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ESTRATEGIAS DE CONSERVACIÓN DE SEMEN DE MORUECO EN FUNCIÓN DEL ORIGEN ESPERMÁTICO.

Memoria que presenta para optar al grado de Doctor el Ingeniero en Agronomía con la especialidad en Producción Animal Tropical.

D. JULIO RENAN TAMAYO CANUL

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Ser humilde para con los superiores es un deber;
Para con los iguales, una muestra de cortesía;
Para con los inferiores, una prueba de nobleza.

—Benjamín Franklin (1706-1790) —

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A mi hija, Danallely

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RESUMEN

Resumen

Los protocolos clásicos de conservación de semen ovino deben rediseñarse y optimizarse en función del tipo, origen y destino de las muestras para asegurar la calidad de los espermatozoides almacenados en los bancos de recursos genéticos. En la presente tesis doctoral realizamos una serie de experiencias encaminadas a mejorar la viabilidad y eficacia de los bancos de germoplasma en la especie ovina a partir de la adaptación especifica de los protocolos estándar de criopreservación espermática.

En el primer trabajo, analizamos a las 0, 24, 48 y 72 h post-mortem, el efecto de dos estrategias de almacenamiento a 5 ºC en espermatozoides epididimarios de carnero: (1) almacenamiento de la papilla epididimaria fuera del epidídimo –en tubo- *vs* almacenamiento dentro del epidídimo y (2) espermatozoides del epidídimo extraídos a las 0 h post-mortem y almacenados diluidos en tres medios a 320, 370 y 420 mOsm/kg. La osmolalidad en las muestras mantenidas en la cola del epidídimo aumenta con el tiempo post-mortem pero no en las muestras almacenadas en tubo. La motilidad de los espermatozoides almacenados en el epidídimo es más alta a las 24 h post-mortem y disminuye a las 48 h frente a las muestras mantenidas en tubo en que la disminución ocurre ya a las 24 h. La viabilidad de las muestras intraepididimarias se reduce a las 72 h post-mortem mientras que en las conservadas en tubo a las 48 h. La calidad de las muestras conservadas fuera del epidídimo es inferior a las muestras conservadas en el epidídimo en todos los tiempos post-mortem. En el experimento 2, la motilidad total y progresiva no se vio afectada por el tiempo de almacenamiento post-mortem ni en las muestras almacenadas en el diluyente con una presión osmótica de 320 ni en la cola del epidídimo. La viabilidad es mayor en las muestras almacenadas en el epidídimo que en las diluidas, y en ambas disminuye con el tiempo post-mortem. A las 24 horas el porcentaje de daño acrosomal fue bajo y similar en los 4 medios de almacenamiento, pero aumentó en los medios de 370 y 420 mOsm/kg con el tiempo post-mortem. Almacenar los espermatozoides en el epidídimo es una buena estrategia para mantener la calidad espermática al menos durante las primeras 48 h. El diluyente con presión osmótica 320 preserva la movilidad de los espermatozoides pero no protege frente al daño acrosomal.

En el segundo trabajo, comprobamos el efecto de la criopreservación de muestras espermáticas conservadas a 5 ºC en tres métodos de almacenamiento: en el epidídimo,

fuera del epidídimo sin diluir -en tubo- y diluidas en diluyentes con diferentes osmolalidades (320, 370 y 420 mOsm/kg). A las 0, 24, 48 y 72 horas los espermatozoides fueron criopreservados utilizando el mismo diluyente de conservación. El tiempo post-mortem en el análisis pre y post-descongelación reduce la calidad de los espermatozoides. Algunas combinaciones de almacenamiento x diluyente mejoran la calidad de los espermatozoides con respecto al tiempo post-mortem. Tanto el almacenamiento en el epidídimo y la dilución con un diluyente con una osmolalidad de 420, como el almacenamiento, tras la extracción inmediata (0 h), de los espermatozoides diluidos en un diluyente con una osmolalidad de 320 mejoran la calidad post-descongelación especialmente en tiempos largos de almacenamiento post-mortem. De estos resultados se desprenden aplicaciones prácticas: el almacenamiento en el epidídimo requiere usar un diluyente hiperosmótico para la congelación y el almacenamiento de los espermatozoides diluidos en un diluyente hiposmótico hasta su congelación es otra opción aceptable.

En el tercer estudio, investigamos el efecto de la congelación de dosis seminales de carnero en concentraciones altas (200, 400, 800 y 1600 x 10^6 ml^{-1}) sobre la calidad post-descongelación y la fertilidad. La motilidad total y la velocidad son menores en dosis de 1600 x 10^6 ml^{-1}, mientras que la motilidad progresiva y la viabilidad son más bajas tanto para 800 como para 1600 x 10^6 ml^{-1}. Para evaluar la eficacia *in vivo* se realizan inseminaciones intrauterinas vía laparoscópica con dosis de 200, 400 y 800 x 10^6 ml^{-1} con un número fijo de espermatozoides (25 millones por cuerno uterino) en ovejas sincronizadas. La fertilidad fue similar para el semen congelado a dosis de 200 y 400 x 10^6 ml^{-1}, mientras que fue significativamente menor en dosis congeladas a 800 x 10^6 ml^{-1}.

En el cuarto trabajo, evaluamos la calidad post-descongelación y la fertilidad de espermatozoides recuperados de tres orígenes (eyaculado, electroeyaculado y epidídimo) y criopreservados con dos concentraciones de yema (Y10 y Y20) y dos de glicerol (G4 y G8). Los espermatozoides del epidídimo son más resistentes a la criopreservación, a pesar de que las muestras del eyaculado tienen similar calidad post-descongelación. La concentración de yema de huevo al 20% es más adecuada para congelar espermatozoides de carnero independientemente de su origen. Sin embargo, en relación con el glicerol, es mejor congelar al 4% las muestras recuperadas del eyaculado y electroeyaculado, mientras que el 8% es más adecuado para congelar espermatozoides

del epidídimo. En la prueba de fertilidad, realizada con dosis seminales de los tres orígenes aplicadas por vía laparoscópica, no se encontraron diferencias entre los tres orígenes aunque la fertilidad tiende a ser inferior en las muestras electroeyaculadas.

CAPÍTULO 1

Capítulo 1

Introducción general.

En la especie ovina, la inseminación artificial tiene una difusión limitada en parte porque el uso del semen congelado requiere una técnica de aplicación compleja (inseminación intrauterina laparoscópica). En las razas donde se aplica, la técnica es un instrumento insustituible en los programas de selección y mejora genética ya que permite la conexión entre rebaños y difusión de perfiles genéticos superiores (sementales probados). Por lo tanto, la congelación de semen y su almacenamiento obedece bien a criterios productivos (semen procedente de animales de alto valor genético de los centros de sementales) o a criterios conservacionistas (creación de bancos de recursos genéticos de poblaciones o razas con riesgo). Para el uso en inseminación artificial intrauterina (vía laparoscópica) se congelan dosis seminales a una concentración estándar ($100x10^6$ spz/ml), con un número bajo de espermatozoides (suficiente para garantizar la fertilidad ya que la aplicación es en el útero). Existen pocos estudios sobre congelación a concentraciones más altas que pueden ser útiles e imprescindibles en la aplicación intracervical profunda o transcervical, técnica que está en vías de desarrollo y supondrá, en el futuro, el despegue definitivo de la inseminación artificial en la especie. Por otro lado, el abanico de posibilidades para la obtención de semen no está suficientemente estudiado en esta especie. La congelación de semen recogido en vagina es la opción más clásica y eficiente en animales entrenados para tal fin, pero la obtención de espermatozoides ya sea mediante electroeyaculación o después de la muerte del animal (post-mortem) suponen fuentes complementarias o únicas que amplían las vías de entrada de gametos masculinos a los bancos de recursos genéticos.

En España, existe una amplia gama de recursos zoogenéticos debido a la diversidad de sus condiciones geográficas y climáticas. En ocasiones, estos recursos se ven amenazados por la introducción de razas exóticas con altos índices de producción que desplazan las razas autóctonas. En otros casos, la baja productividad y el abandono del medio rural de las zonas desfavorecidas son las causas de los descensos tan acusados de las poblaciones autóctonas. En las últimas décadas, la conservación de razas ovinas con pocos censos ha sido muy difícil, y algunas de ellas se encuentran en riesgo o casi han

desaparecido [1], de hecho actualmente se considera que existen 33 razas ovinas españolas oficialmente declaradas en peligro de extinción (Tabla 1).

Tabla 1. Relación de razas ovinas en peligro de extinción.

Razas	Ubicación	Censo	Estatus
Alcarreña	Provincias de Cuenca y Guadalajara	5974	X
Aranesa	Pirineo central	1700	X
Ansotana	Pirineo aragonés	1500	X
Canaria	Archipiélago canario	51000	X
Canaria de Pelo	Archipiélago canario	3500	X
Carranzana (variedad negra)	Valle de Karrantza (Bizkaia)	500	X
Cartera	Provincia de Teruel	9000	X
Castellana (variedad negra)	Provincia de Castilla y León	1540	X
Chamarita	Provincia de la Rioja	11900	X
Churra Lebrijana	Huelva y provincia de Sevilla	1000	X
Churra Tensina	Pirineo central de Huesca	6500	X
Colmenareña	Zona norte comunidad de Madrid	4025	X
Gallega	Comunidad autónoma de Galicia	1680	X
Guirra	Comunidad Valenciana	3824	X
Ibicenca	Islas de Eivissa y Formentera	300	X
Lojeña	Comarca de Loja	68000	X
Maellana	Provincias de Zaragoza, Teruel y Tarragona,	4919	X
Mallorquina	Isla de Mallorca	9000	X
Manchega (variedad negra)	Submeseta Sur Central de la Península Ibérica	2500-3000	X
Menorquina	Isla de Menorca	16000	X
Merina (variedad negra)	Comunidades de Extremadura y Andalucía	No hay datos	X
Merina de Grazalema	Provincias de Cádiz y Málaga	No hay datos	X
Montesina	Montes de Granada	4000	X
Ojalada	Provincia de Soria	5000	X
Palmera	Isla de la Palma	350	X
Ripollesa	Noreste de Catalunya	70000	X
Roja Mallorquina	Isla de Mallorca	1000	X
Roya Bilbilitana	Provincia de Zaragoza	25000	X
Rubia del Molar	Zona norte de la comunidad de Madrid	1330	X
Sasi Ardi	Gipuzkoa y Navarra	1050	X
Talaverana	provincias de Toledo, Cáceres y Ávila	3800	X
Xalda	Asturias	1499	X
Xisqueta.	Comarca de Pallars Jussá	12000-15000	X

Fuente: Barona, 2009. Guía de campo de las razas autóctonas españolas.

Esta situación ha motivado el desarrollo de un programa nacional para la conservación, mejora y fomento de las razas en riesgo (Real Decreto 2129/2008) que recomienda el uso y establecimiento de bancos de germoplasma sobre todo en aquellas con mayor pérdida de variabilidad genética. Para la creación de estos bancos de germoplasma, lo ideal sería disponer de material que se pueda recoger sistemáticamente de animales vivos. Sin embargo, la muerte repentina de animales genéticamente valiosos crea la necesidad de desarrollar protocolos de recogida de espermatozoides post-mortem. La cola del epidídimo es el principal reservorio de espermatozoides en el aparato genital masculino y el lugar de obtención ideal post-mortem. El epidídimo es una estructura

tubular (larga y contorneada) dividida anatómicamente en cabeza, cuerpo y cola, y entre sus múltiples funciones tiene el complejo proceso de maduración espermática [2,3]. La papilla epididimaria es un buen recurso para la obtención de espermatozoides viables con capacidad para fecundar ovocitos [4–7] y que, por lo tanto, se pueden conservar en los bancos de recursos genéticos, lo que es muy útil en diversas situaciones (animales de alto valor genético que mueren repentinamente o individuos pertenecientes a poblaciones que se encuentran en vías de extinción). Sin embargo, los espermatozoides son viables durante un determinado período de tiempo después de la muerte del animal y tras ésta se van degenerando rápidamente [8,9]. Los primeros cambios degenerativos del epidídimo parece que ocurren a las 12 post-mortem [4] y la desestructuración de los túbulos comienza a las 24 horas. Estudios realizados en varias especies han indicado que el almacenamiento de los epidídimos a 5 ºC puede ser una forma adecuada para mantener la motilidad de los espermatozoides y la capacidad de fecundar durante varios días, retrasando los efectos de la degeneración tisular (Ratones: [10]; Cerdo: [11]; Ciervo: [12]; Perro: [9].

Existen pocos trabajos sobre la recuperación de espermatozoides post-mortem en la especie ovina, así como del posterior uso de los mismos; en comparación con otras especies domésticas y silvestres [8,13,14]. En esta Tesis doctoral, en primer lugar, se presentan (capítulo 3-1ª publicación), los resultados de la conservación (5 ºC) de las muestras espermáticas obtenidas del epidídimo, hasta 72 horas después de la muerte del animal, en diferentes métodos de almacenamiento: dentro del epidídimo, fuera del epidídimo -en un tubo- o diluidas en diluyentes con diferente osmolalidad.

La congelación de espermatozoides, sea cual sea su origen, tiene indudables ventajas como técnica básica que permite su almacenamiento indefinidamente en el tiempo para su uso en inseminación artificial (herramienta básica de los programas de mejora genética) o para formar parte de un banco de germoplasma (razas con pocos efectivos) que puede generar, en el futuro, nuevos individuos. Sin embargo, el proceso de congelación y descongelación causa daños físicos y químicos en la membrana de los espermatozoides, estos daños son atribuidos a las alteraciones en la transición de fase, al aumento de la peroxidación lipídica de la membrana inducido por las especies reactivas de oxigeno (ROS) y a la tensión mecánica en la membrana celular por estrés osmótico, así como al estrés térmico de la congelación y descongelación [15,16]. Además, estos cambios provocan efectos negativos, en términos de estructura de los

espermatozoides, daños bioquímicos y funcionales que resultan en una reducción de la motilidad de los espermatozoides, integridad de la membrana y la capacidad para poder fertilizar [14,17].

Los espermatozoides epididimarios tienen además ciertas peculiaridades que pueden afectar a su congelación, de hecho el medio epididimario tiene una presión osmótica superior al semen eyaculado [18]. Existen algunos trabajos que evalúan la resistencia de los espermatozoides del epidídimo a la crioconservación [19,20]. En este sentido, los espermatozoides epididimarios que son colectados en las primeras 24 horas después de la muerte del animal podrían estar mejor preparados para los desafíos de los diluyentes de congelación y para la adaptación a una osmolalidad inferior [21–23]. El tiempo transcurrido desde la muerte del animal, así como el tipo de conservación, son factores que influyen decisivamente en la calidad y procesamiento de las muestras. Un aspecto muy importante de los espermatozoides epididimarios es que parecen ser más resistentes que los espermatozoides del eyaculado al menos en la especie porcina [11], aunque no hayan completado la evolución funcional y morfológica [24–26]. En el capítulo 4 (2ª publicación) de la presente memoria, evaluamos cómo puede influir, en la calidad espermática post-descongelación, la osmolalidad de los diluyentes usados en la crioconservación de espermatozoides epididimarios almacenados a 5 ºC durante diferentes periodos de tiempo antes de la congelación.

Los espermatozoides procedentes del semen eyaculado normalmente se congelan a una concentración estándar e ideal para su uso en inseminación intrauterina (25 $x10^6$ spz/dosis) como ocurre en otras especies. Sin embargo, el uso de semen congelado a esta concentración en la especie ovina obliga, en la actualidad, a la aplicación intrauterina por vía laparoscópica. Esta técnica es compleja y lo ideal sería la aplicación intrauterina transcervical lo que requiere el uso de concentraciones más altas de espermatozoides. En este sentido, otro de los aspectos evaluados en esta memoria, es la congelación de espermatozoides a altas concentraciones. En la actualidad, los estudios realizados sobre la congelación espermática, intentan mejorar la viabilidad post-descongelación [16,27–29]. Sin embargo, como ya se ha comentado, el espermatozoide sufre modificaciones durante la congelación y la descongelación. Las células que son sometidas al proceso de congelación-descongelación están expuestas a la cristalización de hielo extracelular que produce un incremento en la concentración de solutos y por lo tanto unas condiciones de hiperosmolalidad [30,31].

Los resultados obtenidos en IA por vía vaginal con semen descongelado han resultado, hasta ahora, insatisfactorios. La baja fertilidad puede estar debida a la baja viabilidad que se obtiene a la descongelación y posibles trastornos subletales en la proporción de los espermatozoides que sobreviven al proceso de la congelación [32]. Los daños ocasionados en la membrana de los espermatozoides durante el proceso de congelación, alteran la función metabólica del espermatozoide, reduciendo así el número de células viables y ocasionando una capacitación espermática prematura [33]. Consecuentemente, los espermatozoides sólo serían viables en un corto período de tiempo en el tracto reproductor de la hembra, por lo tanto, tendrán una menor oportunidad de fecundar los ovocitos [34]. Debido a estos daños en los espermatozoides, en la inseminación artificial por vía vaginal es necesario contar con un determinado número de espermatozoides plenamente competentes para lograr un éxito en la fertilización en el período periovulatorio de las hembras.

Además, uno de los problemas en la inseminación artificial vaginal, es la complejidad anatómica del cuello uterino que impide depositar la dosis seminal más allá de la entrada del mismo. El cérvix, de naturaleza fibrosa, está conformado por anillos cervicales (2-7 anillos) con forma de embudo y en el 75% de los casos existe un desalineamiento entre el 2º y 3º anillo [35]. Los anillos suponen una barrera que bloquea la penetración de catéter hasta el útero obligando a realizar una inseminación cervical superficial o vaginal en la mayoría de los casos ya que el semen es expulsado caudalmente. Una manera de compensar esta pérdida espermática hacia la vagina es el uso de dosis seminales con altas concentraciones que junto con un incremento de la profundidad de inseminación (diferentes estrategias) pueden suponer una mejora importante en los resultados de inseminación con semen descongelado. Por ello es importante la puesta a punto de protocolos adecuados para la crioconservación de dosis seminales de altas concentraciones espermáticas con el fin de usarlas en la inseminación transcervical. El estudio del efecto de la congelación espermática en altas concentraciones (200, 400, 800 y 1600x10^6 spz/mL concentraciones) sobre la calidad postdescongelación y fertilidad (usando IA laparoscópica) es abordado en el capítulo 5 (3ª publicación).

El éxito de la congelación espermática puede ser diferente si se trata de espermatozoides eyaculados o recogidos directamente del epidídimo después de la muerte del animal. En este aspecto, y considerando que puede ser necesario congelar espermatozoides de

ambos orígenes incluso en un mismo macho por diversas circunstancias, se hace necesario definir un protocolo de conservación adaptado a cada tipo de muestra. El método de obtención de la muestra espermática con vagina artificial requiere un período previo de adaptación del macho [36]. En los centros de sementales existen moruecos, que no se adaptan a la recogida seminal en vagina artificial o por lesiones orgánicas no pueden saltar y en los que es necesario recoger el semen mediante electroeyaculación [37]. En estos casos la recogida mediante la técnica de electroeyaculación puede proporcionar una muestra de características fisicoquímicas y calidad diferente a las muestras eyaculadas o epididimarias y se requiere de nuevo adaptar un protocolo de manejo y congelación a este tipo de muestras. En este contexto, nos planteamos en la presente memoria en el capítulo 6 (4ª publicación) el estudio combinado de 4 diluyentes diferentes (con dos porcentajes de yema y dos de glicerol) para la congelación de espermatozoides obtenidos de las tres fuentes espermáticas. La yema de huevo y el glicerol representan componentes indispensables en la elaboración de diluyentes para la conservación de espermatozoides ya sea en estado líquido o congelado. La interacción de los espermatozoides con el diluyente es un factor crucial que afecta la integridad de los espermatozoides y la capacidad de fertilización. El uso de la yema de huevo ha demostrado que tiene un efecto beneficioso sobre la criopreservación de espermatozoides como protector de las membranas disminuyendo las lesiones producidas por el shock por frío, en asociación con otros componentes de los diluyentes [38–41].

El glicerol contribuye a la integridad de los espermatozoides en el proceso de criopreservación. Una concentración óptima aún no se ha definido, ya que los estudios comparativos se han llevado a cabo utilizando generalmente diluyentes con muchos componentes (azúcares, estabilizadores de membrana, concentración de glicerol, etc.), lo que hace difícil tomar una decisión acerca de la concentración de glicerol más adecuada. Algunos autores han descrito un amplio rango de concentraciones, del 4 a 10% [16,19,25,42,43], pero el resultado final parece depender más bien de la interacción con otros componentes de los diluyentes o de los protocolos seguidos antes que de la concentración de glicerol. Estos hechos justifican la necesidad de desarrollar protocolos criobiológicos básicos para las diferentes fuentes espermáticas. Por otro lado, todos los métodos de recogida seminal: vagina artificial, electroeyaculación y epididimaria son útiles y complementarios en la creación de los bancos de recursos

genéticos. La disponibilidad de diferentes métodos optimizados para el sistema de crioconservación de espermatozoides de carnero adaptados al origen de las muestras, es muy importante para la preservación de material genético valioso, así como para la protección de la biodiversidad genética de esta especie [13,44,45].

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CAPÍTULO 2

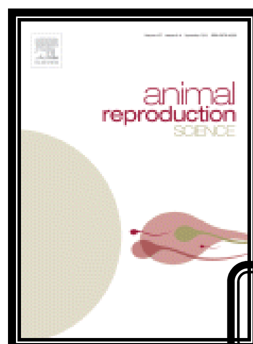
Capítulo 2

Objetivos.

El objetivo general es conseguir una metodología adaptada y óptima que permita la crioconservación eficiente de espermatozoides de carnero obtenidos de diferentes orígenes (epidídimo, eyaculado y electroeyaculado) contemplando las condiciones de recogida, aspectos específicos de cada tipo de fuente espermática y aspectos prácticos sobre la aplicación de las muestras congeladas.

Los objetivos específicos que se plantean en esta tesis son:

1. Analizar el efecto del tipo de almacenamiento (epidídimo vs papilla espermática) a 5 ºC y el tiempo de almacenamiento postmortem (0, 24, 48 y 72 horas) sobre la calidad de los espermatozoides del epidídimo de carnero, así como el efecto de la conservación del semen a 5 ºC diluido en un medio con diferentes osmolalidades (320, 370 y 420).
2. Evaluar el efecto de tres diluyentes de congelación (diferentes osmolalidades) y tres métodos de almacenamiento (epidídimo, semen sin diluir y semen diluido), en la calidad posdescongelación de espermatozoides epididimarios de carnero obtenidos a diferentes tiempos post-mortem.
3. Estudiar la calidad espermática post-descongelación y la fertilidad del semen ovino (eyaculado) congelado en cuatro concentraciones (200, 400, 800 y $1600x10^6$ spz/ml).
4. Evaluación de la calidad post-descongelación y la fertilidad del semen de carnero obtenido de tres fuentes espermáticas (eyaculado, electroeyaculado y epididimario) y congelado con cuatro diluyentes diferentes que combinan dos concentraciones de yema de huevo (10 y 20%) y dos concentraciones de glicerol (4 y 8%) con vistas a identificar la posible especificidad de los diluyentes en función del origen espermático.



CAPÍTULO 3

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Capítulo 3

Undiluted or extended storage of ram spididymal spermatozoa as alternatives to refrigerating the whole epididymes.

J. Tamayo-Canul[a,b], M. Alvarez[a,b], E. López-Urueña[a,b], M. Nicolas[a,b], F. Martinez-Pastor[a,c], E. Anel[a,b], L. Anel[a,b], and P. de Paz[a,c].

[a]ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain, and [b]Animal Reproduction and Obstetrics, University of León, 24071 León, Spain, and [c]Molecular Biology, University of León, 24071 León, Spain.

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3.1 Abstract.

The effect of storage procedure at 5°C on the quality of ram spermatozoa from the cauda epididymis was analyzed. Two strategies were tested at 0, 24, 48 and 72 h post-mortem: (1) spermatozoa held in the epididymal fluid and stored either in the cauda epididymis (In-EPID) or in vitro (Ex-EPID), (2) epididymal spermatozoa extended in three media at 320, 370 and 420 mOsm/kg (D320, D370, D420). Analyzed parameters were: osmolality, pH, motility, acrosomal status and viability. In experiment 1, osmolality of the In-EPID samples, but not in Ex-EPID, increased with post-mortem time. Motility of In-EPID spermatozoa in samples, after 24 h post-mortem, was higher compared to the Ex-EPID samples, although differences decreased at 48 and 72 h. In experiment 2, total (TM) and progressive motility (PM) were not significantly affected by storage time for D320 and In-EPID samples. However, the motility of D370 and D420 samples significantly decreased with time. TM and PM of D320 were significantly higher than D370 and D420 at 72 h. At 24 h, sperm viability was higher for In-EPID (80.7 ± 3.4%) than for the extended samples (44.8 ± 2.9%, 37.7 ± 3.9% and 48.6 ± 6.0% for D320, D370 and D420, respectively), which also decreased faster with time. At 24 h, the percentage of damaged acrosomes was low and similar for the four methods of storage, but damaged acrosomes increased with time for D320 and D370. Storing the spermatozoa in the epididymis is a good strategy for maintaining sperm quality in ram, at least for 48 h. The D320 extender preserve motility of epididymal spermatozoa but does not protect the status of the acrosome.

Keywords: Ram; Epididymis; Refrigerated storage; Osmolality; Extender; Sperm quality.

3.2 Introduction

In the last decade the conservation of rare breeds of domesticated species has been very difficult and many of them have disappeared or are going to become extinct (Canali, 2006). This loss of biodiversity has been due to a poor management policy of genetic resources. In Spain, there is a wide range of animal genetic resources due to its specific geographical and climatic characteristics. These resources are threatened by the introduction of alien breeds with high production rates, and as an example we can cite the 34 sheep breeds officially declared endangered. This situation has motivated the

implementation of a National Programme for conservation, improvement and promotion of livestock breeds (Royal Decree 2129/2008). This programme recommended establishing a germplasm bank for certain sheep breeds with increased risk of losses of genetic variability. This bank would ideally contain germplasm systematically collected from living animals. However, genetically valuable animals may die unexpectedly, calling for a methodology to collect their germplasm post-mortem. In that event, the post-mortem collection of epididymal spermatozoa allows the conservation of valuable genetic material that would otherwise be lost (Saragusty et al., 2006). Epididymal spermatozoa provide a sufficient quantity of viable spermatozoa to be used to fertilize oocytes with the resulting zygotes being able to develop into live young (Songsasen et al., 1998; Sankai et al., 2001; Soler et al., 2003a; Martins et al., 2009).

However, sperm in the epididymis are viable only for a certain period of time and then degenerate rapidly (Hishinuma et al., 2003; Kaabi et al., 2003; Soler et al., 2003b; Yu and Leibo, 2002). Previous studies in several species have indicated that storage of epididymides at 5 °C may be an appropriate way to maintain sperm motility and fertilizing capacity for several days (mice: An et al., 1999; cat: Ganan et al., 2009; boar: Kikuchi et al., 1998; mouse: Kishikawa et al., 1999; red deer: Martinez-Pastor et al., 2005a; bull: Martins et al., 2009; dog: Yu and Leibo, 2002).

Kaabi et al. (2003) preserved ram epididymides at 5 °C, finding good sperm viability until 48 h post-mortem, although their in vitro fertility potential declined significantly after 24 h. Similarly, Martinez-Pastor et al. (2005b) found that deer sperm quality obtained from the epididymis was mostly maintained for the first two days.

In studies examining the conservation of epididymal sperm, the epididymides are usually transported to the laboratory from the site of slaughter at 5 °C. In this period, alterations in the physical environment of the epididymal sperm may cause a loss in sperm quality. Histological examination of mouse epididymides revealed that distinct degenerative changes did not occur until 12 h postmortem (Songsasen et al., 1998) when the epithelial cells became pyknotic and released their intracellular contents into the lumen of the epididymides. By 24 h postmortem, the structure of the epididymal tubule appeared to be breaking down. Martinez-Pastor et al. (2005b) found that osmolality of the epididymal media in red deer increased with postmortem time.

If the epididymis changes its structure after 12 h postmortem, it is possible that the epididymal microenvironment then begins to be harmful to sperm. We hypothesized

that extracting the spermatozoa in the field, refrigerating them either undiluted or extended, and keeping them so for several days post-mortem might represent an advantage, comparing to the traditional methodology of storing the whole epididymes. Therefore, these could be alternative strategies for maintaining the quality of post-mortem samples, when immediate cryopreservation is not possible (Sankai et al., 2001; Fernandez-Santos et al., 2009).

The objective of this study was to analyse the effect of storage at 5 °C on the quality of ram epididymal sperm by: (1) evaluation of spermatozoa held in the epididymal fluid and stored either in the organ or in vitro, (2) analysis of epididymal spermatozoa extended in the same medium at different osmolalities.

3.3 Material and Methods

3.3.1. Reagents.

All the products were obtained from Sigma (Madrid, Spain), except for the SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1 fluorescence probes, which were acquired from Invitrogen (Barcelona, Spain).

3.3.2. Animal and sample collection.

Testes were collected from fifteen rams (Churra breed) after slaughter, and transported in a refrigerated cooler (5 °C) to the laboratory (University of León) within the first 2–4 h post-mortem. In a cold room, the caudae epididymides were isolated, the superficial vessels were cut to reduce blood contamination and the organ was kept at 5 °C.

3.3.2.1. Experimental design.

Two experiments were carried out. In experiment 1, we compared sperm quality, osmolality and pH among intra-epididymal and undiluted extra-epididymal storage (9 + 9 epididymides). In experiment 2, we tested the effect of dilution of the epididymal spermatozoa in three extenders of increasing osmolality (320, 370 and 420 mOsm/kg), using intra-epididymal stored samples as the control group (6 epididymes for control and 6 for dilution in the three extenders). These three osmolalities were chosen

according to previous experiment and represent a hypo-osmotic, iso-osmotic and hyper-osmotic medium, respectively, comparing to the physiological osmolality of the caudal epididymal fluid (experiment 1: the mean value is 365 (isotonic value), with a range of distribution of 355 (25th percentile) to 381 (75th percentile)).

3.3.2.2. Sample collection.

In each cauda epididymes, two symmetrical parts were isolated by a clamp. Experiment 1: one part was maintained without manipulation and the sperm mass was obtained at the different sampling times (0, 24, 48 and 72 h) performing sequential cuts in defined areas of the surface with a scalpel (Intra epididymal sample, In-EPID). The other portion was processed by cuts to obtain the total sperm mass in a glass tube where it was kept undiluted (Extra epididymal sample, Ex-EPID) and samples were obtained sequentially in different time periods (24, 48 and 72 h).

Experiment 2: one part of each cauda was kept as the control (In-EPID) and was manipulated as described in the previous experiment. In the other portion, the total sperm mass was obtained by cuts and this sample was aliquoted and diluted with the same volume of each TTF media (TES-Tris-fructose, pH 7.2, 20% egg yolk and 8% glycerol) with different osmolalities (320, 370 and 420 mOsm/kg, obtained by varying fructose). These diluted aliquots were coded as D320, D370, D420, respectively. Each aliquot was sampled sequentially for analysis each 24 h (24, 48 and 72 h). TTF media containing glycerol, since the epididymal spermatozoa were prepared for freezing.

Between sampling times, the portions of epididymides used for the control samples (In-EPID) were wrapped with gauze moistened with saline, and put inside a plastic bag, which was stored in a refrigerator at 5 °C. At each sampling time (at 24, 48 and 72 h), we recorded the osmolality and the pH of the different samples obtained from the cauda epididymis (In-EPID and Ex-EPID). Tubes with semen were sealed with Parafilm and kept at 5 °C in a refrigerator until sampling.

3.3.3. Evaluation of sperm quality.

Immediately after collection, volume was recorded and sperm concentration was assessed using Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) and

evaluating the information through CASA (Sperm Class Analyzer; Microptic, Barcelona, Spain). In experiment 1, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany) and the pH value was determined by a CG 837 pHmeter (Schott Instruments, Main, Germany).

Motility: the motility was analyzed out using a computer-assisted sperm analysis system (CASA) (Sperm Class Analyzer; Microptic, Barcelona, Spain). A 5-µL drop was taken from each of the different sampling tubes (In-EPID, Ex-EPID, D320, D370, D420), and placed in a Makler counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at 10× (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard settings were set at 25 frames/s, 20–90 μm^2 for head area and VCL > 10 µm/s to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analyzed afterwards. Reported parameters were curvilinear velocity (VCL, µm/s) and linearity (LIN, %). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 µm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 µm/s and STR > 80% (straightness, also provided by the system).

3.3.4. Viability and acrosome status.

These parameters were assessed simultaneously using fluorescence probes and flow cytometry. Briefly, samples were diluted in PBS at 5×10^6 spermatozoa/mL and incubated for 15 min with 24 µM of propidium iodide (PI) and 1 µg/mL of PNA-FITC (peanut agglutinin). PI stains membrane-damaged spermatozoa red, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (non-viable sperm, intact acrosoma), green (viable sperm, damaged acrosome) red and green (non-viable sperm, damaged acrosome) or not-stained (viable sperm, intact acrosome).

To evaluate sperm viability, the double stain SYBR-14/PI was used (LIVE/DEAD Sperm Viability Kit: Invitrogen, Barcelona, Spain). Sperm samples were diluted with PBS down to 5×10^6 spermatozoa/ml, and incubated with 24 µM PI and 1.5 100 nM SYBR-14. The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to live spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red).

Evaluation of flow cytometer parameters was carried out using a FACScalibur flow cytometer (Becton Dicknson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Data corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10,000 spermatozoa were recorded.

3.3.5. Statistical analysis.

Statistical analyses were carried out using the R statistical package (http://www.r-project.org). Data were fitted to linear mixed-effect models by maximizing the log-likelihood (ML method). Male was always included as random effect and time (covariate) and treatment (factor with levels: storage in the epididymis and in extender with different osmolalities) as fixed effects. Results are given as mean ± SEM.

3.4 Results.

3.4.1. Experiment 1: comparison of intra-epididymal versus undiluted extra-epididymal conservation

3.4.1.1. Osmolality and pH of epididymal samples.

The osmolality of the In-EPID sample increased with post-mortem time (Fig. 1). At 0 h, osmolality was 365 ± 6 mOsm/kg, which increased to 375 ± 8, 403 ± 10 and 421 ± 12 at 24, 48 and 72 h, respectively ($P < 0.0001$). However, the Ex-EPID samples stored undiluted in a glass tube did not show any significant differences with post-mortem time (365 ± 5 mOsm/kg at 0 h and 369 ± 6 at 72 h). Similarly, pH did not vary significantly in the In-EPID samples (6.29 at 6.40,) or in the undiluted Ex-EPID ones (6.34 at 6.48) (Fig. 1).

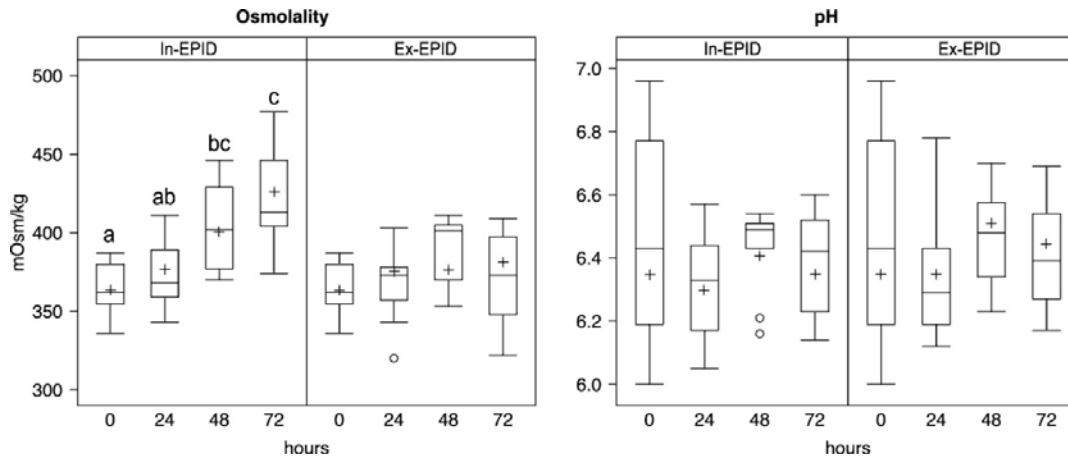

Figure 1. Osmolality and pH of the epididymal samples, Intra-epididymal (In-EPID) and Extra-epididymal undiluted (Ex-EPID), at each sampling time (0, 24, 48 and 72 h). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median. The whiskers reach the maximum and the minimum values of the range. The mean is shown with a cross. Within each treatment, sampling times not sharing letters differ significantly ($P < 0.05$).

3.4.1.2. Spermatozoa quality.

The results of this experiment are shown in Table 1. After 24 h post-mortem, the In-EPID samples yielded higher motility (TM, PM, VCL and LIN) than the Ex-EPID ones stored undiluted in a glass tube ($P < 0.05$). These differences were not observed at 48 and 72 h. Linearity (LIN) among these storage methods at any time. The spermatozoa stored in the epididymis showed higher viability and lower damaged acrosomes, being significantly different to the samples stored in glass tubes at 48 h ($P < 0.05$).

For the Ex-EPID sample, TM, PM and VCL decreased significantly at 24 h in comparison with the control, VIAB decreased at 48 h, and LIN at 72 h; ACR increased significantly at 48 h in comparison with the control. In the In-EPID samples, TM decreased significantly at 48 h, and PM, VCL and VIAB decreased only at 72 h in comparison with the control. LIN and ACR did not significantly differ in these samples.

3.4.2. Experiment 2: effect of extender osmolality in diluted epididymal spermatozoa.

The total motility of the spermatozoa in the diluted samples was higher than in the other treatments at 24 h (Fig. 2); thus, the TM of D370 was significantly higher than in the

control sample stored in epididymis. There were no significant differences among extenders for other motility parameters at 24 h. At 48 h post-mortem, only LIN was different among treatments, with the control samples being significantly higher (54.3 ± 3.5%) than D370 (41.8 ± 3.7%) and D420 (38.1 ± 3.6%). At 72 h post-mortem, motility (TM, PM and VCL) in the samples stored in D320 extender was significantly higher than in the other extenders.

Table 1. Comparison of the two storage methods at 5 °C (Intra-epididymal -In.EPID- versus undiluted extra-epididymal -Ex-EPID-) during 24, 48 or 72 h post-mortem in comparison with a control (0 h). Results are shown as mean ± SEM.

	0 h	24 h		48 h		72 h	
		In-EPID	Ex-EPID	In-EPID	Ex-EPID	In-EPID	Ex-EPID
TM	67.0±8.6^{A}	49.2±7.6^{aAB}	27.6±6.5^{bB}	42.2±9.4^{B}	25.7±7.6^{B}	32.8±7.1^{B}	23.9±4.3^{B}
PM	32,2±5.3^{A}	22.0±5.1^{aAB}	11.1±3.6^{bB}	20.2±6.6^{AB}	10.1±5.7^{B}	11.3±4.0^{B}	5.7±1.2^{B}
VCL	68.8±9.7^{A}	67.2±6.0^{aA}	53.2±6.3^{bB}	57.6±6.4^{AB}	51.1±9.0^{B}	49.1±3.5^{B}	52.9±8.5^{B}
LIN	55.3±4.2^{A}	50.0±3.5^{A}	48.0±2.9^{AB}	50.4±3.7^{A}	44.2±3.7^{AB}	46.2±3.0^{A}	41.7±4.5^{B}
ACR	3.8±0.7^{A}	5.3±2.4^{A}	6.6±1.9^{AB}	3.2±1.2^{aA}	7.7±1.3^{bB}	5.7±1.2^{A}	7.4±2.2^{B}
VIAB	79.7±2.1^{A}	80.7±3.4^{A}	75.6±4.1^{A}	82.3±1.9^{aA}	68.7±4.0^{bB}	68.2±2.6^{B}	62.3±7.0^{B}

TM, total motility (%); PM, % progressive motility; VCL, curvilinear velocity (μm/s); LIN, linearity index (%); ACR, damaged acrosomes (%); VIAB, viability spermatozoa (%).

ab, different letters in the same row indicate that the storage method (In-EPID and Ex-EPID) differs significantly within each storage time.

AB, different letters in the same row indicate that the values for storage time (0, 24, 48 and 72 h) differ significantly within each storage method.

The time of post-mortem storage had no significant effect on TM and PM for D320 and control (epididymis-stored) samples. However, the motility of the spermatozoa stored in D370 or D420 extender decreased significantly in time. The kinetics parameters VCL and LIN showed a reduction only in D370 and D420, being significantly lower at 72 h.

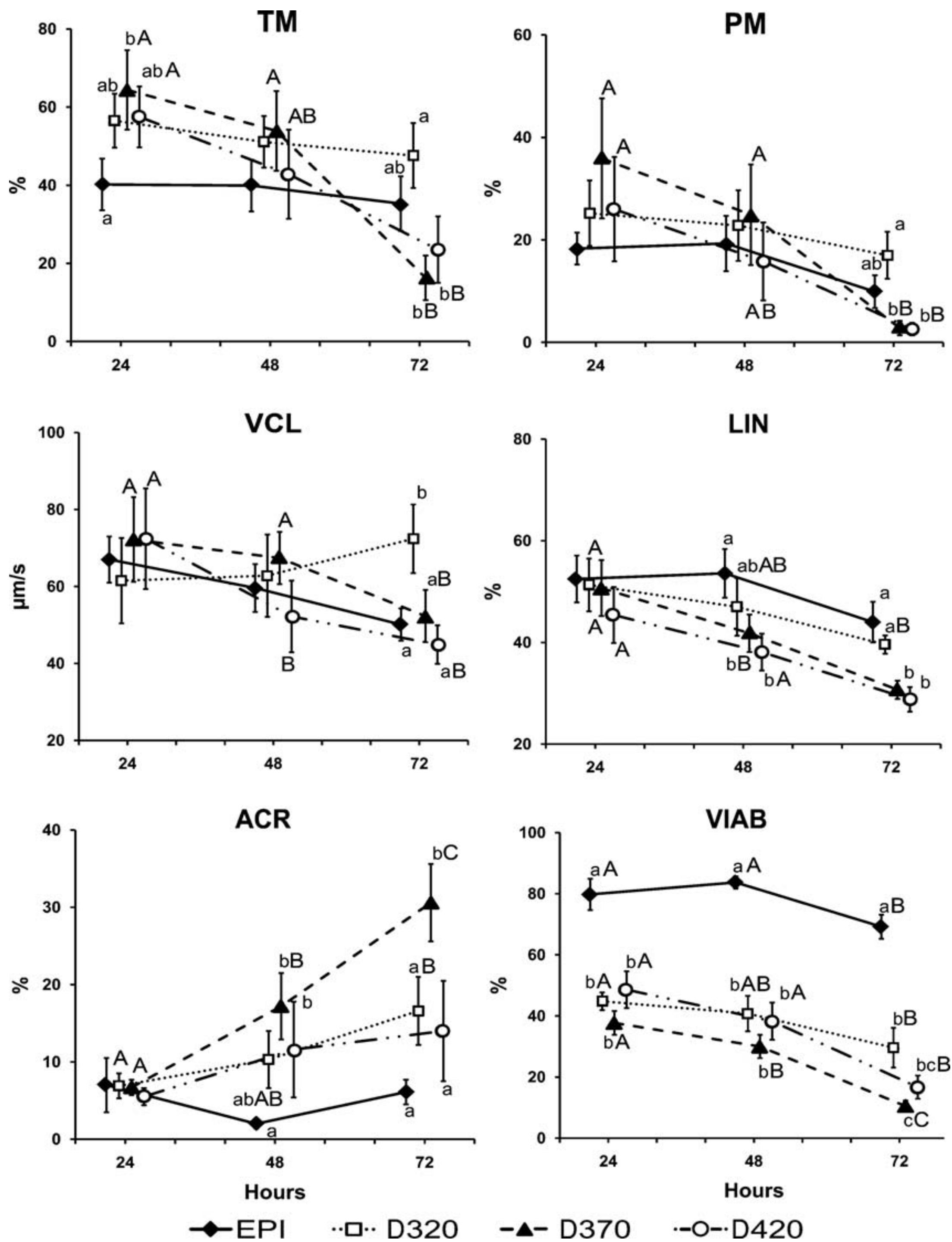

Figure 2. Effects of extenders (D320, D370 or D420) on epididymal sperm quality stored at 5 °C for 24 and 48 and 72 h (mean ± SEM), applying semen from the cauda epididymis (In-EPID) as control. TM: total motility; PM: progressive motility; VCL: curvilinear velocity; LIN: linearity index; ACR: damaged acrosome; VIAB: viability. ab: different letters indicate differences ($P < 0.05$) between extenders for each time period (24 and 48 and 72 h). AB: different letters indicate differences ($P < 0.05$) between storing periods (24 and 48 and 72 h) for each extender.

At 24 h, the proportion of damaged acrosomes has no differences, but sperm viability was considerably higher for epididymis-stored samples (79.8 ± 5.1%) than for the extended samples (between 37.7 ± 3.9 to D370 and 48.6 ± 6.0% to D420). At 48 h and 72 h, the proportion of damaged acrosomes increased for the extended samples, D370 and D420 being significantly higher than the control samples. The viability of spermatozoa declined in the extended samples with increasing storage time.

3.5. Discussion.

Two main conclusions can be extracted from our results. First, quality of ram epididymal sperm is kept storing the spermatozoa in the epididymis at 5 °C, at least for 48 h. Second, an extender of 320 mOsm/kg can preserve the motility of sperm extracted of epididymides but damage to acrosomes is observed.

In this study, we observed that the osmolality of freshly obtained samples, but not the pH, increased in successive sampling times. Therefore, extracting the sample as soon as possible and storing it, could prevent these deleterious changes. Previously Martinez-Pastor et al. (2005b) observed that the osmolality and pH of the semen samples obtained from the epididymides of red deer and roe deer was increased with time post-mortem. Moreover, while collecting the sample from the epididymis, tissue, blood and other fluids can be mixed with the spermatozoa, decreasing their quality (Martinez-Pastor et al., 2006a). This suggest that the presence of tissue debris resulting from the collection of semen may speed up the process of deterioration of stored cells, although this process is equalized after 48 h in our test.

We observed in epidydimal storage a sustained decrease of motility at 48 h and 72 h and a reduction in cell viability at 72 h. Others authors have observed that motility of spermatozoa was adversely affected by post-mortem storage in the epididymis (ram: Kaabi et al., 2003; red deer: Martinez-Pastor et al., 2005c; dog: Yu and Leibo, 2002). However, unlike what was observed in our study, several authors conclude that post-mortem storage does not affect sperm membrane integrity (red deer: Soler et al., 2003b and Martinez-Pastor et al., 2005c; dog: Yu and Leibo, 2002).

The loss of epididymal sperm quality may be related to the fertilizing capacity of sperm. A loss of fertilizing ability of epididymal spermatozoa with post-mortem time has been demonstrated in a few studies. Thus, Kaabi et al. (2003) showed that the in vitro

fertilizing ability of epididymal spermatozoa was similar at 2 h (53%) and 24 h (45%) post-mortem, but decreased significantly at 48 h post-mortem (38%). However, other authors have observed that the fertilizing ability of epididymal spermatozoa decreased quickly with post-mortem time (red deer: Soler and Garde, 2003; mice: Songsasen et al., 1998; mouse: Sankai et al., 2001).

The extension of spermatozoa in a buffered medium could prevent some negative effects after collection, diluting unwanted fluids such as blood or interstitial fluid. In fact, the diluted spermatozoa showed better motility at 24 h post-mortem than the samples stored in the epididymis. Medium osmolality is crucial for the performance of spermatozoa (Yeung et al., 2006), and therefore we expected hyperosmotic media (370 or 420 mOsm/kg) to be more appropriate for storing ram epididymal spermatozoa (with a mean osmolality of 365 mOsm/kg in our study) that a medium isotonic with epididymal fluid. For instance, Si et al. (2009) obtained higher fertility when collecting murine spermatozoa in hyperosmotic medium (415 mOsm/kg, isoosmotic with the epididymal fluid) compared with spermatozoa collected in 290 mOsm/kg as assessed by IVF. Other authors have reported better sperm quality after freezing spermatozoa using moderately hyperosmotic extenders for epididymal spermatozoa (deer: Martinez-Pastor et al., 2006b and Fernandez-Santos et al., 2007; bear: Anel et al., 2010). However, we have observed that D320 extender maintains motility better and induces less acrosomal damage than the hyperosmotic extenders at prolonged post-mortem times (72 h).

The epididymal spermatozoa incubated into a extender showed a higher degree of membrane and acrosomal damage than those stored into the epididymides. Moreover, the loss of sperm viability does not correspond to a reduction in motility at the same storage time and extender. These results suggest that sperm motility and viability are affected by different mechanisms during the cold storage of epididymides.

The ability of extenders to modify sperm membranes, especially when containing egg yolk or milk, has been described previously (Bergeron et al., 2007). While conveying some advantages, storing the spermatozoa in extenders could be triggering changes in membranes or in the general physiology of spermatozoa, which might increase their vulnerability to osmotic stress. It is noteworthy that, in our study, epididymal spermatozoa extended in D370 showed the lower results for sperm viability and acrosomes status, but D420 yielded similar results than D320. This suggests that extender osmolality could ameliorate or worsen the osmotic vulnerability of

spermatozoa and confirms that the osmolality appears to exert a complex effect, as stated by Fernandez-Santos et al. (2007). Therefore, the hypothesis that a slightly hyperosmotic extender could provide the best environment for epididymal ram spermatozoa seems to be disproved and we note that a medium hypotonic to caudal epididymal plasma (D320) might be the best option for the conservation of diluted epididymal spermatozoa in ram. This medium can reduce cellular swelling caused by hypo-osmotic stress when epididymal spermatozoa endure the freezing process.

In conclusion, storing the spermatozoa in the epididymis at 5 °C is a good strategy for maintaining sperm quality in ram, at least for 48 h, and is a better option than extracting the sperm mass and keeping it undiluted. We have also shown that extracting the sperm mass and diluting it in an extender of 320 mOsm/kg can preserve sperm motility at least as well as maintaining it in the epididymis. Nevertheless, the reduction of viability and damage to acrosomes in this extender must be analyzed and remediated improving the extender composition.

3.5.1 Acknowledgements

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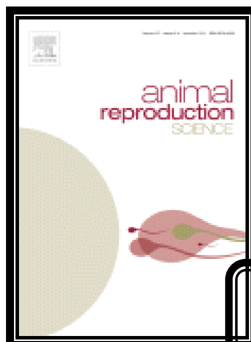

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Capítulo 4

Effect of storage method and extender osmolality in the quality of cryopreserved epididymal ram spermatozoa

Running title: cryopreservation of ram epididymal spermatozoa

J. Tamayo-Canul[1,2], M. Álvarez[1,2], M. Mata-Campuzano[1,3], M. Álvarez-Rodríguez[1,2], P. de Paz[1,3], L. Anel[1,2], F. Martínez-Pastor[1,3*]

[1] ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain, and [2] Animal Reproduction and Obstetrics, University of León, 24071, León, Spain, and [3] Molecular Biology, University of León, 24071, León, Spain

*Corresponding author: +34 9872915679, E-mail: felipe.martinez@unileon.es

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4.1 Abstract

Post-mortem sperm recovery and cryopreservation could be a complement to germplasm banking in sheep, especially for endangered breeds. This study is an attempt to identify factors for improving the success of cryopreserving ram epididymal spermatozoa, considering the decrease of sperm quality with post-mortem time. Epididymal spermatozoa from 9 rams were kept at 5 °C using three storage methods: within the epididymes, undiluted sperm mass, and diluted in extenders of different osmolality (TES–Tris–fructose at 320, 370 or 420 mOsm/kg, 20% egg yolk, 8% glycerol). At 0, 24, 48 and 72 h, spermatozoa were cryopreserved using each extender. Samples were analyzed before and after cryopreservation by CASA (motility) and flow cytometry (viability and acrosomal status). Post-mortem time decreased pre-freezing and post-thawing sperm quality. Some storage × extender combinations improved the effect of post-mortem time on sperm quality. Both epididymis storage combined with the 420 extender, and storing the spermatozoa diluted in the 320 extender improved post-thawing quality, especially at long post-mortem times. Storing the spermatozoa diluted in the 370 extender was detrimental for the acrosomal status. These findings have practical applications. The simplest storage method (within the epididymes) seems to be adequate if hyperosmotic extenders were used for freezing. An alternative method could be storing the spermatozoa diluted in a hypoosmotic extender. These recommendations are limited to the osmolalities tested in this study (420 mOsm/kg and 320 mOsm/kg); other osmolalities should be tested.

Key words: Ram, extender, osmolality, sperm quality, cryopreservation.

4.2 Introduction

Artificial insemination in domestic animals relies almost exclusively on semen obtained by ejaculation (Anel et al., 2003). This is the case of the sheep, most of the doses being obtained by artificial vagina or electroejaculation (Anel et al., 2006). Nevertheless, post-mortem collection could be useful, in the event that a genetically interesting male dies accidentally or must be culled (disease-carriers, physical defects, illness, etc.) (Ehling et al., 2006). For instance, our research group is working with two dairy breeds in

Northwest Spain, Churra and Assaf (143,000 and 153,763 females, respectively, registered in breeding programs), whose genetic improvement programs include the use of post-mortem recovered spermatozoa.

There are many successful reports of collection and cryopreservation of epididymal spermatozoa (buffalo: Lambrechts et al., 1999; dog: Hewitt et al., 2001; ram: Kaabi et al., 2003; boar: Suzuki and Nagai, 2003; red deer: Comizzoli et al., 2001, Soler et al., 2005, Martinez-Pastor et al., 2006a; Spanish ibex: Santiago-Moreno et al., 2006; brown bear: Anel et al., 2011). Ideally, epididymal spermatozoa should be immediately extended and cryopreserved or used for insemination. However, dead males may not be promptly found, genitalia may need to be transported to another facility for processing, or immediate use may not be possible. Kaabi et al. (2003), stored ram epididymides at 5 °C, finding good sperm viability up to 48 h post-mortem, although fertility declined. Using cryopreserved epididymal spermatozoa from the Assaf breed, our group achieved a fertility of 52.8% if frozen before 2 h post-mortem (1054 intrauterine inseminations, unpublished results), similar to cryopreserved ejaculated semen (52.4% in Churra breed; Anel et al., 2003). However, fertility decreased to 46% for samples frozen 24 h post-mortem. Moreover, Martins et al. (2009) showed that bull epididymal spermatozoa maintained an acceptable quality up to 48 h, but fertility (embryo production by IVF) was reduced as soon as 24 h post-mortem. Martinez-Pastor et al. (2005b) found that the quality of epididymal spermatozoa from red deer decreased after the first two days post-mortem. Few studies have analyzed the effect of post-mortem time on the cryopreservation of epididymal spermatozoa. Soler et al. (2005) showed that post-thawing quality was well preserved up to 48 h post-mortem, and Fernández-Santos et al. (2009b) suggested that post-thawing sperm quality could be acceptable up to 96 h post-mortem. Nevertheless, Soler and Garde (2003) showed that the fertility of fresh epididymal spermatozoa (heterologous penetration test) decreased after 12 h post-mortem.

In an attempt to reduce the effect of post-mortem time on the post-thawing quality of ram epididymal spermatozoa, we have aimed at testing different storage methods and extenders. Few studies have focused on comparing storage methods for epididymal spermatozoa (Tamayo-Canul et al., 2011), and there are no reports on their effects on post-thawing quality. Maintaining the spermatozoa in the cauda epididymis have yielded acceptable results in previous studies (Lambrechts et al., 1999 ; Kaabi et al.,

2003 ; Martinez-Pastor et al., 2005b ; Martinez-Pastor et al., 2006a ; Soler et al., 2005 and Anel et al., 2011), but it submits the spermatozoa to a changing environment: disruption of the epididymal tissue (Songsasen et al., 1998), changes in osmolality and pH (Martinez-Pastor et al., 2005b) and changes in components of the epididymal fluid (Jones, 2004). A solution could be storing the sperm mass outside the epididymis – with or without extension. However, spermatozoa are submitted to a higher oxygen pressure outside the epididymis, and the blood and debris concomitant to the extraction could exert a negative effect (Martinez-Pastor et al., 2006a). Diluting the sperm mass immediately after collection with a buffered media containing protective substances (egg yolk) might help protecting spermatozoa, but it might convey disadvantages (osmotic shock, modifications of sperm membrane by egg yolk (Bergeron and Manjunath, 2006), dilution of protective factors from the epididymal medium and long-term exposure to glycerol). Indeed, the collection and extension of epididymal samples might not be advantageous for long-term storage (Fernández-Santos et al., 2009a).

Moreover, the epididymal environment is hyperosmotic comparing to seminal plasma (365 ± 6 mOsm/kg according to Tamayo-Canul et al., 2011). Extender osmolality could influence not only the survival of spermatozoa during the refrigerated storage, but also their resistance to cryopreservation. Studies with red deer (Martinez-Pastor et al., 2006b and Fernández-Santos et al., 2007) showed that epididymal spermatozoa seemed to be better cryopreserved in iso or hyperosmotic extenders (360–430 mOsm/kg).

Therefore, we have tested the effect of three freezing extenders of different osmolalities (320, 370 and 420 mOsm/kg) and three storage methods (epididymis, collected-undiluted and collected-extended) on the loss of quality of ram epididymal spermatozoa throughout post-mortem time. We aimed at obtaining several storage × extender combinations that could improve post-thawing sperm quality after post-mortem storage of ram epididymal spermatozoa.

4.3. Materials and methods

4.3.1. Reagents

All the products were obtained from Sigma–Aldrich Química SA (Madrid, Spain), except fluorescence probes SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1, which were acquired from Invitrogen SA (Barcelona, Spain).

4.3.2. Animals and experimental design

Testes were collected from nine adult rams (Churra breed) just after slaughter, and transported at room temperature (22 °C) to the University of León. Samples were collected from September to November. All procedures were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. Protocols were approved by the ethical committee of the University of León (Spain).

The experimental design is summarized in Fig. 1. The first sperm collection (0 h of experimental time) was carried out within the first 2 h after slaughter. Caudae epididymides were dissected, isolating two symmetrical parts by using a clamp. A sample of epididymal spermatozoa was obtained by performing cuts on one of the parts of the cauda by means of a scalpel.

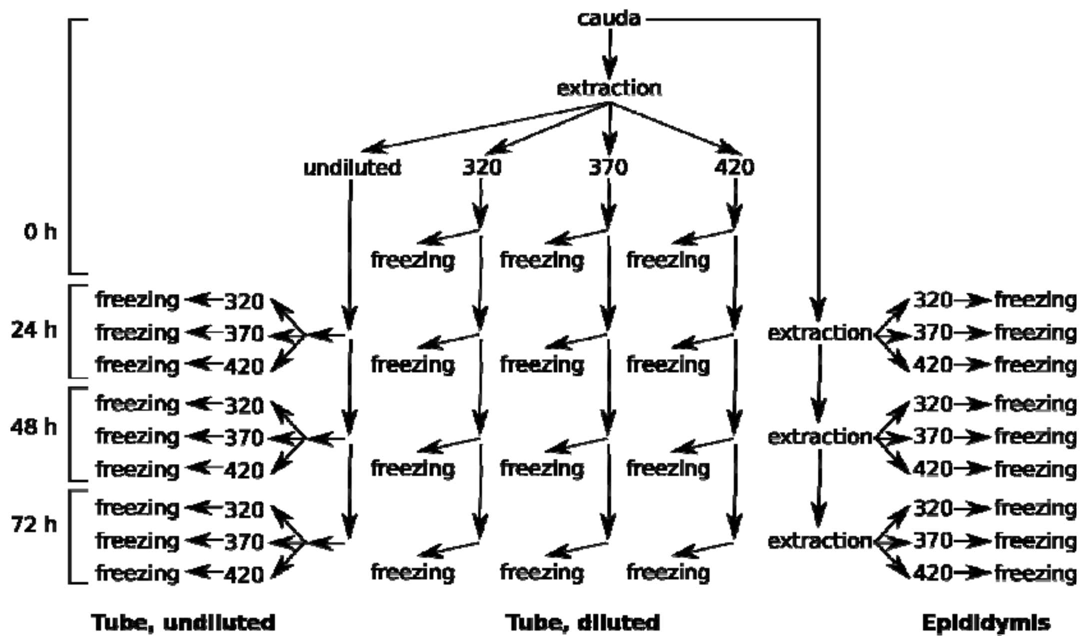

Fig. 1. Experimental design. Samples were stored at 5 °C within the cauda epididymis, undiluted or extended in three extenders with different osmolalities (320, 370 and 420 mOsm/kg, adjusted before adding egg yolk and glycerol). At each time point (0, 24, 48 and 72 h), spermatozoa were cryopreserved using each of the three extenders. Samples were assessed before and after cryopreservation.

The sperm mass obtained at 0 h was split among four aliquots in 10-mL glass tubes. One of them was left undiluted, while the other three were diluted with the same volume of three TTF media (TES–Tris–fructose). These extenders were prepared following instructions for extender M3 in a previous study (Anel et al., 2003). The M3 extender was prepared mixing TES 320 mOsm/kg and Tris 320 mOsm/kg, adjusting the pH at 7.2. Then, a fructose solution at 320 mOsm/kg was added up to 4% of the final volume. In this study, the M3 extender was modified to contain 20% egg yolk and 8% glycerol (more appropriate for epididymal spermatozoa, unpublished data). Moreover, the osmolality of the three TTF media was modified to 320, 370 or 420 mOsm/kg – varying fructose – before adding egg yolk and glycerol. The extenders were clarified by centrifugation (removal of coarse particles from egg yolk). Osmolality was tested using a freezing point osmometer (Osmomat 030, Gonotec, Berlin, Germany). According to a previous study (Tamayo-Canul et al., 2011), 370 mOsm/kg was considered approximately isoosmotic to the epididymal fluid (samples obtained within the first 2–4 h post-mortem).

The epididymides, the tube with the undiluted sperm mass and the three tubes with diluted spermatozoa (320, 370 and 420) were cooled down to 5 °C, and remained at that temperature for the rest of the experiment. An aliquot of the diluted samples was cryopreserved after this cooling step (0 h cryopreservation).

Sperm mass was obtained again at 24, 48 and 72 h, from the untouched half of the caudae. The caudae were handled with care in each extraction, performing small cuts in different locations and trying not to disrupt the external capsule. We took great care to obtain samples as homogeneous as possible between sampling times, and to avoid blood contamination. Between sampling times, the epididymides were wrapped with gauze moistened with saline and put inside a plastic bag, which was stored in a refrigerator at 5 °C.

In each sampling time (Fig. 1), the sperm mass just extracted and an aliquot of the undiluted-stored sperm mass were split, extended with each of the extenders (320, 380, 420) and frozen (see Section 2.4 below). Each of the diluted-stored samples were also extended and frozen.

Sperm quality (CASA and flow cytometry) was assessed after the equilibration time (pre-freezing) and 10 min after thawing (post-thawing).

4.3.3. Sperm cryopreservation and thawing

At each sampling time, we took three aliquots from the sperm mass extracted at that time and other three from the undiluted-stored sperm mass. Each of these aliquots was extended with each of the three extenders (320, 380, 420) at 8% glycerol to achieve a concentration of 200×10^6 mL^{-1}. For the diluted-stored samples, we took an aliquot from each tube and extended them with the same extender, to achieve the same final concentration.

Samples were packed into 0.25-mL plastic straws and equilibrated for 1 h at 5 °C. After the equilibration, the straws were frozen using a programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C. The straws were kept in liquid nitrogen containers for at least one month. Thawing was carried out in a water bath at 65 °C for 6 s.

4.3.4. Motility evaluation.

Motility assessment was carried out using a computer-assisted sperm analysis system (ISAS; V.1.2) (Integrated Semen Analysis System; Proiser, Valencia, Spain). Samples were diluted (10–20 × 10^6 cells/mL) in the same TTF medium that was used for freezing (320, 370 or 420), and warmed on a 37 °C plate for 5 min. Then, a 5-μL drop was placed into a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at ×10 (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard parameter settings were set at 25 frames/s, 20–90 μm^2 for head area and VCL > 10 μm/s to classify a spermatozoon as motile. At least five sequences or 200 spermatozoa were saved and analyzed afterwards. Reported parameters were curvilinear velocity (VCL, μm/s), linearity (LIN, %), and amplitude of lateral head displacement (ALH, μm). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 μm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 μm/s and STR > 80% (straightness, also provided by the system).

4.3.5. Sperm viability and acrosome status.

Viability and acrosomal status were assessed simultaneously using florescence probes and flow cytometry, according to methods described by Martinez-Pastor et al. (2009). Briefly, samples were diluted in PBS at 5 × 10^6 spermatozoa/mL, and incubated for 15 min with 24 μM of propidium iodide (PI) and 1 μg/mL of PNA-FITC (peanut agglutinin). PI stains red membrane-damaged spermatozoa, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (non-viable sperm, intact acrosome), green (viable sperm, damaged acrosome) red and green (non-viable sperm, damaged acrosome) or non-stained (viable sperm, intact acrosome). The proportion of viable spermatozoa (VIAB) and acrosome-intact spermatozoa (ACR) were used in this study.

Evaluation of flow cytometer parameters was carried out using a FACScalibur flow cytometer (Becton Dicknson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Calibration was carried out periodically using standard beads (Calibrites: Becton Dickson). Data

corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10,000 spermatozoa were recorded.

4.3.6. Statistical Analysis.

Unless otherwise stated, data are presented as mean ± SEM and significant values are $P < 0.05$. Statistical analyses were carried out using the R statistical environment (R Development Core Team, 2010). To simplify analysis and interpretation, we assumed that sperm quality was negatively and linearly affected by time, helping to testing if treatments affected the effect of storage time. This assumption was found acceptable for the models tested (using the Akaike Information Criterion – AIC – to compare with alternative models). Therefore, conservation type and extender osmolality were studied in function on their effects on the intercept or slope of this model both before and after cryopreservation. The analysis was performed using linear mixed-effects model (nlme package), with time (treated as a covariate), storage method and extender (and their interactions) in the fixed part of the model. Analyses were carried out with the pre-freezing data and with the post-thawing data, starting with the general model:

$$y_{ij} = \beta_{0i} + \beta_1 \cdot time_{[i]} + \beta_2 \cdot ext + \beta_3 \cdot time \cdot ext_{[i]} + \beta_4 \cdot time \cdot stor_{[i]} + \beta_5 \cdot ext \cdot stor + \beta_6 \cdot time \cdot ext \cdot stor_{[i]} + \beta_7 \cdot male_i + \varepsilon_{ij}$$

where y_{ij} is the observation j in male i, and each β is a vector of coefficients for each covariate, factor or interaction. In this model, both the intercept and the slope of time varied for each male (indicated by the subscript [i] in the terms where time participate), forming the random part of the model. We carried out a model comparison according to the AIC, determining that models following this assumption were more informative that models following the assumption that intercept but no slope varied for each male.

An additional analysis was carried out in post-thawing data including the pre-freezing data as a covariate, in order to identify the influence of the pre-freezing quality on the post-thawing quality. Therefore, helping to assess the effect of time, storage and extender on the freezability of the sample.

4.4. Results

The models obtained for each analysis are summarized in Table 1 and Table 2. In each table, the basic information of the ANOVA table for each model (*F* value, degrees of freedom and resulting *P* value) is shown, with blanks indicating the factors or interactions removed from the models. We obtained complex models for most variables, with many interactions. In order to facilitate the interpretation of the models, Fig. 2, Fig. 3, Fig. 4 and Fig. 5 show the distribution of the real data in the form of box-plots for each time, storage method and extender. For each combination of factors, we overplotted the partial linear models for each storage method and each extender. It is possible to compare the slopes and intercepts of each line (indicating the change of the variable through time) among extenders (different lines within plots) and among storage methods (same lines between plots).

Table 1. Summary of the models selected for analyzing of the response variables within the pre-freezing data. The table shows the *F* value, degrees of freedom and *P* values for each explanatory variable and their interactions, obtained from the ANOVA table of the linear model. Empty cells indicate that the explanatory elements were non-significant and they were removed from the model during its optimization.

		Explanatory variables				
Response variable	Intercept	time	extender	time× storage	extender × storage	time× extender× storage
TM	$F_{1,255}$=527.3 (P<0.001)	$F_{1,255}$=9.2 (P=0.003)		$F_{2,255}$=11.2 (P<0.001)		$F_{6,255}$=3.5 (P=0.003)
PM	$F_{1,259}$=72.7 (P<0.001)	$F_{1,259}$=21.9 (P<0.001)	$F_{2,259}$=6.9 (P=0.001)	$F_{2,259}$=4.7 (P=0.010)		
VCL	$F_{1,259}$=17651.7 (P<0.001)	$F_{2,259}$=3.7 (P=0.027)		$F_{3,259}$=3.7 (P=0.012)		
LIN	$F_{1,261}$=1015.7 (P<0.001)	$F_{1,261}$=19.0 (P<0.001)	$F_{2,261}$=19.0 (P<0.001)			
VIAB	$F_{1,255}$=1100.2 (P<0.001)	$F_{1,255}$=178.2 (P<0.001)	$F_{2,255}$=10.2 (P<0.001)	$F_{2,255}$=18.4 (P<0.001)	$F_{4,255}$=2.4 (P=0.050)	
ACR	$F_{1,249}$=5043.1 (P<0.001)	$F_{1,249}$=277.6 (P<0.001)	$F_{2,249}$=10.6 (P<0.001)	$F_{2,249}$=10.7 (P<0.001)	$F_{4,249}$=3.3 (P=0.011)	$F_{6,249}$=2.3 (P<0.035)

TM: Total motility (%); PM: Progressive motility (%); VCL: Curvilinear velocity (µm/s); LIN: Linearity index (%), VIAB: Viability spermatozoa (%), ACR: Intact acrosomes (%).

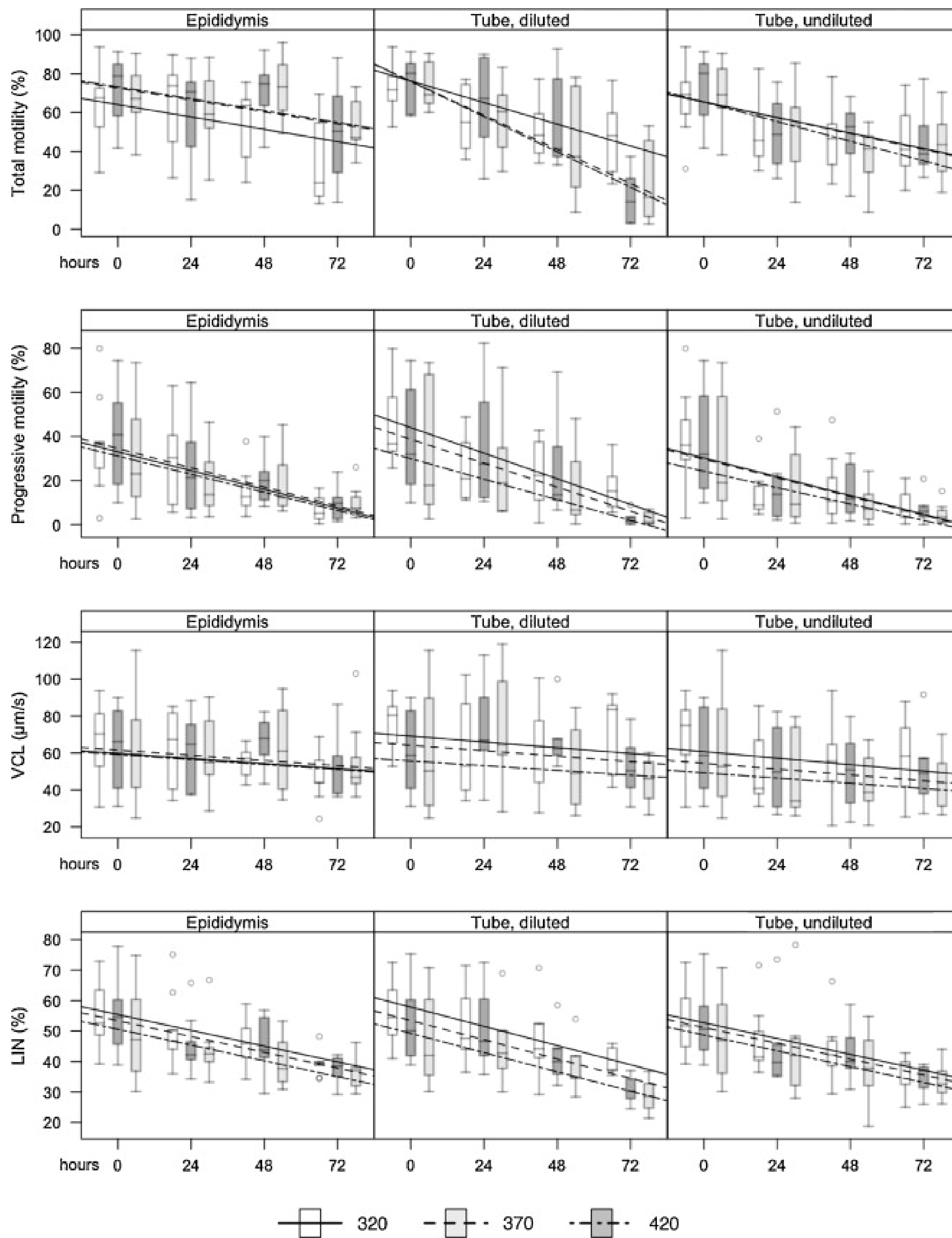

Fig. 2. Motility data yielded by CASA in the pre-freezing assessment. Background box-plots show the distribution of data for each treatment (storage method × extender) and time combination. Boxes span from the 1st to the 3rd quartile, with the inner line showing the median. Whiskers span to 1.5 times the interquartile range, with observations beyond displayed as hollow dots. Lines show the linear estimation of the post-mortem time effect on sperm quality, for each extender within each conservation method (predicted after fitting the actual data). Time significantly decreased motility parameters, except VCL (Table 1). Motility was better preserved by the 320 extender when samples were diluted-stored, but this extender yielded the lowest motility for the samples stored in the epididymis. Extender 370 performed

worse in general, except when samples were stored undiluted in a tube. In general, extending the samples with 320 yielded higher LIN than extending with 420.

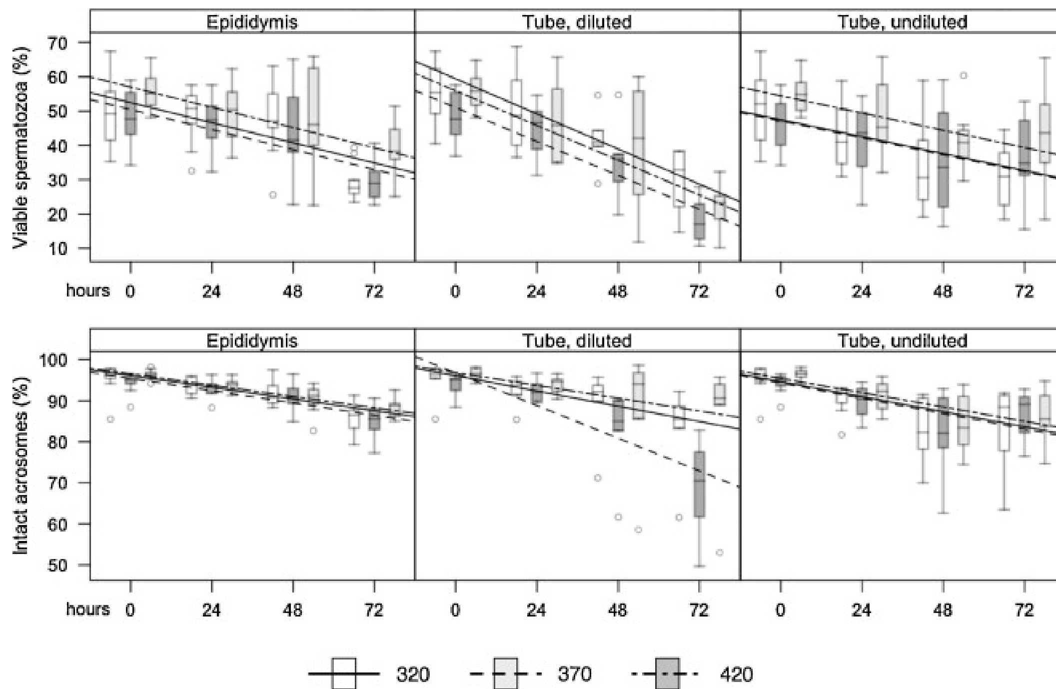

Fig. 3. Viability and acrosomal status yielded by flow cytometry in the pre-freezing assessment. The description of the plots is given in the legend of Fig. 2. Post-mortem time decreased viability in all cases, but the slope was significantly steeper when samples were stored diluted with 370 or 420. Extender 420 yielded a slightly higher viability when combined with the storage in the epididymis or undiluted in a tube, whereas extender 370 yielded lower viability if the samples were diluted-stored. Acrosomal status showed no differences among extenders when spermatozoa were stored in the epididymis or undiluted, but it dropped faster if samples were stored diluted in 370. In general, storing the spermatozoa in the epididymis and diluting with 320 or 420 better maintained acrosomal status at pre-freezing.

Table 2. Summary of the models selected for analyzing of the response variables within the post-thawing data. The table shows the *F* value, degrees of freedom and *P* values for each explanatory variable and their interactions, obtained from the ANOVA table of the linear model. Empty cells indicate that the explanatory elements were non-significant and they were removed from the model during its optimization.

		Explanatory variables					
Response variables	Intercept	time	extender	time× storage	time× extender	extender× storage	time× extender× storage
TM	$F_{1,255}$=741.7 (P<0.001)	$F_{1,255}$=22.4 (P<0.001)		$F_{2,255}$=6.9 (P=0.001)		$F_{6,255}$=2.1 (P=0.052)	
PM	$F_{1,255}$=864.1 (P<0.001)	$F_{1,255}$=47.0 (P<0.001)	$F_{2,255}$=2.6 (P=0.078)	$F_{2,255}$=4.1 (P=0.018)		$F_{4,255}$=3.6 (P=0.072)	
VCL	$F_{1,261}$=895.7 (P<0.001)	$F_{1,261}$=9.2 (P=0.003)		$F_{2,256}$=10.1 (P<0.001)			
LIN	$F_{1,261}$=2194.2 (P<0.001)	$F_{1,261}$=42.9 (P<0.001)	$F_{2,261}$=10.0 (P<0.001)				
VIAB	$F_{1,252}$=2333.2 (P<0.001)		$F_{2,2572}$=5.1 (P=0.006)	$F_{3,252}$=4.3 (P=0.006)	$F_{3,252}$=2.6 (P=0.037)		$F_{3,252}$=2.1 (P<0.051)
ACR	$F_{1,252}$=7984.3 (P<0.001)		$F_{2,252}$=21.9 (P=0.001)	$F_{2,252}$=9.6 (P<0.001)	$F_{2,252}$=3.3 (P<0.020)	$F_{2,252}$=3.3 (P<0.011)	$F_{2,252}$=3.8 (P<0.044)

TM: Total motility (%); PM: Progressive motility (%); VCL: Curvilinear velocity (μm/s); LIN: Linearity index (%), VIAB: Viability spermatozoa (%), ACR: Intact acrosomes (%).

As Fig. 2, Fig. 3, Fig. 4 and Fig. 5 show, sperm quality at pre-freezing and post-thawing decreased with storage time, while the effect of treatment was small. Nonetheless, some treatments stood out because of their higher or lower capacity to maintain sperm quality throughout storage time (described later). Considering the different sampling times, most differences were only noticeable at 48 or 72 h of storage. At 0 h, when the osmolality of the freezing extender was the only experimental factor, only acrosomal status was affected both at pre-freezing (320: 95.2 ± 13%; 370: 94.2 ± 0.8%; 420: 96.4 ± 0.4%; *P* = 0.027) and at post-thawing (320: 82.3 ± 3.7%; 370: 79.4 ± 3.5%; 420: 80.1 ± 3.6%; *P* = 0.025). However these differences were small (see boxplots at 0 h in Fig. 3 and Fig. 5).

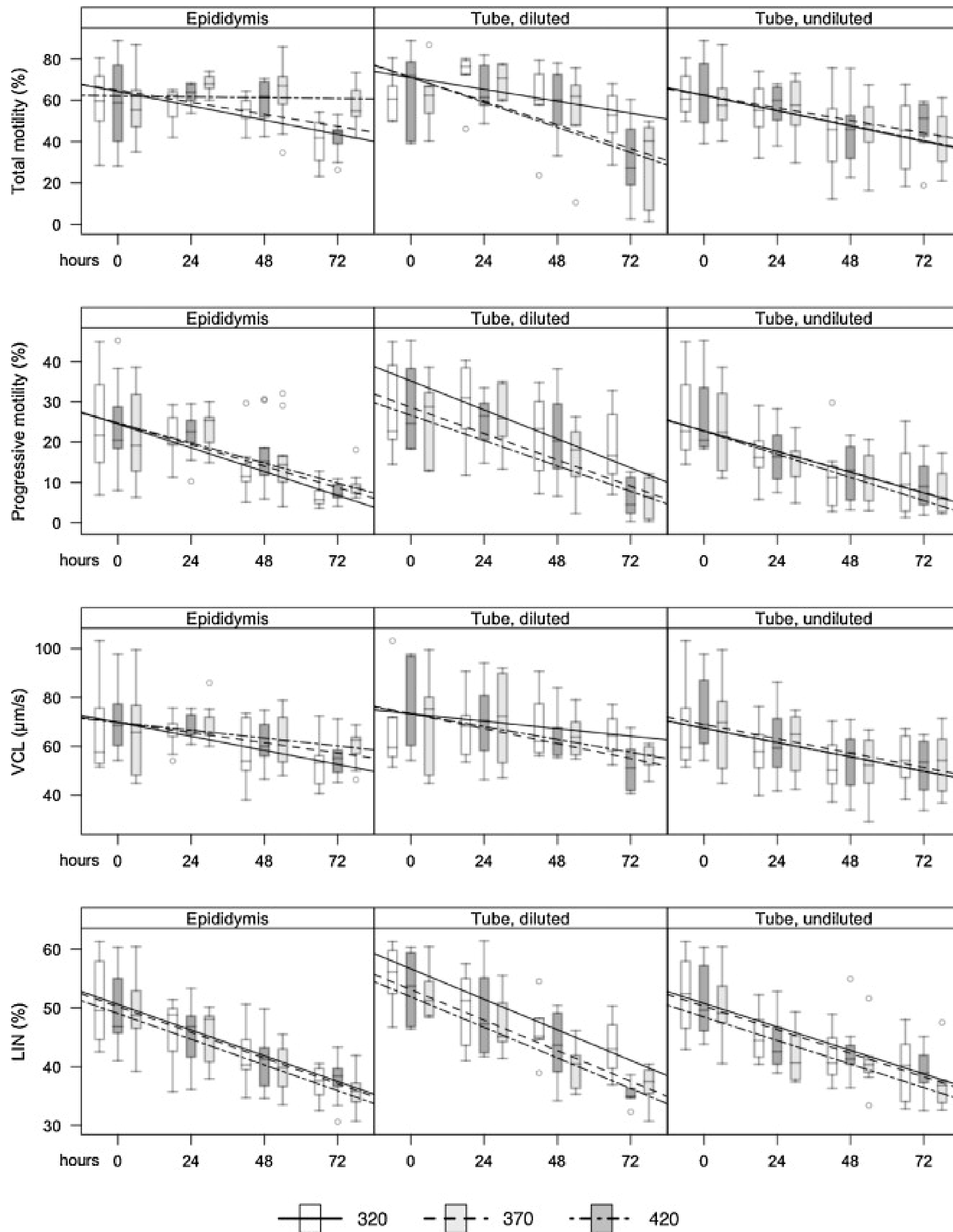

Fig. 4. Motility data yielded by CASA in the post-thawing assessment. The description of the plots is given in the legend of Fig. 2. Total motility post-thawing was maintained throughout post-mortem time by combining epididymal storage and extension in 420, although storing the spermatozoa diluted in 320 yielded similar results. The latter combination (diluted-stored with 320) yielded higher results for the rest of the kinematic parameters.

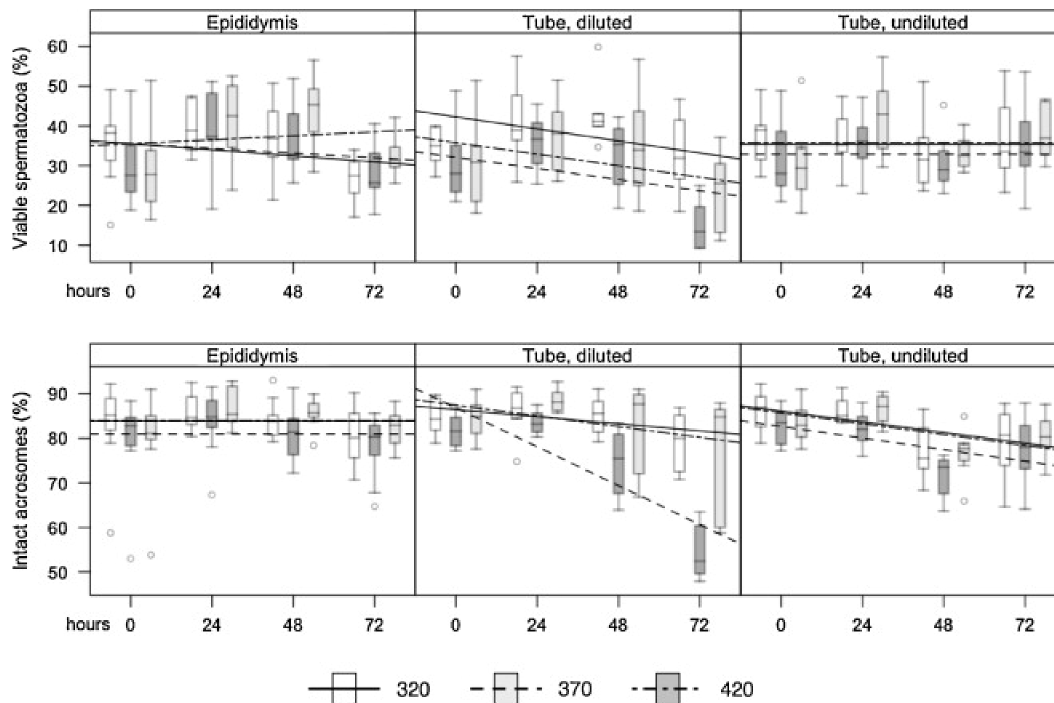

Fig. 5. Viability and acrosomal status yielded by flow cytometry in the post-thawing assessment. The description of the plots is given in the legend of Fig. 2. Post-thaw viability was not significantly affected by storage time or extenders if stored undiluted, and it was only marginally affected (slight decrease with time with improvement by 420) if stored in the epididymis. Viability decreased when stored diluted, but 320 maintained viability similarly to 420 in the other methods. Acrosomal status was not affected significantly by time if stored in the epididymis, whereas storage undiluted in tube slowly decreased acrosomal status (370 slightly worse in both cases). When stored diluted, acrosomal status decreased quickly with extender 370, but it was well preserved if diluted with 320 or 420.

The pre-freezing assessment allowed us to analyze the effect of the storage method and the extender after extending spermatozoa to the final dilution, but without considering cryopreservation. Time had a significant effect on all quality variables (Table 1), as expected in post-mortem storage. The analysis of the different models showed that the quality variables were also influenced by the storage treatments (storage method and extender), although in some cases (TM, VCL), the extender did not have any significant effect. The table shows that storage affected the slope of the model in most cases (interaction with time; expect for LIN), while the effect of the storage method was modulated by the extender (VIAB and ACR) and for TM and ACR a triple interaction was found. These interactions are better visualized in Fig. 2 and Fig. 3. Motility variables (Fig. 2) clearly decreased with storage time in all cases, although it was less

evident for VCL. Total motility underwent a sharper decrease in the samples stored diluted ($P < 0.01$) comparing to the other two methods, except when diluted-stored in 320 (at 72 h, 320: 47.5 ± 3.4% vs. 370: 16.3 ± 2.3% and 420: 23.5 ± 3.5%; $P < 0.01$). Total motility was maintained in the samples diluted-stored with 320 similarly to the samples stored in the epididymis or undiluted. However, when samples were stored in the epididymis, 320 was suboptimal comparing with 370 and 420 ($P < 0.05$; at 72 h, 320: 35.8 ± 2.6% vs. 370: 48.8 ± 3.0% and 420: 53.9 ± 1.6%). There were small differences among storage methods or extenders for the kinematic parameters. VCL was slightly higher in diluted-stored samples extended with 320 (although differences were only evident at 72 h; 72.4 ± 3.6 μm/s vs. overall for others 51.0 ± 0.3 μm/s, $P < 0.05$). LIN decreased with time, being influenced by the extender type (Table 1). In this case, 320 exerted an overall positive effect on LIN, while samples extended with 420 yielded lower LIN.

Flow cytometry parameters of pre-freeze samples are visualized in Fig. 3. Sperm viability was influenced both by extender and storage choice. Viability dropped more sharply when samples were stored diluted ($P < 0.05$), resulting in significantly lower viability at 72 h for 370 and 420, whereas samples diluted-stored with 320 were not significantly different to other storage methods (320: 29.9 ± 1.6% vs. 370: 18.1 ± 1.1% and 420: 22.1 ± 1.2%; overall: 31.7 ± 0.2%). Epididymis and undiluted storage behaved similarly, and extending with 420 kept viability significantly higher than the other extenders in both methods (at 72 h, epididymis: 39.1 ± 0.8% and undiluted: 43.1 ± 1.8%; other extenders: 31.6 ± 0.3%). In the case of acrosomal status, the presence of multiple interactions made the interpretation of the results difficult (Table 1). Thus, when we analyzed samples stored in the epididymis, we found that, despite the overall significance of extender type, when samples were stored undiluted we could not find any difference among the three extenders. When samples were diluted-stored, extending with 370 increased acrosomal damage ($P < 0.05$ after 24 h; at 72 h, 68.8 ± 2.0% vs. 85.7 ± 0.1%, overall for other combinations). Conversely 420 tended to decrease the negative effect of time on acrosomal status ($P = 0.052$). In general, acrosomal status was slightly improved by storing the samples in the epididymis, especially at 48 h (83.7 ± 0.2% vs. 77.5 ± 0.2%, comparing with the other methods, $P < 0.05$), but differences faded at 72 h, excluding samples diluted-stored in 370.

Post-thawing assessment showed significant changes on sperm quality comparing to pre-freezing values. A comparison controlling for treatments indicated a decrease of progressive motility, viability and acrosomal status (mean difference and SEM: −1.6 ± 0.4, −4.2 ± 0.4 and −4.2 ± 0.2, respectively; $P < 0.001$) and an increase of VCL and ALH (mean difference and SEM: −2.2 ± 0.5 and 0.06 ± 0.02, respectively; $P < 0.001$). Nevertheless, both extender and storage choice modulated this decrease with storage time (Table 2). The trends of motility variables with time (Fig. 4) were similar to those of pre-freezing analyses, but we could detect some critical differences. Total motility was better preserved when the samples remained in the epididymis and 420 was used for freezing ($P < 0.05$ at 72 h, 420: 53.9 ± 1.6% vs. 320: 35.8 ± 2.6% and 370: 48.8 ± 3%, overall: 41 ± 0.3%). Interestingly, diluted-stored samples achieved similar total motility when using 320, but not with the other extenders (at 72 h, 47.5 ± 3.4% vs. 370: 16.3 ± 2.3% and 420: 23.5 ± 3.5%). Similarly to the pre-freezing results, storage time caused the decrease of both progressive motility (0 h: 25.0 ± 0.2%; 72 h: 9.0 ± 0.1%) and linearity (0 h: 51.3 ± 0.1%; 72 h: 38 ± 0.1%). Progressive motility was improved by diluted storage and, within this storage method, by using 320 ($P < 0.05$), most noticeably at 72 h (18.7 ± 1.6% vs. 370: 5.9 ± 0.8% and 420: 6.2 ± 0.8%; $P < 0.01$). Contrasting with the pre-freezing assessment, VCL (μm/s) showed a decrease with storage time (0 h: 69.5 ± 0.3; 72 h: 55.5 ± 0.1), which was significantly ameliorated in the combinations epididymis × 420 (72 h: 59.8 ± 0.8) and diluted × 320 (72 h: 64.6 ± 1.5).

Post-thawing viability and acrosomal status (Fig. 5) only underwent small variations with storage time (0 h: 32.2 ± 0.1%, 82.7 ± 0.1%; 72 h: 30.5 ± 0.1%, 77.2 ± 0.1%; overall values for viability and acrosomal status, respectively). The combination diluted × 370 was again the less suitable for both variables (at 72 h, viability: 15.1 ± 1.1% and acrosomal status: 54.4 ± 1.1%). Post-thawing viability did not change significantly with post-mortem time when spermatozoa were stored in the epididymis or undiluted (0 h, epididymis: 31.3 ± 0.4% and undiluted: 33.1 ± 0.4% vs. 72 h, epididymis: 29.4 ± 0.2% and undiluted: 36.7 ± 0.4%), but it decreased if stored diluted (0 h: 32.2 ± 0.5% vs. 72 h: 23.9 ± 0.6%). Moreover, viability was improved by the epididymis × 420 combination ($P < 0.01$ at 72 h, 420: 33.0 ± 0.6% vs. 320: 26.9 ± 0.6% and 370: 28.4 ± 0.8%), and by the diluted × 320 combination ($P < 0.01$ at 72 h, 320: 32.9 ± 1.7% vs. 370: 15.1 ± 1.1% and 420: 23.9 ± 1.7%). In fact, the viability of the diluted × 320

combination was not significantly different in this combination than in the epididymis × 420. The proportion of spermatozoa with intact acrosomes did not change significantly with time when samples were stored in the epididymis or when 320 or 420 were used (within any storage method; 0 h: 82.8 ± 0.1% vs. 72 h: 79.6 ± 0.1%). Acrosomal status decreased with time when samples were stored undiluted ($P < 0.001$; 0 h: 84.1 ± 0.2% vs. 72 h: 79.0 ± 0.3%), although results at 72 h were not different to other methods except for diluted × 370. In all cases, the use of 370 resulted in a significantly lower post-thawing proportion of intact acrosomes, which was more dramatic when spermatozoa had been diluted-stored in 370 (72 h: epididymis: 77.9 ± 0.8%, diluted: 54.4 ± 1.1%, undiluted: 77.5 ± 1.0%; overall except 370: 80.0 ± 0.2%).

When including the pre-freezing values as a covariate in the post-thawing models, the effect of the extender became non-significant for total motility, whereas the effect of the conservation method (interaction with time) became non-significant for viability and acrosomal status. This indicates that the effects of the extender on total motility and conservation method on viability and acrosomal status were exerted pre-freezing, rather than during the freezing-thawing process. Regarding the other factors (Table 2), they remained significant even after including the pre-freezing values as a covariate, suggesting that they might affect not only sperm quality during storage and equilibration, but also the spermatozoa freezability.

4.5. Discussion

Three major conclusions can be extracted from our study, possibly the first one testing storage methods before freezing ram epididymal spermatozoa. First, ram epididymal spermatozoa seem to be resilient cells, which could be stored in different ways while yielding similar quality after cryopreservation. Second, they seem to tolerate well osmotic challenges, and to adapt to media of different osmolality. Third, whenever we detected noticeable differences, they occurred in specific storage × extender combinations, rather than in a specific storage method or extender.

In previous studies on refrigerated storage of epididymal spermatozoa (Tamayo-Canul et al., 2011), we hinted that extender osmolality (prior to addition of egg yolk or glycerol) could have an impact on the storage of epididymal spermatozoa. Deer epididymal spermatozoa yielded higher motility after being cryopreserved with a 380 or

430 mOsm/kg extender rather than with a 320 mOsm/kg extender (Martinez-Pastor et al., 2006b), and similar results were obtained by Fernández-Santos et al. (2007). The osmolality of the epididymal lumen increases from the caput to the cauda epididymis (Cooper and Yeung, 2003), being clearly hyperosmotic in the cauda (deer: 375 ± 8 mOsm/kg (Martinez-Pastor et al., 2005b); ram: 365 ± 6 mOsm/kg at 2 h and 375 ± 8 (Tamayo-Canul et al., 2011); mean ± SEM by 24 h post-mortem). Thus, hyperosmotic extenders could favour sperm cryopreservation, not only because of being isoosmotic to epididymal fluids, but also because they could increase dehydration prior to freezing (Liu and Foote, 1998). Nevertheless, our results did not show any dramatic differences among extenders at 0 h. This suggests that ram epididymal spermatozoa may tolerate a high range of osmolalities undergoing only small changes in pre or post-thawing quality.

Considering storage methods, each of the options that we tested had advantages and disadvantages, as showed in the introduction. Despite these differences, we found a lack of large differences among them. It is possible that the positive and negative effects of each method could compensate themselves, resulting in similar sperm quality pre- and post-freezing. Another explanation, compatible with the previous hypothesis, is that the epididymal samples would be resilient enough to withstand the detrimental effects of post-mortem storage, irrespectively of the storage method. In fact, Fernández-Santos et al. (2009a) tested several storage options for red deer epididymal spermatozoa (epididymis, extended and extended with vitamin C), but, apart from the motility-stimulating effect of vitamin C, differences only appeared at long post-mortem times (96 or 192 h). Larger post-mortem times could have disclosed larger the differences among our treatments, but, for the purposes of the present study, it was impractical.

Notwithstanding the lack of large differences among extenders or storage methods, it was clear that several of their combinations either improved or deteriorated spermatozoa quality (pre and post-thawing) throughout storage time. Thus, 420 improved sperm quality when used to freeze samples stored in the epididymis, while 320 was the most appropriate one for the diluted-stored samples. These two combinations could be considered as the most adequate in the present study, especially at long post-mortem times. Conversely, the diluted-stored method was clearly detrimental whenever 370 or 420 was used to store the samples.

It is difficult to explain the improved performance of the 420 × epididymal storage and 320 × diluted storage combinations. In order to propose an explanation for the first combination, we have to consider that previous studies have demonstrated that the osmolality of the epididymal medium increases with post-mortem time (Martinez-Pastor et al., 2005b and Tamayo-Canul et al., 2011). Thus, epididymal spermatozoa might develop a continuous adaptation while being submitted to an increasingly hypertonic environment. It has been proposed that epididymal spermatozoa undergo intracellular accumulation of osmolytes, which would be useful for facing osmotic challenges upon ejaculation (Cooper and Yeung, 2003 and Yeung et al., 2006). We hypothesise that longer post-mortem times, being concomitant to higher osmolality, would induce these mechanisms. Indeed, extending these spermatozoa in hypoosmotic media (here, 320 extenders) might induce a transient excess of intracellular water, which would render spermatozoa more vulnerable to cryopreservation. Moreover, prolonged storage in the cauda epididymis could cause physiological changes of uncertain consequences (Rodriguez and Bustos Obregon, 1996 and Martinez-Pastor et al., 2005a). Further studies should test this hypothesis, analyzing the changes in sperm osmolytes and cell volume.

The situation of diluted-stored samples was rather the opposite. In this case, the hypoosmotic extender (320) yielded the highest quality. A possible explanation is that, once collected and diluted, epididymal spermatozoa would undergo an adaptation to the new environment, which would be improved by the hypotonicity of the medium. Spermatozoa in the cauda epididymis must contact the hypotonic seminal plasma during ejaculation, and then the much hypotonic (relative to the epididymal medium) female genital tract (Yeung et al., 2006). Epididymal spermatozoa collected shortly after post-mortem might be better prepared to hypoosmotic challenges and to the adaptation to a lower osmolality (Cooper and Yeung, 2003 , Yeung et al., 2006 and Sahin et al., 2009), which might explain the good pre and post-freezing results of samples extended in 320 shortly after the death of the male and stored.

Two questions remain unanswered: why extender choice seems not to be important when the sperm mass is stored undiluted, and why 370 have such a detrimental effect on acrosomal status when samples are diluted-stored. For the first question, we must remind the special status of undiluted-stored spermatozoa, remaining in epididymal medium but not submitted to the osmolality and pH changes of epididymis-stored

spermatozoa. Therefore, undiluted-stored spermatozoa might remain highly adaptable to a wide range of osmotic challenges, thus explaining the similarity of results among extenders after thawing. Nevertheless, undiluted-stored samples did not achieve better post-thawing results, as it would be expected. The second question is also difficult to answer. The acrosome is sensitive to osmotic stress and other challenges (Harrison and Fléchon, 1980). However, acrosomal damage remained very low in all cases, including extenders 320 and 420, which would submit spermatozoa to osmotic stress upon dilution. Therefore, we propose that the 370 extender might cause an osmotic imbalance or modifications in membrane permeability with increasing time, enhancing acrosomal disruption. Further studies are needed.

A caveat must be added. We adjusted the osmolality of the extenders by varying fructose concentration. Spermatozoa can readily use fructose as an energy source, therefore the different fructose concentration might have affected sperm survival beyond its osmotic effects. However, our results suggest that the higher availability of fructose in the 380 and 420 extenders had a minor impact in the study. This makes sense considering that spermatozoa were stored at 5 °C, therefore the metabolic activity would be depressed and fructose may not have been a limiting factor in any extender. Moreover, other methods for varying the osmolality of the extenders would have added more important confounding factors. Increasing the TES/Tris would have modified the buffering capacity of the extenders, possibly impacting the sperm survival at long times. Moreover, using other molecules to increase osmolality would have added confounding factors, making the interpretation of the results more difficult.

In conclusion, this study contributes to the scarce information on the effect of post-mortem time to the post-thawing quality of epididymal spermatozoa. Ram epididymal spermatozoa seemed to endure well the different storage methods and extender osmolalities. However, storing the spermatozoa in the epididymis and then freezing with a hyperosmotic extender (420 mOsm/kg) or collecting the spermatozoa and maintaining them extended in a hypoosmotic extender (320 mOsm/kg) might improve their overall quality and post-thawing results, especially at long times post-mortem. These findings have practical applications, considering the different situations that may involve the preservation of post-mortem samples. In the typical situation, in which a valuable male dies unexpectedly, the scrotum could be simply refrigerated as usual, and these spermatozoa would be frozen using a hyperosmotic extender. However, if samples

were to be sent away for freezing and package volume is important, the sperm mass could be extracted and diluted with a hypoosmotic extender, allowing sending the samples in small tubes; if no extender were available, samples could be simply sent undiluted (taking into account the acceptable results of undiluted-stored samples). Moreover, we can advice not using an extender isoosmotic with the epididymal medium (370 mOsm/kg in this study). A caveat must be added, that these recommendations might be only valid for osmolalities around the ones tested in this study (420 mOsm/kg for the hyperosmotic extenders and 320 mOsm/kg for the hypoosmotic extenders). Extenders with different osmolalities should be appropriately tested.

As described in the introduction, we are currently using epididymal spermatozoa in the production breeds Churra and Assaf, introducing this kind of germplasm source in their genetic improvement programs. The small differences observed in this study could reflect in important changes after applying artificial insemination, which might be critical in the case of spermatozoa from endangered breeds or valuable males.

4.5.1 Acknowledgements

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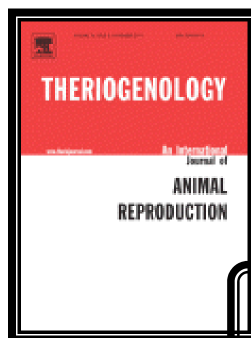

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Capítulo 5

Sperm concentration at freezing affects post-thaw quality and fertility of ram semen

M. Alvarez[1,2], J. Tamayo-Canul[1,2], E. Anel[1,2], J.C. Boixo[4], M. Mata-Campuzano[1,2], F. Martinez-Pastor[1,3], L. Anel [1,2] and P de Paz [1,3].

[1]ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain, and [2]Animal Reproduction and Obstetrics, University of León, 24071, León, Spain, and [3]Molecular Biology, University of León, 24071, León, Spain, and [4]CENSYRA, León, Spain.

Corresponding author:

Felipe Martínez-Pastor

INDEGSAL

Campus de Vegazana

24071 León, Spain

Phone: +34 987291000+5679

e-mail: felipe.martinez@unileon.es

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5.1. Abstract

We have investigated the effect of sperm concentration in the freezing doses (200, 400, 800 and 1600×10^6 mL^{-1}) on the post-thaw quality and fertility of ram semen. Semen was collected from seven adult Churra rams by artificial vagina during the breeding season. The semen was diluted in an extender (TES-Tris-fructose, 20% egg yolk and 4% glycerol), to a final concentration of 200, 400, 800 or 1600×10^6 mL^{-1} and frozen. Doses were analyzed post-thawing for motility (CASA), viability and acrosomal status (fluorescence probes PI/PNA-FITC, SYBR-14/PI and YO-PRO-1/PI). Total motility and velocity were lower for 1600×10^6 mL^{-1} doses, while progressive motility and viability were lower both for 800 and 1600×10^6 mL^{-1}. The proportion of viable spermatozoa showing increased membrane permeability (YO-PRO-1+) rised in 800 and 1200×10^6 mL^{-1}. Intrauterine inseminations were performed with the 200, 400 and 800×10^6 mL^{-1} doses at a fixed sperm number (25×10^6 per uterine horn) in synchronized ewes. Fertility (lambing rate) was similar for semen frozen at 200 (57.5%) or 400x10^6 mL^{-1} (54.4%), whereas it was significantly lower for 800×10^6 mL^{-1} (45.5%). In conclusion, increasing sperm concentration in cryopreserved semen, at least at 800×10^6 mL^{-1} and above, adversely affects the post-thawing quality and fertility of ram semen.

Keywords: Ram, sperm cryopreservation, sperm concentration, sperm quality, fertility

5.2. Introduction

The efficiency of the cryopreservation of ram semen must be improved before widespread application of artificial insemination (AI) in sheep. Acceptable results have been achieved so far using frozen/thawed semen [1–4], but its general use is restricted due to the need of using intrauterine insemination by laparoscopy. Otherwise, AI with frozen semen yields variable and often low fertility results, if applied by vaginal-cervical insemination [4–10]. Another disadvantage of vaginal AI is the high number of spermatozoa required per insemination (100-400×10^6 spermatozoa/dose), whereas laparoscopic AI requires lower sperm numbers (25-50×10^6 spermatozoa/dose) [4,11,12].

In fact, the effect of sperm dose in the cryopreservation of ram semen has been little explored. To our knowledge, the only study was performed by D'Alessandro et al. [11],

who tested two types of diluents (milk-lactose-egg yolk and Tris-fructose-egg yolk), freezing at six different sperm concentrations (50, 100, 200, 400, 500 and 800×10^6 mL^{-1}). They found a variable sperm quality among 50 and 500×10^6 mL^{-1}, but freezing at 800×10^6 mL^{-1} clearly lowered it. They also performed laparoscopic intrauterine insemination with thawed semen, but they did not achieve significant figures. That study showed that freezing ram spermatozoa at concentrations much higher than those used as standard (800×10^6 mL^{-1}) could be detrimental. However, these authors did not reach to definitive conclusions, possibly due to the lack of power in their analyses and to the presence of confounding factors. Although their results suggest a negative effect of increasing sperm concentrations, that trend was not clear. Several studies in different species support this hypothesis. Nascimento et al. [13] evaluated stallion semen doses frozen at different concentrations: 100, 200 and 400×10^6 mL^{-1}, in 0.5 ml and 0.25 ml straws. Those authors found that sperm motility decreased with sperm concentration. Similarly, Peñan and Linde-Forsberg [14], evaluated the effect of freezing dog semen at four different sperm concentrations (50, 100, 200, and 400×10^6 mL^{-1}), in 0.5 ml straws finding sperm motility and viability after thawing was significantly lower in samples frozen at 400×10^6 mL^{-1}.

Increasing the sperm concentration might improve vaginal AI in sheep, by allowing more spermatozoa per dose. Paradoxically, this increase could drive to the opposite effect, if high sperm concentration at freezing would decrease sperm quality. Therefore, we aim at confirming and improving D'Alessandro findings. It is important to confirm and enhance these findings, in order to improve sheep AI. Thus, the objective of this study is to assess the post-thawing sperm quality and fertility of ram semen frozen in different concentrations (200, 400, 800 and 1600×10^6 mL^{-1}) with a possible practical use for AI in sheep. In this study we have tried to avoid confounding factors — equalizing the number of spermatozoa inseminated—, and we have used sensitive techniques (CASA and flow cytometry), in order to reach more definitive conclusions, and the fertility study was carried out using sheep groups large enough to attain a high statistical power.

5.3. Materials and methods.

5.3.1 Reagents

Reagents were obtained from Sigma (Madrid, Spain), except fluorescence probes SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1, which were acquired from Invitrogen (Barcelona, Spain).

5.3.2. Animals and sperm collection.

We used seven adult males (2-9 years old) of the Churra breed, of proven fertility and trained for semen collection by artificial vagina. Ejaculates were collected by artificial vagina at 40 °C (Minitüb, Tiefenbach, Germany), and the tubes were maintained at 35 °C during the initial evaluation of semen quality. The volume was estimated by using the graduation marks of the collection tube. Mass motility was assessed by microscopy (warming stage at 37 °C, ×40; score: 0-5; Labophot 2, Nikon, Tokyo, Japan), and the sperm concentration was assessed by the photocolorimetric method at 540 nm (Spectronic 20, Baush & Lomb, Madrid, Spain), on a specific calibrated scale. Only ejaculates of good quality were used and frozen (volume: ≥0.5 mL; mass motility: ≥4; sperm concentration: $\geq 3000\times10^{6}$ mL^{-1}).

The seven males yielded 18 good-quality ejaculates, which were divided into four aliquots and frozen at 4 different sperm concentrations (200, 400, 800 and 1600×10^{6} mL^{-1}), obtaining a total of 679 straws. Semen collection was performed from September to November (within the breeding season, which spans from July to December). Four males yielded three good-quality ejaculates, whereas and the remaining three yielded two good-quality ejaculates.

5.3.3. Cryopreservation protocol

Semen was diluted with the same volume (1:1) of freezing extender. The freezing extender was of own design (UL) [3], consisting of a TTF medium (TES-Tris-fructose, 320 mOsm/kg, pH 7.2) supplemented with 10% egg yolk and 4% glycerol. The sample was then refrigerated in a cold room at 5 °C for an average of two hours, until the samples reached a temperature of 5 °C. At that point, the sample was divided among four tubes, to whom more extender was added to obtain a concentration of 1600, 800, 400 or 200×10^{6} sperm/ml. Samples were packed into 0.25-mL plastic straws and

equilibrated for 1 h at 5 °C. Then, the straws were frozen using a programmable biofreezer (Kryo 10 Series III; Planer plc. Sunbury-On-Thames, UK) using a rate of -20 K/min down to -100 °C. The straws were kept in liquid nitrogen containers and stored for a minimum of two months until analysis. Thawing was carried out in a water bath at 65 °C for six seconds. Sperm quality parameters were evaluated immediately after thawing.

5.3.4. Spermatozoa evaluation

The assessment of motility parameters was carried out using a computer-assisted sperm analysis system (CASA) (ISAS v. 1.1; Proiser, Valencia, Spain). Samples were diluted ($10–20\times10^6$ cells/ml) in the same TTF medium with 320 mOsm/kg, and warmed on a 37 °C plate for 5 min. Then, a 5-µL drop was placed into a Makler counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at ×10 (negative phase contrast) in a microscope (Eclipse E400, Nikon) with a warmed stage (38 °C). The standard parameter settings were set at 25 frames/s, 20 to 90 μm^2 for head area and VCL > 10 µm/s to classify a spermatozoon as motile [15]. At least five sequences or 200 spermatozoa were saved and analyzed afterwards. Reported parameters were curvilinear velocity (VCL, µm/s), linearity (LIN, %), and amplitude of lateral head displacement (ALH, µm). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 µm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 µm/s and STR > 80% (straightness, also provided by the system).

5.3.5. Sperm viability and acrosome status

Viability and acrosomal status were assessed simultaneously using florescence probes and flow cytometry, according to methods described previously [16]. Briefly, samples were diluted in PBS at 5×10^6 spermatozoa/ml, and incubated for 15 min with 24 µM of propidium iodide (PI) and 1 µg/ml of PNA-FITC (peanut agglutinin). PI stains membrane-damaged spermatozoa red, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (non-viable sperm, intact acrosome), green (viable sperm, damaged acrosome) red and green (non-viable sperm, damaged acrosome) or not-stained (viable sperm, intact acrosome).

As a caveat, the PNA-FITC stain may have a low percentage of false-negatives, since spermatozoa with a completely lost acrosome cannot be stained by PNA-FITC. The percentage of spermatozoa with damaged acrosomes (ACR) was calculated as the sum of viable and non-viable PNA+ spermatozoa.

To evaluate sperm viability, we used the double stain SYBR-14/PI. Sperm samples were diluted with PBS down $5x10^6$ sperm/ml, and incubated for with 24 µM PI and 100 nM SYBR-14. The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to live spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red).

YO-PRO-1/PI was used distinguish three populations of sperm: sperm nucleus with red fluorescence (PI+, dead), spermatozoa with green nucleus indicating intracellular YO-PRO-1 (increased membrane permeability) and unstained spermatozoa (viable). The diluted sample was stained with 100 nM of YO-PRO-1 and 24 µM of PI, and then incubated at 37 °C for ten minutes before being analyzed by flow cytometry. In this analysis, we also calculated the ratio (RATIO) among the proportion of spermatozoa with increased membrane permeability (PI-/YO-PRO-1+) and the proportion of PI-spermatozoa (sum of YO-PRO-1- and YO-PRO-1+).

Evaluation of flow cytometer parameters was carried out using a FACScalibur flow citometer (Becton Dicknson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Calibration was carried out periodically using standard bead (Calibrites: Becton Dickson). Data corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10,000 spermatozoa were recorded for each stain combination.

5.3.6. Insemination procedures

A total 762 adult Churra ewes were used and distributed into three experimental groups (200, 400 and 800×10^6 mL^{-1}) during the breeding season. The 1600×10^6 mL^{-1} treatment showed a clear detrimental effect in the in vitro tests, and it was not included in the fertility trials. The ewe number was estimated through a power analysis, taking into account D'Alessandro et al. [11] results, to detect a difference of at least 13 points in fertility rates, with a statistical power of 0.9 and a signification level of 0.05 (total number of females: 745). These females were subjected to treatment for oestrus

induction and synchronization using intravaginal sponges with 40 mg fluorogestone acetate for 14 days. Then, the sponges were removed and the ewes were treated with 500 IU of eCG (i.m.). Laparoscopic inseminations were performed by two experienced technicians between 62 and 64 hours after the removal of the sponges. The animals, having fasted for the previous 24 hours, were placed on a special cradle (IMV®) adjusted at an inclined plane (45°). The abdominal area in front was shaved and cleaned. Then, two portals (for vision and manipulation/injection) were inserted by performing a pneumoperitoneum (CO_2). The semen, placed in a special applicator (transcap, IMV®), was injected under visual inspection into both uterine horns (0.12 mL per horn). Sperm concentration was equalized to 200×10^6 mL^{-1} just before insemination, using freezing extender, thus 25×10^6 spermatozoa were applied per horn in the three treatments. Pregnancy was diagnosed by ultrasound scan at 35-38 day after insemination. Lambing ewes were noted according to the births registered at 137-154 day post-insemination (lambing rate).

5.3.7. Statistical analysis

Statistical analyses were carried out using the R statistical package, version 2.13.0 (http://www.r-project.org). Data were fitted to linear mixed-effect models (lmer package) by maximizing the log-likelihood (ML method) [17]. Sperm concentration (four levels) was included in the fixed part of the model, whereas male and ejaculate within male were included in the random part of the model. A pairwise comparison among sperm concentrations was performed whenever the effect of sperm concentration was significant, using Tukey contrasts. Fertility results were analyzed by logistic regression. Odd ratios (OR) and 95% confidence intervals (CI) were generated during the logistic regression. Results are given as mean±SEM.

5.4. Results

The results of this experiment are showed in the Table 1. The CASA parameters TM, PM and VCL did not show significant differences between the concentrations 200, 400 and 800×10^6 mL^{-1}, but the highest concentration used in this experiment (1600×10^6 mL^{-1}) yielded significantly lower results (TM and PM: $P<0.001$ for 200, 400 and 800

vs. 1600; VCL: P=0.024 for 200 vs. 1600, P=0.002 for 400 vs 1600, and P<0.001 for 800 vs. 1600). Mean values of LIN and ALH were not significantly different among concentrations.

Table 1. Post-thawing motility (CASA) for the four sperm concentrations (200, 400, 800 and 1600×10^6 mL^{-1}). Results are shown as mean±SD.

Motility variables	Concentration (×10^6 mL^{-1})			
	200	400	800	1600
TM	65.2±4.5^a	63.4±3.5^a	58.3±4.2^a	41.3±4.6^b
PM	39.7±3.5^a	35.9±2.4^{ab}	33.0±2.9^b	22.2±2.4^c
VCL	120.3±4.0^{ab}	123.7±5.1^{ab}	126.8±4.6^a	107.6±3.3^b
LIN	63.5±1.6	62.3±1.7	63.0±1.5	59.2±1.2
ALH	3.3±0.1	3.3±0.1	3.3±0.1	3.1±0.1

TM: Total motility (%); PM: % Progressive motility; VCL: Curvilinear velocity (µm/s); LIN: Linearity index (%), ALH: Amplitude of the lateral head movement (µm). a,b, different superscripts in the same row indicate significant differences among concentrations (P<0.05).

The analysis of physiological parameters using fluorescence probes showed that the highest concentration (1600×10^6 mL^{-1}) yielded significantly lower viability (figures 1, 2 and 3) and a higher "apoptotic ratio" (Figure 3), comparing to the other concentrations. Contrarily, the proportion of damaged acrosomes (ACR) was little affected by sperm concentration, with no significant differences detected among concentrations (Figure 1).

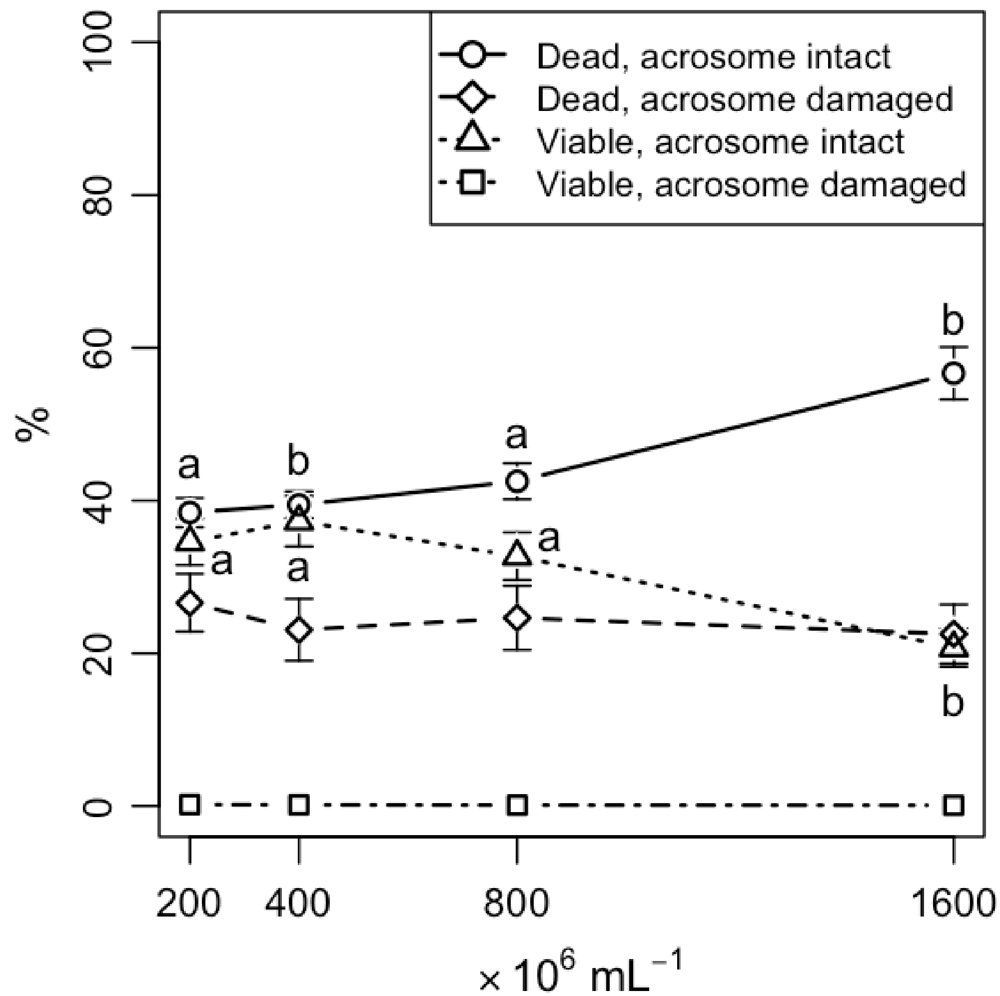

Figure 1. Mean±SEM of four sperm populations obtained from PNA-FICT/PI stain for each of the four concentrations tested, analyzed after thawing: Dead with intact acrosome (PI+/PNA-); dead with damaged acrosome (PI+/PNA+); viable with inctact acrosome (PI-/PNA-); viable with damaged acrosome (PI-/PNA). Different letters indicate differences between sperm concentrations for each sperm population ($P<0.05$).

The proportions of the subpopulations obtained from the SYBR-14/PI and YO-PRO-1/PI stains varied among concentrations, as showed in figures 2 and 3. The viable subpopulation according to both stains showed its highest value for 400×10^6 mL^{-1}, being 800 and 1600×10^6 mL^{-1} significantly lower, especially the latter. Interestingly, while the percentages of dead (PI+) spermatozoa showed little changes among concentrations (being significantly higher for 1600×10^6 mL^{-1}), the percentages of moribund (SYBR-14+/PI+) and spermatozoa with increased membrane permeability

(YO-PRO-1+/PI-) increased significantly when freezing at 800×10^6 mL^{-1} (200×10^6 mL^{-1}, P=0.048; 400×10^6 mL^{-1}, P=0.036) and 1600×10^6 mL^{-1} (P<0.001 comparing with the other three concentrations).

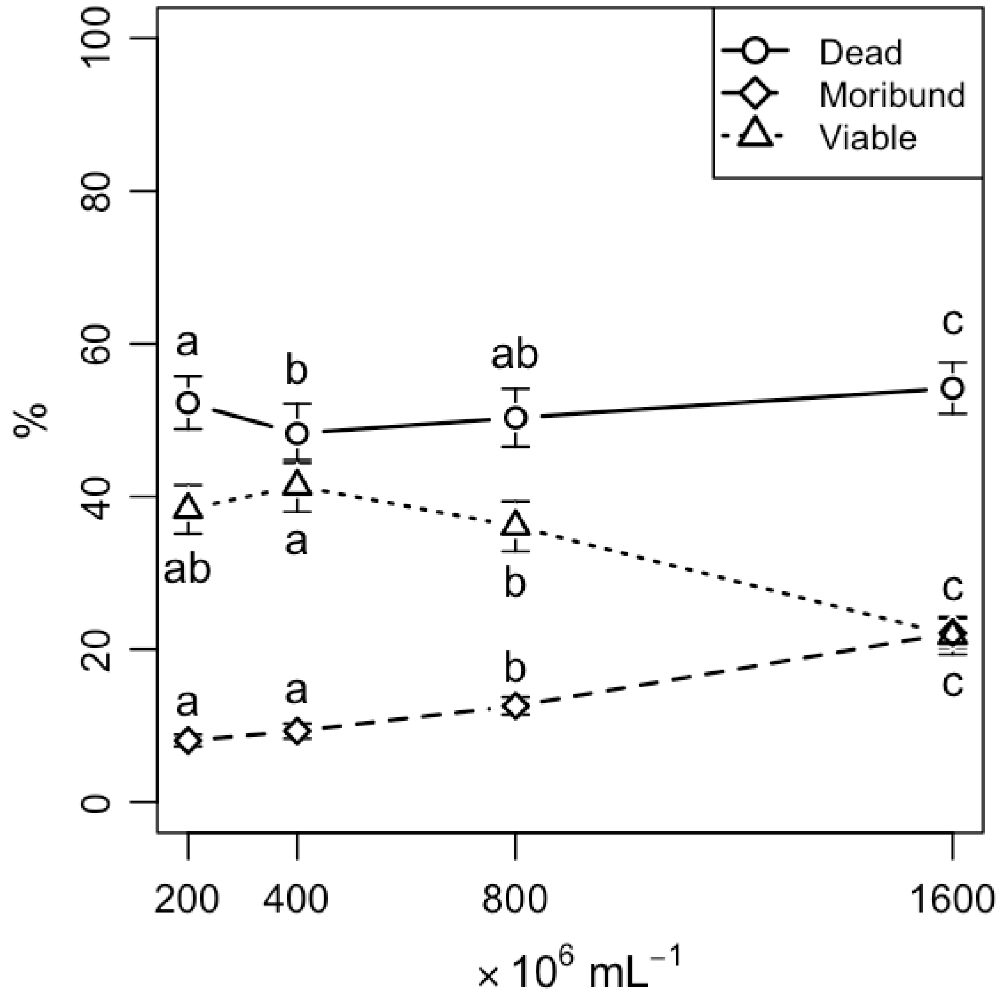

Figure 2. Mean±SEM of the three sperm populations obtained from the SYBR-14/PI stain for each of the four sperm concentrations: viable (SYBR-14+/PI-), moribund (SYBR-14+/PI+) and dead spermatozoa (SYBR-14-/PI+). Different letters indicate differences between sperm concentrations for each sperm population (P<0.05).

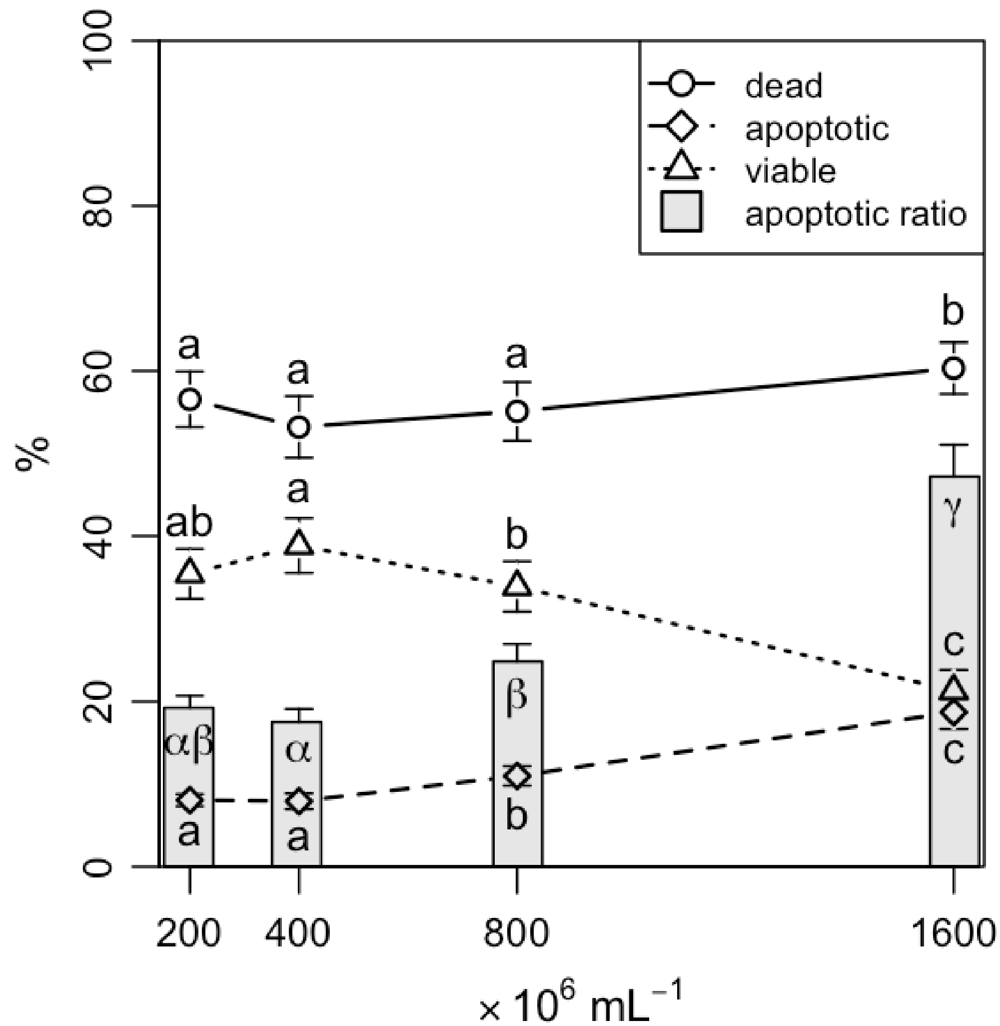

Figure 3. Mean±SEM of the sperm populations obtained from the YO-PRO-1/PI stain for each of the four sperm concentrations: viable (YO-PRO-1-/PI-), increased membrane permeability ("apoptotic", YO-PRO-1+/PI-) and dead spermatozoa (YO-PRO-1+/PI+). The "apoptotic ratio" (YO-PRO-1+/total PI- spermatozoa) is shown as columns. Different letters indicate differences between sperm concentrations for each sperm population, latin letters for YO-PRO-1/PI subpopulations and greek letters for the "apoptotic ratio" ($P<0.05$).

The fertility results after intrauterine insemination (lambing rates) are reported in the Table 2. Fertility was affected by the sperm concentration used for freezing the semen doses. It was significantly higher when the ewes were inseminated with doses frozen at 200 and 400×10^6 mL^{-1} (yielding 57.5 and 54.4% fertility, respectively), comparing with 800×10^6 mL^{-1} (45.5%; $P<0.05$). Females inseminated with 800×10^6 mL^{-1} doses were

0.62 times less likely of getting pregnant than those inseminated with 200×10^6 mL^{-1} doses (odds ratio, 95% CI: 0.44–0.88; P=0.007).

Table 2. Fertility of ewes after intrauterine insemination with semen doses frozen at different sperm concentrations (lambing rates). Insemination dose was fixed at 25×10^6 spermatozoa per uterine horn in all cases. Odds ratios are given taking 200×10^6 mL^{-1} as the reference group.

		200 vs. higher concentration	
Concentration ($\times10^6$ mL^{-1})	lambing rate (%)	Odds ratio (95% CI)	p-value[1]
200	154/268[a] (57.5)	Referent[2]	–
400	135/248[a] (54.4)	0.88 (0.62, 1.25)	0.489
800	112/246[b] (45.5)	0.62 (0.44, 0.88)	0.007

[1] Wald Chi-square for sperm concentration from logistic regression model.

[2] Reference group for odds ratio.

Fertility values with different superscripts differ P<0.05 (Chi-square test).

5.5. Discussion

Semen cryopreservation induces a series of structural and biochemical changes in spermatozoa, thus reducing the integrity of the membrane [18,19], mobility [13,14] and fertilizing capacity [20,21]. Many factors influence the survival and functionality of the frozen/thawed spermatozoa, but sperm concentration has been little explored. The optimization of semen doses and the utilization of sorting technologies has driven interest towards freezing using low sperm concentrations [22,23]. However, freezing at high concentrations might be interesting in some cases, and it could be necessary to increase the absolute number of fertile spermatozoa post-thawing while still managing a small volume. Utilization of very high concentrations is usual in fish species, and it does not seem to be detrimental for the cryopreservation of spermatozoa [24]. However, increasing sperm concentration for freezing could have undesirable consequences in mammals, exceeding the possible advantages of this approach.

Our study follows D'Alessandro et al. [11]. Recapitulating from the introduction, these authors evaluated the survival of sheep semen frozen at several concentrations (50, 100, 200, 400, 500 and 800×10^{6} mL^{-1}) and in two extenders (egg yolk-based —Tris-FY— and milk-based —Milk-LY). Irrespective of the extender, they found that the overall performance (motility, viability and acrosomal status) decreased when freezing at 800×10^{6} mL^{-1}. Nevertheless, results were not entirely conclusive, with a high variability among concentrations. Subjective motility was lower in the 800×10^{6} mL^{-1} samples. In our results, the progressive motility was significantly different among 200 and 800×10^{6} mL^{-1}, although total motility and the kinematic parameters remained similar. Viability also decreased in that study for 800×10^{6} mL^{-1}, although they reported low viability for several of the lower concentrations too. Interestingly, in our results 400×10^{6} mL^{-1} yielded a viability higher than 800×10^{6} mL^{-1}, as assessed using PI/SYBR-14 or PI/YO-PRO-1, while 200×10^{6} mL^{-1} stayed in between, not being significantly different than 800×10^{6} mL^{-1}.

Other studies have detected an effect of high concentrations on cryopreservation yields in other species. Nascimento et al. [13] compared the motility, viability and mitochondrial activity of stallion spermatozoa frozen at 100, 200 and 400×10^{6} mL^{-1}. They found highest motilities at 200×10^{6} mL^{-1}, followed by 400×10^{6} mL^{-1}, and the lowest values at 800×10^{6} mL^{-1}. Similarly, Crockett et al. [25] found higher post-thaw progressive motility in cooled samples and after cryopreservation at concentrations of 50 and 250×10^{6} mL^{-1} (25% and 23%, respectively) than in samples at a concentration of 500×10^{6} mL^{-1} (17%). Peña and Linde-Forsberg [14] obtained diverging results when testing 50, 100, 200 and 400×10^{6} mL^{-1}. Whereas viability was higher at lower concentrations, there were no differences on progressive motility and, after incubating the samples for several hours, 400×10^{6} mL^{-1} yielded both the highest progressive motility and viability. Therefore, the spermatozoa of some species, such as the horse, seems to be sensitive to high concentrations while freezing, whereas others, dog and possibly ovine, seems to be resilient and even being better cryopreserved at moderately high concentrations.

The causes of this decrease are still little know, but could be multifactorial and intertwined: excess of free radicals, modification of the sperm metabolism, changes in the media due to catabolism products, physical changes during the freezing/thawing, etc. Moreover, acrosomal enzymes and toxic products released from damaged

spermatozoa (e.g., free radicals) might contribute to the destabilization of membranes and other structures in live spermatozoa, and it could have a larger effect at higher sperm concentration [26,27]. We aimed at exaggerating any detrimental effects by using the 1600×10^6 mL^{-1} concentration. Indeed, the proportion of spermatozoa with damaged membranes (loss of viability) decreased clearly in that treatment, explaining at least in part the concomitant loss of motility. In equine spermatozoa, Crockett et al. [25] found not only a lower motility, but also a higher percentage of sperm with damaged membranes in the 500×10^6 mL^{-1} doses (45%) than in the 50×10^6 mL^{-1} doses (60%). Interestingly, we could not detect an increase of acrosomal damage, even in the 1600×10^6 mL^{-1} samples. Nevertheless, we must take into account that the absolute quantity of enzymes and other molecules released from damaged acrosomes (and, in general, from dead spermatozoa) increase with sperm concentration. Therefore, at the same proportion of damaged acrosomes, samples with higher sperm concentration would have a higher concentration of these potentially harmful substances, which might explain, at least in part, the lower sperm quality.

The YO-PRO-1 stain has the ability to label spermatozoa that have an increasing membrane permeability that not necessarily implies a loss of continuity [28,29], resembling some phenomena occurring in apoptotic somatic cells. We detected that the proportion of spermatozoa showing these early membrane changes increased with sperm concentration. This increase was more evident when the presence of the "apoptotic" population was expressed as an “apoptotic ratio”. This is an important variable, since these subtle membrane changes could announce a higher vulnerability of inseminated spermatozoa to the oviductal environment and a lower ability to attach to the oviductal epithelium [30]. Therefore, samples frozen at 800 or 1600×10^6 mL^{-1} not only had a lower proportion of viable spermatozoa, but a higher proportion of potentially non-functional spermatozoa [29].

Indeed, the fertility obtained following intrauterine artificial insemination was significantly lower in ewes inseminated with 800×10^6 mL^{-1} doses. D’Alessandro et al. [11] observed that increasing the pre-freezing sperm concentration to 800×10^6 mL^{-1} negatively affected the proportion of pregnant ewes, but their results were not significant. Moreover, inseminations were performed without equalizing sperm numbers among different sperm concentrations, and the excess of spermatozoa when using the doses with higher concentration could compensate in part for the lack of quality. In our

study, we used a larger number of females per insemination group, and utilized the same number of spermatozoa per insemination, irrespectively of the dose concentration. We have achieved an overall fertility above 50%, similarly to previous studies with laparoscopic insemination [3,4]. The best results for fertility were achieved by 200 and 400×10^6 mL^{-1}, related to the highest results for motility, plasma membrane integrity and permeability. The 800×10^6 mL^{-1} doses obtained an odds ratio of 0.62 respect to 200×10^6 mL^{-1} doses. That is, the odds for an insemination with a 800×10^6 mL^{-1} dose to results in a pregnancy are 0.62 times lower than for an insemination with a 200×10^6 mL^{-1} dose.

It is true that pre-insemination extension might have penalized the 800×10^6 mL^{-1} doses. However, we think that our trial is realistic, since dilution would occur in vivo after routine insemination, penalizing these spermatozoa anyway. Moreover, extension sensitivity might be due to sub-lethal membrane damage. Given that samples frozen at 400×10^6 mL^{-1} yielded fertility results similar to the 200×10^6 mL^{-1} doses, we can conclude that these samples were not affected by extension, remarking that detrimental effects occur when samples are frozen at higher concentrations.

In conclusion, increasing sperm concentration in the sperm doses (at least, above 400×10^6 mL^{-1}) affects adversely the post-thawing quality and fertility of ram semen. Sperm quality was slightly affected at 800×10^6 mL^{-1}, but membrane changes (proportion of spermatozoa with apoptotic features) indicated further detrimental effects upon use of the doses in the field. Actually, freezing at those sperm densities affected negatively the fertility of the samples.

One of the purposes of freezing at high concentrations is to increase the number of spermatozoa available in the insemination dose, attempting to improve the odds for achieving a pregnancy. Moreover, this increase of inseminated spermatozoa would compensate for some quality decrease when freezing at high concentrations. Nevertheless, taking into account the results of D'Alessandro et al. [11], it seems that this hypothesis may not be correct, since their insemination results tended to decrease with the 800×10^6 mL^{-1} doses, even though they inseminated with a higher number of spermatozoa (not at a fixed number, like in our case). Therefore, trying to improve AI results in sheep by freezing and inseminating at a higher sperm concentration might not compensate the excess spermatozoa used. Testing this hypothesis should be an objective in future studies. Nevertheless, it is still open to further research if there is any advantage freezing at 400×10^6 mL^{-1} (or higher) and inseminating with the full dose.

Since freezing at 200 and 400×10^6 mL^{-1} have yielded similar performance for in vitro quality and in the fertility trial, it is reasonable to think that ram semen could be frozen at least at that concentration, thus increasing the odds of pregnancy.

5.5.1 Acknowledgements

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CAPÍTULO 6

Enviado a Animal Reproduction Science

Capítulo 6

Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate and epididymis).

M Álvarez[1,2], J Tamayo-Canul[1,2], C Martínez-Rodríguez[1,3], E. López-Urueña[1,2], S. Gomes-Alves[1,2], L Anel[1,2], F. Martínez-Pastor[1,3], P de Paz[1,3]

[1] ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain

[2] Animal Reproduction and Obstetrics, University of León, 24071, León, Spain

[3] Molecular Biology, University of León, 24071, León, Spain

Corresponding author: +34 9872915679, E-mail: ppazc@unileon.es

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6.1 Abstract

The objective of this study was to identify a possible specificity in the extender formulation for the cryopreservation of ram spermatozoa recovered from three origins (ejaculate, electroejaculate or epididymis), by evaluating the post-thawing sperm quality and the fertility. Ejaculated, electroejaculated or epidydimal spermatozoa samples obtained from identical rams (8) were cryopreserved in four different extender (TES-Tris-Fructose with one of two egg yolk concentrations: 10% -Y10- and 20% -Y20-, and with one of two glycerol rates: 4% -G4- and 8% -G8-). Samples were analyzed after and before to cryopreservation by CASA (motility) and flow cytometry (viability with SYBR-14/PI and acrosomal status with PNA/PI). Spermatozoa obtained by electroejaculation yielded lower quality after freezing/thawing, being needed to optimize protocols for these samples. Egg yolk at 20% was more appropriate for freezing sperm from any of the sources. In general, 4% glycerol improved the quality of post-thawing samples recovered from ejaculate and electroejaculate, while 8% glycerol was more appropriate for samples recovered from the epididymis. Based on these results, an analysis of fertility was conducted. Fertility rates were similar between ewe groups inseminated with post-thawing sperm obtained from two sources: ejaculate (cryopreserved in Y20+G4), and cauda epididymis (Y20+G8), and this rate was lower in the electroejaculated sample (Y20+G4).

Keywords: Spermatozoa, ejaculate, electroejaculate, epididymis, ram, cryopreservation, egg yolk, glycerol.

6.2 Introduction

The availability of efficient cryopreservation methods for animal domestic has benefitted the beef and dairy industries for over half a century. In the ram, the improvement in cryopreservation methods may be of value by preserving valuable genetic stock in breeding programs (Anel et al., 2006) as well as to establish a semen bank for sheep breeds with increased risk of losses of genetic variability due to the selection programmes (Ehling et al., 2006; Nel-Themaat et al., 2006). Unfortunately, the success archived with the cryopreservation of bull sperm has not been translated into

most commonly raised farm animal species including the ram (Salamon y Maxwell, 2000). Thus, artificial insemination programs with cryopreserved ram spermatozoa do not satisfy the demand of the sheep industry (Gillan et al., 1999), because only acceptable results of fertility were obtained when using laparoscopic intrauterine insemination, a complex technique unsuitable for field conditions (Anel et al., 2006).

It has been well know that sperm undergoes considerable cellular, biochemical and osmotic changes during epididymal maturation and after addition of seminal fluids (James et al., 1999; Martinez-Pastor et al., 2006; Yeung et al., 2006; Tamayo-Canul et al., 2011b). These changes include a significant variation in the plasma membrane components of ram spermatozoa (Hammerstedt y Parks, 1987) that subsequently affect the cryobiological properties of semen. Understanding the effects of cryoprotective agents on cellular system during cryopreservation procedure is a prerequisite for developing improved protocols for freezing (Curry y Watson, 1994). Although several studies have been conduced regarding osmotic tolerance, cold shock sensitivity and hydraulic conductivity of ram ejaculated sperm during freezing (Curry y Watson, 1994; Anel et al., 2003), these properties have not been fully investigated for electroejaculated or epididymal ram sperm.

Appropriate retrieval and optimal cryopreservation of epididymal spermatozoa following accidental deaths ram and other endangered species would also greatly help preserving biodiversity (Kaabi et al., 2003; Hishinuma et al., 2003; Martinez-Pastor et al., 2009; Fernández-Santos et al., 2006). For that reason there is a need to investigate and compare the fundamental cryobiologic characteristics of ejaculates, electroejaculated and epididymal sperm (Holt, 2000).

The egg yolk and glycerol represent the indispensable compounds for practically all media used for sperm conservation in liquid or frozen states in domestic animal. The egg yolk has been shown to have a beneficial effect on sperm cryopreservation and it is speculated that the egg yolk components associates with sperm membranes and provides protection against cold shock (Moussa et al., 2002; Manjunath et al., 2002; Amirat et al., 2004; Hu et al., 2010). The glycerol contributes to sperm integrity conservation in cryopreservation procedures. An optimal concentration has not been defined, since comparative studies in different species were generally carried out using extender varying on many parameters (sugar, membrane stabilizer, glycerol concentration, etc), thus making difficult to make decisions about the most adequate

glycerol concentration. Some authors have reported concentration range from 4 to 10% (Martinez-Pastor et al., 2006; Anel et al., 2003; Forouzanfar et al., 2010; de Paz et al., 2011; Alvarez-Rodríguez et al., 2011), but result seem to depend rather on other components or on the followed protocols than on glycerol concentration.

In this study, we evaluated the effect of four types of extenders, obtained from the combination of two egg yolk concentrations (10% and 20%) with two glycerol concentrations (4% and 8%), on freezing of semen from three different sources (ejaculated, electroejaculated and epididymis) obtained from identical rams.A fertility trial, using semen samples obtained from the three sources, was carried out.

6.3 Material and Methods

6.3.1. Reagents

All the products were obtained from Sigma (Madrid, Spain), except fluorescence probes SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1, which were acquired from Invitrogen (Barcelona, Spain).

6.3.2. Animal

Adult Assaf rams, aged between 5 and 7 years, were used (8 for in vitro evaluation and 3 for fertility test) They were of proven fertility and belonged to ASSAFE (Spanish Assaf Breeders Association).

6.3.3. Semen collection

Semen samples were obtained during breeding season from three sources (ejaculate, electroejaculate or epididymis), taking samples from the same males sequentially (8 males for in vitro study and 3 males for fertility trial). In a first phase, we obtain sperm from the ejaculate (see 2.3.1); then, after a rest period of 7 days, was obtained semen by electroejaculation (see 2.3.2) and after 7 days of rest, epididymal spermatozoa (see 2.3.3) was obtained.

6.3.3.1. Ejaculate recovery

Ejaculates were collected by artificial vagina at 40 °C (Minitüb, Tiefenbach, Germany), and the tubes were maintained at 35 °C during initial evaluation of semen quality. The volume was estimated by using the graduation marks of the collection tube. Mass motility was assessed by microscopy (warming stage at 38 °C, x40; score: 0-5; Labophot 2, Nikon, Tokyo, Japan), and the sperm concentration was assessed by the photocolorimetric method at 540 nm (Spectronic 20, Baush & Lomb, Madrid, Spain), on a specific calibrated scale. Only ejaculates of good quality were used and frozen (volume: ≥ 0.5 ml; mass motility: ≥ 4; sperm concentration: ≥ 3000×10^6 mL^{-1}).

6.3.3.2. Electroejaculation procedure

Previously to electroejaculation, the rams were restrained and anaesthetized with intravenous injection of xylacine -0,1 mg/kg- and ketamine -2,5 mg/kg- (Rompum® 2%, Bayer and Imalgene 1000®, Merial). Rectum was cleaned from feaces and the prepucial area was shaved and washed with physiological saline serum. Electroejaculation was carried out using a 3 electrode prove (250 mm x 30 mm) connected to electroejaculator which allowed voltage and amperage control. Ejaculation occurred at average values of 4 V and 90 mA (average time to ejaculation: 3 min).

6.3.3.3. Post-mortem sperm recovery

The testicles and epididymis were transported at room temperature (22 °C) and semen collection was carried out in the first 2 h after the slaughter of the ram. The epididymis-testicle complexes were dissected and cauda epididymis was isolated. Sperm was obtained by deep slicing of the cauda epididymis tissue with a scalpel; the fluid was collected and its volume was estimated. To avoid blood contamination, superficial blood vessels were previously cut and their contents were wiped out.

6.3.4. Criopreservation procedure

6.3.4.1. Preparation of the extenders

The extenders were based in a TES-Tris-Fructose adjusted to 320 mOsm/kg and pH 7.2 (TTF, Anel et al. 2003) and complemented with one of two egg yolk concentrations (Y10: 10% and Y20: 20%) and one of two glycerol rates (G4: 4% and G8: 8%). In order to prevent variations due egg yolk, it was obtained from fresh hen eggs in aseptic conditions, and all the yolks were pooled.

6.3.4.2. Cryopreservation protocol

Samples were diluted 1:1 in the corresponding extender. This dilution was carried out at ambient temperature for different samples. The tubes with diluted sperm samples were then put in glass containing 50 ml of water at ambient temperature and transferred to a cold room at 5 °C for an average of two hours, until the samples reached a temperature of 5 °C, so temperature decreased smoothly. After, the sample was further diluted with the same extender down $100x10^6$ sperm/ml, packed in 0.25 ml french straws and equilibrated for 1 h at 5 °C. Then, the straws were frozen using a programmable biofreezer (Kryo 560-16® Planer™, Planer plc., Sunbury-On-Thames, UK) using a rate of -20 °C/min down to -100 °C. The straws were kept in liquid nitrogen containers. Thawing was carried out in a water bath at 65 °C for six seconds. Sperm quality parameters were evaluated immediately after thawing.

6.3.5. Spermatozoa evaluation

6.3.5.1. Motility parameters

The assessment of motility was carried out using a computer-assisted sperm analysis system (CASA) (ISAS® v. 1.1; Proiser, Valencia, Spain). Samples were diluted (10–20×10^6 cells/ml) in the same TTF medium with 320 mOsm/kg, and warmed on a 37 °C plate for 5 min. Then, a 5-µL drop was placed into a Makler® counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at ×10 (negative phase contrast) in a microscope (Eclipse E400®, Nikon) with a warmed stage (38 °C). The standard parameter settings were set at 25 frames/s, 20 to 90 μm^2 for head area and VCL > 10 µm/s to classify a spermatozoon as motile (Tamayo-Canul et al.,

2011a). At least five sequences or 200 spermatozoa were saved and analyzed afterwards. Reported kinetic parameters were curvilinear velocity (VCL, µm/s) and linearity (LIN, %),. Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 µm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 µm/s and STR > 80% (straightness, also provided by the system).

6.3.5.2. Sperm viability and acrosome status

Acrosomal status were assessed using florescence probes (PNA/PI) and flow citometry, according to methods described by (Martinez-Pastor et al., 2009). Briefly, samples were diluted in PBS at 5×10^6 spermatozoa/mL, and incubated for 15 min with 24 µM of propidium iodide (PI) and 1 µg/mL of PNA-FITC (peanut agglutinin). PI stains membrane-damaged spermatozoa red, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (non-viable sperm, intact acrosoma), green (viable sperm, damaged acrosome) red and green (non-viable sperm, damaged acrosome) or not-stained (viable sperm, intact acrosome). The percentage of spermatozoa with damage acrosomes (dACR) was calculated as the sum of viable and not-viable PNA+ spermatozoa.

To evaluate sperm viability, the double stain SYBR-14/PI was used (LIVE/DEAD Sperm Viability Kit®: Invitrogen, Barcelona, Spain). Sperm samples were diluted with PBS down $5x10^6$ spermatozoa/ml, and incubated for with 24 µM PI and 100 nM SYBR-14. The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to live spermatozoa -VIAB- (green), moribund spermatozoa (red + green) and dead spermatozoa (red).

Evaluation was carried out using a FACScalibur® flow citometer (Becton Dicknson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Calibration was carried out periodically using standard bead (Calibrites®: Becton Dickson). Data corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10,000 spermatozoa were recorded.

6.3.6. Fertility trials

A fertility trial was carried out using samples of three males obtained from the three sperm sources. The semen was frozen with the method that best results showed in the in vitro study (TTF-Y20- G4 for ejaculate and electroejaculate and TTF-Y20-G8 for epididymal). Adult Assaf ewes (356 females from 11 commercial farms) were subjected to treatment for oestrus induction and synchronization using intravaginal sponges with 20 mg fluorogestone acetate (Chronogest®, MSD) over 14 days. The sponges were removed and 500 IU of eCG injected (Folligon®, MSD). Laparoscopic inseminations were performed between 62 and 64 h after the removal of the sponges. The animals, having fasted for the previous 24 h, were tied to a special cradle (IMV), placed on an inclined plane (45°) and the area in front of the teat was shaved and cleaned. Local anaesthesia (mepivacaine HCL 2%, Braun™) was applied to the puncture points. Then two portals (for vision and manipulation/injection) were inserted by performing a pneumoperitoneum (CO_2). The semen, placed in a special applicator (Transcap®, IMV), was injected under visual inspection into each uterine horn (0.12 mL, 12.5×10^6 spz). Fertility was evaluated in lambing ewes according to the births registered at 137–154 days post-insemination.

6.3.7. Statistical analysis

Statistical analyses were carried out using the R statistical package version 2.13.0 (http://www.r-project.org). Data were fitted to linear mixed-effect models by maximizing the log-likelihood (ML method) (R Development Core Team., 2010). Male was always included as random effect. Extender and sperm source were the fixed effects. Results are given as mean±SEM.

6.4 Results

6.4.1. Effect of the semen source on pre-freezing and post-thawing quality

The source of the spermatozoa influenced both pre-freezing and post-thawing quality. The results of the pre-freezing analysis (samples extended and equilibrated at 5 °C) are

shown in Table 1. The method of collection of the sample had no significant effect on the pre-freezing total motility, but significantly affected the progressive motility and kinematic parameters. Electroejaculated samples showed lower values for progressive motility, whereas epididymal samples showed the highest values for VCL and viability, and the lowest proportion of damaged acrosomes.

The results of post-thawing quality are displayed in Table 2. There were several changes from pre-freezing results, with electroejaculated samples having the lowest results for kinematic parameters, being significant for total and progressive motility, and VCL. Ejaculated and epididymal samples did not differ significantly regarding CASA parameters. In general, there was an increase in damaged acrosomes and a severe decrease in the proportion of viable spermatozoa, comparing to pre-freezing values. Epididymal samples showed again the lowest values for the proportion of damaged acrosomes, and the highest ones for viable spermatozoa.

6.4.2. Effect of egg yolk and glycerol concentration within each source

The effect of egg yolk and glycerol on sperm quality and your interactions, both at pre-freezing and post-thawing, were showed in Figures 1-4. In pre-freezing ejaculated samples, the total and progressive motility, curvilinear velocity and damaged acrosome were significant affected by the egg yolk concentration. In pre-freezing electroejaculated samples, only the damaged acrosome was significantly affected by the egg yolk. Finally, the curvilinear velocity and viability of semen from epididymal samples were affected significantly by egg yolk. The glycerol concentration affect significantly the progressive motility of pre-freezing electroejaculated samples. The linearity was significantly affected by glycerol in the three sperm sources and the viability was only significantly affected in ejaculated samples.

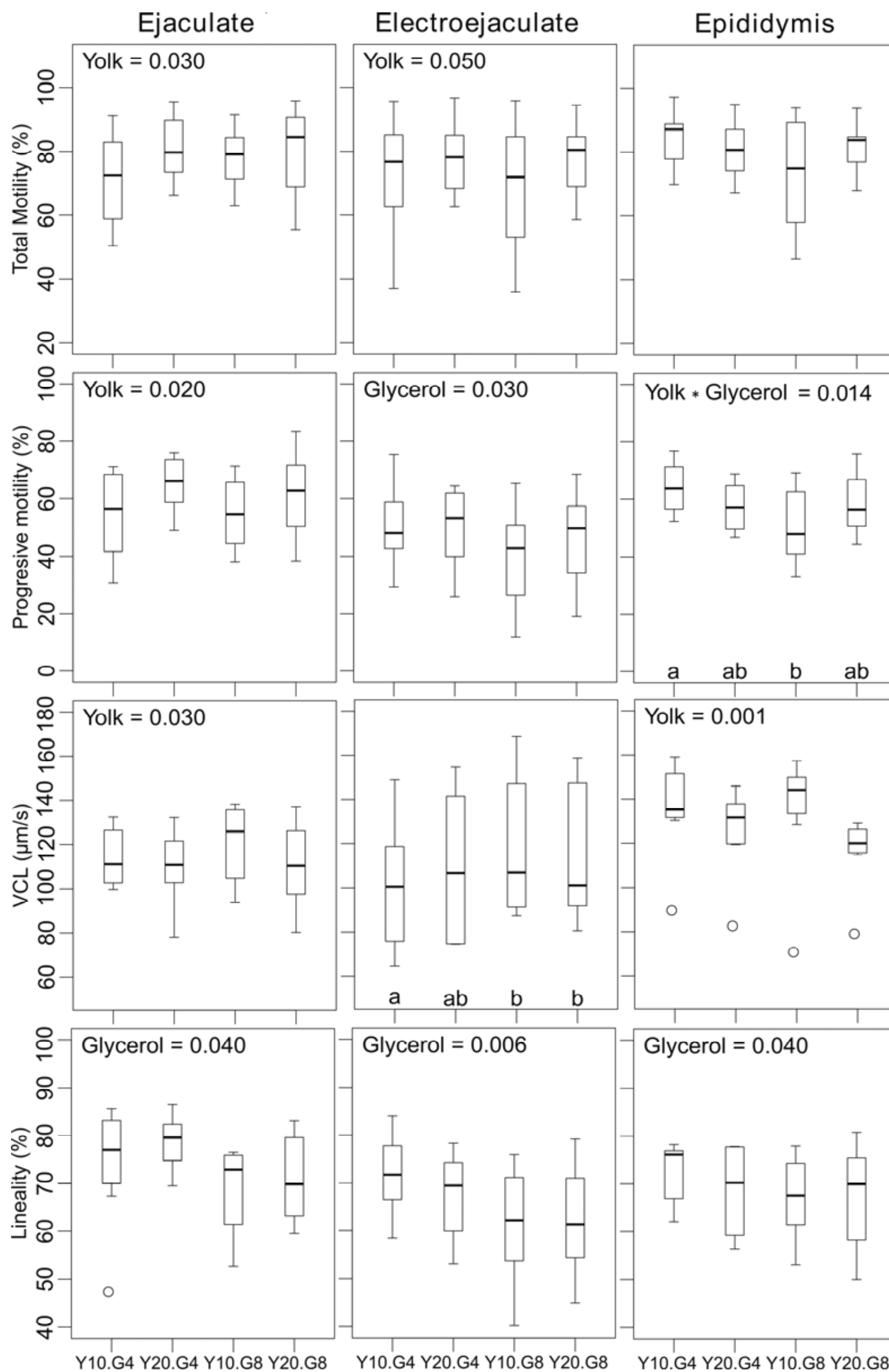

Figure 1. Pre-freezing motility and kinetic parameters (CASA). The boxplot shows the parameters of semen obtained from three sources (ejaculate, electroejaculate or cauda epididymis) evaluated in four extender (two concentrations of egg yolk (Y10: 10%, Y20: 20%) and two concentration of glycerol (G4: 4%, G8: 8%). Insets show if the main effects (yolk or glycerol) or interactions (yolk*glycerol) were

significant. Whenever a significant interaction was found, we performed a pairwise comparison of the 4 extender (different letters indicate significant differences, P<0.05). The median is indicated by a horizontal line.

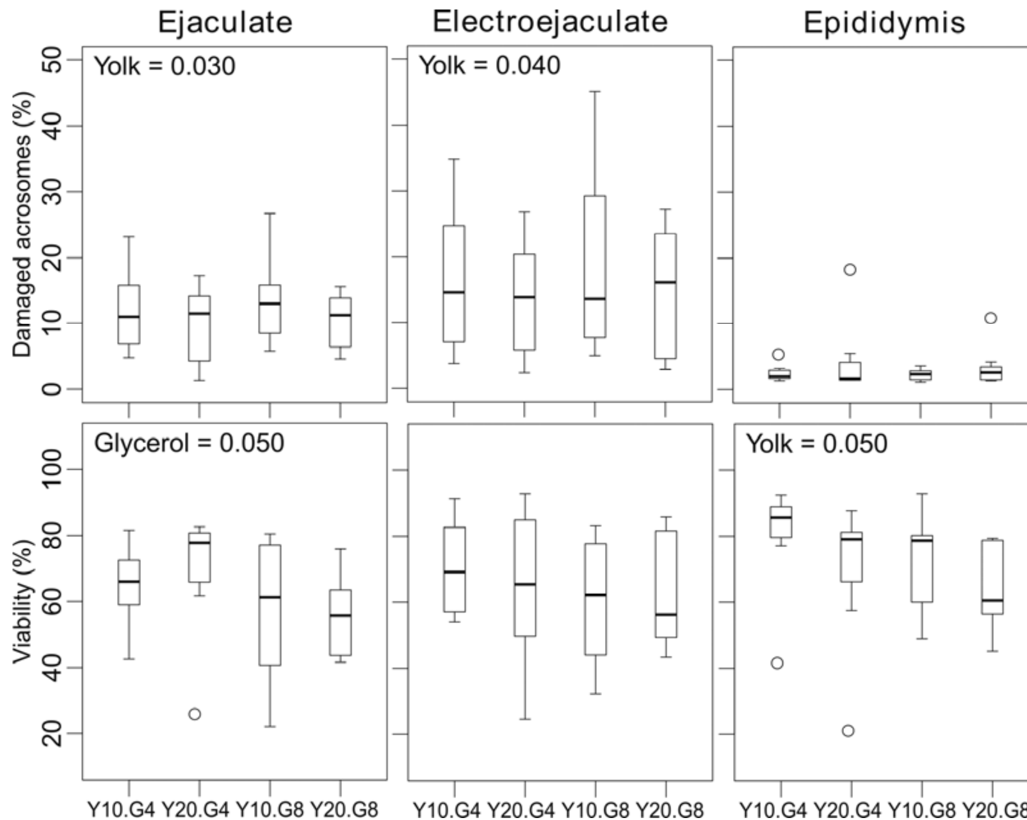

Figure 2. Pre-freezing viability parameters (flow cytometry). The boxplot shows the parameters of semen obtained from three sources (ejaculate, electroejaculate or cauda epididymis) evaluated in four extender (two concentrations of egg yolk (Y10: 10%, Y20: 20%) and two concentration of glycerol (G4: 4%, G8: 8%). Insets show if the main effects (yolk or glycerol) or interactions (yolk*glycerol) were significant. Whenever a significant interaction was found, we performed a pairwise comparison of the 4 extender (different letters indicate significant differences, P<0.05). The median is indicated by a horizontal line.

After thawing, the ejaculated samples was affected by egg yolk concentration in lineality, damaged acrosomes (highest concentration, lower damage) and viability (highest concentration, greater viability). Egg yolk concentration significantly affect to total and progressive motility (highest concentration, greater motility), damaged acrosomes and viability (a higher concentration, more viability) in the electroejaculated samples. The total and progressive motility of epididymal samples was also affected by egg yolk concentration with the same trend.

Glycerol affect significantly total motility post-thawing in epididymal samples. The linearity was negatively affected by glycerol concentration in the three sources evaluated: ejaculate, electroejaculate and epididymis. Glycerol concentration increased significantly the damage acrosomes in ejaculate and electroejaculated samples, and only the viability in ejaculated samples.

Table 1. Effect of ram semen source (ejaculate, electroejaculate or cauda epididymis) on quality in pre-freezing samples (mean±SEM).

	TM (%)	PM (%)	VCL (µm/s)	LIN (%)	dACR (%)	VIAB (%)
Ejaculate	77.5±12.7	59.0±13.5^{a}	113.8±16.4^{a}	73.2±9.7^{a}	11.4±5.7^{a}	62.0±16.9^{a}
Electroejaculate	74.3±15.8	46.6±15.8^{b}	110.8±30.6^{a}	65.6±10.6^{b}	16.0±10.8^{a}	64.6±18.2^{a}
Cauda Epididymis	79.9±11.8	57.6±11.0^{a}	128.4±21.8^{b}	68.9±8.8^{ab}	3.1±3.4^{b}	71.9±16.8^{b}

TM: Total motility; PM: Progressive motility; VCL: Curvilinear velocity; LIN: Linearity; dACR: Damaged acrosomes; VIAB: Viable spermatozoa.

^{a,b} Different superscripts within columns indicate significant differences among sources (P<0.05).

Table 2. Effect of ram semen source (ejaculate, electroejaculate or cauda epididymis) on quality in frozen-thawed samples (mean±SEM).

	TM (%)	PM (%)	VCL (µm/s)	LIN (%)	dACR (%)	VIAB (%)
Ejaculate	59.1±14.4^{a}	38.7±9.0^{a}	118.1±14.7^{ab}	68.4±5.4	33.5±15.0^{a}	29.3±12.9^{a}
Electroejaculate	48.7±20.9^{b}	27.6±10.8^{b}	113.4±18.4^{a}	65.3±10.9	34.2±18.5^{a}	25.5±12.9^{a}
Cauda Epididymis	62.0±16.2^{a}	41.5±11.5^{a}	126.3±16.8^{b}	68.5±8.2	11.6±6.8^{b}	35.7±14.5^{b}

TM: Total motility; PM: Progressive motility; VCL: Curvilinear velocity; LIN: Linearity; dACR: Damaged acrosomes; VIAB: Viable spermatozoa.

^{a,b} Different superscripts within columns indicate significant differences among sources (P<0.05).

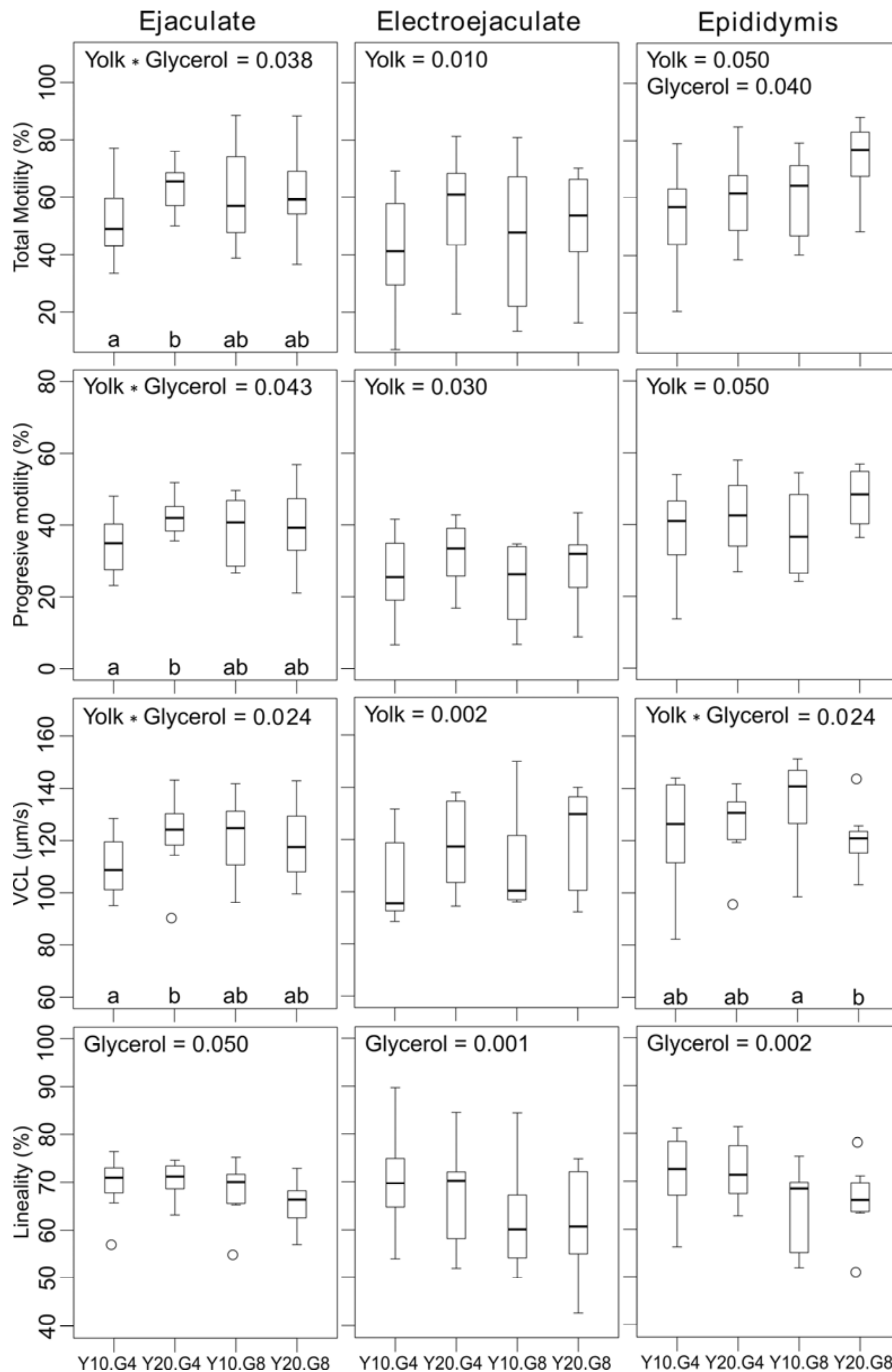

Figure 3. Post-thawing motility and kinetic parameters (CASA). The boxplot shows the parameters of semen obtained from three sources (ejaculate, electroejaculate or cauda epididymis) evaluated in four extender (two concentrations of egg yolk (Y10: 10%, Y20: 20%) and two concentration of glycerol (G4: 4%, G8: 8%). Insets show if the main effects (yolk or glycerol) or interactions (yolk*glycerol) were

significant. Whenever a significant interaction was found, we performed a pairwise comparison of the 4 extender (different letters indicate significant differences, P<0.05). The median is indicated by a horizontal line.

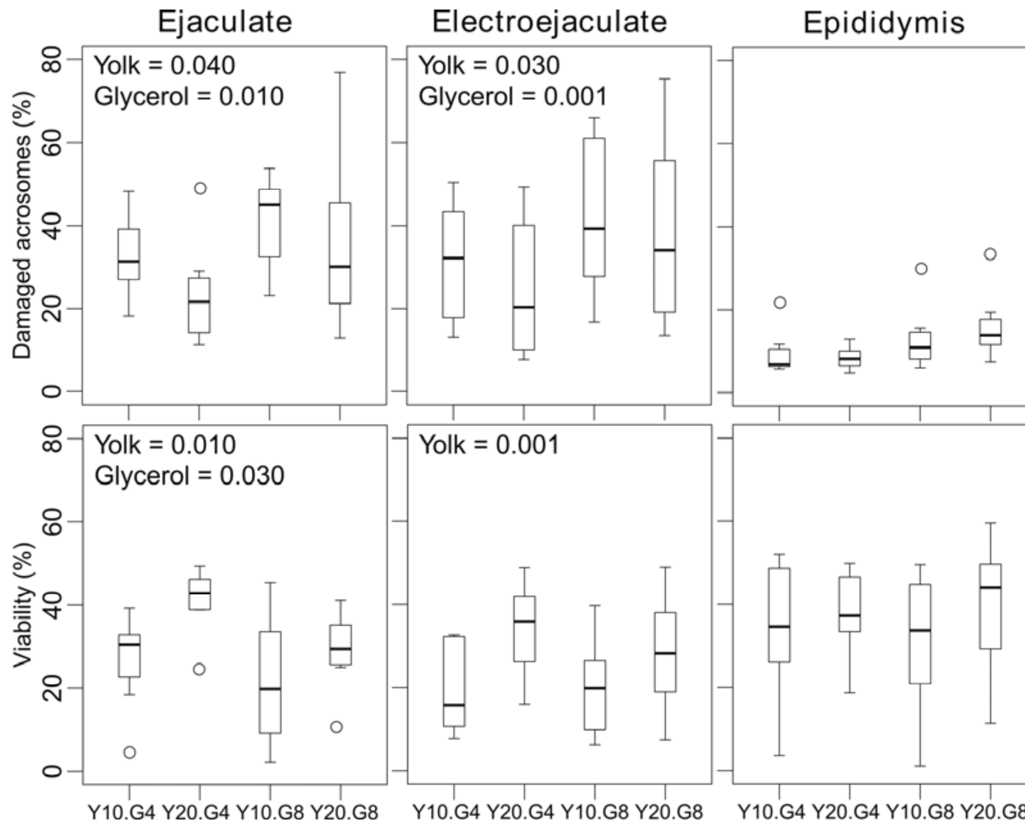

Figure 4. Post-thawing viability parameters (flow cytometry). The boxplot shows the parameters of semen obtained from three sources (ejaculate, electroejaculate or cauda epididymis) evaluated in four extender (two concentrations of egg yolk (Y10: 10%, Y20: 20%) and two concentration of glycerol (G4: 4%, G8: 8%). Insets show if the main effects (yolk or glycerol) or interactions (yolk*glycerol) were significant. Whenever a significant interaction was found, we performed a pairwise comparison of the 4 extender (different letters indicate significant differences, P<0.05). The median is indicated by a horizontal line.

6.4.3. Fertility trials for the three sources of semen

There were not significant differences among the fertility obtained from three sperm sources (Table 3). However, the electroejaculated samples showed a lower fertility (44.4% vs. ejaculated (53.8%, P=0.062) and epididymal (55.8%, P=0.082) samples.

Table 3. Fertility results (lambed/inseminated). A Chi squared test did not show significant differences among sources (ejaculate vs. electroejaculate: P=0.062; epididymis vs. electroejaculate: P=0.082; ejaculate vs. epididymis: P=0.246).

Source	Lambed ewes	Inseminations	Fertility (%)
Ejaculated	63	117	53.8
Electroejaculate	56	126	44.4
Cauda Epididymis	63	113	55.8

6.5. Discussion.

This study directly compare the pre-freezing and post-thawing quality of sperm samples obtained from three sources from the same rams. Other studies have compared epididymal samples with either ejaculated or electroejaculated in other species. For instance, Monteiro et al., (2011) observed that the viability and fertility of stallion cauda epididymal sperm are similar to those of ejaculated sperm, and Rath and Niemann (1997) concluded that boar epididymal spermatozoa can easily be frozen in small containers with higher resultant motility and fertilization rates than with ejaculated semen.

The post-mortem sperm recovery is an useful method to rescue germoplasm of dead animals that otherwise would be lost, especially for the creation germoplasm banks and to preserve endangered breeds, contributing to the preservation of biodiversity (Kaabi et al., 2003). The post-thawing results obtained by Fernandez-Santos at al. (2006) on the red deer samples recovered from the epididymis showed a higher motility (62.0±16.2%) than those reported in other studies for red deer (Martinez-Pastor et al., 2006), bull (Martins et al., 2009) and ram (García-Álvarez et al., 2009). In the present study, the pre-freezing and post-thawing results showed that epididymal spermatozoa yielded motility values as good or higher than ejaculated and electroejaculated samples, respectively. In fact, electroejaculated samples yielded lower motility and viability post-thawing. These results contrast with those by Marco-Jiménez et al. (2005), who evaluated the effect of collection methods (ejaculation and electroejaculation) in the cryopreservation of Guirra rams spermatozoa. These authors found no differences in total motility and linearity. These results could be due to differences in the cryopreservation methods, which may have equalized the differences among sources. García-Álvarez et al. (2009) found considerable differences in total motility, after

cryopreservation, in ram samples recovered from the epididymis and electroejaculated (57.5±4.8% and 36.7±7.6%, respectively). Recent studies have shown that electroejaculated samples had lower resistance than did epididymal spermatozoa when submitted to cryobiologically stressful conditions such as chilling, osmotic stress, and addition and removal of cryoprotective agent (Varisli et al., 2009). This could also explain our fertility results, which showed a lower fertility of cryopreserved electroejaculated semen. These results indicate that cryopreservation protocols should be optimized for electroejaculated semen in ram, in order to obtain fertility similar to those observed with semen from two other sources.

There were differences on the suitability of extenders to cryopreserve samples recovered from these three sources. Other researchers have made similar reports on other species. For instance, Martinez-Pastor et al. (2006) described that epidydimal spermatozoa from red deer were better cryopreserved using 8% glycerol, whereas 4% was more adequate for electroejaculated samples. Nevertheless, similarly to our results, there were small differences between glycerol concentrations, especially for epididymal samples. In fact, these authors highlighted than epididymal spermatozoa could be resilient to different glycerol concentrations, possibly due to higher osmotic resistance (Monteiro et al., 2011). Ejaculated and electroejaculated sperm seem to be less tolerant to high glycerol concentration. However, Anel et al. (2010) did not find differences in sperm quality by using two glycerol concentrations (4% and 8%) in electroejaculated samples recovered in brown bear to the post-thawing, although de Paz et al. (2011) determined that 6% of glycerol could be more adequate than 4% or 8%. It is possible that the susceptibility to different glycerol concentrations could vary among species, and that ram electroejaculated semen could be more sensitive to glycerol.

The vulnerability of electroejaculated spermatozoa to cryopreservation and glycerol concentrations could be due to differences in seminal plasma composition. The addition and removal of a variety of protein during epididymal maturation and at ejaculation have important roles in the capacitation of sperm and fertility of the egg (Yanagimachi, 1994). Barrios et al. (2000) suggested that the acquisition of seminal plasma proteins by adsorption to the sperm cell surface modifies the functional characteristics of damage spermatozoa. Since electroejaculation varies from the physiological ejaculation, the composition of seminal plasma could be different, inducing changes in spermatozoa that could make its membranes less resilient (Marco-Jiménez et al., 2008). So,

electroejaculated samples were less suited for cryopreservation, possibly due to the variation in the composition and proportion of seminal plasma due to the stimulation of accessory glands, and this might have a negative effect in the freezability and fertility of these samples.

Cheng et al. (2004), working with electroejaculated sperm from *Cervus nippon* and *Cervus unicolor*, tested five different extender with glycerol concentrations ranging 5 to 8%, and their results suggested that the efficacy of each extender was rather due to presence of specific components and their interactions, than to glycerol concentration alone. We have detected several interactions among glycerol and egg yolk, indicating that, for these parameters, the effect of one of them depended on the concentration of the other. Nevertheless, in many cases only the main effects were significant, with no interaction.

Regarding egg yolk concentrations, our results indicate that cryopreservation of ram spermatozoa obtained from the three sources were better cryopreserved using an egg yolk concentration of 20%. These results are similar to those obtained in other studies in ruminants. For instance, Fernández-Santos et al. (2006) showed that the post-thawing quality of epididymal deer spermatozoa was higher using 20% egg yolk than 5% or 10%. Anel et al. (2010) evaluated two egg yolk concentrations, 10% and 20%, in electroejaculated sperm from brow bear, and they observed that the post-thawing sperm motility, viability and acromosomal status was higher using an extender with egg yolk concentration at 20%. We noted that it has been suggested that egg yolk's effects differ depending on the composition of the extender buffer. Thus, the higher egg yolk concentration in a raffinose-based diluent resulted in a better preservation of motility and membrane integrity during the cooling of spermatozoa in Cuvier's gazelle (Garde et al., 2008) or in red deer (Fernández-Santos et al., 2007). Although, some authors have reported negative effects of high concentrations of egg yolk on the preservation of motility and acrosome integrity in Mohor gazelle spermatozoa cryopreserved in TEST-yolk diluent (Holt el al., 1996).

In conclusion, this study is the first one simultaneously comparing the cryopreservation of epididymal, ejaculated and electroejaculated spermatozoa from identical ram, and assessing the effect of different concentrations of glycerol and egg yolk. Epididymal spermatozoa seemed to be more resilient to cryopreservation, while ejaculated samples showed better quality post-thawing than electroejaculated samples. However, the higher

in vitro quality of thawed epididymal samples does not imply a better outcome of fertility in the field, and therefore we assume that other factors besides motility, viability and acrosome status, influence this result.

Regarding the concentration on egg yolk and glycerol, a 20% egg yolk concentration was more adequate for freezing ram spermatozoa irrespectively from its origin. We recommend freezing ejaculated or electroejaculated samples with 4% of glycerol, whereas 8% of glycerol seems to be more adequate for freezing epididymal spermatozoa, possibly because their higher resistance to its toxic effects would allow these samples to beneficiate from its protective effects. These results could improve the cryopreservation of ram spermatozoa in different collection scenarios.

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CAPÍTULO 7

Capítulo 7

Discusión general.

La creación de bancos de germoplasma en la especie ovina, bien con fines de gestión productiva (animales de alto valor genético) o de conservación de razas en peligro de extinción debe de estar sustentada en bases metodológicas que garanticen la calidad de las muestras almacenadas. De esta manera, los protocolos deberían de ser optimizados y estandarizados en base al origen de la muestra a almacenar, y a sus diferentes particularidades (ej. el tiempo transcurrido desde la muerte del animal al procesado de la muestra seminal; la concentración espermática de la muestra a congelar, la necesidad de un almacenamiento precongelación, etc). Esto supone adaptar el protocolo de recogida de las muestras, el procesado, el diluyente de conservación, la criopreservación y la posterior aplicación de las mismas. Consideramos que el presente trabajo permite evaluar diferentes protocolos de actuación en las muestras que serán conservadas en los bancos de germoplasma y podrán ser utilizadas en un futuro en técnicas de inseminación artificial en las explotaciones ganaderas de la especie ovina.

Este grupo de experiencias podría considerarse como un modelo extrapolable a otras especies domésticas o silvestres de rumiantes, con vistas a dotar a sus bancos de germoplasma de herramientas útiles y fiables, que permitan rentabilizar al máximo todos los recursos disponibles al efecto.

7.1 Evaluación de métodos para la conservación a 5 ºC de los espermatozoides epididimarios de carnero

La obtención de los espermatozoides almacenados en la cola del epidídimo puede ser la única opción para preservar el material genético de un macho de alto valor genético que muere de una forma repentina, especialmente si no se ha podido obtener semen por otros métodos. Este hecho es especialmente relevante en animales en peligro o vías de extinción. La recuperación post-mortem de espermatozoides del epidídimo es una herramienta sumamente interesante para resguardar el material genético, así como para crear bancos de germoplasma.

En el capítulo 3 de la presente memoria, evaluamos el método de conservación de los espermatozoides a 5 °C, ya sea en el epidídimo o en tubo, sin diluir. En un segundo experimento, evaluamos la conservación de las muestras epididimarias diluidas en diluyentes con diferentes osmolalidades.

En este trabajo, observamos que la osmolalidad de las muestras, pero no el pH, aumentó en los tiempos sucesivos de muestreo. Coincidiendo con nuestros resultados, Martinez-Pastor., et al [1] observaron que la osmolalidad y el pH de las muestras espermáticas de los epidídimos de ciervo y corzo se incrementó con el tiempo post-mortem. Estas observaciones motivaron que comparásemos la conservación en el epidídimo y la recogida inmediata post-mortem y la conservación de la muestra en un tubo.

En este trabajo, observamos una disminución gradual de la motilidad en el almacenamiento en el epidídimo a las 48 y 72 horas, así como una reducción en la viabilidad de las células a las 72 horas. Otros autores han observado que la motilidad de los espermatozoides es afectada negativamente por el almacenamiento post-mortem en el epidídimo (carnero: [2]; ciervo: [3]; perro: [4]). Sin embargo, a diferencia de lo observado a las 48 horas en nuestro estudio, varios autores concluyen que el almacenamiento post-mortem no afecta la integridad de la membrana espermática (ciervo: [3,5]; perro: [4]). No obstante, nuestros resultados indican que la conservación a 5 °C en tubo es inferior a la conservación de los epidídimos y la extracción posterior. En las muestras conservadas en tubo, observamos una disminución significativa de la motilidad a las 24 horas, de la viabilidad a las 48 horas así como un aumento de los daños en los acrosomas a las 48 horas. En la extracción de la masa espermática epididimaria, se produce una contaminación de la muestra por fluidos, tejidos y sangre, lo cual podría disminuir su calidad [6]. Los restos de tejidos que resultan de la recolección de semen pueden acelerar el proceso de deterioro de las células almacenadas en tubo.

La dilución de los espermatozoides en un medio tamponado podría evitar algunos efectos negativos después de la recolecta, diluyendo los líquidos no deseados, tales como sangre o líquido intersticial. En nuestro trabajo, se observó que los espermatozoides diluidos con una osmolalidad de 370 mOsm/kg mostraron una mejor motilidad a las 24 horas post-mortem que las almacenadas en el epidídimo. La osmolalidad del medio es crucial para el funcionamiento de los espermatozoides [7], por lo tanto se espera que los medios de dilución hiperosmóticos (370 y 420 mOsm/kg) sean

los más apropiados para el almacenamiento de los espermatozoides del epidídimo de carnero (en nuestro estudio tiene una osmolalidad media 365 mOsm/kg) ya que son medios isotónicos con el líquido del epidídimo. Sin embargo, hemos observado que el diluyente con 320 mOsm/kg mantienen la mejor motilidad y provoca menos daño acrosomal que los diluyentes hiperosmóticos en prolongados tiempos post-mortem (72h). La capacidad de los diluyentes de modificar las membranas de los espermatozoides, sobre todo la yema de huevo o leche, se ha descrito anteriormente [8]. Si bien cuenta con algunas ventajas, el almacenamiento de los espermatozoides en los diluyentes podría estar provocando cambios en las membranas o en la fisiología en general de los espermatozoides, lo que podría aumentar la vulnerabilidad a distintos tipos de estrés.

Es de destacar que, en los resultados de nuestro estudio, los espermatozoides que se almacenaron diluidos en el diluyente a 370 mOsm/kg mostraron los resultados más bajos en viabilidad y en el estado de los acrosomas, mientras que los mejores resultados se obtuvieron con el diluyente a 320 mOsm/kg, siendo similares los del diluyente a 420 mOsm/kg. Esto sugiere que la osmolalidad de los diluyentes podría modificar la vulnerabilidad de los espermatozoides del epidídimo, y se confirma que la osmolalidad parece ejercer un efecto complejo, como dice [9]. Por lo tanto, la hipótesis de que un diluyente ligeramente hiperosmótico podría proporcionar el mejor ambiente para los espermatozoides del epidídimo de carnero parece ser refutada y, mientras que un medio hipotónico respecto del fluido de la cola del epidídimo (D320) podría ser más adecuado. No obstante, la mejor opción para preservar la calidad de los espermatozoides epididimarios a 5 °C, de las comparadas en este trabajo, ha resultado ser la refrigeración del epidídimo completo.

7.2 Evaluación del almacenamiento y del diluyente de congelación en la criopreservación de los espermatozoides epididimarios de carnero

Los espermatozoides epididimarios parecen ser más tolerantes a los retos osmóticos que los eyaculados [1,10,11], y podrían adaptarse mejor a los cambios osmóticos resultantes de la dilución y congelación/descongelación. En el capítulo 4 de esta memoria, evaluamos el efecto del método de conservación y la utilización de diluyentes de

congelación a diferentes presiones osmóticas en la congelación de espermatozoides epididimarios de carnero.

En estudios anteriores sobre almacenamiento refrigerado de espermatozoides del epidídimo de carnero [11], encontramos que la osmolalidad de los diluyentes (antes de la adición de la yema de huevo y el glicerol) podría tener un impacto en el almacenamiento. Martinez-Pastor, et al. [12] evaluaron diferentes diluyentes con diferentes presiones osmóticas para congelar espermatozoides del epidídimo de ciervo, encontrando una mayor motilidad después de la crioconservación con un diluyente de 380 y 430 mOsm/kg, comparando con un diluyente a 320 mOsm/kg. Estos resultados fueron similares a los observados por [9]. La osmolalidad del fluido epididimario aumenta de la cabeza a la cola [13], siendo éste claramente hiperosmótico en la cola. Por lo tanto, nos planteamos que los diluyente hiperosmóticos podrían favorecer la crioconservación de este tipo de muestras, no solamente por ser isoosmótica a los fluidos del epidídimo, sino también porque podrían tener efectos positivos durante la congelación [14].

No obstante, nuestros resultados no mostraron grandes diferencias entre los diluyentes al congelar muestras epididimarias obtenidas casi después de la muerte del macho. Esto podría ser debido a la buena tolerancia osmótica [9,11,12] de los espermatozoides epididimarios de carnero.

Al analizar los resultados a distintos tiempos post-mortem, no observamos grandes diferencias entre los tratamientos ensayados (métodos de conservación y diluyentes), ni en la calidad a la precongelación ni a la descongelación. Sin embargo, varias combinaciones de tratamientos y diluyentes de congelación resultaron ser más beneficiosas o perjudiciales sobre la calidad espermática, especialmente en tiempos post-mortem largos (48–72 h). Observamos que los dos métodos más eficientes fueron el almacenamiento de los epidídimos, si posteriormente se utilizaba un diluyente con una presión osmótica de 420 mOsm/kg, o la conservación diluida, si para almacenar y congelar se utilizaba un diluyente con una presión osmótica de 320 mOsm/kg. Estas dos combinaciones pueden ser consideradas como las más adecuadas para el presente estudio, sobre todo en tiempos post-mortem largos. Curiosamente, en el almacenamiento diluido los diluyentes con presiones osmóticas de 370 o 420 mOsm/kg fueron claramente perjudiciales.

Podemos interpretar la eficacia de la combinación epidídimo×420 considerando antes que algunos estudios han demostrado que la osmolalidad del epidídimo aumenta con el tiempo post-mortem [1,11]. Es posible que los espermatozoides epididimarios desarrollen una adaptación mientras su entorno se hace más hipertónico. Los espermatozoides podrían acumular osmolitos. Éste es un mecanismo fisiológico ya descrito, y que podría servir para afrontar los desafíos osmóticos de la eyaculación [7,13].

En cambio, la explicación para los resultados de la combinación conservación diluida×320 podría ser muy distinta. En este caso, los espermatozoides del epidídimo, en un medio moderadamente hiperosmótico, sufrirían una adaptación al nuevo entorno hipotónico. Este proceso tal vez sea similar a la que ocurre cuando, durante la eyaculación, los espermatozoides en la cauda del epidídimo entran en contacto con el plasma seminal y, seguidamente, luego con el medio más hipotónico del tracto genital femenino [7]. Los espermatozoides recogidos poco después de la muerte del animal, podrían estar mejor preparados para estos desafíos hipoosmóticos y la adaptación a una osmolalidad menor [7,13,15], explicando el mejor resultado a largo plazo de la conservación y posterior congelación en un diluyente de 320 mOsm/kg.

7.3 Congelación de espermatozoides de carnero a altas concentraciones

La criopreservación de semen induce una serie de cambios estructurales y bioquímicos en los espermatozoides, reduciendo así la integridad de la membrana [16], la motilidad [17,18] y la capacidad fecundante [19]. La optimización de las dosis de semen y la utilización de tecnologías reproductivas complejas (inseminación intrauterina vía laparoscópica) han despertado el interés por congelar espermatozoides a bajas concentraciones [20] que funcionan con éxito cuando son depositados en el útero. De la misma manera, la congelación de altas densidades espermáticas puede resultar muy interesante para incrementar la fertilidad de las dosis seminales utilizadas en inseminación artificial transcervical.

Por esta razón, en el capítulo 5 de esta memoria, evaluamos la calidad post-descongelación de semen de carnero congelado a altas concentraciones. La congelación

de dosis con alta densidad espermática busca mejorar la inseminación artificial cervical, incrementando las probabilidades de lograr una mayor fertilidad.

Nuestro trabajo sigue al publicado por [21] en la especie ovina. Estos autores evaluaron la supervivencia de semen de carnero congelado a varias concentraciones (50, 100, 200, 400, 500 y 800×10^6 mL^{-1}), usando dos tipos de diluyentes (a base de yema de huevo —Tris-FY— o leche —Leche-LY). Independientemente de los diluyentes, encontraron que el rendimiento global de las muestras espermáticas (motilidad, viabilidad y estado acrosomal) se redujo al congelar a la concentración más alta, de 800×10^6 mL^{-1}. Sin embargo, los resultados no fueron concluyentes, observando una alta variabilidad entre las concentraciones que evaluaron. Además, los resultados de fertilidad reportados por estos autores no difirieron entre las distintas concentraciones, aunque sugieren una menor fertilidad de las dosis congeladas a 800×10^6 mL^{-1}. Hay que destacar que estos autores no inseminaron con cantidades fijas de espermatozoides, sino con volúmenes fijos, por lo que se inseminaron más espermatozoides con las dosis más concentradas.

En nuestro trabajo, observamos que la calidad espermática post-descongelación fue más alta en las dosis de 200 y 400×10^6 mL^{-1} que en las de 800 y 1600×10^6 mL^{-1}. El efecto negativo de la congelación con alta densidad espermática no se conoce, aunque se podría atribuir al exceso de radicales libres, la modificación del metabolismo de los espermatozoides, los cambios en el medio debido al metabolismo espermático, interacción célula-célula o cambios físicos durante la congelación/descongelación. Además, las enzimas y los productos tóxicos liberados por los espermatozoides dañados, que podrían tener un efecto mayor en las concentraciones de espermatozoides altas, podrían contribuir a la pérdida de calidad espermática [22,23]. En espermatozoides de caballo, Crockett, et al. [24] encontraron no sólo una motilidad menor, sino también un mayor porcentaje de espermatozoides con acrosomas dañados en dosis de 500×10^6 mL^{-1} (45%) que en dosis de 50×10^6 mL^{-1} (60%). Posiblemente debido a diferencias entre las especies, nosotros no hemos detectado un aumento del daño acrosomal en las muestras congeladas a 800 o 1600×10^6 mL^{-1}.

El aporte más destacado de este trabajo fue el estudio de fertilidad, que reveló que, inseminando con un número fijo de espermatozoides (al contrario que en el trabajo de D'Alessandro, et al. [21], la concentración de 800×10^6 mL^{-1} disminuyó significativamente la fertilidad. Nuestros resultados sugieren que la ventaja de congelar

a altas concentraciones (inseminar con un número mayor de espermatozoides) queda ampliamente contrarrestada por la menor fertilidad de las dosis seminales. Dado que las dosis de 200 y 400×10^6 mL^{-1} difirieron muy poco tanto en calidad como en fertilidad, se podría sugerir un incremento hasta al menos 400×10^6 mL^{-1}, a la espera de nuevos estudios que exploren el rango entre 400 y 800×10^6 mL^{-1}.

7.4 Especificidad de los diluyentes de congelación seminal en función del origen espermático (eyaculado, electroeyaculado y epididimario).

La disponibilidad de métodos optimizados para la criopreservación de espermatozoides de carnero en función del origen espermático tendría un impacto significativo en la mejora de la eficiencia en los sistemas de producción ovina en todo el mundo. Esto permitiría la preservación de material genético valioso, así como la protección de la diversidad genética de razas ovinas y otros pequeños rumiantes [25–28]. Por lo tanto, en el capítulo 6 de esta tesis, estudiamos la calidad de los espermatozoides antes y después de la congelación de muestras de espermatozoides obtenidas de tres diferentes fuentes (eyaculado, electroeyaculado y epidídimo) criopreservadas con dos concentraciones de yema de huevo (10% y 20%) y de glicerol (4% y 8%).

Nuestros resultados en la precongelación y post-descongelación, mostraron que la calidad de la motilidad de los espermatozoides fue similar en las muestras recuperadas del eyaculado y el epidídimo (con mejores parámetros cinéticos en este último), pero los electroeyaculados tuvieron resultados más bajos. Por otra parte, la viabilidad fue mayor y los daños en los acrosomas menores para los espermatozoides recuperados del epidídimo. La criopreservación, como era de esperar, redujo sustancialmente los parámetros de motilidad y viabilidad de los espermatozoides en las tres fuentes de recuperación evaluadas. No obstante, la movilidad tras la descongelación fue inferior en las muestras de electroeyaculación, y la integridad acrosomal y viabilidad fueron superiores en las muestras epididimarias. Todos estos resultados no tienen repecursión en la fertilidad, ya que en la inseminación con los mejores protocolos ensayados para cada origen, no se observan diferencias significativas aunque si se aprecia una tendencia negativa en los resultados obtenidos con las muestras electroyaculadas respecto a los

otros dos orígenes. Estudios recientes han demostrado que los espermatozoides recuperados del electroeyaculado tiene menor resistencia que los espermatozoides del epidídimo, especialmente en condiciones criobiológicamente estresantes como el estrés por el frío, el estrés osmótico y la adición y eliminación de agentes crioprotectores [10]. Es posible que los espermatozoides epididimarios, por no contactar con el plasma seminal, sean más resistentes al estrés osmótico y térmico. Asimismo, las muestras obtenidas por electroeyaculación podrían ser más vulnerables debido a que la estimulación de las glándulas accesorias podría producir una composición desequilibrada del plasma seminal.

El uso de la yema de huevo en los diluyentes proporciona un efecto protector para el semen frente al choque frío y la criopreservación [29]. En nuestro estudio observamos que la concentración de yema de huevo del 20% fue más eficiente en la criopreservación de espermatozoides de las tres fuentes evaluadas. La yema de huevo ha sido utilizada a varias concentraciones para semen ovino. En cuanto a las muestras epididimarias, éstas han sido muy estudiadas en ciervo rojo, encontrándose que una concentración del 20% es más adecuada [30].

En lo que respecta a la evaluación del glicerol realizada en este estudio, encontramos que la congelación con un 4% de glicerol era más adecuada para los espermatozoides procedentes de eyaculación y electroeyaculación. En cambio, los espermatozoides epididimarios parecen ser más resistentes a dosis altas de glicerol, resultando más adecuada una concentración del 8% [12]. Es posible que los espermatozoides epididimarios sean más resistentes al estrés osmótico y otros efectos tóxicos del glicerol, pudiéndose beneficiar durante la congelación del efecto protector de una concentración mayor de glicerol.

7.5 Consideraciones finales.

Los estudios que hemos realizado han proporcionado información útil, que puede ser aplicable para la creación de bancos de germoplasma de animales de alto valor genético o razas en peligro. Así, si no es posible procesar inmediatamente la muestra post-mortem, los espermatozoides se deben conservar en el epidídimo a 5 °C. En caso de que se desee congelar la muestra espermática, tras su conservación en el epidídimo, la mejor opción sería congelar con un diluyente de una osmolalidad de 420 mOsm/kg. Cuando el

procesado rápido no es posible, hemos encontrado que otra opción aceptable sería extraer la muestra espermática inmediatamente tras la muerte del animal y conservarla en refrigeración diluida en un medio con una osmolalidad de 320 mOsm/kg, y congelando en estas mismas condiciones.

Otro resultado relevante ha sido la constatación de que la congelación a altas concentraciones espermáticas determina una disminución de la calidad post-descongelación, así como de la fertilidad tras la inseminación. En caso de requerir un mayor número de espermatozoides por dosis de inseminación, se podría utilizar una concentración de 400×10^{6} mL^{-1}. Se requieren más estudios para explorar el rango entre la concentración de 400 y 800×10^{6} mL^{-1}, esta última que ha dado resultados negativos en nuestro estudio.

Por último, en nuestro estudio comparativo de congelación de muestras espermáticas de carnero obtenidas mediante eyaculación, electroeyaculación y del epidídimo se encuentran diferencias en función del origen espermático. Los espermatozoides del epidídimo son más resistentes a la criopreservación, aunque en la prueba de fertilidad no se confirman estas diferencias. Existe una especificidad de la concentración del agente crioprotector en los medios de congelación para cada origen espermático: en los espermatozoides recuperados del epidídimo es conveniente congelar con una concentración alta de glicerol (8%) mientras que en los espermatozoides eyaculados y electroeyaculados, se obtienen mejores resultados con una concentración más baja (4%).

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CAPÍTULO 8

Capítulo 8

Conclusiones

A continuación se enumeran las conclusiones derivadas de los resultados obtenidos en los trabajos presentados en esta tesis doctoral.

1. La conservación intraepididimaria hasta 48 horas tras la muerte del carnero, es el método ideal para el almacenamiento precongelación de espermatozoides obtenidos post-mortem.

2. El almacenamiento de espermatozoides ovinos en el epidídimo y la congelación con un diluyente hiperosmótico (420 mOsm/kg) o la dilución post-mortem y su mantenimiento y congelación con un diluyente hipoosmótico (320 mOsm/kg) resultaron ser las combinaciones más adecuadas para mantener la calidad espermática post-descongelación tras un periodo post-mortem de almacenamiento prolongado.

3. La congelación a altas concentraciones espermáticas (800 y 1600×10^6 mL^{-1}) afecta negativamente a la calidad post-descongelación y a la fertilidad post-inseminación.

4. Considerando la valoración in vitro, los espermatozoides epidídimarios de carnero, son más resistentes a la criopreservación que los espermatozoides procedentes de otros orígenes (eyaculación y electroeyaculación).

5. La eficacia y tolerancia del glicerol como crioprotector, presenta especificidad en función del origen espermático (8% para muestras epididimarias y 4% para muestras eyaculadas y electroeyaculadas). Para todos los orígenes, una concentración alta de yema de huevo (20%) ofrece los mejores resultados post-descongelación.

APÉNDICE GRÁFICO

Apéndice gráfico

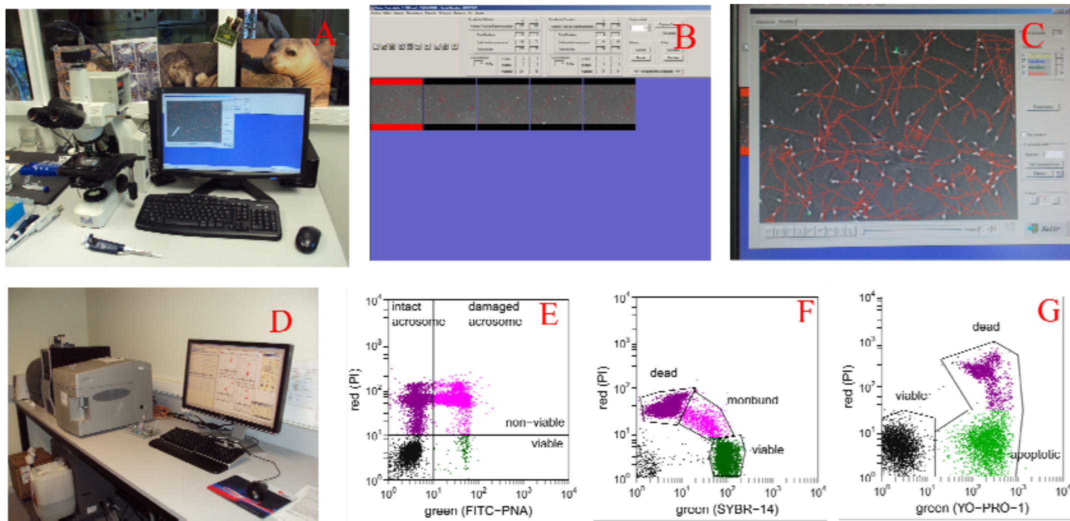

Fig. 1. Captura y análisis de muestras espermáticas. **A**) Asistente computarizado para análisis de semen (CASA). **B**) Captura de información mediante el uso del sistema CASA. **C**) Análisis de las muestras capturadas con el CASA. **D**) Citómetro de flujo. **E**) Análisis IP/PNA-FITC (cuatro poblaciones espermáticas, rojo: No viables con el acrosoma intacto; verde: viables con el acrosoma dañado; rojo + verde: no viables con el acrosoma dañado; no teñidos: viables con el acrosoma intacto). **F**) Análisis SYBR-14/IP (tres poblaciones espermáticas, verde: vivos; rojo + verde: moribundos; rojo: muertos). **G**) Análisis YO-PRO-1/IP (tres poblaciones, rojo: muertos; verde: apoptóticos y sin teñir: viables). Las fotos **E**, **F** y **G** son obtenidas de Reprod Dom Ani 45(Suppl. 2), 67-78 (2010).

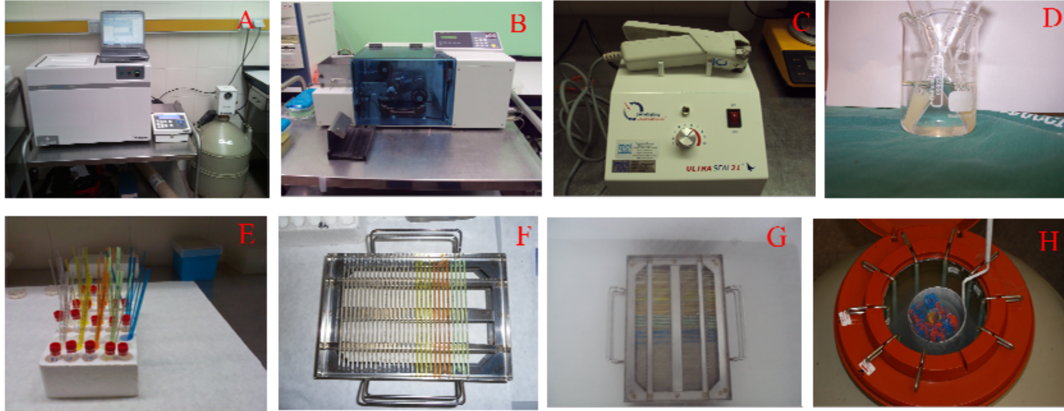

Fig. 2. Equipo para congelación. **A**) Biocongelador (Planner Krio II). **B**) Impresora de pajuelas **C**) Selladora de pajuelas **D**) Conservación de las muestras espermáticas a 5 ºC. **E**) Empajuelado de las muestras para la congelación. **F**) Colocación de las muestras en los racks antes de la congelación. **G**) Extracción de las pajuelas del biocongelador e inmersión en N_2. **H**) Conservación de las pajuelas en N_2 líquido hasta su posterior descongelación.

Fig. 3. Métodos de recogida seminal. **A**) Macho ovino. **B**) Monta de la hembra señuelo para la recogida de la muestra. **C**) Recogida seminal por vagina artificial. **D**) Muestra obtenida por vagina artificial. **E**) Recogida seminal mediante electroeyaculación. **F**) Detalle de emisión seminal por electroeyaculación. **G**) Muestra obtenida por electroeyaculación. **H**) Obtención de los testículos de animales post-mortem. **I**) Recogida seminal de la cola del epidídimo. **J**) Muestra obtenida de la cola del epidídimo.

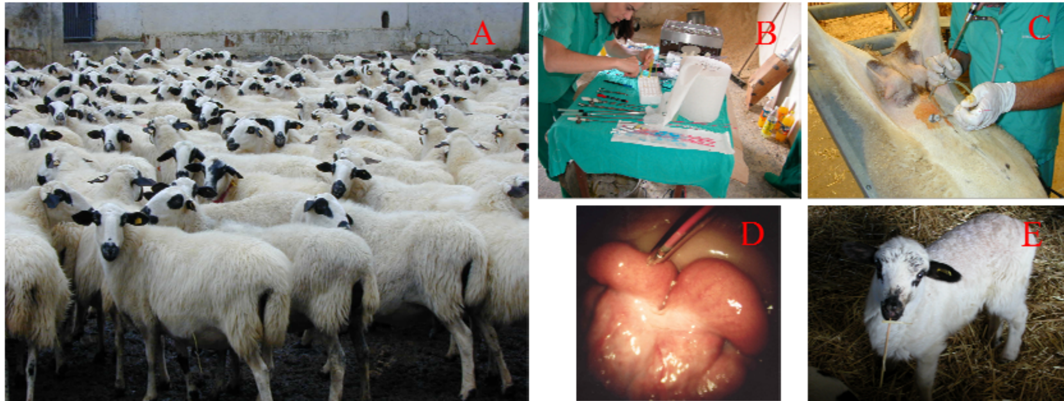

Fig. 4. Inseminación artificial. **A**) Lote de ovejas preparadas para inseminación artificial. **B**) Preparación de las pajuelas para inseminar. **C**) Inseminación artificial intrauterina por vía laparoscópica. **D**) Depósito de la dosis espermática en los cuernos uterinos. **E**) Cordero nacido por inseminación artificial.

APÉNDICE

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Undiluted or extended storage of ram epididymal spermatozoa as alternatives to refrigerating the whole epididymes

J. Tamayo-Canul[a,b], M. Alvarez[a,b], E. López-Urueña[a,b], M. Nicolas[a,b], F. Martinez-Pastor[a,c], E. Anel[a,b], L. Anel[a,b], P. de Paz[a,c,*]

[a] ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain
[b] Animal Reproduction and Obstetrics, University of León, 24071 León, Spain
[c] Molecular Biology, University of León, 24071 León, Spain

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ABSTRACT

The effect of storage procedure at 5 °C on the quality of ram spermatozoa from the cauda epididymis was analyzed. Two strategies were tested at 0, 24, 48 and 72 h post-mortem: (1) spermatozoa held in the epididymal fluid and stored either in the cauda epididymis (In-EPID) or in vitro (Ex-EPID), (2) epididymal spermatozoa extended in three media at 320, 370 and 420 mOsm/kg (D320, D370, D420). Analyzed parameters were: osmolality, pH, motility, acrosomal status and viability. In experiment 1, osmolality of the In-EPID samples, but not in Ex-EPID, increased with post-mortem time. Motility of In-EPID spermatozoa in samples, after 24 h post-mortem, was higher compared to the Ex-EPID samples, although differences decreased at 48 and 72 h. In experiment 2, total (TM) and progressive motility (PM) were not significantly affected by storage time for D320 and In-EPID samples. However, the motility of D370 and D420 samples significantly decreased with time. TM and PM of D320 were significantly higher than D370 and D420 at 72 h. At 24 h, sperm viability was higher for In-EPID ($80.7 \pm 3.4\%$) than for the extended samples ($44.8 \pm 2.9\%$, $37.7 \pm 3.9\%$ and $48.6 \pm 6.0\%$ for D320, D370 and D420, respectively), which also decreased faster with time. At 24 h, the percentage of damaged acrosomes was low and similar for the four methods of storage, but damaged acrosomes increased with time for D320 and D370. Storing the spermatozoa in the epididymis is a good strategy for maintaining sperm quality in ram, at least for 48 h. The D320 extender preserve motility of epididymal spermatozoa but does not protect the status of the acrosome.

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1. Introduction

In the last decade the conservation of rare breeds of domesticated species has been very difficult and many of them have disappeared or are going to become extinct (Canali, 2006). This loss of biodiversity has been due to a poor management policy of genetic resources. In Spain, there is a wide range of animal genetic resources due to its specific geographical and climatic characteristics. These resources are threatened by the introduction of alien breeds with high production rates, and as an example we can cite the 34 sheep breeds officially declared endangered. This situation has motivated the implementation of a National Programme for conservation, improvement and promotion of livestock breeds (Royal Decree 2129/2008). This programme recommended establishing a germplasm bank for certain sheep breeds with increased risk of losses of genetic variability. This bank would ideally contain

* Corresponding author at: Molecular Biology, University of León, 24071 León, Spain. Tel.: +34 9872911320; fax: +34 987291322.
E-mail address: ppazc@unileon.es (P. de Paz).

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Journal Manager
Animal Reproduction Science

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Effect of storage method and extender osmolality in the quality of cryopreserved epididymal ram spermatozoa

J. Tamayo-Canul [a,b], M. Álvarez [a,b], M. Mata-Campuzano [a,c], M. Álvarez-Rodríguez [a,b], P. de Paz [a,c], L. Anel [a,b], F. Martínez-Pastor [a,c,*]

[a] ITRA-ULE, INDEGSAL, University of León, 24071, León, Spain
[b] Animal Reproduction and Obstetrics, University of León, 24071, León, Spain
[c] Molecular Biology, University of León, 24071, León, Spain

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ABSTRACT

Post-mortem sperm recovery and cryopreservation could be a complement to germplasm banking in sheep, especially for endangered breeds. This study is an attempt to identify factors for improving the success of cryopreserving ram epididymal spermatozoa, considering the decrease of sperm quality with post-mortem time. Epididymal spermatozoa from 9 rams were kept at 5 °C using three storage methods: within the epididymes, undiluted sperm mass, and diluted in extenders of different osmolality (TES–Tris–fructose at 320, 370 or 420 mOsm/kg, 20% egg yolk, 8% glycerol). At 0, 24, 48 and 72 h, spermatozoa were cryopreserved using each extender. Samples were analyzed before and after cryopreservation by CASA (motility) and flow cytometry (viability and acrosomal status). Post-mortem time decreased pre-freezing and post-thawing sperm quality. Some storage × extender combinations improved the effect of post-mortem time on sperm quality. Both epididymis storage combined with the 420 extender, and storing the spermatozoa diluted in the 320 extender improved post-thawing quality, especially at long post-mortem times. Storing the spermatozoa diluted in the 370 extender was detrimental for the acrosomal status. These findings have practical applications. The simplest storage method (within the epididymes) seems to be adequate if hyperosmotic extenders were used for freezing. An alternative method could be storing the spermatozoa diluted in a hypoosmotic extender. These recommendations are limited to the osmolalities tested in this study (420 mOsm/kg and 320 mOsm/kg); other osmolalities should be tested.

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1. Introduction

Artificial insemination in domestic animals relies almost exclusively on semen obtained by ejaculation (Anel et al., 2003). This is the case of the sheep, most of the doses being obtained by artificial vagina or electroejaculation (Anel et al., 2006). Nevertheless, post-mortem collection could be useful, in the event that a genetically interesting male dies accidentally or must be culled (disease-carriers, physical defects, illness, etc.) (Ehling et al., 2006). For instance, our research group is working with two dairy breeds in Northwest Spain, Churra and Assaf (143,000 and 153,763 females, respectively, registered in breeding programs), whose genetic improvement programs include the use of post-mortem recovered spermatozoa.

There are many successful reports of collection and cryopreservation of epididymal spermatozoa (buffalo: Lambrechts et al., 1999; dog: Hewitt et al., 2001; ram: Kaabi et al., 2003; boar: Suzuki and Nagai, 2003; red

* Corresponding author at: Molecular Biology, University of León, 24071, León, Spain. Tel.: +34 98729I5679.
E-mail address: felipe.martinez@unileon.es (F. Martínez-Pastor).

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Sperm concentration at freezing affects post-thaw quality and fertility of ram semen

M. Alvarez[a,b], J. Tamayo-Canul[a,b], E. Anel[a,b], J.C. Boixo[c], M. Mata-Campuzano[a,b], F. Martinez-Pastor[a,d,*], L. Anel[a,b], P. de Paz[a,d]

[a] ITRA-ULE, INDEGSAL, University of León, León, Spain

[b] Animal Reproduction and Obstetrics, University of León, León, Spain

[c] CENSYRA, León, Spain

[d] Molecular Biology, University of León, León, Spain

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Abstract

We have investigated the effect of sperm concentration in the freezing doses 200, 400, 800, and 1600 $\times$ 10^6 mL^{-1} on the post-thaw quality and fertility of ram semen. Semen was collected from seven adult Churra rams by artificial vagina during the breeding season. The semen was diluted in an extender (TES-Tris-fructose, 20% egg yolk, and 4% glycerol), to a final concentration of 200, 400, 800, or 1600 $\times$ 10^6 mL^{-1} and frozen. Doses were analyzed post-thawing for motility (computer-assisted sperm analysis system [CASA]), viability, and acrosomal status (fluorescence probes propidium iodide [PI]/PNA-FITC, SYBR-14/PI [Invitrogen; Barcelona, Spain] and YO-PRO-1/PI [Invitrogen; Barcelona, Spain]). Total motility and velocity were lower for 1600 $\times$ 10^6 mL^{-1} doses, while progressive motility and viability were lower both for 800 and 1600 $\times$ 10^6 mL^{-1}. The proportion of viable spermatozoa showing increased membrane permeability (YO-PRO-1+) rose in 800 and 1200 $\times$ 10^6 mL^{-1}. Intrauterine inseminations were performed with the 200, 400, and 800 $\times$ 10^6 mL^{-1} doses at a fixed sperm number (25 $\times$ 10^6 per uterine horn) in synchronized ewes. Fertility (lambing rate) was similar for semen frozen at 200 (57.5%) or 400 $\times$ 10^6 mL^{-1} (54.4%), whereas it was significantly lower for 800 $\times$ 10^6 mL^{-1} (45.5%). In conclusion, increasing sperm concentration in cryopreserved semen, at least at 800 $\times$ 10^6 mL^{-1} and more, adversely affects the postthawing quality and fertility of ram semen.
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Keywords: Ram; Sperm cryopreservation; Sperm concentration; Sperm quality; Fertility

1. Introduction

The efficiency of the cryopreservation of ram semen must be improved before widespread application of artificial insemination (AI) in sheep. Acceptable results have been achieved so far using frozen/thawed semen [1–4], but its general use is restricted due to the need of using intrauterine insemination by laparoscopy. Otherwise, AI with frozen semen yields variable and often low fertility results, if applied by vaginal-cervical insemination [4–10]. Another disadvantage of vaginal AI is the high number of spermatozoa required per insemination (100–400 $\times$ 10^6 spermatozoa/dose), whereas laparoscopic AI requires lower sperm numbers (25–50 $\times$ 10^6 spermatozoa/dose) [4,11,12].

In fact, the effect of sperm dose in the cryopreservation of ram semen has been little explored. To our knowledge, the only study was performed by D'Alessandro, et al. [11], who tested two types of

* Corresponding author: Tel.: +34 987291000 ×5679.
E-mail address: felipe.martinez@unileon.es (F. Martinez-Pastor).

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