional freezing in static LN_2 vapor [15]): from +5 °C to −35 °C at 40 °C/min, from −35 °C to −65 °C at 17 °C/min, from −65 °C to −85 °C at 3 °C/min, and then transfer into LN_2 to cool to −196 °C.

Protocol 2 (n = 79 straws, from 31 semen ejaculates of 12 males): Three-step accelerating cooling rate: from +5 °C to −5 °C at 4 °C/min, from −5 °C to −110 °C at 25 °C/min, from −110 °C to −140 °C at 35 °C/min, and then transfer into LN_2 to cool to −196 °C.

Protocol 3 (n = 79 straws, from 31 semen ejaculates of 12 males): Two-step accelerating cooling rate: from +5 °C to −10 °C at 5 °C/min, from −10 °C to −130 °C at 60 °C/min, and then transfer into LN_2 to cool to −196 °C.

The temperatures of the freezing chamber (T_{ch}) and inside the straws (T_{in}) were assessed. For the latter purpose, the thermocouple (1.5 mm diameter) of the biological freezer (temperature sensor of sample) was introduced to a dummy straw (1.6 mm diameter, IMV, France) containing freezing medium (TEST). When the temperature inside the straws approached the fixed temperature of the freezing chamber (5 °C), each protocol was started (time 'zero'). Consequently, the thermocouple inside the dummy straw allows to register the occurrence of ice nucleation and to roughly estimate the duration of the subsequent dissipation of the latent heat of fusion from each freezing protocol (Fig. 2).

Both straws freezing process and semen collection session procedures from each Protocol were carried out weekly. All frozen sperm samples were thawed after three months by placing the straws in a water bath at 37 °C for 30 s. The contents were poured into dry Eppendorf tubes and

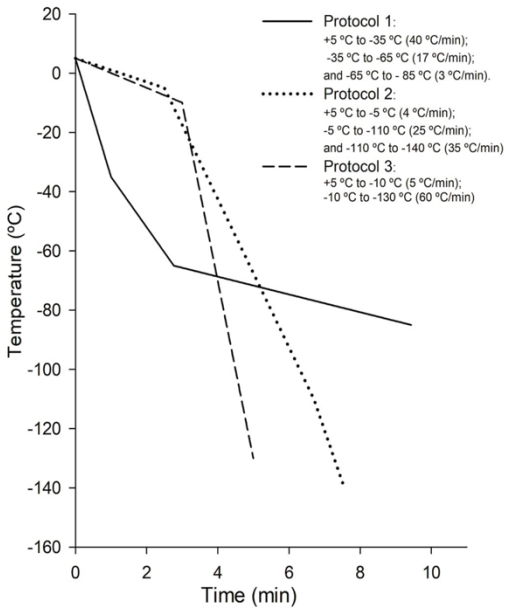

Fig. 1. Three programmable freezing protocols for the cryopreservation of ram semen using two- or three-step controlled cooling rates.

incubated for 5 min at 37 °C. Sperm motility, status of sperm membranes and DNA fragmentation were subsequently evaluated.

2.3. Sperm analysis

Sperm motility analysis was assessed using a CASA system running Sperm Class Analyzer® 1999 v.4.0 software (Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) as previously described by Galarza et al. (2018) [19]. Values were recorded for the percentage of sperm motility (SM), percentage of progressive sperm motility (PSM), straight line velocity (VSL, μm/s), linearity (%LIN), straightness (%STR), and the amplitude of lateral head displacement (ALH, μm).

Plasma, acrosome and mitochondrial membrane status were assessed using a triple association of fluorescent probes - propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut *(Arachis hypogaea)* agglutinin (PNA-FITC, Sigma L7381), and Mitotracker Green *FM*® (MITO, Invitrogen M7514) - according to Galarza et al. (2019) [20]. A total of 200 sperm cells per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450–490 nm, and emission: 520 nm) and eight subpopulations of cells quantified, i.e., those showing in percentages: (1) intact plasma membrane/intact acrosome/intact mitochondrial membrane (IPIAIM); (2) intact plasma membrane/intact acrosome/damaged mitochondrial membrane (IPIADM); (3) intact plasma membrane/damaged acrosome/intact mitochondrial membrane (IPDAIM); (4) intact plasma membrane/damaged acrosome/damaged mitochondrial membrane (IPDADM); (5) damaged plasma membrane/intact acrosome/intact mitochondrial membrane (DPIAIM); (6) damaged plasma membrane/intact acrosome/damaged mitochondrial membrane (DPIADM); (7) damaged plasma membrane/damaged acrosome/intact mitochondrial membrane (DPDAIM); and (8) damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane (DPDADM). In addition, the total percentage of cells presenting an intact plasma membrane (IPM: IPIAIM + IPIADM + IPDAIM + IPDADM), intact acrosomal membrane (IAM: IPIAIM + IPIADM + DPIAIM + DPIADM), and intact mitochondrial membrane (IMM: IPIAIM + IPDAIM + DPIAIM + DPDAIM) were calculated.

DNA fragmentation of frozen-thawed ram sperm was assessed by TUNEL assay. For this purpose, we used the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with few modifications. In brief, after thawing semen samples were fixed in 4% formaldehyde (v/v) in PBS solution at a

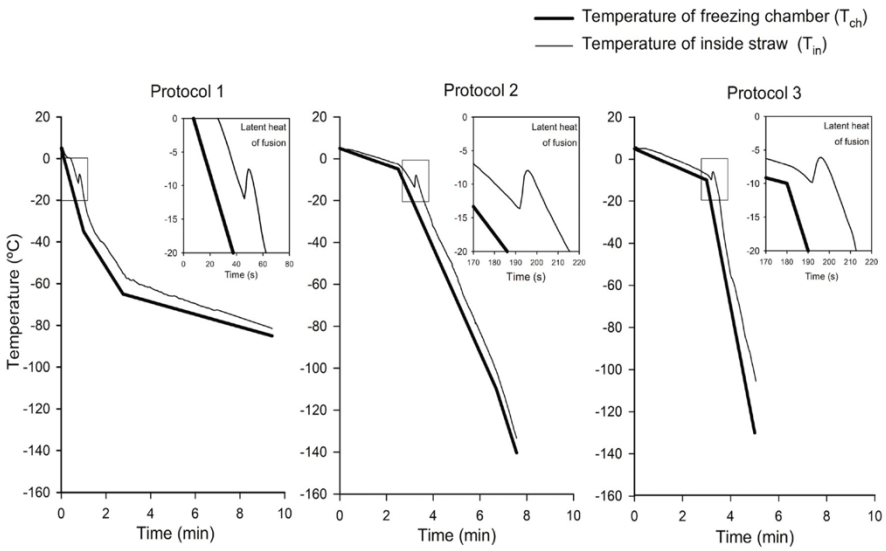

Fig. 2. Time courses of programmed temperature in the freezing chamber (T_{ch}, thick line) and typical examples of time courses of measured temperature inside the straws (T_{in}, thin line) in the different freezing protocols. The duration time (s) and temperature at which latent heat of fusion (ice nucleation) occurs inside straws are shown in the small box (thin line) of each freezing protocol.

concentration of 5×10^6 sperm/mL for 30 min. Then 10 μL of each samples were placed on a glass slide previously demarcated by Liquid-repellent slide marked pen for staining procedures and leave to dry on a thermic plate set at 37 °C. The slides were permeabilized with 0.1% Triton X-100 (v/v) (Sigma X100) for 5 min in a humidified chamber at room temperature and washed with PBS. Subsequently, the slides were incubated for 1 h in a humidified chamber in the dark at 37 °C with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus TMR-Red label. After labeling, samples were washed with PBS, stained with Hoechst 33342 (1 mg/mL), mounted with Fluoromount aqueous mounting medium (Sigma F4680) and sealed with nail polish. Then, the samples were analyzed immediately by Nikon Eclipse E200 epifluorescence microscope [40 × magnification, with an excitation: 510–560 nm and emission: 590 nm for TUNEL; and an excitation: 330–380, and emission: 420 nm for Hoechst]. As positive control for TUNEL (TUNEL +), sperm were treated with DNase I, 1 mg/ml, for 60 min at room temperature, fixed on the slides and then treated with TUNEL reaction mixture. In each sample at least 200 sperm were evaluated. Each microscopic field was evaluated first under UV-light to determine the total number of sperm and then under green-light to determine the positive TUNEL sperm.

2.4. Statistical analysis

The results are presented as mean ± SEM. All statistical analyses were performed using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by Shapiro-Wilk test, were transformed to *arcsine* (percentages values) or log*10* (numeric values) before analysis. The effects of cooling rate protocols on post-thawed sperm quality were then compared by one-way ANOVA using the General Linear Model procedure. In addition, the male factor as covariable was included in this analysis due to variability between some rams. When ANOVA revealed a significant effect, the values were compared by Tukey's multiple range test. Significance was set at $p < .05$.

3. Results

Typical examples of temperature time courses of the freezing protocols are showed in Fig. 2. Ice nucleation occurred in the first ramp in Protocol 1 and in the second ramp in Protocols 2 and 3, at registered T_{in} of approximately −10 to −14 °C. The onset of ice nucleation occurred at 47 s (at −11,95 °C in first-step to 40 °C/min), 193 s (at −13.63 °C in second-step to 25 °C/min), and 192 s (at −9,86 °C in second-step to 60 °C/min) for protocols, 1, 2 and 3, respectively. In addition, the apparent durations of the dissipation of heat of fusion (here defined as the time between ice nucleation and return to the same T_{in}) were 8 s, 13 s, and 10 s, for protocols, 1, 2, and 3, respectively.

After freezing and thawing, significant reductions were recorded for all sperm quality variables in frozen, compared with fresh sperm, for all freezing protocols used. Pre-freeze and post-thaw values for the sperm motility variables are shown in Table 1. Both SM and PSM percentages were greater ($p < .05$) after freezing with Protocol 3 than Protocol 1 and 2. Moreover, the VSL value was greater ($p < .05$) after freezing with Protocol 3 than Protocol 1.

Data for status of sperm membranes and DNA fragmentation are included in Table 2 and Fig. 3. The cryopreservation process affected sperm plasma membranes and mitochondrial membranes, but the percentage of sperm affected was lower with Protocol 3 followed by Protocol 2, and Protocol 1 was the most deleterious (Table 2 and Fig. 3). In addition, the percentage of sperm with DNA damage after thawing was lower ($p < .05$) with Protocol 3 than with Protocol 1 (Table 2).

Indeed, after cryopreservation, the percentage of sperm with intact plasma, acrosome and mitochondrial membranes (IPIAIM) was greater using Protocol 3 than Protocol 2 ($p < .05$) and Protocol 1 ($p < .01$). Protocol 2 showed a higher ($p < .05$) percentage of IPIAIM than Protocol

Table 1
Sperm motility variables assessed by CASA, of ram semen before (fresh) and after freezing/thawing using three different freezing protocols.

Sperm parameters	Fresh samples (n = 98)	Protocol 1 (n = 78)	Protocol 2 (n = 79)	Protocol 3 (n = 79)
SM (%)	87.8 ± 0.8^{a}	44.5 ± 1.9^{c}	47.9 ± 2.0^{c}	61.4 ± 1.9^{b}
PSM (%)	34.8 ± 1.0^{a}	18.0 ± 1.0^{c}	20.8 ± 1.1^{c}	27.2 ± 1.0^{b}
VSL (μm/s)	85.1 ± 1.5^{a}	55.1 ± 2.0^{c}	59.2 ± 1.7^{bc}	62.8 ± 2.1^{b}
LIN (%)	51.1 ± 0.7^{b}	63.3 ± 0.8^{a}	63.5 ± 0.8^{a}	63.5 ± 0.7^{a}
STR (%)	66.5 ± 0.7^{b}	75.1 ± 0.7^{a}	76.1 ± 0.6^{a}	75.1 ± 0.5^{a}
ALH (μm)	4.7 ± 0.1^{a}	2.5 ± 0.1^{b}	2.7 ± 0.1^{b}	2.6 ± 0.1^{b}

Data are mean ± SEM. Different superscripts within a same row differ significantly ($p < .05$ for $^{a–b}$, and $^{b–c}$; and $p < .01$ for $^{a–c}$). SM: total sperm motility; PSM: progressive sperm motility; VSL: straight line velocity; LIN: linearity index; STR: straightness; and ALH: amplitude of lateral head displacement.

Table 2
Percentages of sperm in the various categories of membrane integrity (assessed with fluorescent markers PI/PNA-FITC/MITO) and percentage of sperm with fragmented DNA (TUNEL +), in ram semen before (fresh) and after freezing/thawing using three different freezing protocols.

Fluorescence parameters	Fresh samples (n = 98)	Protocol 1 (n = 78)	Protocol 2 (n = 79)	Protocol 3 (n = 79)
IPIAIM (%)	74.0 ± 0.6^{a}	41.7 ± 1.3^{d}	45.8 ± 1.5^{c}	58.4 ± 1.1^{b}
IPIADM (%)	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
IPDAIM (%)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.1 ± 0.2
IPDADM (%)	0.0 ± 0.0	0.04 ± 0.0	0.0 ± 0.0	0.01 ± 0.0
DPIAIM (%)	13.3 ± 0.6^{c}	18.1 ± 0.6^{a}	15.5 ± 0.6^{b}	11.8 ± 0.4^{c}
DPIADM (%)	6.5 ± 0.4^{c}	11.8 ± 0.5^{a}	10.6 ± 0.6^{a}	8.4 ± 0.4^{b}
DPDAIM (%)	3.3 ± 0.3^{c}	22.9 ± 0.9^{a}	22.3 ± 1.1^{a}	16.4 ± 0.8^{b}
DPDADM (%)	0.2 ± 0.1^{b}	3.6 ± 0.4^{a}	4.2 ± 0.5^{a}	3.1 ± 0.3^{a}
TUNEL+ (%)	-	7.1 ± 1.2^{a}	5.4 ± 1.1^{ac}	2.3 ± 0.5^{c}

Data are mean ± SEM. Different superscripts within a same row differ significantly ($p < .05$ for $^{a–b}$, and $^{b–c}$; $p < .01$ for $^{a–c}$; and $p < .001$ for $^{a–d}$). IPIAIM: intact plasma membrane/intact acrosome/intact mitochondrial membrane; IPIADM: intact plasma membrane/intact acrosome/damaged mitochondrial membrane; IPDAIM: intact plasma membrane/damage acrosome/intact mitochondrial membrane; IPDADM: intact plasma membrane/damaged acrosome/damaged mitochondrial membrane; DPIAIM: damage plasma membrane/intact acrosome/intact mitochondrial membrane; DPIADM: damaged plasma membrane/intact acrosome/damaged mitochondrial membrane; DPDAIM: damaged plasma membrane/damage acrosome/intact mitochondrial membrane; DPDADM: damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane.

1. Lower ($p < .05$) percentages of sperm with damaged plasma membranes were observed after freezing with protocol 3 compared with protocols 1 and 2 in each of the following categories: sperm with damaged plasma membrane, intact acrosome and mitochondrial membrane (DPIAIM), sperm with damaged plasma membrane, intact acrosome and damaged mitochondrial membrane (DPIADM), and sperm with damaged plasma and acrosome membranes, and intact mitochondrial membrane (DPDAIM). Moreover, total values of sperm with intact plasma membrane (IPM, $p < .01$), intact acrosome membrane (IAM, $p < .01$) and intact mitochondrial membrane (IMM, $p < .05$) were greater with Protocol 3 than Protocol 2 and 1 (Fig. 3).

4. Discussion

The results of the present study clearly showed that Protocol 3 was less harmful to ram sperm cells than Protocols 1 and 2, yielding higher values for motility variables, integrity of plasma, acrosome and mitochondrial membranes, and a lower percentage of DNA damage.

The Protocol 3 demonstrated the lowest cryoinjury response which may be attributed to the second-step cooling rate (from −10 to −130 °C at 60 °C/min) that could be sufficiently rapid to minimize exposure to the deleterious effects of unfrozen, extracellular, hypertonic solutions

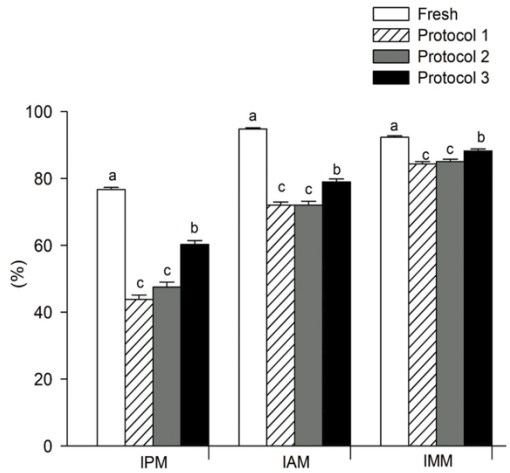

Fig. 3. Percentage of sperm with intact plasma membrane (IPM), intact acrosome (IAM) and intact mitochondrial membrane (IMM) in fresh (n = 98) and in frozen-thawed sperm using three freezing protocols: Protocol 1 (n = 78), Protocol 2 (n = 79), Protocol 3 (n = 79). See main text for details of the three freezing protocols.

during this critical temperature range and ice growth occurs.

Different semen freezing rates have been studied in rams and other ruminant species with variable results. Kumar et al. (2003) [26] recommended a controlled linear cooling rate for ram semen in the temperature range of ice nucleation and ice formation (−5 to −25 °C) of 30 °C/min. Also Fang et al. (2016) [16] and Vichas et al. (2018) [38] found relatively low optimal cooling rates (25–40 °C/min) for ram sperm. However, Fiser and Fairfull (1984) [17] in an elaborate study, as well as Duncan and Watson (1992) [13] found that higher cooling rates were permissible or better for ram semen. Similarly, for bull semen, it was reported that optimal cooling rates used may be as high as 76–140 °C/min [42].

As explained in chapter Introduction, in traditional static LN_2 vapor freezing, cooling rate, after the dissipation of heat of fusion, may decrease already at still relatively high subzero temperatures. This is the opposite of what may be considered optimal, as Woelders and Chaveiro (2004) [43] argued on the basis of mathematical simulations that cooling rates after ice nucleation can and probably should accelerate. In addition to a decelerating cooling rate, static vapor freezing features a rapid cooling in the phase prior to ice nucleation, which may lead to 'cold shock' damage. Indeed, a number of studies have shown that static vapor freezing of ram semen gave poorer results than controlled rate methods [2,3,38]. Our present study confirms these observations, as post-thaw sperm quality appeared lowest in Protocol 1, which protocol had characteristics of freezing in static LN_2 vapor, but with the better control of a controlled rate freezer.

The factor that sets Protocol 3 apart from Protocols 2 and 1 is its relatively high cooling rate (60 °C/min) after the dissipation of heat of fusion until straws were plunged at −130 °C. This shortens the time it takes before the cells are stabilized by reaching the glass transition (but only little compared with Protocol 2). But, perhaps more importantly, cells may become less strongly shrunken and dehydrated. Moreover, even though the total fraction of ice formed only depends on the subzero temperature, the higher cooling rate may also influence forms and shapes of ice crystals and ice masses [5,7] and therewith possibly the mechanical stresses the cells endure [14].

Freezing and thawing can also affect sperm functionality by alteration of DNA integrity [32] and disruption of mitochondrial membrane function [33]. Damage of the mitochondria can cause a decreased oxidative phosphorylation (ATP synthesis) [41], which can affect sperm motility [6] and sperm motility kinetic variables [3]. Previous reports demonstrated a positive correlation between the mitochondrial function and motility of thawed bull sperm, both after freezing in static nitrogen vapor and after a two-step protocol with an accelerating freezing rate [18]. The results of the present study showed that values of motility variables, the percentage of sperm with intact plasma, acrosome and mitochondrial membranes were greater for semen frozen with Protocol 3 than with Protocols 2 and 1. Even sperm subpopulation with damaged plasmalemma and intact acrosome showed the same percentage of sperm with intact mitochondrial membrane than fresh samples when Protocol 3 is used. Moreover, Protocol 3 showed less cryoinjury of sperm with damaged plasmalemma and acrosome, and intact mitochondrial membrane than Protocols 2 and 1. These findings suggest that Protocol 3 produced less damage to mitochondrial membranes allowing better motility than the other protocols.

Cryopreservation increases apoptosis-like manifestations including mitochondrial membrane potential, caspase activation, membrane permeability and phosphatidylserine externalization [27]. It is suggested that sperm DNA fragmentation is associated with an increase in oxidative stress and ROS production during cryopreservation [37]. Said et al. (2010) [34] proposed that an alteration in the mitochondrial membrane fluidity occurs during cryopreservation, which then would lead to a rise in mitochondrial membrane potential and the release of ROS that cause DNA damage in sperm.

In conclusion, the results of the current study indicate that a cooling rate of 60 °C/min around and after the time point of ice nucleation provided better post thaw survival and function of ram sperm than lower and/or decelerating cooling rates. In addition, the results suggest that a rapid cooling in the initial phase of the protocol (i.e. from the holding temperature of +5 °C to just above ice nucleation temperature) may not be beneficial.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.10.007.

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DISCUSIÓN GENERAL

Los métodos de selección de espermatozoides para su empleo en técnicas de reproducción asistida (TRA) consisten en la recuperación de espermatozoides del fluido seminal con el mayor rendimiento posible y sin introducir efectos iatrogénicos que puedan disminuir la motilidad, la viabilidad y, en última instancia, el potencial de capacidad de fertilización del esperma. Los resultados obtenidos en el Capítulo 1 indican que la filtración mediante columnas de Sephadex (ver Anexo 3) muestra ciertas ventajas cinéticas en comparación con los métodos de DGC, después del proceso de selección espermática de semen de morueco (recogido en época no reproductiva) fresco-diluido o almacenado por 24 h a 5 °C. Sin embargo, el semen de morueco colectado durante la época reproductiva y seleccionado mediante el uso de columnas de Sephadex, no mejoró la funcionalidad de los espermatozoides almacenados en refrigeración a largo plazo (96 h), independientemente del diluyente o la adición del glicerol (ver Capítulo 2).

Todos los tratamientos de selección de espermatozoides redujeron el porcentaje de anomalías y el porcentaje de espermatozoides con membrana plasmática y acrosoma dañado (%PDAD) (ver Anexo 5), después de purificar el semen fresco-diluido. El uso de DGC con Accudenz tuvo un marcado efecto negativo en la calidad del esperma (ej. motilidad y colas anormales). El uso de Accudenz en el semen ovino, no es por tanto un método recomendado, al menos según los hallazgos encontrados en el Capítulo 1. Estos resultados no coinciden con los obtenidos con la DGC con Accudenz en esperma humano, debido a la mejora de calidad espermática obtenida luego del proceso de selección (Sbracia et al., 1996).

La filtración con Sephadex produjo una mejor selección de espermatozoides mótiles normales en el semen fresco-diluido y refrigerado por 24 h. De hecho, el porcentaje de espermatozoides con motilidad progresiva fue superior con la filtración de Sephadex en comparación con los métodos DGC. Además, varias de las características de motilidad de CASA fueron mayores con la filtración de Sephadex (ej. %LIN y %STR) que con los métodos de selección mediante DGC. Esto sugiere que la filtración con Sephadex da como resultado una calidad de esperma más deseable que con el uso de los procedimientos de DGC tanto en semen fresco como refrigerado.

El fundamento de la selección de espermatozoides mótiles normales obtenidos mediante la filtración de columnas de Sephadex se centra en que los espermatozoides morfológicamente funcionales y anormales pueden aumentar la producción de ROS, lo que podría provocar un mayor daño del esperma normal en el semen (Henkel, 2011) durante el almacenamiento en frío, la congelación y después de la inseminación. El mecanismo por el cual las perlas de Sephadex atrapan espermatozoides anormales, indeseables, o con membranas espermáticas degeneradas que suponen una capacidad de fertilización menor, aun no se conoce exactamente. Investigaciones previas han sugerido que en el mecanismo de captura de células indeseables puede estar involucrada una unión más específica (ej. espermatozoides capacitados) o espermatozoides con una membrana acrosomal dañada (Graham and Graham, 1990; Maurya and Tuli, 2003).

Dado que la centrifugación del semen ovino es muy dañina para los espermatozoides (García-López et al., 1996), investigaciones previas han sido dirigidas al uso de procedimientos de selección espermática sin centrifugación, que podrían ser beneficiosos para la calidad del esperma del semen fresco o diluido. En este sentido, varios métodos alternativos a DCG han sido previamente investigados para purificar semen ovino con resultados favorables. Estos incluyen el swim-up con dextrano, lavado con sacarosa y la

filtración en Millipore (Marti et al., 2006). El uso de la filtración con columnas de Sephadex ha resultado ser un método eficiente para seleccionar esperma de humano, toro, caballo y cerdo (Bussalleu et al., 2008; Drobnis et al., 1991; Januskauskas et al., 2005; Sieme et al., 2003). En esperma ovino, la purificación espermática con columnas de filtración de Sephadex ha resultado igual de eficaz que el método de DCG con Percoll, mostrado un incremento de la motilidad total y la integridad de la membrana plasmática después de seleccionar muestras astenospérmicas (Valcárcel et al., 1996).

La ventaja del método de filtración mediante columnas de Sephadex en comparación con los métodos DGC utilizados en el presente estudio, es que el uso de Sephadex atrapa más eficazmente el esperma inmóvil, y los métodos DGC causan más daño a las células espermáticas (ej. fuerzas mecánicas causadas por la centrifugación).

Se ha sugerido que los valores de ALH mayores son inversamente proporcionales al movimiento progresivo de los espermatozoides y estos valores aumentan durante la capacitación espermática (Santiago-Moreno et al., 2017). Se requiere un movimiento progresivo efectivo del espermatozoide para superar las barreras anatómicas y fisiológicas en el tracto genital femenino de la oveja, como los pliegues del cuello uterino y el moco cervical, entre otros (Aitken et al., 1985). Esto sugiere que las poblaciones de espermatozoides seleccionados deben ser menores, ya que los métodos de purificación capturan espermatozoides prematuramente capacitados. Los valores de ALH obtenidos con el método de DGC de Accudenz (a pesar de una baja motilidad) y la filtración con Sephadex fueron menores después del uso de todos los tratamientos de selección de espermatozoides en el presente estudio. Por lo tanto, nuestros resultados indican que los porcentajes mayores de motilidad total y progresiva, así como los de linealidad y rectitud, junto con valores menores de ALH, sugieren que la filtración con Sephadex selecciona

esperma de morueco no capacitado con mayor movilidad progresiva y mayor capacidad para cruzar las barreras cervicales de las ovejas.

En contraste al efecto de cinética mejorada demostrado con la filtración de Sephadex, el uso de los métodos DGC en semen fresco (sin refrigerar) dio como resultado porcentajes menores de espermatozoides con la membrana plasmática dañada y acrosoma dañado (%PDAD).

Dado que el porcentaje de morfología de los espermatozoides normales se asocia positivamente con los resultados de fertilidad (Chenoweth, 2005), los métodos de selección espermática estudiados en el presente estudio fueron efectivos para reducir el porcentaje de algunos tipos de anomalías morfológicas. Un factor importante a considerar, es que las muestras espermáticas (provenientes de eyaculados normospérmicos) presentaban una frecuencia de anomalías espermáticas menor antes de la selección. A pesar de eso, el Sephadex disminuyó el porcentaje de cabezas anormales en el semen almacenado en frío al igual que lo reportado por (Graham and Graham, 1990; Januskauskas et al., 2005); Accudenz disminuyó el porcentaje de cabezas sueltas en el semen recién extendido; y Percoll disminuyó el porcentaje de anomalías totales en el semen fresco-diluido.

Por otro lado, la DGC con BoviPure no demostró mejorar parámetros cinéticos, integridad de membranas plasmática y acrosomal, ni reducir porcentajes de anomalías espermáticas estudiadas después de seleccionar muestras normospérmicas en fresco o refrigeradas. De hecho, en algunos parámetros fue igual de eficaz que la filtración con Sephadex, al mostrar un porcentaje menor de colas en látigo en comparación con el Percoll y Sephadex. Este hallazgo es consistente con resultados publicados en ovinos silvestres (muflón, *Ovis musimon*), donde también se usó DGC con partículas de sílice coloidal recubiertas con silano (Santiago-Moreno et al., 2014). Una ventaja muy notable

del BoviPure con respecto al Sephadex y otros métodos de DGC, fue que después del proceso de centrifugación se recupera una mayor concentración espermática pudiéndose ajustar la concentración de las dosis de esperma seleccionado a un número más deseable de células normales y funcionales. Dentro de los métodos mediante DGC, BoviPure parece ser el método que mejor selecciona los espermatozoides de morueco, sin embargo, hacen falta más estudios para identificar tal efecto.

Tras determinar que el Sephadex proporcionó ventajas cinéticas de esperma ovino en comparación con los métodos DCG, se utilizaron las columnas de Sephadex para evaluar su efecto sobre la funcionalidad del esperma ovino enfriado a 5 °C y almacenado hasta 96 h. El semen filtrado y sin filtrar fueron mezclados con diluyentes a base de TEST-yema de huevo o a base de leche desnatada UHT-yema de huevo, suplementados o no con 2% de glicerol. El proceso de filtración supone la selección de una población de espermatozoides morfológicamente normales, con mejor motilidad e integridad de sus membranas espermáticas (Valcárcel et al., 1996). La adición de bajas concentraciones de glicerol a los medios diluyentes supone un efecto crioprotector bajo condiciones de refrigeración (Gil et al., 2011), lo que, a priori, podría prolongar la integridad y funcionalidad espermática bajo condiciones de refrigeración a largo plazo. Sin embargo, bajo estas condiciones, los resultados mostraron que ni la selección de espermatozoides mediante el uso de columnas de Sephadex, ni la adición de glicerol, mejoran la funcionalidad de los espermatozoides diluidos tanto en TEST o UHT y almacenados en frio. El hecho de que los eyaculados al inicio del estudio se recolectaron durante la temporada reproductiva (septiembre–diciembre) y que por tanto presentaban una buena calidad inicial, puede ser la causa de que la filtración con Sephadex no tuvo un efecto positivo sobre muestras normospérmicas. A diferencia de nuestro estudio, muestras normospérmicas, astenospérmicas, o congeladas-descongeladas de morueco, fueron

mejoradas en las variables de motilidad e integridad de membranas, después de ser filtradas en columnas de Sephadex (Valcárcel et al., 1996). No obstante este estudio, no especifica la época de recogida de semen, tal como está especificado en el Capítulo 1 (Época desfavorable: primavera), por lo tanto, se podría especular que la selección espermática mediante la filtración solo parece ser eficaz fuera de la estación reproductiva.

Por otro lado, los resultados mostraron que enfriar el semen de morueco diluido con UHT, sin filtrar ni suplementado con glicerol, conserva la motilidad del esperma, la membrana plasmática, la integridad del acrosoma, la función mitocondrial y la integridad morfológica, mejor que el diluyente TEST durante las 96 h de almacenamiento. De hecho, este semen mantuvo la capacidad de fertilización similar a la del semen fresco hasta 48 h de almacenamiento a 5 °C según los resultados obtenido de fertilización *in vitro* (Ver Tabla 1 de Capítulo 2)

Estudios previos han apuntado que una baja concentración de glicerol (2%) adicionada a diluyentes sintéticos, facilita un efecto crioprotector a espermatozoides mantenidos a bajas temperaturas (Colas, 1975). De hecho, la adición de glicerol a diluyentes de base no sintética (UHT) o sintética (Tris-fructuosa-ácido cítrico) ha demostrado proporcionar un efecto crioprotector al semen ovino (Gil et al., 2011) y caprino (Yotov, 2015), respectivamente, cuando las muestras han sido almacenadas en refrigeración. Los resultados obtenidos en el Capítulo 2 indican que el glicerol no proporcionó ningún beneficio adicional al esperma de morueco sin filtrar y almacenado en frío. Sin embargo, cuando la muestra de semen se filtró previamente con Sephadex, el glicerol mejoró las motilidades (%SM y %PSM) y la velocidad rectilínea (VSL). Estos hallazgos sugieren que la filtración con Sephadex podría seleccionar subpoblaciones de células espermáticas capaces de metabolizar el glicerol, lo que la convierte en una fuente adicional de energía (Mohri and Masaki, 1967). De hecho, Papa et al. (2015) demostraron

una mejoría de VCL y fertilidad del semen de toro cuando se usó 7% de glicerol. Por otro lado, dado que el glicerol acelera la reacción acrosómica en los espermatozoides ovinos almacenado a 5 ºC (Slavík, 1987), la vida útil de las células puede disminuir (Ellington et al., 1999). El porcentaje de espermatozoides con membrana plasmática intacta / acrosoma dañado / membrana mitocondrial intacta (%IPDAHM) (ver Anexo 6) fue relativamente bajo hasta las 96 h de almacenamiento en todos los diluyentes, esto implica que dicha población de espermatozoides tiene un porcentaje menor de espermatozoides capacitados.

Al igual que en el Capítulo 1, en el Capítulo 2 la filtración con Sephadex produjo una población de espermatozoides con motilidad total y progresiva alta, así como un bajo valor de ALH. Estas características cinéticas son muy importantes para superar barreras fisiológicas como la impuesta por el moco cervical y penetrar entre las capas de células del cúmulo de los ovocitos. De hecho, estudios anteriores han revelado una correlación entre variables de motilidad y fertilidad en semen de morueco (Yániz et al., 2015) y humano (Aitken et al., 1985). Los valores de motilidad (total y progresiva), velocidades (VCL y VSL) y de ALH obtenidos con el diluyente UHT en el semen sin filtrar fueron superiores a los obtenidos por el TEST durante los 96 h de almacenamiento, la misma tendencia fue observada en el semen filtrado, excepto que las motilidades empezaron a disminuir a partir de las 72 h. En el semen filtrado diluido con TEST o UHT (ya sea con o sin glicerol), los valores de ALH obtenidos fueron más bajos que en el semen no filtrado y esto concuerda con estudios previos sobre el semen caprino (Santiago-Moreno et al., 2017). Un incremento en el valor de ALH aumenta considerablemente el número de células espermáticas hiperactivas que se someten a capacitación, la filtración de Sephadex, por lo tanto, parece facilitar la selección de espermatozoides no capacitados.

La fecundación *in vitro* (FIV) heteróloga, reveló que la capacidad fecundante de espermatozoides diluidos en UHT se mantuvo hasta las 48 h. De hecho, el presente estudio reveló una capacidad de los espermatozoides, tanto frescos como refrigerados, para unirse por igual a los ovocitos de bovino con zona pelúcida intacta, penetrarlos, fertilizarlos y formar pronúcleos (ej. 18 a 26 horas post fertilización) así como el porcentaje de división de embriones híbridos (ver Anexo 7 y 8). Estudios anteriores han demostrado que la FIV heteróloga que usa esperma ovino (refrigerado o congelado-descongelado) y ovocitos bovinos, es una herramienta eficaz para evaluar la capacidad fecundante de espermatozoides (García-Álvarez et al., 2009; Makarevich et al., 2011). La interacción ovocito-espermatozoide resultó ser similar entre grupos heterólogos y sus grupos de control, esto significa que los espermatozoides de morueco mantenidos en diluyente UHT son capaces de pasar a través de las células del cúmulo y atravesar la zona pelúcida. Una respuesta diferente ha sido observada con esperma de delfín (*Tursiops truncatus*) (Sánchez-Calabuig et al., 2015) y cabra montés (*Capra pyrenayca*) (Pradiee et al., 2018) al evidenciar una mayor interacción entre los espermatozoides y ovocitos bovinos con zona pelúcida intacta (con células del cúmulo).

La tasa de división celular (embriones híbridos con 2 a 8 células) fue similar entre los grupos de semen fresco y refrigerado durante 24 o 48 h (42,3–48,8%). La mayoría de los protocolos de IA usan semen fresco almacenado durante 6 h, aunque algunos recomiendan usar el semen ovino dentro de las 12 h de almacenamiento (Paulenz et al., 2010). Estudios previos han reportado tasas de fertilidad in vivo de 29,0–39,0% o 47,0–49,0% en ovejas después de IA cervical, utilizando esperma enfriado y diluido con Tris-ácido cítrico-Fructuosa (Álvarez et al., 2012) o UHT (Olivera-Muzante et al., 2011), respectivamente. Por lo tanto, los valores de FIV obtenidos en el presente estudio sugieren que los espermatozoides de morueco conservados en el diluyente UHT hasta 48 h,

mantienen su capacidad de fertilización, lo cual es una información útil para futuros ensayos de IA cervical en ovinos.

En el Capítulo 3 se utilizó el diluyente UHT, debido a su eficacia mostrada cuando está suplementado con L-carnitina (LC). Los resultados de este tercer experimento (ver Fig.1 del Capítulo 3) indicaron que la suplementación ya sea con 1, 2,5, 5, 7,5 y 10 mM de LC al diluyente UHT, mejoró el porcentaje de motilidad, los valores de las velocidades (VCL y VSL) y el porcentaje de IPIAIM (espermatozoides con membrana plasmática intacta, acrosoma intacto, y membrana mitocondrial intacta) de semen ovino almacenado líquido a 5 °C durante 96 h. De hecho, la concentración de 5 mM de LC mostró una ligera ventaja sobre las otras concentraciones de LC con valores superiores para motilidad progresiva e inferiores para ALH, lo que indica una mayor desplazamiento en línea recta de espermatozoides no capacitados (Aitken and Baker, 2006; Santiago-Moreno et al., 2017) y por tanto más deseables debido a su relación con la fertilidad después de una IA cervical (Yániz et al., 2015). En efecto, los resultados obtenidos en el Capítulo 1 y 2 sugirieron que los valores más bajos de ALH son compatibles con el movimiento progresivo efectivo (desplazamiento en línea recta) del esperma de morueco almacenado en frío.

Publicaciones anteriores han reflejado que la suplementación del semen de morueco con antioxidantes como catalasa, glutatión reducido (GSH), taurina, trehalosa, quercetina, palmitoleato e hidroxitolueno butilado, kinetina e idebenona, mejora las características de motilidad y la integridad de las membranas de los espermatozoides (plasma y acrosoma) cuando se almacenan en líquido a 4–5 °C (Banday et al., 2017; Bucak and Tekin, 2007; Câmara et al., 2011; Eslami et al., 2019, 2017; Rather et al., 2016). Recientemente, se han publicado los resultados de suplementación con LC a diluyentes sintéticos a base de Tris para esperma ovino y estos no reflejan un efecto

consistentemente beneficioso después del proceso de congelación-descongelación (de Souza et al., 2019). Sin embargo, hasta el momento, no existen estudios de la suplementación con LC a diluyentes de base no sintética (ej. leche desnatada) sobre parámetros de calidad espermática y fertilidad bajo condiciones de refrigeración.

Los efectos antioxidantes de la LC suplementada a diluyentes de base sintética para la criopreservación de espermatozoides de algunos mamíferos ha sido previamente demostrada mejorando la motilidad y la integridad de las membranas (plasma y acrosoma) después del proceso de congelación-descongelación en espermatozoides de humano (Banihani et al., 2014) y rumiantes (toro, búfalo y cabra) (Bucak et al., 2010a, 2010b; El-Raey et al., 2016; Hufana-Duran et al., 2017). Incluso, el efecto estimulador de motilidad de la LC ha sido demostrado en espermatozoides de ratón cuando fue comparada con pentoxifilina (Aliabadi et al., 2012). El esperma de caballo diluido en leche desnatada, suplementado con LC o piruvato, y almacenado en líquido a 20 °C durante 72 h muestra un incremento de la motilidad total y progresiva (Gibb et al., 2015).

El incremento de la producción de ROS puede alterar la motilidad del esperma a través de la interrupción de la producción de ATP o la fosforilación del axonema flagelar (Aitken and Baker, 2006). El incremento de los radicales libres también pueden disminuir la disponibilidad de energía mitocondrial y alterar la motilidad de los espermatozoides (Lamirande and Gagnon, 1992). El efecto antioxidante de la LC puede aumentar la motilidad del esperma al cambiar el metabolismo de los ácidos grasos, lo cual puede proveer energía disponible a los espermatozoides, actuando como un facilitador para el transporte de ácidos grasos activados hacia la matriz mitocondrial para la β-oxidación (Agarwal and Said, 2004). El efecto antioxidante de la LC se debe a un mecanismo de reparación mediante el cual se elimina el acetil-coenzima A (A-CoA) tóxica intracelular y/o de reemplazamiento de los ácidos grasos en fosfolípidos de membrana (Kalaiselvi

and Panneerselvam, 1998; Vicari and Calogero, 2001). Los resultados obtenidos por la interacción del diluyente UHT (6% de yema de huevo) y la LC pueden ser compatibles con la producción mitocondrial de ATP, al tiempo que se minimiza el agotamiento de ATP y los efectos dañinos de los productos metabólicos como los radicales libres, tal como fue demostrado por Gibb et al. (2015). Los resultados *in vitro* claramente mostraron que la LC adicionada al diluyente UHT, permite preservar a largo plazo el semen ovino refrigerado, mejorando las variables de motilidad y protegiendo con éxito las membranas plasmáticas, el acrosoma y la membrana mitocondrial.

Los resultados de fertilidad en la IA realizada durante la primavera (abril–mayo) en 3 lotes de ovejas multíparas inseminadas cervicalmente (ver Anexo 4) con semen de morueco diluido en UHT-5 mM de LC, mostraron resultados prometedores (>50%) con semen fresco bajo las mismas condiciones que los protocolos de IATF habituales (semen ovino diluido con INRA-96® y mantenido a 15 °C hasta el momento de la inseminación [1 a 6 h]).

Los resultados de fertilidad utilizando la IATF en ganado ovino se caracterizan por un alto grado de variación debido a los múltiples factores que intervienen en el protocolo, tipo de tratamiento hormonal, época del año, estatus reproductivo de las ovejas, momento de inseminación etc., por ello para valorar los resultados *in vivo* con el diluyente UHT y 5 mM de LC, se llevaron a cabo las inseminaciones en los mismos lotes y en el mismo momento que las inseminaciones con el diluyente comercial INRA-96® (leche desnatada libre de yema de huevo).

Se utilizó un tratamiento de sincronización con esponjas intravaginales de poliuretano impregnadas con 20 mg de FGA (Chronogest®) durante 14 días y la administración de 400 UI de eCG (IM), (Folligón®) en el momento de la retirada del tratamiento progestativo. La inseminación se realizó a las 55–56 horas después de la eCG, con una

dosis de 400×10^6 espermatozoides/oveja en pajuelas mantenidas a 15 °C entre 1 a 6 h dependiendo de la distancia a cada explotación.

Los resultados de fertilidad en los lotes inseminados con INRA-9® (40%) fueron similares a la media recogida en los datos de estas explotaciones de raza Assaf dentro del programa de selección de Ovigen (Zamora) en los últimos 3 años que se sitúa entre el 38% y 42% (Mantecón, 2019), sin embargo en el lote tratado con semen fresco diluido en UHT, 5mM LC, los porcentajes de fertilidad aunque no significativos se incrementaron hasta el 52%

Cuando se utilizó este mismo diluyente para semen conservado durante 24 h a 5 °C y se inseminaron las ovejas bajo esas mismas condiciones, sólo uno de los tres lotes inseminados no mostró diferencias significativas cuando fue comparado con el semen fresco a 15ºC, (52,3 vs 42,8%), sin embargo en los otros dos lotes la fertilidad fue más baja y cercana al 20%. Por tanto se puede considerar que el uso del diluyente UHT suplementado con LC, mejora las características espermáticas de calidad *in vitro* e *in vivo*, cuando se utiliza para IATF con semen fresco refrigerado a 15 °C. La fertilidad obtenida a las 24 h de almacenamiento fue muy variable entre lotes, sugiriendo un cierto papel del componente "granja" en la efectividad del diluyente en condiciones de refrigeración. Será necesario un mayor número de inseminaciones en estación favorable y desfavorable para llegar a validar estos resultados y concluir sobre las posibilidades de aplicación práctica de este protocolo.

En el Capítulo 4 se compararon tres protocolos de congelación diseñados en un biocongelador con velocidades de enfriamiento controladas: uno (Protocolo 1) que usaba tres velocidades de enfriamiento por deceleración; y otros dos (Protocolo 2 y 3) que usaban velocidades de aceleración bajas al inicio (−4 a −5 ºC/min) o altas (−25 y −60 °

C/min en los Protocolos 2 y 3, respectivamente) en el rango de temperatura en el que se produce la nucleación y crecimiento del hielo. Los resultados obtenidos mostraron claramente que el Protocolo 3 provocaba menos daños celulares que los Protocolos 1 y 2, determinando valores más altos para las variables de motilidad, integridad de la membrana plasmática, acrosomal y mitocondriales, y un menor porcentaje de fragmentación del ADN. Los mejores resultados del Protocolo 3 se pueden atribuir a la velocidad de enfriamiento del segundo paso (de −10 a −130 °C a 60 °C / min) que podría ser lo suficientemente rápida para minimizar la exposición a los efectos perjudiciales provocados por las soluciones hipertónicas, extracelulares y no congeladas, durante el rango crítico de temperatura (− 5 a −25 °C), momento en el que se produce el crecimiento de hielo.

Diferentes velocidades de congelación pueden provocar respuestas variables en la criopreservación de semen de morueco. Publicaciones previas indicaron que velocidades de enfriamiento altas permitirían congelar mejor tanto el semen ovino (Fiser and Fairfull, 1984; Duncan and Watson, 1992) como bovino (Woelders et al., 1997). Asimismo, se han utilizado velocidades de enfriamiento lineal controlado de 30 °C/min durante el período de temperatura de la nucleación y la formación de hielo para congelar semen de morueco (Kumar et al., 2003). Sin embargo, más recientemente, se han utilizado velocidades de enfriamiento óptimas de 25 a 40 °C/min (Fang et al., 2016; Vichas et al., 2018).

Dado que durante la congelación, la temperatura dentro de la pajuela es diferente a la temperatura de la cámara del congelador, las velocidades de enfriamiento van a diferir en el tiempo. La evaluación de la temperatura dentro de la pajuela durante el proceso de congelación nos permitió identificar la disipación del calor latente de fusión que ocurre durante el rango crítico de temperatura. Investigaciones previas usando modelos de

simulación matemática, reflejan que deberían acelerarse las velocidades de enfriamiento después de la nucleación del hielo (Woelders and Chaveiro, 2004). Contrariamente a lo recomendado, el Protocolo 1 proporcionó velocidades de enfriamiento por desaceleración, debido a que la congelación por vapores de LN_2 estático utilizan una velocidad de enfriamiento inicial relativamente alta (40 ºC/min) durante la nucleación de hielo y la disipación del calor latente de fusión, seguidas de velocidades de enfriamiento más bajas (17 y 3 ºC/min). Por lo tanto, el esperma ovino pueden sufrir daños por "choque de frío" (Anel et al., 2003; Ashrafi et al., 2011; Vichas et al., 2018) y disminuir la supervivencia celular por la desaceleración de la velocidad de enfriamiento (inicio con 40 °C/min, seguido de 17 y 3 °C/min).

Otros posibles factores de lesiones criogénicas son la reducción y la deshidratación severa de las células, y estos factores podrían explicar las diferencias después de la descongelación entre los Protocolos 2 y 3 los cuales presentan un enfriamiento lento antes de la nucleación del hielo. Después de la nucleación, el Protocolo 3 parece tener un crecimiento de hielo algo más rápido (que el Protocolo 2 pero más lento que el Protocolo 1), sin embargo, los resultados del Protocolo 3 fueron supriores a los resultados de los Protocolo 1 y 2. La mejor respuesta espermática del Protocolo 3 con respecto al Protocolo 1 después de la congelación puede deberse a la alta velocidad usada luego después de la disipación del calor latente de fusión, por lo tanto se evitaría una excesiva deshidratación celular, además otros factores revelados bajo microscopía electrónica de barrido y transmisión, pueden influir durante la congelación, tales como las formas y tamaños de los cristales de hielo (Bóveda et al., 2018; Cook and Hartel, 2010) y las posibles injurias mecánicas que soportan los espermatozoides (Ekwall et al., 2007).

El proceso de congelación-descongelación además puede afectar a la funcionalidad del esperma mediante la alteración de la integridad del ADN (Nur et al., 2010), y la

alteración de la función de la membrana mitocondrial (Peris et al., 2004). Consecuentemente, esto determina una disminución de la fosforilación oxidativa y síntesis de ATP (Windsor, 1997) con la correspondiente disminución de variables cinéticas del espermatozoide (Celeghini et al., 2007). La fragmentación del ADN espermático está asociada con un aumento en el estrés oxidativo y la producción de ROS durante la crioconservación (Said et al., 2010; Thomson et al., 2009). Los resultados obtenidos demuestran que el Protocolo 3 produjo menos fragmentación de ADN (ver Anexo 9 y 10) comparado con la congelación en vapores de NL_2 estático, después de la descongelación.

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CONCLUSIONES

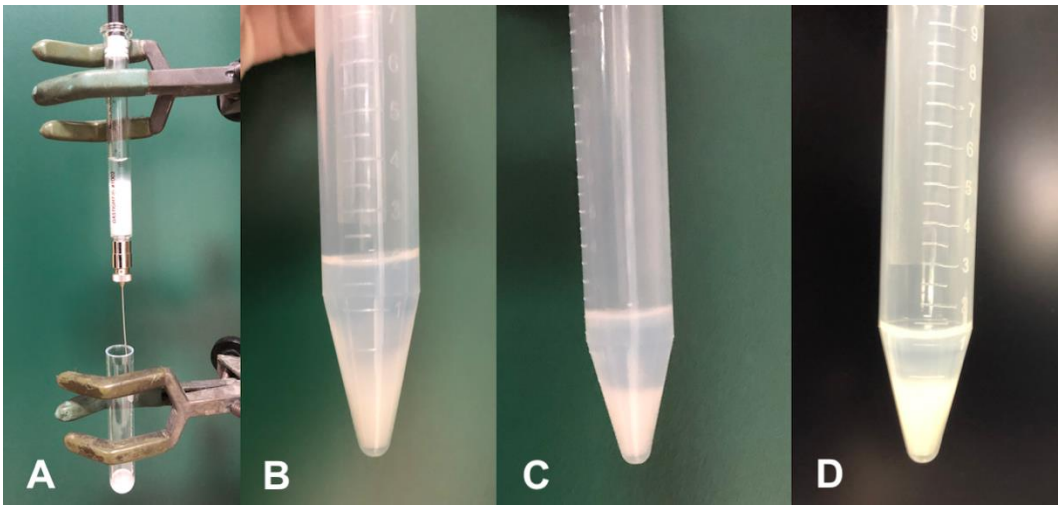
1. La filtración mediante columnas de Sephadex es recomendable para seleccionar espermatozoides de morueco frescos o almacenados a 5 ºC por 24 h, recogidos fuera de la estación reproductiva, con una ventaja en las variables cinéticas en comparación con los métodos de centrifugación de gradientes de densidad.
2. El diluyente UHT preservó la calidad y funcionalidad espermática mejor que el diluyente TEST en condiciones de refrigeración (5 °C) durante 96 h; además permite mantener una adecuada capacidad fecundante hasta las 48 h de refrigeración.
3. La filtración de esperma ovino con Sephadex dentro de la temporada reproductiva no mejoró la calidad espermática; el glicerol no proporcionó protección extra al semen no filtrado.
4. La L-carnitina suplementada al diluyente UHT mejoró las variables de motilidad e integridad de las membranas espermática (plasmática, acrosomal y mitocondrial) así como la fertilidad del semen de morueco almacenado en líquido a medio plazo.
5. El protocolo de congelación en dos fases, con una velocidad de enfriamiento inicial de 5 °C/min, seguida de 60 °C/min alrededor y después del momento de la nucleación del hielo, ofrece los mejores resultados de supervivencia y función celular a la descongelación.

ANEXOS

Anexo 1. Producción científica resultante de la presente tesis doctoral, presentada (y/o aceptada) en congresos nacionales e internacionales y publicadas en proceedings, memorias o revistas:

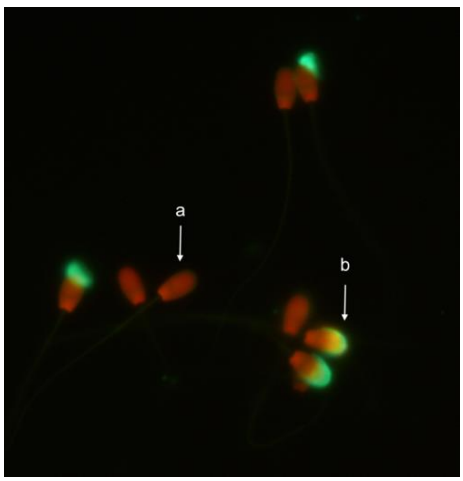
- Second Annual Meeting of IMAGE project- Horizon 2020, Marzo 2018, Viena, Austria. Disertación "*Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics*"
- IV Session PhDay (VETINDOC), junio 2018, Madrid, España. Disertación "*UHT skim milk as alternative to synthetic-based extenders for cold-stored ram semen, previously filtered with Sephadex G-15®"*.
- 34th AETE Scientific Meeting, Septiembre 2018, Nantes, France. Póster "*Sperm selection by density-gradient centrifugation of Merino ram semen cold-stored up to 48 h improves viability and membrane integrity*"
- 45th IETS Annual Conference, Enero 2019. New Orleans, Lousiana, USA. Póster "*Assessment of fertilizing ability of Merino ram semen cold-stored up to 48h by heterologous in vitro fertilization of bovine oocytes*".
- XVIII Jornadas sobre Producción Animal, AIDA – ITEA, Mayo 2019, Zaragoza, España. Trabajo "*Efecto sinérgico de L-carnitina y el diluyente a base de leche desnatada en almacenamiento de semen de carnero refrigerado*"
- 35th AETE Scientific Meeting, Septiembre 2019, Murcia, España. Póster "*L-carnitine supplementation to UHT skimmed milk-based extender improves motility and membranes integrity of chilled ram sperm up to 96 h*"
- 15º Congreso Internacional AERA, Noviembre 2019, Toledo, España. Póster ""*Effectiveness of two-step accelerating cooling rate on post-thaw characteristics of ram sperm*".

Anexo 2. Proceso de entrenamiento y recolección de semen de morueco de raza Merina

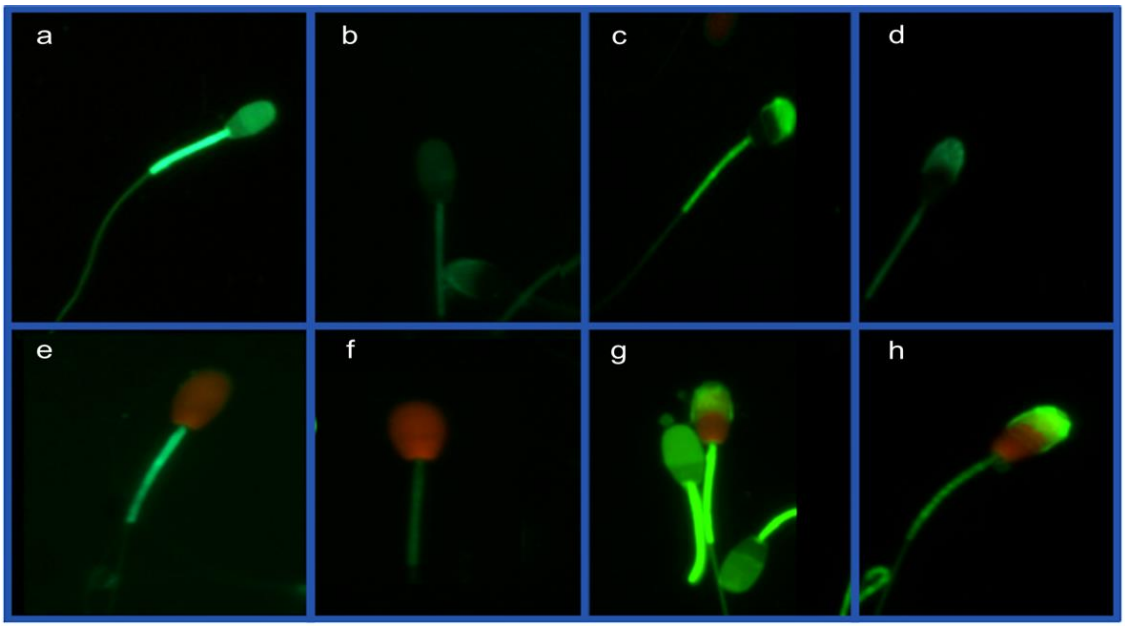


Anexo 3. Métodos de selección espermática mediante (A) filtración de columnas de Sephadex o centrifugación de gradientes de densidad (DGC) usando (B) BoviPure®, (C) Percoll®, o Accudenz®.

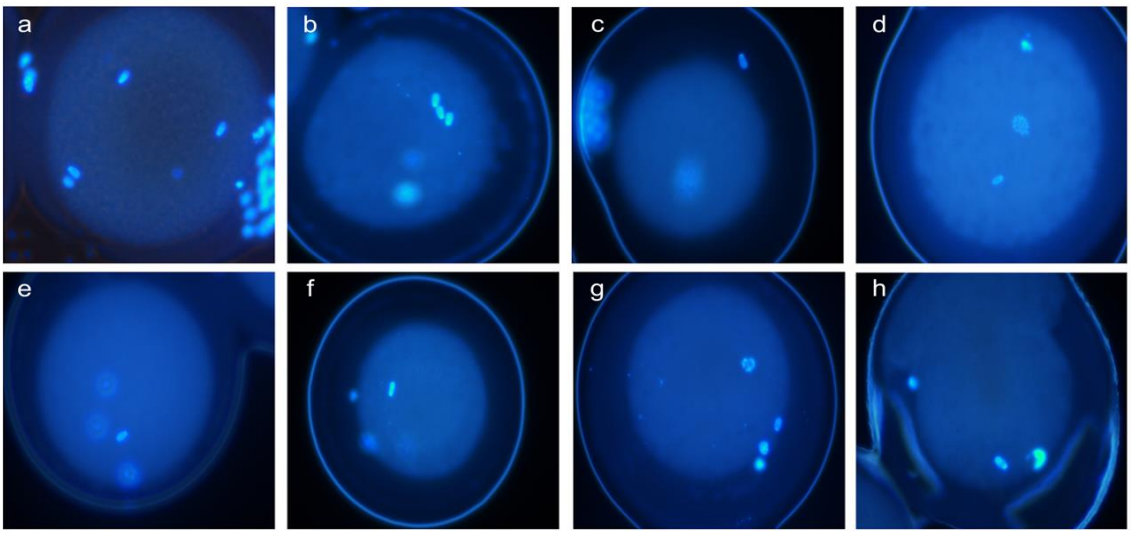
Anexo 4. Proceso de Inseminación artificial a tiempo fijo (IATF) vía cervical, en lote de ovejas de raza Assaf usando esperma ovino diluido con UHT-5 mM L-carnitina, ya sea fresco o refrigerado por 24 h.



Anexo 5. Evaluación del estado de las membranas espermáticas mediante la doble asociación (*PI/PNA-FITC*) de marcadores de fluorescencia (40× magnificación, B-2A, excitación: 450–490 nm y emisión de 520 nm), que muestra (a) espermatozoides con membrana plasmática dañada y acrosoma intacto (%PDIA) y (b) espermatozoides con membrana plasmática dañada y acrosoma dañado (%PDPA).

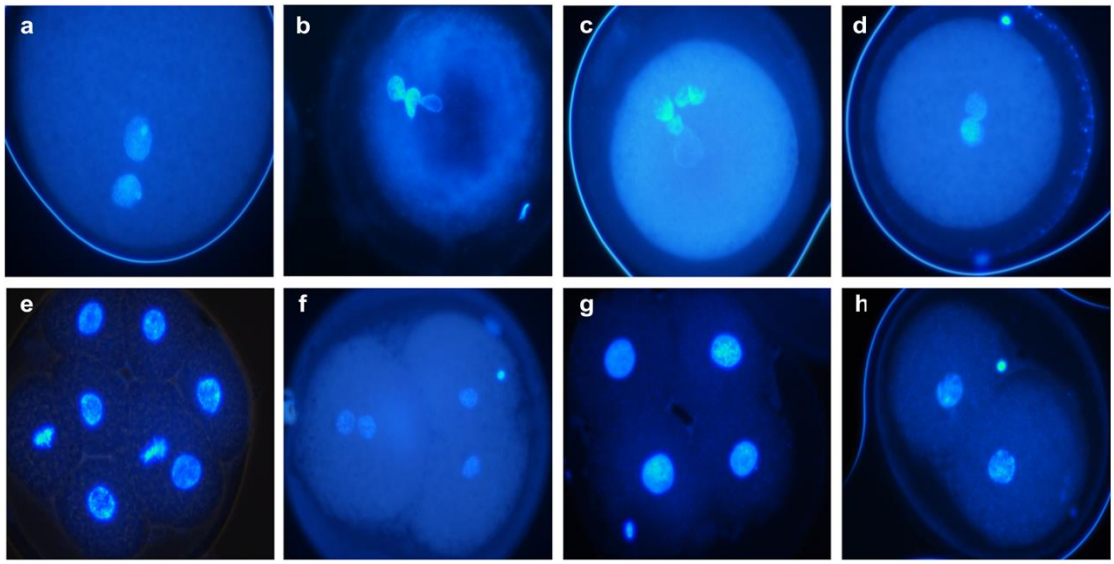


Anexo 6. Evaluación del estado de las membranas espermáticas mediante la triple asociación (*PI/PNA-FITC/Mitotracker*) de marcadores de fluorescencia (40× magnificación, B-2A, excitación: 450–490 nm y emisión de 520 nm) con ocho subcategorías: (a) membrana plasmática intacta / acrosoma intacto / membrana mitocondrial intacta (IPIAIM); (b) membrana plasmática intacta / acrosoma intacto / membrana mitocondrial intacta (IPIADM); (c) membrana plasmática intacta / acrosoma dañado / membrana mitocondrial intacta (IPDAIM); (d) membrana plasmática intacta / acrosoma dañado / membrana mitocondrial dañada (IPDADM); (e) membrana plasmática dañada / acrosoma intacto / membrana mitocondrial intacta (DPIAIM); (f) membrana plasmática dañada / acrosoma intacto / membrana mitocondrial dañada (DPIADM); (g) membrana plasmática dañada / acrosoma dañado / membrana mitocondrial intacta (DPDAIM); y (h) membrana plasmática dañada / acrosoma dañado / membrana mitocondrial dañada (DPDADM).

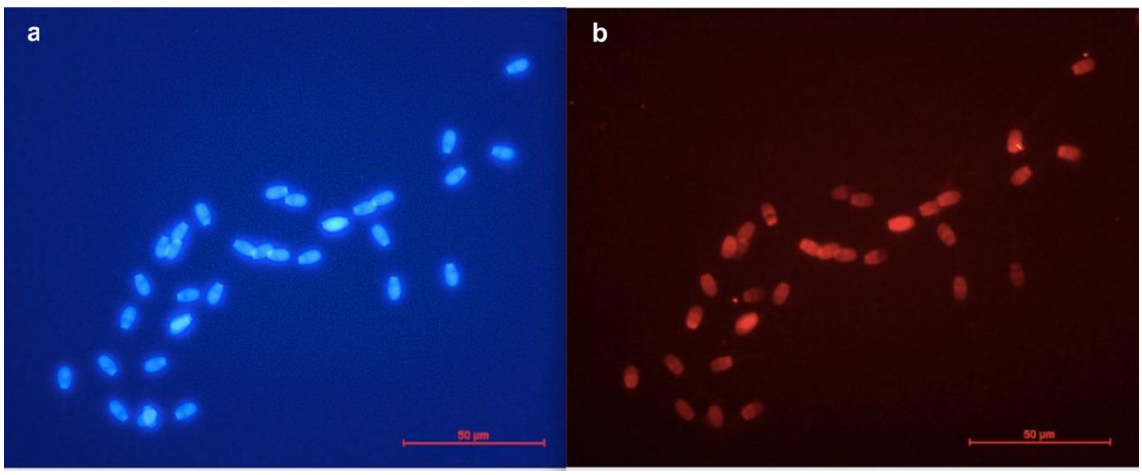


Anexo 7. Unión espermatozoide-ovocito y penetración producida en FIV heteróloga (espermatozoide de morueco diluidos con leche desnatada UHT y ovocitos bovinos) y evaluada bajo un microscopio de epifluorescencia (40× magnificación, UV-2A, excitación: 330–380 nm,

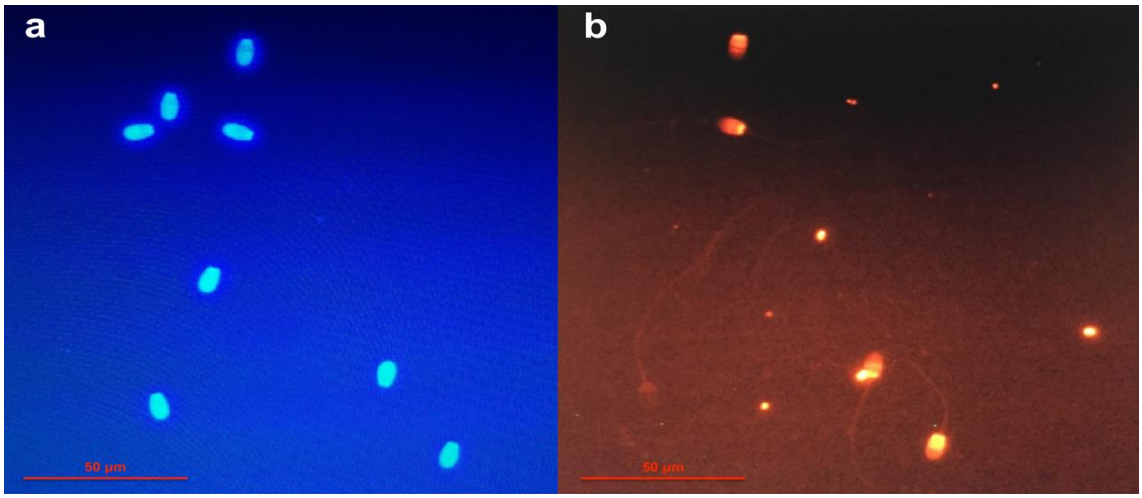
y emisión: 420 nm, Hoechst). Unión espermatozoide y ovocito a las 2,5 h post inseminación, mostrando (a) FIV homóloga (control), y FIV heteróloga usando (b) semen fresco y almacenado a 5ºC por (c) 24 h y (d) 48 h. Penetración de espermatozoides a las 12 h post inseminación, mostrando (c) FIV homóloga (control), y FIV heteróloga usando (f) semen fresco y almacenado a 5ºC por (g) 24 h y (h) 48 h.



Anexo 8. Polispermia, formación pronuclear y división celular producida en una FIV heteróloga (espermatozoide de morueco diluidos con leche desnatada UHT y ovocitos bovinos) y evaluada bajo un microscopio de epifluorescencia (40× magnificación, UV-2A, excitación: 330–380 nm, y emisión: 420 nm, Hoechst). Formación de pronúcleos a 18-26 horas post inseminación (hpi) en (a) FIV homóloga (control), polispermia y formación pronuclear en FIV heteróloga usando (b) semen fresco y almacenado a 5ºC por (c) 24 h y (d) 48 h. División celular (2 a 8 células) a 48 hpi en FIV homóloga (e) y FIV heteróloga usando (f) semen fresco y almacenado a 5ºC por (g) 24 h y (h) 48 h.



Anexo 9. Control positivo de la prueba de TUNEL (+) para análisis de integridad de ADN en espermatozoides de morueco congelados-descongelados analizados bajo microscopía de epifluorescencia (40× magnificación, G-2A, excitación: 510–560 nm y emisión: 590 nm para TMR-Red; UV-2A, excitación: 330–380 nm y emisión: 420 nm Hoechst). Células teñidas con Hoechst (a) y luego tratadas con DNase I (1 mg/ml) que muestran el 100% de su ADN fragmentado



Anexo 10. Prueba de integridad de ADN mediante el ensayo de TUNEL en espermatozoides de morueco congelados-descongelados y analizados bajo microscopía de epifluorescencia (40× magnificación, G-2A, excitación: 510–560 nm y emisión: 590 nm para TMR-Red; UV-2A, excitación: 330–380 nm y emisión: 420 nm Hoechst). Células teñidas con hoechst bajo campo UV-2A (a) y sólo células que muestran su ADN fragmentado bajo campo G-2A (b).